MAIL, a novel nuclear IκB protein that potentiates LPS-induced IL-6 production

Hiroshi Kitamura[a,*], Katsushi Kanehira[a], Keisuke Okita[a], Masami Morimatsu[b], Masayuki Saito[a]

[a] *Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[b] *Department of Veterinary Physiology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan*

Received 25 September 2000; accepted 9 October 2000

First published online 2 November 2000

Edited by Marco Baggiolini

Abstract We have identified and characterized a novel member of the ankyrin-repeat family named 'molecule possessing ankyrin-repeats induced by lipopolysaccharide' (MAIL). The C-terminal portion of MAIL shared high sequence homology with the IκB family. Intraperitoneal injection of lipopolysaccharide (LPS) into mice rapidly (< 0.5 h) induced MAIL mRNA in various tissues, particularly in the spleen, lymph node, and lung. Ectopically expressed MAIL was localized in the nucleus, and remarkably potentiated the LPS-induced mRNA expression and secretion of interleukin (IL)-6 in Swiss 3T3 cells. These findings indicate that MAIL is one of the nuclear IκB proteins and an activator of IL-6 production.

Key words: Lipopolysaccharide; Interleukin 6; Bcl3; IκB; Ankyrin-repeat; Inflammation

1. Introduction

Lipopolysaccharide (LPS) is a component of the bacterial cell wall, and elicits numerous inflammatory responses. Recent studies have demonstrated that the signaling cascade from Toll-like receptors to NF-κB and AP-1 plays a pivotal role in inflammatory responses to LPS [1,2] and IκB proteins, namely that IκB-α, -β, -γ/p105, -δ/p100, -ε and Bcl3, are known to be representative molecules that interact with NF-κB. They have 5–7 ankyrin-repeat domains [3,4], which form a functional unit able to interact with the Rel region of NF-κB proteins, and modulate their transactivation [3,4]. Bcl3 also associates with the basic leucine zipper domain of c-Jun and c-Fos, and potentiates AP-1 activity [5]. Furthermore, mutants of the genes encoding IκB proteins are known to show various abnormal phenotypes of immune and inflammatory reactions [4]. For example, Bcl3-deficient mice have a defect of antigen-specific antibody production and are void of germinal centers in the spleen [6,7]. These facts indicate that IκB proteins are one of the critical determinants of progression of anti-microbial responses.

During a differential display search for inflammation-responsive genes in the mouse brain, we studied 1500 genes and identified 11 whose mRNA levels were elevated by intraperitoneal injection of LPS. We sequenced one of the LPS-inducible genes, 142.5, and proved it to be a novel IκB protein. We report here its structure, mRNA expression pattern and a stimulatory role for interleukin (IL)-6.

2. Materials and methods

2.1. Cloning of MAIL and construction of expression plasmid

A 379 bp cDNA fragment of gene 142.5 was isolated in a differential display search of genes whose mRNA expression was raised in the mouse brain after LPS injection. Using an α^{32}P-dCTP labeled 142.5 cDNA fragment, mouse spleen λ TriplEX (Clontech, Palo Alto, CA, USA) and λ ZAP (Stratagene, La Jolla, CA, USA) cDNA libraries were screened and two clones were isolated. Further screening using these clones as probes detected three additional clones. Obtained clones were sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan). The 5′ and 3′ remaining sequences were determined with RACE kits (Gibco, Gaithersburg, MD, USA).

cDNA fragments encoding MAIL-L and MAIL-S (see below) were obtained from mouse spleen RNA by RT-PCR, and cloned into pDONR201 (Gibco) and pEGFP-N2 (Clontech). Subcloning of fragments from pDONR201 into pDEST12.2 was performed with the Gateway system (Gibco). All plasmid constructs were confirmed by DNA sequencing.

2.2. Treatments of animals

Male C57BL/6 mice (8 weeks old, SLC, Shizuoka, Japan) were injected intraperitoneally with 3 mg/kg of LPS (*Escherichia coli* 055:B5, Difco, Detroit, MI, USA). They were sacrificed by cervical dislocation, and tissues were excised for RNA preparation. The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

2.3. Northern blot analysis

Total RNA was extracted with Trizol solution (Gibco). Poly(A)$^+$ RNA was prepared using oligo-d(T) cellulose columns (Clontech). RNA was separated on 1% agarose/formaldehyde gel, and transferred to a nylon membrane (Amersham Pharmacia, Piscataway, NJ, USA). The membranes were hybridized with an α^{32}P-dCTP labeled 142.5 cDNA fragment and exposed to X-ray films (Fuji Film, Tokyo, Japan) for 1 week. The radioactivity was quantified using a BAS-1000 bioimage analyzer (Fuji Film, Tokyo, Japan). The membranes were also hybridized with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH, +566–+1017 bp) cDNA as a reference.

2.4. Treatments of cells

Swiss 3T3 cells (Riken Cell Bank, Tsukuba, Japan) were transfected with an expression plasmid containing MAIL and GFP-tagged MAIL using FuGENE reagent (Roche, Branchburg, NJ, USA) for 48 h. Transfection by itself did not have any effect on the viability of the

*Corresponding author. Fax: (81)-11-757 0703.
E-mail: ktmr@vetmed.hokudai.ac.jp

Abbreviations: LPS, lipopolysaccharide; IL, interleukin; AA, amino acids

0014-5793/00/$20.00
PII: S0014-5793(00)02185-2

cells. The cells transfected with MAIL were stimulated with LPS (10 μg/ml). The cells transfected with GFP-tagged MAIL were observed with the FULOVIEW system (Olympus, Tokyo, Japan). Selective localization of GFP-tagged MAIL in the nucleus was confirmed by staining with Hoechst 33258.

2.5. Assay of IL-6

The concentration of IL-6 in the culture medium was measured by proliferation assay using an IL-6-dependent cell line, MH60. BSF2 (a gift from Dr. T. Matsuda, Osaka University, Suita, Japan) as described previously [8]. The minimum detectable concentration of IL-6 in the medium was 2.0×10^{-2} U/ml (4.0 pg/ml of recombinant human IL-6). Preliminary experiments confirmed that the biological activity was abolished with an antibody against mouse IL-6 (R and D, Minneapolis, MN, USA).

The level of IL-6 mRNA was evaluated by RT-PCR. Mouse IL-6 primers used were: 5′-CATCCAGTTGCCTTCTTGGGA-3′ and 5′-CTGAAGGACTCTGGCTTGTC-3′. PCR for IL-6 was conducted for 25 cycles, with denaturing at 95°C for 2 min, annealing at 56°C for 30 s, and extension at 72°C for 1 min. The GAPDH mRNA level was also analyzed using commercially available primers (Clontech) as a reference.

2.6. Statistics

All values were expressed as means ± S.E.M. Statistical comparison was made by analysis of variance, followed by Scheffe's *F* test.

3. Results

3.1. cDNA cloning of MAIL

We searched for inflammatory-responsive genes in the mouse brain by differential display analysis. The mRNA expression of one gene, 142.5, remarkably increased after intraperitoneal injection of LPS. The full-length cDNA of 142.5 was isolated as described in Section 2. All sequence data were registered in the database of EMBL (accession number, AB020974). The full-length 142.5 cDNA had an open reading frame of 2187 bp encoding a polypeptide of 728 amino acids (AA) (Fig. 1). The C-terminal half (AA 453–728) contained six typical ankyrin-repeats, and shared high homology with IκB proteins; IκB-α (AA 73–294), -β (AA 57–278), -γ (AA 538–747), -δ (AA 487–706), -ε (AA 122–343) and Bcl-3 (AA 121–349), at 40, 34, 42, 44, 41 and 42%, respectively. Thus, we termed this novel protein Molecule possessing Ankyrin-repeats Induced by LPS (MAIL). In the cloning process, we obtained a clone lacking nucleotides −63–+286 bp of the full-length sequence, suggesting that two isoforms of MAIL with 728 AA and 629 AA may exist. We designed these isoforms MAIL-L and MAIL-S, respectively.

```
MIVDKLLDDSRGGEGLLDAAGDCGLMTSPLNLAYFYGASP  40
PSAPGAGDTGYLSAVPSAPGSPGSDSSDFSSTSSVSSCGA  80
VESRPRGGARAERPQVEPHMGVGRQQRGPFQGVRVKNSVK 120
ELLLHIRSNKQKASGQPVDEFKTQSVNIEQLTDLKSAVSA 160
VGKRKGPDPLSDGPVCKRPALLPSHFVTSPQTPTPGESME 200
DVRHSESKLDSSAALLQNIINIKNECNPVSLNTVQVSWMS 240
PTVPQNSPRDQCQDFHGGQAFSPPQKYQPFQVSGSPQMMD 280
QASMYQYSPQTQNMQQPPPLPPQQQHQQNYPHNSPLQFSP 320
YSRMSQSPKYDSNLFDTHEPQFCTGQSFVSLLTGPGEPES 360
LAVPVPAPTSIPPQTETQLQTFSLMPSNACEAVVGVHDVG 400
SHSLGTSLSLQNIMGSPMNTTQLGKSFFQWQVEQEESKLA 440
NIPQDQFLARDGDGDTFLHIAVAQGRRALSYVLARKMNAL 480
HMLDIKEHNGQSAFQVAVAANQHLIVQDLVNLGAQVNITD 520
CWGRTPLHVCAEKGHSQVLQAIQKGAVRSNQFVDLEATNY 560
DGLTPLHCAVVAHNAVVHELQRNRQSHSPEVQDLLLRNKS 600
LVDTIKCLIQMGAAVEAKDRKSGRTALHLAAEEANLELIR 640
LFLELPSCLSFVNAKAYNGNTALHVAASLQYRVTQLDAVR 680
LLMRKGADPSTRNLENEQPVHLVPDGPVGEQIRRILKGKS 720
IQQRAPPY                                  760
```

Fig. 1. AA sequence of MAIL-L deduced from 2187 bp cDNA (accession number AB020974). An asterisk indicates the initial methionine of a putative truncated isoform (MAIL-S). The ankyrin-repeat domains are boxed. The sequence corresponding to clone 142.5 is underlined.

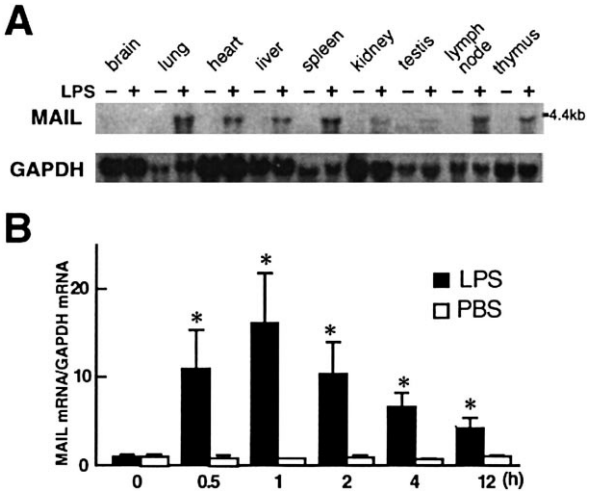

Fig. 2. LPS-induced mRNA expression of MAIL in the mouse. A: At 2 h after intraperitoneal injection of LPS (3 mg/kg), 40 μg of total RNA was extracted and subjected to Northern blot analysis. Results are representative of three independent experiments. B: Time-course of MAIL mRNA expression in the spleen after LPS injection. The MAIL mRNA level was normalized by those of GAPDH, and expressed as relative to the average of control mice (0 h after PBS injection). Values are means ± S.E.M. for three mice. $^*P<0.05$ vs. the PBS control.

3.2. MAIL mRNA expression after LPS administration

The mRNA expression of MAIL was investigated in mouse tissues by Northern blot analysis. When 40 μg of total RNA was applied, the MAIL mRNA levels were under detectable level in all tissues except the testis in the PBS-injected control mice. However, it had greatly increased at 2 h after intraperitoneal injection of LPS in various tissues (Fig. 2A). The LPS-induced MAIL expression was greatest in the spleen, lung and lymph node and lesser in the thymus, liver, heart and testis. MAIL mRNA was not affected in the brain even after LPS injection, but extensive induction by LPS was confirmed when 10 μg of poly(A)$^+$ RNA was used (data not shown). A smaller band, which was not influenced by LPS injection, also existed in the testis. Time-dependent expression of MAIL mRNA after LPS injection was also studied in the spleen. As shown in Fig. 2B, the MAIL mRNA level increased more than 10-fold at 30 min after LPS injection, reached a peak at 1 h, and was sustained at higher levels than in the control until 12 h.

3.3. MAIL potentiates LPS-induced IL-6 production in vitro

To explore possible functions of MAIL, we examined whether ectopic expression of MAIL altered the production of a typical inflammatory cytokine, IL-6, in mouse embryonic

fibroblasts, Swiss 3T3 cells. As shown in Fig. 3A, transfection with MAIL itself did not show any significant effect on IL-6 contents in the culture medium compared with the cells transfected with the control vector. When the cells were stimulated with LPS for 24 h, the medium IL-6 content increased ~26- and ~58-fold in the cells transfected with MAIL-L and MAIL-S, respectively, but only 1.6-fold in the control cells. The mRNA level of IL-6 was also evaluated by RT-PCR (Fig. 3B). In the control cells, a faint band of IL-6 mRNA was detected only 1 h after LPS stimulation. On the other hand, in the MAIL-transfected cells, denser bands were found at 1–6 h. Hence, MAIL potentiated LPS-induced IL-6 production through increased and prolonged accumulation of IL-6 mRNA.

3.4. Nuclear localization of MAIL

To determine the intracellular location of MAIL, we incorporated a green fluorescent protein (GFP)-tag into MAIL and transiently expressed the tagged protein in Swiss 3T3 cells (Fig. 4). Observation with a confocal laser-scanning microscope revealed that GFP itself was diffusely distributed throughout the intracellular space. In contrast, GFP-tagged MAIL-L and MAIL-S were strictly localized in the nucleus. Fluorescent signals of MAIL in the nucleus seemed granulated rather than constant. GFP-tagged MAIL also potentiated LPS-induced IL-6 production after LPS stimulation, indicating that the GFP-tag did not alter the normal MAIL activity (data not shown). These results collectively indicated that MAIL was a nuclear protein.

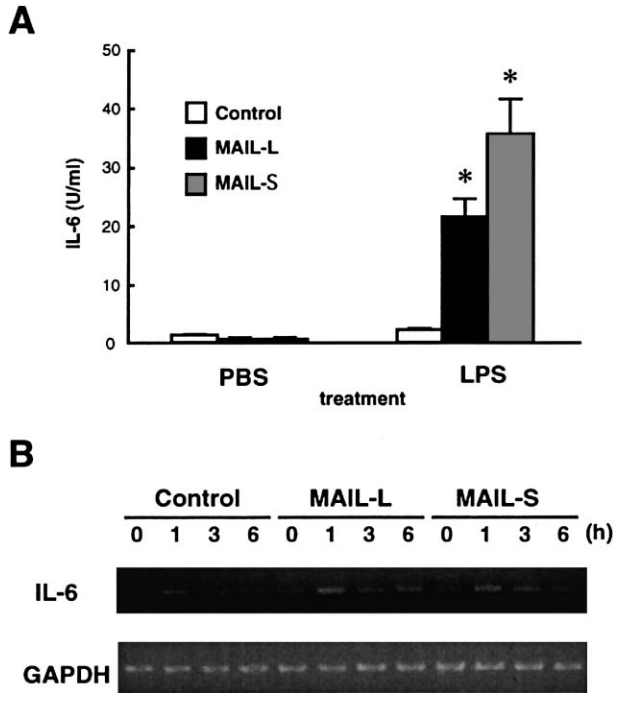

Fig. 3. IL-6 responses to LPS in Swiss 3T3 cells expressing MAIL. A: Cells transfected with MAIL cDNA were stimulated with LPS or PBS for 24 h. IL-6 concentrations in the culture medium were determined. Values are means ± S.E.M. of four separate wells. *$P < 0.05$ vs. the control transfected with empty vector. B: RNA was extracted at 0–6 h after LPS stimulation and subjected to RT-PCR analysis. Results are representative of four independent experiments.

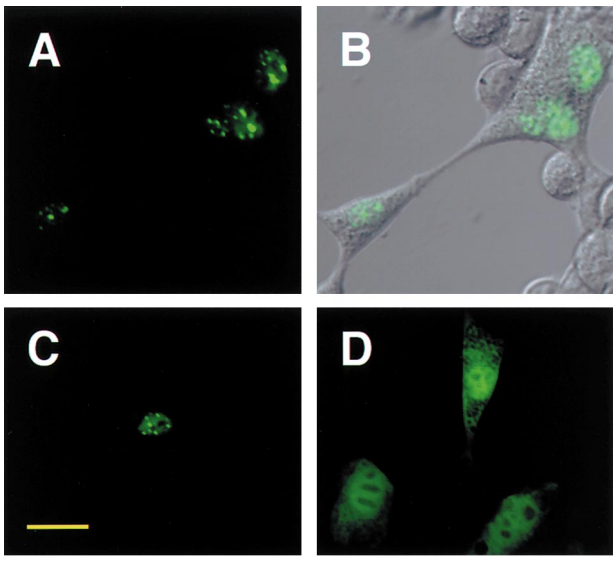

Fig. 4. Subcellular localization of MAIL. Swiss3T3 cells were transfected with GFP-tagged MAIL-L (A and B), GFP-tagged MAIL-S (C), or GFP alone (D). B: Fluorescent images were overlaid on difference-interference contrast images. Bar, 10 μm (A–D).

4. Discussion

In this study, we cloned cDNA of a novel ankyrin-repeat protein, and named it MAIL. The C-terminal region of mouse MAIL had six ankyrin-repeats and shared about 40% homology with those of IκB proteins such as IκB-α and Bcl3.

The most striking findings were that MAIL mRNA was expressed at under detectable levels in almost all tissues of normal mice, but induced rapidly after injection of LPS. It is notable that the LPS-induced MAIL expression was remarkable in the spleen, lung and lymph node, where macrophages and B cells are profuse. In accordance with these in vivo observations, in preliminary experiments, we found that LPS intensely induced MAIL mRNA expression in cultured macrophages and B cells. It is well established that macrophages and B cells exhibit a wide variety of responses to LPS stimulation. For example, LPS stimulates macrophages for phagocytosis and production of various cytokines, and B cells for proliferation and immunoglobulin secretion [9–11]. Thus, MAIL may participate in the cellular responses of the immunocompetent cells during LPS-induced inflammation.

Although the precise roles of MAIL in macrophages and B cells are not clear at present, we demonstrated an apparent stimulatory effect of MAIL on the production of a typical inflammatory cytokine, IL-6, in fibroblast cell line Swiss 3T3: that is, LPS-induced IL-6 secretion was enhanced more than 20-fold by transfection with MAIL cDNA. Since MAIL potentiated the accumulation of IL-6 mRNA by LPS, a probable role of MAIL is as a regulatory factor of IL-6 gene transcription. In support of this idea, we found strict nuclear localization of ectopically expressed GFP-tagged MAIL.

In spite of the nuclear localization of MAIL, typical DNA binding domains were not identified in its AA sequence. Thus, MAIL does not seem to act as a direct transcriptional regulatory factor. It is well known that IκB proteins interact with several transcriptional regulatory factors, modulate their activity, and thereby regulate protein syntheses. For example,

Bcl3 and IκB associate with NF-κB, and modulate NF-κB-dependent gene transcription [4,12,13]. It has also been reported that Bcl3 plays a role as a coactivator of AP-1 and the retinoid X receptor [5,14]. In the 5′-upstream region of the mouse IL-6 gene, there are AP-1, NF-IL6, and NF-κB binding sites [15]. Collectively, it seems likely that MAIL enhances IL-6 production through activation of these factors. The IL-6 promoter region also contains a cAMP-responsive element, which is one of the major *cis*-acting regulatory elements of the IL-6 gene [15,16]. Isumi et al. demonstrated that IL-6 secretion from Swiss 3T3 cells was greatly up-regulated by adrenomedullin [17]. Adrenomedullin is known to act its specific receptor coupled with adenylate cyclase and to elevate intracellular the cAMP level in Swiss 3T3 cells [18]. Thus, it is also possible that MAIL activates the cAMP-dependent signaling pathway and consequently enhances IL-6 production. However, further studies are needed to clarify the action mechanisms of MAIL, particularly the intracellular and intranuclear molecules interacting with MAIL. Such information would be helpful for understanding molecular events in LPS-induced inflammation.

Acknowledgements: This work was supported in part by a Grant-in-aid of the Recombinant Cytokine Project (RCP-1999-4230) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

References

[1] Aderem, A. and Ulevitch, R.J. (2000) Nature 406, 782–787.
[2] Foletta, V.C., Segal, D.H. and Cohen, D.R. (1998) J. Leukoc. Biol. 63, 139–152.
[3] Baeuerle, P.A. and Baltimore, D. (1996) Cell 87, 13–20.
[4] Chen, F., Casranova, V., Xianglin, S. and Demers, L.M. (1999) Clin. Chem. 45, 7–17.
[5] Na, S-Y., Choi, J.-E., Kim, H.-J., Jhun, B.-H., Lee, Y.-C. and Lee, J.W. (1999) J. Biol. Chem. 274, 28491–28496.
[6] Schwarz, E.M., Krimpenfort, P., Berns, A. and Verma, I.M. (1997) Genes Dev. 11, 187–197.
[7] Franzoso, G., Carlson, L., Scharton-Kersten, T., Shores, E.W., Epstein, S., Grinberg, A., Tran, T., Shacter, E., Leonardi, A., Anver, M., Love, P., Sher, A. and Siebenlist, U. (1997) Immunity 6, 479–490.
[8] Matsuda, T., Hirano, T. and Kishimoto, T. (1988) Eur. J. Immunol. 18, 956–957.
[9] Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) J. Immunol. 162, 3749–3752.
[10] Ogata, H., Su, I., Miyake, K., Nagai, Y., Akashi, S., Mecklenbrauker, I., Rajewsky, K., Kimoto, M. and Tarakhovsky, A. (2000) J. Exp. Med. 192, 23–29.
[11] Zhang, P., Nelson, S., Summer, W.R. and Spitzer, J.A. (2000) Shock 13, 34–40.
[12] Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W. and Scheidereit, C. (1992) Proc. Natl. Acad. Sci. USA 89, 2489–2493.
[13] Bundy, D.L. and McKeithan, T.W. (1997) J. Biol. Chem. 272, 33132–33139.
[14] Na, S.-Y., Choi, H.-S., Kim, J.W., Na, D.S. and Lee, J.W. (1998) J. Biol. Chem. 273, 30933–30938.
[15] Tanabe, O., Akira, S., Kamiya, T., Wong, G.G., Hirano, T. and Kishimoto, T. (1988) J. Immunol. 141, 3875–3881.
[16] Dendorfer, U., Oettgen, P. and Libermann, T.A. (1994) Mol. Cell. Biol. 14, 4443–4454.
[17] Isumi, Y., Minamoto, N., Kubo, A., Nishimoto, N., Yoshizaki, K., Yoshioka, M., Kanagawa, K. and Matsuo, H. (1998) Biochem. Biophys. Res. Commun. 244, 325–331.
[18] Withers, D.J., Coppock, H.A., Seufferlein, T., Smith, D.M., Bloom, A.R. and Rozengurt, E. (1996) FEBS Lett. 378, 83–87.