Induction of Activating Transcription Factor 1 by Nickel and Its Role as a Negative Regulator of Thrombospondin 1 Gene Expression

Konstantin Salnikow,1 Sheng Wang,3 and Max Costa

Departments of Environmental Medicine [K.S.S., S.W., M.C.] and Pharmacology [M.C.] and The Kaplan Cancer Center [M.C.], New York University Medical Center, New York, New York 10016

ABSTRACT

Thrombospondin 1 (TSP 1) is an extracellular matrix glycoprotein that influences cell adhesion, motility, and growth. On the basis of its effects on endothelial cell proliferation, TSP 1 has attracted interest as a potential regulator of solid tumor growth through modulation of tumor blood supply. The regulation of TSP 1 expression is of critical importance for designing new approaches in tumor therapy. Recently, we have shown that TSP 1 expression is lost in nickel-transformed cells. In this paper, we identified an activating transcription factor (ATF)/cAMP-responsive element-binding protein binding site as a negative regulatory site in the 5′-flanking sequence of mouse TSP 1 promoter. We identified ATF-1 as a major component of the ATF/cAMP-responsive element-binding protein binding complex. This M, 35,000 nuclear ATF-1 protein was shown to be present in higher amounts in nickel-transformed 3T3 cells that do not express TSP 1. Acute treatment of 3T3 cells with NiCl2 resulted in the induction of this transcription factor, and this induction was correlated temporally with the suppression of TSP 1 expression in the same cells. These results show that nickel exposure causes accumulation of the ATF-1 transcription factor, which is responsible for the down-regulation of transcription of TSP 1, and possibly other tumor suppressor genes during nickel-induced cellular transformation.

INTRODUCTION

TSP1 is a multifunctional, large extracellular matrix glycoprotein (Mr 420,000) that is synthesized by a variety of cells, such as neutrophils, monocytes, macrophages, endothelial cells, fibroblasts, keratinocytes, and smooth muscle cells (1–3). Because TSP 1 protein influences cell-to-cell (4) and cell-to-matrix interactions, it plays an important role in embryogenesis (5, 6), wound repair (2, 7), inflammation (2, 8), and tumorigenesis (2, 9–14). Loss of TSP 1 expression has been found in carcinogen- and oncogene-transformed cells (15–17). It was shown further that TSP 1 inhibits angiogenesis, and there was good correlation between lower levels of TSP 1 expression in tumor cells and angiogenic activity (9, 10, 12–14). TSP 1 inhibits proteases, including plasmin and urokinase, which depress metastatic activity of tumor cells (18). Overexpression of TSP 1 in tumor cells restored the normal cellular phenotype and suppressed their tumorigenesis in vivo by preventing growth of blood vessels (11, 13, 14).

Numerous factors that affect TSP 1 gene expression were studied over the last decade. It was found that TSP 1 expression can be superinduced by cyclohexamide in a manner similar to the induction of c-fos and other immediate early growth-regulatory genes (20). Thus far, little is known about the transcriptional regulation of TSP 1 gene expression, although some attempts have been made to understand the mechanism of regulation of constitutive TSP 1 gene expression and to identify important transcription factors that are involved in its regulation. There is evidence that TSP 1 gene expression is dependent on yet-unidentified tumor suppressor gene(s) (10, 23). Two known tumor suppressor genes, Rb and p53, were shown to be involved in the positive regulation of TSP 1 gene expression (16, 24).

Functional analysis of the human TSP 1 promoter region was carried out using a chimeric gene construct containing a −2004 to +749 fragment of the TSP 1 promoter fused to the promoterless CAT gene (25). This analysis did not reveal any important regulatory elements in the promoter region spanning from −2004 to −234, whereas an important element(s) was localized in the region of the first intron between positions +576 and +727 (25). However, the importance of the first intron for TSP 1 expression was questioned in experiments with chimeric TSP-bovine growth hormone constructs (21). Potential binding sites for AP-1, AP-2, SP-1, NF-IL6, ATF/CREB, EGR-1, and SRE transcription factors were identified on human and mouse TSP 1 promoters (11, 25–27). In spite of the sequence similarities between human and mouse TSP 1 promoters, some important differences exist. For example, the mouse TSP 1 promoter did not contain a c-fos direct repeat that was present in the human promoter, and the mouse TSP 1 promoter contained EGR-1, NFI, and NF-κB sequences not present in the human promoter. It is not yet known how these differences in structure of the promoter will be translated into differences in regulation of the gene in mouse and human cells.

In the present study, we were interested in studying the molecular mechanisms leading to inactivation of TSP 1 expression by nickel in 3T3 cells. We have shown that methylation of TSP 1 promoter is not involved in silencing of the gene, and all effects of nickel are exerted at the transcriptional levels.

MATERIALS AND METHODS

Cell Culture. The BALB/c 3T3 mouse fibroblast cell line from American Type Culture Collection and the derived nickel-resistant and transformed B200 cells were used in this study (28). The transformed phenotype (i.e., ability to grow in soft agar, and so forth) of B200 cells has been described previously (29). The 3T3 and B200 cells were cultured in α-MEM supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO2. B200 cells were resistant to the growth suppression of 200 μM NiCl2. Because these cells inherently maintain their nickel resistance and transformed phenotype, all of the experiments reported here were carried out without culturing B200 cells in the presence of NiCl2.

3T3 cell viability after treatment with 1 mM of NiCl2 for 24 h was monitored by trypan blue staining and was found to be 80–85%.

Analysis of Methylation Status of TSP 1 Promoter Region by Southern Blot. Genomic DNA from 3T3 and B200 was digested with a 4-fold excess of restriction enzymes (MspI, HpaII, and CfoI). Ten μg of digested DNA were fractionated in a 1% agarose gel and transferred to nylon Nitran membranes.
NEGATIVE REGULATORS OF THROMBOSPONDIN I EXPRESSION

(Chen and Schueller, Keene, NH) in 20X SSC (3 mM NaCl and 0.3 mM sodium citrate (pH 7.0)) buffer overnight. Hybridization of the blot was carried out as recommended by the manufacturer. The 1.4-kb mouse TSP I promoter probe was labeled with [32P]dCTP using a random primer kit (United States Biochemical Corp., Cleveland, OH).

Analysis of Methylation Status of TSP I Promoter Region by PCR. Equal amounts of genomic DNA from 3T3 and B200 cells were first digested with methylation-sensitive enzymes AflII, Aval, Mvel, and HaelII. After purification and ethanol precipitation, the DNA concentration was measured again for each sample. Equal quantities of digested DNA were amplified by PCR using a specific TSP I/PCR primer set, 5'—AGTTGAAGATGCTTTGATG and 5'-ATTGCCCTGAAGAATTCCGAAT and Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) under the following conditions, as shown above. The PCR products were then cloned into the pCR II vector (Invitrogen, San Diego, CA), and the insertion orientation was checked by SacI and AflII digestion. Sequence identity of the whole fragment was confirmed by using a Sequenase kit (United States Biochemical). The fragment was recloned into the HindIII and XbaI site of a promoterless pCAT vector (Promega, Madison, WI). Three deletion fragments of the TSP promoter were also obtained by PCR using the pCR II plasmid containing an insert of the 1.4-kb promoter region as template. Primers used for preparation of the deletion fragments contained a HindIII-cuting site at the 5' end and the XbaI site at the 3' end, as follows: upper primer 1, 5'-CATGGAACAGACCTTAG-GATCGCGAGCATGCTTACG-3'; upper primer 2, 5'-CATGAAAGACGACTTGACG-AATGGGCGCGAGCATGCTTACG-3'; upper primer 3, 5'-CATGGAACAGACCTTAG-GATCGCGAGCATGCTTACG-3'; and lower primer (for all), 5'-CAGACTAAGCTCTAGAAATGCAGATGCGCGGACAGGGG-3'.

One deletion mutant was obtained by the Exc/Mung digestion of the pCR II plasmid, and it was recloned into promoterless CAT reporter vector. The 1.4-kb TSP I-CAT plasmid with a mutant ATG site was obtained by site-specific mutagenesis protocol (30). The various TSP I-promoter deletion constructs are shown in Fig. 3.

Transient Transfections and Quantitation of CAT and β-Gal Activity. Cells were transfected with 5 μg of plasmid DNA (pTSP-CAT; deletion mutants of pTSP-CAT; and control, pCAT vector) and 5 μg of pCH110, which codes for β-gal. β-Gal expression was used to quantify the efficiency of transfection among the various experiments. Cells (3 x 10^4) were plated into a 100 mm tissue culture dish and cultured for 16 hours at 37°C. The cells were washed with α-MEM without serum, cultured in α-MEM supplemented with 5% newborn calf serum for 4 h, and then transfected with the vectors using calcium phosphate precipitation. Cells were washed with α-MEM without serum, cultured in α-MEM supplemented with 5% newborn calf serum for 4 h, and then transfected with the vectors using calcium phosphate precipitation. Cells were washed with α-MEM without serum 16 h later and were then cultured in complete medium for an additional 36–48 h to allow expression. The cell lysates were obtained by four cycles of freeze-thawing in PBS buffer. CAT and β-gal expression were measured using CAT and β-gal ELISA kits (Boehringer Mannheim), and the results were obtained using a microplate reader (MR 5000; Dynatech Laboratories, Inc., Chantilly, VA). All CAT activities were normalized to β-gal activities. Four transfection experiments each (in duplicate) were performed to assure reproducibility of the data.

Electrophoretic Mobility Shift Assays. Nuclear protein extracts from 3T3 and B200 cells were prepared by the method of Dignam et al. (31). The protein concentration of the extracts was measured using a protein determination kit (Bio-Rad Laboratories, Hercules, CA) with BSA used as a standard. The normal NF-1, SRE, and ATF (ACTGTCCGGGGCTGACCTCGAATAAA-GAGAT) and mutated ATF (T—C) (ACTGTCCGGGGCTGACCTCGAATTAAGAGAT) fragments of TSP I promoter containing potential binding sites were purchased from National Biosciences, Inc. (Plymouth, MN). These oligonucleotides were labeled with [γ—32P]ATP (Amersham Life Science, Arlington Heights, IL) and T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, MA). The electrophoretic mobility shift assay was performed with labeled probe (5 x 10^4 cpm) added last to a 20-μl reaction mixture containing nuclear extract (4 μg of nuclear proteins), 7.5% glycerol, 0.5 μg of poly(deoxyguanosin deoxyctydilic acid), 5 mM MgCl2, 60 mM NaCl, and 10 mM KCl, in 10 mM HEPES buffer (pH 7.9). The double-stranded unlabeled ATF/CREB oligonucleotides was used as a specific competitor.

For supershift experiments, 8 μg of nuclear proteins were incubated for 1 h with antibodies at 4°C prior to the oligonucleotide addition. Anti-ATF-1, anti-ATF-2, anti-ATF-3, anti-ATF-4, anti-CREB-1, and anti-CREB-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Samples were electrophoresed in a 6% nondenaturing polyacrylamide gel in 0.5X Tris-borate-EDTA buffer (10X TBE, 0.89 mM Tris-borate, 0.025 mM EDTA) for 3 h at 12 V/cm. The gels were visualized dried and exposed to X-ray film (Eastman Kodak Co., Rochester, NY).

Western Blot Analysis. Fifteen μg of the same nuclear extracts that were used for a mobility shift assay were separated on 10% SDS-PAGE, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Nonspecific binding was blocked by soaking the membrane in 5% nonfat dry milk in TBS buffer [20 mM Tris—HCl (pH 8.0), and 150 mM NaCl with 0.1% Tween 20] overnight at 4°C. The membrane was washed (four times for 15 min each) with TBS buffer and incubated for 1 h with anti-ATF-1 antibodies at room temperature. After washing (four times for 15 min each) with TBS buffer, the membrane was incubated with a second antiserum peroxidase-conjugated antibody (Cappel, Durham, NC) and then washed again with TBS buffer (four times for 15 min each). Chemiluminescent substrate Lumi-GLO (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added, and the membrane was exposed to X-Oмат HAR film (Eastman Kodak Co.). After the exposure, the membrane was rinsed with water and silver stained to evaluate protein levels. The molecular weight of ATF-1 was determined using prestained molecular weights markers (Bio-Rad Laboratories).

Northern Blot Analysis. Total RNA was isolated using an RNA prepara-tion kit (RNAzol; Cinna/Biotech Laboratories, Inc., Houston, TX). The RNA (15 μg) was fractionated in a 1.0% formaldehyde agarose gel and then transferred to nitrocellulose membranes (BA-85; Schleicher & Schuell). The membranes were hybridized with a 32P-labeled TSP I cDNA probe (16). Ethidium bromide staining was used to evaluate the nucleic acid loading of each lane.

RESULTS

Low Levels of TSP I Transcription in Nickel-transformed B200 Cells. The TSP I transcription levels in 3T3 and B200 cells were examined by Northern blot analysis using hamster TSP I cDNA as a probe. The analysis showed that the TSP I transcription in B200 cells was almost negligible compared to that of 3T3 cells (Fig. 1). The inactivation of TSP I expression could be accounted for either by hypermethylation of TSP I promoter or by infringement of transcriptional regulation in B200 cells.

Analysis of Methylation Status of the 5' End of TSP I in 3T3 and B200 Cells. To analyze the methylation status of the TSP I promoter region in 3T3 and B200 cells, genomic DNA was digested with the methylation-sensitive restriction endonuclease HpaII and CfoI and the methylation-insensitive restriction endonuclease MspI. Southern blot analysis did not reveal differences in methylation pattern between 3T3 and B200 cells. The analysis was repeated three times with identical results. A representative autoraph is shown in Fig. 2a. The methylation status of some other CpG dinucleotides was examined further using the methylation-sensitive restriction endonucleases AavIII, Aval, Mvel, and HaelII followed by PCR. Again, no differences were found between the two cell lines (not shown). The HaelII site overlapped with the CfoI site, which is located at position 210. This site was methylated as judged by Southern blot analysis. Digestion of genomic DNA with HaelII followed by PCR amplifica-tion of this region confirmed the methylation status of this site.

The region of promoter from +82 to +111 contained three recognition sites for HpaII and one for CfoI within very small distances. Therefore, these sites could not be analyzed by Southern blot. We used sodium bisulfite analysis to measure whether these CpG sites were methylated differently in 3T3 and B200 cells. The method uses bisulfite-induced modification of genomic DNA under conditions
expression. The total RNA (15 μg) from 3T3 cells, 3T3 cells treated with 1.0 mM NiCl2
obtained using methylation-sensitive restriction endonucleases. CpG dinucleotides are also located within the
I promoter shown in Fig. 2b summarizes the obtained results.

whereby cytosine is converted to uracil, but methylated cytosine remains nonreactive. The target DNA was amplified by PCR and
sequenced. The results obtained with this method again did not show
differences in the methylation status between cell lines (data not shown).
The map of methylated and nonmethylated CpG sites on TSP
promoter as a probe. B. map of methylated (m) and
nonmethylated (n) CpG sites in TSP I promoter
shown). The map of methylated and nonmethylated CpG sites on TSP
region, as well as all deletion
whereas to DNA methylation (not shown).

Potential Binding Sites for Transcription Factors on the Mouse TSP I Promoter. The location of potential binding sites for transcription
factors in the mouse TSP I promoter has been published by
Lawler et al. (26). The region from −1400 to +52 contains almost all
known important regulatory sequences, including six SP1
(CCCGCCCC) sites at −1210, −644, −180, −80, −55, and −40; two
AP-1 sites (TGAG/CTCA) at positions −527 and −430; five AP-2
binding sites (CCCT/AG/CCG) at positions −1220, −673, −447, −354, and −24; two NF-1 binding sites (TGGC/A(N)GCCA) at
positions −1265 and −1158; one NFkB-binding site (GGG
GACTCT) at position −260; one SRE (TCCTTATITGG) at position −1188; and one ATF/CREB (TGACGTCTT) responsive element at
position −1098.

Using a MacVector 4.5 computer program, we have analyzed the
promoter region of mouse TSP I and have identified two additional
recognition sites for NF-IL6 transcription factor at positions −865
and −827. The consensus sequence for this transcription factor,
TTNNGNAAT, was also found in the promoters of MDRI genes from
hamster to human (32) and has been suggested to be activated via a
ras-dependent signal transduction pathway (33). There is also a Rb
control element (CCACCCC; Ref. 34) within this fragment of TSP I
promoter. This Rb element overlapped with the AP-2-binding site at
position −447. Rb regulatory elements have also been identified in
the promoter region of the c-fos and c-myc proto-oncogenes and
transforming growth factor β, and insulin-like growth factor II, as
well as other genes (35). We also found that mouse TSP I promoter
contained three potential p53-binding sites (T/C/TGCTC) at positions
−590, −10, and +34. p53 has been shown to be very important in the
regulation of human TSP I expression (24). The structure of the 1.4-kb
portion of mouse TSP I promoter region, as well as all deletion
fragments used in this study, is depicted in Fig. 3.

Identification of Binding Sites for Negative Regulators of TSP I
Expression. A 1.4-kb fragment corresponding to the 5' end of mouse
TSP I was obtained by PCR from the genomic DNA of 3T3 cells. This
fragment was cloned into promoterless CAT plasmid (pTSP-CAT1)
as described in “Materials and Methods.” Four additional constructs with
smaller fragments of the TSP I promoter were also prepared. Potential
binding sites and the activity of each fragment in driving
CAT expression are shown in Fig. 3. Transient transfection of these
constructs into 3T3 cells revealed an approximately 4-fold stimulation
of CAT amount when the first 400 bp of the 5' end of the 1.4-kb
fragment was deleted, suggesting the presence of a negative regula-
tory element within the region deleted (Fig. 3). Additional deletions of
the promoter, up to −200 bp, resulted in a decrease of CAT activity;
however, all plasmids (pTSP-CAT2–pTSP-CAT5) showed higher
activity compared to the original 1.4-kb fragment (pTSP-CAT1) (Fig.
3).

Six potential binding sites for transcription factors were located in

Fig. 1. Expression of TSP I in 3T3 and B200 cells. A. Northern blot analysis of TSP
expression. The total RNA (15 μg) from 3T3 cells, 3T3 cells treated with 1.0 mM NiCl2
for 24 h, B200 cells, and B200 cells treated with 1.0 mM NiCl2 for 24 h was fractionated in a 1.0% agarose gel and then transferred to a membrane. The membrane was hybridized
with 32P-labeled Chinese hamster TSP I cDNA probe. B. ethidium bromide staining of the
same gel was used to evaluate nucleic acid loading in each lane.

Fig. 2. Analysis of methylation status of the 5' end of the TSP I gene. A. 3T3 and B200 genomic
DNAs were cut using 4-fold excess of the enzymes
MspI, HpaII, and CfoI. Ten μg of digested DNA
were fractionated in 1% agarose gel and transferred
to a membrane. The membrane was hybridized
with a 32P-labeled 1.4-kb fragment of mouse TSP I
promoter as a probe. B. map of methylated (m) and
nonmethylated (n) CpG sites in TSP I promoter
obtained using methylation-sensitive restriction endo-
nucleases and sodium bisulfite treatment.

Downloaded from cancerres.aacrjournals.org on August 16, 2017. © 1997 American Association for Cancer Research.
elements were tested in gel mobility shift assays for binding with observe more binding of a negative transcription factor in extracts

because AP-2 had four sites and Spl had five sites within the 1.4-kb
amount of CAT obtained for the intact construct pTSP-CAT1. The data are expressed as
determined as described in 'Materials and Methods" and is expressed relative to the
I promoter. A 1.4-kb promoter fragment construct, pTSP-CAT1, and the shortened
represented a nickel-transformed and resistant clone from 3T3 cells
deleted region of the TSP T promoter.

unique responsive elements that were present in the first 400-kb of the
region of the TSP I promoter. We focused our studies on the three
promoter mutant reporter plasmid (pTSP-CAT1M) that contained a
responsible for negative regulation of TSP I, we constructed a 1.4-kb
efficiently competed by a 50-fold molar excess of unlabeled specific
nucleotides (Fig. 4).

transformed B200 cells compared with parental TSP I expressing 3T3
cells. We tested whether NiCl2 treatment would result in the
down-regulation of TSP expression by nickel (Fig. 6A). Similar results were obtained with nuclear extracts from B200 cells, confirming that ATF-1 was the factor involved in binding to ATF/CREB site in both cell lines (data not shown).

To substantiate further that ATF-1 is a negative regulator of TSP I expression, we analyzed nuclear extracts from 3T3 and B200 cells by Western blot. The molecular weight range of ATF-1 was shown to be between M, 35,000 and 40,000 (39). Using antibodies against ATF-1, we found that a nuclear protein with the molecular weight of approximately M, 35,000 was present at higher levels in nuclear extracts of B200 cells (Fig. 6B).

Acute Treatment of 3T3 Cells with Nickel Induces ATF-1 Transcription Factor and Suppresses TSP I Expression. Acute treatment of 3T3 cells with 1.0 mM NiCl2 for 24 h resulted in suppression of TSP I gene expression (Fig. 1). It is interesting to note here that in acutely treated cells, we did not observe complete inactivation of TSP I gene expression (Fig. 1). This suggested that another negative regulator(s) is also involved in inactivation of TSP I expression in B200 cells. We tested whether NiCl2 treatment would result in the

Fig. 3. Structure and activity of various intact and deleted fragments of the mouse TSP I promoter. A 1.4-kb promoter fragment construct, pTSP-CAT1, and the shortened promoter fragment constructs pTSP-CAT2-pTSP-CAT5 are depicted beneath the 1.4-kb promoter region of the TSP I promoter. We focused our studies on the three unique responsive elements that were present in the first 400-kb of the deleted region of the TSP T promoter.

Oligonucleotides representing sequences of these three responsive elements were tested in gel mobility shift assays for binding with nuclear extracts isolated from 3T3 and B200 cells. Clone B200 represented a nickel-transformed and resistant clone from 3T3 cells that did not express TSP I (see Ref. 16 and Fig. 1). We expected to observe more binding of a negative transcription factor in extracts from B200 cells if this factor was involved directly in the down-regulation of TSP I. No differences in binding of nuclear extracts were found for NF-1 and SRE oligonucleotides (data not shown); however, when an oligonucleotide probe for the ATF/CREB-binding site was tested, more binding was found in nuclear extracts from nickel-transformed B200 cells compared with parental TSP I expressing 3T3 cells (Fig. 4). One specific DNA-protein complex was demonstrated in gel shift assays for both nuclear extracts isolated from 3T3 and B200 cells (Fig. 4). This complex was specific, because it was efficiently competed by a 50-fold molar excess of unlabeled specific nucleotides (Fig. 4).

To obtain additional evidence that the ATF/CREB-binding site was responsible for negative regulation of TSP I, we constructed a 1.4-kb promoter mutant reporter plasmid (pTSP-CAT1M) that contained a single-point mutation at the ATF/CREB-binding site (T→C) in the TSP I promoter (see "Materials and Methods"). The high level of expression of this plasmid when transfected into 3T3 cells was comparable to the expression of the fragment that did not contain an
ATF-binding site (pTSP-CAT2) and was much higher than the whole promoter without this point mutation (Fig. 5). Additionally, nickel exposure down-regulated the expression of the intact TSP I promoter (see below) but did not down-regulate the TSP I promoter that had a point mutation in the ATF/CREB-binding element (Fig. 5). To verify that this base substitution indeed resulted in less protein binding to DNA, we tested the mutated oligonucleotide in a gel mobility shift assay. Fig. 4 shows significantly less binding of transcription factor(s) from nuclear extracts of both 3T3 and B200 cells to the mutated ATF/CREB oligonucleotide.

ATF-1 Transcription Factor Is a Negative Regulator of TSP I. Because any one of the large family of ATF/CREB transcription factors can potentially bind to the ATF/CREB-binding element (36–38), we used antibodies raised against each of the six members of the family to identify those involved in DNA binding. Only antibodies against transcription factor ATF-1 showed a supershift, indicating that this transcription factor was involved in DNA binding to the ATF/CREB site, and most likely, this ATF-1 transcription factor was involved in the down-regulation of TSP expression by nickel (Fig. 6A).
REGULATORS OF THROMBOSPONDIN I EXPRESSION

Fig. 5. The ATF/CREB-binding site is involved in down-regulation of TSP I in 3T3 cells. 3T3 cells were transfected with plasmids pTSP-CAT1 (1), pTSP-CAT1 followed by exposure to 1.0 mM NiCl₂ for 24 h after transfection (2), pTSP-CAT1M (same plasmid as pTSP-CAT1 but with a point-mutated ATF/CREB site (T→C; 3), pTSP-CAT1M followed by exposure to 1.0 mM NiCl₂ for 24 h after transfection (4), and pTSP-CAT2 (5). The CAT activity of each plasmid was determined and expressed relative to the amount of CAT obtained for construct pTSP-CAT1. The data are expressed as the means (bars, SD) from four independent experiments (for details, see “Materials and Methods”).

Fig. 6. ATF-1 transcription factor binds to the ATF/CREB site of TSP I promoter in 3T3 cells. A, nuclear extracts from 3T3 cells were incubated with specific antibodies against ATF-1, ATF-2, ATF-3, CREB-1, and CREB-2 and then tested for binding to the ATF/CREB element using a mobility gel shift assay, as described in “Materials and Methods.” B, nuclear extracts from 3T3 and B200 cells were analyzed by Western blot using antibodies against ATF-1.

DISCUSSION

To analyze the molecular mechanisms of inactivation of TSP I in nickel-resistant and -transformed B200 cells, we have cloned a 1.4-kb fragment of the 5′ end of TSP I and carried out Southern blot and PCR analyses using methylation-sensitive restriction endonucleases. Earlier, we have provided strong evidence that nickel silenced expression of some genes by enhancing DNA methylation (40), and we expected to find similar effects in the 3T3 cell system.

No differences, however, were found in the restriction patterns of B200 cells compared with parental 3T3 cells. In addition, detailed sodium bisulfite analysis did not reveal any differences in the methylation status of the TSP I promoter regions of 3T3 and B200 cells. Finally, 5-azacytidine treatment did not reactivate TSP I expression in B200 cells, providing additional support for the conclusion that methylation was not involved in silencing of TSP I in B200 cells.

A functional analysis of TSP I promoter allowed us to identify a negative regulatory region of the TSP I promoter in mouse 3T3 cells and to find that ATF-1 transcription factor was a negative regulator of TSP I gene expression in these cells. The promoter region of mouse TSP I was first analyzed by Lawler et al. (26). A computer analysis of the 5′ region of the gene revealed the presence of several potential regulatory elements, including AP-1, AP-2, SP-1, SRE, and ATF/CREB, but the significance of each of these elements in the regulation of TSP I expression in different tissues was not studied. All known transcription factors (i.e., serum induced) involved in regulation of TSP I are considered to be positive regulators, and negative regulators have not as yet been described in the literature. There is, however, some indirect evidence suggesting the existence of such negative regulator(s). Superinduction of TSP I expression by inhibition of protein syntheses using cyclohexamide (20) is suggestive of the presence of a negative regulator(s), because one explanation for a superinduction mechanism involves the blockage of protein synthesis for a negative regulator(s).

Earlier, we have shown that TSP I is inactivated in Chinese hamster and mouse nickel-transformed cells (16). Using two different parts of the human TSP I promoter, −1659 to +749 and −234 to +749, linked to CAT, we found that both constructs were down-regulated in nickel-transformed Chinese hamster cells, with the longer construct being suppressed more efficiently. These data showed that the human TSP I promoter was down-regulated at the transcriptional level in nickel-transformed hamster cells. The fact that both constructs were down-regulated in nickel-transformed hamster cells suggested that more than one negative regulator may exist. In this paper, we found one site with negative regulatory activity in the promoter area of mouse TSP I spanning from −1396 to +52. However, we cannot exclude the possibility that there were additional sites with negative regulatory activity upstream of −1396 or in the region of the first exon and intron spanning up to +750. We also cannot exclude the existence of additional negative regulatory sites within the analyzed region of the promoter, and more constructs with smaller deletions will need to be obtained to further characterize this region. The possibility that more than one negative regulator is functional in mouse B200 cells is supported by our demonstration of incomplete inactivation of TSP I in acutely treated 3T3 cells.

Carcinogenic nickel compounds exhibit weak mutagenic activity in all mutation assays tested (41–44); however, they are at the same time potent human and animal carcinogens. For example, nickel chloride has been shown to be the most potent chemical agent that immortalized Syrian hamster fibroblasts in vitro, surpassing such well-known
chemical carcinogens as N-methyl-N-nitrosourea, dimethylsulfate, and benz(a)pyrene diol epoxide (45). A single dose of 0.25 mM NiCl₂ for 24 h was sufficient to produce 6 of 10 and 9 of 10 immortal cultures in two separate experiments, with no spontaneous immortalization occurring in these experiments (45). In experiments carried out in our laboratory, a single dose of 1 mM NiCl₂ for 24 h was sufficient to produce morphological transformation and even anchorage-independent growth of Chinese hamster embryo fibroblasts (46). This implied that nickel treatments described in this paper (1 mM of NiCl₂ for 24 h) could potentially result in transformation of 3T3 cells. Thus, activation of ATF-1 expression may be an early event in nickel-induced transformation.

The mechanism by which the ATF-1 transcription factor is induced by nickel is not known. It is unlikely to be due to a direct effect of nickel, because there are no known nickel-responsive elements that regulate gene expression in any form of life. Because nothing is known about the effects of nickel on mRNA stability, it is difficult to speculate that this may be how nickel enhances the levels of ATF-1 transcripts. Stabilization of mRNAs could be one of the epigenetic mechanisms by which nickel exerts its carcinogenic activity, but additional work is required to understand how nickel may have positive effects on gene expression. Interesting features of the up-regulation of ATF-1 in mouse cells are that it can be caused by direct exposure of cells to nickel, and this change is persistent even in the absence of nickel in transformed cells.

Little is known about the regulation of gene expression of ATF-1, the stability of its mRNA, or the half-life of the protein in different cells. ATF-1 was found to be a major protein purified from HeLa cells grown in suspension (39), and ATF-1 transcripts were found to be more abundant in these cells compared to ATFα, ATF-2, and CREB transcripts (47). However, because no comparison with normal human epithelial cells has been done, it is not clear whether the high level of ATF-1 expression was characteristic of epithelial tissues or this high level of expression was related to the transformed state of HeLa cells. ATF-1 was found to be overexpressed in human lymphomas and in activated lymphocytes, suggesting an important role of this transcription factor in cell growth and malignant transformation (48). In lymphomas, overexpression of ATF-1 was not due to enhanced transcription but rather resulted from increased stability of ATF-1 mRNA in these cells (48).

Previously, we have shown that Rb is an important positive regulator of TSPI expression (16), and we found low levels of Rb expression in human and mouse nickel-transformed cells. It is interesting to note that an ATF-binding site was also identified in the promoter of Rb and was shown to be important for its expression (49). Thus, it is conceivable that ATF-1 transcription factor can also be a negative regulator of Rb expression. p53 has been shown to be another positive regulator of TSPI gene expression (24). Because p53 was found to be mutated in nickel-immortalized human epithelial cells (50), it is also possible that p53 is inactivated or mutated in nickel-transformed B200 cells, resulting in down-regulation of TSP I expression.

In summary, we have found that the ATF/CREB site functions as a negative regulatory site in the mouse TSP I promoter. We also have shown that ATF-1 transcription factor is a negative regulator that binds to ATF/CREB-binding site in mouse 3T3 cells. This transcription factor was also involved in down-regulation of TSP I in nickel-transformed cells. Additionally, acute exposure of 3T3 cells to nickel resulted in induction of ATF-1, and this was correlated temporally with the down-regulation of TSP I.

ACKNOWLEDGMENTS

We thank Dr. C. Klein for critical reading of the manuscript.

REFERENCES

NEGATIVE REGULATORS OF THROMBOSPONDIN I EXPRESSION


Induction of Activating Transcription Factor 1 by Nickel and Its Role as a Negative Regulator of Thrombospondin I Gene Expression

Konstantin Salnikow, Sheng Wang and Max Costa


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/22/5060

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.