The Ubiquitin-like Protein MNSFβ Regulates ERK-MAPK Cascade*

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MNSFβ is a ubiquitously expressed member of the ubiquitin-like family that has been implicated in various biological functions. Previous studies have demonstrated that MNSF covalently binds to intracellular proapoptotic protein Bcl-G in mitogen-activated murine T cells. In this study, we further investigated the intracellular mechanism of action of MNSFβ in macrophage cell line, Raw 264.7 cells. We present evidence that MNSFβ-Bcl-G complex associates with ERKs in non-stimulated Raw 264.7. We found that MNSFβ-Bcl-G directly bound to ERKs and inhibited ERK activation by MEK1. In Raw 264.7 cells treated with MNSFβ small interfering RNA (siRNA) lipopolysaccharide (LPS)-induced ERK1/2 activation was enhanced and LPS-induced JNK and p38 activation was unaffected. SiRNA-mediated knockdown of MNSFβ was enhanced and LPS-induced JNK and p38 activation was unaffected. This article must therefore be hereby marked “in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The covalent attachment of ubiquitin to proteins is an important cellular function that is required for protein degradation, DNA repair, cell cycle control, stress response, transcriptional regulation, signal transduction, and vesicular traffic (1–6). The attachment of a single ubiquitin polypeptide (monoubiquitination) is important for cellular regulation (7). Polyubiquitination targets proteins for destruction by the proteasome. Polyubiquitin chains are formed via isopeptide bond linkages between the C-terminal Gly-76 of ubiquitin and the side chain -NH2 from Lys-48 of another. In addition to ubiquitin, it is evident that several ubiquitin-like proteins have been found to be covalently or noncovalently attached to target proteins (8–12). Interestingly, small ubiquitin-like modifier 1 (SUMO-1) conjugation of kB occurs on the same residues used for ubiquitination, thus making the protein resistant to proteasome-mediated degradation and consequently inhibiting NFkB activation (13).

Monoclonal nonspecific suppressor factor (MNSF), a lymphokine produced by murine T cell hybridomas, possesses pleiotropic anti-gen-nonspecific suppressive functions (14). We have cloned a cDNA encoding a subunit of MNSF, which was termed MNSFβ (15). MNSFβ cDNA encodes a protein of 133 amino acids consisting of a ubiquitin-like protein (36% identity with ubiquitin) fused to the ribosomal protein S30. The ubiquitin-like moiety of MNSFβ shows MNSF-like biologic activity without cytotoxic action (16). Interferon γ (IFNγ) is involved in the mechanism of action of MNSFβ. We have demonstrated that Ubi-L specifically binds to cell surface receptors on mitogen-activated lymphocytes and the T helper type 2 clone, the D.10 cells (17).

We have also shown that MNSFβ covalently conjugates to acceptor proteins and forms MNSFβ adducts including 33.5-kDa protein in concanavalin A- and IFNγ-stimulated D.10 cells (18). Recently, we found that this MNSFβ adduct consists of 8.5-kDa ubiquitin-like protein and Bcl-2-like protein (19), murine orthologue of previously cloned human BCL-G gene product with proapoptotic function (20). The BCL-G gene is a proapoptotic p53 target gene (21). Murine Bcl-G mRNA was highly expressed in testis and significantly in spleen (19).

In this study, we investigated the intracellular mechanism of action of MNSFβ in murine macrophage cell line, Raw 264.7 cells. We observed that MNSFβ siRNA increased ERK activation and TNFα production by LPS-stimulated Raw 264.7 cells. We will show that the MNSFβ is implicated in the regulation of the ERK-MAPK cascade.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibodies to p38, ERK1, and JNK (SAPK) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to phospho-p38 and phospho-JNK were from Promega, and rabbit anti-phospho-ERK1/2 antibodies were from Sigma. Rabbit polyclonal antibodies to MNSFβ and Bcl-G were prepared as described (15, 19). MNSFβ-Bcl-G complex was prepared as described (19).

Immunoprecipitation—Immunoprecipitation was performed with a horseradish peroxidase-conjugated antibody that recognizes native rabbit IgG (TrueBlot™, eBioscience, San Diego, CA) according to the manufacturer’s instructions. RIPA buffer (50 mM Tris, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, containing 1 μg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin) extracts of Raw cells were precleared with 50 μl of antibody-agarose beads for 1 h on ice. Subsequently, 5 μg of primary antibody to MNSFβ or ERK1 was added to precleared lysates and incubated on ice for additional 1 h. Samples were then incubated overnight at 4 °C with 50 μl of anti-rabbit IgG bead. The beads were washed with five times with RIPA buffer, and immunoprecipitates were released from the beads by 10 min boiling in NuPAGE LDS sample buffer (Invitrogen) buffer. Immunoblotting was carried out with anti-ERK1 or anti-Bcl-G antibody. A rabbit IgG TrueBlot was employed as a second antibody.

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2 The abbreviations used are: SUMO-1, small ubiquitin-like modifier 1; MNSF, monoclonal nonspecific suppressor factor; RANTES, the regulated on activation normal T cell expressed and secreted; siRNA, small interfering RNA; PAK2, p21-activated kinase 2; IFN, interferon; TNF, tumor necrosis factor; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; FBS, fetal bovine serum; RNAI, RNA interference.
Ubiquitin-like Protein Regulates ERK Activity

Western Blot Analysis—The protein concentrations of the cell lysates were determined by Bradford assay (Bio-Rad). Equal amounts of protein were loaded onto an SDS-polyacrylamide gel (10% acrylamide), resolved by electrophoresis, and transferred onto polyvinylidene fluoride membranes. The membrane was incubated overnight at 4 °C in a Tris-buffered saline solution with 5% milk to block nonspecific binding sites. Membranes were incubated with the primary antibodies for a minimum of 2 h at room temperature in Tris-buffered saline with 0.1% Tween 20 (Tris/Tween). Horseradish peroxidase secondary antibodies were incubated for 1 h at room temperature in Tris/Tween with 5% milk. Labeled proteins were visualized by chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences).

In-gel Digestion and MALDI-TOF—The 33.5-kDa MNSF adduct was purified to homogeneity from Raw 264.7 cell extracts by a combination of ion exchange chromatography, anti-MNSF affinity chromatography and hydroxylapatite chromatography as described previously (19). In-gel digestion and MALDI-TOF were performed as described (19). Briefly, silver-stained spots were cut out of the gels and digested with 5 μg/ml V8 protease (Sigma) in 25 mM ammonium bicarbonate. Peptide mass fingerprinting was performed using a PerkinElmer Life Sciences/PerSeptive Biosystems Voyager-DE-RP MALDI-TOF mass spectrometer.

Peptide Affinity Chromatography—For affinity chromatography on peptide columns, synthetic peptides described above were coupled to Hi-Trap N-hydroxysuccinimide-activated agarose columns (Amersham Biosciences). Purified MNSF-Bcl-G was incubated with peptide columns, washed extensively, and eluted with 50 mM triethylamine, pH 11. The eluates were neutralized with 100 mM Tris-HCl, pH 7.4, subjected to SDS-PAGE, and detected by immunoblotting with anti-MNSF antibody.

Kinase Assay—To examine the effect of MNSF-Bcl-G complex on ERK activation, MEK kinase assay was performed. Activated GST-MEK1 (Upstate Biotechnology) was incubated with unphosphorylated GST-ERK2 (Upstate Biotechnology) in the presence or absence of MNSF-Bcl-G complex (0.5–2 μg) in a buffer containing 20 mM MOPS, pH 7.2, 5 mM EGTA, 10 μM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 500 μM ATP, 75 mM, for 30 min at 30 °C. The reaction mixture was immunoblotted with using anti-phospho-ERK1/2 antibody.

Cell Culture, the siRNAs, and Transfection of Cells—The Raw 264.7 macrophage-like cell line (ATCC TIB-71) was cultured routinely in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C and 5% CO2. SiRNA duplexes (siRNAs) were synthesized and purified by Qiagen, Inc. (Chatsworth, CA). The target sequences were as follows: MNSF siRNA-332 (5’-CCCAAAGTGCCAAACACG-GGA-3’), MNSF siRNA-437 (5’-CCACCCGCTATGCTAATAAA-3’), Bcl-G siRNA (5’-AGCATAATGGTGTGTTAA-3’). Scramble siRNA directed against 5’-GGACTCGACGCAATGGCGTCA-3’ was the negative control. Cells were treated with siRNA according to the instructions provided with the RNAiFect™ transfection reagent (Qiagen, Inc.). Raw 264.7 cells (1.2 × 106) were treated with 3 μg of siRNA in RPMI 1640 medium supplemented with 10% FBS in the presence of the RNAiFect™ transfection reagent. After a 48-h incubation at 37 °C, the medium containing the mixture of RNAiFectTM and siRNA was replaced by Dulbecco’s...
modified Eagle’s medium that contained 10% FBS and cells were incubated for a further 24 h.

Reverse Transcription (RT)-PCR—RT-PCR was performed for 30 cycles as described previously (19). The PCR primers used to detect mRNA are as follows: MNSFβ, CGCCCAAGGAACATCACC (sense) and GCCGCTG-TACCTCCAGAAGG (antisense) (222 bp); Bcl-G, CCCAAGCTCTCCAGAACAA (sense) and CTGAGCTCGGATCTCCTTTG (antisense) (213 bp). All short amplified PCR products were isolated and sequenced to verify their identity. PCR products were separated in 2% agarose gel electrophoresis and stained with ethidium bromide. In some experiments, signals were quantitated by densitometry and optical densities for MNSFβ and Bcl-G were normalized to the corresponding values for glyceraldehyde-3-phosphate dehydrogenase.

Mutagenesis and Transfection—Mutant MNSFβ (G76A) was generated by replacing the codon for glycine 76 with the codon for alanine by utilizing QuikChange site-directed mutagenesis (Stratagene). cDNAs encoding MNSFβ and Bcl-G were subcloned into the vector pcDNA3.1(+) (Invitrogen Corp.). Transient DNA transfections were conducted using Lipofectamine Plus reagent (Invitrogen) with the protocol provided by the manufacturer and 8 µg of plasmid DNA per 6-well plate.

Quantification of Cytokines—Murine cytokines were measured using sandwich ELISA (R&D System, Minneapolis, MN). The lower limits of detection for the cytokines were TNFα, 10 pg/ml; RANTES (regulated on activation normal T cell expressed and secreted), 20 pg/ml.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared following the methods of Dignam et al. (22). Protein-DNA complexes were detected using biotin end-labeled double-stranded DNA probes. The sequence for the NFκB site was: GGGGACCTTCCC. Oligonucleotides were labeled in a reaction using terminal deoxynucleotidyl transferase and biotin-14-dCTP (Pierce). The binding reaction was performed using the LightShift kit according to the manufacturer’s instructions (Pierce). The reaction products were separated on a 5% polyacrylamide gel in 0.5% Tris borate-EDTA, transferred onto a nylon membrane, and fixed on the membrane by UV cross-linking. The biotin-labeled probe was detected using chemiluminescence (LightShift kit; Pierce).

RESULTS

MNSFβ Complex Is Associated with ERKs—It has been reported that ubiquitin-like protein, ISG15, covalently binds to ERK1 (8). Thus, first we addressed whether MNSFβ could associate with the MAPK family including ERK, JNK, and p38 MAPK. Cell lysates were prepared from non-stimulated Raw 264.7 cells and immunoprecipitated with antib-

**TABLE 1**

Assignments of peptide fragments from a Staphylococcus V8 protease digest of the 33.5-kDa MNSFβ adduct

The 33.5-kDa MNSFβ adduct was digested by V8 protease and subjected to MALDI-MS analysis. The data in the second column are the mass values obtained experimentally, whereas the results in the third column are those calculated from the V8 protease fragmentation of the gene products of Bcl-G and MNSFβ. The fourth column indicates the number of the first and last amino acid of the identified Bcl-G and MNSFβ peptides, whereas the fifth shows the corresponding amino acid sequences. The amino acid sequences derived from MNSFβ are shown in italic.
MNSFβ siRNA increases LPS-induced TNFα production. A, Raw cells were transfected with RNAiFect™ transfection reagent alone or siRNA directed against MNSFβ, Bcl-G, or scramble siRNA. After 72 h of siRNA transfection, Raw cells were stimulated with 100 ng/ml LPS for 4 h. Then the concentration of TNFα in the supernatant was determined by ELISA as described under “Experimental Procedures.” The data represent one of three independent experiments with similar results. Values are shown as the mean ± S.D. of triplicate samples. *, p < 0.05 versus untreated; **, p < 0.01. B, transfection experiments were performed as described above, although Raw cells were stimulated with LPS for 12 h. The concentration of RANTES in the supernatant was determined by ELISA. The data represent one of three independent experiments with similar results. Values are shown as the mean ± S.D. of triplicate samples. *, p < 0.05 versus untreated; **, p < 0.01. C, MNSFβ and Bcl-G mRNA expression was analyzed by RT-PCR after treatment with siRNAs for 48 h. D, 33.5-kDa MNSFβ-Bcl-G complex was determined by Western blot analysis with antibody directed against MNSFβ after transfection with siRNAs for 72 h. The data represent one of three independent experiments with similar results. Lane 1, no siRNA; lane 2, scramble; lane 3, MNSFβ; lane 4, Bcl-G. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ies directed against MNSFβ, and associated proteins were analyzed by Western blot analysis by using anti-ERK1, anti-JNK, and anti-p38 antibodies. As shown in Fig. 1A, MNSFβ associated with ERKs, but not with other members of the MAPK family, under non-stimulated conditions. It should be noted that anti-ERK1 polyclonal antibodies recognize both the 44-kDa ERK1 and the 42-kDa ERK2. Immunoprecipitation of cell lysates with normal IgG followed by Western blot analysis revealed no detectable association of ERKs, indicating the specificity of MNSFβ with ERKs. In addition, concomitant immunoprecipitation with anti-ERK1 antibody and immunoblot analysis with anti-MNSFβ antibody confirmed the association between ERKs and the MNSFβ adduct (Fig. 1B). It should be pointed out that anti-MNSFβ antibody does not recognize free 8.5-kDa MNSFβ in murine T helper type 2 clone, D.10 cells (18). In Raw macrophages as well as D.10 cells, anti-MNSFβ antibody recognized several bands including a band of 33.5-kDa protein (Fig. 1A). We have demonstrated that Bcl-G, a novel proapoptotic member of the Bcl-2 family, is post-translationally modified by MNSFβ (19). Thus, it seemed likely that the 33.5-kDa MNSFβ adduct was the MNSFβ-Bcl-G complex. To determine this, we carried out Western blot analysis with anti-Bcl-G antibody. As depicted in Fig. 1B, antibody directed against Bcl-G reacted with the 33.5-kDa MNSFβ adduct, indicative of the covalent interaction of MNSFβ and Bcl-G as described (19). To confirm the interaction between MNSFβ and Bcl-G in Raw cells, MALDI-TOF was performed. The 33.5-kDa MNSFβ adduct was purified to homogeneity from Raw cell lysates by a combination of ion exchange chromatography, anti-MNSFβ affinity chromatography, and hydroxyapatite chromatography as described (19). The purified MNSFβ adduct was digested by V8 protease and subjected to MALDI-MS analysis. Table 1 shows the peptide masses of observed by MALDI-TOF mass fingerprinting of 33.5-kDa MNSFβ adduct purified from Raw cells. The resulting sets of peptide masses were then used to search the NCBI data base for potential matches, confirming the MNSFβ adduct as MNSFβ-Bcl-G complex. MNSFβ may conjugate to Bcl-G with a linkage between the C-terminal Gly-74 and Lys-110, as described (19). These results indicate that covalent MNSFβ-Bcl-G complex can specifically associate with ERKs in unstimulated Raw macrophages. MNSFβ-Bcl-G Directly Binds to ERK2—We next investigated the nature of the binding of MNSFβ-Bcl-G to ERKs. Purified MNSFβ-Bcl-G complex was incubated with GST or GST-ERK2 bound to GSH-Sepharose. The bead matrices were extensively washed before eluting bound proteins off of the bead matrices. MNSFβ-Bcl-G complex was separated by SDS-PAGE and detected by immunoblotting with anti-MNSFβ antibody. As shown in Fig. 2A, MNSFβ-Bcl-G bound to GST-ERK2 but not to GST. Excess peptide for the MEK dual phosphorylation site of ERK2 inhibited 50–60% this association, compared with a control peptide (mapping at the C terminus of ERK2), indicating that MNSFβ-Bcl-G might bind to the phosphorylation site. To confirm these results, we carried out peptide affinity chromatography. MNSFβ-Bcl-G was incubated with peptides derived from ERK2 immobilized on agarose columns. Bound and eluted MNSFβ-Bcl-G was resolved by SDS-PAGE and immunoblotted with anti-MNSFβ antibody. As can be seen in Fig. 2B, MNSFβ-Bcl-G bound to a column of immobilized the competitive peptide but not of control peptide, albeit this binding was not complete.

MNSFβ-Bcl-G Inhibits ERK Activation—To investigate whether ERK function is directly modified by MNSFβ-Bcl-G, we carried out MEK kinase assay. GST-MEK1 activated with c-Raf was employed in this assay. Activated GST-MEK1 was incubated with unphosphorylated GST-ERK2 in the presence or absence of MNSFβ-Bcl-G as described under “Experimental Procedures.” The reaction mixture was immunoblotted with using anti-phosho-ERK1/2 antibody. As depicted in Fig. 2C, ERK activation by MEK1 was significantly inhibited in the presence of MNSFβ-Bcl-G. These observations were consistent with the results of GST pulldown experiments showing that MNSFβ-Bcl-G directly binds to the phosphorylation site of ERKs (Fig. 2A).

MNSFβ siRNA Increases LPS-stimulated TNFα Production by Raw Cells—It has been reported that ERK pathway is involved in the regulation of TNFα production (23–26). Because our data suggested that covalent MNSFβ-Bcl-G complex affects ERK activation, it seemed likely that inhibition of MNSFβ expression would result in increased or decreased TNFα synthesis. Raw cells were transfected with scramble siRNA or siRNA directed against MNSFβ. After 72 h of siRNA transfection, Raw cells were stimulated with 100 ng/ml of LPS for 4 h. Then the concentration of TNFα in the supernatant was determined by ELISA. Production of the TNFα in LPS-stimulated Raw cells transfected with MNSFβ siRNA-437 was significantly (over 2-fold) up-regulated, compared with the cells with scramble siRNA (Fig. 3A). RT-PCR analysis demonstrated that MNSFβ siRNA-437, but not control scramble siRNA, specifically reduced the expression of MNSFβ but not glycerol-3-phosphate dehydrogenase (GAPDH) (Fig. 3C). Western blot analysis demonstrated that MNSFβ siRNA-437 reduced complex formation of MNSFβ with Bcl-G (33.5-kDa MNSFβ adduct) (Fig. 3D, lane 3). Like MNSFβ siRNA, Bcl-G siRNA also inhibited the complex formation of MNSFβ with Bcl-G (Fig. 3D, lane 4). We also determined whether Bcl-G siRNA would affect TNFα production by LPS-stimulated Raw cells. As can be seen in Fig. 3A, Bcl-G siRNA caused a significantly increased TNFα production, although the effect was less than that seen with MNSFβ siRNA. We did not observe a synergistic effect of transfection with both siRNA. To explore the RAII expression at the mRNA level, we performed RT-PCR on total RNA isolated from siRNA-transfected Raw cells. MNSFβ siRNA-437 up-regulated 60% TNFα expression (data not shown). We also investigated whether MNSFβ siRNA would affect RANTES, a member of C-C chemokine superfamily, production by LPS-stimulated Raw cells. MNSFβ siRNA-437 caused a significantly increased RANTES production (Fig. 3B), indicative of the involvement of MNSFβ in RANTES production by LPS-stimulated Raw cells.

MNSFβ-Bcl-G Association with ERK Is Decreased in a LPS-dependent Manner—To determine whether the interaction of MNSFβ-Bcl-G with ERK could be altered by treatment with LPS, Raw cells were stimulated with 100 ng/ml of LPS followed by immunoprecipitation with antibody against ERK1 or IgG and Western blot analysis to detect MNSFβ-Bcl-G complex. The binding of MNSFβ adduct with ERKs was decreased at both 15 and 45 min post-LPS stimulation (Fig. 4, upper panel). This blot was subsequently re-probed to ensure equal loading of ERKs from each condition (Fig. 4, middle panel). Control immunoprecipitates with normal rabbit IgG did not demonstrate an association of MNSFβ adduct

![Figure 4](image-url)

**Figure 4.** MNSFβ-Bcl-G association with ERK is decreased after LPS stimulation. Raw cells were treated with 100 ng/ml LPS for 5, 15, 45, or 90 min. Immunoprecipitation (IP) assays were performed with either anti-ERK antibody followed by Western blot (WB) analysis with either anti-MNSFβ antibody (top panel) or anti-ERK antibody (middle panel). Immunoprecipitation assays were also performed with normal IgG followed by Western blot analysis with anti-MNSFβ antibody (bottom panel).
with ERKs (Fig. 4, bottom panel). Taken together, these results, which were seen in three independent experiments, demonstrated that LPS treatment results in decreased interaction of MNSF/H9252/H18528 Bcl-G and ERKs.

**MNSFβ Inhibits LPS-induced ERK Activation**—LPS is known to activate three major MAPKs, ERK1/2, p38, and JNK, which play an important role in LPS-induced cellular effects. We determined whether MNSFβ is involved in these pathways by treating cells with an MNSFβ-specific siRNA. In Raw 264.7 cells, LPS-stimulated ERK1/2 phosphorylation peaked at 20 min after LPS stimulation and was detected slowly over 1 h (Fig. 5A). Phosphorylation of p38 and JNK also peaked at 20 min...
and returned quickly to basal levels (Fig. 5, B and C). In Raw 264.7 cells treated with MNSFβ siRNA-437 LPS-induced ERK1/2 phosphorylation was enhanced (Fig. 5A), and LPS-induced p38 and JNK phosphorylation were unaffected (Fig. 5, B and C). Forty-eight h after transfection, cell lysates were immunoprecipitated with anti-Bcl-G antibody and were analyzed by Western blot analysis with antibody directed against MNSFβ. Lane 1, Raw cells transfected with vector alone; lane 2, transfected with Bcl-G together with MNSFβ; lane 3, Bcl-G together with MNSFβ (G74A). Raw cells were transfected with MNSFβ together with Bcl-G. Forty-eight h after transfection, cells were stimulated with 100 ng/ml LPS, and then the phospho-ERK1/2 and total ERK1/2 in the cell lysates were detected by Western blot. The intensity of the signals as determined by densitometric scanning is expressed as fold change relative to that of the untreated cells. Open bars represent vehicle-treated control cell. Closed bars represent transfected cells. Values are given as mean ± S.D. (n = 3). A representative autoradiograph is shown. *, p < 0.05 compared with transfected cells.

**MNSFβ Inhibits LPS-induced TNFα Production**—The role of MNSFβ in regulating LPS-induced TNFα production was addressed in transfection studies. As can be seen in Fig. 6A, transfection with pcDNA3.1-MNSFβ resulted in a significant inhibition of LPS-induced TNFα production. In contrast to MNSFβ, Bcl-G alone did not show any inhibitory effect. However, co-transfection with MNSFβ and Bcl-G greatly decreased the TNFα production. Unlike co-transfection with wild-type MNSFβ, co-transfection of a mutant MNSFβ (G74A) and Bcl-G did not result in a decrease in the TNFα production. These results suggest that Bcl-G may enhance and/or stabilize the inhibitory activity of MNSFβ by complex formation. Further supporting this idea is the observation that overexpression of mutant MNSFβ (G74A) fails to form
a MNSFβ-Bcl-G complex (Fig. 6B, lane 3). We also determined whether ERK activation is affected by transfection with pcDNA3.1-MNSFβ. Raw cells co-transfected with MNSFβ and Bcl-G significantly decreased the ERK1/2 phosphorylation at 20–40 min (Fig. 6C). These results are in good accordance with findings by siRNA experiments (Fig. 5).

MNSFβ Inhibits LPS-induced NFκB Activation—NFκB is involved in the LPS signaling cascade leading to TNFα production. Gel shift assay was performed to determine whether MNSFβ is relevant to LPS-induced NFκB activation. Raw cells were transfected with either siRNA directed against MNSFβ or scramble siRNA. After 72 h of siRNA transfection, Raw cells were treated with 100 ng/ml LPS for 60 min. EMSAs were performed using a consensus NFκB probe in the presence or absence of excess of unlabeled competitor. A representative blot is shown in the upper panel. The means ± S.D. of six experiments are shown in the bottom panel. *p < 0.05 versus control (LPS plus scramble).

![Image](https://example.com/image.png)

**FIGURE 7.** MNSFβ siRNA slightly enhances LPS-induced NFκB activation. Raw cells were transfected with either MNSFβ siRNA-437 or scramble siRNA. After 72 h of siRNA transfection, Raw cells were treated with 100 ng/ml LPS for 60 min. EMSAs were performed as described under “Experimental Procedures.”

DISCUSSION

LPS induces the signaling pathways leading to the activation of the mitogen-activated protein kinases, including ERK1/2, JNK, and p38 (27–29). LPS-induced ERK activation is required for the expression of TNFα, a potent proinflammatory cytokine, in Raw cells (23, 25, 26). In this study, we present data that the MNSFβ regulates LPS-mediated ERK activity in Raw cells. First, MNSFβ siRNA increases LPS-stimulated TNFα and RANTES production (Fig. 3). Second, MNSFβ siRNA up-regulates LPS-mediated ERK activation (Fig. 5). Third, transfection with MNSFβ expression construct significantly decreased ERK1/2 phosphorylation and TNFα production (Fig. 6). These results suggest that MNSFβ functions as a negative regulator of the ERK pathway likely by down-regulating ERK activity following LPS treatment.

We have previously demonstrated that Bcl-G, a novel proapoptotic member of the Bcl-2 family, is covalently modified by MNSFβ in concanavalin A-stimulated D.10 G4.1 cells, a murine T helper clone type 2 (19). In this study, we showed that MNSFβ covalently binds to Bcl-G in unstimulated Raw 266.7 macrophage cell line. Thus, the mechanism of modification of Bcl-G by MNSFβ may differ in each cell type. We also demonstrated that MNSFβ conjugates to Bcl-G with a linkage between the C-terminal Gly-74 and Lys-110 as previously described in D.10 cells (19). We presented data showing that this isopeptide bond is important for MNSFβ-Bcl-G interaction in Raw cells (Fig. 6).

We showed that MNSFβ-Bcl-G directly binds to ERK inhibiting ERK phosphorylation by MEK (Figs. 1 and 2). This association is decreased in a LPS-dependent manner (Fig. 4). Interestingly, blocking peptide for the phosphorylation site on ERK2 inhibited this association, suggesting that this phosphorylation site is critical for the association with the MNSFβ adduct (Fig. 2). It cannot be ruled out that another molecule(s) might be implicated in the complex formation in vivo. This is an intriguing possibility that needs to be carefully addressed experimentally. Shin et al. (30) mentioned that the p21-activated kinase 2 (PAK2) directly binds to ERKs. The formation of a multimeric complex consisting of ERK/PAK2-PI3-kinase interacting exchange factor (PIX) is required for fibroblast growth factor-induced neurite outgrowth (30). In addition, complex formation among ERK, 14-3-3, and Heat shock factor 1 during stress is evident (31).

We presented the evidence that the MNSFβ adduct directly regulates ERK activation (Fig. 2). It should be pointed out that MNSFβ is an aggregable polypeptide (14, 15). Even recombinant MNSFβ has a tendency to form aggregation (15). Thus, it might be inferred that Bcl-G functions as a stabilizer of this aggregable polypeptide. Indeed, knock-down experiments using Bcl-G siRNA showed a significant increased TNFα production (Fig. 3). In addition, transfection experiments using a point mutation showed that covalent interaction between MNSFβ and Bcl-G enhances the inhibitory activity (Fig. 6). Interestingly, ubiquitin-related BAG-1 (Bcl-2-associated athanogene-1) interacts with anti-apoptotic protein, Bcl-2, and enhances the anti-apoptotic activity of Bcl-2 (32). Thus, it is possible that MNSFβ may regulate the pro-apoptotic protein, Bcl-G. We are currently investigating whether MNSFβ is involved in the mechanism of apoptosis.

Like MNSFβ, other ubiquitin-like proteins are also implicated in MAPK pathway. The ubiquitin-related BAG-1 described above binds to and activates the kinase Raf-1 (33). Modification of Smad4 with the ubiquitin-like protein SUMO-1 is enhanced by TGFβ-induced activation of the p38 MAP kinase pathway (34). Recently, Malakhov et al. (8) demonstrated that ubiquitin-like protein ISG15 modifies three key regulators of signal transduction, phospholipase Cγ1, Jak1, and ERK1. ISG15 conjugates to these target proteins via an isopeptide bond in a manner similar to ubiquitin and other ubiquitin-like proteins. In contrast to ISG15, MNSFβ non-covalently binds to ERK. In addition, MNSFβ failed to bind to Jak1 (data not shown). It might be inferred that ubiquitin-like proteins are differentially involved in the regulation of the ERK-MAPK cascade. Interestingly, MNSFβ, ISG15, and FAT10, ubiquitin-like protein involved in apoptosis, are induced by IFNγ (16, 35–38).

It is well known that NFκB and MAPK signaling proteins are activated by LPS. Many reports have demonstrated that inhibition of both NFκB and ERK-MAPK signaling affects LPS-mediated TNFα production in Raw cells (23, 25, 26). In this study, we demonstrated that MNSFβ affects ERK-MAPK cascade rather than NFκB signaling (Figs.
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