A Splicing Variant of NME1 Negatively Regulates NF-κB Signaling and Inhibits Cancer Metastasis by Interacting with IKKβ*

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Dong-Joo You†, Cho Rong Park‡, Hyun Bok Lee†, Mi Jin Moon†, Ju-Hee Kang§, Cheolju Lee*, Seong-Hyun Oh‖, Curie Ahn**, Jae Young Seong†, and Jong-Ik Hwang†

From the †Graduate School of Medicine, Korea University, 73 Incheon-ro, Seongbuk-gu, Seoul 136-791, Korea, the ‡National Cancer Center, Goyang-si, Gyeonggi-do 410-769, Korea, the §Life Sciences Division, Korea Institute of Science and Technology, Seongbuk-gu, Seoul 136-791, Korea, the ¶College of Pharmacy, Gachon University, Incheon 406-840, Korea, and the **Transplantation Research Institute, Cancer Research Institute, Seoul National University, Yongun-dong, Jongno-gu, Seoul 110-799, Korea

Background: We found NME1L to be an IKKβ-interacting protein. The physiological functions of NME1L in NF-κB signaling and cellular responses should be studied.

Results: Specific interaction between NME1L and IKK-β negatively regulates NF-κB activation and metastatic activity of cancer cells.

Conclusion: NME1L may be a useful weapon to repress cancer development.

Significance: These findings should help in understanding the antitumor mechanism of NME1L.

IKKβ functions as a principal upstream activator of the canonical NF-κB pathway by phosphorylating IκB, leading to its proteasomal degradation. Because IKKβ is considered a therapeutic target, understanding its regulation may facilitate the design of efficient regulators of this molecule. Here, we report a novel IKKβ-interacting molecule, NME1L, a splicing variant of the NME1 protein. NME1 has attracted attention in cancer research because of its antimetastatic activity and reduced expression in multiple aggressive types of cancer. However, the effect was just moderate but not dramatic in anti-cancer activities. We found that only NME1L interacts with IKKβ. Exogenous expression of NME1L resulted in a potent decrease in TNFα-stimulated NF-κB activation, whereas knockdown of NME1/NME1L with shRNA enhanced activity of NF-κB. NME1L down-regulates IKKβ signaling by blocking IKKβ-mediated IκB degradation. When NME1L was introduced into highly metastatic HT1080 cells, the mobility was efficiently inhibited. Furthermore, in a metastasis assay, NME1L-expressing cells did not colonize the lung. Based on these results, NME1L is a potent antimetastatic protein and may be a useful weapon in the fight against cancers.

The nuclear factor-κB (NF-κB) family of proteins was initially characterized as a group of transcription factors critical in the immune response to pathogens and other foreign bodies. Subsequently, these proteins have been found to regulate expression of a variety of genes responsible for diverse biological processes, including cell proliferation, migration, survival, and stress response (1, 2). Many of these genes play critical roles in cancer development and progression (3). Five members of the NF-κB family are expressed in mammals, and they interact to form homo- or heterodimers (4, 5). In the basal states, NF-κB stays in the cytoplasm by interacting with IκB, an inhibitory protein. IκB phosphorylation, ubiquitination, and subsequent proteasomal degradation frees NF-κB to enter the nucleus, where it activates the expression of target genes. Thus, diverse upstream signaling pathways converge at the IκB kinase (IKK) complex, which phosphorylates IκB and leads to NF-κB activation.

The IKK complex contains the serine/threonine protein kinases IKKα and IKKβ as well as a regulatory protein, NEMO (6, 7). Catalytic activation of IKK is dependent on the phosphorylation of serine residues in its kinase domain, either by upstream kinases, such as cyclin-dependent kinase 9 (TAK), an atypical class of protein kinase C enzymes (PKC), and protein kinase B (Akt), or, in certain circumstances, by autophosphorylation (8–11). The activation process also depends on nondestructive Lys63-linked ubiquitination of NEMO (12). Furthermore, temporary interactions with the chaperones heat shock protein 90 (HSP90) and Cdc37 appear to be essential for the maturation of de novo synthesized IKKs into enzymatically competent kinases (13, 14). HSP90 may also help IKK escape autophagy-mediated degradation. Our previous work provides evidence for this function, demonstrating that HSP90 competes with Kelch-like ECH-associated protein 1 (KEAP1), which inhibits IKKβ phosphorylation and mediates autophagy-dependent degradation (15).

As crucial factors in NF-κB signaling, we expect IKKs to be regulated by many cytosolic proteins. Without strict regulation, IKKs could elicit aberrant cellular responses, leading to disease. In particular, IKKβ has been reported to induce tumor development via NF-κB activation and the phosphorylation-dependent inhibition of tumor suppressors (16, 17). In many cancers, including lymphoma and prostate cancer, persistent activation...
of the IKK complex results in the constitutive release of NF-κB, driving expression of genes with important functions in cell cycle progression, tropism, or tumor cell migration (18, 19). Consequently, pathophysiological effects mediated by IKKs have grabbed the attention of many researchers, and the protein complex is considered a promising target for modulating aberrant responses related to NF-κB signaling. Despite extensive study of IKK activation, the molecular mechanisms involved in IKK deactivation and negative regulation remain poorly understood.

Some regulators of proliferation, migration, and programmed cell death also have a critical role in determining the fate of the cell. Non-metastatic cells 1 (NME1), also called NM23-H1, was the first metastasis suppressor gene discovered (20). The 18-kDa protein it encodes is ubiquitously expressed in most cellular compartments, and it functions as a nucleotide diphosphate kinase, histidine/aspartic acid-specific protein kinase, and 3′-5′ exonuclease (21–23). NME1 tends to undergo homo- and heterohexameric oligomerization, which is necessary for enzymatic activity. In vitro research suggests that metastasis suppression depends on the integrity of the kinase activity of NME1; this subject is still under debate, however, because mutations targeting kinase activity may affect other molecular functions as well, such as other enzymatic activities and molecular interactions with binding partners (24). The 3′-5′ exonuclease activity of NME1 has also been reported to be essential for antimetastatic activity in human melanoma (25). Exonucleases edit and proofread DNA; NME1 uses its exonuclease activity to promote repair of ultraviolet (UV) radiation-induced DNA damage and mutagenesis, suppressing UV-induced skin cancer (26, 27). However, this enzymatic activity is not enough to explain the antimetastatic function of NME1 in all cancers; NME2, which is homologous to NME1, has similar enzymatic activity, but its expression is not associated with cancer cell migration and invasion (28, 29).

Studies of NME1-binding proteins indicate that metastasis suppression by NME1 is probably due to interaction with signaling molecules. GTP-binding proteins play a pivotal role in cell growth and migration. Active Rad (GTP-Rad), for example, activates downstream effectors, such as calmodulin, calmodulin kinase II, and β-tropomyosin, which are responsible for cytoskeletal organization and cell motility (30). Direct interaction with the Rad protein enables NME1 to regulate Rad GDP/GTP cycling by recruiting either a GTPase-activating protein or a guanine exchange factor (31, 32). In addition, NME1 negatively regulates Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 (Cdc42) by interacting with and inhibiting their specific exchange factors, T-cell lymphoma invasion and metastasis 1 (Tiam1) and Dbl-1, respectively (33). NME1 also represses the Ras/mitogen-activated protein kinase (MAPK) signaling pathway by interacting with kinase suppressor of Ras (KSR) (34). Transforming growth factor-β (TGF-β) signaling is well known to stimulate the invasion and metastasis of cancer cells. NME1 interacts with serine/threonine kinase receptor-associated protein (STRAP) through an intermolecular disulfide bond, resulting in the down-regulation of TGF-β signaling (35). Finally, gene silencing experiments using RNA interference have demonstrated that NME1 is implicated in maintaining cell-cell adhesion mediated by E-cadherin (28).

There have been reports of decreased NME1 levels in many cancer cells capable of invasion and metastasis (36, 37). However, repeated Western blots have revealed that, in actuality, NME1 levels are still readily detectable compared with cells that exhibit low metastatic activity. This suggests that protein quantity may not fully explain the antimetastatic activity of NME1. Interestingly, the NME1 gene produces two splicing variants. With the exception of 25 additional N-terminal amino acids in the long form, their amino acid sequence is identical. However, most functional studies of NME1 have been done on the short form, perhaps due to its abundance in the cell. The long form may be relatively rare, but it may play roles that the short form does not.

In the present study, we identified the long form of NME1 (NME1L) as a novel IKKβ-binding partner. We demonstrated that the NME1L N-terminal region is the specific binding target of IKKβ. Ectopic regulation of NME1 influenced TNFα-stimulated NF-κB activation by inhibiting the activity of IKKβ. Furthermore, NME1L down-regulated expression of TNFα target genes involved in cancer cell motility.

**Materials**—All primers for gene cloning and materials for expression vector construction were obtained from Cosmo-genetech (Seoul, Korea), and DNA sequencing was conducted by the same company. HEK293 and HT1080 cells were purchased from the American Type Culture Collection (Manassas, VA). Cell culture media, including Dulbecco’s modified eagle medium (DMEM) and Opti-MEM, were obtained from Invitrogen and WELGENE Inc. (Daegu, Korea). Human recombinant TNFα was purchased from R&D Systems, protease inhibitor mixture was purchased from Roche Applied Science, and anti-NME1 and anti-tubulin antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-IKKβ antibodies and anti-phospho-IKKα/β antibodies were from Cell Signaling Technology. All other chemical reagents and antibodies, such as anti-hemagglutinin (HA) and anti-FLAG antibodies, were purchased from Sigma.

**Plasmid Constructs and Small Hairpin RNAs (shRNAs)**—All IKKβ constructs, including fragments and epitope-tagging forms, have been described previously (15, 38). cDNAs for NME1L, NME2, and NME3 were cloned via the reverse transcription polymerase chain reaction (RT-PCR), using RNA from MCF7 cells. PCR with the appropriate primers and a subsequent restriction enzyme digest were performed to insert the genes in mammalian expression vectors. The short form of NME1, designated NME1L throughout the rest of this work, was obtained via PCR using NME1L as a template because the former is an N-terminal deletion form of the latter. To characterize NME1L expression, the entire cDNA sequence, including the 3′-untranslated region (3′-UTR), was obtained via RT-PCR and cloned into pcDNA3.1. The genetic sequence encoding the 25 N-terminal amino acids was obtained via PCR and subse-
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fluently inserted into the Nhel/BamHI site of a pcDNA3.1zeo expression vector containing the green fluorescent protein (GFP) gene. All constructs were confirmed by DNA sequencing. Three shRNAs for human NME1, constructed in the pLKO.1 vector, were obtained from Thermo Fisher Scientific; their inhibitory efficiency was examined by transfecting them into HEK293 cells with pcDNA3 HA/NME1 plasmids. shRNA-resistant forms of NME1 and NME1L were constructed by site-directed mutagenesis using PCR and inserted into pcDNA3 HA vector.

**Immunoprecipitation and Western Blotting**—Cells were maintained in DMEM in the presence of 10% fetal bovine serum (FBS) and penicillin/streptomycin. A day before transfection, HEK293 cells were plated in 600-mm dishes at 1 × 10⁶ cells/dish. The relevant plasmids were transfected with Lipofectamine 2000 transfection reagent (Invitrogen), following the manufacturer’s instructions. After 36 h, cells were washed with ice-cold PBS and solubilized with 1 ml of lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor mixture, for 30 min on ice. The lysates were centrifuged at 15,000 rpm for 15 min at 4 °C, and then supernatants were incubated with anti-FLAG antibody-conjugated beads (Sigma) at 4 °C for 2 h. The beads were washed four times with lysis buffer. Bound proteins were eluted by boiling in SDS sample buffer and separated by SDS-PAGE. To examine the expression of specific proteins, 20 μg of clarified lysate was loaded into each well of the gel. HEK293, MCF7, HT1080, PC-3, and HeLa cells grown in DMEM or RPMI1640 containing 10% FBS were lysed with buffer containing 1% Triton X-100, and HeLa cells grown in DMEM in the presence of 10% fetal bovine serum were lysed with buffer containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture to investigate NME1L expression. Proteins were transferred onto a nitrocellulose membrane and probed with the relevant antibodies and then detected using the ECL assay kit (GE Healthcare). To determine phosphorylation of IKKβ, cells were starved for 24 h, treated with TNFα for 15 min, and applied for Western blotting with phospho-specific antibodies.

**Reporter Gene Assays**—Analysis of TNFα-stimulated luciferase activity, driven by a synthetic NF-κB promoter, was performed as described previously (38). Cells (80,000/well) were cultured overnight in 24-well plates and transfected with a liposomemay optimum containing pGL3/NF-κB-luciferase reporter gene (50 ng), pCMV/β-gal (20 ng), and other relevant plasmids (400 ng). After 48 h, cells were treated with TNFα (10 ng/ml; R&D Systems) for 6 h, washed with PBS, and solubilized with lysis buffer. The luciferase activity of cell extracts was determined using the standard luciferase assay system from BioTek Instruments, Inc. (Winooski, VT). To determine transfection efficiency, luciferase activity was normalized to β-galactosidase (β-gal) activity. All data points were calculated from at least three independent experiments and normalized to untreated groups.

**Lentivirus Generation and Establishment of NME1 or NME1L-expressing Cells**—Wild-type NME1L and HA-tagged forms of NME1L and NME1 were cloned into a lentiviral FUGW vector containing a ubiquitin promoter (39). Cells were infected with lentivirus produced by transfecting the expression and accessory plasmids into HEK293T cells using calcium phosphate precipitation. The supernatant of virus-producing cells was harvested 48 h post-transfection and concentrated on Amicon Ultra columns (Millipore, MA). An enhanced GFP-expressing lentivirus was used as a control to monitor viral titer. A 4:1 virus/host cell ratio was used to infect HT1080 cells. Protein expression was characterized using Western blots.

**Immunofluorescence Assays**—HEK293 cells were grown on Matrigel-coated glass coverslips in 24-well plates. Cells were transfected with 1 μg of NME1 or NME1L plasmids. The next day, cells were cultured with 1% FBS-containing DMEM overnight, treated with 10 ng/ml TNFα for 30 min, and then fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 min and then incubated with anti-HA antibodies and anti-p65 antibodies in PBS containing 10% (v/v) FBS for 2 h at room temperature. Coverslips were washed and incubated with Cy3-conjugated anti-mouse IgG antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG for 1 h. Staining was analyzed with a confocal LSM510 microscope (Carl Zeiss Microimaging Inc.). HEK293 cells were infected with lentivirus containing HA-NME1L or HA-NME1 to establish stably expressed genes. These cells were used to examine the effect of NME1L on TNFα-mediated NF-κB translocation.

**Cell Growth Assay**—To investigate cell proliferation, we used Cell Counting Kit-8 (CCK-8) from Dojindo Molecular Technologies, Inc. (Rockville, MD). HT1080 cells were seeded in 96-well plates at 1,000 cells/well. Cells were cultured for 48, 72, or 96 h, and then cells were incubated with 10 μl of CCK-8 solution for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader.

**Transwell Migration and Invasion Assays**—HT1080 cells were incubated with 10 ng/ml TNFα for 24 h. A single cell suspension of HT1080 cells was obtained via trypsin-EDTA treatments for 2 min at 37 °C. Prior to the migration assay, transwell inserts with 8-μm pores (Corning Inc.) were coated with 100× diluted Matrigel (Invitrogen) for 2 h at 37 °C. Cells (5 × 10⁴) in DMEM containing 0.1% BSA were added to the transwell inserts. The bottom of the wells was filled with DMEM containing 10% FBS. Plates were maintained in a humidified incubator with 5% CO₂ at 37 °C for 24 h. For the invasion assay, 20 μl of 1:6 diluted Matrigel was applied to the upper surface of the transwell inserts, and the wells were placed in an incubator to allow the Matrigel to solidify. Cells were added to the upper chamber in 0.1% bovine serum albumin (BSA) medium. The bottom chambers contained DMEM supplemented with 10% FBS. Cells were allowed to invade at 37 °C for 24 h. Transwell inserts were washed in PBS, and cells in the inner well that had not migrated were removed with a cotton swab. Membranes were fixed in 4% formalin and stained with hematoxylin, and then cells that had migrated were counted in five high power fields under the microscope.

**Quantitative RT-PCR**—To quantitate mRNA levels, total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was prepared using reverse transcriptase from Promega, and real-time quantitative PCR was performed using IQ™ SYBR Green Supermix and an iCycler PCR thermocycler (Bio-Rad) with gene-specific primer sets designed by Beacon Designer version 2.1 (Biosoft Interna-
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FIGURE 1. The N-terminal region of NME1L is essential for interaction with the leucine zipper domain of IKKβ. A and B, interaction between IKKβ and NME1L. Lysates of HEK293 cells cotransfected with the FLAG- or HA-tagged forms of each gene were immunoprecipitated (IP) with anti-FLAG antibodies and analyzed by Western blot (IB), using anti-FLAG or anti-HA antibodies. LC, immunoglobulin light chain. C, alternative splicing results in the expression of two forms of NME1. Analysis of the coding region shows that the long form has an ATG codon in the second exon that does not exist in the short form. Both variants have an identical amino acid sequence, except for 25 additional residues in the N terminus of the long form. RT-PCR with a primer pair common to both forms produced a strong signal for the short form and a very weak signal for the long form. The reaction with a forward primer from exon 2 and a common reverse primer produced a long form-specific signal. A Western blot of cell lysates shows that faint signals on strong NME1 bands were detected with anti-NME1 antibodies by expanded exposure time of x-ray film. aa, amino acids. D, NME1 does not interact with IKKβ. Lysates of HEK293 cells expressing IKKβ and NME1 were immunoprecipitated with anti-FLAG antibodies and analyzed by Western blot. E, a form of NME1L with no tag also interacts with IKKβ. To exclude an effect of the HA epitope on the interaction, NME1L was expressed with FLAG-IKKβ in HEK293 cells. Cell lysates were subjected to immunoprecipitation and a subsequent Western blot with anti-FLAG antibodies. F, NME1L was cotransfected into HEK293 cells with IKKβ fragments (V, vector; W, whole protein; N, kinase domain; M, leucine zipper domain; C, helix-loop-helix domain and NEMO-binding domain). Cell lysates were subjected to immunoprecipitation and Western blots with specific antibodies. LC, immunoglobulin light chain; HC, immunoglobulin heavy chain.

RESULTS

The N-terminal Region of NME1L Binds IKKβ—We identified NME1L as a novel binding partner of IKKβ in a yeast two-hybrid screen of a human lymphocyte cDNA library (data not shown). To confirm the interaction between NME1L and IKKβ in mammalian cells, we subjected HEK293 cells expressing FLAG-IKKβ and HA-NME1L to immunoprecipitation with anti-FLAG antibodies. In a subsequent immunoblot analysis, HA-NME1L was detected in theFLAG-IKKβ precipitate (Fig. 1A). The interaction was also confirmed via immunoprecipitation of HEK293 cells expressing HA-IKKβ and FLAG-NME1L (Fig. 1B). According to the NCBI database, there are two splic-
ing variants of NME1. The coding sequence for the long form, NME1L, consists of six exons, with the translation start codon located in the second exon; this results in a 177-amino acid protein. The second exon is missing in the short form’s coding sequence; this 152-amino acid protein (NME1) is translated starting at an ATG codon near the beginning of the third exon (Fig. 1C). Thus, NME1L has 25 more amino acids than NME1 in the N-terminal region. To examine the expression of these variants, RT-PCR was performed on HEK293 cells using a forward primer from exon 1 and a reverse primer from exon 5. Expression of the short form was abundant, but the long form produced only a very faint band. A reaction with primers specific for the long form produced an obvious band, however, implying that both forms are transcribed. Western blots using NME1-specific antibodies revealed that NME1 is highly expressed in most cell lines, including HEK293, MCF7, PC-3, U87MG, and HeLa cells. However, NME1L was barely detected as a separated band with a long exposure time, implying that NME1L may exist in a tiny amount compared with NME1 (Fig. 1C). The long term exposure revealed an obvious band below the endogenous NME1. This band may be another splicing form. However, we could not find any other splicing form from GenBankTM database analysis. Another possibility is that this may be NME2 or just nonspecifically detected by the antibody, because this band was not decreased by shRNA for NME1 and NME2 (data not shown). To confirm that the translation start site in NME1L is valid, we cloned the entire cDNA of the gene and expressed it in HEK293 cells. Western blots showed that although expression efficiency was a bit low, NME1L was readily expressed, suggesting that the N-terminal region of NME1L may be involved in interaction with IKKβ. Interestingly, when NME1L was overexpressed in HEK293 cells, endogenous NME1, as well as NME1L, was coprecipitated with FLAG-IKKβ using FLAG antibodies (Fig. 1E). Because NME proteins usually exist as homo- or hetero-oligomers with other NME isoforms, it is reasonable to believe that NME1L readily undergoes complex formation with NME1.

To determine the region of IKKβ involved in NME1L interactions, immunoprecipitation was carried out on HEK293 cells expressing NME1L and various IKKβ fragments. The resulting immunoblots showed that the leucine zipper domain of the IKKβ regulatory region is responsible for interaction with NME1L (Fig. 1F).

NME1L Negatively Regulates TNFα-stimulated NF-κB Signaling Pathways—Because IKKβ is a major upstream mediator of NF-κB activation, we investigated the effect of the NME1L-IKKβ interaction on NF-κB signaling using a luciferase reporter gene. We found that exogenous NME1L dramatically repressed TNFα-stimulated NF-κB activation; NME1 also inhibited NF-κB activation, although with less potency (Fig. 3A). Furthermore, NF-κB activity was down-regulated by NME1L in an expression-dependent manner (Fig. 3B). The results of our binding experiments and reporter gene assays indicated that the N-terminal sequence of NME1L could play a pivotal role in the regulation of IKKβ-mediated NF-κB signaling. To investigate, we examined changes in TNFα-stimulated NF-κB activity in response to the expression of a GFP fusion protein containing the 25 N-terminal amino acids of NME1L (N25-GFP). The fusion protein had no effect on NF-κB activity and did not coprecipitate with IKKβ (data not shown). These data imply that the N-terminal alone does not constitute a functional
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FIGURE 3. NME1L inhibits NF-κB signaling pathways. A, ectopic expression of NME1L suppresses TNFα-stimulated NF-κB activation. HEK293 cells were transfected with NF-κB luciferase reporter plasmids, pCMV/β-gal, and either NME1L or NME1. Two days later, cells were treated with TNFα (10 ng/ml) for 6 h, and then reporter gene assays were performed. NT, not treated. B, NME1L inhibits TNFα-stimulated NF-κB activation in an expression-dependent manner. HEK293 cells were transfected with 0.1 μg of NF-κB luciferase reporter plasmids and varying amounts of NME1L plasmids, and then reporter gene assays were performed as described in A. C, NME1L partially blocks IkB degradation. HEK293 cells were transfected with expression plasmids for NME1L and FLAG-IkB. After 48 h, cells were treated with TNFα (10 ng/ml) for 30 min and harvested with lysis buffer. SDS-PAGE followed by Western blots with anti-IkB or anti-FLAG antibodies. Signal intensity was measured using the ImageJ program. D, HEK293 cells on coverglass were transfected with HA-NME1L. The next day, cells were serum-starved overnight and treated with TNFα (10 ng/ml) for 30 min. After fixation with 4% paraformaldehyde, cells were stained with anti-HA antibodies (red) and anti-p65 antibodies (green) and observed under the confocal microscope. Arrowheads, exogenous NME1L-expressing cells. All experiments were done three times. A and B, data from three independent experiments, done in triplicate, were combined and are presented as the mean ± S.E. (error bars). *, compared with TNFα-treated in vector, p < 0.01.

motif, although it may play a physiological role in combination with other regions of NME1L or as an oligomeric complex of proteins.

IKK phosphorylates IkB, which then undergoes proteasomal degradation, allowing NF-κB to translocate to the nucleus (7). To examine the role of NME1 in IkB degradation, we infected HEK293 cells with a lentivirus containing HA-NME1 or HA-NME1L and then treated the cells with TNFα. Immunoblots showed that IkB was readily degraded in response to TNFα stimulation, and the degradation rate fell dramatically in the presence of NME1L (Fig. 3C). In addition, nuclear translocation of p65, a subunit of the NF-κB transcription complex, decreased in the presence of NME1L (Fig. 3D). These results demonstrate that NME1L negatively regulates TNFα-stimulated NF-κB activation by directly interacting with IKKβ.

To determine whether endogenous NME1L modulates NF-κB signaling, we knocked down NME1L/NME1L expression using plasmid-based shRNAs. First, we selected three shRNA constructs with different inhibitory activity for NME1L/NME1L expression (Fig. 4A). Next, we cotransfected shRNA plasmids with a luciferase reporter gene into HEK293 cells and treated the cells with TNFα. NME1/L/NME1L knockdown cells showed higher basal luciferase activity and augmentation of TNFα-induced activity, observations tightly associated with the inhibitory efficiency of the shRNA (Fig. 4B). Luciferase activity was also enhanced in an shRNA inhibition dose-dependent manner (Fig. 4C). HEK293 cells lacking endogenous NME1s were established by infecting them with lentivirus containing shRNA. NF-κB activity stimulated with TNFα in these cells was markedly enhanced, which confirms precious results from transient expression system. To explore whether this is an NME1L-specific effect, we constructed a shRNA-resistant genes and transfected them into the cells expressing shRNA. A reporter gene assay revealed that expression of shRNA-resistant NME1L down-regulated TNFα-stimulated NF-κB activation (Fig. 4, D and E). Although, because the shRNAs down-regulated expression of both forms of NME1, the effect we observed cannot be ascribed to NME1L only, the results from the shRNA-resistant mutant may highlight the specific role of NME1L in IKKβ-mediated signaling.

IKKs are readily phosphorylated by various stimuli, resulting in NF-κB activation and/or overexpression (8, 9, 11). To investigate the effect of NME1L on IKK phosphorylation, we introduced IKKβ and varying amounts of NME1L plasmids into HEK293 cells. As shown in Fig. 5A, phosphorylation of IKKβ decreased in the presence of NME1L. However, NME1L had no effect on IKKβ phosphorylation (Fig. 5B). We employed extracellular stimuli to explore the effect of NME1L on IKKβ phosphorylation further. Endogenous IKKβ was phosphorylated by TNFα, whereas IKKβ phosphorylation was markedly down-regulated in the presence of exogenous HA-NME1L, indicating that NME1L inhibits IKKβ phosphorylation via upstream kinases (Fig. 5C).

NME1L Inhibits Migration of HT1080 Cells—We employed HT1080 cells to investigate migration, because they are highly metastatic, and it has also been reported that NME1 expression is down-regulated in them (42). First, we infected the cells with a lentivirus harboring NME1L or NME1 and investigated whether they responded to TNFα stimulation. TNFα-triggered NF-κB activation was inhibited by exogenous NME1L in
HT1080 cells (Fig. 6A). Compared with other cell lines, including HEK293, HeLa, and MCF7, NME1 expression was slightly decreased in HT1080 cells, consistent with a previous report (42). However, the protein was sufficiently abundant to be easily detected via Western blot with specific antibodies (data not shown) (Fig. 6B). The data also showed that expression of exogenous HA-NME1L was lower than HA-NME1. This was quite similar to the expression pattern of endogenous genes, suggesting that the N-terminal sequence of NME1L may affect translation efficiency or protein stability. The inhibitory effect of wild-type and HA-tagged NME1L on NF-κB activity was identical, indicating that the HA epitope does not interfere with NME1L function (data not shown). To investigate IKKβ activation, each cell line was treated with TNFα and applied for Western blotting with anti-phospho-IKKβ-specific antibodies. The data show that phosphorylation of IKKβ was hardly detected in cells expressing HA-NME1L, suggesting that NME1L blocks IKKβ activation in HT1080 cells (Fig. 6B). However, exogenous expression of both NME1 proteins had no effect upon cell proliferation (Fig. 6C). This may be because 1) potent proliferation

FIGURE 4. NF-κB activity is enhanced in NME1/NME1L knockdown cells. A, to examine the efficiency of shRNA inhibition of NME1L expression, HEK293 cells were transfected with HA-NME1L and shRNA plasmids. Western blots (IB) with anti-HA antibodies were performed on cell lysates. B, HEK293 cells were transfected with a reporter gene and NME1/NME1L shRNA plasmids. Reporter gene assays were performed after TNFα treatment. sc, scrambled RNA; sh, shRNA. C, HEK293 cells were transfected with a reporter gene and varying amounts of the sh3 plasmid. Then the reporter gene assay was performed. D, top, HEK293 cells infected with lentivirus containing scramble or sh3 were used for Western blotting; bottom, HEK293 cells containing sh3 were transfected with an shRNA-resistant form of NME1 or NME1L and used for Western blotting. E, HEK293 cells expressing sh3 with or without resistant genes were applied for a TNFα-induced reporter gene expression assay. NME1Lm and NME1m, shRNA-resistant forms. Data from three independent experiments, done in triplicate, were combined and are presented as the mean ± S.E. (error bars). * (B and C), compared with TNFα-treated in scrambled RNA; * (E), compared with TNFα-treated in scrambled RNA; # (E), compared with TNFα-treated in sh3, p < 0.01. NT, not treated.

FIGURE 5. IKKβ phosphorylation is inhibited by NME1L but not NME1. A and B, HEK293 cells were transfected with 0.5 μg of FLAG-IKKβ and varying amounts of HA-NME1L or HA-NME1 plasmids. After 48 h, cells were treated with 10 ng/ml TNFα for 15 min and then harvested and subjected to Western blots. C, HEK293 cells infected with a lentivirus containing HA-NME1L were treated with TNFα, and a Western blot (IB) was performed on the cell lysates.
signals override NME1 regulation in HT1080 cells or 2) NF-κB signaling is dispensable for cell growth. In contrast, when HT1080 cells were treated with TNFα, which may stimulate expression of the genes involved in cell migration and metastasis, migration of the cells expressing exogenous NME1L toward serum was impaired (Fig. 6, D and E), implying that NME1L may down-regulate TNFα-stimulated gene expression by inhibiting IKKβ-mediated NF-κB activation.

NME1L also inhibited cell migration, although efficiency was quite a bit lower. To explore invasiveness, HT1080 cells were added to transwell inserts coated with Matrigel. As shown in Fig. 6F, fewer NME1- or NME1L-expressing cells were able to pass through the Matrigel-coated transwell, yielding a pattern similar to that observed for the migration assay. These results suggest that both forms of NME1 are negative regulators of cell migration, regardless of expression level, although NME1L is also able to inhibit cell movement.

**NME1L Alters Transcription of TNFα-stimulated and Metastasis-related Genes**—Given that TNFα induces expression of genes facilitating metastasis and that NME1L inhibits TNFα-stimulated NF-κB activation, we were interested in whether expression of endogenous NF-κB-responsive, prometastatic genes was also affected by NME1L. To determine the effect of NME1L at the mRNA level, total RNA was isolated from HT1080 cells transfected with NME1 or NME1L and treated with TNFα for 24 h; real-time quantitative RT-PCR analysis was then performed. mRNA levels of uPA and its cognate receptor, uPAR, were slightly lower in cells expressing exogenous NME1L. Expression in NME1L-expressing cells did not change in response to TNFα stimulation, although it was enhanced in control cells and NME1-expressing cells. In the presence of exogenous NME1L and NME1, mRNA levels of PAI-1 and MMP2 did change in response to TNFα. mRNA levels of the chemokines CCL7 and IL-8 were markedly enhanced by TNFα stimulation in control cells, but NME1L expression inhibited this response (Fig. 7). The inhibitory effect of NME1L on the expression of these genes is likely to help explain its inhibitory role in cell migration and metastasis. Expression of some genes was also influenced by NME1, which may explain its partial regulation of HT1080 cell migration.

**NME1L Efficiently Inhibits Lung Metastasis of HT1080 in an in Vivo Model**—To test the effect of NME1L upon metastasis, we established a lung metastasis model using HT1080 cells. H1080 cells overexpressing NME1L or NME1 were injected into the left flank of female NOD/SCID mice. Tumors were surgically resected when they reached 600—700 mm³. 49 days after transplantation, bioluminescent imaging was employed, and then the animals were sacrificed for histological analysis (Fig. 8A). Marked signals of lung metastasis were detected in
the control group; by contrast, only half of the NME1 group and only one mouse in the NME1L group showed signals in the lung region (Fig. 8B). Histological analysis of luciferase activity in the lungs revealed that the control and NME1 groups had nodules of similar size. Small nodules were observed in the single NME1L mouse (Fig. 8C), implying that NME1L may be a potent inhibitor of metastasis in this cell line.

**DISCUSSION**

IKK activation is believed to be a pivotal step in the path from extracellular stimuli to NF-κB activation, which modulates the expression of various genes. According to recent studies, however, IKK catalytic subunits also affect the activity of many other signaling molecules, especially in cancer development (43). Although IKKα and IKKβ are structurally similar to one another, with 50% sequence identity, and both can make a functional complex with NEMO, IKKβ is known as the dominant kinase in the canonical NF-κB signaling pathways that induce expression of genes involved in cell proliferation, angiogenesis, cell survival, invasion, metastasis, and the epithelial-mesenchymal transition, processes underlying cancer development (44, 45). Moreover, IKKβ regulates many non-IκB targets. For example, FOXO3a, a tumor-suppressive forkhead transcription factor that inhibits cell cycle progress and promotes cellular apoptosis, is phosphorylated by IKKβ (16). Phosphorylated FOXO3a undergoes translocation to the cytosol and ubiquitination. In multiple cancers, cytoplasmic FOXO3a is correlated with up-regulation of IKKβ activity (46). IKKβ has also been reported to phosphorylate tuberous sclerosis 1 (TSC1), resulting in enhancement of mammalian target of rapamycin (mTOR) activity, which plays a role in tumorigenesis (17, 47). Finally, phosphorylation of docking protein 1 (DOK1) by IKKβ relieves DOK1-mediated repression of cell migration and proliferation by up-regulating MAPK pathways (48, 49). In this fashion, IKKβ is likely to play a crucial role in tumor development by modulating NF-κB-dependent and independent signaling pathways. Furthermore, many types of cancer, such as prostate cancer, melanoma, colon cancer, and hepatocellular carcinoma, display constitutive activation of NF-κB and elevated expression of IKKβ but not IKKα. For this reason, IKKβ and NF-κB have been considered targets for pharmacological interference in cancer and immune diseases.

Due to the idea that various signals converge on IKKs to stimulate the transcriptional activity of NF-κB, much effort has gone into elucidating the mechanisms regulating IKK activity. CUE domain-containing protein 2 (CUEDC2) has been identified as an IKKβ-binding protein (50). It interacts with both IKKα and IKKβ, enhancing their dephosphorylation and deactivation by recruiting protein phosphatase PP1. The catalytic domain of IKKβ, but not IKKα, contains unique sequences (E(T/S)GE and DLG) that serve as binding targets for Kelch domains. We and other groups have reported that KEAP1 interacts with these motifs through its C-terminal Kelch domain, negatively regulating NF-κB signaling by leading to IKKβ degradation. Whether IKKβ is degraded through an autophagic or proteasomal pathway is still under debate; however, despite the interaction of KEAP1 with Culin3 in the E3 ubiquitin ligase complex, the KEAP1-IKKβ interaction is IKKβ-specific and is thought to down-regulate the tumorigenic effects of IKKβ (15, 51).

In the present study, we identified NME1L, a splicing variant of NME1, as a novel IKKβ-binding protein. NME1 belongs to the NME family, which is composed of nine members that contain nucleoside diphosphate kinase domains. Although there have been reports that their kinase domains are responsible for their antimitostatic activity, NMEs are primarily thought to regulate cancer cell mobility by interacting with various signaling molecules (31, 33, 34). Some NMEs are small proteins that contain only a nucleoside diphosphate kinase domain, which is responsible for interaction with other proteins and homo- or hetero-oligomerization with other NME members (52).

Despite the lack of any functional domain other than nucleoside diphosphate kinase domains, NME1, as a novel IKKβ-binding protein, might play a crucial role in tumor development. In this study, we demonstrate that NME1L is a potent inhibitor of metastasis in cancer cell lines.
NME1L Regulates NF-κB Signaling

Unfortunately, functional differences in NF-κB regulation between the forms could not be assessed because a long form-specific shRNA was not identified. However, reporter gene assays showed that NF-κB activity was down-regulated in proportion to the inhibition efficiency of various shRNAs on NME1/NME1L expression, suggesting that NME1L is an inhibitor of NF-κB signaling.

Phosphorylation and the subsequent conformational change to a catalytically competent structure is necessary for protein kinases to phosphorylate the target residues of their substrates; therefore, inhibition of phosphorylation or acceleration of dephosphorylation may suppress kinase activity. According to previous reports on IKK function, KEAP1 binds to the catalytic domain and suppresses phosphorylation, whereas CUEDC2 and GβL terminate IKK activation by forming a complex with protein phosphatases (38, 50). NME1L interacts with IKKβ via the latter molecule’s leucine zipper domain, which is responsible for IKK dimerization. This interaction may inhibit IKK complex formation, resulting in suppression of autophosphorylation by overexpressed IKKβ molecules. However, a binding experiment with different epitope-tagged forms of IKKβ revealed that exogenous NME1L did not inhibit IKKβ dimerization (data not shown), indicating that NME1L inhibits IKKβ phosphorylation via a mechanism independent of dimerization. The effect of IKKβ overexpression on autophosphorylation may not reflect the unique role of NME1L in vivo results may reflect the unique role of NME1L in the regulation of kinase activity.

NME1 has been shown to inhibit cancer cell metastasis. Under certain conditions, TNFα promotes metastasis by enhancing expression of genes related to metastatic progression (53, 54). We found that exogenous NME1L was a potent inhibitor of TNFα-stimulated NF-κB activation in HT1080, an aggressive cancer cell line. Furthermore, the expression of pro-metastatic genes modulated by TNFα was significantly down-regulated in the presence of NME1L. Some genes were also affected by NME1 overexpression; this may underlie that protein’s antimetastatic activity. However, the effects of NME1L on gene expression were more dramatic than those of NME1. This may explain the inhibition of metastasis and migration in cells expressing the long form.

The antimetastatic activity of NME1 has been demonstrated using animal models. For example, exogenous NME1-expressing melanoma cells show reduced cytokine responsiveness and reduced metastatic potential to the lung (55). NME1 also reduces metastasis of colon cancer cells to the liver, potentially by regulating myosin light chain phosphorylation (56). A recent study indicates that a newly developed cell-permeable form of NME1 probably blocks pulmonary metastasis of several cancer cell lines (57). All of the experiments of Lim et al. (57), conducted with NME1, reveal that the short form of the protein has moderate but efficient antimetastatic activity, a finding confirmed in our model of metastasis. However, we observed that NME1L dramatically inhibited pulmonary metastasis of cancer cells. Our in vivo results may reflect the unique role of NME1L in migration, invasiveness, and gene expression.

Here, we have demonstrated a potential mechanism by which NME1L regulates IKKβ-mediated signaling. Our results indicate that NME1L is a key inhibitor of cancer cell metastasis via its inhibition of IKKβ. These findings suggest that NME1L may represent a weapon with which to halt cancer progression.

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FIGURE 8. NME1L suppresses lung metastasis of HT1080 cancer cells. A, H1080 cells overexpressing NME1L or NME1 were injected into the left flank of female NOD/SCID mice, and tumors were surgically resected when they reached 600–700 mm3. At 4 and 7 weeks, d-luciferin (7.5 mg/ml) was injected intraperitoneally. Bioluminescence imaging with a charge-coupled device camera was initiated 30 min after the injection for 1–60 s, as appropriate. B, the number of tumor nodules observed on the surface of the lung was counted using a stereomicroscope. C, tumor tissue isolated from each treatment group was examined via hematoxylin and eosin staining. V, control virus-infected cells; NT, not treated.
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