Regulation of Human LZIP Expression by NF-κB and Its Involvement in Monocyte Cell Migration Induced by Lkn-1*

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Human LZIP is a transcription factor that is involved in leukocyte cell mobility. Expression of LZIP is known to differentially regulate monocyte cell migration induced by CCR1-dependent chemokines. However, its transcriptional regulation has not been characterized. Our results indicate that Lkn-1 induces LZIP expression in a time- and dose-dependent manner, and the induction of LZIP shows an immediate early response to Lkn-1. We identified and cloned ~1.4 kb of the LZIP promoter from a human genomic DNA. To identify regulatory elements controlling restricted expression of LZIP, deletion mutants were constructed from the 1469-bp LZIP promoter region (~1219/+251) linked to the luciferase reporter gene. Maximal promoter activity was contained within 613 bp from the tentative transcription initiation site and was sharply reduced in a truncated construct (~338/+251). This promoter sequence contained consensus NF-κB- and Sp-1-binding sites.

Results from an inhibitor assay showed that NF-κB is involved in Lkn-1-induced LZIP expression, but Sp-1 is not. We also demonstrated that NF-κB binds to the LZIP promoter and that the binding is specific, as revealed by an electrophoretic mobility shift assay and a mutation analysis. Chemotaxis analysis showed that LZIP expression because of the NF-κB subfamily is specifically involved in Lkn-1-induced chemotaxis. Our findings suggest that transcription factor NF-κB plays an important role in regulation of LZIP expression, and LZIP expression regulates the monocyte cell migration induced by Lkn-1.

The human leucine zipper protein LZIP binds to canonical cAMP-responsive elements (CREs) and can activate transcription from CRE-containing reporter genes (1–3). LZIP also binds to the CCAAT enhancer-binding protein element (1). LZIP is known to be involved in the regulation of cell growth by binding to the human host cell factor, which is involved in cell proliferation (4). It has also been suggested that LZIP serves a novel cellular tumor suppressor function that is targeted by the hepatitis C virus core protein (4). We have recently reported that LZIP associates with CC chemokine receptor 1 (CCR1) and up-regulates leukotactin-1 (Lkn-1)-induced chemotactic activity, indicating that LZIP functions as a positive regulator in leukocyte cell migration induced by chemokines (5). However, the exact biological roles and the natural target genes of LZIP remain to be characterized.

The human LZIP gene is located on chromosome 9p and consists of nine exons and eight introns. Sequence analysis of the 5′-flanking region of the LZIP gene has revealed that LZIP promoter has potential binding sites for transcription factors involved in inflammatory processes and regulation of the immune system, including nuclear factor κB (NF-κB), nuclear factor of activated T-cells, specific protein-1 (Sp-1), and activator protein-2 (AP-2) (6). However, the regulatory mechanism of the transcriptional activation of LZIP is still obscure.

Chemokines are a large family of chemotactic cytokines that provide key signals for trafficking and homing of specific subpopulations of leukocytes and other cells in both physiological and pathological processes (7, 8). The human chemokine Lkn-1 that belongs to the CC chemokine family binds CCR1 and CCR3 and functions on neutrophils, monocytes, and lymphocytes (9). Recent studies suggest that Lkn-1 is involved in human atherosclerosis and angiogenesis (10, 11). Lkn-1 induces chemotaxis through Gs/Gt protein, phospholipase C (PLC), protein kinase C δ (PKCδ), and NF-κB, and newly synthesized proteins are required for Lkn-1-induced chemotaxis (12). Because LZIP is involved in Lkn-1-induced chemotaxis, we characterized the regulatory mechanism of LZIP expression in response to CCR1-dependent chemokines by analyzing the promoter region of LZIP. We cloned the human LZIP promoter and defined its core sequences. We showed that the NF-κB-binding site in 613 bp of the LZIP core promoter region is important for LZIP expression in response to Lkn-1, and that NF-κB is a specific modulator of Lkn-1-induced LZIP expression.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Invitrogen. Lkn-1, human macrophage inflammatory protein (hMIP)-1α, regulated on activation, normal T cell expressed and secreted (RANTES), HCC-4, and eotaxin were obtained from R&D Systems (Minneapolis, MN). Rat tail collagen type I was a product of Sigma. Anti-p65 and anti-p50 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-LZIP antibody was raised...
against an N-terminal peptide (amino acids 2–29) conjugated to keyhole limpet hemocyanin. SN50 and WP631 were obtained from Calbiochem.

**Cell Culture**—Stable human osteogenic sarcoma cells expressing CCR1 (HOS/CCR1) (12) were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and puromycin (0.5 μg/ml). HeLa and HEK 293 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). THP-1 and U937 cells were grown in RPMI with 10% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml).

**Western Blot Analysis**—Cells were lysed in 10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 0.1 mM Na3VO4, and protease inhibitors. Protein samples (30 μg of each lane) were separated by a 10% SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with anti-LZIP antibody and developed with the enhanced chemiluminescence detection system (Amersham Biosciences).

**Promoter Cloning and Plasmid Constructions**—Genomic DNA was isolated from human blood. Genomic DNA was extracted from 3 ml of EDTA-peripheral blood leukocytes (13). A 1469-bp fragment of human LZIP promoter was generated by a GeneAmp PCR system thermocycler (Applied Biosystems, Foster City, CA) with human genomic DNA as a template. This approach used a forward primer from the 5’-upstream genomic sequence with an XhoI site (5’-AAGCTTAATCATCTACTTCCCAGTCGCTCGGTACCT; for 5’-CTCGAGCCTGATTCCTGTTGTTGTTGACCCTTAACC-3’). The amplimers were digested with XhoI/HindIII and subcloned into the pGL3 basic vector (Promega, Madison, WI) located upstream of the luciferase reporter gene. The construct was named p-1219-luc. The promoter was generated by PCR-amplified T-vector containing the 1219-bp fragment with a HindIII site in the downstream initiator methionine codon with a HindIII site (5’-CTCGAGCTGATTCCTGTTGTTGACCCTTAACC-3’). The oligonucleotides for 20 min before addition of the radiolabeled oligonucleotides. For supershift assays, the antibodies were added to the reaction mixture for an additional 30 min. After incubation, the reaction mixture was then separated by electrophoresis on a 10% SDS-PAGE gel and dried for autoradiography.

**Transient Transfection**—HOS/CCR1 cells were plated at a density of ~4 × 10⁵ cells/well in a 6-well plate. After 18–24 h, cells were cotransfected with 1 μg of reporter gene plasmid and 500 ng of pGL3 basic control plasmid using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). THP-1 cells were transfected in a 6-well plate by Lipofectamine according to the manufacturer’s instructions (Invitrogen). After 24 h of transfection, cells were grown in the same medium supplemented with 0.5% FBS for 24 h. Serum-starved cells were used for the assay.

**Luciferase Reporter Gene Activity Assay**—Luciferase assays were performed using the luciferase assay system (Promega). Briefly, the transfected cells were lysed in the culture dishes with reporter lysis buffer, and the lysates were centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge. Ten μl of the supernatant fraction were incubated with 50 μl of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent (Thermo Labsystems Oy, Helsinki, Finland). Luciferase activity was normalized with β-galactosidase activity. All data are presented as the mean ± S.D. of at least three independent experiments.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from confluent flasks of Lkn-1-treated or -untreated THP-1 cells. The oligonucleotides corresponding to several potential transcription factor binding sites on the hLZIP promoter were as follows: NF-κB-581 (underlined, 5’-CGTCTCTGGGAAACGTCCTCCCCCCACC-3’), NF-κB-304 (5’-GGCCGAGGCGTGCTCCCCTACA-3’), Sp1-1564 (5’-ACCAGGCGGCTGGCCTTGAGTCCC-3’), Sp1-1564 (5’-GGCCGAGGCGGCTGGCCTTGAGTCCC-3’), and Mu-κB (5’-GGCCGAGGCGGCTGGCCTTGAGTCCC-3’). The oligonucleotides were 5’-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Unincorporated nucleotides were removed by passage over a Bio-Gel P-6 spin column (Bio-Rad) as described by the manufacturer’s instructions. Nuclear extracts (5 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 MgCl2, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM dithiothreitol, and 50 μg/ml poly(dI-dC)/poly(dI-dC). Gels were pre-electrophoresed in 0.5× TBE for 30 min prior to loading, and electrophoresis continued for ~3 h until the bromphenol blue dye approached the bottom of the gel. Dried gels were visualized by autoradiography.

In competition experiments, binding reactions 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 MgCl2, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM dithiothreitol, and 50 μg/ml poly(dI-dC)/poly(dI-dC). Gels were pre-electrophoresed in 0.5× TBE for 30 min prior to loading, and electrophoresis continued for ~3 h until the bromphenol blue dye approached the bottom of the gel. Dried gels were visualized by autoradiography.
phoresis through a 4% native polyacrylamide gel, and the results were recorded by autoradiography.

Confocal Microscopy—THP-1 cells transiently transfected with pcDNA-p65 and pcDNA-p50 were grown on coverslips. After 36 h, cytospin preparations were obtained on super-frost slides by centrifugation for 5 min. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were washed with phosphate-buffered saline and incubated with monoclonal anti-p65, anti-p50, and polyclonal anti-LZIP antibodies for 1 h. Cells were then washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse and TRITC-conjugated anti-rabbit antibodies for 30 min. Cover slides were washed with phosphate-buffered saline, mounted, and examined using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar GmBH, Germany).

Chemotaxis Assay—Migration of cells was monitored using a 48-well microchamber (Neuro Probe Inc., Cabin John, MD) as described previously (12). Briefly, the lower wells were filled with 28 μl of buffer alone or with buffer containing Lkn-1, and the upper wells were filled with 50 μl of THP-1 cells at 1 × 10^6 cells/ml in RPMI 1640 medium containing 1% bovine serum albumin and 30 mM HEPES. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuro Probe Inc.) with 5-μm pores that was precoated with RPMI 1640 medium containing rat tail collagen type I at 4 °C overnight. After incubation for 6 h at 37 °C, the filters were removed from the chamber, washed, fixed, and stained with Diff-Quick (Baxter, Deerfield, IL). 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). The chemotactic index was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as chemotactic index >2.

RESULTS

LZIP Is Induced by CCR1-dependent Chemokines in THP-1 and HOS/CCR1 Cells—It is known that LZIP is ubiquitously expressed in most tissues (1). We examined the basal expression level of LZIP in different human cell lines. Western blotting data indicated that all cells tested showed LZIP expression (Fig. 1, A and B). Low levels of LZIP expression were observed in U937 cells, whereas THP-1 monocyte cells showed a high level of LZIP expression (Fig. 1, A and B). Therefore, we used THP-1 monocyte cells to analyze inhibitory and CC chemokine effects and HOS/CCR1 cells to analyze CCR1 ligand stimulatory effects. Because chemokines play...
important roles in diverse biochemical and physiological events, including directed migration, activation of immunity, and hematopoiesis of leukocytes (7, 8), we examined whether LZIP expression is affected by various CC chemokines in THP-1 and HOS/CCR1 cells. Serum-starved THP-1 and HOS/CCR1 cells were treated with and without 100 ng/ml of Lkn-1, MIP-1α, RANTES, and HCC-4. All CCR1-dependent chemokines induced LZIP expression by 2.5–6-fold in THP-1 and HOS/CCR1 cells (Fig. 2, A and C). We also examined the effects of pro-inflammatory cytokines and growth factors in LZIP expression. However, these stimulators did not affect expression of LZIP (data not shown).

Lkn-1 Induces LZIP in a Time- and Dose-dependent Manner—We next investigated the time and dose dependence of Lkn-1 on LZIP expression in HOS/CCR1 cells. Serum-starved HOS/CCR1 cells were treated with 1–500 ng/ml of Lkn-1, and the expression level of LZIP was determined by Western blotting. Lkn-1 induced LZIP expression at the same concentration as the maximum chemotactic activity (5). We also treated HOS/CCR1 cells with 100 ng/ml of Lkn-1 for different times, and the LZIP level was determined. The level of LZIP was increased in a time-dependent manner with the maximum increase at 15 min in response to Lkn-1 (Fig. 3C), indicating that Lkn-1 induces LZIP expression at the same concentration as the maximum chemotactic activity (5). We also treated HOS/CCR1 cells with 100 ng/ml of Lkn-1 for different times, and the LZIP level was determined. The level of LZIP was increased in a time-dependent manner with the maximum increase at 15 min in response to Lkn-1 (Fig. 3C), indicating that Lkn-1 induces LZIP expression at the same concentration as the maximum chemotactic activity (5). We also treated HOS/CCR1 cells with 100 ng/ml of Lkn-1 for different times, and the LZIP level was determined. The level of LZIP was increased in a time-dependent manner with the maximum increase at 15 min in response to Lkn-1 (Fig. 3C), indicating that Lkn-1 induces LZIP expression at the same concentration as the maximum chemotactic activity (5). We also treated HOS/CCR1 cells with 100 ng/ml of Lkn-1 for different times, and the LZIP level was determined. The level of LZIP was increased in a time-dependent manner with the maximum increase at 15 min in response to Lkn-1 (Fig. 3C), indicating that Lkn-1 induces LZIP expression at the same concentration as the maximum chemotactic activity (5).
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and four consensus sequences for Sp-1. To examine the transcriptional promoter activity, multiple constructions containing different lengths of the 5′-flanking region coupled to the firefly luciferase reporter gene were generated (Fig. 5A). The constructs containing different lengths of the LZIP promoter were transiently transfected into HOS/CCR1 cells. Cells were serum-starved for 24 h prior to induction of protein expression with 100 ng/ml Lkn-1 for 6 h. Cell extracts were assayed for luciferase activity and determined as relative luciferase activity. Activity values are relative to the pGL3 basic plasmid. Luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean ± S.D. for three independent experiments.

NF-κB Binding Is Necessary for LZIP Expression in THP-1 Cells—Because NF-κB- and Sp-1-binding sites are involved in the LZIP promoter activity, we examined whether the transcription factors NF-κB and Sp-1 induce LZIP protein expression. The selective Sp-1 inhibitor bisanthracycline (WP631) (14, 15) and the NF-κB peptide inhibitor SN50 were used to analyze involvement of these transcription factors. The basal LZIP expression level was not affected by treatment with 1 μM WP631, whereas SN50 slightly decreased the LZIP level (Fig. 6, A and B). Lkn-1 increased LZIP expression 4-fold, compared with control cells. However, SN50 completely abolished LZIP induction in response to Lkn-1 (Fig. 6, A and B). WP-631 did not affect Lkn-1-induced LZIP expression (Fig. 6, A and B). These results indicate that Lkn-1-induced LZIP expression is because of activation of NF-κB.

NF-κB Binds to the LZIP Promoter—To evaluate the DNA binding activity of NF-κB to the LZIP promoter in response to Lkn-1, we performed an electrophoretic mobility shift assay (EMSA) using a 32P-labeled NF-κB DNA binding sequence in the LZIP promoter (−589 to −568) as a probe. THP-1 cells were treated with 100 ng/ml Lkn-1 for different times. Results showed that the DNA binding activity of NF-κB to the LZIP promoter increased in a time-dependent manner, and maximum NF-κB activation was observed at 60 min (Fig. 7A). To determine the specificity of NF-κB binding activity, nuclear extracts at 60 min were incubated with the labeled NF-κB binding probe in the absence and presence of either a 10-fold molar excess of unlabeled NF-κB binding probe or a mutant probe. Results from competition experiments showed that the NF-κB binding complex competed with the unlabeled NF-κB binding probe but not with mutant NF-κB binding probe (Fig. 7B), indicating that NF-κB binding activity is specific. NF-κB is a family of dimeric transcription factors, and various combinations of the Rel/NF-κB protein constitute an active NF-κB heterodimer that binds to a specific sequence of DNA (16). We confirmed the authenticity of the NF-κB band using supershift EMSA by incubation of nuclear extracts with antibodies against either the p50 (NF-κB1) or the p65 (RelA) subunit. Fig. 7C shows that the p50 and p65 subunits of NF-κB were supershifted, indicating that the band represented a p65/p50 heterodimer. We also examined the effects of WP631 and SN50 on Lkn-1-induced
NF-κB DNA binding. The DNA binding activity of NF-κB was not affected by treatment with WP631, whereas SN50 inhibited Lkn-1-induced DNA binding of NF-κB (Fig. 7D). These data indicate that NF-κB binds to the LZIP promoter and that the DNA binding activity increases in response to Lkn-1.

**NF-κB Subunits Increase the Transcriptional Activation of LZIP**—NF-κB transcription factors are protein dimers of several possible subunits, including p65, p50, p52, RelB, and c-Rel (17–20). These proteins dimerize to form heterodimers or homodimers, depending on the combination, and may act as transcriptional activators or repressors (21–25). To investigate the regulatory mechanism of NF-κB in transcriptional activation of LZIP, we cloned the p65 and the p50 genes into pcDNA3.1. These plasmids were transfected into THP-1 cells, and LZIP expression was determined using confocal microscopy. As shown in Fig. 8A, p65 increased the LZIP expression level, whereas p50 had little effect on LZIP expression. THP-1 cells cotransfected with both p65 and p50 showed an increased LZIP expression level (Fig. 8A). These results suggest that the p65 homodimer and/or the p65/p50 heterodimer enhances LZIP protein expression, whereas the p50 homodimer does not. We also cotransfected p65 and/or p50 plasmids with the reporter gene containing the p-613 and/or p-338 promoter segments into THP-1 cells, and the luciferase activity was determined. THP-1 cells cotransfected with the reporter gene and p50 showed no effect. Furthermore, cotransfection of both p65 and p50 also increased LZIP promoter activity, but to a lesser extent than p65 alone (Fig. 8B).
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These results indicate that the p65 homodimer and/or the p65/p50 heterodimer probably induce LZIP expression.

**Mutation Analysis of LZIP Promoter Activity**—To determine whether NF-κB DNA binding is required for LZIP expression, the NF-κB consensus sequence in the LZIP promoter was mutated, and the luciferase activity was determined (Fig. 9A). This mutation resulted in a significant decrease in the basal luciferase activity compared with the wild-type LZIP promoter (Fig. 9B). THP-1 cells cotransfected with the mutated reporter gene and p65 failed to induce luciferase activity (Fig. 9B). Lkn-1 induced LZIP promoter activity in cells transfected with the wild-type NF-κB binding sequence by 6.5-fold, compared with cells transfected with the mock vector (Fig. 9B). However, cells transfected with the mutated NF-κB reporter increased LZIP promoter activity 1.8-fold in response to Lkn-1 (Fig. 9B). These results indicate that NF-κB DNA binding is involved in up-regulation of LZIP expression.

NF-κB-induced LZIP Expression Is Involved in Lkn-1-induced Chemotaxis—Because NF-κB is involved in LZIP expression, we investigated whether NF-κB-induced LZIP expression affects the chemotactic activity of Lkn-1. THP-1 cells were cotransfected with p65, p50, LZIP, or siRNA-LZIP (siLZIP) (5), and the chemotactic activity of Lkn-1 was determined. The chemotactic activity of Lkn-1 in cells transfected with LZIP was increased by ~2.6-fold, compared with cells transfected with the mock vector (Fig. 10). Cells transfected with p65, p50, and p65/p50 showed increased chemotactic activities of 2.5-, 2.3-, and 1.8-fold, respectively (Fig. 10). However, the chemotactic activity of cells transfected with siLZIP was decreased in response to Lkn-1 (Fig. 10). Also, siLZIP inhibited the chemotactic activity of Lkn-1 that was enhanced in cells transfected with p65/p50 (Fig. 10). We also examined the chemotactic activity of MIP-1α. The chemotactic activity of MIP-1α was not affected by the NF-κB subfamilies or LZIP (Fig. 10). These results indicate that NF-κB-induced LZIP expression is involved in Lkn-1-induced chemotaxis.

**DISCUSSION**

Transcription factors in mammalian cells play important roles in regulation of inducible transcription of a large number of target genes involved in many signal pathways and intracellular events. LZIP is an uncharacterized transcription factor that is probably involved in immune responses, including leukocyte cell migration and tumor suppression (4, 5). In this contribution, we cloned and analyzed the promoter region of the human LZIP gene in order to investigate the regulatory mechanism of LZIP expression and subsequent effects on monocyte cell migration in response to chemokines. We demonstrated the following: 1) LZIP is induced by CCR1-dependent chemokines in THP-1 and HOS/CCR1 cells; 2) Lkn-1 induces LZIP in a time- and dose-dependent manner; 3) NF-κB binding is necessary for LZIP expression and NF-κB binds to the LZIP promoter; 4) NF-κB subunits increase the transcriptional activa-

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**Figure 9.** Mutation analysis of LZIP promoter activity. A, the NF-κB-binding site of the −613/+251 reporter construct was mutated as indicated. B, wild-type (WT, solid bar) or mutated (Mut, open bar) constructs were transfected into THP-1 cells, which were either cotransfected with p65 or treated with Lkn-1. Cell extracts were assayed for luciferase activity and determined as relative luciferase activity. Activity values are relative to the pGL3 basic plasmid. Luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean ± S.D. for three independent experiments.

**Figure 10.** NF-κB-induced LZIP expression is involved in Lkn-1-induced chemotaxis. THP-1 cells were cotransfected with the indicated plasmids. After 36 h, cells were applied to Lkn-1 (solid bar) or MIP-1α (open bar) in a microchamber and were allowed to migrate for 6 h. The number of cells that migrated was counted microscopically in two randomly selected fields per well. The chemotactic index (CI) was calculated from the number of cells migrating to the test chemokines divided by the number migrating to the controls. Results are expressed as the mean chemotactic index ± S.E. of six replicate measurements from a single experiment, and it is representative of three separate experiments.
tion of LZIP; and 5) NF-κB-induced LZIP expression is involved in Lkn-1-induced chemotaxis.

To investigate regulation of LZIP protein expression, we examined the expression level of LZIP in mammalian cells, including U937, THP-1, HeLa, HOSS/CCR1, and HEK 293 cells. The level of LZIP was elevated in THP-1 cells compared with all other cell types. This result is consistent with our previous report that LZIP functions as a positive regulator of monocyte cell migration in response to Lkn-1 (5). Although all CCR1-dependent chemokines induced LZIP expression, Lkn-1 showed the highest level of LZIP induction, indicating a special relationship between LZIP and Lkn-1. Time dependence results for Lkn-1-induced LZIP expression showed that Lkn-1 induced LZIP expression in 10 min. Upon stimulation, cells rapidly activate the very first set of genes, known as immediate early genes, which include transcriptional regulators jun, fos, myc, and c-erg (26, 27). Immediate early induction of LZIP indicates the presence of a novel Lkn-1-mediated regulatory mechanism during cell migration and other immune responses.

We identified a putative transcriptional activation site for the LZIP gene and analyzed a 1469-bp human genomic DNA fragment containing 1219 bp of the LZIP gene promoter region. The 1.2-kb long 5′-flanking region of the LZIP gene contains multiple binding sites for transcription factors, including consensus binding sequences for NF-κB, Sp-1, CREB, and Oct-1. These sites were mainly dispersed at position −180 to −589 containing five sites for Sp-1, 2 sites for NF-κB, 1 site for CREB, and 1 site for Oct-1. Promoter activity studies showed that a 1219-bp sequence enhances transcription up to 9.5-fold in response to Lkn-1. Stepwise deletion from the 5′-end resulted in decreased promoter activity, indicating the presence of regulatory sequences that enhance transcription. A short promoter region −613/+251 was as active as the full-length promoter. However, further deletion (−338/+251) caused a loss of promoter activity of up to 70%, suggesting that this fragment is critical for transcriptional regulation of LZIP expression in response to Lkn-1. Because the −613/+251 portion showed high promoter activity in response to Lkn-1, we believe that the −613/−339 sequence constitutes an important part of the LZIP promoter. This fragment contains one binding site for NF-κB and four binding sites for Sp-1. Inhibitor analysis showed that the NF-κB inhibitor SN50 completely suppressed Lkn-1-induced LZIP expression. However, the Sp-1 inhibitor WP631 did not affect LZIP expression, suggesting the importance of NF-κB DNA binding to the LZIP promoter. This was confirmed by EMSA analysis demonstrating that Lkn-1 increased NF-κB binding activity to the cognate sequence in the LZIP promoter. We also analyzed Sp-1 consensus sequence oligonucleotides, as revealed by EMSA. Nuclear translocation of Sp-1 was not affected by Lkn-1 (data not shown).

NF-κB is a transcriptional factor that regulates expression of genes involved in inflammation, angiogenesis, chemotaxis, and cell survival (28–31). NF-κB is composed of homo- and heterodimers of Rel family proteins, including p65/RelA, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (32). NF-κB, which binds to DNA as a dimer composed primarily of p65 and p50, is usually sequestered in the cytoplasm by a family of inhibitory proteins termed the IkBs (33, 34). Promoter elements that bind NF-κB have been identified in numerous genes, including cytokines, chemokines, and adhesion molecules, some of which influence the processes of angiogenesis, chemotaxis, and metastasis (35–39). NF-κB also regulates the expression of chemokines (30). Therefore, we investigated the character of NF-κB in Lkn-1-induced LZIP expression. LZIP promoter activity and LZIP expression were both increased in THP-1 cells transfected with p65 but not in cells transfected with p50. Cotransfection with both p50 and p65 increased promoter activity and LZIP protein expression, indicating that the p65/p65 homodimer and/or the p50/p65 heterodimer probably promote LZIP expression, whereas the p50 homodimer does not. These results are consistent with a report that demonstrated the p50 homodimer lacks a transactivation domain, whereas heterodimers consisting of p65 and p50 represent the primary activated form of NF-κB (32, 40–42). The importance of the NF-κB-binding site for LZIP expression was further supported by experiments containing mutations of the NF-κB-binding site in the LZIP promoter. Reporter constructs carrying a mutated NF-κB site failed to increase promoter activity after transfection with p65 and/or p50 (data not shown) or after treatment with Lkn-1. Chemotaxis analysis showed that the chemotactic activity of Lkn-1 was decreased in cells transfected with p65/p50 and siLZIP, but siLZIP did not affect the activity of MIP-1α, indicating that LZIP expression because of the NF-κB subfamily is specifically involved in Lkn-1-induced chemotaxis. Taken together, these results indicate that NF-κB binding is important for Lkn-1-induced LZIP expression and chemotaxis.

We have previously reported that the pertussis toxin-sensitive Gi/Gi protein, PLC, and PKCδ are all involved in cell migration through CCR1 induced by Lkn-1, and newly synthesized proteins are required for Lkn-1-induced chemotaxis (12). Lkn-1 also activates the extracellular signal-regulated kinase (ERK) pathway by transducing the signal through Gi/Gi protein, PLC, PKCδ, and Ras, and it may play a role for cell proliferation, differentiation, and regulation of gene expression for other cellular processes (43). Because LZIP functions as a positive regulator of Lkn-1-dependent signaling through CCR1 (5) and shows an immediate early response to Lkn-1, Lkn-1 probably induces LZIP expression via Gi/Gi protein, PLC, PKCδ, and NF-κB and subsequently increases the chemotactic activity.

Although its role in chemotaxis remains unclear, LZIP appears to be a potent activator of monocyte mobility. This study is the first to characterize the functional roles of the LZIP promoter. Critical control of LZIP expression resides in a segment within the promoter that carries the NF-κB- and Sp1-binding sites, and binding of NF-κB to this site is important for basal and Lkn-1-induced LZIP expression. Further characterization of the promoter region of this gene will help to elucidate mechanisms of LZIP regulation and chemotaxis in response to chemokines under physiological and pathological conditions. These chemotax attractants act as immediate mediators of inflammatory responses by regulating leukocyte recruitment, infiltration, homing, and trafficking as well as their development and function (44). Although inflammation plays an important role in host defense, uncontrolled inflammatory reactions are
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responsible for initiation and progression of many human diseases, including atherosclerosis, ischemia-reperfusion injury, virus-induced myocarditis, rheumatoid arthritis, allergic reactions, psoriasis, and other inflammatory skin conditions, and even tumorigenesis and tissue-targeted metastasis (45–49). However, a lack of specific therapeutic agents has impeded effective treatment of these inflammatory conditions. Therefore, a better understanding of the regulation of leukocyte chemotaxis by chemoattractant-induced LZIP will lead to development novel therapeutic targets and strategies to treat these diseases.

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