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Involvement of Two NF-κB Binding Sites in PMA-induced Expression of the Human Leukotactin-1/CCL15 Gene in U937 Monocytoid Cells

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Leukotactin-1 (Lkn-1)/CCL15, is a recently cloned chemotactic chemokine that appears to play important roles in the inflammatory process by recruiting immune cells to inflammatory sites. Expression of the Lkn-1/CCL15 gene is inducible in monocytes but its transcriptional regulation has not been studied. To identify Lkn-1/CCL15 regulatory sequences in monocytic cells, U937 cells were transiently transfected with the luciferase reporter gene linked to various deletions of the Lkn-1/CCL15 promoter region. The region –269 to –43 bp from the transcription start site proved to be important for induction by PMA. This region contained two potential NF-κB sites: one between –191 and –182 bp, and the other between –60 and –51 bp. Mutation of either element reduced PMA-induced expression and electrophoretic mobility shift assays revealed that NF-κB recognized both potential NF-κB sites. In addition, PMA-induction of Lkn-1/CCL15 in transiently transfected U937 cells was blocked by proteasome inhibitor 1. These observations demonstrate that the two NF-κB binding sites are essential for PMA-induced Lkn-1/CCL15 expression in human monocytes.

Keywords: CCL15; Chemokine; Gene Expression; Leukotactin-1; Monocytoid Cell; NF-κB.

Introduction

Chemokines are small chemotactic (CC) cytokines involved in a variety of biological processes such as inflammation, hematopoiesis, and angiogenesis (Baggiolini, 2001; Wang *et al.*, 1998). They are arbitrarily divided 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_3C (Oppenheim *et al.*, 1991; Zlotnik and Yoshie, 2000). Leukotactin-1 (Lkn-1)/CCL15 belongs to a family of CC chemokines (Forssmann *et al.*, 2001; Murphy *et al*, 2000; Youn *et al.*, 1997); it was identified independently by a number of investigators and named variously NCC-3, MIP-5, and HCC-2 (Coulin *et al.*, 1997; Naruse *et al.*, 1996; Pardigol *et al.*, 1998). Lkn-1/CCL15 induces chemotaxis and calcium influx in human neutrophils, monocytes, eosinophils, and lymphocytes (Coulin *et al.*, 1997; Forssmann *et al.*, 2001; Lee *et al.*, 2002a; Pardigol *et al.*, 1998; Zhang *et al.*, 1999). It is a chemotactic attractant for leukocytes, and induces pro-atherogenic cytokines and tissue factors, suggesting that it may play a part in the pathogenesis of inflammatory diseases (Baggiolini, 2001). Many different tissues, such as liver, gut, lung, heart, and skeletal muscle contain Lkn-1/CCL15 mRNA (Forssmann *et al.*, 2001; Pardigol *et al.*, 1998) and expression of Lkn-1/CCL15 has been detected in regions of plaques rich in foam cells (Lee *et al.*, 2002b).

The human Lkn-1/CCL15 gene is located in a CC chemokine gene cluster on chromosome 17q11-q21 (Naruse *et al.*, 1996), and consists of four exons and three introns with a single TATA box (Forsmann *et al.*, 2001; Pardigol *et al.*, 1998). Sequence analysis of the 5′-flanking region has revealed several potential binding sites for factors involved in inflammatory processes and regulation of the immune system, including nuclear factor (NF)-κB, Nuclear Factor of activated T-cells (NFAT), and AP-1 (Forsmann *et al.*, 2001), but little is known about the transcriptional regulation of Lkn-1/CCL15. In order to identify sequences responsible for transcriptional regulation in response to phorbol myristate acetate (PMA), we introduced luciferase reporter plasmids containing serial 5′-deletions of the Lkn-1/CCL15 promoter region into the

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PMA-responsive human monocytoid cell line, U937. We show that regions –191 to –182 bp, and –60 to –51 bp, which contain potential binding sites for NF-κB, are essential for induction by PMA.

Materials and Methods

Cell culture Human monocyte-like cell line U937 was grown in complete RPMI 1640 medium containing 10% fetal bovine serum (Gibco-BRL), 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C under humidified 5% CO_2.

Plasmids A series of segments of the 5′-promoter region of the human Lkn-1/CCL15 gene were prepared by PCR using the oligonuclotide primers listed in Table 1. The amplified PCR products were fused to the firefly luciferase gene (Luc) in plasmid pGL3-Basic (Promega). The forward and reverse primers used in the PCR contained Mlu I and Sma I sites, respectively, for cloning the PCR products into pGL3-Basic.

Transfection and luciferase reporter assays Transient transfections were carried out by the DEAE-dextran method as described (Kunch *et al.*, 1994). Briefly, 5×10^6 U937 cells per plate were seeded in 100 mM-diameter plates 24 h prior to transfection. One or other Lkn-1/Luc reporter plasmid together with pUC19 (total DNA 6 ug per plate) was mixed with DEAE-dextran (average MW ~500,000; Sigma). The DNA/DEAE-dextran mix was added to the cells and they were incubated in RPMI 1640 medium/chloroquine (0.1 mM) for 30 min at 37°C, washed in unsupplemented medium and resuspended in 10 ml of complete medium. After 24 h, the cells were exposed to PMA (10 ng/ml; Sigma) for 24 h unless indicated. In some cases the drug, proteasome inhibitor 1 (PS1; Calbiochem), at a concentration of 1 μM was added to the transfected cells with the PMA. After harvesting, the cells were lysed in Report lysis buffer (Promega) and luciferase activity was measured with the Luciferase Assay System (Promega). Experiments were repeated at least three times and values are expressed as means ± standard errors (SE).

Electrophoretic mobility shift assays (EMSA) Nuclear extracts were prepared and EMSA was performed as described previously (Chen *et al.*, 2003; Lee *et al.*, 2003). The following double-stranded oligonucleotide probes were used: NF-κB site 1: 5′- CTG GGA CTT CCT GG-3′, NF-κB site 2: 5′-TCG GGA AAT CCA C-3′. The NF-κB consensus oligonucleotide, 5′-GGG ACT TCC C-3′, and the respective NF-κB site 1 or site 2 oligonucleotides, were used as specific competitors. An oligonucleotide containing the AP1 consensus sequence 5′- TTC CGG CTG ACT CAT C-3′ was used as non-specific competitor. The probe was labeled with [γ-^{32}P] ATP (3000 Ci/mmol; Amersham) and incubated for 30 min on ice with 10 μg of nuclear extract from either unstimulated or PMA-stimulated U937 cells. For super-shift analysis, antibodies specific for p65 and p50 (Santa Cruz Biotechnology) were added after incubating the probe with extract. The reaction products were separated on 5% polyacrylamide gels in 0.25× Tris-borate EDTA buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

Table 1. Oligonucleotide primers for PCR.

Name	Sequence
LK-2613[a]	GGTACGCGTGGACCCACCAACAGCTCCTGCG
LK-1567	GCTACGCGTCCCTATTCTGAAGGAC
LK-1247	CAGACGCGTCACCTGAGGTCAGTAG
LK-535	CACACGCGTGGCACTTTTCCAAG
LK-418	GGCTACGCGTGACTGATATGGATTCACCACTGC
LK-265	AGGCACGCGTGATTAGATTAGATTAATCTCCTGGAGGG
LK-150	TGTACGCGTCTTTCTGTTGCACTCTCAG
LK-43	CGCGTCTTCTTATAAATACAAGGGCAGAGCTGGTATCCC
LK-3R[b]	CCCGGGATACCAGCTCTG

[a] The numbers indicate the position of primers from the transcription initiation site.

[b] R Represents reverse primer for Lkn-1 promoter.

Western blot analysis Whole cell extracts and nuclear extracts were prepared as described previously (Kim *et al.*, 2003). Western blot analysis was performed using mouse monoclonal anti-NF-κB-p65 and p50 antibodies (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) as secondary antibody. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and analyzed by autoradiography.

Site directed mutagenesis Mutations were introduced into the putative NF-κB sites in pGL3-269 using a Quick-change site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Mutagenic primers were as follows: NF-κB site 1-mut 5′ GCA TTC TGA CCT CTG CCC TGC CAC TTG GTG GAT CCT CCT CTT C, NF-κB site 2-mut 5′ GGA CAA AGA TGA CTT GAA CTG TCC CTG TCC ATG GCC CGA ATT ATC CC. The mutations were confirmed by sequencing.

Results and Discussion

Identification of the DNA sequences required for Lkn-1/CCL15 gene expression To characterize the sequences involved in Lkn-1/CCL15 induction by PMA, we fused serially 5′-deleted promoter sequences with the luciferase reporter gene as depicted in Fig. 1. The chimeric constructs were transfected into U937 cells and the cells exposed to PMA for 24 h. Deletions up to –269 bp did not affect induction whereas deletion up to –43 bp resulted in the almost complete loss of both basal and induced activity.

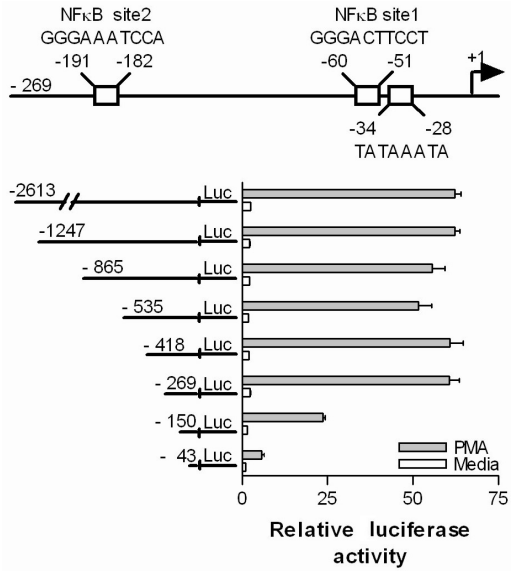

Fig. 1. Location of two potential NF-κB binding sites, and transcriptional activities of 5′-deleted constructs of the Lkn-1/CCL15 promoter in the presence and absence of PMA. The positions and nucleotide sequences of the two potential NF-κB binding sites and TATA box are shown. The arrow indicates the initiation site and direction of transcription. Eight deletion constructs are shown, together with their transcriptional activity in U937 cells. Luciferase activities were measured in duplicate transfections in at least three independent experiments.

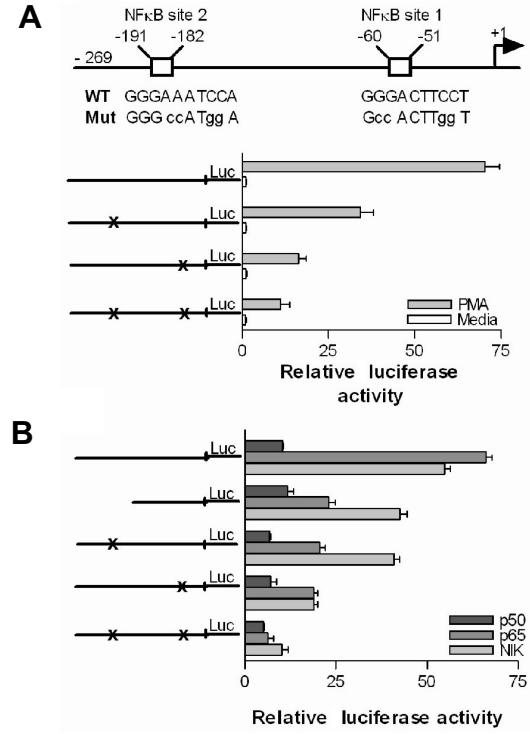

Fig. 2. Effects of point mutations within the NF-κB sites on PMA-inducibility. **A.** The mutated NF-κB sequences used are shown along with the corresponding wild type sequences. Wild type and mutant constructs linked to the reporter gene were transfected into U937 cells, and the cells stimulated with PMA for 24 h and assayed for luciferase activity. **B.** Constructs harboring the wild type or mutated NF-κB sites were cotransfected with expression plasmids for NIK, p65 or p50, and the transfected cells incubated for 24 h. and assayed for luciferase.

Two NF-κB sites are required for induction by PMA The region from –269 to –43 bp contains two sequences resembling NF-κB binding sites, between –191 and –182 bp and between –60 and –51 bp. NF-κB plays a central role in coordinating the expression of a variety of cytokine genes that control immune responses (Li and Verma, 2002; Richmond, 2002). Expression of chemokine genes such as IL-8, RANTES, MIP-1, and MCP-1 is mediated by NF-κB in response to stimuli including proinflammatory cytokines and PMA (Fessele *et al.*, 2001; Grove and Plumb, 1993; Mukaida *et al.*, 1990; Wang *et al.*, 2000). To test whether the two potential NF-κB sites in the Lkn-1/CCL15 promoter region are essential for responsiveness to PMA, we introduced point mutations into them as shown in Fig. 2A, and transfected the corresponding constructs into U937 cells. Mutations in either element reduced PMA-induced expression substantially and mutations in both sites virtually abolished induction (Fig. 2A), indicating that both sites are required for PMA-induced expression.

To confirm the importance of the two potential NF-κB sites, the Lkn-1(-269)/Luc reporter plasmid was cotransfected with a plasmid expressing either NF-κB-inducing kinase (NIK) or the NF-κB p65 subunit. Both NIK and p65 activated luciferase expression from the unmutated Lkn-1(-269)/Luc plasmid but not from the doubly mutated derivative (Fig. 2B). The Lkn-1 promoter with one mutated NF-κB site was also activated by NIK and p65, though less efficiently than the wild type promoter. The other Rel protein, which forms p50 homodimers, lacks a transactivation domain but still binds to NF-κB sites, while the p65 (RelA) NF-κB subunit has a carboxy-terminal transactivation domain (Li and Verma, 2002). The main activated form of NF-κB is a heterodimer of the p65 subunit associated with either a p50 or p52 subunit. As expected, over-expression of the NF-κB p50 subunit did not activate transcription from either the wild type or the mutated promoter (Fig. 2B).

Nuclear factors bind to each NF-κB sites To characterize the nuclear factors that bind to the two potential NF-κB sites of the Lkn-1/CCL15 promoter, we performed electrophoresis mobility shift assays (EMSA) with specific oligonucleotide probes containing the NF-κB sequences at sites 1 and 2. A basal level of DNA-protein complex was formed with nuclear extracts from unstimulated U937 cells (Figs. 3A and 3B), and nuclear extracts

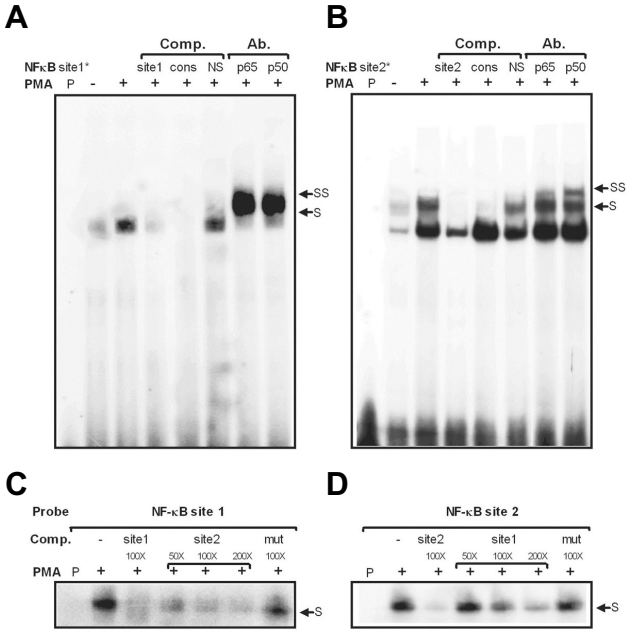

Fig. 3. Electrophoretic mobility shift assays. Double stranded, end-labeled oligonucleotides containing NF-κB binding sites 1 or 2 were mixed with 10 μg of nuclear extract from control U937 cells or cells stimulated with PMA for 90 min in the presence or absence of a 100-fold excess of the corresponding unlabeled oligonucleotides. DNA-protein complexes were separated by non-denaturing polyacrylamide gel electrophoresis and visualized with a phosphoimager. Supershift experiments were performed by preincubating nuclear extracts with 2 μg of antibody against p65 or p50. NF-κB consensus oligonucleotides (5′-GGG ACT TCC C-3′) and the respective NF-κB site 1 or site 2 oligonucleotides, were used as specific competitors, and an oligonucleotide containing the AP1 consensus sequence 5′-TTC CGG CTG ACT CAT C-3′ was used as non-specific competitor. **A.** EMSA with labeled oligonucleotide probe containing the potential NF-κB binding site 1. **B.** EMSA with labeled oligonucleotide probe containing NF-κB binding site 2. **C.** EMSA with the labeled oligonucleotide probe containing the potential NF-κB binding site 1 competed with different concentrations of NF-κB binding site 2. **D.** EMSA with the labeled oligonucleotide probe containing potential NF-κB binding site 2 competed with different concentrations of NF-κB binding site 1. cons, consensus competitor; NS, non-specific competitor; S, specific band; SS, supershifted band; P, probe.

of PMA-stimulated cells produced a much higher yield of complex. Competition assays using an excess of unlabeled wild type oligonucleotides corresponding to site 1, site 2, or the NF-κB consensus sequence abolished the labeled DNA-protein complex, whereas the corresponding mutant oligonucleotides had no effect. The band formed with site 1 was strongly supershifted by antibodies against NF-κB p65 and p50 subunits whereas the complex with site 2 was only weakly supershifted (Figs. 3A and 3B).

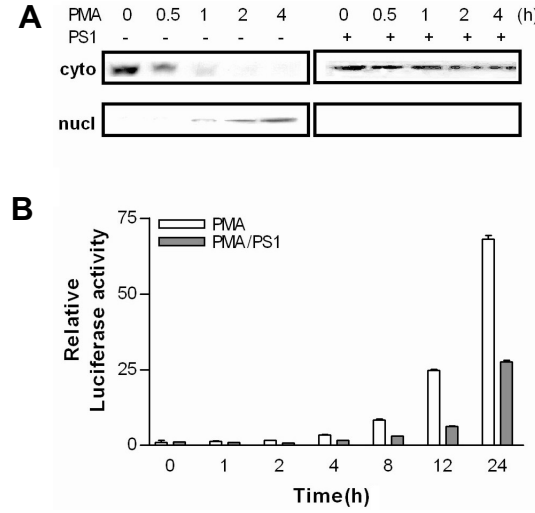

Fig. 4. Effect of the proteasome inhibitor PS1 on Lkn-1/CCL15 gene expression induced by PMA. **A.** Western blot showing nuclear localization of p65 and simultaneous disappearance of cytoplasmic p65 in PMA-stimulated U937 cells. Nuclear localization was inhibited by PS1. Times of PMA treatment are indicated at the top of the blot. **B.** Effect of PS1 on the PMA-induced promoter activity. U937 cells transfected with the Lkn-1(-269)/Luc reporter gene were stimulated with PMA in the presence or the absence of PS1 (1 μm) for the indicated periods and assayed for luciferase.

We sometimes observed an additional band with the site 2 DNA fragment; this was significantly reduced by the respective cold competitor but not by the NF-κB consensus sequence (Fig. 2B), and may be a binding site for another transcription factor. Site 2 appears to have higher affinity for NF-κB than site 1 as judged by results with competitors containing the opposite binding site (Figs. 3C and 3D). Our findings demonstrate that NF-κB sites 1 and 2 are recognized by the NF-κB p65/p50 heterodimeric complex. Together with the results of the mutational analysis of the NF-κB sites, our observations strongly suggest that the two NF-κB elements are essential for transcriptional activation in response to PMA, although we cannot rule out the possibility that other DNA elements are also involved.

Proteasome Inhibitor 1 (PS1) inhibits PMA-induced transcription of Lkn-1/CCL15 Activation via the NF-κB pathway generally requires degradation of NF-κB inhibitors (IκBs) by the 26S proteasome (Anest *et al.*, 2003; Yamamoto *et al.*, 2003); this releases NF-κB dimers from cytoplasmic NF-κB-IκB complexes and allows them to translocate to the nucleus. p65 protein was easily detected by immunoblotting in the cytoplasm, but not in the nucleus, of unstimulated cells (Fig. 4A), and there was a substantial increase in nuclear p65 after an hour of stimulation with PMA. In order to confirm the contribution of

NF-κB proteins to the expression of the Lkn-1/CCL15 gene, we assessed the effect of PS1, an inhibitor of IκB degradation via the proteasome. PS1 indeed inhibited translocation of NF-κB from cytoplasm to nucleus in response to PMA (Fig. 4A), and markedly inhibited expression of the reporter gene (Fig. 4B).

NF-κB is rapidly activated in response to a wide variety of stimuli, including pathogens, stress signals and pro-inflammatory cytokines such as TNF-α and IL-1β (Li and Verma, 2002; Richmond, 2002). Lkn-1/CCL15 gene is activated by TNF-α, IL-1β and LPS with similar kinetics to those observed with PMA (data not shown). The two NF-κB binding sequences may also be involved in the responses to TNF-α and IL-1β.

Additional elements may be required for Lkn-1/CCL15 expression because mutation of both NF-κB sites did not completely abolish PMA-induced expression. The Lkn-1/CCL15 promoter region contains a putative AP-1 binding site between –76 and –65 bp and a putative NFAT site between –207 and –197 bp. AP-1 and NFAT are expressed in most immune cells and play important roles in the transcription of cytokine genes and other genes critical for the immune response (Rao *et al.*, 1997). The DNA-binding domains of NFAT proteins resemble those of Rel-family proteins, and NF-κB and NFAT proteins show some overlap in their ability to bind to certain regulatory elements of cytokine genes (Jain *et al.*, 1995). The possible roles of other transcription factors and their interaction with NF-κB in activating Lkn-1/CCL15 require further investigation.

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