Physical Interaction of p73 with c-Myc and MM1, a c-Myc-binding Protein, and Modulation of the p73 Function*

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p73 shares high sequence homology with the tumor suppressor p53. Like p53, ectopic overexpression of p73 induces cell cycle arrest and/or apoptosis, and these biological activities are linked to its sequence-specific transactivation function. The COOH-terminal region of p73 is unique and has a function to modulate DNA-binding ability and transactivation activity. To identify and characterize cellular proteins that interact with the COOH-terminal region of p73 and regulate its activity, we employed a yeast-based two-hybrid screen with a human fetal brain cDNA library. We found MM1, a nuclear c-Myc-binding protein, was associated with p73α in both yeast two-hybrid and in vitro pull-down assays. In mammalian cells, MM1 co-immunoprecipitated with p73α, whereas p73β and tumor suppressor p53 did not interact with MM1. Overexpression of MM1 in p53-deficient osteosarcoma SAOS-2 cells enhanced the p73α-dependent transcription from the p52/p73-responsive Bax and PG13 promoters, whereas p73β- and p53-mediated transcriptional activation was unaffected in the presence of MM1. MM1 also stimulated the p73α-mediated growth suppression in SAOS-2 cells. More importantly, we found that c-Myc was physically associated with p73α and significantly impaired the transcriptional activity of p73α on Bax and p21\(^{\text{waf1}}\) promoters. Expression of MM1 strongly reduced the c-Myc-mediated inhibitory activity on p73α. These results suggest that MM1 may act as a molecular partner for p73 to prevent the c-Myc-mediated inhibitory effect on its activity.

p73 is a new member of the p53 gene family (1). Like p53, p73 is a nuclear transcription factor, which carries an NH\(_2\)-terminal transactivation domain, sequence-specific DNA-binding domain, and oligomerization domain. As expected from the significant amino acid sequence homology in the sequence-specific DNA-binding domain between p73 and p53, p73 recognizes and binds to the p53-responsive elements found within the promoter regions of the various p53-target genes. In transiently transfected mammalian cells, p73 transactivates the transcription from a variety of p53-responsive promoters to various degrees (1–8). Artificially introduced mutation within the DNA-binding domain has significantly reduced the transactivation activity of p73, suggesting that the structural integrity of this domain is required for this activity (1, 2). Similarly to p53, the cellular transcriptional coactivator p300/CBP interacts with the NH\(_2\)-terminal transactivation domain of p73, resulting in stimulation of its activity (9). Furthermore, ectopic overproduction of p73 induces the cell cycle arrest and/or apoptosis in p53-deficient cultured cells (1–6, 10). Recently, it has been shown that p73 is stabilized and its apoptotic activity is enhanced in response to ionizing radiation or genotoxic agents such as cisplatin in a pathway depending on c-Abl (11–13). In addition, the endogenous level of p73 is increased during retinoic acid-induced differentiation in cultured neuroblastoma cells, and overexpression of p73 but not p53 caused neuronal differentiation (14). Fang et al. (7) have found that overexpression of p73 induces the growth arrest and the senescence-like phenotypes in human bladder carcinoma cells.

p73 is assigned to chromosome 1p36.3, which is a candidate tumor suppressor locus in a variety of human cancers (1). Although p73 mimics p53 in transcriptional activation as well as induction of apoptosis, p73 is infrequently mutated in many human tumors (15). In contrast to p53-knockout mice, p73 deficiency in mice did not lead to an increased susceptibility to spontaneous tumorigenesis (16). In addition, the elevated level of p73 expression was detected in some primary tumors, including breast and ovarian cancers (17, 18). Subsequent work from several laboratories has shown that forced expression of cellular and viral oncoproteins, such as E2F-1, c-Myc, and E1A, stimulated expression of the p73 gene (19–22). Thus, there have been conflicting reports about the role of p73 in cellular function, although the reasons remain unclear.

Unlike p53, p73 encodes at least six distinct isoforms (α, β, γ, δ, ε, and ζ) that are generated as a result of the alternative splicing of the primary p73 transcript (1, 3, 8, 23). These splicing isoforms possess different COOH-terminal extensions not found in p53, and their expression patterns vary among normal tissues (3, 8, 23). Intriguingly, these COOH-terminal splicing isoforms show different transcriptional and biological properties (3–5, 8). Indeed, p73α transactivated a variety of the p53-responsive promoters to a greater degree than p73α (2, 3, 7, 8, 24, 25). Similarly, the ability of p73β to inhibit cell growth in p53-deficient cells was stronger than p73α (3). These observations suggest that the COOH-terminal region of p73 may possess a regulatory role, which modulates its transactivation ability as well as its biological activity.

Accumulating evidence indicates that homotypic and hetero-
typic interactions among p53 family members regulate their activities. The various p73 splicing isoforms interacted with each other with various efficiency (1, 3). Di Como et al. (5) found that tumor-derived p53 mutants but not wild-type p53 were associated with p73α and thereby reduced its transactivation and pro-apoptotic function. Likewise, p53 mutant also interacted with the remaining p73 splicing isoforms (β, γ, and δ) through its DNA-binding domain, and abrogated their transcriptional activities (26). The ability of mutant p53 to interact with p73 was regulated by the status of a common p53 polymorphism at codon 72 (27). Recently, Yang et al. (16) discovered the truncated p73 isoform (ΔNp73),1 which lacks the NH2-terminal transactivation domain. ΔNp73 was generated from an alternative promoter located within the intron 3 of the p73 gene and lost the transactivation ability toward the p53-responsive promoter. Of note, ΔNp73 was predominantly expressed in the developing brain and sympathetic neurons, and inhibited the pro-apoptotic function of p53 by hetero-oligomerization (29). These homotopic and heterotypic interactions among p53 family members give a complexity to the understanding of the p73 signaling in vivo.

Various lines of evidence suggest that the extreme COOH-terminal region of p53 has a function of negative regulator. The COOH terminus of p53 directly binds and masks its central DNA-binding domain (30, 31). Indeed, its inhibitory effect was removed by structural modifications such as phosphorylation, glycosylation, acetylation, or deletions (32–36). Additionally, physical interaction of Ref-1 (previously identified as the AP-1-stimulating protein (37)) or 14-3-3 with the COOH-terminal region of p5353 caused the significant increase in its DNA-binding and transactivation activities (8, 25, 40). These observations suggest that the p73-DNA crystal structure might be similar to that of p53, although the COOH-terminal region of p73 does not share amino acid sequence similarity with that of p53.

The purpose of this study was initially to isolate and characterize cellular protein(s) that could associate with the COOH-terminal region of p73α. By using a yeast-based two-hybrid screening, we identified MM1, which had been reported to be a c-Myc-binding protein (41), as a p73α-COOH-terminal region-binding protein. We found that overexpression of MM1 stimulated the p73α-mediated transcription from some p53/p73-responsive promoters as well as growth suppression. Moreover, c-Myc bound to p73α and inhibited the p73α-dependent transactivation. Of interest, overexpression of MM1 antagonized the inhibitory effect of c-Myc on p73α.

**Experimental Procedures**

**Cell Culture**—Human osteosarcoma SAOS-2, COS7, and human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY) and penicillin (100 U/mL)/streptomycin (100 μg/mL). IL60 cells were grown in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum and antibiotic mixture. Cultures were maintained at 37 °C in a water-saturated atmosphere of 5% CO2 in air.

**Transfection**—Transient transfection of SAOS-2 cells was performed by LipofectAMINE Plus reagent according to the manufacturer's recommendations (Invitrogen). COS7 and 293 cells were transfected with the indicated expression plasmids using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the manufacturer's specifications.

**Yeast Two-hybrid Screening**—The MATCHMAKER Two-Hybrid System was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The cDNA encoding the extreme COOH-terminal region of p73α (amino acid residues 551–636) was amplified by PCR-based strategy using the full-length p73α cDNA as a template. The PCR product, which was produced by an additional EcoRI site in 5′-upstream and BanHI site in 3′-downstream, was digested completely with EcoRI and BanHI and subcloned in-frame into the identical restriction sites of pS2-1-p73α-COOH. The resulting plasmid was used to isolate the cDNA for p73α-binding protein from a cDNA library derived from human fetal brain or 293 cells in the pACT yeast expression vector (CLONTECH Laboratories, Inc.). Both plasmids were introduced into the Saccharomyces cerevisiae strain CG1945 (MATa, ura3–52, his3–200, ade2–101, lys2–801, trpl–901, leu2–3,112, gal1–54, gal80–538, cyh2, LYS2::GAL1-UAS::GAL1-PATA::HIS3, URA3:: GAL1-TATA::CYC1-TEAD::LacZ) using the lithium acetate/heat-shock protocol (42). The transformed yeast cells were plated on SD medium lacking tryptophan, leucine, and histidine in the presence of 20 μg 3-amino-1,2,4-triazole and incubated for 1 week at 30 °C. Positive colonies were picked up and assayed for lacZ activity using a filter β-galactosidase assay, as described previously (43). The interacting cDNA clones were rescued from the selected yeast transformants. β-Galactosidase activity in the yeast two-hybrid system was determined by a liquid assay using an o-phenylenediamine galactoperoxidase according to the manufacturer's instructions. The nucleotide sequences of the positive cDNA clones were determined by the dyeodeoxy terminator cycle sequencing method using an automated Prism 377 DNA sequencer (PerkinElmer Life Sciences and Applied Biosystems, Foster City, CA) and nucleotide sequence data bases were searched for homologous sequences using the BLAST program.

**Pramida Constructs**—The mammalian expression plasmid encoding hemagglutinin (HA) epitope-tagged p73α or p73β was a generous gift from Dr. Mourad Khash (Sanofi Recherche, Paris, France). The p53 expression plasmid and the p53/p73-responsive reporter constructs were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The truncated form of p73α was generated as described previously (40). The full-length cDNA encoding human MM1 was amplified by PCR using the C42 cDNA present in pACT2 as a template. The primers used for PCR were: sense, 5′-CCAGGAATTC-GGGGTGTATGTGATGACTGTAGTCGGCCTTCCCAACATGGCAGTC; antisense, 5′-CGTGAGTCGGACCTGTCACTGTTACATCAAGCGGAGCTGAAAT-3′ (the EcoRI recognition site is underlined). The PCR product was subcloned into pGEM-T Easy (Promega), and its nucleotide sequence was verified by automated dideoxy terminator cycle sequencing. The PCR product, which was produced by additional EcoRI site in 5′-upstream and XhoI site in the 3′-downstream, was digested with EcoRI and XhoI and subcloned into the restriction sites of the pcDNA3-FLAG (kindly provided by Dr. Toshiharu Suzuki, University of Tokyo, Tokyo, Japan) to give pcDNA3-FLAG-MM1.

**Generation of a Bacterial Expression Construct**—The cDNA clone (C42) encoding the 150-amino acid sequence of human MM1 (amino acid residues 18–167) was digested with EcoRI and XhoI and subcloned into the EcoRI and XhoI restriction sites of the pcDNA3-FLAG-MM1. The MBP-fusion vector (New England Biolabs Inc., Beverly, MA), to create pMAL-MM1-(18–167). Cultures of Escherichia coli DH5α harboring the pMAL-cR or pMAL-M1 (18–167) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 37 °C. Cells were harvested by centrifugation at 4000 rpm for 5 min at 4 °C, and the bacterial pellet was resuspended in NETN buffer (50 mM Tris-C1, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA). After the brief sonication, the lysate was centrifuged at 12,000 rpm for 30 min at 4 °C to remove insoluble materials. The cleared supernatant was incubated with 1/10th volume of pre-equilibrated amylose resin (New England BioLabs Inc.) for 30 min at 4 °C. MBP fusion proteins bound to the beads were recovered with an elution buffer containing 10 mM maltose, 150 mM NaCl, and subcloned into the pMAL-cR recognition site of the pcDNA3-FLAG (kindly provided by Dr. Toshiharu Suzuki, University of Tokyo, Tokyo, Japan) to give pcDNA3-FLAG-MM1.

**In Vitro Interaction Assay**—p73α or p53 was generated in *vitro* in the presence of [35S]methionine using the quick-coupled *in vitro* transcription-translation system. The expressed protein was labeled with 35S-methionine using the T7 transcription-translation system and the labeled proteins were incubated with the bacterially expressed MBP-MMP1 fusion protein. The MBP-MMP1 fusion protein was subsequently purified using amylose resin (New England BioLabs Inc.). The MBP-MMP1 fusion protein was recovered with an elution buffer containing 10 mM maltose, 150 mM NaCl, and subcloned into the pMAL-cR recognition site of the pcDNA3-FLAG (kindly provided by Dr. Toshiharu Suzuki, University of Tokyo, Tokyo, Japan) to give pcDNA3-FLAG-MM1.

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1 The abbreviations used are: ΔNp73, truncated p73 isoform lacking the NH2-terminal transactivation domain; COS7 cells, SV40-transformed kidney cells from African green monkey; FITC, fluorescein isothiocyanate; HA, hemagglutinin; HDAC, histone deacetylase; MBP, maltose-binding protein; MM1, Myc modulator 1; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonil fluoride; TBS, Tris-buffered saline; TK, thymidine kinase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GST, glutathione S-transferase; RT, reverse transcriptase.
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FIG. 1. Binding of the human MM1 to the COOH-terminal region of p73 in yeast. A, schematic representation of p53 and p73. TA, transactivation domain; DB, sequence-specific DNA-binding domain; OD, oligomerization domain; SAM, sterile α motif domain. The extreme COOH-terminal region of p73α used for two-hybrid screening is indicated by a filled box. The COOH-terminal deletion mutants of p73α are also shown. Numbers indicate amino acid position. B, schematic diagram of the cDNA clone (C42) found in the two-hybrid screening. Sequencing of the insert revealed that it encoded amino acids 18–167 of MM1. LZ, a putative leucine zipper structure. Numbers indicate amino acid position. C, binding of C42 with the COOH-terminal region of p73α was quantified by the liquid β-galactosidase assay. Results shown are an average of three independent experiments, and the error bar indicates S.D. β-Galactosidase activity is indicated in Miller units.

A

B

C

FIG. 2. Interaction of MM1 with p73 in vitro. A, Coomassie Blue-stained MBP fusion protein used for the pull-down assays. MBP or MBP-MM1 (18–167) was expressed in E. coli DH5α and purified by amylose resin as described under “Experimental Procedures.” Each protein (1 μg) was analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. The positions of molecular mass markers are shown in kDa. B, binding of in vitro-translated p73 to MBP-MM1 fusion protein. [35S]Methionine-labeled p73α or p53 was generated in the coupled transcription/translation system, and incubated with 1 μg of purified MBP (lanes 3 and 5) or MBP-MM1 (lanes 4 and 6) for 2 h at 4°C. Protein complexes were collected on the amylose resin, washed extensively with binding buffer, and then boiled in SDS sample buffer. 35S-Labeled bound proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. The 1/2 volumes of the radiolabeled p73α (lane 1) and p53 (lane 2) used for the pull-down assays were applied in the same gel. The positions of molecular mass markers are shown in kDa.

1:500, Medical and Biological Laboratories, Nagoya, Japan), and the monoclonal anti-FLAG M2 antibody (diluted 1:50, Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. After incubation with primary antibodies, cells were washed and incubated with FITC- or rhodamine-conjugated secondary antibodies (Invitrogen) diluted 1:200 for 1 h at room temperature. Cells were finally washed in PBS, the coverslips were removed from the dishes, mounted onto slides, and observed under Fluoview laser scanning confocal microscope (Olympus, Tokyo, Japan).

Production of Polyclonal Anti-MM1 Antibody—The polyclonal anti-MM1 antibody was raised against the glutathione S-transferase (GST)-MM1 (1–167) fusion protein. The specificity of the antibody was checked on its ability to immunoprecipitate MM1 expressed in 293 cells and its ability to detect MM1 by Western blot analysis.

Immunoblotting—COS7 cells were transfected with 2 μg of the indicated expression plasmid and harvested at 48 h after transfection. Cells were washed with ice-cold PBS, lysed in 400 μl of EBC buffer (50 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM PMSF) containing protease inhibitor mixture (Sigma), and the extracts were sonicated for 10 s and centrifuged at 15,000 rpm for 10 min to remove insoluble materials (2). The protein concentrations were determined by

A

B

C

D
the Bradford protein assay (Bio-Rad Laboratories), using bovine serum albumin as a standard. Protein samples were boiled in the SDS-sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis, and then electrotransferred onto a nitrocellulose membrane in blotting buffer containing 20% methanol, 20 mM Tris, and 150 mM glycine at room temperature for 1 h. The membrane was blocked with TBST (50 mM Tris-Cl (pH 7.6), 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 1 h, and subsequently incubated for 1 h with the monoclonal anti-p73 (Ab-4, NeoMarkers, Inc., Fremont, CA), the monoclonal anti-c-Myc antibody (C-33, Santa Cruz Biotechnologies, Santa Cruz, CA), the polyclonal anti-actin antibody (20-33, Sigma), or the polyclonal anti-MM1 antibody in TBST, followed by an incubation with the horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (diluted 1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA). Protein bands were visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Amersham Biosciences, Inc., Piscataway, NJ).

**Co-immunoprecipitation**—COS7 cells were transiently transfected

**Fig. 3. Co-immunoprecipitation and co-localization of MM1 and p73.** A, COS7 cells transiently expressing FLAG-MM1 (lane 1), HA-p73α (lane 2), or FLAG-MM1 and HA-p73α (lane 3) were lysed in EBC buffer, and the total cell lysates were immunoprecipitated (IP) with the polyclonal anti-MM1 antibody. The immunocomplexes were resolved by 10% SDS-polyacrylamide gel electrophoresis and detected by Western blotting (WB) with the monoclonal anti-p73 antibody (Ab-4) (top). Total cell lysates were monitored on Western blot for expression of these components (middle and bottom panels). B, COS7 cells were transiently transfected with the indicated combinations of the expression plasmids. Whole cell lysates were immunoprecipitated with the polyclonal anti-MM1 antibody and then analyzed by immunoblotting using the anti-p73 antibody (top). Whole cell lysates were monitored on Western blot for expression of these components (middle and bottom panels). C, COS7 cells were transiently cotransfected with the expression plasmids for FLAG-MM1 (lane 1), HA-p73β (lane 2), or FLAG-MM1 and HA-p73β (lane 3). Whole cell lysates were immunoprecipitated with the polyclonal anti-MM1 antibody and subjected to the immunoblot analysis with the monoclonal anti-p73 antibody (top). Whole cell lysates were immunoblotted with the monoclonal anti-p73 or polyclonal anti-MM1 antibody to show the expression of HA-p73α or FLAG-MM1, respectively (middle and bottom panels, respectively). D, COS7 cells, which expressed a large amount of endogenous p53, were transiently transfected with the empty plasmid (pCDNA3) (lane 1), or the expression plasmid encoding FLAG-MM1 (lane 2). Whole cell lysates were immunoprecipitated with the monoclonal antibodies to p53 (DO-1 and PAb1801) and subjected to the immunoblot analysis using the polyclonal anti-MM1 antibody (top). The expression of endogenous p53 and FLAG-MM1 was monitored by Western blot analysis (middle and bottom panels, respectively). E, nuclear co-localization of MM1 and p73α. COS7 cells were transiently cotransfected with the expression plasmids for HA-p73α and FLAG-MM1. Forty-eight h post-transfection, cells were fixed, and incubated with the polyclonal anti-HA and the monoclonal anti-FLAG antibodies. Expression of HA-p73α and FLAG-MM1 was visualized with FITC-conjugated anti-rabbit IgG (green) and with rhodamine-conjugated anti-mouse IgG (red), respectively. The merged image suggests the nuclear co-localization of MM1 and p73α.
with 1 μg of the expression plasmid for HA-p73α, and 1 μg of pcDNA3-FLAG-MM1 using FuGENE 6 reagent. Forty-eight hours post-transfection, cells were harvested and cell lysates were prepared in 400 μl of the EBC buffer, which were spun at 15,000 rpm for 20 min at 4 °C to remove insoluble materials. The precleared supernatants were mixed with the polyclonal anti-MM1 antibody and incubated for 2 h at 4 °C. Protein A-Sepharose beads were then added to the reaction mixtures and incubated for 1 h at 4 °C. The immune complexes were washed with the lysis buffer three times at 4 °C, and the bound proteins were eluted by boiling in the SDS-sample buffer. Proteins were then analyzed by 10% SDS-polyacrylamide gel electrophoresis, semi-dry transferred onto nitrocellulose membrane, and probed with the monoclonal anti-p73 antibody (Ab-4, NeoMarkers, Inc.). Immuno-complexes were detected by ECL (Amersham Biosciences, Inc.).

Luciferase Assays—SAOS-2 cells were plated for transfection at a density of 5 × 10^4 cells/well in a 12-well tissue culture dish for 24 h. Cells were co-transfected with 100 ng of the indicated p53/p73-responsive reporter plasmid (p21^−/−, MDM2, Bax, or PG103), 10 ng of pRL-TK Renilla luciferase cDNA, and 25 ng of the indicated expression plasmid (p53, p73α, p73α (1-427), or p73β) in the presence or absence of the increasing amounts of the expression plasmid for FLAG-MM1. The total amount of DNA was kept constant (510 ng) with pcDNA3 (Invitrogen, Carlsbad, CA) per transfection. Forty-eight hours post-transfection, cells were lysed and luciferase activity was measured by using the dual-luciferase reporter assay system (Promega, Corp.) according to the manufacturer’s instructions. The transfection efficiency was standardized against Renilla luciferase.

Cell Growth Assay—SAOS-2 cells were plated on a 12-well tissue culture dish (5 × 10^4 cells/well) and transiently transfected with 125 ng of the reporter plasmid pCH110 (Amersham Biosciences, Inc.), which encodes E. coli β-galactosidase, plus 12.5 ng of the expression plasmid for p53 or p73 in the presence or absence of 187 ng of the expression plasmid encoding FLAG-MM1 by the LipofectAMINE procedure. The total amount of transfected plasmid DNA was kept constant (500 ng) per transfection by adding pcDNA3. At 48 h post-transfection, cells were fixed with 0.25% glutaraldehyde in PBS, the transfected cells were washed with the lysis buffer four times at 4 °C, and the bound proteins were eluted by boiling in the SDS-sample buffer. Proteins were then analyzed by 10% SDS-polyacrylamide gel electrophoresis, semi-dry transferred onto nitrocellulose membrane, and probed with the monoclonal anti-p73 antibody (Ab-4, NeoMarkers, Inc.). Immuno-complexes were detected by ECL (Amersham Biosciences, Inc.).

Isolation of MM1 as a p73α-binding Protein in a Two-hybrid Screen—To isolate one or more cellular proteins that could interact with the unique COOH-terminal region of p73α and regulate its activity, we used a yeast-based two-hybrid system to screen a human fetal brain cDNA library with a β-galactosidase expressing cell line (pAS2-1-p73α/COOH) encoding the extreme COOH-terminal portion of p73α (amino acid residues 551–636) of p73α (Fig. 1A). A yeast strain (CG1945) was co-transformed with the bait plasmid and the human fetal brain cDNA library, and the yeast colonies showing positive signals for nutrient (His) selection and β-galactosidase activity were selected. Of a total of 1 × 10^6 primary transformants, 34 colonies grew on the selection medium lacking tryptophan, leucine, and histidine, and 10 out of the 34 His-positive transformants formed blue colonies. Plasmids carrying these 10 positive candidates were rescued into E. coli, and their nucleotide sequences were determined. One clone, termed C42, contained a partial human cDNA for MM1, which had previously been reported to be a c-Myc-binding protein (41). The cDNA insert of this clone encoded a peptide ranging from amino acids 18 to 167 of MM1 (Fig. 1B). To assess the binding activity of C42 with the COOH-terminal region of p73α, yeast cells were co-transformed with various combinations of the constructs, and the β-galactosidase activity (Miller units) of each transformant was measured and compared. Transformants lacking the p73α COOH-terminal region or C42 were negative for the β-galactosidase activity, whereas those expressing both constructs induced the enzymatic activity (Fig. 1C). These data suggested that MM1 interacts with p73α in yeast.

Specificity of the MM1-p73α Interaction in Vitro—To confirm the results obtained from the yeast two-hybrid assay, we first examined the MM1-p73α interaction in vitro pull-down assay using MBP-MM1 (18-167) fusion protein (Fig. 2A). Amlyose resin bearing MBP or MBP-MM1 (18–167) was incubated with [35S]methionine-labeled full-length p73α generated in vitro in the coupled transcription/translation system. After washing the beads extensively with the binding buffer, the MBP- or MBP-MM1 (18–167)-bound proteins were eluted by boiling. Following the SDS-polyacrylamide gel electrophoresis, protein complexes were detected by autoradiography. As shown in Fig. 2B, we observed that the MBP fusion protein containing MM1 (18–167) was associated with radiolabeled p73α, whereas the MBP control was not. We also tested MM1 for its ability to interact with p53. As seen in Fig. 2B, we were unable to detect the interaction between MBP-MM1 (18–167) and radiolabeled p73α. These results suggested that p73α but not p53 directly binds to MM1.

In Vivo Interaction between MM1 and p73α—To demonstrate the interaction of MM1 with p73α in mammalian cells, human full-length MM1 tagged with the FLAG peptide on its NH2 terminus (FLAG-MM1) was prepared and introduced into COS7 cells. Whole cell lysates from COS7 cells transiently overexpressing HA-p73α, FLAG-MM1, or HA-p73α, and FLAG-MM1 were immunoprecipitated with the polyclonal anti-MM1 antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with the monoclonal anti-p73 antibody. As shown in Fig. 3A, HA-p73α was co-immunoprecipitated with FLAG-MM1. The expression of HA-p73α and FLAG-MM1 in COS7 cells was confirmed by immunoblotting with anti-p73 and anti-MM1 antibody, respectively (Fig. 3A, lower panels). To determine the MM1-binding region of p73α, p73α deletion mutants were used for the immunoprecipitation experiments with MM1. As expected, these COOH-terminal deletion mutants failed to co-immunoprecipitate with MM1 (Fig. 3B). We also tested MM1 for its ability to interact with p73β or p53 by co-immunoprecipitation analysis. As shown in Fig. 3 (C and D), FLAG-MM1 was not co-immunoprecipitated with HA-p73β or p53. These results suggested that p73α but not p53 and p73β interacted with MM1 in mammalian cells, and the extreme COOH-terminal region of p73α was required for this interaction.

To confirm the above co-immunoprecipitation analysis, we examined the subcellular distribution of MM1 and p73α. COS7 cells were transiently co-transfected with the expression plasmids for FLAG-MM1 and HA-p73α, and were double-stained with anti-FLAG and anti-HA antibodies. The transfected cells were then observed under a confocal laser scanning microscope. As reported previously (2, 41), MM1 or p73α was almost exclusively located in the nucleus (Fig. 3E). Upon close inspection of
Fig. 4. Differential effects of MM1 on the p73α-mediated transcriptional activation. p53-deficient SAOS-2 cells (5 × 10⁴ cells/well) were transiently co-transfected with 25 ng of the expression plasmid for p73α (A), p73β (B), p73α-(1–427) (C), or p53 (D) along with the luciferase
Effect of MM1 on the p73-mediated Transcriptional Activation and Growth Suppression—To determine whether or not MM1 affects the p73- or p53-dependent transcriptional activation in cells, p53/p73-responsive Bax, PG13, which carries 13 copies of the consensus p53-responsive element, p21<sup>wt/1</sup>, or MDM2-luciferase reporter construct was co-transfected with the expression plasmid for HA-p73α, HA-p73β, HA-p73sα-(1-427), or p53, in the presence or absence of the increasing amounts of FLAG-MM1 expression plasmid into p53-deficient human osteosarcoma SAOS-2 cells. Under our experimental conditions, endogenous p73 and MM1 could not be detected, and the levels of the ectopically expressed proteins were not affected in the presence of MM1 (data not shown). Expression of p73α, p73β, p73α-(1-427), or p53 successfully activated transcription of each of those p53/p73-responsive reporters compared with the empty plasmid controls, and MM1 alone failed to induce these reporters (Fig. 4). The ability of p73α to drive transcription from Bax and PG13 reporter was enhanced by MM1 in a dose-dependent manner (Fig. 4A), whereas MM1 did not elevate the luciferase activity of those reporters induced by p73β or p53 (Fig. 4, B and D). These observations were consistent with the results showing that MM1 interacted with p73α but not with p73β or p53. In addition, MM1 failed to affect the transcription activation function of p73α-(1-427), which lacked the COOH-terminal region of p73α (Fig. 4C). On the other hand, MM1 did not enhance the MDM2- or p21<sup>wt/1</sup>-driven transcription mediated by p73α (Fig. 4A). Similarly, p53-dependent luciferase activity driven by the p53/p73-responsive p21<sup>wt/1</sup> or MDM2 promoter was not affected in the presence of exogenously expressed MM1 (data not shown). These results indicated that MM1 might determine the differential response of target genes to p73, although the precise mechanism defining those specificities remained to be determined.

In view of the ability of MM1 to modulate cellular function of p73α, we next examined whether MM1 could affect the p73α-mediated growth inhibition. To this end, SAOS-2 cells were co-transfected with the expression plasmid for p53 or p73α in the presence or absence of MM1 expression plasmid together with β-galactosidase expression construct (pCH110) to identify transfected cells. The number of β-galactosidase-positive cells was measured 48 h post-transfection. As shown in Fig. 5 (top panel), ectopic overexpression of p73α or p53 led to the significant decrease in number of β-galactosidase-positive cells compared with that transfected with the empty plasmid alone (compare lane 1 with lanes 3 or 6), whereas the number of β-galactosidase-positive cells expressing MM1 was similar to that observed in the empty plasmid-transfected cells (compare lane 1 with lane 2). At expression plasmid concentrations ranging from 12.5 to 375 ng, p73α and p53 decreased the number of β-galactosidase-positive cells in a dose-dependent manner (data not shown). To facilitate the detection of the possible effect of MM1 on p73α or p53, SAOS-2 cells were transfected with 12.5 ng of p73α or p53 expression plasmid, together with the expression plasmid for MM1. Under these experimental conditions, endogenous p73α and MM1 were undetectable, and the ectopically expressed proteins were readily detected by Western analysis (Fig. 5, bottom panel). As seen in Fig. 5 (top panel), co-expression of p73α with MM1 significantly reduced the number of β-galactosidase-positive cells compared with that transfected with p73α expression plasmid alone, whereas expression of MM1 had no significant effect on the growth suppression induced by p53, suggesting that MM1 might cooperate with p73α in inhibiting the cell growth.

**Physical and Functional Interaction among p73, MM1, and c-Myc**—As reported previously, MM1 bound to the NH2-terminal transactivation domain of c-Myc and inhibited its E-box-dependent transcriptional activity (41). Recently, Ceballos et al. have found that c-Myc counteracts the transactivation of p21<sup>wt/1</sup> promoter mediated by p53 (45). These observations prompted us to examine the role of c-Myc on p73 function. To determine the functional relevance of the p73-c-Myc interaction, we examined the effect of c-Myc on the p73α-mediated transcriptional activation. SAOS-2 was co-transfected with the expression plasmid encoding p73α or p53 along with the p53-/p73-responsive Bax or p21<sup>wt/1</sup> promoter luciferase reporter construct in the presence of c-Myc expression plasmid. Consistent with the previous results (45), p53-dependent transcriptional activation was repressed by co-expression with c-Myc (Fig. 6). Similarly, c-Myc abrogated the p73α-mediated transcriptional activation. Intriguingly, unlike c-Myc, overexpression of N-Myc exhibited no detectable effect on the transcription function of p73α and p53. The protein levels of ectopically expressed c-Myc and N-Myc were easily detected by

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**Fig. 5.** MM1 stimulates the p73α-induced growth suppression. SAOS-2 cells (5 × 10<sup>4</sup> cells/well) were transiently co-transfected with 12.5 ng of the expression plasmid encoding p53 or p73α together with 125 ng of the reporter plasmid (pCH110), which encodes <i>E. coli</i> β-galactosidase, in the presence or absence of 187 ng of the expression plasmid for MM1. As a control transfection, 100 ng of the expression plasmid for p73α (lane 3) or p53 (lane 6) were transfected into SAOS-2 cells. The total amount of transfected plasmid DNA was adjusted to 500 ng/well by adding pcDNA3. Forty-eight h after transfection, transfected cells were detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The relative percentage of β-galactosidase-expressing cells represents the ratio of the number of β-galactosidase-positive cells to the number of those transfected with pcDNA3 alone. Results shown are the mean value from three independent experiments (top panel). Whole cell lysates prepared from each transfected SAOS-2 cells were analyzed by immunoblotting with the monoclonal anti-p73 (Ab-4), the monoclonal anti-p53 (DO-1), or the polyclonal anti-MM1 antibody (bottom panel).

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*reporter constructs* (100 ng) as indicated, and 10 ng of the Renilla luciferase plasmid (pRL-TK) in the presence or absence of the increasing amounts of pcDNA3-FLAG-MM1 (125, 250, or 375 ng). The total amount of plasmid DNA per transfection was kept constant (510 ng) with pcDNA3. All transfections were performed in triplicate. Forty-eight h after transfection, cells were lysed, and the firefly luciferase activities were determined. The transfection efficiency was standardized against Renilla luciferase. Results are shown as -fold induction of the firefly luciferase activity compared with control cells transfected with pcDNA3 alone.
Western analysis, whereas endogenously expressed c-Myc and N-Myc could not be detected (data not shown). We then examined whether MM1 could antagonize the inhibitory effect of c-Myc on the p73 function. To this end, we performed the luciferase reporter assays in transiently transfected SAOS-2 cells using the Bax promoter luciferase reporter construct. As expected, co-expression of MM1 with c-Myc and p73/H9251 apparently rescued the reporter activity of p73/H9251 suppressed by c-Myc (Fig. 7A). Unlike p73/H9251, c-Myc-mediated repression of the reporter activity of p53 was not affected in the presence of MM1 expression (Fig. 7B). These studies raised the possibility that MM1 is associated with p73/H9251 and/or c-Myc and suppresses the functional interaction between p73/H9251 and c-Myc.

To determine whether p73α could physically bind to c-Myc, and/or whether p73α could be a part of the c-Myc-MM1 complex, co-immunoprecipitation analysis was carried out. COS7 cells were transfected with the indicated combinations of the expression plasmids, and the whole cell lysates were immunoprecipitated with antibody to MM1 or p73 followed by immunoblotting with antibody to c-Myc. Consistent with the previous report (41), the anti-MM1 immunoprecipitates contained c-Myc (Fig. 8A). Analysis of the anti-p73 or the anti-c-Myc immunoprecipitates also indicated that c-Myc can form a physical complex with p73/H9251 in vivo (Fig. 8B). In contrast, we could not detect the physical interaction between p73/H9251 and N-Myc (data not shown). To evaluate their interaction by confocal immunofluorescence microscopy, HA-p73/H9251 was co-expressed with FLAG-c-Myc in COS7 cells. Fig. 8C shows the confocal images from the same cell expressing both proteins. A significant co-localization of p73/H9251 and c-Myc to the nucleus was observed when both images were overlaid. To determine whether c-Myc could be associated with p73α in the presence of MM1, HA-p73α and FLAG-c-Myc were co-expressed with the increasing amounts of MM1 in COS7 cells. As shown in Fig. 8D, the amounts of p73α co-precipitated with c-Myc was decreased in the presence of the increasing amounts of MM1 (compare lanes 1, 2, and 3). Taken together, these results suggested that MM1 might prevent the c-Myc-p73α interaction and/or directly bind to c-Myc to inhibit its activity and thereby stimulate the p73α activity.

Etoposide Induces Expression of p73 and Down-regulation of c-myc—Recently, it has been shown that expression of p73 is significantly induced in response to various DNA-damaging agents.
agents such as etoposide, doxorubicin, and camptothecin (46).

To determine the effect of etoposide on p73, MM1, and c-myc, HL60 cells (which do not contain p53 protein) were treated with etoposide, and the expression levels of p73, MM1, and c-myc were examined by RT-PCR analysis. As described previously (47), incubation of HL60 cells with etoposide resulted in the remarkable increase in the number of cells in sub-G1 phase indicating the apoptosis (Fig. 9A). Under our experimental conditions, sub-G1 fractions were not increased by the treatment with the dimethyl sulfoxide solvent alone (data not shown). We then prepared the total RNA from HL60 cells at the indicated time points after the treatment with etoposide, and RT-PCR analysis was carried out. In agreement with the previous reports, the expression level of p73 was significantly increased in response to etoposide (Fig. 9B). On the other hand, the level of MM1 was unaffected in the presence of etoposide, whereas etoposide treatment caused a significant decrease in c-myc level. Similar results were also obtained by Western blot analysis (Fig. 9C).

DISCUSSION

Our present results have revealed for the first time that p73 is directly associated with c-Myc and its repressor MM1. However, the functional interactions among them appear to be finely regulated. MM1 is able to bind only to p73/H9251 but not to p73/H9252 and stimulates transactivation ability of the former. Only c-Myc but not N-Myc binds to p73α and inhibits its transcriptional activity. In addition, the regulatory effect of MM1 on p73α function seems to be dependent on each target gene promoter. Nevertheless, our finding of a direct link through
The extreme COOH-terminal region of p53 is closely involved in its pro-apoptotic function by protein inter-
action and regulation. The SAM domain seems to be a protein-protein interaction module found in a variety of proteins involved in developmental regulation (52). Recently, it has been shown that Yes-associated protein is associated with p73α via its PPPPY motif and enhances the transcriptional activity of p73α (53). In addition, Minta et al. (54) have found that p73α but not p73β bound to SUMO-1, and the extreme COOH-terminal Lys residues of p73α (at position 627) is the major site for SUMO-1 modification. SUMO-1 modification alters the subcellular distribution of p73, although it did not affect the transcriptional activity of p73. Furthermore, the extreme COOH-terminal region of p73α is suggested to act as a negative regulator of its own function (8, 40). In conjunction with those observations reported, our data suggest that the interaction of MM1 with the extreme COOH-terminal region allows p73α to take the conformation competent to express its activity, although the precise mechanism remains to be clarified.

Our present data have shown that the ability of p73α to drive the transcription from the Bax and the PG13 promoter is enhanced by overexpression of MM1. However, the effect of MM1 on the p73α-mediated transcription from the MDM2 and p21promoter is barely detectable. The similar pattern of target gene induction is also observed in some other cases. Mts1, which is associated with the extreme COOH-terminal region of p53, differentially regulates the transcription function of p53 (55). Mts1 significantly inhibited the p53-dependent transcription from the p21promoter as measured in transient reporter assays, whereas its effect on the Bax promoter was negligible. Similarly, WT1 exhibited an inhibitory effect on the p53-regulated activation of the MDM2 promoter, whereas the repression of the Bax promoter by WT1 was not significant (56). Recently, Thonborow and Manfredi (57) have reported that there exists an Sp1-binding site immediately adjacent to the p53-responsive element of the Bax promoter, and p53 may require the cooperation of Sp1 to activate Bax promoter. In contrast, the p53-Sp1 complex may function in an antagonistic manner for other promoters (58). These findings suggest that MM1-mediated differential regulation of the p53/p73-responsive promoters is due to a requirement for additional cofactors specific to each promoter, although this requires further elucidation.

Recently, Ceballos et al. (45) have found that c-Myc significantly inhibits the transactivation and pro-apoptotic function of p53. Consistent with their report, our data have shown that co-expression of p53 with c-Myc results in a remarkable reduction of the p53-mediated transcriptional activation of the Bax and p21promoter. The use of immunoprecipitation and the luciferase reporter assays, we have demonstrated that c-Myc physically interacts with p73α and thereby inhibits its transactivation function. In contrast, N-Myc, the other member of myc family, is unable to affect the p73α- or p53-dependent transcriptional activation. As reported previously, targeted homozygous disruption of the c-myc or N-myc gene resulted in embryonic lethality (59–61). When c-myc was replaced by N-
mice did not show any evident defects (62), suggesting that c-Myc and N-Myc are functionally redundant. However, c-myc and N-myc have shown a distinct expression pattern in the tissues or organs during embryogenesis. Therefore, our present results may suggest that there is tissue-specific signaling or regulation in the p73 function, to which c-Myc and N-Myc are differently concerned.

MM1 has been originally identified as a c-Myc-binding protein and blocked the E-box-dependent transcriptional activation of c-Myc (41). Recently, Satou et al. (28) found that MM1 recruits an HDAC complex and thereby inhibits the c-Myc-mediated transactivation. According to our present results,
MM1 attenuated the c-Myc-dependent down-regulation of p73α, and the amount of p73α co-precipitated with c-Myc was decreased in the presence of MM1. It is unclear at this time whether HDAC-mediated repression of the transcriptional activity of c-Myc is required for MM1 to abolish the ability of c-Myc to inhibit p73α.

Intriguingly, when HL60 cells (which do not contain p53 protein) were exposed to etoposide, the expression level of p73α protein clearly increased 4 h after the treatment and continued to increase at least until 8 h, being strongly associated with increase in the number of cells in sub-G1. On the contrary, the levels of c-Myc expression decreased 4 h after the treatment with etoposide. Given that c-Myc inhibits the transcriptional activity of p73α, it is possible that MM1 interferes with the ability of c-Myc to inhibit the pro-apoptotic function of p73. It is necessary to be clarified whether the c-Myc-binding site in p73α is localized close to the MM1-binding site within the extreme COOH-terminal region. However, MM1 may not only bind to p73α but also inhibit the interaction between c-Myc and p73α and thereby allow p73α to change to a conformation that is competent to express its activity.

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