Truncation of NH$_2$-terminal Amino Acid Residues Increases Agonistic Potency of Leukotactin-1 on CC Chemokine Receptors 1 and 3*

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Leukotactin-1 (Lkn-1) is a human CC chemokine that binds to both CC chemokine receptor 1 (CCR1) and CCR3. Structurally, Lkn-1 is distinct from other human CC chemokines in that it has long amino acid residues preceding the first cysteine at the NH$_2$ terminus, and contains two extra cysteines. NH$_2$-terminal amino acids of Lkn-1 were deleted serially, and the effects of each deletion were investigated. In CCR1-expressing cells, serial deletion up to 20 amino acids (Δ20) did not change the calcium flux-inducing activity significantly. Deletion of 24 amino acids (Δ24), however, increased the agonistic potency ~100-fold. Deletion of 27 or 28 amino acids also increased the agonistic potency to the same level shown by Δ24. Deletion of 29 amino acids, however, abolished the agonistic activity almost completely showing that at least 3 amino acid residues preceding the first cysteine at the NH$_2$ terminus are essential for the biological activity of Lkn-1. Loss of agonistic activity was due to impaired binding to CCR1. In CCR3-expressing cells, Δ24 was the only form of Lkn-1 mutants that revealed increased agonistic potency. Our results indicate that posttranslational modification is a potential mechanism for the regulation of biological activity of Lkn-1.

Chemokines are a family of small cytokines that induce migration and activation of leukocytes. They contain 4–6 conserved cysteine residues, and have been classified into four subfamilies based on the configuration of the first two cysteine residues near the amino terminus: CXC(α), CC(β), C(γ), and CX$_2$C(1–3). Chemokines exert their biological activities via binding to specific surface receptors, which belong to seven-transmembrane G protein-coupled receptors (4–6). Some of the biological activities of chemokines include induction of leukocyte migration, immunoregulation, suppression of hematopoietic stem cell proliferation, and suppression of human immunodeficiency virus (HIV) infection (7–10).

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1 The abbreviations used are: HIV, human immunodeficiency virus; CCR, CC chemokine receptor; HCC, hemofiltrate CC chemokine; Lkn-1, Lkn-1 is a human CC chemokine that binds to both CCR1 and CCR3 and induces chemotaxis and calcium influx in human neutrophils, monocytes, eosinophils, and lymphocytes (11). Chemotaxis of neutrophils distinguishes Lkn-1 from other CCR1 agonists such as human macrophage inflammatory protein-1α (MIP-1α) (12). Lkn-1 is a member of a human CC chemokine subfamily that contains four conserved cysteines (11). Lkn-1, however, is distinct from other human CC chemokines in that it has two extra cysteines, which may form a third disulfide bond. Lkn-1 is also distinct from other human CC chemokines in that it has long amino acid residues preceding the first cysteine at the NH$_2$ terminus. The mature form of Lkn-1 consists of 92 amino acids and has 31 amino acid residues preceding the first cysteine, whereas most human as well as mouse CC chemokines have 10 or fewer amino acid residues preceding the first cysteine (13).

Recombinant Lkn-1 produced in Escherichia coli also shows several distinguished characteristics. In contrast to other CC chemokines such as monocyte chemoattractant protein 1 (MCP-1), recombinant Lkn-1, which contains additional methionine and six histidines at its NH$_2$ and COOH termini, respectively, shows almost normal biological activities (11). In addition, purified recombinant Lkn-1 undergoes a spontaneous site-specific cleavage producing a 24-amino acid shorter protein than the intact form of Lkn-1 (11). The biological significance of the spontaneous site-specific cleavage is not known yet. In the present study, we produced a series of NH$_2$-terminal deletion mutants of Lkn-1 including the site-specific cleaved form, and the effects of each deletion were investigated in CCR1- or CCR3-expressing cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—HOS (human osteogenic sarcoma) cells expressing CCR1 and CCR3 and HEK (human embryonic kidney) 293 cells expressing CCR3 were provided by Dr. Ji-Ming Wang (National Cancer Institute, Frederick, MD). The cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HOS transfectant cells were selected regularly with 1 μg/ml puromycin (Sigma), and HEK transfectant cells were selected with 400 μg/ml G418 (Invitrogen).

**Production and Purification of NH$_2$-terminal Deletion Mutants of Lkn-1**—A plasmid containing full-length CDNA of Lkn-1 was used as a template in PCR reactions to create Lkn-1 mutants with progressive amino acid deletions at the NH$_2$ terminus. The sequences of the oligonucleotide primers used for amplification are shown in Table I. All of leukotactin-1; MCP, monocyte chemoattractant protein; MIP-1α, macrophage inflammatory protein-1α; Ni-NTA, nickel-nitriilotriacetic acid; RANTES, regulated on activation normal T cell expressed protein.
The forward primers contained overhanging nucleotide sequences for glycine-isoleucine-glutamic acid-glycine-arginine (GIEGR) at the 5' end of the target gene, and the reverse primer contained stop codon of Lkn-1 cDNA. The PCR products were cloned into the E. coli expression vector, pET30Xa/LIC (Novagen, Madison, WI), and transformed into E. coli strain BL21(DE3). Expression of Lkn-1 mutant proteins was induced by isopropyl-1-thio-D-galactopyranoside (Sigma). The mutant proteins were purified from bacterial lysate with Ni-NTA spin columns (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Eluted proteins were folded by gradually removing denaturants in the protein preparation by stepwise dialysis. Overhanging extra amino acid residues at the NH2 terminus including the polyhistidine tag were removed by cleavage with factor Xa (Novagen). For cleavage of 1 μg of target protein, 0.04 unit of factor Xa was added, and the mixture was incubated at 22 ℃ for 7 h. Final purification of the cleaved proteins was performed by chromatographic separation in Superdex Peptide HR 10/30 column (Amersham Biosciences) attached to AKTApurifier (Amersham Biosciences). The proteins were eluted with a 0.02 M phosphate buffer containing 0.25 M NaCl. Finally, purified mutant proteins were free of endotoxin by a Limulus amoebocyte assay (Associates of Cape Cod, Woods Hole, MA). The concentrations of the proteins were determined by micro-bicinchoninic acid assay kit (Pierce) according to the manufacturer's instructions using bovine serum albumin as standard protein.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed as described previously (14). The gels were either stained with a 0.025% Coomassie Brilliant Blue R-250 or silver nitrate, or they were transferred onto nitrocellulose membrane (Haake Buchler Instruments, Saddle Brook, NJ). The membrane was probed with polyclonal rabbit anti-Lkn-1 and then with alkaline phosphate-labeled goat anti-rabbit Ig (Bio-Rad). The membrane was developed by the addition of enzyme substrates, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium (Bio-Rad).

Calcium Influx Assay—Receptor activation was assessed by real time measurement of intracellular calcium concentration in cells labeled with Fura-2/AM (Molecular Probes, Eugene, OR) in MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ) as described previously (4, 15). Receptor desensitization was tested by monitoring intracellular calcium changes in cells upon repeated chemokine stimulation at 100-s intervals. Results were expressed as excitation ratios at 340 and 380 nm.

Chemotaxis Assay—Chemotactic activities were performed in a 48-microwell Boyden chamber (Neuroprobe, Cabin John, MD) as described previously (15). The lower wells were filled with 27 μl of buffer alone or...
NH₂-terminal Deletion Mutants of Leukotactin-1

Production and Purification of NH₂-terminal Deletion Mutants—The intact form of Lkn-1 and its mutants lacking NH₂-terminal 5, 10, 15, 20, 24, 27, 28, and 29 amino acid residues, respectively, were produced in E. coli using the T7 polymerase-based expression vector, pET-30 Xa/LIC. Scheme I depicts the NH₂-terminal deletion mutants of Lkn-1 produced as well as the expression system used in the present study.

Recombinant fusion proteins were cleaved with Factor Xa and then separated by gel filtration using a Superdex peptide HR 10/30 column (Amersham Biosciences) to remove tagged peptides and uncleaved fusion proteins. Here, we show purification of Δ28 fusion proteins as a representative example. A, Factor Xa-treated Δ28 fusion proteins were separated by gel filtration on a Superdex peptide HR 10/30 column, and the eluted proteins were detected at 214 nm. B, the protein in the single dominant peak in A was collected and separated in SDS-PAGE, and the gel was stained with silver nitrate. Lane 1 shows total proteins before the chromatographic separation, and lane 2 shows the finally purified Factor Xa-treated proteins. Std indicates the protein size marker.

Induction of Ca²⁺ Mobilization in CCR1- and CCR3-expressing Cells—Recombinant proteins of the NH₂-terminal deletion mutants were compared for calcium mobilization in CCR1 transfectant cells. When calcium flux-inducing activity was compared at several different concentrations with that of MIP-1α, it was evident that agonistic potency decreases dramatically between Δ28 and Δ29 (Fig. 3A). To further confirm that deletion of one more amino acid from Δ28 results in an almost complete loss of agonistic potency, calcium flux-inducing activity of Δ29 was compared at several different concentrations with that of Δ28 as well as that of MIP-1α. As shown in Fig. 3B, calcium flux-inducing activity of Δ28 was dose-dependent, reaching a plateau at 10 nM. In contrast, Δ29 elicited maximal response at

with buffer containing chemokines, and the upper wells were filled with 50 μl of cell suspension (6 × 10⁵ cells/ml). The two wells were separated by a fibronectin (Sigma)-coated polyvinylpyrrolidone-free polycarbonate filter (Neuroprobe) with 10-μm pores. Human MIP-1α and human eotaxin were purchased from PeproTech Inc. (Rocky Hill, NJ). Receptor Binding Assay—Lkn-1 mutant proteins were labeled radioactively with ¹²⁵I using the chloramine T method (16). The specific activities of the labeled Lkn-1 mutants were ~ 8 × 10⁶ cpm/μg protein. CCR1- or CCR3-transfected cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at a density of 10⁵ cells/ml. The two wells were separated by a nitrocellulose membrane by electroblotting (C).

RESULTS

Purification and Removal of Tags—E. coli were incubated at 4°C for 90 min with continuous rotation. Incubation was terminated by centrifuging the cell suspension over 1 ml of 10% sucrose (Sigma) cushion. Cell pellets were cut from the tubes, and cpm were counted using a gamma counter.

FIG. 1. Identification of progressive deletion of NH₂-terminal region. A, recombinant fusion proteins expressed in the bacterial lysate were separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. B and C, recombinant fusion proteins purified from the bacterial lysate with Ni-NTA spin columns (Qiagen) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (B) or probed with rabbit polyclonal anti-Lkn-1 antibody after transfer to nitrocellulose membrane by electroblotting (C). Std indicates the protein size marker. Arrows indicate truncated forms of recombinant Lkn-1.

FIG. 2. Removal of tags and final purification of the recombinant proteins. Recombinant fusion proteins were cleaved with Factor Xa and then separated by gel filtration using a Superdex peptide HR 10/30 column (Amersham Biosciences) to remove tagged peptides and uncleaved fusion proteins. Here, we show purification of Δ28 fusion proteins as a representative example. A, Factor Xa-treated Δ28 fusion proteins were separated by gel filtration on a Superdex peptide HR 10/30 column, and the eluted proteins were detected at λ = 214 nm. B, the protein in the single dominant peak in A was collected and separated in SDS-PAGE, and the gel was stained with silver nitrate. Lane 1 shows total proteins before the chromatographic separation, and lane 2 shows the finally purified Factor Xa-treated proteins. Std indicates the protein size marker.

(Fig. 2B, lane 2). Likewise, other fusion proteins were also cleaved with Factor Xa, and then the cleaved proteins were purified by a chromatographic separation. The purified proteins contained undetectable level of endotoxin (data not shown).
100 nM, but the level was at most comparable with that of 0.1 nM of Δ28. These results show that at least 3 amino acid residues preceding the first cysteine are required for the agonistic activity. Potential differences in the agonistic potency were further examined for all of the NH₂-terminal deletion mutants by varying protein concentration. We found that deletion of NH₂-terminal amino acid residues up to 20 did not change agonistic potency for CCR1; the calcium flux-inducing activities of Δ5, Δ10, Δ15, and Δ20 were almost the same as that of Δ0 (Fig. 3). Lkn-1 lacking NH₂-terminal 24 amino acids (Δ24), however, induced robust calcium flux responses compared with Δ0. Δ24 induced maximal calcium flux response at ~1 nM, which is an ~100-fold lower concentration than that required to induce similar level of response for Δ0 (Fig. 3D). EC₅₀ of Δ24 and Δ0 was ~0.2 and ~4.0 nM, respectively. Deletion of 3 or 4 more amino acid residues from Δ24 (i.e. Δ27 and Δ28) also increased agonistic potency to the same level shown by Δ24.

**Fig. 3.** Comparison of calcium flux-inducing activity of the NH₂-terminal deletion mutants on CCR1 transfectants. Calcium influx was measured in Fura-2/AM-loaded CCR1-HOS cells. Calcium flux-inducing activity was compared at a fixed concentration of 100 nM. A, the arrow indicates the time of addition of the indicated chemokine. B, dose-response curve of calcium flux-inducing activity of Δ28, Δ29, and MIP-1α. C, flux-inducing activity of the deletion mutants was further compared in a dose-response study, and some of the representative data are shown. D, the EC₅₀ and the potency of the activity for each of the mutants were summarized. Relative activity was calculated from the peak amplitude elicited at the indicated concentration. The results show the mean ± S.D. of three separate experiments.

**Fig. 4.** Comparison of calcium flux-inducing activity of the NH₂-terminal deletion mutants on CCR3 transfectants. Calcium influx was measured in Fura-2/AM-loaded CCR3-HOS cells. A, calcium flux-inducing activity was compared at a fixed concentration of 100 nM. The arrow indicates the time of addition of the indicated chemokine. B, calcium flux-inducing activity of Δ0, Δ10, Δ24, and Δ28 was compared in a dose-response study with that of eotaxin. C, the EC₅₀ and the potency of the activity for each of the mutants were summarized. Relative activity was calculated from the peak amplitude elicited at the indicated concentration. The results show the mean ± S.D. of three separate experiments.

**Fig. 5.** Cross-desensitization of calcium mobilization in CCR1- and CCR3-HOS cells. Calcium flux was measured in Fura-2/AM-loaded cells, which were stimulated sequentially at 100-s intervals. A, Fura-2/AM-loaded CCR1-HOS cells were exposed sequentially to the indicated chemokine at 100 nM (upper panel) or exposed first with increasing concentration of Δ29 and then with 100 nM Δ28 (lower panel). B, Fura-2/AM-loaded CCR3-HOS cells were stimulated sequentially with eotaxin and Δ24 at the same concentration (100 nM). The arrows indicate the time of addition of the indicated chemokine. The results are representative of three separate experiments.
The calcium flux-inducing activity of Δ28 as well as Δ24 appears to be stronger than that of MIP-1α (Fig. 3B).

Anergic potency on CCR3 was also examined for all of the NH2-terminal deletion mutants using CCR3 transfectant cells, and some of the data are shown in Fig. 4. In CCR3 transfectant cells, Δ24 was the only form of the NH2-terminal deletion mutants that showed enhanced agrinogenic potency; the EC50 of all the other NH2-terminal deletion mutants as well as Δ0 was in the range of 40 to ∼55 nM. In contrast, the EC50 of Δ24 was ∼22 nM. The agrinogenic potency of Δ24, however, was lower than that of eotaxin, which showed a peak response at 50 nM with an EC50 of ∼18 nM. At a 50 nM concentration, Δ24 exhibited ∼50% of the calcium flux response to eotaxin. It is noteworthy that the EC50 of Δ24 on CCR1 was ∼0.2 nM, which is ∼100-fold lower than that on CCR3.

Desensitization Experiments—To test whether loss of agrinogenic activity of Δ29 is due to inability to bind to CCR1, desensitization experiments were performed in CCR1 transfectant cells. As shown in the upper panel of Fig. 5A, MIP-1α was not able to desensitize CCR1 transfectant cells completely to subsequent exposure to Δ0 or Δ28. However, stimulation of CCR1 transfectant cells with Δ0 or Δ28 completely abolished responsiveness to a second stimulation with MIP-1α. These data indicate that Δ28, as well as Δ0, shares receptors with MIP-1α and has a stronger desensitizing capability than MIP-1α. Then, we examined whether Δ29 could desensitize Δ28. Stimulation of CCR1 transfectant cells with Δ29 did not affect the ability of Δ28 to induce the subsequent calcium response, even at a concentration as high as 400 nM (Fig. 5A). These results suggest that Δ29 may not be able to bind to CCR1.

Desensitization experiments were also performed in CCR3 transfectant cells to compare desensitizing capability of Δ24 with that of eotaxin. As shown in Fig. 5B, stimulation of CCR3 transfectant cells with eotaxin abolished responsiveness to a subsequent stimulation with Δ24. In contrast, stimulation with Δ24 did not completely abolish the ability of eotaxin to induce a calcium response.

Induction of Chemotaxis in CCR1- and CCR3-expressing Cells—Chemotactic activities of the NH2-terminal deletion mutants of Lkn-1 were evaluated on CCR1 transfectant cells, and the results are shown in Fig. 6A. Consistent with the calcium flux results, chemotactic activity decreased dramatically when 29 amino acids were deleted. Δ29 did not show significant chemotactic activity even at a concentration of 10 nM.

Potential differences in the chemotactic activity were also examined for all of the NH2-terminal deletion mutants at different protein concentrations, and some of the results are shown in Fig. 6A. Chemotactic activities of Δ5, Δ10, Δ15, and Δ20 were almost the same as that of Δ0. In contrast, consistent with the calcium flux results, Δ24, or Δ28 exhibited increased chemotactic activity on CCR1 transfectants. EC50 values for Δ0, Δ5, Δ10, Δ15, and Δ20 were within a range of 0.51 to ∼0.58 nM, whereas EC50 values for Δ24, Δ27, and Δ28 were 0.09 ± 0.01, 0.17 ± 0.01, and 0.14 ± 0.02, respectively.

In CCR3 transfectant cells, Δ24 was again the only form of the NH2-terminal deletion mutant of Lkn-1 that showed increased agrinogenic activity (Fig. 6B). The chemotactic activity of Δ24, however, was lower than that of eotaxin. The EC50 of Δ24 was 33.0 ± 2.5, whereas that of eotaxin was 13.0 ± 1.2. Furthermore, at a concentration of 100 nM, at which eotaxin showed a peak response, Δ24 exhibited 58% of the chemotactic activity of eotaxin.

Receptor Binding Assay—Binding affinities of Lkn-1 mutant proteins with CCR1 and CCR3 were determined with 125I-labeled ligands (Fig. 7). In this experiment, we focused mainly on three proteins: Δ0, which is the intact form of Lkn-1; Δ24, which is the deletion mutants with enhanced agrinogenic activities on CCR3 as well as CCR1; and Δ29, which essentially lacks the biological activity. Fig. 7, A and B, depicts the binding isotherm. Conversion of the data by Scatchard analysis revealed a Kd of 1.00 ± 0.02 nM (Δ0) and 0.38 ± 0.01 nM (Δ24) to the CCR1 (Fig. 7A), and 2.41 ± 0.08 nM (Δ0) and 1.05 ± 0.09 nM (Δ24) to the CCR3 (Fig. 7B). The relative affinity of the Lkn-1 mutants was investigated further in a cross-competition binding assay using CCR1 transfectant cells (Fig. 7C). Δ24 was more effective in inhibiting the binding of 125I-labeled Δ0 with CCR1 than Δ0. The IC50 of Δ24 was ∼26 nM, whereas that of Δ0 was ∼42 nM. Δ29, at the highest concentration tested, inhibited the binding of 125I-labeled Δ0 by only 20%.

### DISCUSSION

It has been found that recombinant Lkn-1 produced in insect cells undergoes a spontaneous site-specific cleavage at the NH2-terminus.
terminus, producing a 24-amino acid shorter protein than the intact form (11). The significance of this autolysis, however, has remained unknown. In the present study, the role of the NH₂-terminal domain for agonistic activity was studied with a series of NH₂-terminal deletion mutants of Lkn-1, including the spontaneously cleaved form (Δ24). Our preparations of NH₂-terminal deletion mutants of Lkn-1 do not have any foreign amino acid residues at either the NH₂ or COOH terminus of the recombinant proteins. Although recombinant Lkn-1, which has additional methionine and six histidines at its NH₂ terminus, has biological activities (11), we found that truncation of the foreign amino acid residues at the NH₂ terminus of the recombinant proteins increases the biological activity by ~10-fold (data not shown).

In contrast to other CC chemokines such as MCP-1 and RANTES, deletion of NH₂-terminal amino acids up to 20 residues from the natural form of Lkn-1 did not cause noticeable alterations in agonistic potency on CCR1. This feature is unique for Lkn-1, because for CC chemokines such as MCP-1 and RANTES, minimal truncation or modification of the first few NH₂-terminal amino acids leads to significant changes in receptor binding and functional activity (17–20). Deletion of the pyroglutamate residue at the NH₂ terminus of the natural form of MCP-1 results in an at least 50-fold decrease in agonistic activity on monocytes and basophils (17, 18). Deletion of 2 amino acid residues, MCP-1-(3–76), leads to total loss of agonistic activity on eosinophils and basophils (18). RANTES loses agonistic potency and becomes a potent antagonist of chemokine binding when the first amino acid residue has been modified artificially by the addition of methionine or treatment with aminooxypentane (19, 21). Deletion of NH₂-terminal amino acid residues can even result in changes in receptor specificity (18, 22). In particular, MCP-1 acquires agonistic activity on CCR3 only when the first residue at the NH₂ terminus, pyroglutamate, is deleted (18). Similar to artificially modified chemokines, posttranslationally modified natural forms of MCP-1 such as MCP-1-(5–76) and MCP-1-(6–76) are also devoid of bioactivity (20). The naturally cleaved form of MCP-2, MCP-2-(6–76), is also devoid of bioactivity and blocks the chemotactic effects of MCP-2 as well as that of MCP-1.
MCP-3, and RANTES (20). Thus, the integrity of the NH2-terminal region of CC chemokines appears to be critical for receptor binding and biological function. In fact, the search for NH2-terminal variants as receptor antagonists has been one of the major areas of interest since the discovery that chemokines inhibit HIV-1 infection (9, 23–25).

The situation is quite different for Lkn-1. Our results show that deletion of up to 20 amino acids from the intact form of Lkn-1 does not affect agonistic potency on CCR1. More interestingly, recombinant Lkn-1, which contains additional methionine and six histidines at its NH2 terminus, is only 10-fold less active than the intact form of Lkn-1. These observations suggest that the receptor binding and biological function of Lkn-1 is more dependent on the downstream amino acid residues of the NH2-terminal domain. Deletion of more amino acid residues, 24 amino acids (Δ24), increases the calcium flux-inducing activity almost 100-fold on CCR1 transfectants. These results may explain why recombinant Lkn-1 undergoes a spontaneous site-specific cleavage at the NH2 terminus producing a 24-amino acid shorter protein than the intact form (10). For Lkn-1, posttranscriptional modification may be a mechanism to augment chemokine potency on CCR1. This is also true for CCR3; Δ24 shows increased calcium flux-inducing activity in CCR3 transfectants compared with that of the intact form of Lkn-1. Deletion of 27 or 28 amino acids (Δ27 or Δ28) also produces a protein that has increased calcium flux-inducing activity on CCR1 transfectants. Deletion of 29 amino acid residues (Δ29), however, abolishes the calcium flux-inducing activity almost completely, showing that at least 3 amino acid residues preceding the cysteine are essential for the biological activity of Lkn-1.

Because deletion or proteolytic cleavage of the NH2-terminal region usually results in derivatives that still recognize the receptor but do not induce functional responses, we were curious to know whether Δ29 acts as an antagonist of Lkn-1. This issue was addressed by receptor binding experiments as well as desensitization experiments. As shown in Fig. 6A, Δ29 was unable to desensitize calcium flux-inducing activity of Δ28 even at 400 nM concentration. Furthermore, receptor binding experiments showed that Δ29 was not able to bind to CCR1 effectively. Thus, it was obvious that deletion of 29 amino acids at the NH2 terminus inactivates the receptor binding capability of Lkn-1. This feature is unique for Lkn-1 in that it does not produce antagonists by truncations of the NH2-terminal domain.

Although cleavage of the NH2-terminal region increases the agonistic potency on CCR1, the length of the NH2-terminal region does not proportionally affect agonistic potency; Δ24, Δ27, and Δ28 show similarly potent biological activity in both calcium flux assays and migration assays. This feature is in contrast with that of hemofiltrate CC chemokine (HCC)-1, a recently cloned CC chemokine that is structurally similar to MIP-1α (26). Comparison of the three NH2-terminal truncated variants of HCC-1 revealed that the rank order of potency was inversely correlated with the length of the protein. Thus, the shortest form of HCC-1 was the most potent, and the longest form of HCC-1 was the least potent (27).

To examine whether increased agonistic potency shown by the deletion mutants is due to increased binding affinity to receptors, we also performed receptor binding assays for Δ0 and Δ24. Based on the \( K_d \) values, Δ24 appeared to have an ~2.6-fold higher binding affinity on CCR1 than Δ0. Cross-competition experiments using labeled Δ0 also showed that Δ24 had higher binding affinity on CCR1 than Δ0. Because both Δ27 and Δ28 have similarly strong agonistic potency as Δ24 on CCR1, it is reasonable to assume that Δ27 and Δ28 would also have similar binding affinity as Δ24 on CCR1. In CCR3 transfectants, Δ24 showed an ~2.3-fold higher binding affinity than Δ0. Thus, it appears that the increased agonistic potency of the NH2-terminal deletion mutants is due, at least in part, to the increased binding capability of receptors.

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REFERENCES

1. Oppenheim, J. J., Zachariae, C. O. C, Mukaida, N., and Matsushima, K. (1991) Annu. Rev. Immunol. 9, 617–648
2. Kelm, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largespadas, D. A., Jenkins, N. A., Copenland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994) Science 266, 1395–1399
3. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Grevvus, D. R., Zlotnik, A., and Schall, T. J. (1997) Nature 385, 640–644
4. Murphy, P. M. (1994) Annu. Rev. Immunol. 12, 593–633
5. Baggolin, M., Dewald, B., and Moser, B. (1997) Annu. Rev. Immunol. 15, 675–705
6. Neste, K., Digregorio, D., Mark, J. Y., Horuk, R., and Schall, T. J. (1993) Cell 72, 415–425
7. Schall, T. J., and Bacon, K. B. (1994) Curr. Opin. Immunol. 6, 865–873
8. Broxmeyer, H. E., Sherry, B., Lu, L., Cooper, S., Oh, K-O., Tokamp-Olsen, P., Kwon, B. S., and Cerami, A. (1990) Blood 76, 1110–1116
9. Coccia, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1985) Science 228, 1811–1815
10. Wolpe, S. D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D. G., Nguyen, H. T., Moldawer, L. L., Nathan, C. F., Lowry, S. F., and Cerami, A. (1998) J. Exp. Med. 187, 570–581
11. Youn, B.-S., Zhang, S. M., Lee, E. K., Park, D. H., Broxmeyer, H. E., Murphy, P. M., Locati, M., Pease, J. E., Kim, K. K., Antol, K., and Kwon, B. S. (1997) J. Immunol. 159, 5201–5205
12. Zhang, S. M., Youn, B.-S., Gao, L. L., Murphy P. M., and Kwon, B. S. (1999) J. Immunol. 162, 4928–4942
13. Clark-Lewis, I., Kim, K.-S., Rajarathnam, K., Gong, J.-H., Dewald, B., Moser, B., Bagnoli, M., and Sykes, B. D. (1995) J. Leukocyte Biol. 57, 703–711
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Sarafi, M., Garcia-Zepeda, E., MacLean, J., Charu, I. F., and Luster, A. D. (1997) J. Exp. Med. 185, 99–110
16. Hunter, W. M., and Greenwald, B. (1962) Nature 194, 495–498
17. Gong, J. H., and Clark-Lewis, I. (1995) J. Exp. Med. 181, 631–640
18. Weber, M., Uguccioni, M., Bagnoli, M., Clark-Lewis, I., and Dahinden, C. A. (1990) J. Exp. Med. 182, 5201–5205
19. Proudfoot, A. E. I., Power, C. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994) Science 266, 1395–1399
20. Proost, P., Struyf, S., Dugare, M., Lusso, P. (1995) J. Clin. Immunol. 15, 680–685
21. Simmons, G., Chalmpe, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N., and Proudfoot, A. E. I. (1997) Science 276, 276–279
22. Gong, J. H., Uguccioni, M., Dewald, B., Bagnoli, M., and Clark-Lewis, I. (1996) J. Biol. Chem. 271, 10521–10527
23. Oppermann, M., Mark, M., Proudfoot, A. E. I., and Olbrich, H. (1999) J. Biol. Chem. 274, 8875–8885
24. Wang, J. M., and Oppenheim, J. J. (1999) J. Exp. Med. 190, 591–595
25. Howdard, O. M. Z., Oppenheim, J. J., and Wang, J. M. (1999) J. Clin. Immunol. 19, 280–292
26. Schulz-Knappe, P., Magert, H.-J., Dewald, B., Meyer, M., Cetin, Y., Kubbies, M., Tomomukochi, J., Kirchhoff, K., Raida, M., and Andermann, K. (1996) J. Exp. Med. 183, 295–299
27. Tsou, C.-L., Gladue, R. P., Carroll, L. A., Paradis, T., Boyd, J. G., Nelson, R. T., Neote, K., and Charo, I. F. (1998) J. Exp. Med. 188, 603–608