Activation of Nuclear Factor κB: Potential Role in Metallothionein-mediated Mitogenic Response

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Abstract

The antiapoptotic response and enhanced cellular proliferation observed in neoplastic cells on overexpression of metallothionein (MT) have been well documented. We have investigated the mechanisms associated with this phenomenon by using MT inducers that increased MT transcripts and stimulated growth in MCF-7 cells. A MT antisense phosphorothioate oligonucleotide inhibited growth induction by >50%, suggesting a potential role of MT in mediating the mitogenic effects of these agents. Mobility shift assays using oligonucleotides encompassing the consensus nuclear factor κB (NFκB) binding site and anti-MT antibody revealed activation and a specific interaction of NFκB with MT. Cotransfection experiments using expression and reporter constructs demonstrated that MT causes transactivation of NFκB. Gel shift assays using purified proteins showed a specific interaction between MT and the p50 subunit of NFκB. These data indicate that MT may be involved in the interaction of NFκB with the DNA-binding domain and further suggest a potential role for NFκB in mediating the antiapoptotic effects of MT.

Introduction

MTs are ubiquitous low molecular weight proteins with high binding affinity to bivalent metal ions. A number of studies have demonstrated the presence of enhanced synthesis of MT in rapidly proliferating normal cells (1), regenerating cells (2), and cancer cells (3). These studies have suggested a relationship between high expression of MT protein and the presence or enhanced synthesis of MT in rapidly proliferating normal cells. A number of studies have demonstrated that down-regulation of MT in MCF-7 cells with a 18-mer antisense phosphorothioate oligonucleotide inhibited growth induction by >50%, suggesting a potential role of MT in mediating the growth induction of these agents. Mobility shift assays using oligonucleotides encompassing the consensus nuclear factor κB (NFκB) binding site and anti-MT antibody revealed activation and a specific interaction of NFκB with MT. Cotransfection experiments using expression and reporter constructs demonstrated that MT causes transactivation of NFκB. Gel shift assays using purified proteins showed a specific interaction between MT and the p50 subunit of NFκB. These data indicate that MT may be involved in the interaction of NFκB with the DNA-binding domain and further suggest a potential role for NFκB in mediating the antiapoptotic effects of MT.

Materials and Methods

Cell Culture and Reagents. MCF-7 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids, sodium pyruvate, streptomycin (100 μg/ml), and penicillin (100 units/ml). MCF-7 cells were initially serum-starved in culture medium containing 1% fetal bovine serum for 24 h and subsequently stimulated with zinc sulfate (100 μM), 17β-estradiol (10 nM), or TPA (100 nM) for various time intervals as indicated in the text. All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated in the text.

Growth Assay. Serum-starved MCF-7 cells were initially transfected with an 18-mer antisense MT phosphorothioate oligonucleotide or an 18-mer reverse antisense oligomer according to the procedure reported earlier from our laboratory (7). The cells were subsequently subjected to stimulation by zinc sulfate (100 μM) or 17β-estradiol (10 nM) for up to 24 h. [3H]Thymidine (0.5 μCi/ml) was then added and allowed to incorporate into DNA for 8 h. Cells were then harvested and lysed, and DNA was precipitated in 5% trichloroacetic acid and resuspended in NaOH. The incorporation of [3H]thymidine was determined in triplicates by scintillation counting.

Transcriptional Activation Studies. To examine the transcriptional activation effect of MT on NFκB, MCF-7 cells were initially seeded to 70–80% confluence and subsequently transfected (1:5) with MT eukaryotic expression vector pGECNeo-sMT-IIA harboring the hMT-IIA and pHIV-LTR-CAT mutant construct (12). The deletion mutant reporter construct contained only the NFκB-binding site in the long terminal repeat promoter region immediately upstream from the CAT reporter gene. Transfection was performed using electroporation with a single pulse of 250 V/960 μF (Gene Pulser; Bio-Rad Laboratories, Hercules, CA). To monitor transfection efficiency, cells were transfected with pCMV-βGal plasmid followed by in situ detection of β-galactosidase activity (7). Cell lysates were prepared, and 100 μg of total protein were assayed for CAT activity as described previously (7).

RNA Isolation and RT-PCR Amplification. Total cellular RNA was isolated from the cells after experimental manipulations using RNAzol (Tel-Test, Inc.; Friendswood, TX) and prepared for RT-PCR. Total RNA (200 ng) was primed with 12–18-mer oligodeoxynucleotidylic acid (Promega, Madison, WI) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in the presence of RNasin (20 units; Promega) in a 20-μl total reaction volume. MT gene
amplification was performed using 1 μl of the synthesized cDNA with 35 cycles of two-temperature PCR amplification using Taq DNA polymerase (Promega) in a Perkin-Elmer Corp. GeneAmp 9600 thermal cycler. PCR cycles included denaturation for 15 s at 95°C and annealing and extension for 15 s at 59.3°C. Based on the mRNA sequence of the human MT-IIA gene, a pair of primers, 5'-GCTCGCCATGGATCCCAACTG-3' (sense) and 5'-TTGTCCGACGCCCTTTG-3' (antisense), was synthesized in our laboratory to amplify a fragment encompassing the coding region of the MT gene to generate a PCR product of 171 bp (7). For quantitative analysis, amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was performed using the amplimer set 5'-GAAGGTGAGGTCGGAGTCAACG (sense) and 5'-TGCCATGGGTGGAATCATATTGG (antisense; Clontech Laboratories, Inc., Palo Alto, CA). The PCR products were subsequently resolved by electrophoresis on a 2% agarose gel containing ethidium bromide, visualized under UV light, and analyzed by densitometric scanning (Model GA-700; Bio-Rad Laboratories).

**Gel Retardation Experiments.** Nuclear extracts were prepared by initially harvesting cells on ice, which were then washed with PBS, resuspended in hypotonic buffer, and subsequently subjected to Dounce homogenization. After centrifugation, the nuclei were isolated and lysed after a suspension in low-salt buffer that was followed by adjustment of the KCl concentration to 300 mM using high-salt buffer. Nuclear extracts were then obtained by centrifugation (25,000 × g, 30 min at 4°C) and optimized by dialysis in a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; dithiodiethylidichlororothiophene. Total proteins were determined (BCA; Pierce) in the supernatants, and samples were stored at -80°C. Crude nuclear extracts (10 μg) were preincubated with 4 μg of poly(dI-dC) in a reaction mixture containing 12% glycerol, 12 mM HEPES, 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, and 1 mM DTT at 30°C for 15 min. [32P]ATP-labeled oligonucleotides containing consensus binding sites for NFκB or AP-1 (Promega) were added and incubated for an additional 30 min. DNA probes were prepared by end-labeling the positive- and negative-strand oligonucleotides using [γ-32P]ATP and T4 polynucleotide kinase. Competition assays were performed by incubating the extracts with 50-200-fold molar excess with unlabeled nucleotides. Polyclonal rabbit antihorse MT antibodies raised in our laboratory as described previously (7) were then added and incubated for an additional hour. Samples were then resolved on a 6% nondenaturing polyacrylamide gel. Gels were dried and exposed to Kodak XAR film for 12-48 h at -70°C, and autoradiograms were tested for supershifts. Similar experiments using 200 ng of purified MT (Sigma) and 200 ng (1.6 gel shift units) of rhp50 protein (Promega) were performed.

**Results**

**Induction of MT Gene and Activation of NFκB.** MCF-7 cells were initially exposed to MT-inducing agents 17β-estradiol and zinc sulfate at concentrations (10 nM and 100 μM, respectively) that can stimulate cellular proliferation. The RT-PCR data demonstrated an increase in MT transcripts in a time-dependent manner as early as 4 h, with peak induction occurring at 16 and 24 h, respectively (Fig. 1a). The induction of NFκB by these agents was also examined under conditions identical to those used for stimulating cellular proliferation. Gel mobility shift experiments using nuclear extracts and a [γ-32P]ATP-labeled double-stranded oligonucleotide containing the consensus κB-binding site as a probe showed an early activation (within 4 h) of NFκB in MCF-7 cells (Fig. 1b). These results demonstrate that both the induction of MT and the activation of NFκB occur in MCF-7 cells on exposure to mitogenic agents, suggesting that both events are probably involved in mediating the growth-stimulatory response.

**Blockade of Zinc- and 17β-Estradiol-induced Growth-stimulatory Response by MT Antisense.** To further characterize whether growth-stimulatory effects of zinc and estradiol can be mediated by induced MT, MCF-7 cells were initially transfected with an 18-mer phosphorothioate antisense oligonucleotide and subsequently stimulated with these agents. Cells treated with a reverse sequence of the 18-mer phosphorothioate antisense oligonucleotide were also used in these studies as control. The ability of the antisense oligonucleotide to down-regulate MT protein synthesis in this cell line has been reported previously (7). The results depicted in Fig. 2 indicate that the growth stimulation observed on treatment with these factors was abrogated by approximately 50% in cells transfected with the MT antisense as opposed to those transfected with the reverse oligomer. These findings suggest a role for MT in mediating the mitogenic response of these factors in this cell line.

**Transactivation of NFκB by MT.** To dissect whether NFκB can also be activated by MT in the absence of stimulatory factors, MCF-7 cells were transfected with eukaryotic expression plasmid pBAcNEO-SMT-IIA and mutant reporter plasmid pHIV-LTR-CAT. The former is an expression vector carrying hMT-IIA under the control of the β-actin promoter (7), and the latter is a deletion mutant construct with sequences encompassing the NFκB site upstream of a CAT enzyme (12). Cotransfection experiments with these plasmids indicated a 3-fold activation of NFκB as determined by the induction of CAT activity by MT in the absence of stimulatory factors when compared to that of controls (Fig. 3).

**Interaction of MT with NFκB.** Gel mobility shift assays using nuclear extracts from zinc-stimulated cells were initially performed to determine NFκB activation (as shown in Fig. 1b). Both the p50/RelA and [p50]2 dimers could be observed in stimulated cells (data for [p50]2 are not shown in Fig. 4a); however, very little NFκB-binding activity was observed in nonstimulated control cells. The specificity for DNA binding was examined by the addition of 100-fold molar excess of unlabeled probe that abrogated the binding observed in zinc-stimulated cells. To
further confirm the specificity of NFκB binding, nuclear extracts were treated with an AP-1 oligonucleotide probe. The results showed no activation of AP-1 transcription factor (data not shown). The addition of MT antibodies to the DNA/protein complex resulted in a supershift with a significant decrease in the p50/RelA complex. Subsequent gel shift assays using 17β-estradiol as a mitogenic agent demonstrated similar interaction, but to a lesser magnitude (data not shown). This may presumably be attributable to differences in the potency of MT and NFκB inducibility by these factors. No binding activity was observed between MT antibody and NFκB oligonucleotide probe, thus excluding the possibility of nonspecific interactions. To dissect the specificity of MT/NFκB interaction, purified MT and rhp50 proteins were used. Gel mobility shift assays indicated a specific interaction between MT and rhp50 protein, as evidenced by a supershift on the addition of MT antibody (Fig. 4b). However, the addition of MT to the oligonucleotide probe did not show binding activity, indicating that there is no nonspecific interaction (Fig. 4b). Similarly, nonspecific interactions of the MT antibody with rhp50 were also not observed in a separate set of experiments using nuclear extracts from the stimulated MCF-7 cells (data not shown).

Discussion

The current series of experiments was designed to characterize the role of MT in inducing cellular proliferation and to identify the potential mechanisms that could mediate such an effect. Zinc, estradiol, and TPA were used as MT-inducing agents (13–15) in this study. Besides inducing MT transcription, these agents are known to promote cellular growth (16–18) by mechanisms that are not yet fully understood. By using nuclear extracts from stimulated MCF-7 cells, we have shown that zinc, estradiol, or TPA can not only induce MT gene expression but can also cause activation of NFκB in these cells. These findings were further confirmed by using purified MT and rhp50 proteins, which showed a specific interaction as demonstrated by a supershift with a MT antibody (Fig. 4b). Although the intricate signaling mechanisms by which these agents can stimulate cellular proliferation or exert their antiapoptotic effects are still fragmentary, our results of MT antisense experiments (Fig. 2) in this report clearly suggest that MT is involved in inducing these effects. These findings are consistent with our recent study using both MT antisense and MT

Fig. 2. Inhibition of growth stimulation by an antisense MT oligonucleotide. Serum-starved cells were transfected with MT antisense oligonucleotide (AO) or reverse antisense oligonucleotide (RO) as a control using electroporation and were subsequently subjected to 100 μM zinc sulfate or 10 nm 17β-estradiol for 24 h. [3H]Thymidine was added to a final concentration of 0.5 μCi/ml for 8 h before harvesting the cells. Cells were washed two times in PBS and lysed as described in “Materials and Methods.” DNA was precipitated and resuspended in NaOH. The incorporation of [3H]thymidine was then measured by liquid scintillation.

Fig. 3. Transactivation of NFκB by MT. MCF-7 cells were cotransfected (5:1) with hMT-IIA expression plasmid pBAcNEO-sMT-IIA and pHIV-LTR-CAT, a deletion mutant reporter construct encompassing the NFκB binding site upstream from the CAT gene. Cell-free extracts were prepared 48 h after transfection as described in “Materials and Methods.” The total proteins were measured. CAT activity was determined in cell extracts containing equal amounts of protein (100 μg) by measuring the incorporation of tritiated acetate in acetyl chloroamphenicol with a 3-6-h assay mixture. The results were expressed as a fold increase in the CAT activity as a percentage of that of the control. pHMT-IIA, cells transfected with hMT-IIA expression vector pBAcNEO-sMT-IIA; pHIV-LTR-CAT, cells transfected with the deletion mutant construct encompassing the NFκB binding site upstream from the CAT gene; pHIV-LTR-CAT, cells cotransfected with pBAcNEO-sMT-IIA and pHIV-LTR-CA.

Fig. 4. a, interaction between MT and NFκB in stimulated MCF-7 cells. Cells were stimulated with zinc for 4, 6, or 16 h as described in the Fig. 1a legend. Nuclear protein extracts (10 μg) were added to a reaction mixture containing a [32P]labeled double-stranded DNA oligonucleotide (5'-AGTTGAGGGGACTrTCCCAGGC-3') encompassing the NFκB binding site, poly(dI-dC), and BSA in binding buffer and allowed to incubate at 30°C for 15 min. Competitive assays were performed by the addition of molar excess of unlabeled oligonucleotides. Anti-MT antibodies were added, and incubation continued for an additional hour. The reaction mixtures were then fractionated onto 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to Kodak X-ray film for 48 h at −70°C. Autoradiograms were analyzed by densitometric scanning (a, a supershift; b, p65/p50 complex). A, interaction between the MT protein and rhp50 subunit. MT protein (200 ng) and rhp50 protein (200 ng, 1.6 gel shift units) were added to the reaction mixture and incubated as described above. Supershifts were determined by the addition of anti-MT antibodies. Products were fractionated and analyzed as shown above (a, a supershift; b, p65/p50 complex).

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overexpression experiments (7). As an apoptosis inhibitor (16), zinc is a ubiquitous component of chromatin and enzymes involved in transcription and accessory transcription factors that relay a variety of intra- and intercellular signals to the nucleus and to the transcriptional apparatus. The fact that MT serves as a zinc-specific storage site with no recognizable direct DNA-binding site implies that it can induce its biological effects via activation and/or donation of zinc to zinc fingers or twists of transcription factors, thereby stabilizing their DNA binding and subsequently leading to transactivation of the genes. To this end, a recent study (19) demonstrated that MT is perhaps required for the binding of estrogen receptor to its DNA-binding site, which subsequently results in transactivation of other genes.

Based on other reports showing that MT-mediated growth induction is associated with its translocation to the nucleus (2), we further examined the potential interaction of MT with NF-κB in zinc-stimulated MCF-7 cells. Our study showed that in stimulated cells, MT can activate and interact with NF-κB, a finding that was confirmed with purified proteins (Figs. 3 and 4, a and b). The fact that only a 3-fold transactivation of NF-κB was observed on cotransfection of MCF-7 cells with hMT-IIA and pHIV-LTR-CAT may be attributed to the fact that more than one MT isoform can be induced by zinc, estradiol, and TPA, and differential transactivation may be observed by other isoforms. No significant shift was observed on the interaction of MT with rhp50; this may be ascribed to the relatively small size of MT (6–7 kDa). However, the increase in the intensity of p50 binding to its binding site in the presence of MT suggests that MT specifically interacts with the p50 subunit of the p50/RelA dimer and that MT may be required for stabilization of its DNA complex formation, thus allowing potent transactivation of target genes. Whether the MT-mediated enhanced DNA binding of NF-κB is attributed to the formation of high-order structures, cooperative DNA binding on apparent adjoining sites, or other unknown mechanism(s) certainly warrants further investigation. Our findings are in accord with a recently emerging mechanism by which NF-κB and other DNA-binding factors may physically combine to induce or repress transcription. This may be exemplified by the interaction between NF-κB and c-Jun and/or c-Fos with either an NF-κB binding site or an AP-1 binding site, suggesting that one of the factors contacts the DNA, whereas the other seems to act as an accessory factor without binding to DNA (20).

The activation and interaction of NF-κB with MT may be further strengthened by the fact that these two factors share many properties suggestive of their role in growth and proliferation. These include (a) up-regulation of these proteins during development (21, 22); (b) translocation of these proteins to the nucleus on activation or induction (2, 23); and (c) both activation of NF-κB (24) and induction of MT gene expression (2) have been shown to occur during rapid tissue regeneration after partial hepatectomy in vivo. Furthermore, both MT and NF-κB have been shown independently to possess antiapoptotic properties in various cell systems including abrogation of the cytotoxic effects of TNF-α (6, 11), protection from ionizing radiation (25, 11), and resistance to anticancer drugs (4, 26). Moreover, recent evidence (2, 5) suggests that the MT-mediated antiapoptotic and cytoprotective effects are contingent on its nuclear localization. Therefore, it is apparent that such cooperation between MT and NF-κB may explain their protective effects by the activation/suppression of other genes involved in proliferation and/or apoptosis such as c-myc, bcl2, and p53. The results presented here suggest that inhibitors of both MT and NF-κB may have potential therapeutic application in inducing apoptosis in neoplastic cells.

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References

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