0022-3565/04/3102-589–598$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 310, No. 2
66126/1156274
JPET 310:589–598, 2004
Printed in U.S.A.

Metallothionein Mediates the Level and Activity of Nuclear Factor κB in Murine Fibroblasts

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Received January 27, 2004; accepted March 23, 2004

ABSTRACT

The zinc-binding protein metallothionein (MT) is associated with resistance to apoptosis. We examined whether MT regulates the zinc-dependent antiapoptotic transcription factor nuclear factor κB (NF-κB), which is up-regulated under many conditions that lead to elevated MT expression. NF-κB protein levels and NF-κB-dependent reporter gene activity were examined in clonal MT(+) (MT-WT) and MT(−) (MT-KO) fibroblastic cell lines. The amount of cellular NF-κB p65 protein in MT-KO was less than 20% of the amount in MT-WT cells, in accord with increased sensitivity of MT-KO cells to apoptosis. NF-κB p65 mRNA levels, and NF-κB p50 subunit and IκBα protein levels, were unchanged. NF-κB activity assessed by expression of a transfected NF-κB reporter construct was less than half that observed in MT-KO cells. Decreased nuclear localization of NF-κB p65 in MT-KO clones was not responsible for differences in activity. In fact, MT-KO cells had higher nuclear levels of NF-κB p65 than did MT-WT cells, despite a lower cellular NF-κB level and function, suggesting that metallothionein mediated the specific activity of NF-κB. Reconstitution of MT by stable incorporation of an MT-1 expression vector in MT-KO cells resulted in increased NF-κB p65 (but not IκBα or NF-κB p50), increased NF-κB-dependent reporter activity, and increased resistance to apoptosis. These data support the hypothesis that metallothionein positively regulates the cellular level and activity of NF-κB.

Metallothioneins (MTs) are small (<10 kDa) metal-binding proteins found in all eukaryotes. MTs bind a variety of metal ions (Ag, Hg, Cu, Cd, and others) but are associated primarily with zinc in most mammalian cells (reviewed in DeMoor et al., 2001). MTs are highly homologous and of similar structure, implying common biological function. However, an essential function for MTs has yet to be identified. Among the four MT isoforms identified, MT-1 and MT-2 are expressed in virtually all mammalian cells. MT-3 is limited to brain, pancreas, and intestine (Ebadi et al., 1995) and MT-4 to squamous epithelial cells in skin and tongue (Quaife et al., 1994). MT-3 and MT-4 are constitutively expressed, whereas MT-1 and MT-2 are both constitutive and highly inducible. MTs are associated with proliferation in human tumor cells in culture (Koropatnick et al., 1995) and in situ (Kontozoglou et al., 1989), compensatory renal growth (Zalups et al., 1995), and monocyte activation (Leibbrandt and Koropatnick, 1994; Leibbrandt et al., 1994). MT expression and intracellular localization is altered in some cells and tissues undergoing differentiation (Koropatnick and Duerksen, 1987; Quaife et al., 1994) Thus, MTs are associated with a broad range of physiological events. Roles in response to cellular and tissue stress have been attributed to MT, including detoxification of heavy metals, protection against oxidative injury (including apoptosis), and homeostatic regulation of essential metals (Koropatnick and Zalups, 2000). This has led to the hypothesis that MT mediates gene expression by donating zinc, directly or indirectly, to transcription factors and other proteins that contribute to chemo- and radio-resistance by inhibiting apoptosis.

This work was supported by grants from the Canadian Institutes of Health Research to J.K. and from the National Institute of Environmental Health Research to R.K.Z. (Grants ES05157 and ES05980) and to R.K.Z. and J.K. (Grant ES11288).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.104.066126.

ABBREVIATIONS: MT, metallothionein; NF-κB, nuclear factor of the κ-enhancer in B cells; TNF, tumor necrosis factor; KO, knockout; WT, wild-type; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; DTT, dithiothreitol; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; TBH, *tert*-butylhydroperoxide; ANOVA, analysis of variance.

The antiapoptotic, zinc-dependent, Rel family NF-κB transcription factors are candidates for regulation by MT. They are dimers, with the p50/p65 complex the most abundant form in mammalian cells (Siebenlist et al., 1994). NF-κB interacts with an inhibitory protein (an IκB family member) in cytoplasm to block nuclear localization (Karin, 1999). In response to diverse stimuli, IκBs are degraded to allow NF-κB to enter the nucleus, bind target DNA elements, and regulate transcription of genes involved in multiple events including immune response, growth, and apoptosis (Siebenlist et al., 1994). NF-κB is required to protect cells from the apoptotic cascade induced by TNF and other stimuli (Beg and Baltimore, 1996) through activation of antiapoptotic genes such as TRAF-1 and -2, c-IAP-1 and -2, A1/Bfl-1, IEX-1, and XIAP (Wang et al., 1999).

Both MT and NF-κB are induced by stress and mediate antiapoptotic processes, including abrogation of the cytotoxicity of TNFα (Sciavolino et al., 1992; Van Antwerp et al., 1996), protection from ionizing radiation (Thornalley and Vasak, 1985; Van Antwerp et al., 1996), and resistance to anticancer drugs (Lazo and Pitt, 1995; Wang et al., 1996). Undefined associations between MT proteins and NF-κB activity have been investigated. However, reported results are contradictory. On the one hand, MT may positively regulate NF-κB: increasing MT by transfection of an MT expression vector into a human breast carcinoma-derived cell line (MCF-7) (Abdel-Mageed and Agrawal, 1998) or zinc induction of L929 cells (Kanekiyo et al., 2001) increased NF-κB binding to DNA and reporter gene activity, and NF-κB-mediated gene expression was down-regulated in peritoneal macrophages isolated from MT-null mice (Kanekiyo et al., 2002). On the other hand, MT may inhibit NF-κB function, since MT has been reported to inhibit TNF-induced degradation of IκBα, and NF-κB-dependent gene expression (Sakurai et al., 1999). In addition, splenocytes from MT-null mice had elevated NF-κB activity compared with those from wild-type controls (Crowthers et al., 2000), and antisense down-regulation of MT-2 in a mitomycin C-resistant hamster cell line (V-H4) partially restored the capacity of those cells to activate NF-κB in response to mitomycin C (Papouli et al., 2002).

To characterize the role of MT in regulating NF-κB, we generated MT-null and wild-type fibroblastic cell lines and multiple clonal cell lines with and without reconstituted MT, and assessed the effect on NF-κB. We report, for the first time, that renal fibroblastic cells lacking MT have lower cellular levels of NF-κB p65, but not p50, and lower NF-κB-mediated reporter activity. Changes in nuclear localization of either the p65 or p50 subunits of NF-κB were not responsible for the effects of MT on NF-κB activity. Rather, decreased NF-κB activity in the absence of MT resulted from decreased capacity of existing NF-κB subunits to participate in NF-κB function. Furthermore, genetic reconstitution of MT-1 expression (without induction of MT expression by external agents) increased the cellular level of NF-κB p65 and enhanced NF-κB activity. Overall, these data strongly support a role for MT-1 and/or MT-2 in mediating NF-κB activity.

Materials and Methods

Mammalian Cell Lines and Culturing Conditions

MT-KO1, MT-KO2, and MT-WT cells were generated from 129/Sv-Mt1Mt2 MT-null mice with disrupted translation of mRNA from MT-1 and MT-2 genes (Masters et al., 1994) and parental strain mice with wild-type MT expression (129/Sv). Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Kidneys from neonates were minced in tissue culture dishes, and cells were cultured for 2 to 4 weeks in DMEM (Invitrogen, Carlsbad, CA) plus 10% FBS, penicillin/streptomycin, and fungizone (Invitrogen). Kidney cell cultures from three separate neonates spontaneously displayed accelerated, continuous growth (immortalization) and fibroblast-like appearance. Clonal isolates of each of the three cultures were maintained in DMEM/10% FBS without antibiotics at 37°C, 5% CO_2.

MT-KO2(+mt) and MT-KO2(−mt) clonal cell lines were generated by stable cotransfection of a 10:1 mixture of 1) an MT-1 expression vector [pRc/CMV-MT-1, containing a 372-bp mouse MT-1 cDNA including 12 bp of pSP72 DNA (Leibbrandt et al., 1994) inserted into the NotI cloning site of the pRc/CMV vector (Invitrogen)], or an empty pRc/CMV control vector; and 2) a pcDNA3.1hygro$^+$ (hygromycin-resistance) vector (Invitrogen). Effectene (QIAGEN, Mississauga, ON, Canada) was used to enhance transfection according to the manufacturer's instructions. Colonies growing in the presence of hygromycin B (Sigma-Aldrich, St. Louis, MO) (300 μg/ml) were selected and assessed for the presence of MT-1 vector in genomic DNA using PCR primers complementary to sequences 5′ and 3′ to the NotI site and spanning the MT-1 insert (5′ primer: 5′CTGCAGATATCCATCACACTG3′, 3′ primer: 5′CCCTCTAGATGCATGCTCGA3′). Several control cell lines [MT-KO2(−mt), containing the empty pRc/CMV vector plus pcDNA3.1hygro$^+$], and cell lines with reconstituted MT-1 expression [MTKO1(+mt), containing pRC/cMV-MT-1 plus pcDNA3.1hygro$^+$) were derived as clonal isolates and assessed for MT, NF-κBα, and IκBα expression, and NF-κB activity.

Human epithelial MCF-7 (breast carcinoma-derived) and mouse NIH 3T3 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and were grown and maintained in DMEM plus 10% fetal bovine serum (37°C/5% CO_2, without antibiotics).

Plasmids

PCMVβ, a mammalian β-galactosidase expression vector (BD Biosciences Clontech, Mississauga, ON, Canada); pNF-κB-LUC, a mammalian NF-κB-responsive luciferase reporter vector) (Stratagene, La Jolla, CA); pcDNA3.1hygro$^+$ and pRc/CMV (Invitrogen); pT7T3-Pac-FSP-1, containing 510 bp of a human fibroblast-specific protein-1 cDNA, with a 600-bp fragment containing the FSP-1 cDNA insert excisable by NotI/XhoI digestion [the kind gift of Dr. G. DiMattia, London Regional Cancer Centre Cancer Research Laboratories, London, ON, Canada); human FSP-1 and mouse FSP-1 cDNAs are identical in sequence (Strutz et al., 1995)]; and pM-GAP, containing a 1050-bp mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, excisable by PstI digestion (Denhardt et al., 1987), were used.

Treatments

Treatment with Metal Salts. Cells were plated on 90-mm tissue culture dishes and fresh medium containing either 100 μM $ZnCl_2$ or 1 μM $CdCl_2$ (Sigma-Aldrich) was added. After 6 or 24 h, media were removed, and cells were rinsed twice in phosphate-buffered saline (PBS) and collected by scraping. Cell pellets were centrifuged and stored at −80°C until used for protein lysate preparation or nucleic acid isolation.

RNA Isolation and Analysis

Cell pellets or mouse tissues were lysed in TRIzol reagent (Invitrogen), and total cellular or tissue RNA was isolated, dissolved in RNase-free water, and stored at −80°C until use according to the manufacturer's instructions.

Northern Blots. MT-1 and MT-2 transcripts were detected by Northern blot analysis using a radiolabeled mouse MT-1 cDNA (400 bp) as a probe (Koropatnick and Pearson, 1990). The close sequence

similarity and size of MT-1 and MT-2 transcripts resulted in detection of both by the same probe. FSP-1 (600-bp cDNA fragment) and GAPDH (1050-bp cDNA fragment) were radiolabeled and used as probes for some RNA samples. Hybridized bands were visualized using a PhosphorImager SI (Amersham Biosciences Inc., Piscataway, NJ).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection of Specific Transcripts. RNA (1 μg) was lyophilized, redissolved in 20 μl of reverse transcription mix [10 μl of sterile water, 1 μl of random hexanucleotide primers (10 mM stock), 4 μl of 5× reverse transcription buffer, 1 μl of random primers (100 μM stock), 1 μl of Moloney murine leukemia virus reverse transcriptase, and 2 μl of DTT (all reagents from Invitrogen)], and incubated 1 h at 37°C, followed by 5 min at 95°C. Five microliters of the resulting cDNA products were amplified by PCR (45 s at 95°C, annealing for 30 s at 55°C, and extension for 1.5 min at 72°C) in 50 μl of PCR mix [5 μl of cDNA product, 5 μl of *Taq* polymerase buffer, 1.5 μl of $MgCl_2$ (50 mM stock), 1 μl of mixed deoxynucleoside-5′-triphosphates (10 mM), 0.5 μl of each cDNA-specific primer (50 pmol/μl stock), 0.25 μl of *Taq* polymerase, and 36.25 μl of sterile water (all reagents from Invitrogen)]. Analysis of products from both 23 and 28 PCR cycles was performed to confirm qualitative results and to exclude the possibility of "plateau" effects. Primers to amplify MT-3, MT-4, NF-κB p65, and GAPDH cDNAs were synthesized by the Molecular Biology Core Facility, London Regional Cancer Centre, and were as follows: MT-3 cDNA (183-bp cDNA amplification product), sense primer (5′-atggaccctgagacctgcccctgtcct-3′), antisense primer (5′-ggcctctgccttggccccctcttcadd-3′); MT-4 cDNA (100-bp cDNA amplification product), sense primer (5′-acaacctgcagctgtaaaacctgtcgt-3′), antisense primer (5′-tgaaccccctttgcagatgcagccccg-3′); NF-κB p65 mRNA (330-bp cDNA amplification product), sense primer (5′-gaagaagcgagacctggag-3′), antisense primer (5′-tccggaacacaatggccac-3′); GAPDH cDNA (752-bp cDNA amplification product), sense primer (5′-tattgggcgcctggtcacca-3′), antisense primer (5;-ccaccttcttgatgtcatca-3′). RT-PCR products were visualized after electrophoresis through 1% agarose gels and staining with ethidium bromide, visualized under UV light, and (where quantification was desired) analyzed by densitometric scanning (ImageQuant; Amersham Biosciences).

Protein Preparation and Analysis

MT Protein Measurement. Cells were lysed in 300 μl of 1% Tween 20 (BDH, Toronto, ON, Canada) in PBS by five freeze/thaw cycles, and insoluble protein was removed by centrifugation. Total soluble protein concentrations were determined by a Bio-Rad Protein Assay using the method provided by the manufacturer (Bio-Rad, Hercules, CA). A sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) was used to quantify MT-1 and MT-2 protein (Butcher et al., 2003) using a mouse monoclonal antibody targeting MT-2, but with affinity for both MT-1 and MT-2 (Leibbrandt et al., 1991). Standard rabbit MT-2 (Sigma-Aldrich), mouse monoclonal antibody to MT (DakoCytomation California Inc., Carpinteria, CA), europium-labeled anti-mouse IgG antibody (PerkinElmer Wallac, Turku, Finland), and DELFIA Enhancement solution (PerkinElmer Wallac) were used according to the manufacturer's instructions.

Western Blot Analyses. Cells were lysed by sonication in 2% SDS, 0.1 M DTT, and 0.06 M Tris, pH 6.8. Nuclear extracts were prepared as described previously (Scheinman et al., 1995). Briefly, cells were washed in PBS and lysed on ice for 5 min in buffer E [0.3% Nonidet P-40, 10 mM Tris-HCl, pH 8.0, 60 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Nuclei were pelleted by centrifugation (5 min, 2500 rpm, 4°C), washed in buffer E lacking Nonidet P-40, and resuspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride). NaCl was added to a final concentration of 0.4 M; then, nuclei were gently shaken for 20 min at 4°C and centrifuged for 10 min at 12,000 rpm and 4°C. Nuclear extracts were stored at −80°C until use. Total cellular protein or nuclear protein (10 μg, as determined by Bio-Rad Protein Assay) was separated by electrophoresis through 12% SDS-polyacrylamide and transferred to nitrocellulose membranes using standard methods; probed with a rabbit polyclonal NF-κB p65 antibody, a rabbit polyclonal NF-κB p50 antibody, a rabbit polyclonal IκBα, or a mouse monoclonal histone H1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and detected by chemiluminescence (ECL; Amersham Biosciences AB, Uppsala, Sweden) using the method described by the manufacturer. To quantify specific proteins against total soluble proteins, identical protein samples were separated by electrophoresis on identical gels and stained with Coomassie Blue [0.25% Coomassie Brilliant Blue (Sigma-Aldrich), 45% methanol, and 10% acetic acid] overnight. All Coomassie-stained protein bands and protein bands visualized by phosphorimaging of chemiluminescence were quantified by densitometric analysis. The amount of antibody-detected specific protein was an indication of relative (but not absolute) specific protein levels, and amounts are reported as the ratio of antibody-detected over total protein.

NF-κB Activity Measurement

Cells were plated in 60-mm culture plates and transiently transfected with an NF-κB luciferase reporter gene (pNF-κB-LUC, directing luciferase expression under the control of a TATA box and 5 tandem repeats of an NF-κB binding element) (Stratagene) or a β-galactosidase expression vector (pCMVβ, to determine transfection efficiency), using Effectene Transfection Reagent and the protocol recommended by the manufacturer (QIAGEN).

Transfection Efficiency. Cells transfected with pCMVβ were washed and fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min at 4°C, 48 h after transfection. Analysis for β-galactosidase activity was performed by washing with PBS and incubating with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BioSource International, Camarillo, CA) substrate solution [77 mM Na_2HPO_4, 23 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, in dimethyl formamide] at 37°C overnight for blue color development. Stained cells were counted at 40× and 100× magnification, and transfection efficiency was calculated as the fraction of blue-stained cells (mean of five measurements ± S.E.M.).

NF-κB Activity. Cells were rinsed with PBS 24 h after addition of pNF-κB-LUC, lysed in Reporter Lysis Buffer (Promega, Madison, WI), and centrifuged at 12,000*g* (1 min, 4°C) to pellet cell debris, and the supernatant was transferred to sterile 0.5-ml Microfuge tubes and stored at −70°C. Luciferase assays were performed by adding a volume of room temperature supernatant containing 5 μg of soluble protein (determined by the Bio-Rad Protein Assay) to 96-well polystyrene plates (PerkinElmer Wallac) adjusted to a constant volume of 10 μl per well with Reporter Lysis Buffer. A plate-reading luminometer (PerkinElmer Wallac 1420 VICTOR^2 Multilabel Counter) was programmed to automatically dispense 50 μl of Luciferase Assay Reagent (Promega) into each well and to measure luminescence 1 s after mixing. Four replicate wells were measured for each sample, and luminescence was expressed as "linear units of counts per second" (cps) (mean of four measurements ± S.E.M.).

***tert*-Butylhydroperoxide (TBH) Resistance.** MT-KO1, MT-KO2, MT-WT, clonal MT-KO2(+mt), and MT-KO2(−mt) clonal cell lines were seeded in 24-well tissue culture plates (2×10^4 cells per well), and a range of concentrations of TBH were added such that the number of cells generated by proliferation 48 h later (measured by Coulter counter) was reduced by 10 to 90%. The IC_{50} (concentration of TBH inhibiting proliferation of cells by 50%) was determined by interpolation in three independent experiments, and the mean IC_{50} ± S.E.M. was calculated from those data. To determine sensitivity to apoptosis, cells were treated with TBH (20 μM, 4 h, 5×10^6 cells per ml of tissue culture media + 10% FBS) in three separate experiments and stained with propidium iodide (50 μg/ml; Sigma-Aldrich)

and Annexin V-FITC (1 μl; BD Biosciences PharMingen, Mississauga, ON, Canada), according to the manufacturer's protocol. Annexin V-positive, propidium iodide-negative cells (i.e., those actively undergoing apoptosis) were measured by flow-cytometric measurement of two-color fluorescence data on 10,000 cells. The increase in apoptosis induced by 25 μM TBH was calculated as the percentage increase in apoptotic cells per 10,000 assessed cells relative to untreated control cells.

Statistical Analyses. Data are presented as mean ± S.E.M., with n as indicated for each analysis. Assessment of differences among means was carried out using a one-way analysis of variance (ANOVA), followed, when comparing differences between multiple experimental means and a single mean control value, by a Dunnett's multiple comparison post hoc procedure. When multiple experimental values were compared, ANOVA was followed by the Bonferroni post hoc test. The level of significance for all statistical analyses was chosen a priori to be $p < 0.05$.

Results

Characterization of MT-Expressing and MT-Null Cell Lines. Two clonally derived cell lines with genetically ablated MT-1 and MT-2 genes (MT-KO1 and MT-KO2) and one wild-type (MT-WT) cell line were generated from kidneys of newborn MT knockout mice (which harbor translation stop signals) and their wild-type parental strain 129/Sv, respectively, as described under *Materials and Methods*. All three cell lines had the morphological appearance of fibroblasts, similar cell size at comparable cell densities when grown in culture (data not shown), and rates of proliferation that were not significantly different ($p = 0.645$, one-way ANOVA) [doubling times (T_d) were: 17.0 ± 0.9 h (MT-KO1), 15.5 ± 1.3 h (MT-KO2), and 16.5 ± 1.1 h (MT-WT)]. FSP-1 is a filament-associated, calcium-binding protein transcribed in fibroblasts, but not epithelial or mesangial cells or embryonic endoderm (Strutz et al., 1995). All three cell lines expressed detectable amounts of fibroblast-specific protein (FSP-1 mRNA), suggestive of fibroblast origin (Fig. 1A). None of the three cell lines expressed detectable levels of two other members of the MT gene family expressed in brain (MT-3) and differentiated stratified squamous epithelial cells, including tongue tissue (MT-4), that could conceptually compensate for loss of MT-1 and MT-2 (Fig. 1B). Both MT-3 and MT-4 are not inducible by metal salts. All three cell lines had zinc- or cadmium-inducible levels of MT-1 plus MT-2 mRNA (Fig. 2A), as expected, since both wild-type and MT-null cells had intact promoter/enhancer-regulatory sequences (Masters et al., 1994). However, both MT-KO1 and MT-KO2 cells had significantly lower basal and metal-inducible MT protein levels, and no significant increase in MT protein levels was detectable in either MT-KO cell line after metal salt induction (Fig. 2B). Therefore, MT-null and wild-type cells generated for analysis of NF-κB activity had true differences in basal and inducible MT expression without obvious differences in morphology or growth capacity.

NF-κB Level and Activity, and Resistance to Toxicity in MT-Null and Wild-Type Cell Lines. When assessed for NF-κB component levels by Western blot analysis, MT-KO1 and MT-KO2 cells had approximately 20% and 11%, respectively, of the level of total cellular NF-κB subunit p65 measured in MT-WT cells (Fig. 3). In accordance with the decreased p65, the capacity of a transiently transfected NF-κB reporter construct to drive luciferase expression was significantly lower in MT-KO1 and MT-KO2 cells compared with MT-WT cells (less than 50% and approximately 25%, respectively), with no significant difference in transfection efficiency among the three cell lines (Fig. 4). In accordance with the role played by MT in mediating resistance to toxic agents, both MT-KO cell lines were more sensitive to induction of apoptosis and growth inhibition by TBH. MT-KO1 and MT-KO2 cells were 5-fold and 3-fold more sensitive to apoptosis induced by 20 μM TBH, with TBH IC_{50} values approximately 50% and 20% (respectively) of the TBH IC_{50} of MT-WT cells (Fig. 4). Exposure to zinc (100 μM) or cadmium (1 μM) for 24 or 48 h did not increase NF-κB p65 levels or NF-κB activity in either MT-WT or MT-KO cells, and NF-κB activity remained significantly higher in MT-WT cells compared with both MT-KO cell lines regardless of whether cells were induced with metal salts (data not shown). Interestingly, NF-κB subunit p50 levels were not significantly different among MT-KO and MT-WT cell lines (one-way ANOVA, $n = 4$, $p \leq 0.05$, Fig. 5A), nor were levels of the cytoplasmic NF-κB inhibitory binding partner IκBα (one-way ANOVA, $n = 4, p \leq 0.05$, Fig. 5B), suggesting that NF-κB subunit p65 is the critical protein dependent on MT-1.

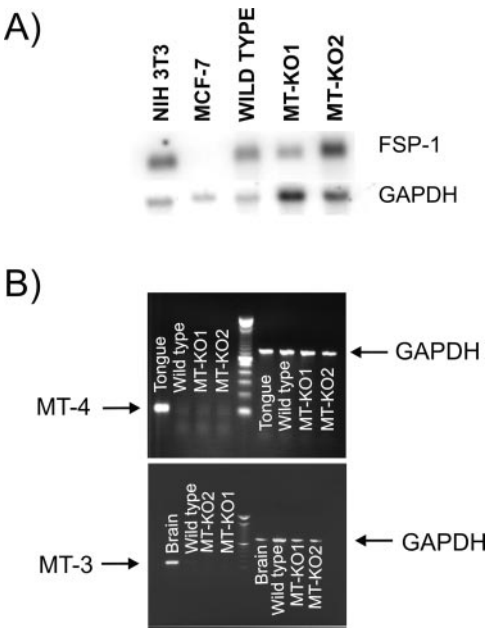

Fig. 1. Wild-type and MT-KO cell lines do not express MT-3 or MT-4 and accumulate a fibroblast-associated marker mRNA. A, Northern blot of fibroblast-specific protein (FSP-1) mRNA in mouse NIH 3T3 fibroblasts (positive control) and human MCF-7 breast carcinoma-derived epithelial cells (negative control), and wild-type and MT-KO clonal cell lines. GAPDH mRNA detection was used as the loading control. B, RT-PCR assessment of MT-3 and MT-4 mRNA in wild-type and MT-KO cell lines and in tissues obtained from wild-type mice (mouse tongue as a positive control for MT-4 mRNA; mouse brain as a positive control for MT-3 mRNA). All three fibroblast-like cell lines were negative for MT-3 mRNA and MT-4 mRNA.

Nuclear NF-κB p65 Localization. The possibility that MTs mediate nuclear localization of NF-κB without altering total cellular NF-κB was assessed by measuring NF-κB p65 in nuclear lysates from MT-KO and MT-WT cell lines. In nuclear proteins (containing nuclear histone H1 and lacking detectable cytoplasmic IκBα), NF-κB p65 was not lower in MT-KO1 and MT-KO2 cells than in MT-WT cells (Fig. 6). In fact, MT-WT cells had significantly less nuclear NF-κB p65

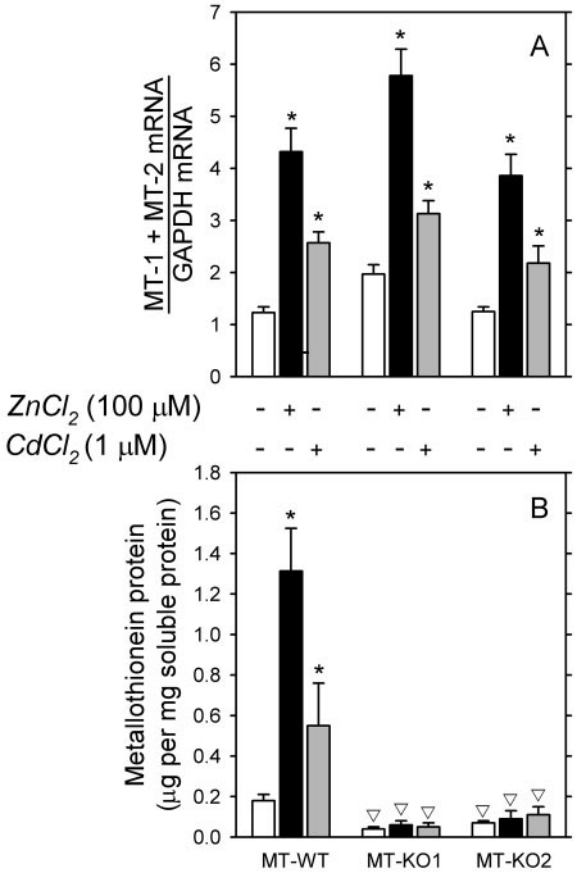

Fig. 2. Metal salts induce MT mRNAs in both wild-type and MT-KO mouse fibroblast-like cell lines, but basal and metal-induced MT protein levels are reduced in MT-KO cells. Cells were treated with zinc or cadmium salts, or control vehicle. A, Northern blot analysis of MT-1/MT-2 expression, using GAPDH mRNA as a comparitor. B, DELFIA measurement of MT protein in the same cell samples. Each bar indicates the mean ± S.E.M. of four independent treatments with metal salts or vehicle. Asterisks (*) indicate values higher than those obtained from cells treated with control vehicle alone. Triangles (▽) indicate values lower than those obtained in similarly-treated MT-WT cells.

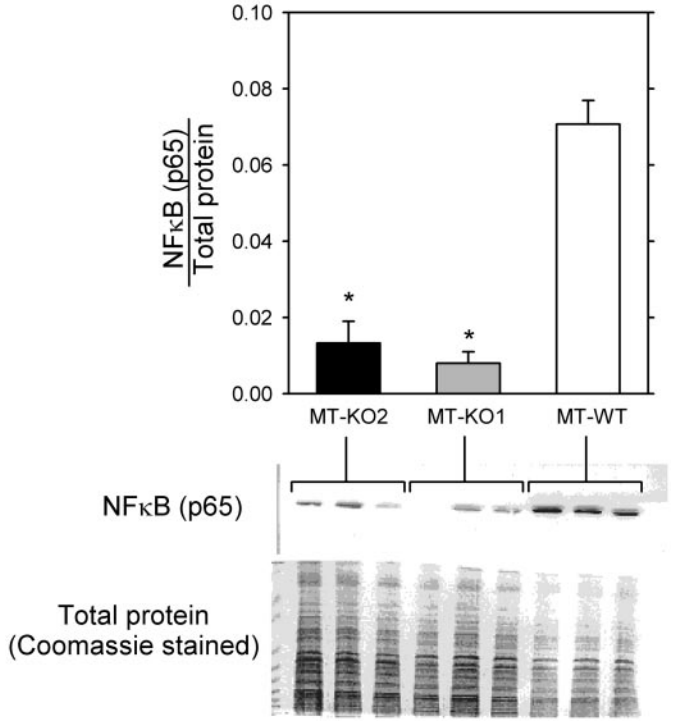

Fig. 3. MT-KO cells contain lower levels of NF-κB (p65 subunit) than wild-type cells. Soluble protein from each of three independent protein preparations from MT-WT, MT-KO1, and MT-KO2 cell lines were subjected to polyacrylamide gel electrophoresis and Western blot detection of the p65 subunit of NF-κB, or Coomassie-staining to visualize total protein. Bars represent the mean ± S.E.M. of three assays. Asterisks indicate lower p65 subunit levels in MT-KO cell lines than in MT-WT cells ($n = 3$, $p \leq 0.01$, one-way ANOVA).

than did either MT-KO1 or MT-KO2. In the context of the 2- to 3-fold greater activity of NF-κB in MT-WT cells compared with MT-KO1 and MT-KO1 (Fig. 4), the higher nuclear levels of p65 but decreased activity of NF-κB in MT-KO1 and MT-KO2 suggest that the specific activity of NF-κB (activity per p65 NF-κB subunit), and not only the amount of p65, is dependent on MT-1/MT-2.

NF-κB p65 mRNA. The possibility that lack of MT resulted in decreased NF-κB p65 by reducing p65 gene transcription and/or p65 mRNA stability was assessed by measurement of cellular p65 mRNA levels relative to GAPDH mRNA levels in MT-KO and MT-WT cell lines by RT-PCR. There were no detectable differences in that ratio among MT-KO1 (0.86 ± 0.09), MT-KO2 (0.97 ± 0.08), or MT-WT (1.14 ± 0.10) ($n = 4$, one-way ANOVA, $p = 0.143$). In combination with the observation of decreased p65 level and specific activity, this finding indicates that MT mediation of NF-κB activity is post-transcriptional and/or post-translational.

Reconstitution of MT Expression, NF-κB Activity, and Resistance to TBH in MT-KO Cells. DNA isolated from clonal isolates transfected with the MT expression vector [MT-KO2(+mt), transfected with pRc/CMV-MT-1] or the control vector [MT-KO2(−mt), transfected with pRc/CMV] was assessed for the presence of stably incorporated vector, empty or containing mouse MT-1 cDNA, by PCR analysis (data not shown). Three putative MT-KO2(+mt) clones (4, 7, and 10) and four MT-KO2(−mt) clones (2, 5, 8, and 9) were selected, and whole-cell soluble protein lysates were assessed for MT (by DELFIA) and for NF-κB p65 and IκBα protein (by Western blot) as described under *Materials and Methods*. All three clones containing transfected mouse MT-1 cDNA expressed higher levels of protein than did all three clones containing the control vector (Fig. 7A) and 10- to 15-fold higher levels of NF-κB p65 (Fig. 7B). There were no apparent differences in IκBα between MT-KO2(+mt) and MT-KO2(−mt clones) (Fig. 7). In addition to the increase in NF-κB p65 protein levels in MT knockout clones following reconstitution of MT expression, NF-κB activity (as measured by NF-κB-mediated transcription of a transiently transfected luciferase reporter construct) was significantly elevated (2- to 3-fold) in all three MT-KO2(+mt) compared with MT-KO2(−mt) clones (Fig. 8, bottom graph). Furthermore, resistance to TBH, in the form of elevated TBH IC_{50} and decreased apoptosis in response to treatment with 20 μM TBH, was restored in all three MT-KO2(+mt) clones relative to all four MT-KO2(−mt) clones (Fig. 8, top graphs).

Discussion

A limited number of studies have assessed the effect of MT on the capacity of NF-κB to mediate one or more steps in transcription, and the conclusions drawn cannot be easily reconciled. Several studies support a role for MT in enhanc-

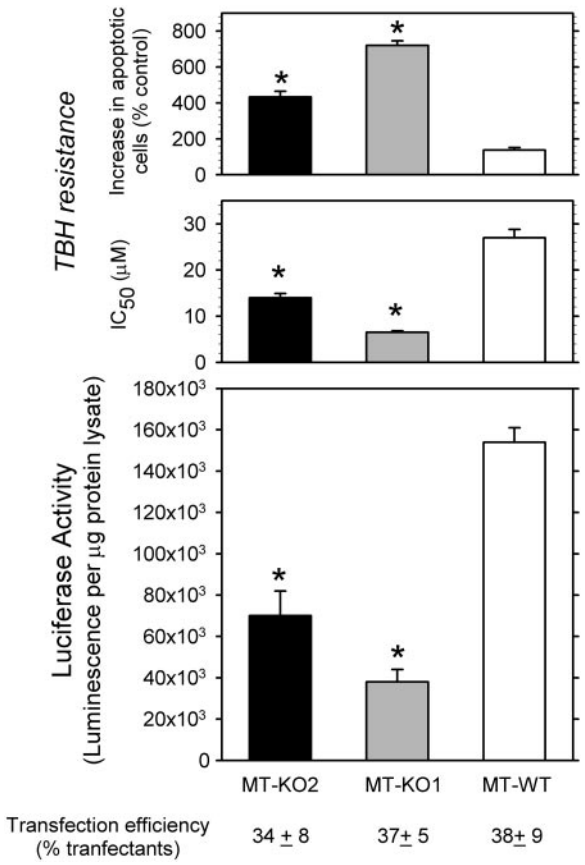

Fig. 4. Resistance to TBH toxicity and NF-κB activity is lower in MT-KO cells than in wild-type cells. MT-WT, MT-KO1, and MT-KO2 cells were transiently transfected with a NF-κB-driven luciferase reporter construct, comparative transfection efficiencies were determined, protein lysates were prepared from three independent transfectant populations for each cell line, and NF-κB-dependent luciferase activity (luminescence) was measured. At the same time, the IC_{50} of TBH, and apoptosis induced by 20 μM TBH, was determined in all three cell lines (untransfected with reporter). Bars represent the mean ± S.E.M. of three assays. Asterisks indicate lower NF-κB-responsive reporter activity, lower TBH IC_{50}, and higher TBH-induced apoptosis in MT-KO cell lines than in MT-WT cells ($n = 3$, $p \leq 0.01$, one-way ANOVA).

ing NF-κB activity (Abdel-Mageed and Agrawal, 1998; Kanekiyo et al., 2001, 2002), whereas others indicate that MTs are inhibitory (Sakurai et al., 1999; Crowthers et al., 2000; Papouli et al., 2002). None of the studies quantitatively assessed total cellular or nuclear NF-κB levels, or the capacity of NF-κB to induce transcription when MT genes were functionally deleted in, and when an MT expression vector was introduced into, MT knockout cells. We applied these strategies to assess whether MT-1 and MT-2 positively or negatively affect the level and activity of NF-κB. In addition, we assessed whether reconstitution of MT-1 in MT-null cells reversed the effect. We found that a small subpopulation of embryonic kidney cells from MT-null mice (lacking expression of MT-1 and MT-2) spontaneously immortalize after growth in culture for several weeks, without use of transforming vectors. A spontaneous immortalized clonal population from wild-type control mice was similarly obtained. Both expressed FSP-1, indicative of fibroblastic characteristics, and neither expressed detectable MT-3 or MT-4 that could, conceivably, functionally compensate for MT-1 and MT-2. To our knowledge, this is the first report of spontaneously immortalized MT-KO and MT-WT cell lines. As expected, both MT-WT and MT-KO cell lines had elevated levels of MT mRNA after induction with zinc or cadmium; the knockout construct used to generate the MT-null mice ablated MT-1 and MT-2 protein but not mRNA (Masters et al., 1994). However, only MT-WT cells were capable of responding to metal induction by increasing MT protein. Basal MT levels in both MT-KO cell lines were lower than those in MT-WT cells.

Previous studies of the relationship between NF-κB and MT used indirect measures of NF-κB, including NF-κB binding to DNA (Abdel-Mageed and Agrawal, 1998; Crowthers et al., 2000; Kanekiyo et al., 2002) and NF-κB-dependent expression of reporter (Abdel-Mageed and Agrawal, 1998; Sakurai et al., 1999; Kanekiyo et al., 2002; Papouli et al., 2002) or endogenous genes (Kanekiyo et al., 2001). When we measured the level of the NF-κB p65 subunit directly by Western blot, we found that it was reduced in both MT-KO cell lines to less than 20% of the level in MT-WT cells. Resistance to TBH-induced growth inhibition and cell death was correspondingly lower in both MT-KO cell lines, in accord with the connection of both MT and NF-κB to toxicity resistance. This decrease was not due to decreased p65 gene transcription since p65 mRNA levels were unchanged in MT-KO cells compared with MT-WT cells. Rather, post-transcriptional events (inhibited p65 mRNA translation or enhanced degradation) are the likely cause. The capacity of the two MT-KO cell lines to mediate transcription of an NF-κB luciferase reporter construct was correspondingly reduced, to less than 50% of the NF-κB-responsive luciferase activity observed in MT-WT cells under conditions of similar efficiency of reporter transfection. The mechanism by which low MT levels decreased NF-κB activity is not known. Low intracellular zinc decreased NF-κB activity in a rat glioma cell line (Ho and Ames, 2002), and a diet depleted of zinc decreased NF-κB activity in rat testes (Oteiza et al., 2001). NF-κB requires zinc to bind DNA and mediate transcription (Zabel et al., 1991), and MT has been implicated as a zinc donor, directly or indirectly, for transcription factors and enzymes requiring zinc for activity (Leibbrandt and Koropatnick, 1994: Leibbrandt et al., 1994; Koropatnick and Zalups, 2000). Taken together, these observations suggest that MT could mediate zinc availability to NF-κB to maintain NF-κB function. In fact, a physical interaction of MT with the p50 subunit of NF-κB has been reported and suggested to stabilize the association of NF-κB with DNA (Vasak and Hasler, 2000). In the cell lines we studied, that putative interaction was not assessed and, if it occurred, would be unlikely to mediate p50 stability or level, since we did not observe a difference in the amount of p50 in MT-KO and MT-WT cells.

The dependence of cellular levels of NF-κB p65 and NF-κB activity on MT suggests that nuclear NF-κB levels would be significantly lowered in MT-KO cell lines compared with those in MT-WT cells. Surprisingly, this was not the case, for either p50 or p65 subunits. Nuclear p50 levels were not different among MT-KO and MT-WT cells, and nuclear p65 levels were elevated approximately 2-fold in both MT-KO1 and MT-KO2 cells compared with MT-WT. Therefore, the dramatic decrease in NF-κB activity under conditions where MT was absent was not due to lack of nuclear p50 or p65, indicating that MT influences both the specific activity (activity per molecule) of NF-κB or its subunits, and their levels within cells. It is possible that MT influences the association of p50 and p65 with each other, affects the ability of dimeric

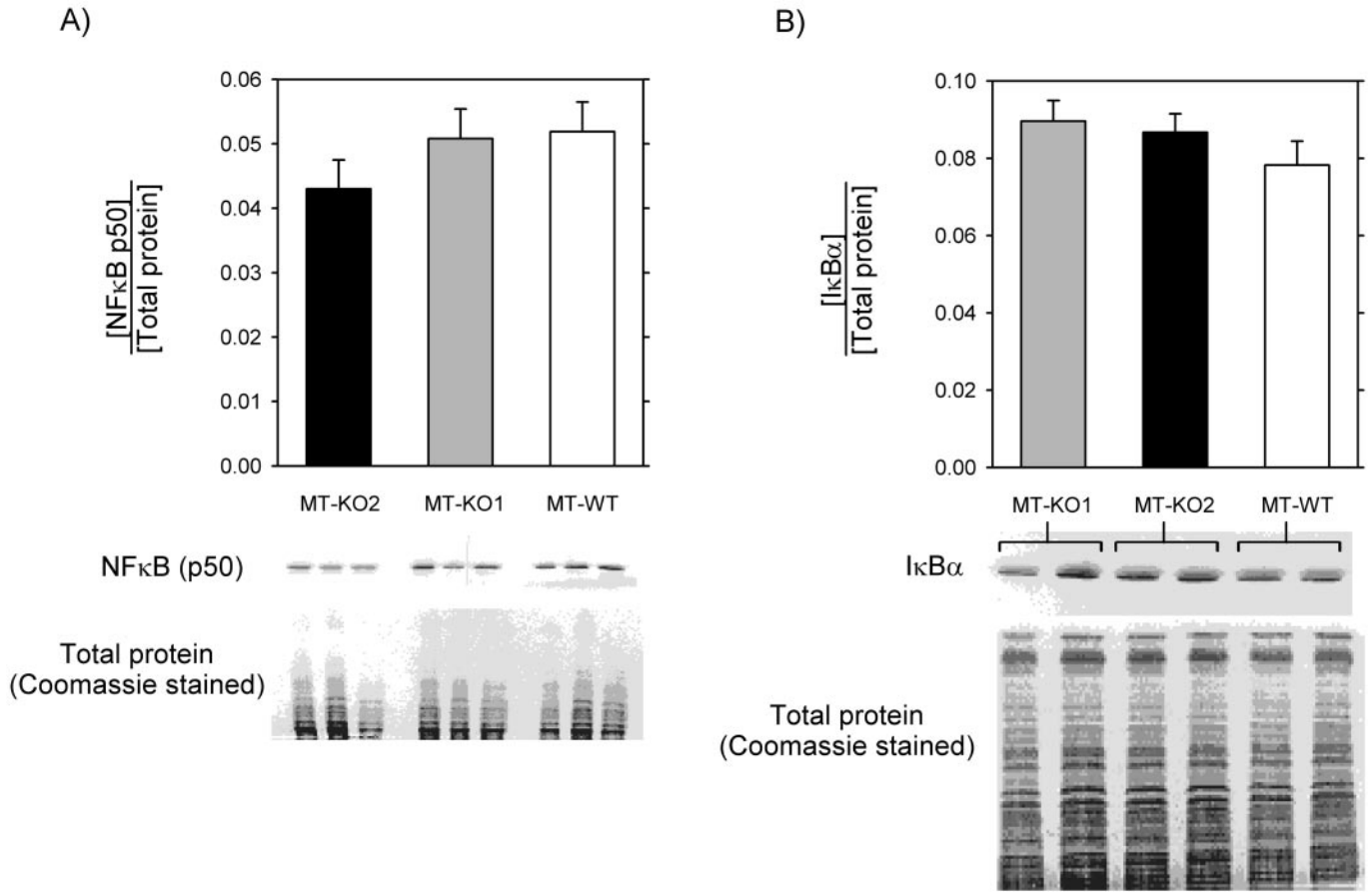

Fig. 5. NF-κB p50 subunit and IκBα levels in MT-KO cells are not different from those in MT-WT cells. Three independent protein isolates were analyzed for NF-κB p50 levels (panel A), and four independent protein isolates were analyzed for IκBα levels (panel B; data from two representative isolates shown) by polyacrylamide gel electrophoresis and Western blot. Bars indicate the mean ± S.E.M. of data from four assays.

p50/p65 NF-κB to subsequently bind to DNA and mediate transcription, or both. Studies are in progress to assess these possibilities.

Decreased NF-κB level and activity in MT-KO cell lines strongly suggested dependence of NF-κB on basal MT. However, differences in other proteins or physiological processes could have arisen following functional ablation of MT-1 and MT-2 genes. Therefore, changes in NF-κB in MT-null cells could be attributable to events unrelated, or only indirectly related, to MT. To explore this possibility, mouse MT-1 mRNA and protein were reconstituted in three independent clonal populations of MT-KO2 cells. NF-κB p65 level, NF-κB transcriptional activity, and resistance to toxic events were assessed in these cells and compared with those in four control clonal cell lines transfected with an "empty" expression vector lacking the mouse MT-1 cDNA, but generated identically in all other respects. All three MT-1-expressing clonal populations had significantly elevated MT protein, p65 protein levels, NF-κB transcriptional activity, and resistance to TBH-induced apoptosis and growth inhibition (as expected in light of the association of both NF-κB and MT with resistance to toxicity). These observations indicate that differences between MT-null cells and MT-WT cells other than MT status were unlikely to be responsible for differences in NF-κB.

MT has been reported to inhibit TNF-induced IκBα degradation, but not basal IκBα levels, in SV40-transformed MT-null mouse embryo cells (Sakurai et al., 1999). This would have the potential to attenuate TNF-mediated NF-κB activation by reducing translocation from the cytoplasm to the nucleus, and would involve an inhibitory role for MT in NF-κB activation. We did not observe reduced basal IκBα levels in relation to total cellular protein in MT-KO cells compared with MT-WT cells, nor did reconstitution of MT-1 expression in MT-KO2 cells lead to increased IκBα levels, in agreement with the observations of Sakurai et al. (1999). However, our results are different with respect to basal NF-κB activity. They saw no change in uninduced NF-κB transcriptional activity (NF-κB-dependent luciferase reporter function) in MT-null cells, whereas we report a dramatic drop in p65 levels and NF-κB activity under similar circumstances. It is possible that the influence of SV40 TAg expression in the cells assessed by Sakurai et al. (1999), or differences in the characteristics of the MT-null cells used in the Sakurai study and those generated by us, could account for the difference. Notably, the MT-null mice we used as the source of our cells (Masters et al., 1994) were generated in a mouse strain different from those used by Sakurai et al. (1999) (Michalska and Choo, 1993). This raises the possibility that basal NF-κB activity may, in the absence of MT, be protected from reduction by physiological characteristics that remain undefined.

Although the mechanism by which MTs may mediate NF-κB activity is not known, there is a wealth of circumstantial evidence that they regulate zinc availability to proteins

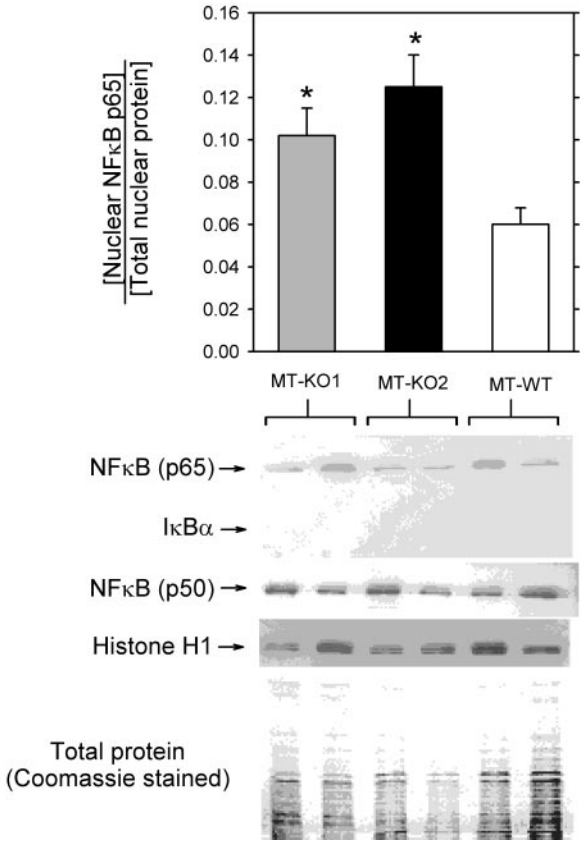

Fig. 6. Nuclear NF-κB p65 levels are not lower in MT-KO cells than in MT-WT cells. Nuclear extracts were prepared from MT-WT, MT-KO1, and MT-KO2 cells and analyzed for NF-κB p65 and p50 subunits, IκBα, and histone H1 by Western blot. The absence of cytoplasmic IκBα and the presence of nuclear histone H1 indicate low cytoplasmic contamination and expected nuclear protein, respectively. Photographs of two representative nuclear extracts (out of a total of four) are shown. Bars indicate the mean ± S.E.M. of four independent measurements. Asterisks indicate lower NF-κB p65 levels in MT-WT cell nuclei than in MT-KO1 or MT-KO2 cell nuclei ($n = 3$, $p \leq 0.01$, Student's t test).

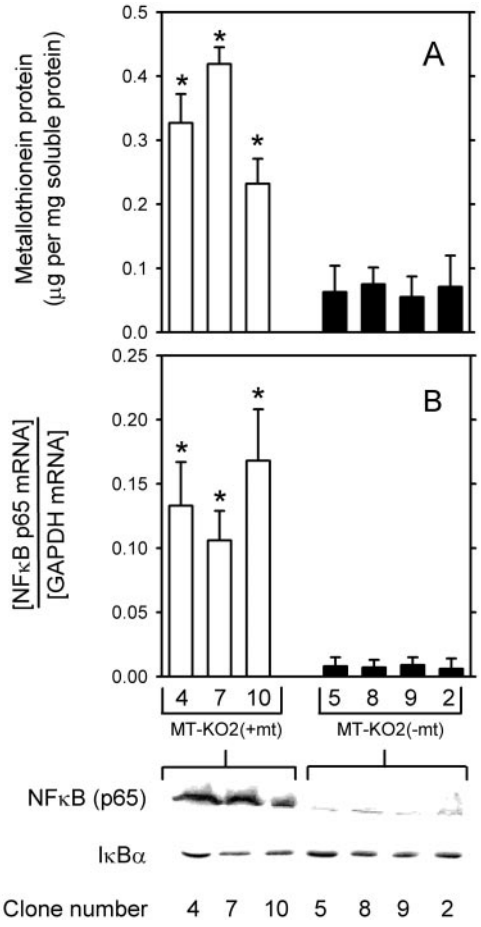

Fig. 7. Stable incorporation of a mouse MT-1 expression vector into MT-KO2 cells increases the level of MT protein (panel A) and NF-κB p65 (panel B). Three independent stable MT-1-expressing transfectant clones [MT-KO2(+mt) clones 4, 7, and 10] and four independent stable control clones [MT-KO2(−mt) clones 5, 8, 9, and 2] were assessed for NF-κB p65 subunit and IκBα protein levels by Western blot and MT protein by DELFIA. Bars indicate the mean of three independent MT (panel A) and NF-κB p65 protein measurements ± S.E.M. A representative Western blot of NF-κB p65 and IκBα is shown. Asterisks indicate MT-KO2(+mt) values significantly different from MT-KO2(−mt) values ($n = 4$, one-way ANOVA, $p \leq 0.05$).

(Koropatnick and Zalups, 2000; DeMoor et al., 2001). However, MT is capable of either donating or sequestering zinc, depending on the affinity of MT for zinc compared with other proteins, overall zinc status within cells, and cellular location of MTs and putatively dependent proteins (Koropatnick and Zalups, 2000). Different groups have reported apparently contradictory effects of altering MT levels on NF-κB, but none have evaluated their observations in terms of the dual sequestering/donating capability of MT. For example, MT-1 and MT-2 normally sequester seven zinc atoms, but only one or two zinc atoms are readily available for transfer to other proteins (Maret, 2003). The remainder require disruption of the zinc-thiol coordinate covalent bonds for zinc release, and it is conceivable that variation in the number of zinc atoms associated with MTs in different cell systems could account for differences in observations. Considering the minority of zinc atoms associated with MT that are readily available for transfer, high levels of intact MT could conceivably act as a "zinc sink" with the overall effect of reducing levels of available zinc. Furthermore, apoMT with an incomplete zinc complement has been reported in vivo (Pattanaik et al., 1994), and zinc in this state is extraordinarily tightly associated and unavailable. ApoMT has been postulated to be a physiological zinc chelator capable of reducing the activity of zinc-dependent proteins (Zeng et al., 1991). Thus, although low levels of MT fully associated with zinc could act as physiological zinc donors to maintain the activity of certain zinc-requiring proteins (including NF-κB), higher MT levels could, conceivably, have either no effect on the activity of such proteins or an opposite effect due to sequestration of zinc in an unavailable form. These possibilities must be taken into consideration in future studies of the role of MT in the activity of NF-κB and other zinc-requiring proteins.

In conclusion, we provide evidence that loss of MT-1 and MT-2 expression in MT-null cell lines leads to decreased p65, but not p50, NF-κB subunit levels, and decreased NF-κB transcriptional activity. Reconstitution of MT-1 expression in MT-null cells restores NF-κB levels and activity, indicating that the MT is the key mediator of reduction in NF-κB activity concomitant with loss of MT. Apparently contradictory evidence supporting both positive and negative regulation of NF-κB by MT, in different cell types with MT levels altered using different strategies, has been observed. The apparent discord among observations should be examined in

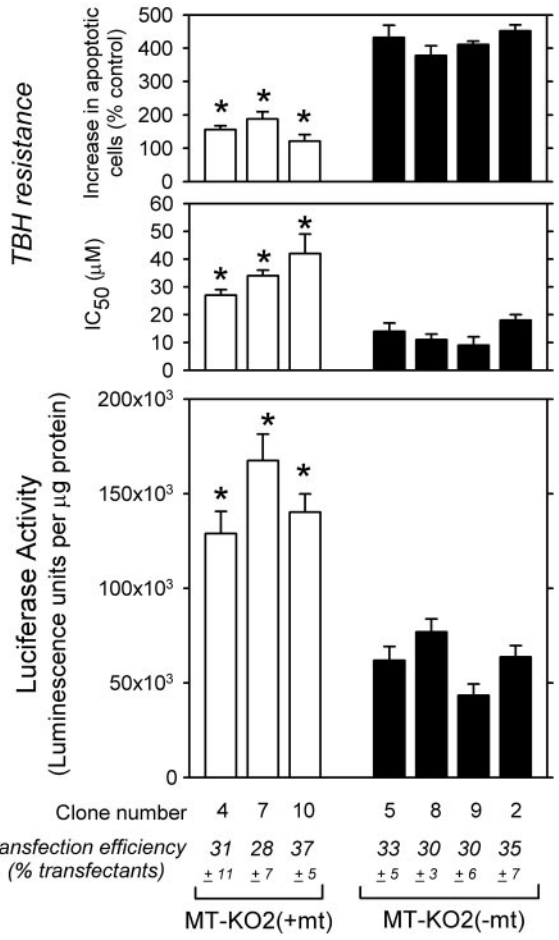

Fig. 8. Resistance to TBH (growth inhibition and induction of apoptosis shown in upper graphs) and NF-κB activity (lower graph) are higher in MT-KO2(+mt) clones expressing a stably transfected mouse MT-1 expression vector (white bars) than in control MT-KO2(−mt) clones (black bars). The IC_{50} of TBH and apoptosis induced by 20 μM TBH in three MT-KO2(+mt) and four MT-KO2(−mt) clones was measured, and NF-κB-dependent reporter activity (luciferase-generated luminescence) was assessed. Bars represent, for each clone, the mean ± S.E.M. of values from four assays. Asterisks indicate higher IC_{50} levels and lower apoptotic levels (indicative of greater resistance to TBH), and increased reporter activity in MT-KO2(+mt) clones compared with control MT-KO2(−mt) clones ($n = 4$, $p \leq 0.01$, one-way ANOVA). The transfection efficiency of each experiment is indicated (mean ± S.E.M., $n = 4$).

light of the dual capacity of MT to both sequester and donate zinc under different circumstances, particularly, the number of metal ions associated with MT molecules, cellular localization of MT, and the amount of zinc available in diet or culture media. Delineation of the role of MT in mediating NF-κB activity (including the intriguing possibility of either up- or down-regulation under different circumstances) has the potential to lead to targeted alteration of cellular sensitivity to apoptosis, including therapeutic apoptosis in human tumor cells.

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