TATA-binding Protein-associated Factor 7 Regulates Polyamine Transport Activity and Polyamine Analog-induced Apoptosis*

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Identification of the polyamine transporter gene will be useful for modulating polyamine accumulation in cells and should be a good target for controlling cell proliferation. Polyamine transport activity in mammalian cells is critical for accumulation of the polyamine analog methylglyoxal bis(guanylhydrazone) (MGBG) that induces apoptosis, although a gene responsible for transport activity has not been identified. Using a retroviral gene trap screen, we generated MGBG-resistant Chinese hamster ovary (CHO) cells to identify genes involved in polyamine transport activity. One gene identified by the method encodes TATA-binding protein-associated factor 7 (TAF7), which functions not only as one of the TAFs, but also as both an activator for c-Jun. TAF7-deficient cells had decreased capacity for polyamine uptake (20% of CHO cells), decreased AP-1 activation, as well as resistance to MGBG-induced apoptosis. Stable expression of TAF7 in TAF7-deficient cells restored transport activity (55% of CHO cells), AP-1 gene transactivation (100% of CHO cells), and sensitivity to MGBG-induced apoptosis. Overexpression of TAF7 in CHO cells did not increase transport activity, suggesting that TAF7 may be involved in the maintenance of basal activity. c-Jun NH2-terminal kinase inhibitors blocked MGBG-induced apoptosis without alteration of polyamine transport. Decreased TAF7 expression, by RNA interference, in androgen-independent human prostate cancer LNCaP104-R1 cells resulted in lower polyamine transport activity (25% of control) and resistance to MGBG-induced apoptosis. Taken together, these results reveal a physiological function of TAF7 as a basal regulator for mammalian polyamine transport activity and MGBG-induced apoptosis.

Polyamines are ubiquitous cellular components that affect a variety of biochemical processes, especially those involving synthesis of macromolecules (1). The growth of mammalian cells requires polyamines, and the optimal intracellular concentration is regulated by multiple pathways, including synthesis from amino acid precursors, cellular uptake mechanisms, as well as stepwise degradation and efflux.

The importance of polyamines for cell proliferation has led to the development of various inhibitors of the biosynthetic pathway or analogs that disrupt normal polyamine functions (2). One inhibitor, methylglyoxal bis(guanylhydrazone) (MGBG),1 is a structural analog of the natural polyamine spermidine, and it is a potent inhibitor of S-adenosylmethionine decarboxylase, an enzyme that supplies propylamino groups for synthesis of spermidine and spermine (3). MGBG is transported into cells by the polyamine transporter (4, 5). Accumulation of MGBG in cells induces apoptosis as well as reduction of cellular polyamine levels (6). Because polyamines are important for cell proliferation, MGBG has been recognized as a potential antineoplastic agent. Despite the long-standing availability of MGBG, the exact mechanism for its cytotoxicity is not clear. Mandel and Flintoff (7) reported that MGBG-resistant Chinese hamster ovary (CHO) cells have decreased polyamine transport activity, indicating that an active mammalian polyamine transport system was a critical factor for the intracellular accumulation of this polyamine analog as well as natural polyamines.

Polyamine transport activity can be assayed using radioactive polyamines (8), and recently, transport activity was visualized using polyamines conjugated to a fluorescent dye (9). Although bacterial and/or fungal polyamine transporter genes have been characterized (10, 11), the mammalian polyamine transporter gene has not been identified, even though the entire mouse and human genomes have been sequenced. Genetic approaches to identify the mammalian polyamine transporter gene have been tried by several research groups. Adair et al. (12) mapped the MGBG resistance locus to part of chromosome Z3 in Chinese hamster ovary cells, and Byers et al. (13) demonstrated that a human DNA fragment could restore MGBG sensitivity to MGBG-resistant cells. Most recently, using a plasmid base gene targeting vector, Shao et al. (14) reported a decrease in polyamine transport activity in the human non-small cell lung carcinoma line NCI H157. Despite these efforts, genes responsible for the transport activity have not been identified. Here we report the identification of a gene involved in polyamine transport activity using retroviral gene trap screening and the characterization of its physiological function.

1 The abbreviations used are: MGBG, methylglyoxal bis(guanylhydrazone); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CHO, Chinese hamster ovary; CMV, cytomegalovirus; HA, hemagglutinin; JNK, c-Jun NH2-terminal kinase; ORN72, ornithine transporter 2; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; TAF7, TATA-binding protein-associated factor 7.

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EXPERIMENTAL PROCEDURES

Materials—Hygromycin B was purchased from Calbiochem. MGBG, spermidine, spermine, ornithine, and Hoechst 33258 were obtained from Sigma-Aldrich. 5-[(3R)-3-(3,4-Dichlorophenyl)ethyl]pyrrolo[2,3-c]pyridine (DPZ) was purchased from Toronto Research Chemicals (San Diego). \(^{13}C\)- and \(^{15}C\)-Spermidine (4.29 GBq/mmol), \(^{12}C\)-Spermidine (4.07 GBq/mmol), \(^{15}C\)-Spermine (1.17 GBq/mmol), and \(^{12}C\)-Spermine (2.05 GBq/mmol) were purchased from Amersham Biosciences. Monoclonal antibody against the HA tag was obtained from Covance (Princeton, NJ). Polyclonal antibodies against cyclin A and eIF-4E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture—Cells were cultured at 37 °C in the indicated media and in the presence of 5% CO\(_2\). The Chinese hamster ovary cell line CHO-CI22 (CHO) was cloned by limiting dilution from wild type CHO-K1 (ATCC, Manassas, VA) in our laboratory. For convenience, the parent cells, CHO-CI22 are described as "CHO cells" in this paper. CHO cells and derivatives were maintained in minimum essential medium-\(a\) (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. Gene trap virus-producing cells, \(2\alpha\)/U3Hygro (15), were grown in Dulbecco’s modified Eagle medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. The human prostate cancer cell line LNCaP104-S and 104-R1 cells were passaged and maintained as described previously (16).

Gene Trap—The retroviral gene trap method was performed as described previously (17). Briefly, \(2\alpha\)/U3Hygro cells were seeded at 2 × 10\(^5\) cells/10-cm dish. After 18 h, the culture medium was removed, and 2 ml of virus was added. After 48 h, the culture medium was collected and then filtered through a 0.22-μm pore size membrane. This medium containing virus was added to 5 × 10\(^5\) CHO cells in a 10-cm dish. Polybrene (Aldrich) was added to a final concentration of 8 μg/ml. After 18 h, the medium was changed, and hygromycin B was added to a 0.6 μg/ml concentration. Hygromycin selection was continued for 10 days, and then hygromycin-resistant colonies were cultured in the presence of 10 μg/ml MGBG. Surviving colonies were picked after a week and expanded in the absence of MGBG.

Cell Proliferation Assay—Assays were performed based on the method of Rago et al. (18). Cells were seeded at 3,000–4,000 cells/well in 96-well tissue culture plates. After 18 h, MGBG was added without changing the medium. At the indicated time, cells were lysed in distilled water and frozen. Cell lysates were incubated with 10 μg/ml Hoechst 33258 in 5 ml Tris-HCl, pH 7.4, 0.5 ml EDTA, and 1 ml NaCl. Fluorescence was measured using a Wallac 1420 Multilabel plate reader (PerkinElmer Life Sciences) with excitation at 355 nm and emission at 460 nm. A standard curve was generated using CHO cells counted using a hemocytometer.

Polyamine Transport Assay—CHO cells were plated at 4 × 10\(^4\) cells/well in a 24-well plate. LNCAp cells were plated at 5 × 10\(^5\) cells/well in a 6-well plate. The growth medium was aspirated, and transport assay buffer consisting of 153 mM NaCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 20 mM Hepes/Hepes-Tris, pH 7.2, and 10 mM glucose (8) was added. After incubation at 37 °C for 10 min, the uptake assay was started by the addition of transport assay buffer containing 3.7 kBq of \(^{14}C\)-labeled polyamine so that the final concentration of labeled polyamine was 5 μM. After the indicated incubation time, the buffer was aspirated, and the cells were washed three times with transport assay buffer containing 10 mM unlabeled substrate. Washed cells were lysed in 0.1 ml NaOH. Aliquots of lysed cells were used for protein determination with the Bradford reagent. Ornithine decarboxylase, S-Adenosylmethionine decarboxylase, and Spermidine/Spermine N\(^{\text{6}}\)-Acetyltransferase Assay—Growing cells were harvested, lysed with ornithine decarboxylase buffer (19), and dialyzed against 500 ml of ornithine decarboxylase buffer to remove endogenous small molecules. Ornithine decarboxylase (19), S-Adenosylmethionine decarboxylase (7), and spermidine/spermine N\(^{\text{6}}\)-Acetyltransferase (20) activities were measured according to published methods.

Measurement of the Cellular Content of Polyamines—Polyamines were extracted from the cells with 5% trichloroacetic acid. Polyamine content was analyzed by high performance liquid chromatography as described previously (21). The cellular content of polyamines was normalized to the amount of total cellular protein determined with the Bradford reagent.

Determination of MGBG in CHO Cells—MGBG content was measured using the method of Sepanek et al. (22). CHO cells were harvested three times with the transport assay buffer and then lysed with 0.1% Nonidet P-40. Before the determination of MGBG, the cell lysates were boiled for 10 min to inactivate endogenous S-adenosylmethionine decarboxylase. A homogenate of LNCaP104-S cells was used as a S-adenosylmethionine decarboxylase source. The boiled CHO lysates were added to the S-adenosylmethionine decarboxylase assay system as described. A series of standards containing known amounts of MGBG was run in parallel, and a standard curve was obtained by plotting MGBG concentration versus the reciprocal of initial velocity. The MGBG content in unknown samples was calculated from the standard curve. The MGBG content was normalized to cellular protein content.

Southern Blot Analysis—Genomic DNA of CHO cells was isolated as described. Digestion with appropriate restriction enzymes, fractionation in 0.7% agarose gels, and transfer to a ZetaProbe nylon membrane (Bio-Rad). A 3'-labeled DNA probe, containing the EcoRI-XbaI fragment of the TAF7 3'-rapid amplification of cDNA ends (RACE) product isolated from pSTBlue-1, was generated by the random priming method using the Prime-It Labeling Kit (Stratagene). Probe hybridization and Southern blotting were performed as described previously (23).

RACE-PCR—Total RNA was extracted using the TRizol Reagent (Invitrogen). 5'-RACE was performed with 1 μg of total RNA using a 5'-RACE system kit (Invitrogen), following the manufacturer’s instructions. The sequence of the polyamine resistance gene primers were 5'-AGCGGGGCTG-TCCGTGTTTCA-3' for cDNA synthesis and 5'-ACATCGCGCCGACAT- TTAGGCATTTTAA-3' for cDNA synthesis. The following program was used for amplification: preamplification at 95 °C for 15 min, 35 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min followed by 72 °C for 8 min. The PCR product was cloned into pBluescript (Stratagene) and sequenced. The sequence was then used for primer design for 3'-RACE. mRNA, isolated from CHO-CI22 cells by using an oligo(dT)-cellulose column, was used for 3'-RACE using a 5'-3' RACE system kit and Advantage-2 polymerase (Clontech), following the manufacturer’s instructions. The sequence of specific trapped gene primer and 3' -counterprimer were 5'-GCTCCGCGCCGCGAGCCGCGTGATAG-3' and 5'-GGCCACCGCTCGACTGATGTC-3', respectively. The PCR cycle parameters were: preheating at 95 °C for 1 min, 30 cycles of 95 °C for 15 s and 68 °C for 3 min, followed by 68 °C for 3 min. The PCR product was cloned into the pSTBlue-1 cloning vector (Novagen, Madison, WI), and the sequence was determined.

Northern Blot Analysis—mRNA (5 μg/lane) was size fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred to Zetaprobe nylon membranes, and probed with 32P-labeled cDNA, as described for Southern blot analysis.

Expression Plasmid Construction—Full-length hamster TAF7 was amplified with 5'-GGATCCGAGAAGCCCTCGATGAGTAAGACATCAGGA-3' (T55-F) and 5'-CTCGAGTCTAAGGACCGAAATCACCAC-3'. After verification by sequencing, the DNA fragment was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Cloning vectors pSG5HA(N-) for amino-terminal HA tagging and pSG5HA(C-) for carboxyl-terminal HA tagging were generated from the pSG5 vector (Stratagene) with the following oligonucleotide DNA pairs: 5'-GATTCC-ATGATCCATACAGGTCGACAGCTACCGTA-3' and 5'-GATCTAGCGATGCATCGCGCTGCTGCTGATGGT-3', respectively.

The following program was used for amplification: preamplification at 95 °C for 1 min, 30 cycles of 95 °C for 15 s and 68 °C for 3 min, followed by 68 °C for 3 min. The PCR product was cloned into the pSTBlue-1 cloning vector (Novagen, Madison, WI), and the sequence was determined.

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An AP-1-responsive luciferase reporter construct (pGL3-73coll.) was generated from Coll.-73CAT (24), which contains the human collagenase promoter region. Briefly, Coll.-73CAT was digested with HindIII, treated with the Klenow-fragment of DNA polymerase and then cut with BamHI. The resulting DNA fragment was
cloned into the BglII/Smal site of pGL3-Basic (Promega, Madison, WI). Cells were plated at 3 × 10^6 cells/well into a 24-well plate and grown overnight. Cells were transiently transfected with 0.5 µg of pGL3-73coll. and 0.5 ng of pRL-CMV (normalization reporter plasmid from Promega) using PolyFect (Qiagen), according to the manufacturer's instructions. After 6 h the medium was changed, and MGBG was added. After a 24–36-h incubation, cells were harvested, and luciferase activity was measured with a commercial kit (Dual-Luciferase, Promega) on a Monolight Luminometer (Pharminingen, San Diego), and their relative activities were compared.

**Caspase-3 Assay**—Cells were treated as indicated, harvested, and lysed in a buffer containing 25 mM Hepes/NaOH, pH 7.4, 5 mM EDTA, 10% CHAPS, 2 mM dithiothreitol, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. Cells were lysed by freezing and thawing one time. Total cell protein in the cell lysate was measured using Bradford reagent. The caspase-3 substrate, DEVD-AFC (ApoAlert, Clontech) was added to the cellular lysate and incubated for 2 h at 37 °C. Fluorescence was measured using a CytoFluor II fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation at 440 nm and emission at 490 nm. The activity was normalized to cellular protein content.

**RNA Interference (RNAi) Experiments**—An RNAi expression vector pH1RP was constructed as described. The human H1 RNA promoter was amplified from LNCaP104-S genomic DNA using primers 5'-CCATGG-AATTCGAACTGCGTACGTC-3' and 5'-GCAAGCTTAGATCTGTGTC-TCGATCACAGACTTAAGGATCCCAG-3'. The PCR product was cloned into pCRII from which the CMV promoter was removed by BamHI-BglII digestion and religation. The RNAi sequence was designed according to the method of Brummelkamp et al. (25) using the design program from OligoEngine (Seattle, WA). The sequences of RNAi for human TAF7 were 5'-GATCCCTTTAAGCTTAGATCTGTGTCGTCCAAGAGAC-ACGGCCAGCTCTACTAAATTTTGGAAGGAA-3' and 5'-ACGGTTTCCAAATTTAGAAGCCTTCTCTCTTTTGAACACAGCAGCGCT-CTACTAAAGG-3'. These 64-mer oligonucleotides were annealed and ligated into the pH1RP vector, and the construct was verified by sequencing. The TAF7-RNAi expression plasmid was stably transfected into LNCaP104-R1 cells using Effectene as described above.

**Real Time Quantitative PCR**—Total RNA was isolated using the TRizol Reagent and was treated with DNA-free, Ambion, Austin, TX). Reverse transcription was performed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Omniscript, Qiagen). The TaqMan primer/probe was designed using Primer Express (Applied Biosystems). The probe for the TAF7 gene was labeled with the fluorescent dye, TAMRA. The sequence of the forward primer, reverse primer, and TaqMan probe were 5'-CCCTGAGCTCTGGA-CACCTG-3', 5'-CAATCCCATGAGCCCCACATC-3', and 6FAM-5'-AACCT-GAAAGACAGACTGACATGATTACACC-3'-TAMRA, respectively. Real time PCR was performed on an ABI Prism 7700 system (Applied Biosystems) using the Quantitect Probe real time PCR protocol (Qiang). The Ribosomal RNA Control Kit (Applied Biosystems) was used to normalize transcript levels between samples.

**RESULTS**

**Screening for MGBG Resistance in CHO Cells Transfected with Gene Trap Retrovirus**—Gene trap screening was performed with U3Hygro (15) to attempt to identify the gene responsible for MGBG resistance and polyamine transport activity. CHO cells were infected with U3Hygro and selected with hygromycin B. CHO cells are functionally hemizygous at a single insertion site. 3'-RACE PCR was performed using primers designed from the trapped sequence (dark boxes). The sequence of the CHO cell endogenous gene (open box) was determined, and then expression studies were performed to analyze the function of the target gene.

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**Fig. 1. Strategy for a genetic screen for polyamine transport involved gene traps.** CHO cells were infected with the retroviral vector U3Hygro and selected for hygromycin resistance. After 10 days, cultures were washed with phosphate-buffered saline and then maintained in medium containing 10 µM MGBG for 7 days. MGBG-resistant colonies were then picked and expanded in medium without MGBG. Candidate clones were selected using a polyamine uptake assay with radiolabeled spermine. Total RNA was isolated from a polyamine transport-deficient clone. 5'-RACE PCR was primed using the hygromycin resistance gene (hatched box) to determine DNA sequence upstream of the insertion site. 3'-RACE PCR was performed using primers designed from the trapped sequence (dark boxes). The sequence of the CHO cell endogenous gene (open box) was determined, and then expression studies were performed to analyze the function of the target gene.
Cells were treated with 3 MGBG (hatched bars) and 432c (open bars) cells. Cells were incubated with 5 μM [14C] spermine and uptake measured every 5 min. The activity was normalized to total cellular protein. C, MGBG uptake in CHO (hatched bars) and 432c (open bars) cells. Cells were treated with 3 μM MGBG for the indicated times. Intracellular MGBG content was normalized to total cellular protein. D, effect of additional spermine in media on the intracellular spermine content. Spermine was added to medium at the indicated concentration with 0.5 mM aminoguanidine to inhibit degradation of spermine by serum amine oxidase. After 12 h, cells were collected, and spermine was analyzed using high performance liquid chromatography and normalized to total cellular protein. The intracellular spermine content without additional spermine in the medium was 7.23 and 11.1 nmol/mg protein for CHO and 432c cells, respectively, and the spermine content of polyamine-treated cells is shown as a percentage of these control cells.

Identification of TAF7 as the Trapped Gene in 432c Cells—Using total RNA from 432c cells, 5′-RACE was performed with priming from the hygromycin resistance gene (Fig. 1). A 495-bp PCR product was obtained, and this was subcloned and sequenced. Sequencing revealed that 226 nucleotides of CHO cDNA (Fig. 2A) were present. The sequence of this DNA did not match any of the currently available genes from the GenBank data base. No open reading frame was found in this sequence. Because we were unable to identify a gene associated with viral insertion based on 5′-RACE data, we designed DNA primers to perform 3′-RACE on the cDNA so that we could determine the sequence of DNA 3′ to the insertion site. Total RNA was extracted from parent CHO cells, reverse-transcribed, and used for 3′-RACE PCR with appropriate primers (Fig. 1). A 1.7-kb PCR product was obtained, subcloned, and sequenced. The cDNA encoded an open reading frame matching human and murine TAF7 cDNA. Also present were the 5′-untranslated region, as well as the poly(A) tail with 3′-untranslated region sequence. The arrowhead indicates the start point of the U3 sequence. Using hamster TAF7 cDNA as a probe, Southern blot analysis was performed to confirm that the TAF7 locus was altered by U3Hygro insertion. Fig. 3B shows that the Southern blot pattern of the TAF7-positive fragment in 432c cells was different from the parent CHO cells. Digestion with PstI (lane 1 versus 4) or SspI (lane 3 versus 6) showed that the TAF7 gene in 432c is modified. The TAF7 gene is most likely hemizygous based on comparisons of the Southern blot patterns of DNA from CHO and 432c cells. Lanes 3 and 6 in Fig. 3B are most informative. The proviral insert is ~6.2 kb in size and lacks an SspI site (15). The faint ~1 kb band in lane 6 corresponds to the single band observed from DNA from wild type CHO cells and may represent a small population of cells that have lost the retrovirus. The expression levels of TAF7 mRNA in 432c cells were also analyzed by Northern blot using TAF7 cDNA as a probe. The insertion of U3Hygro greatly decreased the amount of TAF7 mRNA (Fig. 3C). Although U3Hygro does not disrupt the open reading frame of TAF7, alteration of its 5′-untranslated region appears to be sufficient to silence this gene.

Effect of Ectopic Expression of TAF7 in 432c Cells on Spermine Transport Activity—To determine whether changes in TAF7 expression were responsible for the phenotype of 432c cells, we constructed not only a hamster TAF7 expression plasmid, but also a HA-tagged version that was designed and generated because of the lack of availability of an anti-TAF7 antibody. HA-tagged TAF7 was useful for checking expression by Western blot analysis. To override the intrinsic G418 resistance in U3Hygro (15), we selected stable transformants with a relatively high concentration (0.8 mg/ml) of G418. As shown in Fig. 4A, the ectopic expression of TAF7 as well as HA-tagged TAF7 increased spermine transport activity in 432c cells to 55% of the parent cells. The expression of carboxyl- or aminoterminal HA-tagged TAF7 proteins was confirmed by Western analysis (Fig. 4B). Although the expression levels of these HA-tagged proteins were different, the spermine transport activities in cells expressing their genes were similar. Spermine transport activity was decreased significantly by 30% by overexpression of TAF7 in wild type CHO cells (Fig. 4A), perhaps indicating that optimal levels of TAF7 are required for maximum transport activity. Putrescine and spermidine transport activities were also increased by ectopic TAF7 expression in 432c cells (data not shown). The effect of TAF7 expression on the intracellular polyamine content was also examined. CHO and 432c cells transformed with a control vector showed no significant differences in polyamine content. On the other hand, compared with control transformants, putrescine and spermidine levels in TAF7 transformants were increased 6-fold and 2-fold, respectively. The incomplete restoration of polyamine transport activity by ectopic TAF7 expression in 432c cells may be the result of high intracellular polyamine levels in...
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TAF7 transformants (Table I) because an excess amount of intracellular polyamine suppresses polyamine transport activity (8).

Effect of TAF7 Expression in 432c Cells on MGBG-induced Apoptosis and AP-1 Transactivation—MGBG caused apoptosis in wild type CHO cells (data not shown). Caspase-3, a cell death protease involved in the execution phase of apoptosis (29), was up-regulated by MGBG treatment in a dose-dependent manner and reached a maximum at 36 h after the onset of treatment. As shown in Fig. 5A, treatment with 15 \mu M MGBG increased caspase-3 activity 12.9-fold in the parental CHO cell line, whereas TAF7-deficient 432c cells did not show an increase in caspase-3 activity with MGBG treatment. Ectopic expression of TAF7 increased MGBG-induced caspase-3 activity in 432c cells (Fig. 5A). These results suggest that MGBG-induced CHO cell death is dependent on TAF7 expression.

Recently, it has been reported that the TAF7 protein interacts with c-Jun in vitro as well as in vivo and also stimulates c-Jun-dependent AP-1 transactivation (30). AP-1 activity is a well known modulator of apoptosis, although the exact outcome is highly tissue- or condition-specific (31). To assess the role of TAF7 in CHO cell signaling, AP-1 activity was analyzed in CHO and 432c cells with an AP-1-responsive promoter-reporter plasmid, pGL3-73coll., using transient transfection. Basal activity of AP-1 was lower in 432c than parental CHO cells (Fig. 5). On the other hand, AP-1 activity was restored in cells ectopically expressing TAF7. Treatment with MGBG increased AP-1 activity in TAF7-expressing cell lines similar to its effect on caspase-3 activity (Fig. 5B).

c-Jun NH2-Terminal Kinase (JNK) Inhibitors Suppress MGBG-induced Apoptosis without Altering Polyamine Transport Activity—Because activation of JNK has been reported to be sufficient to induce apoptosis in CHO cells (32), we examined the effect of JNK inhibitors on MGBG-induced apoptosis. JNK inhibitors, SP600125 and D-JNKI-1 are widely used in studies of JNK-mediated signaling pathways (33, 34). Both compounds were added to the culture medium 14 h before starting MGBG treatment. As shown in Fig. 6A, both inhibitors blocked the increase in caspase-3 activity caused by MGBG. SP600125 at 10 \mu M completely blocked the increase in caspase-3 activity, and the activity was as low as without MGBG treatment. Although JNK inhibitors suppressed apoptosis induced by MGBG, spermidine transport activity was not affected (Fig. 6B), and so TAF7 may be involved in polyamine transport activity independently of JNK activity and perhaps AP-1.

**TABLE I**

| Cell line     | Putrescine | Spermidine | Spermine |
|---------------|------------|------------|----------|
| CHOpcDNA      | 0.078      | 4.58       | 8.78     |
| 432pcDNA      | 0.089      | 3.09       | 8.97     |
| CHOTAF7       | 0.597      | 6.35       | 8.28     |
| 432TAF7       | 0.446      | 9.53       | 8.18     |
| 432TAF7-C     | 0.439      | 10.89      | 8.37     |
| 432TAF7-N     | 0.388      | 9.77       | 9.57     |

Values are the means of duplicate determinations.

![Fig. 4. Effect of ectopic expression of TAF7 in 432c cells.](image)

A, spermine uptake activity was measured in parental CHO and 432c and the indicated stable transformants of these cell lines. The TAF7 gene was cloned into expression vector pcDNA3. Amino-terminal-HA tagged (TAF7-N) and carboxyl-terminal-HA tagged (TAF7-C) were also expressed with the same vector. B, expression of HA-tagged TAF7 was confirmed with Western analysis using anti-HA tag monoclonal antibody.

![Fig. 3. Characterization of the effects of insertion of U3Hygro.](image)

A, the cDNA sequence around the insertion site of the gene trap vector U3Hygro. The arrowhead shows the insertion site. Underlined nucleotides are conserved in the published human TAF7 sequence (accession number AF349938). The initiator codon is boxed. B, Southern blot analysis of the TAF7 gene in CHO and 432c cells. Genomic DNA was digested with PstI (lanes 1 and 4), StuI (lanes 2 and 5), and SspI (lanes 3 and 6), respectively, separated on an agarose gel, and transferred to a nylon membrane. The membrane was hybridized with a 32P-labeled TAF7 DNA fragment as described under “Experimental Procedures.” C, expression of TAF7 mRNA in CHO and 432c cells. Total RNA was isolated from both cell lines, separated on a formaldehyde-agarose gel, and transferred to a nylon membrane. Norther blotting was performed with a 32P-labeled TAF7 DNA fragment (upper panel). The lower panel shows the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control.
Effect of Altered TAF7 Expression on Polyamine Transport Activity and Resistance to MGBG-induced Growth Suppression in Human LNCaP104-R1 Cells—To determine whether TAF7-dependent polyamine transport activity is also observed in other cell lines, we used the human prostate cancer cell lines, androgen-dependent LNCaP104-S and its subline, androgen-independent 104-R1 cells (16, 35). We measured the expression levels of TAF7 in both cell lines with real time quantitative PCR, but there was no significant difference between these cell lines (data not shown). We chose 104-R1 cells rather than 104-S cells for further study because of their relatively higher sensitivity to MGBG and polyamine transport activity. We designed an RNAi sequence for human TAF7 and expressed this RNAi in 104-R1 cells using the RNAi expression vector pH1RP, which has an RNA polymerase III-dependent promoter for RNAi expression and a neomycin resistance marker gene, as described under “Experimental Procedures.”

The transformants of pH1RP-TAF7-RNAi showed significant repression of TAF7 mRNA expression compared with pH1RP empty vector-transfected cells as a negative control (Fig. 7A). These TAF7-RNAi transformants had decreased polyamine transport activity (Fig. 7B) and were relatively resistant to MGBG-induced growth suppression (Fig. 7C). These results also demonstrated that TAF7 functions as a regulator of human polyamine transport as well as cell death caused by MGBG.

DISCUSSION

A polyamine transport system is a crucial factor for the uptake of polyamines and synthetic analogs. However, in mammalian cells, the molecular mechanism and the genes involved are poorly understood. In this report, using gene trap screening, we identified TAF7 as a regulator of polyamine transport activity. The cell line 432c is deficient in TAF7 expression because of insertion of the U3Hygro retroviral gene trap vector into a 5'/H11032-­noncoding region of the TAF7 gene. This insertion decreased polyamine transport activity, and restoring TAF7 expression in 432c cells increased polyamine transport activity.

Theoretically, the number of retroviral insertion events in this study was enough to cover the whole genome (15), and the hemizygous character of most CHO genes should facilitate a loss of gene function (26). However, under our experimental conditions, only one clone was isolated that was resistant to MGBG, and this clone was deficient in polyamine transport activity. Unlike chemical mutagenesis, retroviral insertion does not occur as a totally random event because preferable retrovirus insertion sites exist in chromosomal DNA. The gene trap frequency in our study was 1 hygromycin-resistant cell for each 200 CHO cells infected with retrovirus. We then selected a single MGBG clone from 105 hygromycin-resistant CHO cells. These selection frequencies are comparable with those observed by Chang et al. (15) for retroviral inactivation of single copy genes. Larger numbers of cells should be screened to identify other genes involved in polyamine transport and/or resistance to MGBG.

Heaton and Flintoff (4) reported that at least two distinct loci are involved in polyamine transport activity in CHO cells. One
of them, the so-called MBG locus (12), has been localized to chromosome Z3, which consists of portions of Chinese hamster chromosomes 3 and 4 (36). Using the NCBI genomic data base, we found that the human TAF7 gene is located on chromosome 5, and mouse and rat TAF7 genes are on chromosome 18. Based on comparative cytogenic maps of Chinese hamsters, mice, rats, and humans (37), human chromosome 5 and mouse and rat chromosome 18 are homologous to hamster chromosomes 2 and 7. The MBG resistance locus has also been linked to the isocitrate dehydrogenase 2 gene on chromosome Z3 in CHO cells (12). The human isocitrate dehydrogenase 2 gene is located at 15q26.1. Based on these chromosomal relationships, it is unlikely that the TAF7 gene is the same as the MBG resistance locus identified by Adair and Siciliano (12). However, until the chromosomal location of the TAF7 gene in CHO cells is determined, for example by fluorescent in situ hybridization, there remains the possibility that the hamster TAF7 gene is, in fact, present on Z3 and is related to the locus studied by Adair and Siciliano.

Heaton and Flintoff (4) showed that more than one locus controls polyamine transport activity in CHO cells. Control CHO cells had a 90% decrease in plating efficiency in the presence of 0.1 μM MGBG. Multistep selection with MGBG produced CHO cells highly resistant to the toxic effects of MGBG, with cells surviving in the presence of 25 μM MGBG. CHO cells with a low level of resistance were also isolated, and these cells had a 90% decrease in plating efficiency with 1.5 μM MGBG. Analysis of MGBG resistance in hybrid cell lines was consistent with separate genes being responsible for these traits. The highly resistant CHO cells lacked the ability to transport MGBG, whereas the cells with low resistance retained about 10% of wild type MGBG transport activity. Heaton and Flintoff speculated that alteration of a regulator of MGBG transport might be responsible for the low resistance phenotype. The MGBG-resistant 432c cells isolated in this study also retained some polyamine transport activity and were selected for using 10 μM MGBG. The relationship of TAF7 to the low resistance phenotype is unknown, but it is also possible that TAF7 has a role in regulating polyamine transport and MGBG sensitivity independently of acting as a polyamine/MGBG transporter. Residual transport activity in mutants may also be indicative of multiple transport systems for polyamines (13). We speculate that TAF7 is likely a regulatory gene for polyamine transport activity in mammalian cells; because TAF7 also functions as a transcriptional cofactor or coactivator (30, 38). However, at present, it is unclear whether TAF7 directly controls the transcription of a polyamine transporter gene or whether a TAF7 target gene serves as a transcriptional activator for the transporter gene. To address the mechanism of TAF7-dependent polyamine transport activity, we have examined several possible roles of TAF7. Although it has been reported that overexpression of ornithine decarboxylase (27) or decreased expression of eIF-4E (39) modulates polyamine transport activity in mammalian cells, expression of these genes was not altered in TAF7-deficient 432c cells (data not shown). Furthermore, intracellular putrescine and spermidine levels in TAF7 transformants were increased without significant changes in ornithine decarboxylase and spermidine/spermine N\(^1\)-acetyltransferase activity (data not shown). Recently, the endocytic (9) or proteoglycan-mediated pathways (40) have been reported as possible mechanisms of polyamine transport. The involvement of TAF7 in these pathways needs to be explored in the future.

Based on the insertion site of U3Hygro in the 5′-noncoding region of the TAF7 gene, we searched 500 kb upstream and downstream of the same gene in humans, mice, and rats. No likely candidates for a polyamine transporter gene, other than the mitochondrial ornithine transporter 2 (ORNT2) was found. Insertion of U3Hygro into the 5′-noncoding region of the TAF7 gene also affected expression of the ORNT2 gene. Recently, it was shown that ORNT2 transports ornithine into mitochondria and its activity is inhibited by polyamines (41). How retroviral insertion affects ORNT2 expression is unclear. ORNT2 is located 4–5 kb from the TAF7 gene, and both genes are in the same orientation on the chromosome. Retrovirus insertion may disrupt DNA sequences controlling expression of both these genes, or TAF7 may have some role in controlling expression of ORNT2. Both genes appear to be single exon genes and are embedded in a cluster of protocadherin genes in mice and humans (42). The expression level of ORNT2 in 432c cells was less than 10% of the parent CHO cells based on real time quantitative PCR. Ectopic expression of human ORNT2 in 432c...
cells, however, did not restore polyamine uptake activity (data not shown). Therefore, retroviral insertion may have fortuitously affected ORNT2 expression as well as the gene responsible for polyamine transport activity, TAF7.

To confirm our observations in the CHO cell model using another cell line, we chose the human prostate cancer cell because the prostate gland and prostate tumors are unique in that they contain high levels of polyamines (43), and polyamine metabolism has been considered as a potential target for treatment of prostate tumors (44). To assess the effect of differential TAF7 expression on polyamine transport activity, we decreased TAF7 expression in 104-R1 cells with RNAi treatment. However, besides regulating c-Jun, JNK is activated by other stressors, such as DNA damage and growth factor withdrawal, and has been shown to play a role in the regulation of polyamine transport activity (45). Therefore, it is possible that the decreased polyamine transport activity observed in TAF7-deficient 104-R1 cells is due to the loss of c-Jun-mediated activation of polyamine transport genes, such as SLC7A11, the gene encoding the transporter for diethylarginine (46). However, this hypothesis is not supported by the observation that the expression of SLC7A11 is not decreased in TAF7-deficient 104-R1 cells (ots not shown). These results suggest that the effect of TAF7 on polyamine transport activity is independent of c-Jun and JNK.

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