Leukotactin-1/CCL15-induced chemotaxis signaling through CCR1 in HOS cells

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Abstract Leukotactin-1 (Lkn-1)/CCL15 is a recently cloned CC-chemokine that binds to the CCR1 and CCR3. Although Lkn-1 has been known to function as a chemoattractant for neutrophils, monocytes and lymphocytes, its cellular mechanism remains unclear. To understand the mechanism of Lkn-1-induced chemotaxis signaling, we examined the chemotactic activities of human osteogenic sarcoma cells expressing CCR1 in response to Lkn-1 using inhibitors of signaling molecules. Inhibitors of G_i/G_o protein, phospholipase C (PLC) and protein kinase Cδ (PKCδ) inhibited the chemotactic activity of Lkn-1 indicating that Lkn-1-induced chemotaxis signal is transduced through G_i/G_o protein, PLC and PKCδ. The activities of PLC and PKCδ were also enhanced by Lkn-1 stimulation. Chemotactic activity of Lkn-1 was inhibited by the treatment of cycloheximide and actinomycin D suggesting that newly synthesized proteins are needed for chemotaxis. Nuclear factor-κB (NF-κB) inhibitor reduced chemotactic activity of Lkn-1. DNA binding activity of NF-κB was also enhanced by Lkn-1 stimulation. These results suggest that Lkn-1 transduces the signal through G_i/G_o protein, PLC, PKCδ, NF-κB and newly synthesized proteins for chemotaxis. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Chemokine; Protein kinase C; Phospholipase C; Nuclear factor-κB

1. Introduction

Chemokines are a large family of structurally homologous small proteins containing four to six conserved cysteines linked by disulfide bonds. Chemokines and their receptors play important roles in leukocyte trafficking under inflammatory conditions and many other immune responses through the regulation of cell migration and growth [1–3]. Two major chemokine receptor groups, CCR and CXCR, belong to the seven-transmembrane G protein-coupled receptor (GPCR) superfamily. GPCRs transduce signals to the inside of the cell through heterotrimeric G proteins which are grouped into two families depending on the sensitivity to pertussis toxin (PTX). G_i/G_o family G proteins are sensitive to PTX whereas G_q family G proteins are known to be insensitive to

this toxin [4]. Activation of the GPCR-mediated signaling pathway involves the activation of several different effector molecules such as adenylate cyclase, phospholipase C (PLC), PLD, PLA$_2$, phosphoinositide-3 (PI-3) kinase, protein kinase C (PKC) and Ca^{2+} mobilization which initiate a variety of cellular responses [5,6].

Recently, leukotactin-1 (Lkn-1)/CCL15, a member of the CC chemokine family, has been cloned and partially characterized [7,8]. Lkn-1 has been known to bind CCR1 and CCR3, and has a chemoattractant role for neutrophils, monocytes and lymphocytes [7]. Although Lkn-1 is believed to play an important role in the development of inflammation and allergic inflammatory diseases, its biological function and the mechanism of cell migration events remain to be characterized. CCR1 is a GPCR that is expressed by a variety of cells, including lymphocytes, monocytes, basophils, eosinophils, and neutrophils [9]. CCR1-mediated signal transduction pathways have been proposed. For example, it has been reported that myeloid progenitor inhibitor factor-1 signal transduction includes binding to CCR1, transduction by G protein, effector function by PLC, PKC, calcium flux and PLA$_2$, and cytoskeletal remodeling [10]. Although CCR1 and Lkn-1 may play important roles in physiologic processes, little information is available regarding the Lkn-1-induced signal transduction pathway via CCR1 and the relationship of signaling events to the functional response of the cell. In this study, we investigated the mechanism of Lkn-1-induced chemotaxis signal transduction via CCR1 using human osteogenic sarcoma cells expressing CCR1 (HOS/CCR1) as a model system.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). PTX, PD98059, U73122, Ly294002, genistein, rottlerin, Ro-31-8425, SB202190, SN50, cycloheximide (CHX), and actinomycin D (ActD) were obtained from Calbiochem (San Diego, CA, USA). Quinacrine and rat tail collagen type I were products of Sigma (St. Louis, MO, USA). rLkn-1 was the kind gift of Greencross Life Science Corp. (Yongin, Korea). hMIP-1α/CCL3, hMIP-1β/CCL4, anti-CCR1 antibody and anti-CCR5 antibody were obtained from R&D Systems (Minneapolis, MN, USA). Antibody against PKCδ was purchased from Transduction Laboratories (Lexington, KY, USA). [γ-^{32}P]ATP and myo-[2-^{3}H]inositol were purchased from Amersham Pharmacia Biotech. (Piscataway, NJ, USA). Stable HOS cells expressing CCR1 or CCR5 were the kind gift of Dr. O.M. Howard (National Cancer Institute, MD, USA) and were grown in

DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and puromycin (0.5 μg/ml).

2.2. FACS analysis

HOS cells seeded into 100 mm dishes at 5×10^5 cells/dish were cultured in DMEM. Cells were harvested and washed with phosphate-buffered saline (PBS) buffer. After saturation of non-specific binding sites with total rabbit IgG, cells were incubated at 4°C for 30 min with anti-CCR1 or anti-CCR5 antibodies. Baseline staining was obtained by incubating mouse IgG instead of anti-CCR1 or anti-CCR5 antibodies. Following incubation and washings, cells were incubated at 4°C for 30 min with FITC-conjugated goat anti-mouse IgG. Finally, cells were washed and analyzed on a FACSort cytofluorimeter (Becton Dickinson, San Jose, CA, USA).

2.3. Chemotaxis assay

Migration of cells was monitored using a 48-well microchamber (Neuroprobe, Cabin John, MD, USA) as previously described [7]. Briefly, the lower wells were filled with 28 μl buffer alone or with buffer containing rLkn-1, hMIP-1α or hMIP-1β and the upper wells were filled with 50 μl of HOS cells at 5×10^5 cells/ml in RPMI 1640 containing 1% bovine serum albumin (BSA) and 30 mM HEPES. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuroprobe) with 10 μm pores that was pre-coated with RPMI 1640 containing rat tail collagen type I at 4°C overnight. After incubation for 3 h at 37°C, the filters were removed from the chamber, washed, fixed, and stained with Diff-Quick (Baxter, Deerfield, IL, USA). The cells of two randomly selected oil-immersed fields were counted using Axiovert 25 (Carl Zeiss, Jena, Germany) and Visus Image Analysis System (Foresthill Products, Foresthill, CA, USA). The chemotactic index (CI) was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as CI > 2.

2.4. Measurement of PLC activity

HOS cells seeded into 35 mm dishes at 2×10^5 cells/dish were cultured in DMEM. The cells were labeled with myo-[2-^{3}H]inositol (2 mCi/ml) in inositol-free DMEM for 24 h. Subsequently, the labeled cells were washed and pre-treated with 20 mM LiCl for 15 min in DMEM containing 20 mM HEPES (pH 7.2) and 1 mg/ml BSA. Stimulation was initiated by the addition of Lkn-1 for different times, and

terminated by the addition of ice-cold 5% HClO$_4$. After 30 min in an ice bath, extracts were centrifuged and diluted with distilled water, and applied to Dowex AG 1-X8 anion exchange column (Bio-Rad, Hercules, CA, USA). The column was then washed with 10 ml of distilled water followed by 10 ml of 0.06 M ammonium formate containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid.

2.5. PKC translocation assay

HOS cells seeded into 100 mm dishes at 5×10^5 cells/dish were cultured in DMEM. The cells were starved for 24 h in 0.5% FBS. After treatment with Lkn-1, the cells were harvested and washed three times with ice-cold PBS (without Ca^{2+}). The cells then were resuspended in 200 μl of homogenization buffer A (50 mM Tris–HCl, pH 7.4, 25 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM PMSF, and protease inhibitors) and homogenized at 4°C by 10 passages through a 21G needle fitted on a 1 ml plastic syringe. The homogenate was centrifuged at $1000 \times g$ for 5 min at 4°C. The supernatant was collected and centrifuged at $100\,000 \times g$ for 1 h at 4°C. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in 100 μl of homogenization buffer B (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM EGTA, 1 mM PMSF, and protease inhibitors) and sonicated for 10 s. The suspension was centrifuged at $100\,000 \times g$ for 30 min at 4°C. The supernatant was collected as a membrane fraction. Protein concentration of each sample was determined.

2.6. Western Blot analysis

Cells were lysed in 10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na$_3$VO$_4$, and protease inhibitors. Protein samples (15 μg of each) were separated by SDS–PAGE (10%) and transferred to nitrocellulose filters. The blots were incubated with antibodies and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech.).

2.7. Electrophoretic mobility shift assay (EMSA)

For the nuclear factor-κB (NF-κB) gel shift assay, nuclear extracts were prepared as described [11] from HOS/CCR1 cells. A double-
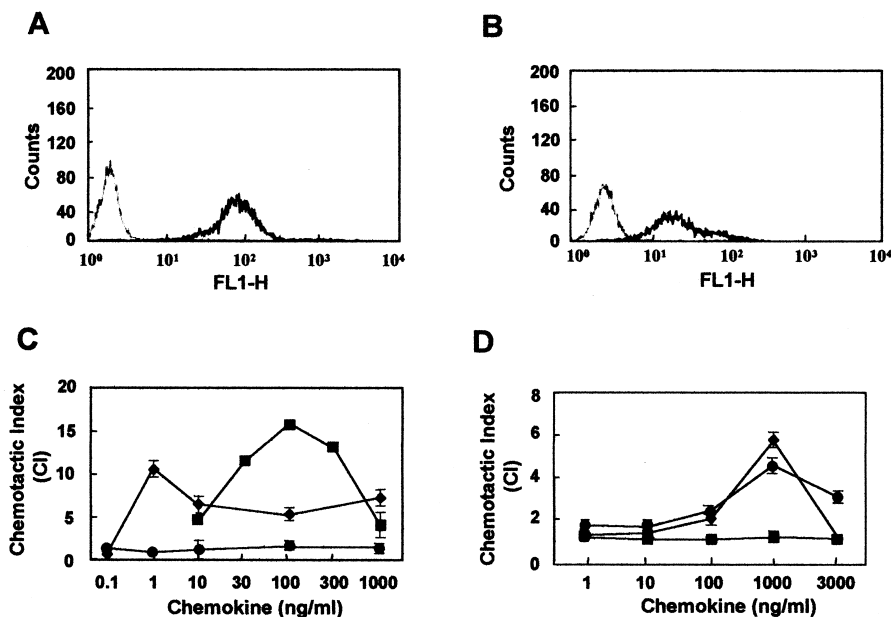

Fig. 1. Lkn-1 induces cell migration in HOS cells expressing CCR1. HOS/CCR1 cells (A) or HOS/CCR5 cells (B) were harvested and analyzed by fluorescence-activated cells sorter using monoclonal anti-CCR1, anti-CCR5 antibodies (thick line) or control mouse IgG (thin line). HOS/CCR1 cells (C) or HOS/CCR5 cells (D) were applied to the indicated concentrations of Lkn-1 (■), MIP1-α (♦) or MIP1-β (●) in a microchamber and were allowed to migrate for 3 h. The number of cells that migrated was counted microscopically in two randomly selected fields per well. The CI was calculated from the number of cells migrating to the test chemokines divided by that migrating to the controls. Results are expressed as mean CI ± S.E.M. of six replicate measurements from a single experiment and it is representative of three separate experiments.

stranded oligonucleotide containing a consensus binding site for NF-κB (underlined), 5′-AGTTGAGGGGACTTTCCCAGGC-3′, was obtained from Promega (Madison, WI, USA). The oligonucleotide was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad) as described by manufacturer's instruction. Nuclear extracts (15 μg of total protein) were incubated with radiolabeled probe for 20 min at room temperature, and protein–DNA complexes were separated from free probe by electrophoresis on a 4% native polyacrylamide gel in 0.5× Tris–borate EDTA (TBE). DNA binding buffer contained 10 mM Tris–HCl, pH 7.5, 1 mM MgCl$_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 μg/ml of poly(dI-dC)•poly(dI-dC). Gels were pre-electrophoresed in 0.5× TBE for 30 min prior to loading and electrophoresis continued for approximately 3 h until the bromophenol blue dye approached the bottom of the gel. Dried gels were visualized by autoradiography. In competition experiments, binding reactions were incubated with 10-fold molar excess of unlabeled NF-κB binding oligonucleotide or NFAT binding oligonucleotide for 20 min before addition of the radiolabeled oligonucleotide.

3. Results

3.1. Lkn-1 induces cell migration in HOS cells expressing CCR1

A stable HOS cell line expressing CCR1 (HOS/CCR1) was established to investigate Lkn-1-induced chemotaxis signaling through CCR1 [7]. HOS/CCR5 was also established and used as a negative control for Lkn-1 stimulation [7]. To examine the surface expression of CCR1 and CCR5 on HOS cells, FACS analysis was conducted. Fig. 1A,B shows that CCR1 and CCR5 are expressed on the surface of HOS cells. To examine the chemotactic activity of Lkn-1 in HOS/CCR1 cells, cell migration assay was performed in a 48 well microchamber. The chemoattractant effect of Lkn-1 was comparable to that of MIP-1α in HOS/CCR1, but MIP-1β did not affect migration of HOS/CCR1 (Fig. 1C). Lkn-1 showed the typical bell-shape curve in HOS/CCR1 chemoattraction with the peak of the curve at 100 ng/ml and MIP-1α showed the maximum activity at 1 ng/ml. Lkn-1 did not show any chemotactic activity in HOS/CCR5 cells whereas MIP-1α and MIP-1β had chemoattractant effects in HOS/CCR5 (Fig. 1D). These data indicate that Lkn-1 shares receptor CCR1 with MIP-1α but not with MIP-1β.

3.2. Lkn-1-induced chemotaxis is mediated via G_i/G_o protein and PLC

To evaluate the coupling mechanism of the Lkn-1 receptor and the nature of possible G proteins involved in the signaling events activated by Lkn-1, HOS/CCR1 was treated with PTX before the stimulation of Lkn-1 or MIP-1α as a control. Fig. 2A shows that PTX has inhibitory effects on the Lkn-1 and MIP-1α-induced chemotaxis. In the presence of PTX, the number of the cells affected by Lkn-1 decreased to only 10–20% of the number that migrated in the absence of PTX. These data indicated that Lkn-1 exerts its effect through a receptor linked to a PTX-sensitive G_i/G_o family G proteins.

To further characterize the chemotaxis signaling pathway implicated in response to Lkn-1, we investigated the possible involvement of PLC. Fig. 2B shows that Lkn-1 and MIP-1α-induced cell migration are inhibited by the treatment of U73122. This result indicates that PLC is involved in Lkn-1-induced chemotaxis. We also determined the activity of PLC in response to Lkn-1. Exposure of HOS/CCR1 to Lkn-1 for different periods of time resulted in increased activities of PLC
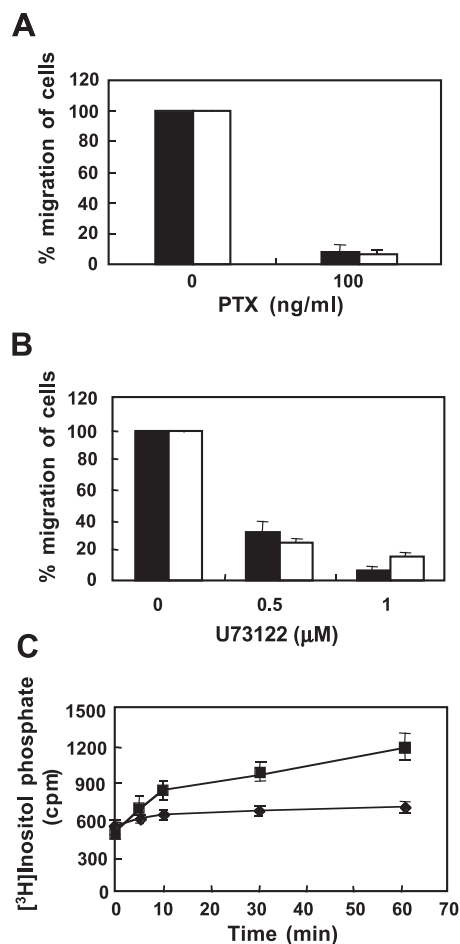
Fig. 2. Lkn-1-induced chemotaxis is mediated via G_i/G_o protein and PLC. A: HOS/CCR1 cells were pre-incubated in the absence (control) or presence of 100 ng/ml PTX for 16 h. Cell migration in response to Lkn-1 (100 ng/ml, black bars) or MIP-1α (1 ng/ml, white bars) was measured as described in Section 2. B: HOS/CCR1 cells were pre-incubated in the absence or presence of U73122 for 30 min at 0.5 and 1 μM. Cell migration in response to Lkn-1 (100 ng/ml, black bars) or MIP-1α (1 ng/ml, white bars) was measured as described in Section 2. C: HOS/CCR1 (■) or HOS/CCR5 (◆) cells were radiolabeled with myo-[2-^{3}H]inositol (2 μ Ci/ml) in inositol-free DMEM for 24 h. Then, HOS cells were stimulated with 100 ng/ml Lkn-1 for the indicated times. Results are expressed as the radioactivity in the total inositol phosphates. Each point represents the average value of three different experiments ± S.E.M.

as assessed by measuring the [^{3}H]IP that was produced by PLC activity (Fig. 2C). Increased activation was observed within 5 min after addition of Lkn-1 to HOS/CCR1, and continued to increase up to 1 h, but no PLC activation was detected in HOS/CCR5.

3.3. PKCδ is involved in Lkn-1-induced chemotaxis signaling

Since G_i/G_o protein and PLC participated in Lkn-1-induced chemotaxis, we examined whether possible downstream regulator PKC is involved in the chemotaxis pathway of Lkn-1. As shown in Fig. 3A, chemotactic activities of Lkn-1 and MIP-1α were inhibited in the presence of rottlerin but not in the presence of Ro-31-8425. Since rottlerin is a PKCδ-specific inhibitor, this result indicates that PKCδ is involved in Lkn-1-induced chemotaxis among various PKC isoforms.

Translocation of PKC to a particulate fraction is the key step for the activation of this enzyme [12]. Determination of
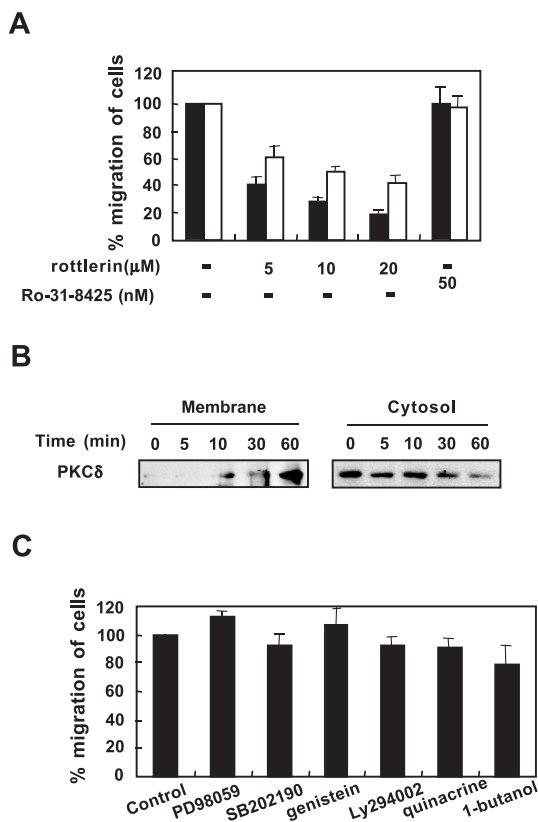
A

B

C

Fig. 3. PKCδ is involved in Lkn-1-induced signaling. A: HOS/CCR1 cells were pre-incubated in the absence or presence of rottlerin (5, 10 and 20 μM) and Ro-31-8425 (50 nM) for 30 min. Cell migration in response to Lkn-1 (100 ng/ml, black bars) or MIP-1α (1 ng/ml, white bars) was measured as described in Section 2. B: Serum-starved HOS/CCR1 cells were stimulated with 100 ng/ml Lkn-1 for the indicated times. Harvested cells were fractionated as described in Section 2, then analyzed by 10% SDS–polyacrylamide gels (15 μg/lane) and transferred to nitrocellulose membrane. PKCδ translocation was detected by Western blotting with anti-PKCδ antibody. C: HOS/CCR1 cells were pre-incubated in the absence (control) or presence of PD98059 (50 μM), SB202190 (20 μM), genistein (10 μM), Ly294002 (10 μM), quinacrine (10 μM), and 1-butanol (0.3%) for 30 min. Cell migration in response to Lkn-1 (100 ng/ml) was measured as described in Section 2.

PKC content in membranes can reflect PKC activity. To investigate whether PKCδ is activated by Lkn-1 stimulation, we analyzed the membrane/cytosol distribution of PKCδ. PKCδ activity began to increase at 10 min after addition of Lkn-1 and the maximum activity of PKCδ was detected at 60 min by the Western blot analysis (Fig. 3B). These results indicate that Lkn-1 transduces the signal to PKCδ for chemotaxis.

To determine other signaling molecules contributing to Lkn-1-induced chemotaxis, we tested the respective effects of inhibitors of MEK (PD98059), p38 (SB202190), tyrosine kinase (genistein), PI-3 kinase (Ly294002), PLA$_2$ (quinacrine), and PLD (1-butanol) on chemotactic activity of Lkn-1. Lkn-1-induced cell migration was not inhibited by the addition of these inhibitors suggesting that these molecules are not involved in Lkn-1-induced chemotaxis (Fig. 3C).

3.4. Lkn-1 enhances NF-κB activation

Since many cellular processes need newly synthesized proteins to carry out distinct functions, we tested the effects of

ActD and CHX on Lkn-1-induced chemotaxis. As shown in Fig. 4A, both inhibitors reduced the chemotactic activities of Lkn-1 and MIP-1α to 20–30% of the control group indicating that chemotaxis induced by Lkn-1 and MIP-1α needs newly synthesized proteins. Inhibition of Lkn-1-induced chemotaxis by transcription/translation inhibitors prompted us to test the activation of transcription factors. Since NF-κB is activated by several chemokines [13,14], we investigated whether Lkn-1 induces the activation of NF-κB in HOS/CCR1 cells. We performed EMSA to evaluate the DNA binding activity of NF-κB in HOS/CCR1 cells treated with Lkn-1 for the indicated times by using a ^{32}P-labeled consensus NF-κB DNA binding sequence as a probe. Fig. 4B shows that there are two shifted NF-κB nuclear complexes bound to the probe, and Lkn-1 induces an increase in the formation of these complexes over time (0.5–4 h). Based on supershift EMSA, the lower band consisted of the p50 homodimer, whereas the upper band is the p65/p50 heterodimer (data not shown). To determine the specificity of NF-κB binding activity, nuclear extracts from the 2 h time point were incubated with the labeled NF-κB binding probes in the absence or presence of a 10-fold molar excess of unlabeled NF-κB or NFAT binding competitor (Fig. 4B). The competition experiments showed that NF-κB binding complex was competed with unlabeled NF-κB binding probes, but not with NFAT binding probes indicating that NF-κB binding activity is specific. DNA binding ability of NF-κB decreased in the presence of inhibitors of G$_i$/G$_o$ protein, PLC and PKCδ (Fig. 4C) demonstrating that these molecules are the upstream regulators of NF-κB. Especially, a PKCδ-specific inhibitor, rottlerin, completely blocked the DNA binding of NF-κB at the concentration of 5 μM. However, Ro-31-8425 which does not inhibit PKCδ had no inhibitory effect on NF-κB activation. We also tested effects of a specific inhibitor of NF-κB nuclear translocation on Lkn-1-induced chemotaxis. Fig. 4D demonstrates that NF-κB inhibitor SN50 decreased the migrated cell number to 50% of the control group at 36 μM. Taken together, our data suggest that Lkn-1-induced chemotaxis requires new proteins to function, and the addition of Lkn-1 enhances NF-κB activity, which is involved in the expression of many regulator proteins.

4. Discussion

Despite substantial recent advances in our understanding of chemotaxis, the precise mechanism through which cells respond to a chemotactic gradient remains unclear. In this contribution, we tried to understand the mechanism of Lkn-1-induced chemotaxis signaling through CCR1 in HOS cells. We have demonstrated that (1) Lkn-1 transduces the signal via PTX-sensitive G$_i$/G$_o$ family G proteins; (2) Lkn-1 activates PLC and PKCδ in a time-dependent manner; (3) Lkn-1 enhances the DNA binding activity of NF-κB; cell migration assay showed that (4) G$_i$/G$_o$ protein, PLC, PKCδ, NF-κB and newly synthesized proteins are involved in Lkn-1-induced chemotaxis, but ERK, p38, tyrosine kinases, PI-3 kinase, PLA$_2$, and PLD are not involved in the chemotaxis pathway of Lkn-1.

Since expression levels of CCR1 in resting neutrophils, lymphocytes, and monocytes are relatively low, CCR1 overexpression HOS cell line was used to examine the Lkn-1-stimulated signal transduction. The sensitivity to chemokines and
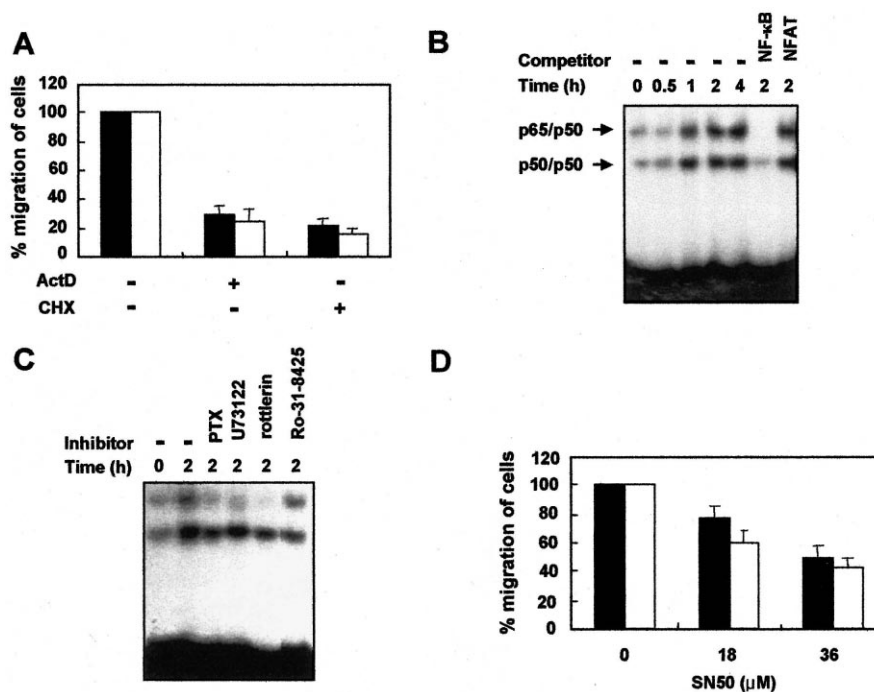
Fig. 4. Lkn-1 enhances the NF-κB activity. A: HOS/CCR1 cells were pre-incubated in the absence or presence of 10 μg/ml ActD or 10 μg/ml CHX for 2 h. After the addition of Lkn-1 (100 ng/ml, black bars) or MIP-1α (1 ng/ml, white bars), migration assay was performed as described in Section 2. B: Serum-starved HOS/CCR1 cells were treated with 100 ng/ml Lkn-1 for the indicated times. Nuclear extracts (15 μg/lane) were prepared and subjected to EMSA for DNA binding activity of NF-κB with ^{32}P-end-labeled oligonucleotides in the absence or presence of 10-fold molar excess of unlabeled competitors. NF-κB, unlabeled NF-κB binding oligonucleotides; NFAT, unlabeled NFAT binding oligonucleotides. Arrows indicate the shifts corresponding to the position of NF-κB protein–DNA complexes. C: Serum-starved HOS/CCR1 cells were pre-incubated in the absence or presence of PTX (100 ng/ml), U73122 (10 μM), rottlerin (5 μM) and Ro-31-8425 (50 nM) for 30 min (16 h for PTX) and were treated with 100 ng/ml Lkn-1 for 2 h. Nuclear extracts (15 μg/lane) were prepared and subjected to EMSA. D: HOS/CCR1 cells were pre-incubated with SN50 (18 and 36 μM) for 30 min and migrated toward 100 ng/ml of Lkn-1 (black bars) or 1 ng/ml of MIP-1α (white bars). Cell migration in response to Lkn-1 or MIP-1α was measured as described in Section 2.

cell migration activity of HOS/CCR1 were comparable to that of leukocytes from human PBMC ([7] and Fig. 1).

Accumulating data have implicated that multiple signaling mechanisms exist to regulate cell migration. Mitogen-activated protein kinase [15,16], PI-3 kinase [13,17,18] and PKC [19] signaling pathways have been shown to regulate the cell migration induced by chemokines or cytokines. Most of these signaling pathways are initiated with GPCR. A classical model for signal transduction by chemokines involves GPCR whose affinity state is increased by conformational changes induced by association with the GDP-bound state of a PTX-sensitive heterotrimeric G protein. Upon ligand binding, the activated receptor catalyzes exchange of GDP for GTP by the G protein α subunit, resulting in dissociation of α from βγ subunits. In turn, βγ activates a phosphoinositide-specific PLC leading to the accumulation of IP$_3$ and DAG in the cytoplasm. These products induce mobilization of calcium and activation of PKC, respectively [20]. Besides the classical model, other pathways for GPCR that have been studied include JAK/STAT, as well as both tyrosine and Ser/Thr kinases [3]. Our data from cell migration assay showed that Lkn-1-induced chemotaxis was sensitive to PTX, indicating that G$_i$/G$_o$ protein is involved in cell migration (Fig. 2A). In addition, PLC and PKC participated in Lkn-1 signaling indicating that Lkn-1 transduces the signal through the classical chemokine signaling pathway (Figs. 2B and 3A). Among the various PKC isoforms, PKCδ was activated by Lkn-1 stimulation and the PKCδ specific inhibitor rottlerin [21] blocked the che-

motactic activity of Lkn-1 (Fig. 3A). However, Ro-31-8425, a PKC inhibitor that does not inhibit PKCδ, showed no inhibitory activity of Lkn-1-induced chemotaxis. As any other novel PKC, PKCδ is activated in a Ca^{2+}-independent manner by DAG, which is produced by activated PLC. To our knowledge, this is the first report that PKCδ is involved in chemokine signaling.

The transcription factor NF-κB is critical for the expression of multiple genes involved in inflammatory response and apoptosis [22]. Proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α) rapidly activate NF-κB in most cell types through the NIK/MEKK-IKK-IκB signal pathway [22,23]. These cytokines induce NF-κB activation by modulating IκB phosphorylation, ubiquitination, and proteolytic degradation and by releasing functional NF-κB dimers to translocate to the nucleus [24]. Chemotactic factors, such as the lipid mediator platelet-activating factor [25] and SDF-1 [13], are also reported to activate NF-κB in leukocytes or murine pre-B cells, respectively. However, the mechanism of chemokine receptor activation of NF-κB is not clear. Our studies suggest that Lkn-1 can cause the activation of NF-κB. Lkn-1-induced cell migration was reduced, but not completely inhibited by the NF-κB inhibitor SN50, reflecting the involvement of NF-κB in Lkn-1-induced chemotaxis (Fig. 4D). Through Lkn-1-induced activation of the NF-κB signaling pathway and possibly in conjunction with other signaling pathways, Lkn-1 probably promotes changes in cellular morphology, collec-

tively known as polarization, required for chemotactic responses.

It has been reported that NF-κB activation in TNF-α-stimulated neutrophils is mediated by PKCδ [26]. At present, we do not know the downstream events regulated by PKCδ and leading to NF-κB activation and chemotaxis in Lkn-1-stimulated HOS/CCR1 cells. One of the critical regulatory steps dictating IκB degradation and NF-κB activation is IκB kinase (IKK) and NF-κB inducing kinase (NIK) [27]. Recent studies demonstrated that IKKβ is involved in NF-κB activation through PKCθ in T lymphocytes [28–30]. Therefore, the possible roles of IKK isoforms or other cellular molecules in Lkn-1-stimulated NF-κB activation are under investigation.

In conclusion, the present study provides the characterization of the chemotaxis signaling pathways activated by Lkn-1 in a model system of HOS/CCR1. We have demonstrated that the binding of Lkn-1 to its G_i/G_o protein coupled receptor CCR1 leads to the specific activation of PLC, PKCδ, and NF-κB, and that the PLC/PKCδ/NF-κB pathway may mediate Lkn-1-induced chemotactic activity. While further studies are required to delineate the signaling pathways leading to chemotaxis through CCR1 stimulation by Lkn-1, this is the first report characterizing the chemotaxis signaling events in response to Lkn-1. Although we investigated Lkn-1-induced chemotaxis signaling in the HOS/CCR1 model system, our preliminary data using neutrophils showed the same results and we are conducting the experiments using primary cells naturally expressing CCR1. From this information, we can begin to understand the molecular mechanism of cell migration and other cellular processes induced by Lkn-1 which are important events in both physiological and pathological processes.

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