Mitogen-activated Protein Kinase Kinase-4 Promotes Cell Survival by Decreasing PTEN Expression through an NFκB-dependent Pathway*‡§

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Mitogen-activated protein kinase kinase-4 (MKK4/SEK1) cooperates with phosphatidylinositol 3-kinase to maintain the survival of non-small cell lung cancer (NSCLC) cells, but the biochemical basis of this phenomenon has not been elucidated. Here we used genetic approaches to modulate MKK4 expression in mouse embryo fibroblasts (MEF cells) and NSCLC cells to identify prosurvival signals downstream of MKK4. Relative to wild-type MEF cells, MKK4-null MEF cells were highly susceptible to apoptosis by LY294002, paclitaxel, or serum starvation. MKK4 promoted the survival of MEF cells by decreasing the expression of phosphatase and tensin homologue deleted from chromosome 10 (PTEN). MKK4 inhibited PTEN transcription by activating NFκB, a transcriptional suppressor of PTEN. MKK4 was required for nuclear translocation of RelA/p65 and processing of the NFκB2 precursor (p100) into the mature form (p52). Studies on a panel of NSCLC cell lines revealed a subset with high MKK4/high NFκB/low PTEN that was relatively resistant to apoptosis. Thus, MKK4 promotes cell survival by activating phosphatidylinositol 3-kinase through an NFκB/PTEN-dependent pathway.

Regulation of phosphoinositides is important to tumorigenesis. PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a dual specificity phosphatase that dephosphorylates the 3'-sites of the phosphoinositides PI(3,4)P₂ and PI(3,4,5)P₃ (1). Endogenous PI(3,4,5)P₃ levels are also regulated by phosphatidylinositol 3-kinase (PI3K), which phosphorylates the D3 position of phosphatidylinositol (PI) on PI(4)P and PI(4,5)P₂ to produce PI(3,4)P₂ and PI(3,4,5)P₃. PI(3,4,5)P₃ and PI(3,4)P₂ recruit the pleckstrin homology domains of specific intracellular proteins to the plasma membrane, an essential event in the activation of PI3K-dependent kinases such as phosphoinositide-dependent kinase-1 and protein kinase B/AKT, which have a key role in cellular survival and transformation (2).

PI3K-dependent signaling is frequently activated in a variety of tumor types, including non-small cell lung cancer (NSCLC). Several genetic events previously described in NSCLC activate PI3K, including amplification of PIK3CA and activating mutations in PIK3CA, EGFR, or K-RAS (3–6). PTEN gene expression is frequently silenced in NSCLC (7), but the mechanisms contributing to the loss of PTEN expression in NSCLC have not been defined. PTEN genetic deletion is a rare event in NSCLC (8), raising the possibility that PTEN is silenced transcriptionally or post-transcriptionally. Of note, PTEN expression is transcriptionally suppressed by tumor necrosis factor-α (TNFα) through NFκB (9, 10), a heterodimeric transcription factor that is constitutively activated in NSCLC (7).

NFκB consists of the transactivation subunits RelA/p65 and the DNA-binding subunits p50 (NFκB1) and p52 (NFκB2), which are processed from the precursors p105 and p100, respectively (11). In unstimulated conditions, NFκB is sequestered in the cytoplasm by inhibitor of NFκB (IκB) and remains transcriptionally inactive. Upon stimulation by inflammatory cytokines or peptide growth factors, IκB is phosphorylated by IκB kinase (IKK), a multiprotein complex consisting of two kinase subunits (IKKα and IκKB) and a regulatory subunit (IKKγ/NEMO), and undergoes proteasome-dependent degradation. The released NFκB translocates into the nucleus and regulates the expression of target genes with key roles in the prevention of apoptosis, promotion of tumor growth, and activation of inflammatory responses (12).

NSCLC cells undergo apoptosis in response to PI3K pathway inhibition (13, 14). We previously found that a stress kinase, phoretic mobility shift assay; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; RNAi, RNA interference; p-, phosphorylated; EV, empty vector; Scr, scrambled.
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mitogen-activated protein kinase kinase-4 (MKK4), activates prosurvival signals in NSCLC cells and can rescue them from the proapoptotic effect of PI3K inhibition (14). MKK4 is a dual specificity kinase that is activated by environmental stresses, including exposure of cells to UV irradiation, DNA damage, growth factors, or inflammatory cytokines (15). Consistent with a prosurvival role, disruption of MKK4 causes embryonic death (16) and increases liver cell apoptosis (17, 18). However, the mechanisms by which MKK4 regulates cell survival have not been fully defined.

In this study, we hypothesized that MKK4 promotes cell survival through interactions with PI3K-dependent signaling, which we addressed by using genetic approaches to modulate MKK4 expression in mouse embryo fibroblasts (MEF) cells and NSCLC cells. We conclude that MKK4 promotes cell survival by activating PI3K through an NFκB/PTEN-dependent pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The NSCLC cell lines H1299, H226B, H226Br, H332, H358, H460, A549, H596, and Calu-6 were obtained from American Type Cell Culture and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen). MKK4-null and wild-type MEF cells (17) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. We purchased LY294002 (Calbiochem), paclitaxel (Bristol-Myers Squibb Co.), TNFα (Leinco Technologies), L-α-phosphatidyl-inositol 4-monophosphate (Sigma), and ImmunoPure immobilized protein A beads (Pierce).

Antibodies—We purchased rabbit polyclonal antibodies against human Ser-473-phosphorylated AKT (p-AKT1), Thr183/Tyr-185-phosphorylated JNK (p-JNK), AKT1, X-chromosome-linked inhibitor of apoptosis protein (Cell Signaling Technology), and PTEN (Cell Signaling Technology and Neomarkers); rabbit polyclonal antibodies to p85α (Upstate Biotechnology); rabbit polyclonal antibodies to p65, p50, cyclin D1, p110α, MKK4, and β-actin (Santa Cruz Biotechnology); and mouse monoclonal antibodies to β-actin (Sigma) and p52 (Santa Cruz Biotechnology).

Plasmids—NFκB-Luc was a gift from Dr. Bing Su (M. D. Anderson Cancer Center). The p65 expression vector was provided by Dr. Paul Chiao (M. D. Anderson Cancer Center) (19). Two PTEN promoter reporter constructs were used. One contains 1,064 base pairs from −1,809 to −745 and was provided by Dr. Alfred Yung (M. D. Anderson Cancer Center) (20). The other contains 1,978 base pairs from −1,978 to translation start site) and was provided by Dr. Jan de Belle (The Burnham Institute). Wild-type MKK4 plasmid constructs were a gift from Dr. Jiale Dai (M. D. Anderson Cancer Center). Retroviral vectors expressing murine MKK4 short hairpin RNA (shRNA) and scrambled control shRNA were purchased (Open Biosystems). Human MKK4 small interfering RNA and scrambled control small interfering RNA oligonucleotides were purchased (Invitrogen). pcDNA3-FLAG-PTEN was constructed by subcloning FLAG-PTEN into pcDNA3 at the KpnI and XbaI sites.

Cell Proliferation and Apoptosis Assays—Cells were seeded in either 96-well plates (1,000 cells/well) for proliferation assays or 24-well plates (4,000 cells/well) for apoptosis assays. These conditions achieved 70% confluence at t = 0. After overnight incubation at 37°C, cells were either treated with LY294002 or paclitaxel at the concentrations indicated for 72 h at 37°C or were washed twice with phosphate-buffered saline and incubated in serum-free medium for the indicated time points at 37°C. After treatment, cell proliferation was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Depending upon the experiment, values were calculated relative to untreated wild-type MEF cells, which were set at 100%. Asterisks indicate values that are significantly different (p < 0.05, MKK4-null versus wild-type MEF cells).

Apoptosis was measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling with the APO-BRDU staining kit (Phoenix Flow Systems, San Diego, CA) or by Hoechst
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were performed using a nonradioactive AKT kinase assay kit (Cell Signaling Technology) according to the manufacturer’s instructions. JNK and PI3K in vitro kinase assays were performed as described previously (14, 21). For PI3K assays, PI3K was immunoprecipitated by anti-p85 antibody. The immunoprecipitates were washed sequentially in (a) phosphate-buffered saline, 100 μM Na3VO4, 1% Triton X-100; (b) 100 mM Tris (pH 7.6), 0.5 mM LiCl, 100 μM Na3VO4; (c) 100 mM Tris (pH 7.6), 100 mM NaCl, 1 mM EDTA, 100 μM Na3VO4; (d) 20 mM Hepes (pH 7.5), 50 mM NaCl, 5 mM EDTA, 30 mM NaPPi, 200 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.03% Triton X-100 and resuspended in 30 μl of kinase reaction buffer (33 mM Tris (pH 7.6), 125 mM NaCl, 15 mM MgCl2, 200 μM adenosine, 15 μM ATP, 30 μCi of [γ-32P]ATP). PI (Sigma) was resuspended in 20 mM Hepes (pH 7.5) at 2 mg/ml and sonicated on ice for 10 min. The PI-3 kinase reaction was initiated by adding 10 μl of the PI suspension. The reaction proceeded for 30 min at room temperature and was terminated by adding 100 μl of 1 M HCl. Lipids were extracted by 600 μl of chloroform:methanol (1:1). The organic phase was washed with H2O, collected, and dried by vacuum centrifugation. The lipids were resuspended in 20 μl of chloroform:methanol (1:1) and resolved on silica gel G-60 thin-layer chromatography (TLC) plates (Merck) in chloroform:methanol:acetone:acetic acid:H2O (60:23:18:11). Radiolabeled phosphatidylinositol phosphate was visualized by autoradiography.

PTEN Phosphatase Assay—Cells were lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100 with protease inhibitors (Sigma). PTEN was immunoprecipitated using a PTEN-specific antibody (Neomarkers). The immunoprecipitates were washed sequentially once with lysis buffer (without protease inhibitors), twice with low stringency wash buffer (20 mM Hepes (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl2) and once with Reaction Buffer without substrate (100 mM Tris·HCl (pH 8.0), 10 mM dithiothreitol). The immunoprecipitates were then incubated in 50 μl of Reaction Buffer with 100 μM diC8-PIP2 (Echelon Research Laboratories) at 37 °C for 40 min with occasional mixing. The beads were staining. For Hoechst staining, cells were serum-starved or treated with LY294002 or paclitaxel for the time periods indicated and then fixed with 4% paraformaldehyde at room temperature for 20 min. Hoechst 33342 (Sigma) was added directly to a final concentration of 10 μg/ml. Cells were stained in the dark at 4 °C overnight and examined under an Olympus IX71 fluorescence microscope the next day. For each treatment, cells with condensed or fragmented chromosomes, a hallmark of apoptosis, were counted in three randomly chosen fields. The percentages of apoptotic cells were then calculated based on the total cell count.

Western Analysis and Kinase Assays—Western blot analysis was performed as described previously (14). AKT kinase assays of PTEN bands were corrected based on the levels of PTEN. Results shown are means of three independent experiments. Error bars indicate S.E. p = 0.0024. PTEnzyme, protein-tyrosine phosphatase. C, PI3K expression and activity were similar in wild-type (+/+) and MKK4-null (−/−) MEF cells. The left panel shows PI3K expression levels by Western blotting of whole-cell lysates using specific antibodies to p110, p85, or β-actin. The right panel shows PI3K activity by PI3K kinase assay. PI3K, phosphorylated phospholipids. D, endogenous PI3P levels were lower in MKK4-null than in wild-type cells. Phospholipids were extracted, and PI3P levels were analyzed by enzyme-linked immunosorbent assay following treatment of wild-type (+/+) and MKK4-null (−/−) MEF cells for 24 h with or without LY294002. E, AKT phosphorylation and activity were lower in MKK4-null than in wild-type cells. Cells were treated with or without LY294002 for two days. The upper four panels show Western blot analyses of whole-cell lysates using specific antibodies to p-AKT, AKT, PTEN, or β-actin. The lower panel shows immunoprecipitation of p-AKT from cell lysates by anti-p-AKT antibody. AKT activity was measured by kinase assay using GST-GSK3 as a substrate. Densitometric values of GST-GSK3 bands are indicated relative to that of untreated wild-type MEF cells, which was set at 1.0. F, with serum starvation, p-AKT declines in MKK4-null cells (+/+) but not MKK4-null null cells (−/−). PTEN expression and AKT expression and phosphorylation by Western blotting following serum deprivation overnight in wild-type (+/+) and MKK4-null (−/−) MEF cells are shown.

FIGURE 2. MKK4 loss increases PTEN expression and decreases PI3K-dependent signaling. MKK4-null MEF cells have higher PTEN expression (A) and phosphatase activity (B) than do wild-type MEF cells. A, PTEN expression levels of MEF cells by Western blotting of whole-cell lysates with specific antibodies to PTEN or β-actin. Densitometric values of PTEN bands were corrected based on β-actin and expressed relative to that of wild-type MEF cells, which was set at 1.0. B, immunoprecipitation of PTEN by anti-PTEN antibody and measurement of PTEN phosphatase activity. Results shown are means of three independent experiments. Error bars indicate S.E. p = 0.0024. PTPase, protein-tyrosine phosphatase. C, PI3K expression and activity were similar in wild-type (+/+) and MKK4-null (−/−) MEF cells. The left panel shows PI3K expression levels by Western blotting of whole-cell lysates using specific antibodies to p110, p85, or β-actin. The right panel shows PI3K activity by PI3K kinase assay. PI3K, phosphorylated phospholipids. D, endogenous PI3P levels were lower in MKK4-null than in wild-type cells. Phospholipids were extracted, and PI3P levels were analyzed by enzyme-linked immunosorbent assay following treatment of wild-type (+/+) and MKK4-null (−/−) MEF cells for 24 h with or without LY294002. E, AKT phosphorylation and activity were lower in MKK4-null than in wild-type cells. Cells were treated with or without LY294002 for two days. The upper four panels show Western blot analyses of whole-cell lysates using specific antibodies to p-AKT, AKT, PTEN, or β-actin. The lower panel shows immunoprecipitation of p-AKT from cell lysates by anti-p-AKT antibody. AKT activity was measured by kinase assay using GST-GSK3 as a substrate. Densitometric values of GST-GSK3 bands are indicated relative to that of untreated wild-type MEF cells, which was set at 1.0. F, with serum starvation, p-AKT declines in MKK4-null cells (+/+) but not MKK4-null null cells (−/−). PTEN expression and AKT expression and phosphorylation by Western blotting following serum deprivation overnight in wild-type (+/+) and MKK4-null (−/−) MEF cells are shown.

Western Analysis and Kinase Assays—Western blot analysis was performed as described previously (14). AKT kinase assays of PTEN bands were corrected based on β-actin and expressed relative to that of wild-type MEF cells, which was set at 1.0. B, immunoprecipitation of PTEN by anti-PTEN antibody and measurement of PTEN phosphatase activity. Results shown are means of three independent experiments. Error bars indicate S.E. p = 0.0024. PTPase, protein-tyrosine phosphatase. C, PI3K expression and activity were similar in wild-type (+/+) and MKK4-null (−/−) MEF cells. The left panel shows PI3K expression levels by Western blotting of whole-cell lysates using specific antibodies to p110, p85, or β-actin. The right panel shows PI3K activity by PI3K kinase assay. PI3K, phosphorylated phospholipids. D, endogenous PI3P levels were lower in MKK4-null than in wild-type cells. Phospholipids were extracted, and PI3P levels were analyzed by enzyme-linked immunosorbent assay following treatment of wild-type (+/+) and MKK4-null (−/−) MEF cells for 24 h with or without LY294002. E, AKT phosphorylation and activity were lower in MKK4-null than in wild-type cells. Cells were treated with or without LY294002 for two days. The upper four panels show Western blot analyses of whole-cell lysates using specific antibodies to p-AKT, AKT, PTEN, or β-actin. The lower panel shows immunoprecipitation of p-AKT from cell lysates by anti-p-AKT antibody. AKT activity was measured by kinase assay using GST-GSK3 as a substrate. Densitometric values of GST-GSK3 bands are indicated relative to that of untreated wild-type MEF cells, which was set at 1.0. F, with serum starvation, p-AKT declines in MKK4-null cells (+/+) but not MKK4-null null cells (−/−). PTEN expression and AKT expression and phosphorylation by Western blotting following serum deprivation overnight in wild-type (+/+) and MKK4-null (−/−) MEF cells are shown.
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A

Vector  -/-  EV  MKK4
MKK4
p-JNK
JNK
PTEN
β-Actin

Apoptosis (%)
Control  LY294002  Paclitaxel

B

+/+

Clone  shRNA
Scr  1  2
MKK4
p-JNK
JNK
PTEN
β-Actin

Apoptosis (%)
Control  LY294002  Paclitaxel

C

+/+

Clone  EV  23  39
PTEN
FLAG
β-Actin

Cell density (%)
LY294002 (μM)

Apoptosis (%)
LY294002 (μM)
centrifuged, and 40 µl of supernatant were transferred to 96-well plates (flat bottom) and incubated at room temperature with 100 µl of malachite green reagent (Echelon Research Laboratories) for 30 min. PTEN phosphatase activity was calculated with a standard curve according to the manufacturer’s instructions.

Phosphatidylinositol 3,4,5-Trisphosphate (PIP₃) Assay—PIP₃ was quantified in cell extracts using a PIP₃ mass enzyme-linked immunosorbent assay kit (Echelon Research Laboratories) as directed by the manufacturer.

Cell Transfection and Reporter Gene Assays—Cells were transfected in 24-well plates with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. To measure luciferase activity for the reporter gene assays, NFκB-Luc or PTEN promoter reporter vector was co-transfected with a pRL-CMV vector, which expresses Renilla luciferase as an internal transfection control. Luciferase activity was measured 48 h after transfection by a dual luciferase reporter system (Promega). Relative luciferase activity was expressed as the firefly luciferase activity normalized by the Renilla luciferase activity. For construction of site-directed mutants of the PTEN promoter, the reporter vector containing 1,064 base pairs of PTEN promoter sequence was used. For all other experiments on the PTEN promoter, the reporter containing 1,978 base pairs of PTEN promoter sequence was used.

For stable selection, cells were seeded in 35-mm plates, transfected, incubated at 37 °C overnight, and then replated into 150-mm dishes, and G418 (Invitrogen) was added. Medium was replaced every 5 days. Up to 48 subclones were chosen for expression screening.

Site-directed Mutagenesis—Mutations in the PTEN gene promoter were created in putative NFκB binding sites at nucleotides −1574 to −1565 (Box 1) and −1450 to −1441 (Box 2) by overlapping PCR as follows. To create a Box 1 mutant, primers used were: primer A, 5′-GGGGTACCCGGATCCTCCTTCA-GTTCATTGATAGTGTC-3′; primer B, 5′-TTTGGCTTAAAGATACCTTCCCTCACATGG-TGTCAG-3′; primer C, 5′-AGGTTGAATCTTATGGCACA-AGGGCTTACAGCTAATCTGGAAAGC-3′; and primer D, 5′-CCCAAGTGTGCGGCGCCGGCTCTCTC-ATCCCTTGGC-3′. The mutant nucleotides are shown in bold.

Using pGL3-PTEN-Luc as template, primers A and B were used to generate a 260-base pair PCR fragment, and primers C and D were used to generate a 710-base pair PCR fragment. The PCR products had 20-base pair overlapping sequences at the 3′-end. The PCR products were used as a template for a third round of PCR using primers A and D. The resulting PCR product, with the mutations in the Box 1 region, was cloned into pGL3-Luc basic vector. The same protocol was used to create mutations in the Box 2 region of PTEN promoter. For this PCR reaction, the primers used were: primer B, 5′-CTGCAAGGAAGAATACAAGCTCCCCCTTGCTCTACCTAGTTCC-3′; and primer C, 5′-GGGATTGTATTTCTCTTG-GAGGACCCTCCGTATTTCTCTTCTAC-3′. Primers A and D for this protocol were the same as those used for making Box 1 mutants.

Northern Blotting—Total RNA (20 µg) was separated on 1.5% agarose gels in 1× MOPS buffer and transferred to Zeta-Probe blotting membranes (Bio-Rad). Probes to glyceraldehyde-3-phosphate dehydrogenase and PTEN were prepared by PCR of cDNA prepared from total cellular RNA. PCR primers were designed previously for glyceraldehyde-3-phosphate dehydrogenase (22). The primers for PTEN were 5′-TTGAA-GACATACACCCACACAC-3′ and 5′-GGCAGACCAAA-CTGAGGATTG-3′. PCR products were labeled using the Rediprime II random prime labeling system (Amersham Biosciences).

Electrophoretic Mobility Shift Assays (EMSA)—Cells were treated with or without TNFα for the indicated times and fractionated into cytoplasmic and nuclear fractions. EMSAs were performed according to a method described previously (19). For typical NFκB binding activity, we used a 30-nucleotide double-stranded κB oligonucleotide from the human immunodeficiency virus long terminal repeat (5′-CTCAACAGAGGGGATTTCCCGAGAGGCCCAT-3′; boldface indicates NFκB binding site). A double-stranded mutated oligonucleotide (5′-CTCAACAGAGTTGACCTTTCGAGAGGCCCAT-3′) was used to examine the specificity of binding of NFκB to DNA. To examine NFκB binding activity in the PTEN promoter, probes were made from two putative NFκB binding sites in that promoter (Box 1, 5′-TTGGGAGAAGGGGATCTCTAG-GCAAAGG-3′; Box 2, 5′-CAAGGGGGGAGGATTCCCTC- TTGGCAGGGA-3′) and their mutants (mutant Box 1, 5′-TTGGGGAAGGTTGACCTTTCGAGAGGCCCAT-3′). As a control, we used Oct-1 probe, which has been described previously (19).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP analysis was performed using a ChIP assay kit (Upstate) as recommended by the manufacturer. Following immunoprecipitation of protein-DNA complexes using anti-p50 (Santa Cruz Biotechnology) antibody or normal rabbit IgG, reversal of the histone-DNA interaction was performed according to the manufacturer's instructions. DNA was precipitated with NaCl and ethanol, and DNA was analyzed by PCR using primers specific for the NFκB binding sites. The expected PCR products were 205 and 215 bp for Box 1 and 220 and 229 bp for Box 2.

FIGURE 3. MKK4 regulates PTEN and promotes MEF cell survival. A, increased MKK4 expression decreased PTEN and attenuated sensitivity to apoptosis. Left panel, Western blotting of MKK4-null MEF cells transiently transfected with MKK4 expression vector or empty vector (EV). Densitometric values of bands were indicated relative to that of MKK4-transfectants, which was set at 1.0. Right panel, Hoechst staining of transient transfectants treated for 48 h with LY29402 or paclitaxel. Asterisks indicate values that are significantly different (p < 0.05, EV versus MKK4 transfectants). B, decreased MKK4 expression increased PTEN and sensitized cells to apoptosis. Left panel, Western blotting of MKK4 wild-type MEF cells transfected with MKK4 shRNA (clones 1 and 2) or scrambled (Scr) control shRNA. Densitometric values of bands were indicated relative to that of scrambled transfectants, which was set at 1.0. Right panel, Hoechst staining of clones cultured for 48 h with LY29402 or paclitaxel. Asterisks indicate values that are significantly different (p < 0.05, Scr versus MKK4 shRNA transfectants). Lower two panels, densities (MMT assays) of clones treated for 3 days with LY29402 or paclitaxel. Results shown are means of three independent experiments performed in triplicate, p = 0.001, Scr versus clone 1 with LY29402; p = 0.001, Scr versus clone 2 with LY29402; p = 0.001, Scr versus clone 1 with paclitaxel; and p = 0.001, Scr versus clone 2 with paclitaxel. C, increased PTEN expression sensitized wild-type MEF cells to LY29402. Left panel, Western blotting of PTEN expression in MKK4 wild-type cells that stably express FLAG-PTEN (clones 23 and 39) or EV. Middle panel, cell densities (MTT assays) of clones treated for 3 days with LY29402. Results shown are means of three independent experiments performed in triplicate, p = 0.0047 (EV versus clone 23) and 0.0027 (EV versus clone 39). Right panel, apoptosis (Hoechst staining) induced by LY29402 in these cells. Asterisks indicate values that are significantly different (p < 0.05, EV versus PTEN transfectants).
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tone-DNA cross-links, and recovery of DNA, purified DNA was then amplified by PCR. The protocol was as follows: denaturation, 95°C for 5 min; amplification, 34 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and extension, 72°C for 7 min. Primers were used (5'-CTGGGGAGCTGTTACA-CAA-3' and 5'-CTCTGTGTTCTGATCTGCC-3') that spanned the mouse PTEN promoter region containing putative NFKB binding sites and generated a PCR product of 181 bp. PCR products were then resolved on a 1.5% agarose gel.

Statistical Analysis—Paired Student's t test using Microsoft Excel was performed on the data from MTT assays, which were conducted in triplicate and repeated three times. Student's t test using Microsoft Excel was performed on the data from reporter gene assays and PTEN phosphate activity assay. p values of 0.05 or less were considered significant.

RESULTS

MKK4 Loss Enhances Sensitivity to Apoptosis—We showed previously that MKK4-null MEF cells are more sensitive than wild-type MEF cells to apoptosis caused by LY294002, a PI3K inhibitor (14). We therefore investigated whether loss of MKK4 confers sensitivity to other proapoptotic stimuli. Basal rates of proliferation (Fig. 1, left panels) and apoptosis (Fig. 1, right panels) were similar in MKK4-null and wild-type MEF cells, indicating that MKK4 loss had no measurable effects on these basal parameters. However, like LY294002 (Fig. 1A), treatment with paclitaxel (Fig. 1B), which is an antimicrotubule agent, or serum starvation (Fig. 1C) induced apoptosis of MKK4-null but not wild-type cells based on Hoechst staining. Apoptotic cells were also quantified by terminal deoxynucleotidyl transferase dUTP nick-end labeling assays, which revealed that MKK4-null MEF cells were more sensitive than wild-type MEF cells to LY294002-induced apoptosis (supplemental Fig. 1).

MKK4 Loss Increases PTEN Expression and Inhibits PI3K-dependent Signaling—Given that MKK4-null MEF cells were more susceptible than wild-type MEF cells to apoptotic stimuli, including treatment with LY294002, which inhibits PI3K, we hypothesized that MKK4 loss alters PI3K-dependent signaling, a key mediator of cellular survival, at the level of protein expression, activity, or both. Indeed MKK4-null MEF cells had higher PTEN expression (Fig. 2A) and lipid phosphatase activity (Fig. 2B) than wild-type MEF cells did. In contrast, PI3K expression (p85 and p110) and lipid kinase activity were similar in MKK4-null and wild-type cells (Fig. 2C), suggesting that basal activity of PI3K-dependent signaling was altered specifically at the level of PTEN.

Because PTEN negatively regulates intracellular levels of PI3P, which is required for the activation of downstream effectors of PI3K, such as AKT, based on the above results we predicted that MKK4-null and wild-type MEF cells would differ with respect to the expression and activity of these downstream effectors basally or in response to LY294002. Whereas basal levels were similar in the two cell types, LY294002-induced PI3P levels (Fig. 2D) and the phosphorylation and kinase activity of AKT (as measured using GST-GSK3 as substrate) (Fig. 2E) were considerably lower in MKK4-null MEF cells than in wild-type cells. LY294002 led to a slight diminution in PTEN expression in MKK4-null cells but remained higher than that of MKK4 wild-type cells (Fig. 2E). Thus, MKK4 loss was associated with an increase in PTEN expression and enhanced sensitivity to treatment with a PI3K inhibitor.

Persistent AKT Phosphorylation in Serum-starved MKK4-null Cells—Because serum contains growth factors that maintain cell survival, in part through AKT activation, we reasoned that serum starvation would prominently inhibit AKT phosphorylation in MKK4-null MEF cells. Surprisingly whereas PTEN expression did not change in either cell type, serum starvation decreased AKT phosphorylation in wild-type cells but not in MKK4-null cells (Fig. 2F). AKT phosphorylation remained high up to 12 h after serum removal (Fig. 2F) at which time apoptotic cells were detectable (data not shown). We concluded that the apoptosis induced by serum starvation did not require AKT inhibition and hence occurred through a distinct mechanism from that of LY294002.

Suppression of PTEN Expression Is a Prosurvival Signal Activated by MKK4—We next sought to rule out the possibility that PTEN expression and susceptibility to apoptosis differed in the two cell types due to MKK4-independent factors. MKK4 was added back to MKK4-null cells by the introduction of an MKK4 expression vector and depleted from MKK4 wild-type cells using shRNA. As a control we examined the phosphorylation of JNK, a substrate of MKK4. MKK4 overexpression in MKK4-null cells enhanced JNK phosphorylation, decreased PTEN expression, and attenuated apoptosis by LY294002 and paclitaxel (Fig. 3A). Conversely MKK4 knockdown in wild-type MEF cells decreased JNK phosphorylation, enhanced PTEN expression, and increased sensitivity to treatment with LY294002 or paclitaxel (Fig. 3B). Thus, the differences of the two cell lines in PTEN expression and sensitivity to LY294002 and paclitaxel were related to differences in MKK4 expression.

We next investigated whether high PTEN expression contributed to the enhanced susceptibility of MEF cells to apoptosis. When PTEN was constitutively expressed in wild-type MEF cells by stable transfection, the PTEN transfectants were more sensitive than control cells to the antiproliferative and apoptotic effects of LY294002 (Fig. 3C), suggesting that high PTEN expression contributed to the enhanced sensitivity of MKK4-null cells to LY294002.

High MKK4 Confers a Survival Advantage in NSCLC Cells—Based on our observations in MEF cells, we hypothesized that the prosurvival effect of MKK4 that we observed previously in NSCLC cells (14) is mediated through the inhibition of PTEN expression. We examined eight NSCLC cell lines, three of which (H460, A549, and H596) expressed low or undetectable MKK4 (Fig. 4A). These three also had the highest PTEN expression (Fig. 4A). H460 cells and H1299 cells were selected from the panel for further characterization as models of low and high MKK4-expressing NSCLC cells, respectively. Consistent with their relative basal MKK4 expression levels, JNK phosphorylation and kinase activity were higher in H1299 cells than in H460 cells (Fig. 4A). H460 cells (which had low MKK4 and high PTEN expression) were more sensitive than H1299 cells (which had high MKK4 and low PTEN expression) to the antiproliferative and apoptotic effects of LY294002 and paclitaxel (Fig. 4B). Thus, consistent with our findings in MEF cells, high MKK4...
FIGURE 4. MKK4 decreases PTEN and promotes survival in NSCLC cells. A, PTEN expression was inversely associated with MKK4 expression in a panel of NSCLC cell lines. Left panel, whole-cell lysates were prepared from the cell line panel and subjected to Western blot analyses with specific antibodies. Right panel, Western blotting and JNK in vitro kinase assays (GST-c-Jun) were performed on cells grown in the presence of serum (+) or after serum starvation for 24 h (−). Densitometric values of bands were corrected based on β-actin (for MKK4 and PTEN) or total JNK (for p-JNK) and were expressed relative to that of H1299 cells, which was set at 1.0. B, low MKK4, high PTEN correlated with sensitivity to LY294002 and paclitaxel. H1299 and H460 cells were treated with LY294002 (left panel) or paclitaxel (middle panel) for 3 days, and relative density of viable cells was measured by MTT assay. Results shown are means of three independent experiments performed in triplicate. LY294002, p = 0.0077; paclitaxel, p = 0.019. Right panel, Hoechst staining of H1299 and H460 treated with 10 μM LY294002 or 20 nM paclitaxel for 48 h. Asterisks indicate values that are significantly different (EV versus H1299). C, increased MKK4 decreased PTEN and attenuated sensitivity to apoptosis. Western blotting (top panel) of H460 cells stably expressing MKK4 (clones 20 and 21) or EV. Densitometric values of bands were corrected based on β-actin and expressed relative to that of EV transfectants, which was set at 1.0. Relative densities (MTT assays) of clones treated with LY294002 (lower left panel) or paclitaxel (lower middle panel) are shown. Results shown are means of three independent experiments performed in triplicate. EV versus clone 20 with LY294002).
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expression correlated with resistance to apoptosis in these NSCLC cell lines.

Despite their similarities to MEF cells, NSCLC cell lines are genetically heterogeneous and, therefore, may differ from the MEF cells with respect to their dependence on MKK4 for cell proliferation and survival. Therefore, we investigated the role of MKK4 in H460 cells (which express low MKK4) by stably transfecting them with MKK4 or empty vector and examining changes in JNK phosphorylation, PTEN expression, and sensitivity to treatment with LY294002 or paclitaxel. Relative to empty vector transfectants, MKK4 transfectants had higher JNK phosphorylation, lower PTEN expression, and decreased sensitivity to the antiproliferative and apoptotic effects of LY294002 or paclitaxel (Fig. 4C). We then performed the converse experiment in H1299 cells (which expressed high MKK4) by subjecting them to RNAi-mediated depletion of MKK4. Relative to the effects of scrambled oligonucleotide controls, MKK4 RNAi increased PTEN expression and enhanced apoptosis by LY294002 or paclitaxel (Fig. 5A). Thus, MKK4 regulated PTEN expression and sensitivity to apoptosis in NSCLC cells.

To examine the role of PTEN in regulating the sensitivity of NSCLC cells to apoptosis, PTEN was stably transfected into H1299 cells, which express low PTEN. PTEN transfectants were more sensitive than empty vector transfectants to the antiproliferative and apoptotic effects of LY294002 and serum starvation (Fig. 5B). Together these findings suggest that the prosurvival pathway activated by MKK4 in MEF cells is also functional in NSCLC cells.

MKK4 Regulates PTEN Promoter Activity—We investigated the mechanism by which MKK4 regulates PTEN expression. We first examined whether MKK4 regulates PTEN transcription. Northern blot analysis revealed higher PTEN mRNA levels in MKK4-null cells than in wild-type MEF cells (Fig. 6A). We examined PTEN promoter activity by transient transfection assays using a luciferase reporter that contained a PTEN genomic fragment including 1,978 bp 5’ of the ATG translation initiation site. PTEN promoter activity was higher in MKK4-null MEF cells than in wild-type MEF cells (Fig. 6B), indicating that MKK4 loss was associated with enhanced PTEN gene transcription.

MKK4 Loss Is Associated with Low NFκB Activity—There are two putative NFκB response elements in the PTEN promoter (9, 10), located at positions −1565 and −1441 (designated in this study as Box 1 and Box 2, respectively), and NFκB is known to be a negative regulator of PTEN expression (9, 10). We therefore investigated whether NFκB contributes to the low basal activity of the PTEN promoter in MKK4 wild-type MEF cells. We first examined NFκB expression and activity in MKK4-null and wild-type MEF cells. NFκB transcripational activity was higher in wild-type MEF cells as shown by transient transfection assays using a reporter construct containing NFκB response elements (Fig. 6C). MKK4 wild-type cells had higher expression of the NFκB family members p50 (NFκB1) and p52 (NFκB2) and known NFκB target genes IkBα and X-chromosome-linked inhibitor of apoptosis protein (Fig. 6D), higher phosphorylation of IkBα (Fig. 6D), and, by EMSA, greater TNFα-induced DNA binding activity to a canonical NFκB binding site in the human immunodeficiency virus long termi-

FIGURE 5. MKK4 decreases PTEN expression and promotes the survival of H1299 NSCLC cells. A, MKK4 depletion increased PTEN expression and sensitized H1299 cells to apoptosis. Left panel, Western blotting of H1299 cells transiently transfected with Scr control RNAi or MKK4 RNAi. Densitometric values of bands were corrected based on β-actin and expressed relative to that of scrambled transfectants, which was set at 1.0. Right panel, Hoechst staining of transfectants treated for 48 h with LY294002 or paclitaxel. Asterisks indicate values that are significantly different (p < 0.05, Scr versus MKK4 RNAi transfectants). B, increased PTEN expression sensitized H1299 cells to LY294002 and serum starvation. PTEN expression was examined by Western blotting (top panel) in H1299 cells (clones 6 and 17) stably expressing FLAG-PTEN or EV. Left middle and lower panels, relative densities (MTT assays) of stable PTEN-expressing clones 6 and 17. Results shown are means of three independent experiments performed in triplicate. p = 0.003, EV versus clone 6 with LY294002; p = 0.006, EV versus clone 17 with LY294002; p = 0.012, EV versus clone 6 with serum starvation; and p = 0.004, EV versus clone 17 with serum starvation. Right Middle and lower panels, apoptosis induced by LY294002 or serum starvation. Asterisks indicate values that are significantly different (p < 0.05, EV versus PTEN transfectants).
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Given the association of MKK4 loss with diminished NFκB DNA binding activity, we investigated the role of MKK4 in NFκB binding to the PTEN promoter in MEF cells and NSCLC cells. MKK4 transfection enhanced basal and TNF-induced Box 1 binding activity in MKK4-null MEF cells (Fig. 7C), and shRNA-mediated depletion of MKK4 from wild-type cells attenuated binding to Box 1 (Fig. 7D). Box 1 binding activity was greater in H1299 cells than in H460 cells (Fig. 7E, lanes 1–4), correlating with the relative expression of MKK4 in these cell lines (Fig. 4A). Stable transfection of MKK4 into H460 cells enhanced basal and TNF-induced Box 1 binding activity (Fig. 7E, lanes 5–10). Conversely depletion of MKK4 from H1299 cells by RNAi decreased Box 1 binding activity (Fig. 7F). Thus, MKK4 enhanced NFκB binding to the PTEN promoter.

NFκB Suppresses PTEN Promoter Activity—Based on the evidence above that MKK4 regulates NFκB binding to the PTEN promoter, we investigated the possibility that NFκB acts as a transcriptional suppressor of PTEN. We first performed transient co-transfection experiments to examine whether NFκB regulates PTEN promoter activity in MKK4-null MEF cells.

Supporting this possibility, transfection of either p50 or p65 suppressed PTEN-Luc activity in MKK4-null MEF cells (Fig. 8A, left panel). Furthermore PTEN-Luc suppression by p50 was enhanced by co-transfection of MKK4 (Fig. 8A, right panel). We concluded that NFκB suppressed PTEN promoter activity, and MKK4 enhanced PTEN transcriptional suppression by NFκB.

We next examined the role of the two putative NFκB binding sites in the regulation of PTEN promoter activity. We performed site-directed mutagenesis of the PTEN promoter at Box 1 and Box 2 (Fig. 8B) to create mutant PTEN promoter constructs that do not bind NFκB. These mutants were the same as those used in EMSA competition experiments that did not compete with wild-type PTEN promoter sequences for binding (Fig. 7A). Transient transfection assays with wild-type and mutant PTEN reporters demonstrated that, relative to the activity of the wild-type promoter, the Box 1 mutant was 3-fold more active, whereas the activity of the Box 2 mutant was similar to that of the wild-type promoter (Fig. 8B), indicating that Box 1 acts as a transcriptional suppressor of the PTEN promoter.

We next investigated binding of NFκB to the two putative NFκB response elements in the PTEN promoter by EMSA. Whereas binding was undetectable in MKK4-null cells, wild-type cells exhibited binding activity to Box 1 and Box 2 (Fig. 7A, left panel), and supershift confirmed the presence of NFκB (Fig. 7A, right panel). TNFα treatment increased NFκB binding to both PTEN promoter elements (Fig. 7A, left panel). Thus, nuclear extracts from wild-type MEF cells demonstrated NFκB binding activity on PTEN promoter response elements. To examine NFκB binding activity in vivo, ChIP assays were performed on wild-type MEF cells using anti-p50 or IgG control antibodies to immunoprecipitate chromatin complexes. Input and immunoprecipitates were then used as templates to PCR amplify PTEN promoter sequences containing the putative NFκB binding sites. The PTEN promoter fragment was readily amplified from both input and p50 immunoprecipitate but not from the IgG control (Fig. 7B), indicating that NFκB bound to the PTEN promoter in vivo.

FIGURE 6. MKK4 loss is associated with enhanced PTEN transcription and attenuated NFκB activity. A, MKK4-null (−/−) cells had higher PTEN mRNA levels than did wild-type (+/+) cells. Total RNA was extracted from MEF cells and subjected to Northern blotting with probes for PTEN (upper panel), fold changes in PTEN are indicated at the bottom) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; middle panel). Control 28 and 18S rRNAs are shown in the lower panel. Densitometric values of bands were corrected based on glyceraldehyde-3-phosphate dehydrogenase and expressed relative to that of wild-type MEF cells, which was set at 1.0. B, MKK4-null cells had higher PTEN promoter activity than did wild-type cells. Luciferase activity was measured 48 h after MEF cells were co-transfected with PTEN-Luc and pRL-CMV (Renilla) reporter constructs. Results shown are means of three independent experiments performed in triplicate. Error bars indicate S.E. p = 0.024. C, MKK4-null cells had lower NFκB activity than did wild-type cells. MEF cells were transfected with NFκB-Luc and pRL-CMV reporter constructs, and luciferase activity was measured as in B. Results shown are means of three independent experiments performed in triplicate. Error bars indicate S.E. p = 0.00012. D, expression of NFκB family and NFκB target genes was lower in MKK4-null than in wild-type cells. Whole-cell lysates were prepared and subjected to Western blot analyses. Densitometric values of bands were corrected based on β-actin and expressed relative to that of MKK4-null MEF cells, which was set at 1.0. E, MKK4-null cells had reduced NFκB binding activity. Cells were stimulated with or without 10 ng/ml TNFα for various times. The left panel shows EMSA of nuclear extracts with 32P-labeled consensus probes to NFκB or Oct-1 (as control). Arrows point to shifted bands. The right panel shows supershift and competition assays with 50× excess cold wild-type or mutant probe. Antibody against cyclin D1 was included as negative control. Arrows point to shifted bands. XIAP, X-chromosome-linked inhibitor of apoptosis protein.
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FIGURE 7. MKK4 regulates NFκB binding to the PTEN promoter. A, disruption of MKK4 associated with reduced binding to putative NFκB binding sites in PTEN promoter. MKK4-null (−/−) and wild-type (+/+ ) cells were stimulated with TNFα for various times, and nuclear extracts were prepared and subjected to EMSA (left panel) or supershift assay (right panel) with 32P-labeled probes for Box 1 and Box 2 in PTEN promoter. Supershift and competition assays were performed with 40× excess cold wild-type or mutant probe. B, NFκB bound to the PTEN promoter in vivo. ChIP assays were performed on wild-type MEF cells to PCR amplify PTEN promoter sequences containing the putative NFκB binding sites from chromatin complexes before (Input) or after immunoprecipitation (anti-p50 or IgG control). Input PCR product was diluted 1:5 prior to gel electrophoresis. Locations of the expected PCR product (arrowhead on right) and the 300- and 150-bp molecular weight markers (MW) are indicated. C, MKK4 enhanced binding to PTEN promoter Box 1 sequences. EMSA of Box 1 binding activity in nuclear extracts prepared from MKK4-null cells (−/−) transiently transfected with EV or MKK4 stimulated with TNFα is shown. D, MKK4 depletion in MEF cells reduced binding to Box 1 sequences. EMSAs of Box 1 binding activity in nuclear extracts prepared from untransfected (Parent) and stably transfected (scrambled and MKK4 shRNA) wild-type MEF cells (+/+ ) stimulated with TNFα are shown. E, MKK4 overexpression in NSCLC cells enhanced binding to Box 1. EMSA of Box 1 binding activity in nuclear extracts prepared from H1299 cells transiently transfected with MKK4 RNAi and then treated with TNFα is shown. F, MKK4 depletion in H1299 cells attenuated binding to Box 1. EMSA of Box 1 binding activity in nuclear extracts prepared from MKK4-null MEF cells; hence MKK4 regulated NFκB through an IKK-independent pathway.

DISCUSSION

Previous studies have shown that MKK4 and its downstream mediator JNK have either oncogenic or tumor-suppressive effects depending upon the cellular context (23). For example, MKK4 mediates survival signals in T lymphocytes (24) and was recently shown to be oncogenic in breast and pancreatic tumors (25). Furthermore, JNK1 disruption in mice causes defective transformation of pre-B cells by the BCR-ABL oncogene, which is associated with reduced expression of the antiapoptotic protein Bcl-2, and the effect of JNK1 loss can be rescued by transgenic expression of Bcl-2 (26). Further supporting a prosurvival role, studies using antisense oligonucleotides demonstrate that JNK inhibition can cause growth arrest or apoptosis of some tumor cells (13, 27, 28). However, other studies suggest an opposite role for MKK4 and JNK in cancer.

MKK4 Is Required for RelA/p65 Translocation and Maturation of NFκB2/p100—We investigated the biochemical basis of the defect in NFκB activation in MKK4-null MEF cells. We examined nuclear translocation of RelA/p65, NFκB1/p50, and NFκB2/p52 following TNFα treatment. Western analysis of fractionated nuclear and cytosolic proteins revealed that TNFα induced nuclear translocation of p52 and p65 in MKK4 wild-type cells, whereas in TNFα-treated MKK4-null MEF cells, p65 nuclear translocation was diminished, and p52 was undetectable in both the cytoplasmic and nuclear fractions (Fig. 9A). In H1299 NSCLC cells, TNFα induced robust nuclear translocation of p65, p50, and p52, whereas in H460 cells, nuclear translocation of p65 was diminished, and p52 was undetectable in both the cytoplasmic and nuclear fractions (Fig. 9B). We concluded that MKK4 loss was associated with defects in p65 nuclear translocation and maturation of NFκB2.
For example, inactivating mutations in JNK and MKK4 have been detected in tumor cells (29–32), and JNK suppresses transformation by oncogenic Ras in vivo (33). Hence a key question that remains unresolved concerns the mechanistic basis for the different roles of MKK4 and JNK in tumors.

We showed previously that MKK4 is a prosurvival mediator that rescued NSCLC cells from apoptosis by PI3K inhibition (14). Here we hypothesized that MKK4 mediated its prosurvival effects through regulation of PI3K-dependent signaling. Findings reported here support this hypothesis. In MEF cells and NSCLC cells, high MKK4 expression correlated with low PTEN expression, and modulation of MKK4 expression (forced expression or knockdown) regulated PTEN expression in a reciprocal direction, whereas cell survival was regulated in a corresponding manner, demonstrating a causal relationship between high MKK4 expression, low PTEN expression, and cell survival. Furthermore introduction of PTEN into wild-type MEF cells or H1299 NSCLC cells enhanced apoptosis by LY294002 or serum starvation, indicating that low PTEN expression is a prosurvival signal in these cell types. Lastly high PTEN expression was associated with a reduction in intracellular phosphoinositides (basal and LY294002-induced), which are required for the activation of phosphoinositide-dependent kinase-1, AKT, and other kinases involved in prosurvival signaling (2). Thus, MKK4 promoted cell survival, in part, through suppression of PTEN expression.

Several lines of evidence presented here and elsewhere show that MKK4 and NFκB are components of a common pathway. In this study, high MKK4 expression correlated with NFκB DNA binding activity and expression of NFκB target genes in MEF cells. Furthermore forced expression or knockdown of MKK4 expression in MEF cells and NSCLC cells induced a corresponding change in NFκB DNA binding activity. Previous studies support the physiological relevance of this pathway. Mice that carry null mutations for either MKK4 or p65 die during embryogenesis due to fulminant hepatic failure, a consequence of massive apoptosis of hepatocytes, whereas other organs develop normally (16, 17, 24, 34), indicating that these mutations lead to embryonic death through apoptosis of identical cell populations.

Genes with NFκB binding sites in their upstream regulatory elements include, among others, Fas ligand-inhibitory protein, inhibitor of apoptosis protein-1 and 2, TNF receptor-associated factor-1 and -2, Bcl-2, Bcl-xL, X-chromosome-linked inhibitor of apoptosis protein, and growth arrest and DNA damage-45β, which encode gene products that contribute to innate immunity, inflammation, and cell survival (11, 12, 35). NFκB activates the transcription of these genes, thereby promoting these cellular functions. In contrast to its role as a transcriptional activator, here we showed that NFκB is a potent transcriptional suppressor of PTEN. Supporting this conclusion, NFκB binding activity was detected on PTEN promoter elements in vitro by EMSA and in vivo by ChIP assay, site-directed mutagenesis of putative NFκB response elements in the PTEN promoter revealed Box 1 to be a transcriptional sup-
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pressor, and overexpression of p65 suppressed PTEN promoter activity. Although these findings support the possibility that NFκB suppresses PTEN transcription through direct effects on the PTEN promoter, we have not completely excluded the possibility that NFκB indirectly suppresses PTEN transcription through effects on other transcription factors. Other genes are negatively regulated through direct interactions of NFκB family members with promoter elements, including H⁺-K⁺-ATPase α2, which is expressed in the distal colon and renal collecting duct and plays a critical role in potassium and acid-base homeostasis, and inducible nitric-oxide synthase, which induces nitric oxide production in response to inflammatory stimuli (36). Thus, NFκB controls diverse cellular functions through transcriptional activation and suppression of target genes.

We characterized the MKK4-dependent defect in NFκB function in MEF cells and NSCLC cells and found diminished nuclear translocation of RelA/p65 and maturation of NFκB2. These events are dependent upon the IKK complex, which phosphorylates and initiates proteasome-dependent degradation of IkB, thereby releasing RelA/p65 to translocate to the nucleus (11). In addition, processing of the NFκB1 precursor into its mature form requires IKKβ, whereas IKKα is required for the processing of the NFκB2 precursor by NFκB-activating kinase (26, 34). These findings raised the possibility that MKK4 is required for IKKα activation by upstream regulators, such as NFκB-activating kinase. Arguing against this possibility, we observed no evidence of a defect in IKK kinase activity in MKK4-null MEF cells. Thus, MKK4 regulated NFκB through an IKK-independent mechanism.

A growing body of evidence supports a role for MKK4 and its downstream mediators NFκB and PTEN in NSCLC. Although their biochemical properties and oncogenic potential have not been reported, point mutations in the MKK4 kinase domain were identified recently in NSCLC biopsies (37). Constitutive activation of MKK4 has been reported in a mouse model of lung cancer induced by oncogenic K-ras (22). NFκB is constitutively activated in a variety of cancer cell types, including NSCLC, and promotes NSCLC cell survival (7, 12, 38). The best characterized activators of NFκB are inflammatory cytokines, which are secreted into the tumor microenvironment by cancer cells and inflammatory cells. Supporting a role for this process in NSCLC, neutrophils and macrophages are prominent stromal components in NSCLC tumor specimens, and NSCLC patients have high serum levels of a variety of cytokines including TNFα (39). PTEN gene expression is frequently silenced in NSCLC (7), but the mechanisms contributing to the loss of PTEN expression in NSCLC have not been defined. Findings presented here in NSCLC cells suggest that PTEN is transcriptionally suppressed by MKK4 through an NFκB-dependent pathway.

Lastly these findings have potential clinical implications. We found that activation of this pathway in NSCLC cells correlated with resistance to paclitaxel, a commonly used chemotherapeutic agent in NSCLC patients. Thus, further studies are justified to investigate whether immunohistochemical evidence for activation of this pathway in tumor tissues correlates with resistance to paclitaxel in NSCLC patients and whether, in selected patients, strategies to inhibit MKK4 or NFκB enhance the efficacy of paclitaxel.

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