0026-895X/06/6903-857–865$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:857–865, 2006

Vol. 69, No. 3
15909/3085573
Printed in U.S.A.

The Amino Terminus and the Third Extracellular Loop of CX3CR1 Contain Determinants Critical for Distinct Receptor Functions[S]

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Received June 24, 2005; accepted November 22, 2005

ABSTRACT

The G protein-coupled receptor CX3CR1 is a specific receptor for the CX3C chemokine fractalkine (CX3CL1 according to the new chemokine nomenclature). The aim of this study was to identify receptor elements that contribute independently to agonist binding and receptor activation. Targeted mutation of selected acidic amino acid residues demonstrated that the binding activity of CX3CR1 was critically dependent on the two negatively charged residues Asp25 and Glu254 located on the N-terminal domain and third extracellular loop, respectively. In addition, mutation of the uncharged polar residue Tyr14 in the amino terminus caused a reduction in the ligand binding affinity. In contrast, the three acidic residues Glu13, Asp16, and Asp266 did not contribute to ligand binding but were crucial for receptor activation. The mutant receptors E13A, D16A, and D266A bound fractalkine with high affinity but were unable to induce signaling events necessary to support chemotaxis. These acidic residues may engage in electrostatic interactions with basic residues on fractalkine that are necessary for receptor function but not for binding. Our data are consistent with a model of chemokine receptor activation consisting of a multistep mechanism. Step one mediates the high-affinity fractalkine binding involving Tyr14, Asp25, and Glu254. The initial interaction then triggers the engagement of Glu13, Asp16, and Asp266, which are necessary for CX3CR1 activation.

Leukocyte trafficking from the circulation to sites of injury or inflammation requires the coordinated action of both cellular adhesion proteins and chemotactic factors. The CX3C chemokine fractalkine (CX3CL1) is of particular interest because of its unique properties to function either as an adhesion molecule or as a chemokine (Bazan et al., 1997; Pan et al., 1997). Unlike other known chemokines, fractalkine is encoded as a transmembrane protein composed of the classic chemokine domain that is tethered to the cell surface via a mucine-like stalk. It is expressed primarily on activated endothelium and functions in this configuration as an adhesion protein for circulating leukocytes (Fong et al., 1998; Haskell et al., 1999). The soluble form of fractalkine is released from the cell surface through proteolytic cleavage by ADAM17 or ADAM10 (Tsou et al., 2001; Hundhausen et al., 2003). In this soluble form, fractalkine behaves like a conventional chemokine and strongly induces chemotaxis.

This work was supported by the National Institutes of Health grant HL56989 (La Jolla Specialized Center of Research in Molecular Medicine and Atherosclerosis).

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.015909.

The functional responses to fractalkine, including chemotaxis and adhesion, are mediated by the G protein-coupled receptor CX3CR1 (Imai et al., 1997). CX3CR1 is expressed primarily on human monocytes, T cells, natural killer cells, and dendritic cells (Imai et al., 1997; Yoneda et al., 2000; Dichmann et al., 2001; Nishimura et al., 2002). CX3CR1 is also expressed in astrocytes and microglia throughout the brain and may mediate the communication with neurons, a major source of fractalkine (Pan et al., 1997; Harrison et al., 1998). The targeted disruption of the CX3CR1 or the fractalkine gene in mice did not produce an obvious phenotype, but studies in appropriate animal models have implicated this chemokine system in several diseases, including atherosclerosis, cardiac allograft rejection, glomerulonephritis, cerebral ischemia reperfusion injury, and antitumor responses (Feng et al., 1999; Haskell et al., 2001; Soriano et al., 2002; Combadiere et al., 2003; Lavergne et al., 2003; Lesnik et al., 2003). Based on these studies, CX3CR1 and fractalkine have emerged as attractive therapeutic targets for the effective treatment of certain inflammatory diseases.

Little is known about the structural requirements for the functional interaction between CX3CR1 and fractalkine. Two single-nucleotide polymorphisms have been identified in the

ABBREVIATIONS: HEK, human embryonic kidney; BSA, bovine serum albumin; ERK1/2, extracellular regulated kinases 1 and 2; PD98059, 2′-amino-3′-methoxyflavone.

open reading frame of the CX3CR1 gene, and analysis of the two receptor variants implicated Ile249 and Met280 in ligand binding (Faure et al., 2000). A somewhat larger body of structural and functional information is available about the ligand fractalkine. The solution structure has been solved, and mutagenesis studies identified a cluster of five basic amino acid residues in the chemokine domain of fractalkine that are critical for binding and function (Mizoue et al., 1999, 2001; Harrison et al., 2001).

In this study, we tested a panel of CX3CR1 mutants consisting of single amino acid substitutions in the amino terminus and the third extracellular loop for ligand binding activity and ability to induce intracellular signaling essential for functional responses. Results with the naturally occurring CX3CR1-T280M mutant showing that this mutation did not alter ligand binding but greatly impaired receptor-mediated responses, including chemotaxis and adhesion (McDermott et al., 2003), and our own work on CCR2 showing that agonist binding and receptor activation occur in separate steps (Han et al., 1999), led us to hypothesize that some amino acid residues may differentially contribute to distinct CX3CR1 functions. We placed our primary efforts on the amino terminus and third extracellular loop because these regions were implicated in receptor function by analogy with other chemokine receptors and human genetic studies on CX3CR1 (Hebert et al., 1993; Monteclaro and Charo, 1997; Faure et al., 2000; McDermott et al., 2003). Because most of the amino acid residues on fractalkine that have been shown to contribute to binding are basic residues, we scanned the selected receptor domains for acidic residues that may engage in electrostatic interactions with the ligand. Our study identified several key residues that differentially contribute to ligand binding and receptor activation.

Materials and Methods

Cell Culture. Human HEK 293T cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 50 μg/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO_2. All of the cell culture components were purchased from Invitrogen (Carlsbad, CA).

Mutagenesis. Human CX3CR1 cDNA was obtained by polymerase chain reaction using reverse-transcribed mRNA from THP-1 cells. The forward and reverse primers (Table 1) were designed to amplify the open reading frame, including the untranslated consensus sequence for initiation of translation, according to the published mRNA sequence (Combadiere et al., 1995). The amplified cDNA was cloned into the pcDNA3.1 expression vector (Invitrogen) using the restriction sites that were included in the primers, and the construct was sequenced. Site-directed mutagenesis was performed by a two-step polymerase chain reaction approach using a pair of complementary primers carrying appropriate nucleotide substitutions (Table 1). In the first step, each of the mutagenic primers was used in combination with the wild-type primers to amplify the 5′ and 3′ fragments of CX3CR1 in separate reactions. In the second step, the two fragments were annealed at the overlap generated by the complementary mutagenic primers, and the full-length mutant was amplified with the two wild-type primers. All mutations were confirmed by sequence analysis. The mutants were subcloned into pcDNA3.1 (Invitrogen) for transient transfection and into the retroviral vector pLXie (a generous gift from Dr. Tuszynski, University of California, San Diego, La Jolla, CA) for stable transfection.

Transient Transfection. HEK 293T cells were grown in six-well tissue culture plates until they reached approximately 50% confluence. The cells were then transfected with wild-type and mutant CX3CR1 cDNA subcloned into pcDNA3.1 (Invitrogen) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's directions. After 48 h, the cells were collected and were used for ligand binding analyses.

Stable Transfection. For stable transfection, the full-length cDNAs encoding wild-type and mutant CX3CR1 were subcloned into the retroviral vector pLXie, which contains an internal ribosomal entry site, the sequence of the enhanced green fluorescent protein, and the 5′-long terminal repeat as a promoter for transgene expression (Lacroix et al., 2002). The retroviral constructs were introduced into the AmphoPack-293 Cell Line (BD Biosciences, Mountain View, CA) to produce retroviral particles, which were subsequently used to

TABLE 1
Sequence of primers used in site-directed mutagenesis
The mutated codons are underlined.

Mutant	Sense Primers
CX3CR1	
Sense	AACAAGCTTTTCACCATGGACCAGTTCC
Antisense	ATTGGATCCTCAGAGAAGTAGCAATGC
D2A	
Sense	CTTAAGCTTTTCACCATG<u>GCC</u>CAGTTCCCTGAATCAGTG
Antisense	CACTGATTCAGGGAACTG<u>GGC</u>CATGGTGAAAAGCTTAAG
E6A	
Sense	ACCATGGACCAGTTCCCT<u>GCA</u>TCAGTGACAGAAAACTTTG
Antisense	AAAGTTTTCTGTCACTGA<u>TGC</u>AGGGAACTGGTCCATGGTG
E10A	
Sense	TCCCTGAATCAGTGACA<u>GCA</u>AACTTTGAGTACGATGATTTGG
Antisense	ATCATCGTACTCAAAGTT<u>TGC</u>TGTCACTGATTCAGGGAAC
E13A	
Sense	TCAGTGACAGAAAACTTT<u>GCG</u>TACGATGATTTGGCTGAGG
Antisense	TCAGCCAAATCATCGTA<u>CGC</u>AAAGTTTTCTGTCACTGATTC
E13Q	
Sense	TCAGTGACAGAAAACTTT<u>CAG</u>TACGATGATTTGGCTGAGG
Antisense	TCAGCCAAATCATCGTA<u>CTG</u>AAAGTTTTCTGTCACTGATTC
Y14A	
Sense	AGTGACAGAAAACTTTGAG<u>GCC</u>GATGATTTGGCTGAGGCC
Antisense	GCCTCAGCCAAATCATC<u>GGC</u>CTCAAAGTTTTCTGTCACTG
D15A	
Sense	ACAGAAAACTTTGAGTAC<u>GCT</u>GATTTGGCTGAGGCCTGTT
Antisense	ACAGGCCTCAGCCAAATC<u>AGC</u>GTACTCAAAGTTTTCTGTC
D16A	
Sense	AAAACTTTGAGTACGAT<u>GCT</u>TTGGCTGAGGCCTGTTATATTGG
Antisense	TAACAGGCCTCAGCCAA<u>AGC</u>ATCGTACTCAAAGTTTTCTG
D16N	
Sense	AAAACTTTGAGTACGAT<u>AAT</u>TTGGCTGAGGCCTGTTATATTGG
Antisense	TAACAGGCCTCAGCCAA<u>ATT</u>ATCGTACTCAAAGTTTTCTG
E19A	
Sense	AGTACGATGATTTGGCT<u>GCG</u>GCCTGTTATATTGGGGAC
Antisense	TCCCCAATATAACAGGC<u>CGC</u>AGCCAAATCATCGTACTC
D25A	
Sense	GCCTGTTATATTGGG<u>GCC</u>ATCGTGGTCTTTGGGACTG
Antisense	TCCCAAAGACCACGAT<u>GGC</u>CCCAATATAACAGGCCTC
D25N	
Sense	GCCTGTTATATTGGG<u>AAC</u>ATCGTGGTCTTTGGGACTG
Antisense	TCCCAAAGACCACGAT<u>GTT</u>CCCAATATAACAGGCCTC
E254A	
Sense	GTTATGATTTTCCTG<u>GCG</u>ACGCTTAAGCTCTATGACTTC
Antisense	ATAGAGCTTAAGCGT<u>CGC</u>CAGGAAAATCATAACGTTGTAGG
E254Q	
Sense	GTTATGATTTTCCTG<u>CAG</u>ACGCTTAAGCTCTATGACTTC
Antisense	ATAGAGCTTAAGCGT<u>CTG</u>CAGGAAAATCATAACGTTGTAGG
D260A	
Sense	ACGCTTAAGCTCTAT<u>GCC</u>TTCTTTCCCAGTTGTGACATG
Antisense	ACAACTGGGAAAGAA<u>GGC</u>ATAGAGCTTAAGCGTCTCCAGG
D266A	
Sense	TTCTTTCCCAGTTGT<u>GCC</u>ATGAGGAAGGATCTGAGGATG
Antisense	CAGATCCTTCCTCAT<u>GGC</u>ACAACTGGGAAAGAAGTCATAG
D266Q	
Sense	TTCTTTCCCAGTTGT<u>AAC</u>ATGAGGAAGGATCTGAGGATG
Antisense	CAGATCCTTCCTCAT<u>GTT</u>ACAACTGGGAAAGAAGTCATAG
D270A	
Sense	TGTGACATGAGGAAG<u>GCT</u>CTGAGGCTGGCCCTCAGTGTG
Antisense	GAGGGCCAGCCTCAG<u>AGC</u>CTTCCTCATGTCACAACTGGG

infect HEK 293T cells. Three days after infection, single cells were sorted by flow cytometry into wells of 96-well plates by virtue of green fluorescent protein expression. Individual clones were grown, and the colonies giving rise to similar expression levels of wild-type and mutant CX3CR1 were expanded and further analyzed by Western blotting and flow cytometry.

Flow Cytometry. Cells were washed twice with ice-cold phosphate-buffered saline containing 0.1% BSA (buffer A) and were incubated for 60 min on ice (5×10^5 cells in 250 μl) with rabbit anti-human CX3CR1 polyclonal antibody directed against the amino terminus or second extracellular loop (ProSci, Poway, CA). After two washes with buffer A, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 30 min on ice. After two additional washes with buffer A, the cells were analyzed by flow cytometry using FACSCalibur equipped with CellQuest software (BD Biosciences, San Jose, CA). List mode files were collected for 50,000 cells from each sample.

Equilibrium Binding Analysis. The binding assay was carried out essentially as described previously (Tangirala et al., 1997). In brief, the transfected HEK 293T cells expressing wild-type or mutant CX3CR1 were harvested and washed with phosphate-buffered saline, and 2×10^6 cells were resuspended in 100 μl of RPMI 1600 medium containing 0.5% BSA (binding buffer). The cells were incubated with various concentrations of ^{125}I-fractalkine (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) for 90 min at room temperature with gentle shaking. To remove unbound ligand, the cells were centrifuged through a mixture (1:1) of dioctylphthalate and dibutylphthalate (Sigma-Aldrich, St. Louis, MO), and the radioactivity associated with the cell pellet was counted. The specific binding of ^{125}I-fractalkine was obtained by subtracting from the total binding the nonspecific binding, determined in the presence of 100 nM unlabeled fractalkine (R&D Systems, Minneapolis, MN). All assays were done in triplicate, and the binding data were examined with the Prism software program (GraphPad Software Inc., San Diego, CA).

Chemotaxis Assay. Chemotaxis assays were performed with stably transfected cells in 24-well Transwell plates (Costar, Acton, MA) using polycarbonate membranes with 8 μm pore size (Becton Dickinson Labware, San Jose, CA). Various concentrations of fractalkine (R&D Systems) were added to the lower chamber in 800 μl of RPMI medium supplemented with 10 mM HEPES and 0.1% BSA (chemotaxis buffer). The stably transfected cells were resuspended in chemotaxis buffer, and 200 μl of the cell suspension (2×10^5 cells) was added to the upper chamber and incubated for 2.5 h at 37°C under a 5% CO_2 atmosphere. The cells that migrated to the lower chamber were collected by centrifugation stained with 0.04% trypan blue and counted under a microscope. The specific, fractalkine-induced chemotaxis was determined by correction for nonspecific cell migration in the absence of the chemokine. All experiments were performed in triplicate.

Western Blot Analysis. The transfected HEK 293T cells expressing wild-type or mutant CX3CR1 were grown in six-well tissue culture plates to confluence and were lysed in 100 μl of NuPAGE LDS sample buffer (Invitrogen). Equal amounts of protein were resolved by electrophoresis on a 10% SDS-polyacrylamide gel electrophoresis gel, transferred to a polyvinylidene difluoride membrane, and probed with rabbit anti-human CX3CR1 polyclonal antibody directed against the second extracellular loop (ProSci). The specificity of the antibody was tested by preabsorption by the peptide antigen (ProSci). Equal volumes of blocking peptide and anti-CX3CR1 IgG were incubated for 30 min at 37°C before Western blotting. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich).

Extracellular Signal-Related Kinases Analysis. For analysis extracellular signal-related kinases 1 and 2 (ERK1/2), the stably transfected cells were grown to 70% confluence, serum-starved for 16 h, and then stimulated with 1 nM fractalkine. The cells were lysed in 200 μl of NuPAGE LDS sample buffer (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Active Motif, Carlsbad, CA). The crude lysates were sonicated for 4 s and were cleared by centrifugation at 13,000*g* for 15 min. The proteins in equal amounts of cell lysates were resolved by electrophoresis on a 10% SDS-polyacrylamide gel electrophoresis gel, transferred to a polyvinylidene difluoride membrane, and probed with rabbit anti-human phospho-ERK1/2 antibody (Thr202/Tyr204) for phosphorylated ERK1/2 and with rabbit anti-human ERK1/2 antibody for total ERK expression (Cell Signaling Technology Inc., Beverly, MA). Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich).

Results

Generation of Transfected HEK 293T Cells Stably Expressing Wild-Type and Mutant CX3CR1. Mutagenesis analysis of fractalkine indicated that amino acid residues with positively charged side chains are essential for chemokine function (Harrison et al., 2001; Mizoue et al., 2001). It is plausible that these residues interact with negatively charged counterparts located within the ligand binding domain of CX3CR1. To test this hypothesis, we generated a series of CX3CR1 mutants containing single amino acid substitutions of the individual acidic amino acid residues in the amino terminus and the third extracellular loop (Fig. 1). Previous studies suggested that sulfation of tyrosine residues within the amino terminus enhances the function of chemokine receptors (Farzan et al., 1999; Fong et al., 2002). Therefore, Tyr14 within the EYDD cluster of acidic amino acid residues was included in our analysis. These residues were mutated individually to alanine, and the mutant receptors were ectopically expressed in stably transfected HEK 293T cells. To reduce the potential of conformational changes as a contributing factor to the observed effects of the mutation, the aspartic and glutamic amino acid residues that were identified in the initial experiments to be critical for ligand binding and receptor activation were more conservatively mutated to asparagine and glutamine, respectively. Surface expression was analyzed by flow cytometry and showed that the expression levels of the wild-type and various mutant receptors were similar (Fig. 2A and Table 2). Total expression was estimated by Western blotting and gave identical results (Fig. 2B). The antibody was highly specific for CX3CR1, as demonstrated by Western blotting using anti CX3CR1 IgG preabsorbed by the peptide antigen (Fig. 2C).

Identification of Residues Critical for Ligand Binding. To assess the effect of the various mutations on the binding affinity, equilibrium binding analyses were performed with the established cell lines stably expressing the mutant receptors. As shown in Fig. 3, the acidic amino acid residues Asp25 located in the amino terminus and Glu254 in the third extracellular loop are critical for fractalkine binding and substitution of either residue with alanine abolished the ligand binding capability. In addition, the mutation of Tyr14 to alanine also impacted ligand binding, causing a significant reduction in the binding affinity compared with wild-type CX3CR1 (K_d = 11.2 ± 2.9 versus 1.9 ± 0.5 nM, respectively). These results indicate that both the N-terminal domain and the third extracellular loop are involved in the ligand binding. None of the remaining negatively charged amino acid residues within these receptor segments seemed to contrib-

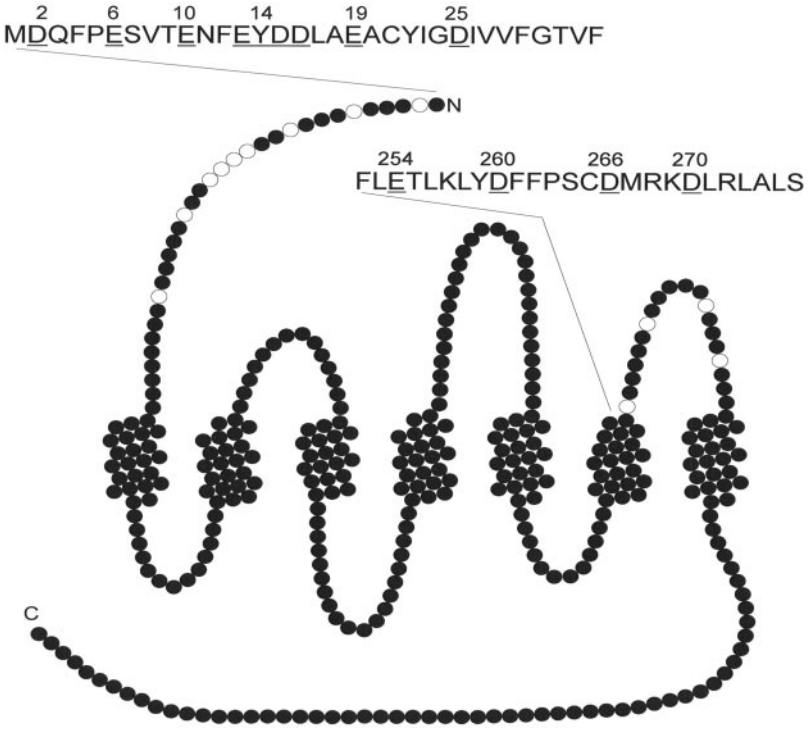
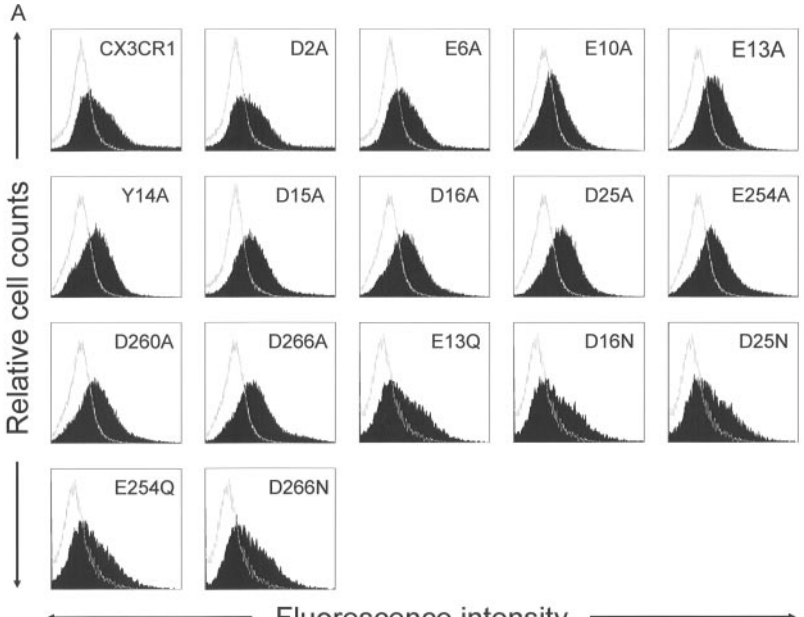


Fig. 1. Structural diagram and partial amino acid sequence of CX3CR1. ●, amino acid residues; ○, residues substituted by alanine. The sequences of the extracellular amino terminus and third extracellular loop are shown with the mutated residues underlined. The numbers specify the positions of the various amino acid residues in the protein. The transmembrane domains are illustrated by the cylindrical arrangements of the amino acids.



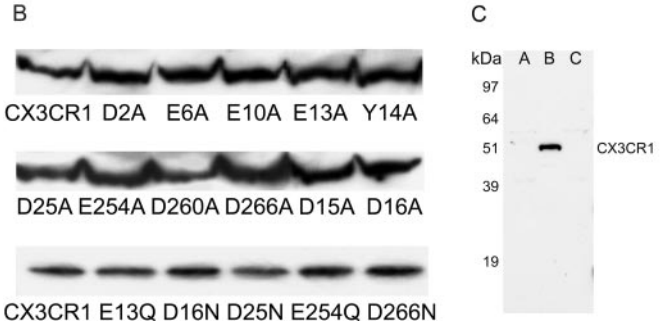


Fig. 2. Expression of wild-type and mutant CX3CR1 in transfected HEK 293T cells. A, HEK 293T cells stably transfected with wild-type CX3CR1 and various mutants were tested for surface expression by flow cytometry. The surface expression was determined on intact cells using anti-human CX3CR1 IgG directed against the second extracellular loop and fluorescein isothiocyanate-conjugated secondary antibody (shaded areas). Nontransfected HEK 293T cells are used as a negative control (fine lines). B, total expression. The total expression of wild-type and mutant CX3CR1 was determined by Western blotting. C, the specificity of the antibody for CX3CR1 was tested by Western blotting using anti-CX3CR1 IgG that was preabsorbed by blocking peptide antigen. Lane A, control transfected HEK 293T cells blotted with anti-CX3CR1 IgG; lane B, CX3CR1 transfected HEK 293T cells blotted with anti-CX3CR1 IgG; lane C, CX3CR1 transfected HEK 293T cells blotted with anti-CX3CR1 IgG preabsorbed by the peptide immunogen.

ute to ligand binding, and all other mutants bound fractalkine with high affinity similar to that of the wild-type receptor (Fig. 3, A and B, and Table 2). We were not able to establish cell lines stably expressing E19A and D270A, but the analysis of the binding isotherms generated with transiently transfected HEK 293T cells demonstrated that these residues were not involved in fractalkine binding (Table 2).

To confirm that the reduction in ligand binding affinity seen with the alanine mutant receptors is a direct result of the removal of negative charges rather than an indirect consequence of conformational changes, the acidic residues associated with ligand binding were mutated more conservatively to amino acid residues of similar structure. As shown in Fig. 3C and Table 2, the binding affinities of the resulting mutant receptors D25N and E254Q were greatly diminished. Despite the fact that these mutants retained some binding ability, qualitatively, the mutations had a significant impact on the ligand binding affinity. As expected from the alanine mutations, the binding affinities of E13Q, D16N, and D266N remained unchanged and were similar to the wild-type receptor (Table 2). These results provide additional support that selected negatively charged amino acid residues on CX3CR1 directly contribute to ligand binding.

Residues Involved in the Stimulation of Receptor-Mediated Cellular Responses but Not Ligand Binding. The primary function of chemokine receptors is to mediate cell migration in response to stimulation. To examine whether the mutations perturbed this receptor function, we performed chemotaxis assays. As shown in Fig. 4 and summarized in Table 2, the mutant receptors with a defective ligand binding capability failed to induce migration. The cells expressing D25A, Y14A, and E254A did not respond to fractalkine, and no chemotactic activity was observed. These results indicated that high-affinity ligand binding is essential for optimal receptor function.

The chemotactic response of cells expressing the three mutant receptors E13A, D16A, and D266A was unexpectedly compromised, despite the fact that these mutants bound fractalkine with an affinity similar to that of the wild-type receptor (Fig. 4A and Table 2). In addition, the more conser-

TABLE 2

Expression and functional responses of wild-type and mutant receptors in transfected HEK 293T cells

The binding affinity was calculated from the binding isotherms obtained with stably transfected cells. Results are expressed as means ± S.D. of three independent experiments. Selected isotherms are shown in Fig. 3. The surface expression was determined by flow cytometry on three individual clones. The representative data are shown in Fig. 2. The expression levels of the mutant receptors were normalized to that of the wild-type receptor (100%). Results are expressed as means ± S.D. of three independent experiments. The binding data for E19A and D270A were obtained with transiently transfected cells. Chemotactic activities shown are the migratory rates to 1 nM fractalkine and were calculated from Fig. 4. All activities were normalized to that induced by the wild-type receptor (100%).

Mutant	Binding Affinity (K_d)	Expression	Chemotaxis	ERK Activation
	nM	%	%	
CX3CR1	1.9 ± 0.5	100.0 ± 15.2	100 ± 35	+
D2A	1.5 ± 0.0	99.5 ± 7.6	139 ± 23	+
E6A	2.3 ± 0.5	107.3 ± 0.7	95 ± 51	+
E10A	1.7 ± 0.3	104.7 ± 11.3	97 ± 45	+
E13A	2.0 ± 0.5	101.6 ± 12.9	31 ± 33	−
E13Q	1.6 ± 0.3	98.8 ± 8.6	24 ± 18	−
Y14A	11.2 ± 2.9	103.4 ± 13.1	21 ± 24	−
D15A	2.0 ± 0.4	97.4 ± 9.3	117 ± 18	+
D16A	2.1 ± 0.4	99.2 ± 9.5	11 ± 29	−
D16N	1.6 ± 0.3	94.5 ± 10.2	33 ± 17	−
E19A	2.0 ± 0.4	N.D.	N.D.	N.D.
D25A	N.B.	96.9 ± 6.2	8 ± 8	−
D25N	15.9 ± 0.2	97.3 ± 11.3	25 ± 11	−
E254A	N.B.	90.5 ± 12.6	5 ± 8	−
E254Q	23.7 ± 0.3	91.8 ± 14.4	31 ± 10	−
D260A	1.9 ± 0.3	99.2 ± 16.3	99 ± 44	+
D266A	1.8 ± 0.4	101.4 ± 16.0	43 ± 30	−
D266N	1.8 ± 0.3	103.4 ± 7.4	36 ± 8	−
D270A	1.9 ± 0.6	N.D.	N.D.	N.D.

N.D., not determined; N.B., no binding.

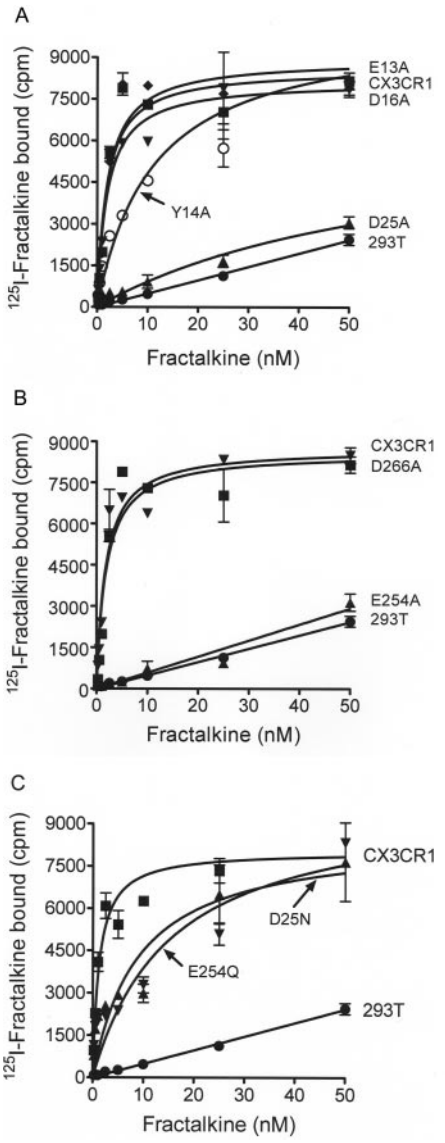


Fig. 3. Ligand binding analysis of the wild-type and mutant receptors. A, fractalkine binding to stably transfected HEK 293T cells expressing wild-type CX3CR1 (■) and the receptors with mutations in the amino terminus E13A (♦), Y14A (○), D16A (▼), and D25A (▲). Nontransfected HEK 293T cells were used as a negative control (●). B, fractalkine binding to transfected HEK 293T cells expressing the wild-type CX3CR1 (■) and the receptors with mutations in the third extracellular loop E254A (▲) and D266A (▼). Nontransfected HEK 293T cells (293T) were used as a negative control (●). C, fractalkine binding to transfected HEK 293T cells expressing the wild-type CX3CR1 (■) and the receptors with more conservative mutations D25N (▲) and E254Q (▼). Nontransfected HEK 293T cells (293T) were used as a negative control (●). Shown is the specific ^{125}I-fractalkine binding after subtraction of the nonspecific binding from the total binding, determined in the presence of a 100-fold excess of unlabeled fractalkine. The affinities were calculated from the binding isotherms and are shown in Table 2. The data shown are the mean ± S.D. of three independent experiments.

vative mutations E13Q, D16N, and D266N also caused functional impairments affecting the chemotactic activity in transfected cells without changing the ligand binding affinity (Fig. 4B and Table 2). These data suggested that the interactions responsible for high-affinity ligand binding are not sufficient for receptor activation, and the induction of functional responses may require a separate set of interactions.

ERK Signaling Is Necessary for CX3CR1-Dependent Chemotaxis. A recent study has demonstrated that fractalkine stimulates ERK1/2 in the human monocyte MonoMac6 cell line (Cambien et al., 2001). Consistent with this, we found that fractalkine also induced ERK1/2 phosphorylation in human monocytic THP-1 cells and in HEK 293T cells transfected with CX3CR1. As shown in Fig. 5, fractalkine induced a rapid increase in the level of phosphorylated ERK1/2 that peaked at approximately 15 min for the THP-1 cells and at approximately 5 min for the CX3CR1-transfected cells. Pretreating the cells with PD98059, a selective inhibitor of the ERK1/2 kinase, effectively blocked ERK1/2 phosphorylation (data not shown).

We next examined the potential involvement of ERK1/2 signaling in chemotaxis induced by endogenous or ectopically expressed CX3CR1. THP-1 monocytes or CX3CR1-transfected HEK 293T cells were pretreated with the ERK1/2 kinase inhibitor PD98059 and then tested for their chemotactic activity in response to fractalkine. As shown in Fig. 6, the treatment with PD98059 ablated the chemotactic response in both cell types, suggesting that CX3CR1-dependent chemotaxis requires ERK1/2 signaling.

Effect of the CX3CR1 Mutations on Activation of the ERK Signaling Pathway. As shown above, the mutant receptors E13A, D16A, and D266A were ineffective mediators of chemotaxis despite displaying a high affinity for fractalkine. These results suggested that specific amino acid residues may be essential for CX3CR1-mediated activation of intracellular signaling pathways without significantly contributing to ligand binding. To examine this hypothesis, we analyzed the various mutant receptors for their ability to stimulate ERK1/2 phosphorylation. The result shown in Fig. 7A demonstrated that the high-affinity ligand binding was essential for stimulating ERK1/2 phosphorylation. As expected, the low-affinity mutants Y14A, D25A, and E254A were unable to induce ERK1/2 phosphorylation. In contrast, the mutants E13A, D16A, and D266A bound fractalkine with high affinity; however, the ability to activate the ERK signaling pathway was significantly impaired. The conservative mutations E13Q, D16N, and D266N gave similar results (Fig. 7B). These mutants were also ineffective in inducing a chemotactic response in the transfected cells despite high-affinity agonist binding (Fig. 4 and Table 2), suggesting that E13, D16, and D266 may play an important role in receptor activation but without contributing to the fractalkine binding. The data shown above were obtained after stimulation of the various mutant receptors with 1 nM fractalkine. To test whether higher receptor occupancy would augment ERK1/2 phosphorylation, specifically by the low-affinity mutants, we increased the fractalkine concentration to 10 nM. However,

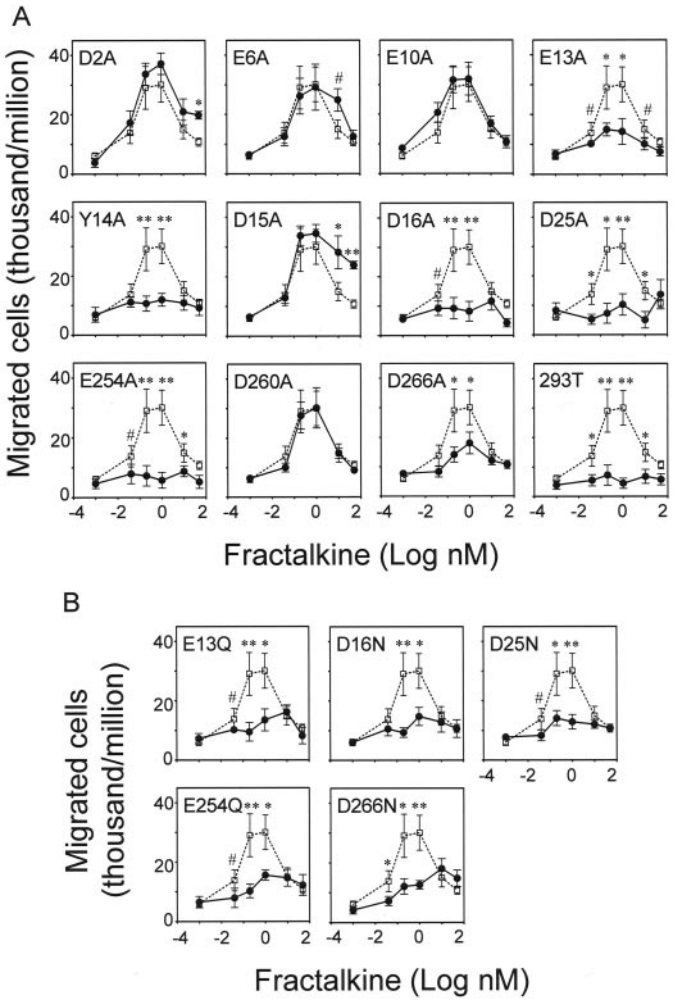


Fig. 4. Effect of the mutations on the chemotactic activity. The chemotactic activities of HEK 293T cells stably transfected with wild type CX3CR1 (broken lines) and mutant CX3CR1 (solid lines) were examined in Transwell assays at the indicated fractalkine concentrations. Nonspecific migration was determined with nontransfected HEK 293T cells (293T). The tracing of the chemotactic response mediated by the wild-type receptor is included in each for better comparison. A, mutants with alanine substitution. B, mutants with the corresponding amide substitution. The data shown are the mean ± S.D. of three independent experiments. The significance of the differences between wild-type and mutant CX3CR1 was calculated for each concentration point using the unpaired Student's t test. **, $p < 0.01$; *, $p < 0.05$; #, $p < 0.1$.

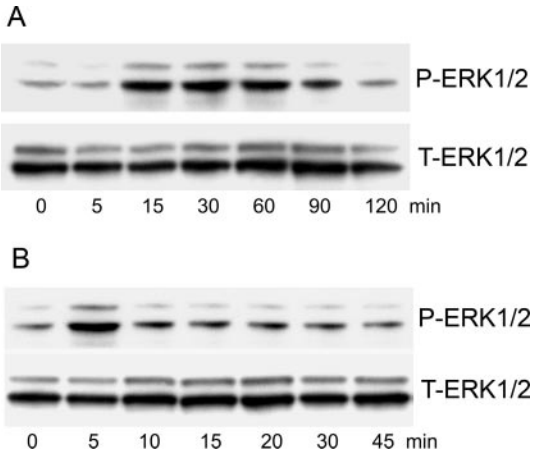


Fig. 5. Time course of mitogen-activated protein kinase phosphorylation stimulated by fractalkine. Serum-starved THP-1 cells (A) or HEK 293T cells stably transfected with CX3CR1 (B) were stimulated with 1 nM fractalkine for the indicated time periods. Equal amounts of cell lysates were subjected to immunoblotting with antibodies against phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2). Representative Western blots from three experiments are shown.

increasing the ligand concentration did not further stimulate ERK1/2, and the phosphorylation levels were identical with the ones achieved with 1 nM fractalkine (Supplemental Fig. S1).

Discussion

A precise knowledge of the structural features regarding the interaction between a receptor and its ligand is fundamental to the understanding of the molecular processes crucial to receptor function. A major goal of the current study was aimed at identifying specific amino acid residues in the N-terminal domain and third extracellular loop of CX3CR1 that are critical for ligand binding and receptor activation. We have tested a series of CX3CR1 mutants for functional responses and found that acidic amino acid residues on the N-terminal domain and third extracellular loop of CX3CR1 differentially contribute to fractalkine binding and receptor activation. Our results are consistent with a two-step mechanism for receptor activation by chemokines. This model involves the initial interaction between chemokine and receptor for high-affinity binding, followed by interactions that are believed to be involved in stabilizing the active conformation of the receptor (Clark-Lewis et al., 1995; Wells et al., 1996; Han et al., 1999).

Structural analysis of fractalkine implicated positively charged amino acid residues in the chemokine function (Mizoue et al., 1999, 2001; Harrison et al., 2001). These findings strongly suggest that these residues interact with negatively charged counterparts on the receptor and that these interactions are essential for ligand binding and functional activation of CX3CR1. Several studies have implicated the N-terminal domain of chemokine receptors in agonist binding. In the present study, we identified four amino acid residues in the amino terminus of CX3CR1 that may engage in electrostatic interactions with the ligand fractalkine. The results show that the acidic residue Asp25 in the amino terminus of CX3CR1 is critical for high-affinity ligand binding, and alanine substitution of this residue significantly impaired ligand binding and the chemotactic function. Like other chemokine receptors, CX3CR1 contains within its N-terminal domain a tyrosine residue adjacent to negatively charged amino acids to form the EYDD acidic cluster. This tyrosine residue can be post-translationally modified by sulfation, which confers a negative charge that may be relevant to chemokine receptor function (Farzan et al., 1999; Fong et al., 2002). Consistent with an important role, substitution of Y14 in CX3CR1 with alanine decreased the binding affinity, and the cells expressing Y14A-CX3CR1 displayed defective signaling and chemotactic responses to fractalkine. The negatively charged residues in the EYDD cluster do not seem to be involved in the primary interaction with fractalkine, and the mutant receptors E13A/Q, D15A, and D16A/N displayed a ligand binding affinity similar to that of the wild-type receptor. Although not critical for binding, E13 and D16 seem to be crucial for receptor function, and substitution of these residues severely impairs the chemotactic response of the transfected cells. These residues may play a role in the stabilization of the active receptor conformation.

Human population genetic studies have identified two polymorphisms, V249I and T280M, in the sixth and seventh transmembrane domains of CX3CR1 as risk factors for coronary artery disease (McDermott et al., 2001; Moatti et al., 2001; Ghilardi et al., 2004). Functional analysis demonstrated that leukocytes from human subjects homozygous for CX3CR1-Ile249/Met280 had a significant decrease in the number of fractalkine binding sites and reduced binding affinity for fractalkine compared with wild-type CX3CR1-Val249/Thr280 cells (Faure et al., 2000). However, subsequent studies with transfected cells showed that the T280M mutation by itself did not change the binding affinity but reduced the kinetics of fractalkine binding (McDermott et al., 2003). The same group also showed that leukocytes from CX3CR1-Met280 homozygotes, which also carry allele Ile249, have defective chemotactic responses to soluble fractalkine, and it was concluded that the T280M mutation causes an impairment of receptor function.

It remains unclear whether fractalkine can interact directly with the transmembrane domain residues associated with these polymorphisms or whether ligand contact sites located on the adjacent extracellular region were perturbed. Furthermore, the studies with the CX3CR1-Met280 mutant showing defective receptor function but intact fractalkine binding suggest that this residue may contribute to receptor activation independently of binding. We hypothesized that additional residues may exist which are important for distinct receptor functions, and we extended our study to the proximal region. Indeed, analysis of our substitution mutants identified two acidic amino acid residues in the third extracellular loop that may be critical for receptor function. The residue E254 is important for ligand binding, and sub-

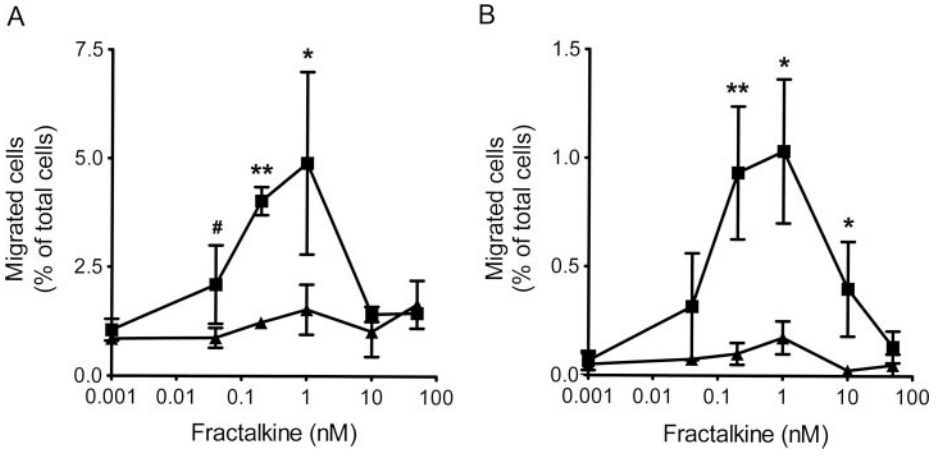


Fig. 6. CX3CR1-mediated chemotaxis involves the ERK1/2 signaling pathway. THP-1 cells (A) or HEK 293T cells stably transfected with CX3CR1 (B) were treated for 2 h with 25 μM PD98059, a specific inhibitor of the direct upstream kinase of ERK1/2. The chemotactic activity of the untreated cells (■) and PD98059-treated cells (▲) was determined as described under *Materials and Methods*. The chemotactic response is expressed as the percentage of cells added to the upper chamber that migrated to the lower chamber of the Transwell. Data shown are the mean ± S.D. of three independent experiments. The significance of the differences between the untreated and PD98059-treated cells was calculated for each fractalkine concentration using the unpaired Student's *t* test. **, $p < 0.01$; *, $p < 0.05$; #, $p < 0.1$.

stitution greatly reduced the binding affinity and chemotactic activity. The mutants D266A and D266N showed normal ligand binding affinity; however, receptor activation mediated by fractalkine was defective, as evidenced by the impaired ability to induce the chemotaxis of the transfected cells. Together, these data implicate the amino terminus and third extracellular loop of CX3CR1 in both ligand binding and receptor activation.

Mitogen-activated protein kinases participate in diverse biological functions, and several studies have indicated that this family of kinases is involved in many aspects of innate and adaptive immune responses (Dong et al., 2002; Roux and Blenis, 2004). Fractalkine has been shown to induce ERK1/2 phosphorylation in monocytic cell lines, but the functional implications of this remained untested (Cambien et al., 2001). Our data demonstrate that fractalkine-mediated chemotaxis is dependent on the activation of ERK1/2 signaling pathway. In both THP-1 cells and transfected cells, inhibition of ERK1/2 phosphorylation ablated the chemotactic response. Moreover, a strong correlation exists between the level of ERK1/2 phosphorylation and the chemotactic response of the various mutants. The observation that the E13A/Q, D16A/N, and D266A/N mutant receptors do not support ERK1/2 phosphorylation despite displaying a high affinity for fractalkine is a further confirmation that these residues may be involved in receptor activation leading to functional intracellular signaling but not in ligand binding.

In summary, we identified three residues critical for ligand binding and an additional three residues that are essential for receptor activation without contributing to the agonist binding affinity. Our results are consistent with a two-step mechanism for receptor function, which dissociates ligand binding from receptor activation. Each step involves distinct amino acid residues and separate sets of interactions. The precise knowledge of the structural elements critical for CX3CR1 activation may form the basis for a rational design of small molecular weight therapeutics targeting receptor function in the treatment of inflammatory diseases.

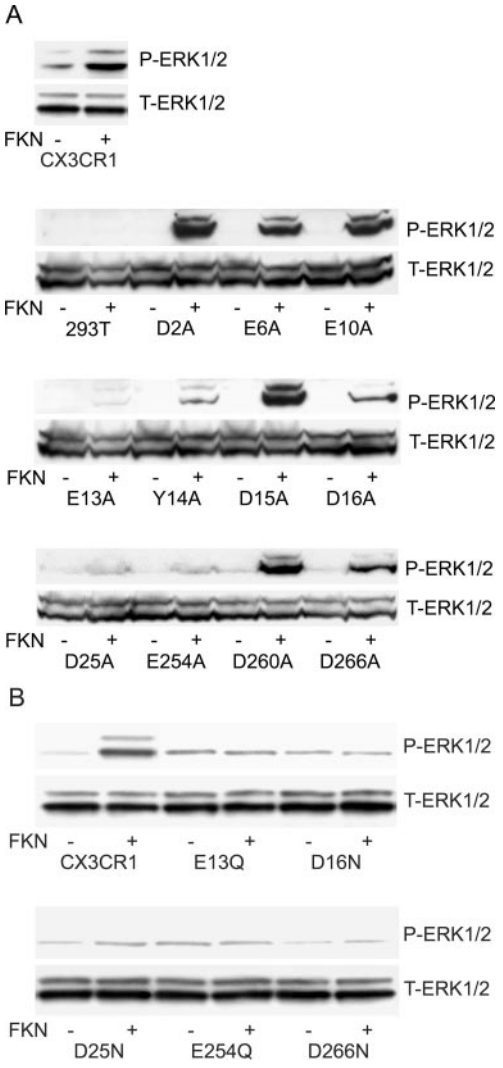


Fig. 7. Effect of CX3CR1 mutations on ERK1/2 phosphorylation. Stably transfected HEK 293T cells expressing the various mutants were stimulated for 5 min with 1 nM fractalkine (FKN). Controls without fractalkine stimulation were included. Equal amounts of cell lysates were subjected to immunoblotting with antibodies against phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2). A, mutants with the alanine substitution. B, mutants with corresponding amide substitution. Representative Western blots of three experiments are shown.

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