

based on the Sos recruitment system. We report here that protein kinase A catalytic subunit β (PKA-C β)¹ bound to p73 α in cells, but not to p53, and that their interaction was mediated by the N- and C-terminal regions of p73 α . *In vitro* kinase assays revealed that the catalytic subunit of PKA phosphorylated p73 α . PKA-C β inhibited the p73 α -mediated transcriptional activation of the p21^{WAF1} and *Bax* promoters and p73 α -dependent apoptosis in response to camptothecin. On the other hand, the kinase-deficient mutant of PKA-C β had little effect on p73 α . Of note, we found that PKA-C β facilitated the intramolecular interaction of p73 α . Our results strongly suggest the PKA-C β -mediated phosphorylation and intramolecular interaction of p73 to be a novel inhibitory mechanism of p73 function.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—SV40-transformed African green monkey kidney cells (COS-7) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. p53-deficient human lung carcinoma H1299 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic mixture. The cells were cultured at 37 °C in a water-saturated atmosphere of 95% air and 5% CO₂.

Transient Transfection—COS-7 cells grown to 50–70% confluence in 60-mm dishes were transfected with the indicated expression plasmids using FuGENE 6 transfection reagent (Roche Applied Science) following the protocol recommended by the manufacturer. H1299 cells transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Yeast Two-hybrid Screening—The CytoTrap two-hybrid system was purchased from Stratagene (La Jolla, CA). The cDNA encoding the full-length open reading frame of human p73 α was amplified by PCR using pcDNA3-p73 α as template. The PCR product, which was produced by additional upstream 5'-BamHI and downstream 3'-SalI restriction sites, was digested completely with BamHI and SalI; purified on agarose gel; and directly inserted in-frame into the identical restriction sites of pSos to give pSos-p73 α . The resulting pSos-p73 α "bait" plasmid was used to identify the cDNA encoding the p73 α -binding protein from a human fetal brain cDNA library cloned into the pMyr plasmid (Stratagene). The screening was carried out according to the manufacturer's instructions. Briefly, a temperature-sensitive yeast strain (cdc25H α) was cotransformed with pSos-p73 α and the cDNA library using the lithium acetate/heat shock procedure as described previously (29). Transformants were allowed to grow on selection medium containing glucose for 2 days at 25 °C and then transferred onto selection medium containing galactose. Plasmid DNAs were isolated from the colonies exhibiting galactose-dependent growth at 37 °C and transformed into *Escherichia coli*. Finally, the nucleotide sequences of the positive cDNA clones were determined by the dideoxy terminator cycle sequencing using an ABI automated DNA sequencer (Applied Biosystems, Foster City, CA).

Western Blot Analysis—Transfected cells were washed twice with phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer A (25 mM Tris-Cl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, and 1% Triton X-100) containing protease inhibitor mixture (Sigma). After a brief sonication, whole cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C to remove insoluble materials, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Protein samples were boiled in SDS sample buffer for 5 min, resolved by 10% SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked overnight with 50 mM Tris-Cl (pH 7.6), 100 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk and then incubated at room temperature for 1 h with anti-FLAG monoclonal antibody (M2, Sigma), anti-green fluorescence protein (GFP) monoclonal antibody (1E4, Medical and Biological Laboratories, Nagoya, Japan), anti-p53 monoclonal antibody (DO-1, Oncogene Research Products, Cambridge, MA), anti-p73 monoclonal antibody (Ab-4, NeoMarkers, Inc., Fremont, CA), anti-p21^{WAF1} monoclonal antibody (Ab-1, Oncogene Research Products), anti-PKA-C α polyclonal

antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-PKA-C β polyclonal antibody (C-20, Santa Cruz Biotechnology, Inc.), followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Following the last wash, horseradish peroxidase-labeled antibodies were detected using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences) according to the manufacturer's instructions.

Immunoprecipitation and Pull-down Assay—For immunoprecipitation, cell lysates were prepared in lysis buffer A. Equal amounts of protein extracts were pre-absorbed with protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, and the precleared lysates were incubated with the indicated antibodies for 2 h at 4 °C, followed by incubation with protein G-Sepharose beads for an additional 1 h at 4 °C. The immune complexes were then washed three times with lysis buffer A, eluted by boiling in SDS sample buffer for 5 min, and subjected to Western blot analysis. For glutathione S-transferase (GST) pull-down assays, GST alone or the indicated GST-p73 α fusion proteins were expressed in *E. coli* strain DH5 α and loaded onto glutathione-Sepharose 4B beads (Amersham Biosciences). PKA-C β was generated *in vitro* in the presence of [³⁵S]methionine using the TNT quick-coupled *in vitro* transcription/translation system (Promega Corp., Madison, WI) according to the manufacturer's instructions. ³⁵S-Labeled PKA-C β was incubated with GST or GST-p73 α fusion proteins bound to glutathione-Sepharose beads for 2 h at 4 °C in a total volume of 400 μ l of binding buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 0.1% Nonidet P-40). Beads were washed extensively with the same buffer, and the radiolabeled proteins were eluted by boiling in SDS sample buffer for 5 min. Following electrophoresis, gels were destained, dried, and exposed to an x-ray film with an intensifying screen at -80 °C.

Cell Fractionation—Transfected COS-7 cells were fractionated into nuclear and cytoplasmic fractions as described previously (30). In brief, cells were washed twice with ice-cold 1 \times PBS and lysed in lysis buffer B containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture for 30 min at 4 °C. Cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C to separate soluble (cytoplasmic) from insoluble (nuclear) fractions. The pellets were washed extensively with lysis buffer B and further dissolved in 1 \times SDS sample buffer. The nuclear and cytoplasmic fractions were analyzed by immunoblotting with anti-lamin B monoclonal antibody (Ab-1, Oncogene Research Products) or with anti- α -tubulin monoclonal antibody (Ab-2, NeoMarkers, Inc.).

Immunofluorescence Microscopy—H1299 cells were grown on coverslips and transiently cotransfected with the expression plasmids for hemagglutinin (HA)-p73 α and FLAG-PKA-C β . Forty-eight hours after transfection, cells were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min. Nonspecific binding sites were blocked by treating cells with PBS containing 3% bovine serum albumin. The cells were incubated with anti-HA polyclonal and anti-FLAG monoclonal antibodies for 1 h, followed by incubation with fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies (Invitrogen). The coverslips were washed with PBS, mounted onto slides, and observed under a Fluoview laser scanning confocal microscope (Olympus, Tokyo, Japan).

Luciferase Reporter Assay—p53-deficient H1299 cells (5 \times 10⁴ cells in a 12-well plate) were transiently cotransfected with a constant amount of the indicated expression plasmid (HA-p73 α , HA-p73 β , or p53), a p53/p73-responsive luciferase reporter construct (p21^{WAF1} or *bax*), and pRL-TK encoding *Renilla* luciferase with or without increasing amounts of the expression plasmid for FLAG-PKA-C β . The total amount of DNA was kept constant (510 ng) with pcDNA3 per transfection. Forty-eight hours after transfection, cells were lysed and assayed for luciferase activity using the Dual-Luciferase reporter assay system (Promega Corp.) according to the manufacturer's recommendations. The transfection efficiency was normalized based on pRL-TK reporter activity.

Reverse Transcription-PCR—H1299 cells were transiently cotransfected with the indicated combinations of expression plasmids. Twenty-four hours after transfection, total RNA was prepared using an RNeasy mini kit (Qiagen Inc.) according to the manufacturer's protocol. One microgram of total RNA was used to synthesize the first-strand cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen). Reverse transcription was carried out at 42 °C for 90 min, and reverse transcripts were amplified by standard PCR with *rTaq* DNA polymerase (Takara, Ohtsu, Japan). The primers used for PCR were as follows: p21^{WAF1}, 5'-ATGAATTACACCCCTTTCC-3' (sense) and 5'-CCCTAGGCTGTGCTCACTTC-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase, 5'-ACCTGACCTGCCGTCTAGAA-3'

¹ The abbreviations used are: PKA-C, protein kinase A catalytic subunit; PBS, phosphate-buffered saline; GFP, green fluorescence protein; GST, glutathione S-transferase; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; Bt₂cAMP, dibutyryl cAMP.

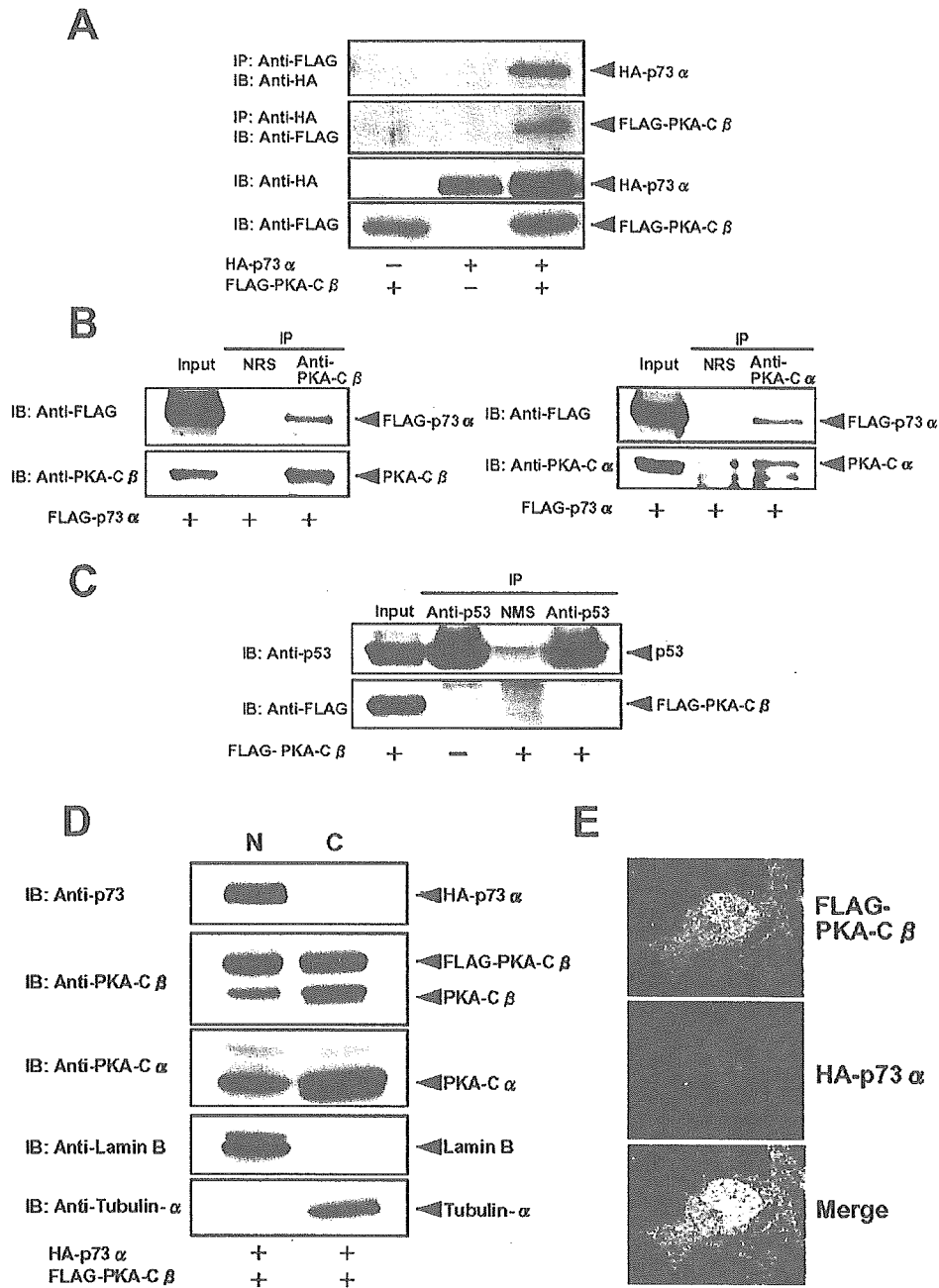


FIG. 1. Interaction between p73 and PKA-C β in mammalian cultured cells. A, p73 α forms a complex with PKA-C β in COS-7 cells. Whole cell lysates prepared from COS-7 cells transiently cotransfected with the indicated combinations of expression plasmids were immunoprecipitated (IP) with anti-FLAG or anti-HA monoclonal antibody. Immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA (first panel) or anti-FLAG (second panel) monoclonal antibody. Whole cell lysates were immunoblotted with anti-HA (third panel) or anti-FLAG (fourth panel) monoclonal antibody to show the expression of HA-p73 α or FLAG-PKA-C β , respectively. B, p73 α binds to endogenous PKA-C in COS-7 cells. COS-7 cells were transiently transfected with the expression plasmid for FLAG-p73 α . Forty-eight hours after transfection, whole cell lysates were prepared and subjected to immunoprecipitation with anti-PKA-C β (left panels) or anti-PKA-C α (right panels) polyclonal antibody. Immunoprecipitation with normal rabbit serum (NRS) was used as a negative control. After immunoprecipitation, coprecipitating FLAG-p73 α was detected by immunoblotting with anti-FLAG monoclonal antibody. C, PKA-C β does not bind to endogenous p53. COS-7 cells were transiently transfected with the empty control plasmid or with the expression plasmid encoding FLAG-PKA-C β . Forty-eight hours post-transfection, whole cell lysates were prepared and subjected to immunoprecipitation with anti-p53 monoclonal antibody or normal mouse serum (NMS), followed by immunoblotting with anti-p53 (upper panel) or anti-FLAG (lower panel) monoclonal antibody. D, subcellular localization of exogenous and endogenous PKA-C β . p53-deficient H1299 cells were transiently cotransfected with the expression plasmids for HA-p73 α and FLAG-PKA-C β (first through third panels). Forty-eight hours after transfection, transfected cells were fractionated into nuclear (N) and cytoplasmic (C) fractions as described under "Experimental Procedures." Each fraction was adjusted to an equal volume, and the aliquots of these fractions were separated by 10% SDS-PAGE, followed by immunoblotting with the indicated antibodies. These fractions were analyzed for lamin B (fourth panel) and α -tubulin (fifth panel) to show the validity of our fractionation technique. E, nuclear co-localization of p73 and PKA-C β . H1299 cells plated on coverslips were cotransfected with the expression plasmids for HA-p73 α and FLAG-PKA-C β and processed for immunocytochemical detection using anti-HA and anti-FLAG antibodies. The merged image shows the nuclear co-localization of p73 α and PKA-C β .

(sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense). PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

In Vitro Kinase Assays—GST or the indicated GST-p73 α fusion pro-

teins bound to glutathione-Sepharose beads were washed three times with kinase buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 12 mM MgCl₂). The washed beads were incubated with 30 μ l of kinase buffer containing 2 units of purified PKA catalytic subunit (Sigma), 2 mM

dithiothreitol, and 10 μ Ci of [γ - 32 P]ATP for 30 min at 4 $^{\circ}$ C. The reaction mixtures were boiled in 2 \times SDS sample buffer for 5 min, and the proteins were separated by 10% SDS-PAGE. The gels were dried and processed for autoradiography.

Cell Survival Assays—H1299 cells were seeded in 6-well plates and allowed to attach. Cells were then cotransfected with the indicated expression plasmids. Twenty-four hours after transfection, cells were exposed to camptothecin (final concentration of 1 μ M) for 24 h. Cell viability was measured by a colorimetric assay with modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as the substrate.

Construction of a Kinase-deficient Mutant of PKA-C β —The K76R mutation was introduced into wild-type PKA-C β by PCR-based mutagenesis using *PfuUltra*TM high fidelity DNA polymerase (Stratagene) according to the manufacturer's protocol. The following oligonucleotide primers were used: 5'-AGGATCTTAGATAAGCAGAAGGTT-3' (the underlined segment encodes Arg at position 76) and 5'-CATGGCATA-ATCTGTTTCAGTGGCT-3'. This yielded the expression plasmid pcDNA3-FLAG-PKA-C β (K76R), which was completely sequenced by the dideoxy chain termination method.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed following a protocol provided by Upstate Biotechnology, Inc. (Lake Placid, NY). In brief, H1299 cells were transiently cotransfected with the expression plasmids for HA-p73 α (1.2 μ g) and FLAG-PKA-C β (4.8 μ g). Thirty-six hours after transfection, cells were cross-linked with 1% formaldehyde in medium for 15 min at 37 $^{\circ}$ C. Cells were then washed with ice-cold PBS and resuspended in 200 μ l of SDS-sample buffer containing protease inhibitor mixture. The suspension was sonicated 10 times for 30 s with a 1-min cooling period on ice between times and precleared with 20 μ l of protein A-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 $^{\circ}$ C. The beads were removed, and the chromatin solution was immunoprecipitated overnight with anti-HA monoclonal antibody at 4 $^{\circ}$ C, followed by incubation with protein A-agarose beads for an additional 1 h at 4 $^{\circ}$ C. The immune complexes were eluted with 100 μ l of elution buffer (1% SDS and 0.1 M NaHCO₃) and formaldehyde cross-links were reversed by heating at 65 $^{\circ}$ C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45 $^{\circ}$ C for 1 h. DNAs of the immunoprecipitates and control input DNAs were purified using a QIAquick PCR purification kit (Qiagen Inc.) and then analyzed by regular PCR using the human p21^{WAF1} and *bax* promoter-specific primers. The primer sequences were 5'-CACCTTTTACCATTCCCCTA-3' and 5'-GCAGCCCA-AGGACAAAATAG-3' for p21^{WAF1} and 5'-AAAGCTCAGAGGCCCAAA-AT-3' and 5'-AGGCTGAGACGGGGTTATCT-3' for *bax*.

RESULTS

Identification of PKA-C β as a Novel Binding Partner of p73—Because the conventional yeast two-hybrid system depends on the DNA binding as well as the transactivation function of Gal4, it is quite difficult to use a full-length transcriptional regulator with a transactivation domain as bait. To identify potential p73-interacting cellular protein(s), we used full-length p73 α as bait in a new CytoTrap yeast two-hybrid screen relying on the Sos recruitment system. A temperature-sensitive yeast strain (*cdc25H α*) was cotransformed with a bait plasmid and a human fetal brain cDNA library. Of a total of 1 \times 10⁶ primary transformants grown on medium containing glucose at 30 $^{\circ}$ C, one clone (termed F115) exhibited galactose-dependent growth at 37 $^{\circ}$ C. The plasmid DNA derived from the cDNA library was introduced into *E. coli*, and its nucleotide sequence was determined. Sequence analysis revealed that the F115 cDNA clone encodes full-length PKA-C β . Of the PKA catalytic subunit isoforms (PKA-C α , PKA-C β , and PKA-C γ), PKA-C β is highly expressed in brain and reproductive tissues, whereas PKA-C α is ubiquitously expressed in mammalian tissues (31, 32).

PKA-C β Associates with p73 in Mammalian Cultured Cells—To confirm the interaction between PKA-C β and p73 detected by the CytoTrap yeast two-hybrid system, co-immunoprecipitation experiments were carried out using whole cell lysates prepared from COS-7 cells expressing exogenous FLAG-PKA-C β and HA-p73 α . As shown in Fig. 1A, the anti-FLAG immunoprecipitates contained HA-p73 α . Used as a control, HA-p73 α was not detectable in the anti-FLAG immuno-

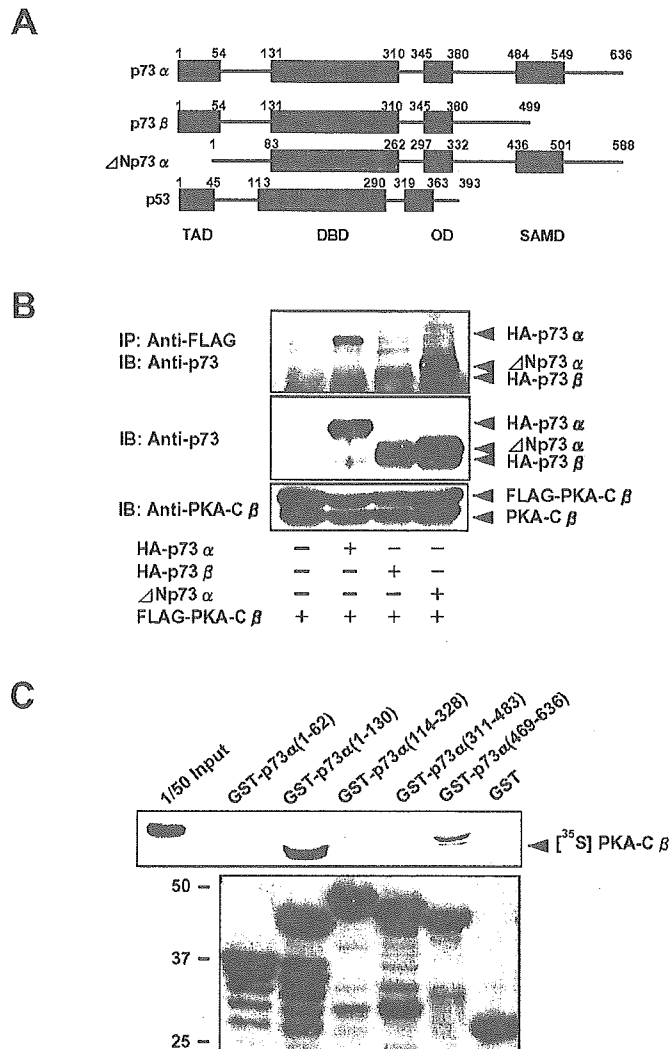


FIG. 2. Interacting region within p73 for PKA-C β . A, domain structures of p73 and p53. The transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD), and sterile α motif domain (SAMD) are indicated. The numbers above the p73 variants and p53 indicate amino acid numbering. B, interaction of PKA-C β with various p73 variants. Whole cell lysates prepared from H1299 cells transiently cotransfected with the expression plasmid for HA-p73 α , HA-p73 β , or Δ Np73 α and with the expression plasmid for FLAG-PKA-C β were immunoprecipitated (IP) with anti-FLAG monoclonal antibody. The immune complexes were analyzed by immunoblotting (IB) with anti-p73 monoclonal antibody (upper panel). The expression levels of p73 variants (middle panel) and PKA-C β (lower panel) in whole cell lysates were monitored by immunoblotting with the indicated antibodies. C, in vitro GST pull-down assays. Bacterially expressed GST or the indicated GST-p73 α fusion proteins were incubated with in vitro translated 35 S-labeled FLAG-PKA-C β and precipitated with glutathione-Sepharose 4B beads (50% slurry). After extensive washing, the bound proteins were separated by 10% SDS-PAGE and processed for autoradiography (upper panel). 1/50 Input indicates the radiolabeled FLAG-PKA-C β used for in vitro pull-down assays that was directly loaded on the same gel as a control. GST and GST-p73 α fusion proteins were stained with Coomassie Brilliant Blue (lower panel). The positions of molecular mass markers are indicated on the left in kilodaltons.

precipitates of COS-7 cells expressing FLAG-PKA-C β or HA-p73 α alone. Analysis of the anti-HA immunoprecipitates also demonstrated that FLAG-PKA-C β co-immunoprecipitated with HA-p73 α . Next, we examined whether endogenous PKA-C β could interact with p73 α . To this end, whole cell lysates prepared from COS-7 cells transfected with the expression plasmid for FLAG-p73 α were immunoprecipitated with normal rabbit serum or with the specific antibody against PKA-C β , followed by immunoblotting with anti-FLAG anti-

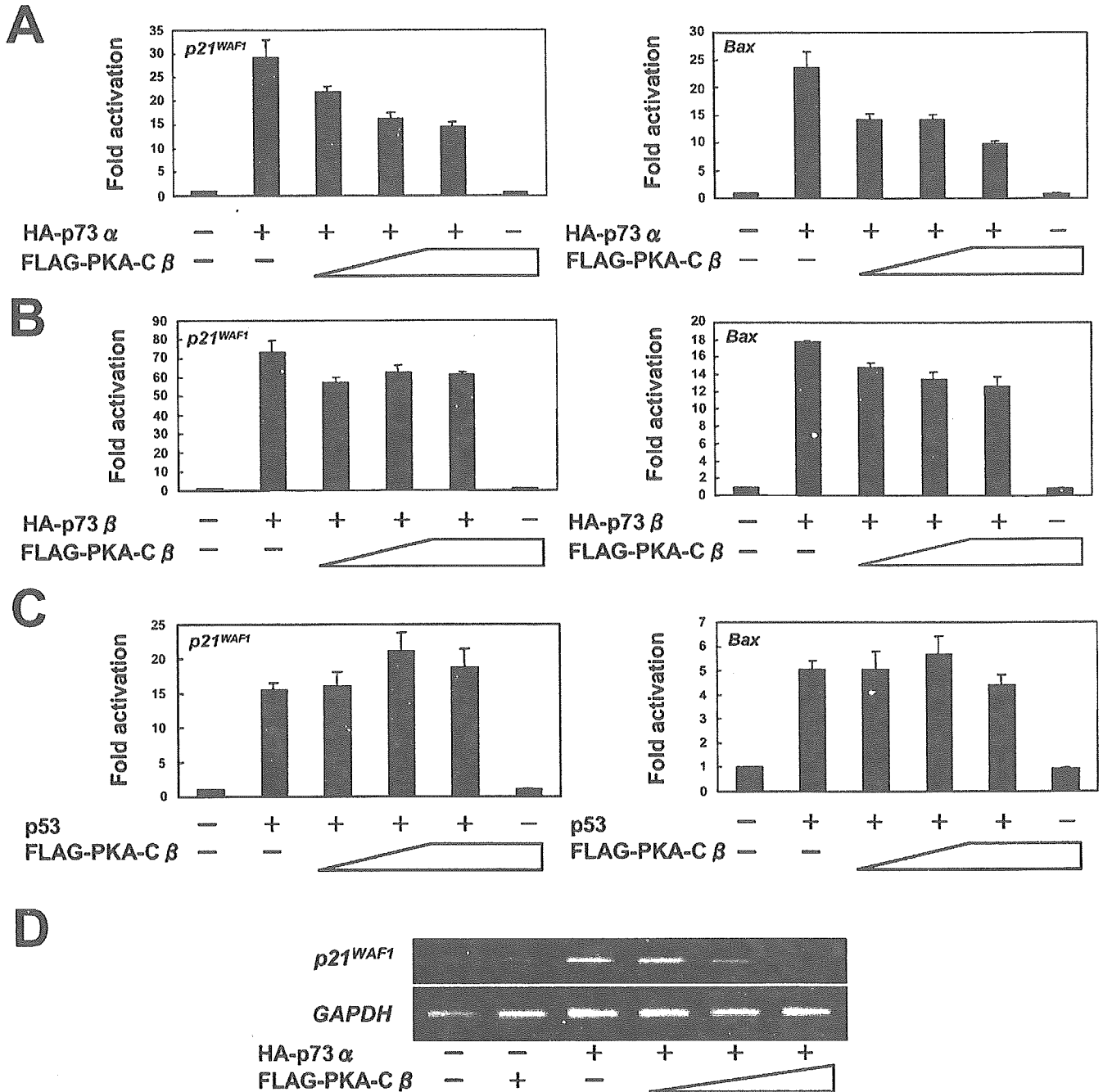


FIG. 3. PKA-C β inhibits p73 α -mediated transcriptional activation. A–C, luciferase reporter assays. H1299 cells (5×10^4 cells/12-well plates) were transiently cotransfected with 25 ng of the expression plasmid for HA-p73 α (A), HA-p73 β (B), or p53 (C); 100 ng of the luciferase reporter construct containing the p53/p73-responsive element derived from the p21^{WAF1} (left panels) or bax (right panels) promoter; and 10 ng of the *Renilla* luciferase plasmid (pRL-TK) with or without increasing amounts of the expression plasmid for FLAG-PKA-C β (25, 50, and 100 ng). The total amount of the plasmid DNA per transfection was kept constant (510 ng) with pcDNA3. All transfections were performed in triplicate. Luciferase activity was measured 48 h post-transfection. The transfection efficiency was standardized for *Renilla* luciferase activity. The -fold increase in luciferase activity is compared with that in cells transfected with pcDNA3 alone. D, reverse transcription-PCR analysis. Total RNA prepared from H1299 cells transiently cotransfected with a constant amount of the expression plasmid for HA-p73 α (200 ng) with or without increasing amounts of the expression plasmid for FLAG-PKA-C β (200, 400, and 800 ng) was subjected to reverse transcription-PCR analysis for endogenous p21^{WAF1} mRNA expression (upper panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were used as an internal control (lower panel).

body. As shown in Fig. 1B (upper panels), FLAG-p73 α co-immunoprecipitated with endogenous PKA-C β . Because the amino acid sequences of PKA-C α and PKA-C β are 91% identical (31), we examined whether endogenous PKA-C α could bind to p73 α . Co-immunoprecipitation experiments revealed that, like PKA-C β , endogenous PKA-C α associated with FLAG-p73 α (Fig. 1B, lower panels). In sharp contrast to p73 α , p53 failed to interact with FLAG-PKA-C β under our experimental conditions (Fig. 1C).

To investigate the subcellular distribution of PKA-C β in the presence of exogenous p73 α , we employed the biochemical fractionation of transfected H1299 cells. H1299 cells transiently cotransfected with the expression plasmids for HA-p73 α and FLAG-PKA-C β were fractionated into nuclear and cytoplasmic fractions, and the fractions obtained were subjected to immunoblotting with the indicated antibodies. The purity of the nuclear and cytoplasmic fractions was verified by immunoblotting with anti-lamin B and anti- α -tubulin antibodies, respec-

tively. As shown in Fig. 1D, HA-p73 α was detected exclusively in the nuclear fractions, whereas FLAG-PKA-C β and endogenous PKA-C β and PKA-C α were present in both the cytoplasmic and nuclear fractions. As expected, confocal microscopy of immunostained H1299 cells expressing FLAG-PKA-C β and HA-p73 α revealed that both proteins co-localized in the cell nucleus (Fig. 1E).

Identification of the Interacting Region within p73—To examine which region(s) of p73 could be engaged in the interaction with PKA-C β , we performed co-immunoprecipitation and GST pull-down experiments. Fig. 2A depicts the domain structures of various p73 variants used for co-immunoprecipitation experiments. Whole cell lysates prepared from H1299 cells transiently cotransfected with the indicated combinations of expression plasmids were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-p73 antibody. As shown in Fig. 2B, HA-p73 α and Δ Np73 α co-purified with FLAG-PKA-C β , whereas the binding of HA-p73 β to FLAG-PKA-C β was significantly weaker than seen with HA-p73 α and Δ Np73 α , suggesting that the C-terminal region of p73 α might be required for the interaction with PKA-C β . To verify these results, *in vitro* GST pull-down assays were carried out using a series of GST-p73 α fusion proteins. *In vitro* translated 35 S-labeled FLAG-PKA-C β was incubated with glutathione-Sepharose beads complexed either with GST alone or with GST-p73 α . The autoradiogram in Fig. 2C (upper panel) shows that GST-p73 α (1–130) and GST-p73 α (469–636) were able to interact with FLAG-PKA-C β . The Coomassie Brilliant Blue staining shown in Fig. 2C (lower panel) revealed that the glutathione-Sepharose beads contained equal amounts of GST-p73 α fusion proteins. Taken together, our results suggest that both the N-terminal (amino acids 63–130) and C-terminal (amino acids 469–636) regions of p73 α might be essential for the interaction with PKA-C β .

PKA-C β Inhibits p73 α -mediated Transcriptional Activation—In view of the ability of PKA-C β to interact with p73 α , we next examined whether PKA-C β could affect p73 α function as a transcriptional regulator. For this purpose, p53-deficient H1299 cells were transiently cotransfected with a constant amount of the expression plasmid for HA-p73 α , HA-p73 β , or p53 together with the luciferase reporter construct controlled by the p53/p73-responsive element from the p21^{WAF1} or *bax* promoter in the presence or absence of increasing amounts of the expression plasmid for FLAG-PKA-C β . All cotransfections included pRL-TK to monitor transfection efficiency, and controls included cotransfections with the empty control plasmid. As shown in Fig. 3A, coexpression of FLAG-PKA-C β and HA-p73 α resulted in marked repression of the p21^{WAF1} and *bax*-luciferase activities induced by HA-p73 α in a dose-dependent manner, and FLAG-PKA-C β alone had no effect on the reporter gene activity. In contrast, FLAG-PKA-C β had no obvious effects on p73 β - and p53-mediated transcriptional activation (Fig. 3, B and C). These results strongly suggest that there is a correlation between the capacity of PKA-C β to interact with p73 or p53 and its ability to inhibit their transactivation function. To determine whether PKA-C β could inhibit the p73 α -mediated transcriptional activation of endogenous p21^{WAF1}, we performed reverse transcription-PCR analysis using total RNA prepared from H1299 cells transiently cotransfected with the indicated combinations of expression plasmids. As shown in Fig. 3D, ectopic expression of HA-p73 α resulted in a remarkable up-regulation of endogenous p21^{WAF1} expression, and coexpression of FLAG-PKA-C β and HA-p73 α inhibited the p73 α -mediated induction of p21^{WAF1} in a dose-dependent manner.

To further confirm the inhibitory effect of PKA-C β on the transcriptional activity of p73 α , H1299 cells were transiently

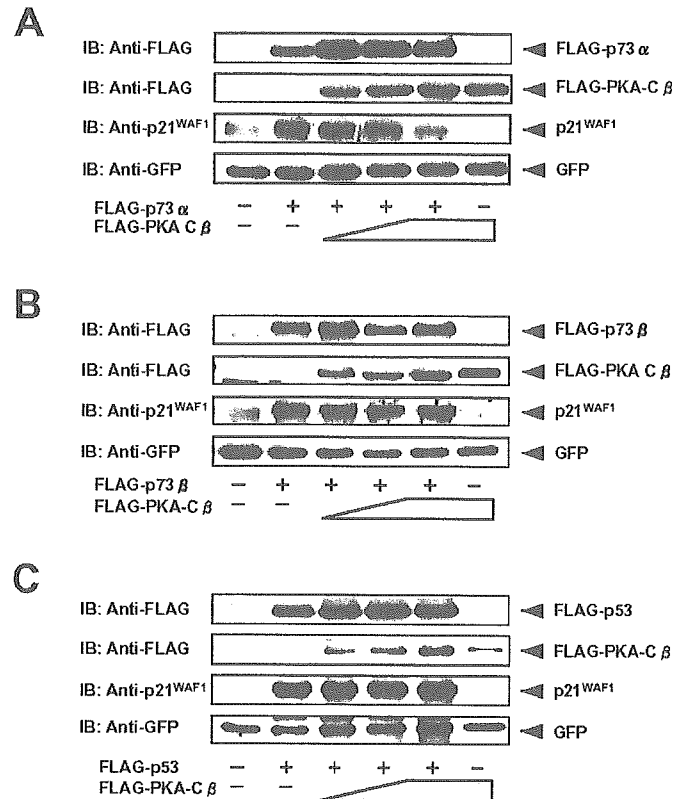
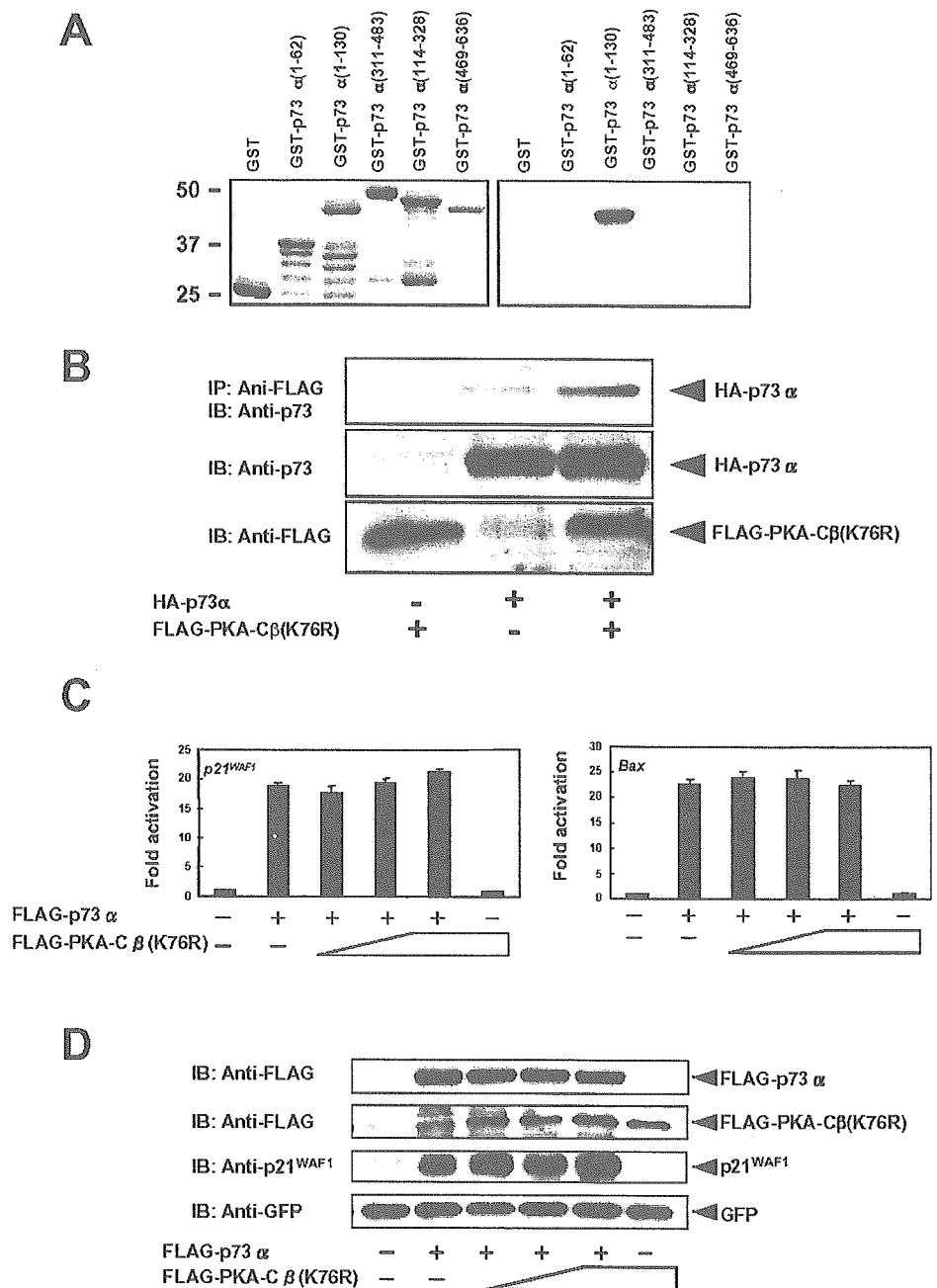


FIG. 4. PKA-C β inhibits the p73 α -dependent accumulation of endogenous p21^{WAF1}. H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for FLAG-p73 α (A), FLAG-p73 β (B), or FLAG-p53 (C) and 50 ng of the GFP expression plasmid with or without increasing amounts of the expression plasmid for FLAG-PKA-C β (200, 400, and 800 ng). Thirty-six hours after transfection, whole cell lysates were prepared and subjected to immunoblotting (IB) with the indicated antibodies (first through third panels). The GFP expression plasmid was included in each transfection as a transfection efficiency control, and the expression levels of GFP were detected with anti-GFP monoclonal antibody (fourth panels).

cotransfected with a constant amount of the expression plasmid for FLAG-p73 α , FLAG-p73 β , or FLAG-p53 with or without increasing amounts of the expression plasmid for FLAG-PKA-C β , and the protein levels of endogenous p21^{WAF1} were determined by immunoblotting. As shown in Fig. 4A, endogenous p21^{WAF1} was increased by ectopic FLAG-p73 α expression, whereas overexpression of FLAG-PKA-C β resulted in a reduction in the level of endogenous p21^{WAF1} induced by FLAG-p73 α , supporting the notion that PKA-C β inhibits the transcriptional activity of p73 α . In contrast, PKA-C β had no detectable effects on the p73 β - or p53-dependent induction of endogenous p21^{WAF1} (Fig. 4, B and C), consistent with the results obtained by luciferase reporter analysis. In addition, coexpression of FLAG-p73 α and FLAG-PKA-C β resulted in a slight increase in the amounts of FLAG-p73 α , whereas FLAG-PKA-C β had a negligible effect on the amounts of FLAG-p73 β and FLAG-p53 (Fig. 4). FLAG-p73 α decayed at slower rates in the presence of FLAG-PKA-C β than in its absence (data not shown); however, its physiological implications remain to be determined.

PKA-C β Phosphorylates p73—To determine whether p73 could be a substrate for PKA-C β , the GST-p73 α fusion proteins used for the *in vitro* pull-down assay were incubated with the commercially available PKA catalytic subunit purified from bovine heart and [γ -³²P]ATP. Of the GST-p73 α fusion proteins tested, only GST-p73 α (1–130) was phosphorylated by the PKA catalytic subunit (Fig. 5A). The N-terminal region of p73 α might be involved in phosphorylation by the PKA catalytic subunit.

FIG. 5. PKA-C β phosphorylates p73 α , and the kinase-deficient mutant of PKA-C β fails to inhibit the transcriptional activity of p73 α . **A**, PKA-C β can phosphorylate p73 α *in vitro*. GST or GST-p73 α fusion proteins bound to glutathione-Sepharose beads were incubated with the purified catalytic subunit of PKA in the presence of [γ - 32 P]ATP for 30 min at 30 °C. Samples were then directly boiled in 2 \times SDS sample buffer prior to loading them onto 10% SDS-polyacrylamide gels. Following electrophoresis, gels were dried and processed for autoradiography (*right panel*). GST and GST-p73 α fusion proteins were stained with Coomassie Brilliant Blue and used for *in vitro* kinase assay (*left panel*). The positions of molecular mass markers are shown on the left in kilodaltons. **B**, kinase-deficient PKA-C β retains the ability to interact with p73 α . Whole cell lysates prepared from COS-7 cells transiently cotransfected with the indicated combinations of expression plasmids were immunoprecipitated (IP) with anti-FLAG monoclonal antibody, and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-p73 monoclonal antibody (*upper panel*). Lysates not subjected to immunoprecipitation were analyzed by immunoblotting with anti-p73 (*middle panel*) or anti-FLAG (*lower panel*) monoclonal antibody. **C**, luciferase reporter analysis. H1299 cells were transiently cotransfected with a constant amount of the expression plasmid encoding FLAG-p73 α , the luciferase reporter construct carrying the p53/p73-responsive element derived from the p21^{WAF1} (*left panel*) or bax (*right panel*) promoter, and pRL-TK in the presence or absence of increasing amounts of the expression plasmid for FLAG-PKA-C β (K76R). Forty-eight hours after transfection, luciferase activity was determined as described in the legend to Fig. 4. **D**, PKA-C β (K76R) has no detectable effect on the p73 α -dependent induction of endogenous p21^{WAF1}. H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for FLAG-p73 α and 50 ng of the GFP expression plasmid with or without increasing amounts of the expression plasmid for FLAG-PKA-C β (K76R) (200, 400, and 800 ng). Thirty-six hours after transfection, whole cell lysates were prepared and analyzed by immunoblotting for the expression levels of endogenous p21^{WAF1}. The GFP expression plasmid was included as a control for transfection efficiency.



Next, we examined whether the inhibitory effect of PKA-C β on the transcriptional activity of p73 α is dependent on its kinase activity. As described previously (33, 34), PKA-C β (K76R), in which Lys-76 within the ATP-binding motif is replaced with Arg, showed very little catalytic activity. We therefore constructed an expression plasmid for FLAG-PKA-C β (K76R) and tested whether PKA-C β (K76R) could bind to p73 α and also repress p73 α -mediated transcriptional activation. Co-immunoprecipitation experiments demonstrated that, like wild-type PKA-C β , the kinase-deficient form of PKA-C β bound to FLAG-p73 α in cells (Fig. 5B). Notably, luciferase reporter analysis revealed that FLAG-PKA-C β (K76R) had little effect on the ability of p73 α to drive transcription from the p21^{WAF1} and bax promoters (Fig. 5C). In accordance with the results from luciferase reporter analysis, FLAG-PKA-C β (K76R) failed to reduce the expression levels of endogenous p21^{WAF1} induced by FLAG-p73 α as examined by immunoblotting (Fig. 5D). Taken together, these results strongly suggest that PKA-C β inhibits p73 α -mediated

transcriptional activation by a kinase activity-dependent mechanism.

Reduction in the Pro-apoptotic Activity of p73 α by PKA-C β upon DNA Damage—To extend the functional consequences of the interaction between p73 α and PKA-C β , we investigated whether PKA-C β could affect the pro-apoptotic function of p73 α in response to DNA damage. For this purpose, we used a low apoptotic dose of camptothecin to facilitate the detection of a potential induction mediated by p73 α . H1299 cells were transiently cotransfected with the expression plasmid for FLAG-p73 α or FLAG-p53 with or without the expression plasmid encoding FLAG-PKA-C β or FLAG-PKA-C β (K76R) and then treated with camptothecin at a final concentration of 1 μ M for 24 h. After camptothecin action, cell viability was examined by cell survival assay. As shown in Fig. 6A, H1299 cells expressing FLAG-p73 α alone exhibited an enhanced sensitivity to apoptosis following exposure to camptothecin, which was consistent with previous observations (35). Of note, coexpression of FLAG-PKA-C β and FLAG-p73 α resulted in a reduction in the cellular

sensitivity to camptothecin, whereas kinase-deficient PKA-C β had no significant effect on cell viability. As was also observed in H1299 cells expressing FLAG-p73 α , ectopic expression of FLAG-p53 enhanced camptothecin-induced apoptosis (Fig. 6B). In sharp contrast to p73 α , wild-type or kinase-deficient PKA-C β had a negligible effect on p53.

cAMP Analog Inhibits p73 α -mediated Transcriptional Activation—Given the inhibitory effect of exogenous PKA-C β on p73 α in transfected cells, we sought to determine whether the activation of PKA attenuates p73 α -mediated transcriptional activation. H1299 cells were transiently cotransfected with or without the expression plasmid for HA-p73 α along with the luciferase reporter construct driven by the p53/p73-responsive element from the p21^{WAF1} or *bax* promoter. Twenty-four hours after transfection, cells were either left untreated or treated with the PKA-activating agent dibutyryl cAMP (Bt₂cAMP) in the presence or absence of the PKA inhibitor H-89. As shown in Fig. 7A, Bt₂cAMP treatment inhibited p73 α -induced p21^{WAF1} and *bax* promoter activation. Intriguingly, the inhibitory effect of Bt₂cAMP was attenuated when cells were exposed to H-89. Under the identical experimental conditions, endogenous p21^{WAF1} was significantly induced by exogenously expressed HA-p73 α (Fig. 7B). Densitometric scanning of the immunoblot revealed that Bt₂cAMP treatment decreased the level of p21^{WAF1} by 29% relative to that induced by HA-p73 α , and the p21^{WAF1} level was partially restored in the presence of H-89, in accordance with the results obtained by luciferase reporter analysis. Thus, it is likely that the elevation of intracellular cAMP and the subsequent PKA activation contribute to the reduction in p73 α -mediated transcriptional activation.

PKA-C β Stimulates the Intramolecular Interaction of p73—To clarify the precise molecular mechanism by which PKA-C β impairs the transcriptional activity of p73 α , we performed ChIP analysis. Cross-linked chromatin prepared from H1299 cells transiently cotransfected with the indicated combinations of expression plasmids was immunoprecipitated with anti-HA antibody, followed by amplification with the indicated promoter-specific primers. Under our experimental conditions, HA-p73 α was efficiently recruited to the p21^{WAF1} and *bax* promoters in the absence of exogenous PKA-C β (Fig. 8A). No significant decrease in chromatin binding was detected in cells expressing HA-p73 α and FLAG-PKA-C β , suggesting that PKA-C β has little effect on the sequence-specific DNA binding activity of p73 α .

It has been reported recently that the extreme C-terminal regions of p73 α and p63 α (another member of the p53 family) have an inhibitory effect on their transactivation potential (7, 36, 37). To assess whether the C-terminal inhibitory domain of p73 α could be involved in the PKA-C β -mediated down-regulation of p73 α , we performed additional luciferase reporter analyses in H1299 cells cotransfected with the expression plasmid for HA-p73 α (1–548) and FLAG-PKA-C β . As shown in Fig. 8B (upper panel), HA-p73 α (1–548), which lacks the extreme C-terminal extension of wild-type p73 α , interacted with FLAG-PKA-C β as determined by co-immunoprecipitation experiments. It is worth noting that, in contrast to wild-type p73 α , FLAG-PKA-C β had no detectable effect on the transcriptional activity of HA-p73 α (1–548) (Fig. 8B, lower panel), indicating that the extreme C-terminal region of p73 α plays a critical role in the PKA-C β -mediated inhibition of p73 α .

Serber *et al.* (37) reported that the extreme C-terminal domain binds to the N-terminal transactivation domain of p63 and inhibits its transactivation potential. Considering that PKA-C β interacts with p73 α through its N- and C-terminal domains, it is possible that PKA-C β could stimulate the intramolecular interaction between the two domains of p73 α , thereby inhibiting its transcriptional activity. To test this pos-

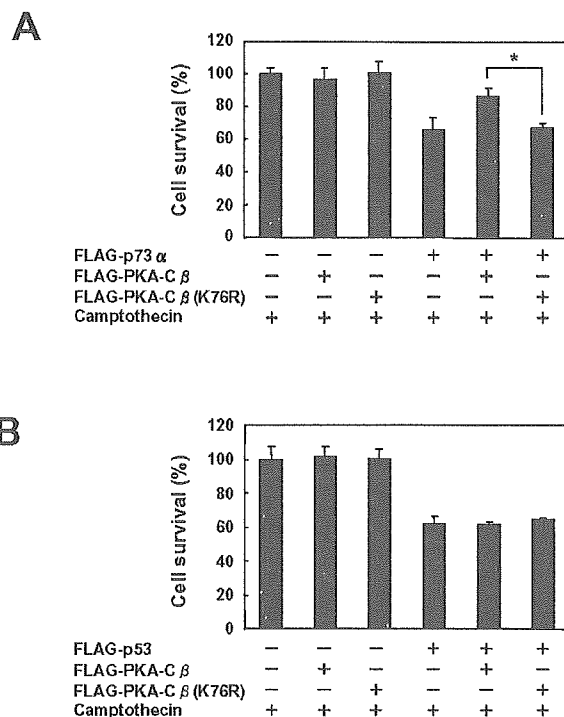


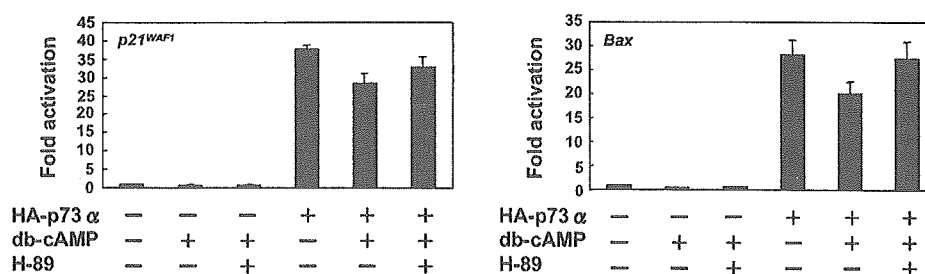
FIG. 6. p73 α -mediated increase in sensitivity to camptothecin is suppressed by wild-type PKA-C β , but not by kinase-deficient PKA-C β . H1299 cells were transiently cotransfected with the expression plasmid for FLAG-p73 α (A) or FLAG-p53 (B) with or without the expression plasmid encoding FLAG-PKA-C β or FLAG-PKA-C β (K76R). Twenty-four hours after transfection, cells were exposed to camptothecin (final concentration of 1 μ M) for 24 h, and their viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *, $p < 0.01$ versus +FLAG-PKA-C β .

sibility, we performed co-immunoprecipitation analysis. Whole cell lysates prepared from COS-7 cells transiently transfected with the indicated combinations of expression plasmids were immunoprecipitated with anti-p73 antibody, followed by immunoblotting with anti-HA antibody, and the possible effect of FLAG-PKA-C β on the complex formation between HA-p73 α (1–247) and FLAG-p73 α (247–636) was examined. The anti-p73 antibody used for this assay recognizes the C-terminal portion of p73 and thus does not detect p73 α (1–247). As shown in Fig. 8C, HA-p73 α (1–247) efficiently co-immunoprecipitated with FLAG-p73 α (247–636) in the presence of FLAG-PKA-C β , whereas FLAG-PKA-C β (K76R) had a negligible effect on the complex formation between HA-p73 α (1–247) and FLAG-p73 α (247–636). The few complexes observed in the absence of FLAG-PKA-C β could be due to endogenous PKA-C β . These results strongly suggest that FLAG-PKA-C β contributes to the intramolecular interaction of p73 α between the N-terminal transactivation and C-terminal inhibitory domains.

DISCUSSION

In this study, we have screened a human fetal brain cDNA library using a new CytoTrap yeast two-hybrid screening method based on the Sos recruitment system and identified, for the first time, PKA-C β as a p73 α -binding protein. PKA-C β associated with p73 α through its N- and C-terminal regions in mammalian cultured cells and significantly inhibited its transactivation function. Under our experimental conditions, PKA-C β , which did not bind to p53, had a negligible effect on p53. Intriguingly, PKA-C β might bridge the N-terminal transactivation and C-terminal inhibitory domains of p73 α , thereby rendering p73 α a latent inactive form. *In vitro* kinase assay demonstrated that PKA can phosphorylate p73, and the kinase-deficient mutant of PKA-C β (PKA-C β (K76R)) failed to

A



B

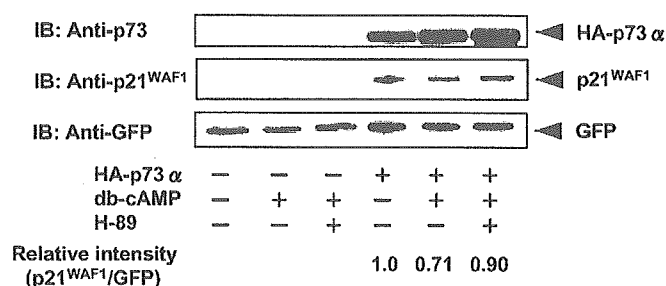


FIG. 7. Effects of Bt₂cAMP on p73 α -mediated transcriptional activation. A, luciferase reporter analysis. H1299 cells were transiently cotransfected with 25 ng of the expression plasmid for HA-p73 α , 100 ng of the luciferase reporter construct containing the p53/p73-responsive element derived from the p21^{WAF1} (left panel) or bax (right panel) promoter, and 10 ng of pRL-TK. Twenty-four hours after transfection, cells were left untreated or were treated with Bt₂cAMP (db-cAMP; 1 mM) or with Bt₂cAMP (1 mM) plus the PKA inhibitor H-89 (10 μ M) for 24 h. Cell lysates were then prepared and subjected to the determination of luciferase activity as described in the legend to Fig. 4. B, immunoblot analysis for p21^{WAF1}. H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for HA-p73 α and 50 ng of the GFP expression plasmid. Twenty-four hours after transfection, cells were treated with or without Bt₂cAMP (1 mM) in the presence or absence of H-89 (10 μ M) for 24 h. Whole cell lysates were then prepared and subjected to immunoblotting (IB) with the indicated antibodies. Densitometry was used to quantify the amounts of p21^{WAF1}, which were normalized to GFP.

reduce the transcriptional activity of p73 α , suggesting that the kinase activity of PKA-C β is required for its inhibitory effect on p73 α . In accordance with these results, the transient activation of the cAMP/PKA signaling pathway by Bt₂cAMP reduced p73 α -mediated transcriptional activation, whereas the inhibitory effect of Bt₂cAMP was attenuated when cells were exposed to H-89, a specific pharmacological inhibitor of PKA. Collectively, our present findings indicate that the PKA-mediated phosphorylation and conformational alteration of p73 α might be a novel inhibitory mechanism of its activity.

As described previously (38), the PKA catalytic subunit family is composed of three isoforms: PKA-C α , PKA-C β , and PKA-C γ . PKA-C α is expressed ubiquitously, whereas PKA-C β is expressed predominantly in brain and reproductive tissues (31, 32). PKA-C β is expressed as at least six variants (C β 1, C β 2, C β 3, C β 4, C β 4ab, and C β 4abc) arising from alternative splicing of the primary transcript (39). These splice variants contain a unique N terminus, but share a common catalytic domain, suggesting that they have similar enzymatic activity. Sequence analysis revealed that the PKA-C β we identified in this study is PKA-C β 4ab. According to our *in vitro* phosphorylation assay using various truncated forms of GST-p73 α as substrates, the N-terminal region of p73 α (residues 1–130) might contribute to phosphorylation by PKA. As described previously (40, 41), the amino acid sequence (R/K)XX(S/T) is a consensus motif for PKA-dependent phosphorylation. Examination of the amino acid sequence of p73 α for a putative PKA recognition site(s) showed three related motifs (⁷⁸RAAS⁸¹, ¹⁶⁴KVST¹⁶⁷, and ⁴⁰²KLPS⁴⁰⁵). Ser-81 exists in the N-terminal region of p73 α . It

is thus likely that this site could be one of the site(s) phosphorylated by PKA, although there is no direct evidence for this possibility. Because PKA-C β (K76R), which retained the ability to bind to p73 α , failed to inhibit p73 α -mediated transcriptional activation, it is conceivable that the PKA-dependent phosphorylation of p73 α might serve to modulate its function. Accumulating evidence suggests that, as for p53, post-translational modifications such as phosphorylation and acetylation regulate p73. In response to DNA-damaging agents, p73 is phosphorylated at Tyr-99, Ser-289, and Ser-47 by c-Abl, the protein kinase C δ catalytic fragment, and Chk1, respectively (16–19, 25). Each of these phosphorylations is associated with the activation of p73. Alternatively, Pin1 recognizes phosphorylated Ser-412, Thr-442, and/or Thr-482 of p73, thereby activating p73 in association with the enhanced levels of its acetylation mediated by p300 (28). On the other hand, cyclin-dependent protein kinase-dependent phosphorylation of p73 at Thr-86 results in a significant reduction of the transcriptional activity of p73 (42). Accordingly, the identification of the precise phosphorylation site(s) of p73 α by PKA is necessary to confirm the functional significance of the PKA-mediated phosphorylation of p73 α .

We (36) and others (7, 43) reported that p73 α exhibits a low level of transactivation ability relative to that of p73 β , suggesting that the C-terminal extension of p73 α exerts an inhibitory effect on the transcriptional activity of p73. Another p53 family member (p63) also showed similar results (44). Intriguingly, three-dimensional analysis demonstrated that the C-terminal region of p53 exists in close proximity to the central DNA-binding domain (45). In addition, it has been shown that the C

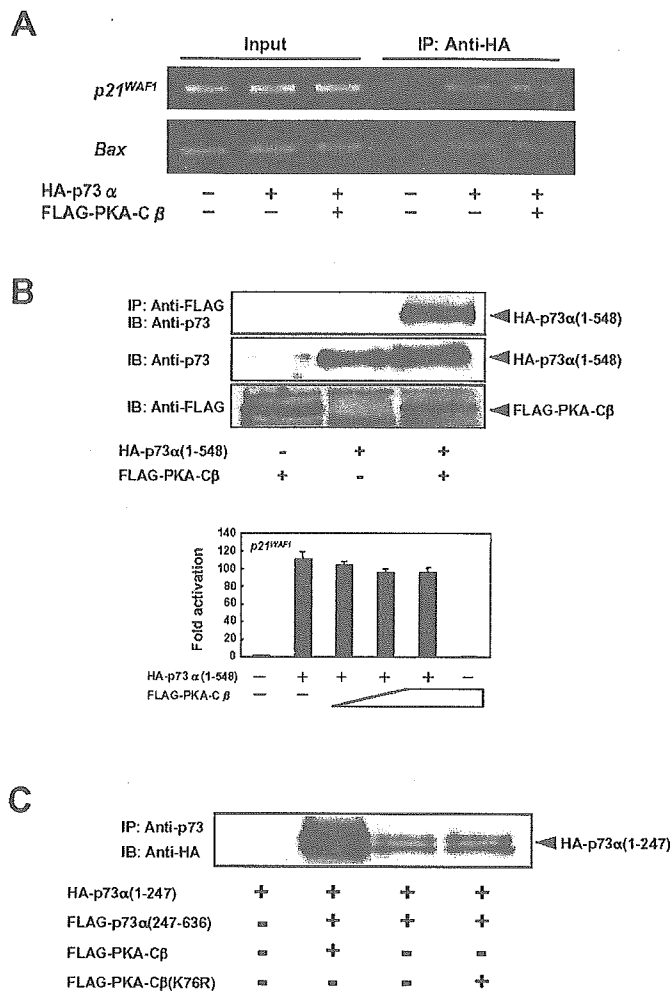


Fig. 8. PKA-C β -mediated intramolecular interaction of p73 α contributes to the down-regulation of the transcriptional activity of p73 α . **A**, ChIP assay. H1299 cells were transiently cotransfected with the expression plasmid for HA-p73 α or HA-p73 α plus FLAG-PKA-C β . Thirty-six hours after transfection, cells were fixed in formaldehyde and lysed, and DNA was sheared into 200–500-bp fragments by sonication. HA-p73 α -bound DNA was immunoprecipitated (IP) with anti-HA monoclonal antibody. The amounts of HA-p73 α bound to the p53/p73-responsive element within the p21^{WAF1} (upper panel) or bax (lower panel) promoter region were analyzed by standard PCR. **B**, PKA-C β fails to inhibit p73 α (1–548). COS-7 cells were transiently cotransfected with the indicated combinations of expression plasmids. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to co-immunoprecipitation (first panel) or immunoblotting (second and third panels) (upper panels). H1299 cells were transiently cotransfected with 25 ng of the expression plasmid for HA-p73 α (1–548), 100 ng of the luciferase reporter construct carrying the p53/p73-responsive element of the p21^{WAF1} promoter, and 10 ng of pRL-TK with or without increasing amounts of the expression plasmid for FLAG-PKA-C β (25, 50, and 100 ng). Forty-eight hours after transfection, luciferase activity was determined as described in the legend to Fig. 4 (lower panel). **C**, the intramolecular interaction of p73 α is stimulated by PKA-C β . Whole cell lysates prepared from COS-7 cells transfected with the indicated combinations of expression plasmids were immunoprecipitated with anti-p73 antibody, followed by immunoblotting (IB) with anti-HA antibody.

terminus of p53 directly interacts with and masks its DNA-binding domain, thereby inhibiting its DNA binding activity (46, 47). Recently, Serber *et al.* (37) found that the transcriptional activity of p63 α is significantly inhibited by an intramolecular interaction. In sharp contrast to p53, the extreme C-terminal region of p63 α binds to the N-terminal transactivation domain, but not to the DNA-binding domain, and abrogates its transactivation potential. Given the high amino acid sequence homology between p73 α and p63 α and their similar domain structure, the transcriptional activity of

p73 might be regulated at least in part by an intramolecular inhibitory interaction. According to our *in vitro* pull-down assay, PKA-C β bound to the N- and C-terminal regions of p73 α . Furthermore, the co-immunoprecipitation experiments demonstrated that p73 α (1–247) efficiently coprecipitated with p73 α (247–636) in the presence of PKA-C β , suggesting that PKA-C β might promote the intramolecular interaction of p73 α to mask the N-terminal transactivation domain rather than the central DNA-binding domain and keep it in an inactive form. Indeed, our ChIP experiments revealed that PKA-C β had no significant effect on the DNA binding activity of p73 α . Because the kinase-deficient mutant of PKA-C β failed to bridge p73 α (1–247) and p73 α (247–636), it is likely that the PKA-mediated phosphorylation of p73 plays an important role in the conformational alteration of p73. However, the precise molecular mechanism by which PKA-mediated phosphorylation could contribute to the inhibition of p73 is currently unknown.

It has been shown previously (48–50) that the activation of PKA has either mitogenic or anti-proliferative effects in mammalian cultured cells and that these opposite responses might be due to the existence of cell type-specific targets of this signaling pathway. Accumulating evidence indicates that the anti-apoptotic effect of PKA might be mediated by the activation of the ERK (extracellular signal-regulated kinase) (51, 52) and phosphatidylinositol 3-kinase/Akt (53, 54) pathways. Recently, Wu *et al.* (55) found that c-Myc enhances the activity of PKA by transactivating the expression of PKA-C β . According to their results, constitutive expression of PKA-C β results in the promotion of colony formation in soft agar medium, and PKA-C β as well as c-Myc-mediated cellular transformation is markedly inhibited by H-89, suggesting that PKA might be one of the downstream mediators of c-Myc function. As described previously (55–57), PKA directly phosphorylates Bad and glycogen synthase kinase-3 β to inhibit their apoptosis-inducing activity. Likewise, our present findings indicate that the PKA-mediated phosphorylation of pro-apoptotic p73 abrogates its function. Thus, it is likely that the anti-apoptotic function of PKA is at least in part due to the inactivation of p73 and the subsequent suppression of apoptotic signaling.

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Prediction of *MYCN* Amplification in Neuroblastoma Using Serum DNA and Real-Time Quantitative Polymerase Chain Reaction

Takahiro Gotoh, Hajime Hosoi, Tomoko Iehara, Yasumichi Kuwahara, Shinya Osone, Kunihiro Tsuchiya, Miki Ohira, Akira Nakagawara, Hiroshi Kuroda, and Tohru Sugimoto

From the Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto; and Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba, Japan.

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Address reprint requests to Takahiro Gotoh, MD, PhD, Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kamigyo-ku, Kyoto 602-8566, Japan; e-mail: takahiro_email@yahoo.co.jp.

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ABSTRACT

Purpose

MYCN amplification (MNA) indicates a poor prognosis in neuroblastoma (NB) and is routinely assayed for therapy stratification. We aimed to develop a diagnostic tool to predict *MYCN* status using serum DNA, which, in cancer patients, predominantly originates from tumor-released DNA.

Patients and Methods

Using DNA-based real-time quantitative polymerase chain reaction, we simultaneously quantified *MYCN* (2p24) and a reference gene, *NAGK* (2p12), and evaluated *MYCN* copy number as an *MYCN/NAGK* (M/N) ratio in 87 NB patients whose *MYCN* status had been determined by Southern blotting. Of these patients, 17 had *MYCN*-amplified NB, and 70 had nonamplified NB.

Results

The serum M/N ratio in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test). The sensitivity and specificity of the serum M/N ratio as a diagnostic test were both 100% when the serum M/N ratio cutoff was set at 10.0. Among six MNA patients whose clinical courses were followed, the serum ratios decreased to the normal range in the patients in remission ($n = 3$), whereas the ratios increased to high levels in the patients who relapsed ($n = 2$) or failed to achieve remission ($n = 1$).

Conclusion

Measurement of the serum M/N ratio seems to be a promising method for accurately assessing *MYCN* status in NB, although a larger set of patients needs to be examined to confirm this result.

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INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome. The clinical heterogeneity has been reported to be associated with a variety of biologic features of NB. One such aberration, *MYCN* amplification (MNA; ie, creation of multiple

copies of the *MYCN* gene in the nuclei of tumor cells), is strongly associated with rapid tumor progression and a poor outcome. MNA is detected in 4% of patients in the early stages of NB, 8% of patients in stage 4S, and approximately 30% of patients in advanced stages. Currently, assessment of *MYCN* status is essential for determining therapy stratification in NB.¹⁻⁶ Having rapid access to selected biologic data for each tumor has become increasingly important in

routing patients to appropriate therapies. Several years ago, fluorescence in situ hybridization (FISH) replaced Southern blotting as the most accurate and timely way of evaluating tumors for MNA. Using FISH, the turnaround time for results was shortened from weeks to days, making its use in clinical trials realistic.

In this study, we describe a real-time polymerase chain reaction (PCR) method for evaluating *MYCN* status that shortens the turnaround for results to just a few hours. Furthermore, to facilitate the evaluation of *MYCN* status of tumors, we used serum DNA for the PCR template, which, in cancer patients, predominantly consists of tumor-released DNA.⁷ Quantification of serum DNA has also been proposed as a screening tool for early detection of lung cancer.⁸ Several groups were able to detect tumor-related aberrations, such as loss of heterozygosity and mutations in the *p53* gene, using the serum DNA of patients with a malignant tumor.⁹⁻¹¹

Recently, Combaret et al¹² reported that high levels of *MYCN* DNA were present in the peripheral blood of patients with *MYCN*-amplified NB. However, they evaluated serum *MYCN* (2p24) dosage based on PCR without a reference gene, so their assay could be influenced by the quality of the template DNA or a numerical change of chromosome 2. To avoid these problems, we used DNA-based real-time quantitative PCR and a single copy reference gene, the *N-acetylglucosamine kinase* gene (*NAGK*; 2p12), so that *MYCN* copy number per chromosome 2 could be evaluated as the *MYCN*/*NAGK* (*M/N*) ratio. *NAGK* was chosen because it is on the same chromosome as *MYCN* but sufficiently distant from the region spanned by the *MYCN* amplicon (2p12 v 2p24)¹³ that a numerical change in chromosome 2 would not affect the *M/N* ratio. The diagnostic performance of the test was evaluated in patients with an NB whose *MYCN* status had been determined by Southern blotting.

PATIENTS AND METHODS

Subjects

Eighty-seven patients diagnosed with NB at the Hospital of the Kyoto Prefectural University of Medicine and Chiba Cancer Center Research Institute were enrolled onto this study with the informed consent of their parents. The studies were conducted under research protocols approved by each institutional review board. At the time of diagnosis, 44 patients were younger than 1 year, and 43 were between 1 and 13 years of age. Seventeen of the patients had MNA, and 70 patients did not have MNA, as determined by Southern blotting. According to the International Neuroblastoma Staging System,⁴ the 17 children with MNA included one patient each in stage 1 and 2B, two in stage 3, and 13 in stage 4, whereas the 70 children without MNA included 22 in stage 1, 18 in stage 2A and 2B, five in stage 4S, seven in stage 3, and 18 in stage 4.

Twelve of the 17 patients with MNA and 33 of the 70 nonamplified patients were also analyzed by dual-color FISH technique,

as previously described,¹⁴ using an *MYCN* probe (pNb101) and a chromosome 2 centromere probe (D2Z). FISH results of these patients were consistent with the Southern blotting results, although three of the patients who were diagnosed as non-MNA by Southern blotting were found to have one to four extra copies of the *MYCN* gene relative to the chromosome 2 centromere number by FISH. This low level of amplification has been defined as *MYCN* gain, which is an intermediate stage between MNA and non-MNA.¹⁵ Because the prognostic significance of *MYCN* gain is still unclear, these patients were classified as non-MNA according to the Southern blotting results.

Sample Preparation

Tumor specimens were surgically resected and immediately stored at -80°C . Peripheral blood was obtained from each patient before any therapy and surgery. To avoid contamination of serum DNA by the DNA from WBCs, we prepared serum exclusively from the liquid fraction of clotted blood after centrifugation at $1,000 \times g$ for 10 minutes and stored it at -20°C until DNA extraction.

DNA Isolation

DNA was extracted from tissues and serum samples by using the QIAmp tissue and blood kits (Qiagen, GmbH, Hilden, Germany), respectively, according to the manufacturer's protocols. For each patient, 200 μL of the stored serum was used for extraction of free DNA.

Real-Time Quantitative PCR

TaqMan PCR was performed using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR mixture contained TaqMan universal PCR master mix (Applied Biosystems), 200 nmol/L of each primer, and 100 nmol/L of fluorogenic probe. The principle of the TaqMan analysis has been described previously in detail.¹⁶⁻¹⁸ In addition to the *MYCN* sequence, *NAGK* (GenBank accession No. NM 017567) located at 2p12 was simultaneously measured as a single-copy reference gene. The sequence of primers and the TaqMan probe used for *MYCN* and *NAGK* are as follows: *MYCN* forward, 5'-GTGCTCTCCAATTCTCGCCT-3'; *MYCN* reverse, 5'-GATGGCCTAGAGGAGGGCT-3'; *MYCN* probe, 5'-FAM-CACATAAAGTTCCTCCACCCTCTCCT-TAMRA-3'; *NAGK* forward, 5'-TGGGCAGACACATCGTAGCA-3'; *NAGK* reverse, 5'-CACCTTCACTCCCACCTCAAC-3'; and *NAGK* probe, 5'-VIC-TGTTGCCCGAGATTGACCCGGT-TAMRA-3'. All PCR reactions were performed with one cycle of 95°C for 5 minutes, followed by PCR amplification with 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were constructed in each PCR run with four-fold serial dilutions containing 20, 5, 1.25, 0.3125, and 0.078125 ng/ μL of a healthy donor's DNA in addition to 20 ng/ μL of salmon sperm DNA, and the dosages of the target genes in each sample were interpolated using these standard curves. The *MYCN* copy number of a sample of DNA was determined by the ratio of the *MYCN* dosage to the *NAGK* dosage (*M/N* ratio). Copy numbers were expressed as the average of two measurements.

Effect of WBC Contamination

To assess the effect of WBC contamination in serum samples on the serum *M/N* ratio, we measured the serum *M/N* ratio using DNA extracted from a series of WBC-contaminated serum samples. The samples were prepared by adding 0, 1×10 , 1×10^2 , 1×10^3 , 1×10^4 , and 1×10^5 of WBCs from a healthy donor to 200 μL of serum from a *MYCN*-amplified patient.

Statistical Methods

The difference in the serum *M/N* ratio between the MNA and non-MNA groups was assessed using the Mann-Whitney *U* test. $P < .05$ was judged as significant.

RESULTS

Serum *M/N* Ratio As a Predictor of MYCN Status of Tumor

Serum *M/N* ratios could be determined in approximately 4 hours by real-time quantitative PCR. Figure 1 shows the distribution of the serum *M/N* ratio in the MNA and non-MNA groups at the time of diagnosis. The serum *M/N* ratio in the MNA group ($n = 17$; median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group ($n = 70$; median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26). In fact, there was no overlap between the two groups in the limited number of patients examined in this study. As a cutoff for the serum *M/N* ratio to distinguish

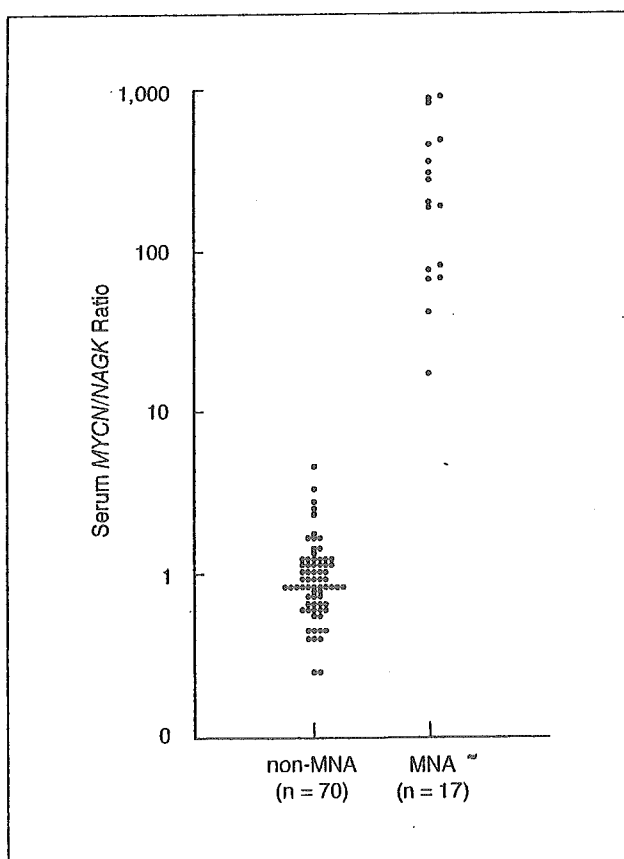


Fig 1. A scatter plot of serum *MYCN/NAGK* ratio in patients with *MYCN*-amplified (MNA) and nonamplified (non-MNA) neuroblastoma. The serum *MYCN/NAGK* ratio was significantly ($P < .001$) higher in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) than in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test).

between MNA and non-MNA patients, we empirically chose a value of 10, which was in the middle of the two ranges. With this value, the sensitivity and specificity of the serum *M/N* ratio as a diagnostic test to distinguish patients with MNA from those without MNA were both 100% for our limited number of patients. That is, the serum *M/N* ratio was in complete agreement with the Southern blotting results. The positive and negative predictive values were 100%. The serum *M/N* ratios were also consistent with results obtained by FISH for 45 of the patients (FISH analyses were performed in 12 of the 17 MNA patients and in 33 of the 70 nonamplified patients). Three of the patients who had one to four extra copies of the *MYCN* gene relative to chromosome 2 centromere number, as determined by FISH, also had slightly elevated serum *M/N* ratios (2.5, 3.3, and 4.6).

Change in Serum *M/N* Ratio Levels During Follow-Up

To evaluate whether an increase in the serum *M/N* ratio can be used as an indicator of relapse, we measured serum *M/N* ratios at several points in the clinical courses of six patients with MNA (Fig 2). In three patients who were in complete remission (patients 1, 2, and 3), the serum *M/N* ratios decreased to the normal range and were consistently low. In contrast, in one patient who failed to achieve remission (patient 4), the serum *M/N* ratio did not decrease to the normal range and remained at a high level until his death. In the other patients who experienced recurrence after remission (patients 5 and 6), the serum *M/N* ratio first decreased to the normal range and then increased beyond the cutoff value by the time of diagnosis.

Effect of WBC Contamination on Serum *M/N* Ratio

We found that a high serum *M/N* ratio could be masked by the presence of WBC. The *M/N* ratio of serum from an *MYCN*-amplified patient decreased with increasing WBC contamination (Fig 3). When 200 μ L of serum was contaminated with 1×10^5 of WBC, corresponding to approximately one fortieth of the WBC concentration in normal whole blood, the serum *M/N* ratio decreased below the cutoff level.

DISCUSSION

Serum markers, such as ferritin,¹⁹ lactic dehydrogenase,²⁰ and neuron-specific enolase,²¹ have been proposed as prognostic markers of NB, although they have shown little prognostic value. Recently, elevated levels of plasma midkine have been reported to correlate with a poor prognosis. However, the significance of this finding is controversial because plasma midkine levels are highest in stage 4S patients.²² Therefore, a noninvasive assay of tumor-related

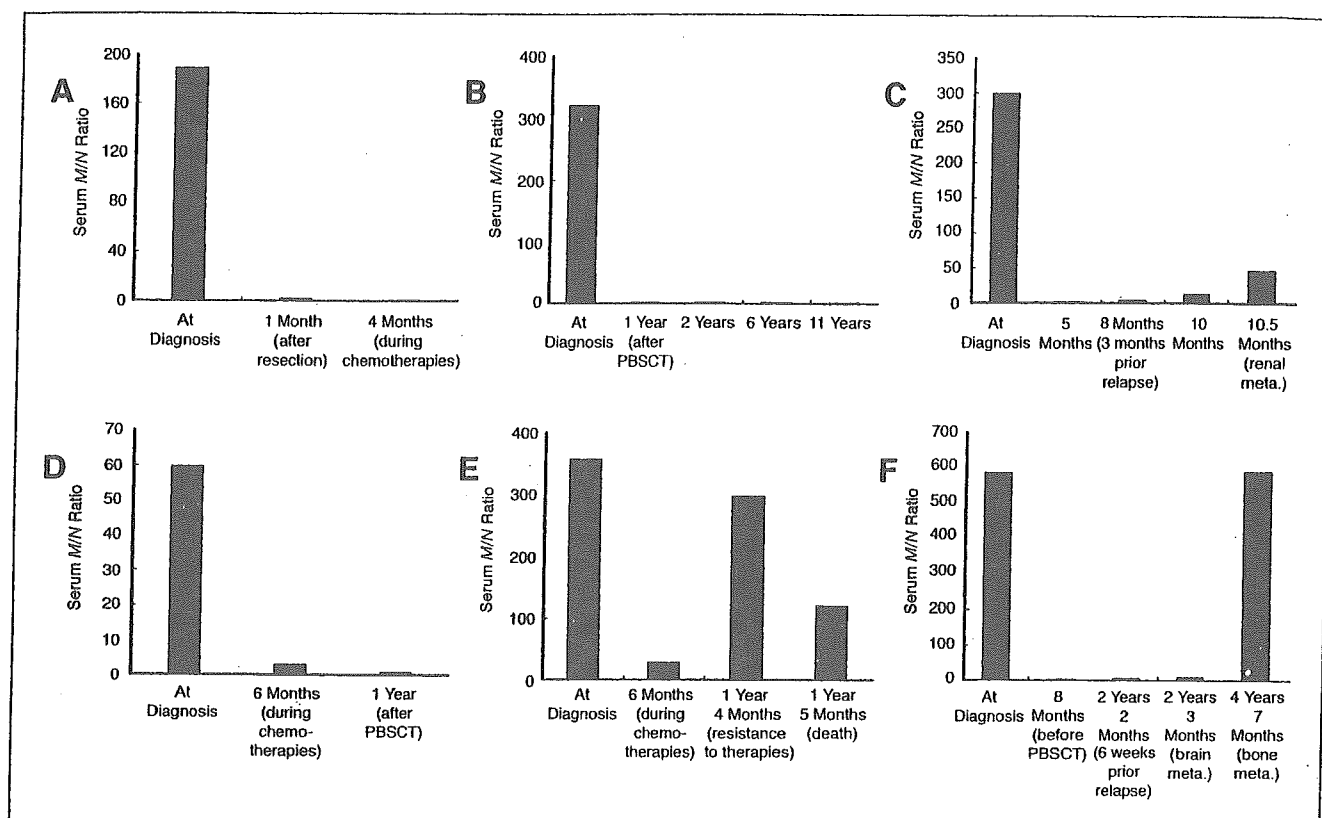


Fig 2. Changes in serum MYCN/NAGK (M/N) ratio levels of six patients with MYCN amplification during follow-up. PBST, peripheral-blood stem-cell transfusion; meta., metastasis. (A) Patient 1; (B) patient 3; (C) patient 5; (D) patient 2; (E) patient 4; (F) patient 6.

genetic aberrations using serum DNA is desirable for the assessment of prognosis and therapy stratification at the time of diagnosis. Among the tumor-related genetic aberrations detected in NB, MNA was of greatest interest to us because of its significant prognostic value.

By using DNA-based real-time quantitative PCR with a single-copy reference gene, we have demonstrated that the M/N ratio in serum DNA is a valuable diagnostic tool to discriminate MNA patients from non-MNA patients. The serum M/N ratio in the MNA group was significantly higher than the ratio in the non-MNA group, without an overlap. The highest sensitivity (100%), highest specificity (100%), highest positive predictive value (100%), and highest negative predictive value (100%) were obtained with a serum M/N ratio cutoff value of 10.0. Furthermore, we found an elevated level of the serum M/N ratio in a stage 1 patient and a stage 2B patient with MNA (188.7 and 901.6, respectively), even though the tumor was localized in these patients. This suggests that tumors could release a significant amount of genomic DNA into the systemic circulation even at an early stage. Furthermore, Sozzi et al²³ reported that the concentration of plasma DNA in 84 lung cancer patients was higher than the concentration in 43 controls, regardless of the tumor stage, and suggested that circulating DNA in peripheral blood was an early event in lung carcinogenesis.

Another clinical benefit of the serum M/N assay is that it could be used as a marker to monitor therapeutic efficacy and recurrence after therapies. The serum M/N ratio decreased to the normal range in the patients in remission but remained at a high level in the patient who failed to achieve remission. Furthermore, in two patients with recurrence after remission, the serum M/N ratio initially decreased to the normal range but then increased beyond the cutoff value by the time of diagnosis. The serum M/N ratio did not increase to the initial level as long as the metastasis was localized in the brain, but it did increase to the initial level when the patient later developed a bone metastasis (patient 6). This is noteworthy because it suggests that a brain metastasis releases genomic DNA into the systemic circulation less easily than extracranial tumors. If this is confirmed by examination of additional patients, then it is possible that tumors localized in brain could be overlooked with diagnostic assays based on serum DNA.

A possible pitfall of our serum M/N assay is that a high serum M/N ratio could be reduced by WBC contamination (Fig 3). This could be a result of dilution of tumor DNA with the WBC DNA, which would be expected to have an M/N ratio of 1. Therefore, the importance of removing WBCs from serum should be addressed in diagnostic assays

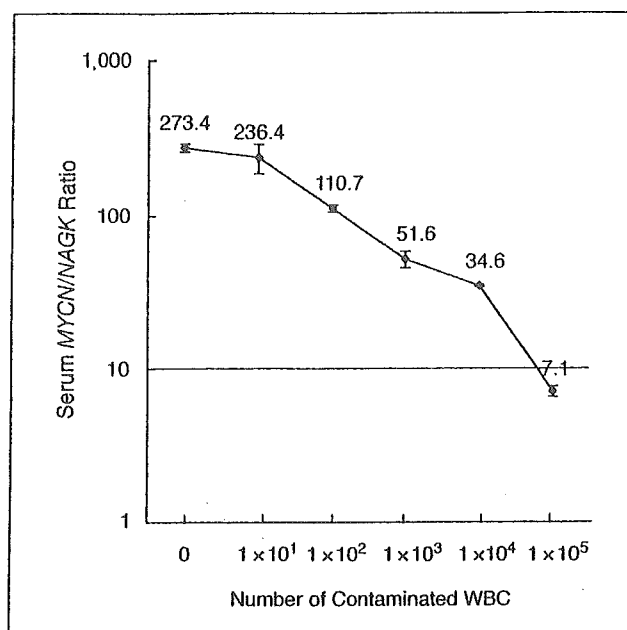


Fig 3. Influence of WBC contamination in serum samples on the serum *MYCN*/*NAGK* ratio. Data are presented as the mean \pm standard deviation of duplicate measurements. The transverse line represents a *MYCN*/*NAGK* ratio cutoff value of 10.0.

that use serum DNA. For the same reason, a predominance of any nontumor DNA in serum may lower an elevated *M/N* ratio of an *MYCN*-amplified patient. However, this assay can be accurate on the premise that, in cancer patients, serum DNA predominantly consists of tumor-released DNA.⁷ In addition, the use of serum DNA as a diagnostic tool in lung cancer patients has

resulted in a diversity of findings, suggesting that these differences likely reflect variations in the manner in which the blood specimens were collected and handled and variations in the methods by which the assay were conducted.²⁴ Therefore, it is necessary to standardize the serum collection procedure to ensure that different laboratories obtain the same result with a given blood sample. An additional high-speed centrifugation step ($16,000 \times g$ for 5 minutes) was found to eliminate cellular contamination even after thawing of stored samples.²⁵ By using the appropriate centrifugation methods, we believe that WBC-free serum can be reliably achieved.

Although a large set of patients needs to be studied to verify the accuracy of this assay and to set an appropriate cutoff, our results are promising and need to be further tested. The advantages of this method are that it takes only 4 hours and much less effort than FISH and Southern blotting, which should make this assay an alternative to these other methods for determining *MYCN* status. A third advantage is that the serum *M/N* ratio seems to be a promising indicator of therapeutic efficacy and relapse in the follow-up of patients with MNA, although more patients need to be examined to confirm its reliability.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Tumorigenesis and Neoplastic Progression

Biological Role of Anaplastic Lymphoma Kinase in Neuroblastoma

Yuko Osajima-Hakomori,^{*¶} Izumi Miyake,^{*†}
Miki Ohira,[‡] Akira Nakagawara,[‡]
Atsuko Nakagawa,[§] and Ryuichi Sakai^{*}

From the Growth Factor Division,^{*} National Cancer Center Research Institute, Chuo-ku, Tokyo; St. Marianna University School of Medicine,[¶] Kawasaki-shi, Kanagawa; Tokyo Metropolitan Geriatric Hospital,^{||} Itabashi-ku, Tokyo; the Department of Pediatrics,[†] Kitasato University School of Medicine, Sagami-hara-shi, Kanagawa; the Division of Biochemistry,[‡] Chiba Cancer Center Research Institute, Cyuo-ku, Chiba; and the Department of Pathology,[§] Aichi Medical University, Aichi-gun, Aichi, Japan

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor originally identified as part of the chimeric nucleophosmin-ALK protein in the t(2;5) chromosomal rearrangement associated with anaplastic large cell lymphoma. We recently demonstrated that the ALK kinase is constitutively activated by gene amplification at the ALK locus in several neuroblastoma cell lines. Forming a stable complex with hyperphosphorylated ShcC, activated ALK modifies the responsiveness of the mitogen-activated protein kinase pathway to growth factors. In the present study, the biological role of activated ALK was examined by suppressing the expression of ALK kinase in neuroblastoma cell lines using an RNA interference technique. The suppression of activated ALK in neuroblastoma cells by RNA interference significantly reduced the phosphorylation of ShcC, mitogen-activated protein kinases, and Akt, inducing rapid apoptosis in the cells. By immunohistochemical analysis, the cytoplasmic expression of ALK was detected in most of the samples of neuroblastoma tissues regardless of the stage of the tumor, whereas significant amplification of ALK was observed in only 1 of 85 cases of human neuroblastoma samples. These data demonstrate the limited frequency of ALK activation in the real progression of neuroblastoma. (*Am J Pathol* 2005, 167:213–222)

Receptor tyrosine kinases (RTKs) play an important role in regulating diverse cellular processes, such as prolifer-

ation, differentiation, survival, motility, and malignant transformation. The activation of RTKs typically requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of the receptors at tyrosine residues.^{1–3} By recruiting specific sets of signal transducer molecules in a phosphorylation-dependent manner, each RTK is capable of inducing individual, specific cellular responses.⁴ On the other hand, activation of RTKs by either mutations or overexpression is frequently found in various human malignancies.^{3,5}

Anaplastic lymphoma kinase (ALK) is a 200-kd tyrosine kinase encoded by the ALK gene on chromosome 2p23. ALK was first identified as part of an oncogenic fusion tyrosine kinase, nucleophosmin-ALK, which is associated with anaplastic large cell lymphoma.^{6,7} It was also found as a form of fusion protein with a clathrin heavy chain (CTCL) in myofibroblastic tumors.⁸ Full-length ALK has the typical structure of an RTK, with a large extracellular domain, a lipophilic transmembrane segment, and a cytoplasmic tyrosine kinase domain.^{9,10} ALK is highly homologous to leukocyte tyrosine kinase (LTK) and is further classified into the insulin receptor superfamily. The LTK gene is mainly expressed in pre-B lymphocytes and neuronal tissues,^{11–13} whereas expression of the normal ALK gene in hematopoietic tissues has not been detected. Instead, it is dominantly expressed in the neural system.^{14,15} In the developing brains of mice, specific expression of ALK was seen in the thalamus, mid-brain, olfactory bulb, and selected cranial regions, as well as the dorsal root, the ganglia of mice,^{9,10,16} suggesting a specific role in the development of the embryonic nervous system. Currently, however, the function of ALK in adult normal tissue or carcinogenesis remains an open question. Several studies have recently indicated pleiotrophin or midkine as possible ligands for ALK.^{17,18} Although they appeared to induce the functional activa-

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Address reprint requests to Ryuichi Sakai, M.D., Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: rsakai@gan2.res.ncc.go.jp.

tion of ALK, it is still unclear whether these molecules are the physiological ligands of ALK.

Neuroblastoma is one of the most common pediatric tumors derived from the sympathoadrenal lineage of the neural crest. Tumors found in patients under the age of 1 year are usually favorable and often show spontaneous differentiation and regression.¹⁹ Amplification of the *N-myc* gene occurs in approximately 25% of neuroblastomas and correlates with the aggressiveness of the disease. In addition to *N-myc* gene amplification, the expression of various genes has significant correlation with the stage of and prognosis for neuroblastoma. A high level of TrkA expression is predictive of a favorable outcome,²⁰ whereas TrkB is highly expressed in immature neuroblastomas with *N-myc* amplification.²¹ High expression of caspase-1, -3, and -8 is correlated with favorable neuroblastomas.^{22,23} On the other hand, survivin, which suppresses caspase and promotes the cell survival signal, is significantly expressed,²⁴ and telomerase is activated²⁵ in unfavorable tumors. There may be a critical difference in the expression of other molecules, including RTKs, in neuroblastoma. A recent paper showed that full-length ALK is detected in almost one-half of the cell lines derived from neuroblastomas and neuroectodermal tumors.²⁶ We have recently shown using mass-spectrometry analysis that ALK is a major phosphoprotein associated with hyperphosphorylated ShcC in several neuroblastoma cell lines.²⁷ In these cells, ALK was markedly activated, and it induced the constitutive phosphorylation of ShcC and mitogen-activated protein kinase (MAPK), regardless of stimulation by epidermal growth factor (EGF) or nerve growth factor.²⁷ These findings strongly suggest that constitutively activated ALK kinase plays a physiological role in the development of neuroblastoma.

In this study, we investigated the biological function of the constitutively activated ALK kinase in neuroblastoma. The RNA interference (RNAi) technique using specific sets of small interfering RNA (siRNA) was induced to inhibit the *ALK* gene expression in human neuroblastoma cells with or without gene amplification of *ALK*. The effects of disrupted ALK expression on cell survival or downstream signaling, such as MAPKs or Akt pathways, are examined to understand the biological meaning of ALK amplification in neuroblastoma cells. We also performed Southern blot analysis of primary neuroblastoma tumors from 85 patients to check whether the *ALK* gene amplification was actually present in neuroblastoma tissues. Furthermore, we sought the *ALK* gene expression in human neuroblastoma tissues using immunohistochemical analysis.

Materials and Methods

Cell Culture

Cell lines of human neuroblastoma were maintained in RPMI 1640 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), penicillin, and streptomycin at 37°C in a humidified 5% CO₂ incubator.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted with ISOGEN (Nippongene Japan, Toyama, Japan) from NB-39-nu and SK-N-MC cells. The PCR primer pair 5'-AGGTTCTGGCTGCAGATGGT-3' and 5'-ACATTGTTCTCTCGAGTGCAGAC-3' corresponding to the cytoplasmic portion of human ALK was prepared. As much as 0.25 µg of total RNA was reverse transcribed and amplified with the SuperScript One-step RT-PCR with the Platinum Taq kit (Invitrogen Life Technologies, Carlsbad, CA) in a total volume of 50l including 2× reaction mix, 0.2 µmol/L of each primer, and 1 µl of RT/Platinum Taq Mix. Amplification conditions consisted of cDNA synthesis and predenaturation at 50°C for 30 minutes and 94°C for 2 minutes followed by 25 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. A final amplification for 7 minutes at 72°C finished the PCR. The product was separated with 1.2% agarose gel electrophoresis and analyzed using the Quality One System (Bio-Rad, Hercules, CA).

Immunochemical Analysis of Proteins

Immunoprecipitation and immunoblotting were performed as described previously.²⁷ The polyclonal antibodies against the CH1 domains of ShcC (amino acids 306–371) and the anti-ALK antibody (αALK) that was against the cytoplasmic portion (amino acid 1379–1524) of human ALK were prepared as described previously.^{27,28} An anti-phosphotyrosine antibody (4G10) was obtained from UBI. Anti-p44/42 MAPKs, anti-phospho-p44/42 MAPKs, anti-Akt, and anti-phospho-Akt antibodies were purchased from Cell Signaling (Beverly, MA). Anti-EGF receptor (EGFR), anti-Ret, and anti-TrkA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *In vitro* kinase assay for ALK was performed as previously described.²⁷ Anti-ALK immunoprecipitates were incubated with or without Poly-Glu/Tyr as an exogenous substrate.

Immunocytochemical Staining

For ALK/TOTO-3, immunostaining using anti-ALK antibody was performed at first, and then nuclei were stained using TOTO-3. The cells seeded on the 24-well plates were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde (methanol free) for 5 minutes at room temperature. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 10 minutes at room temperature. The cells were blocked with 5% goat serum and 3% bovine serum albumin-Tris-buffered saline for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of αALK for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:2000 dilution of Alexa fluor (Molecular Probes, Eugene, OR) and 1:100 dilution of TOTO-3 (Molecular Probes) for

Table 1. Patient Characteristics of Neuroblastoma Tissues with *ALK* Gene Gain or Amplification

Case	Age*	Primary tumor		Copy nos. of <i>ALK</i> [‡]	Amplification of <i>N-myc</i> (n)
		Location	Clinical stage [†]		
1	3y5m	Adrenal gland	IV	2.0 ± 0.2	+
2	5y0m	Peritoneum	IV	1.8 ± 0.1	(35)
3	2y7m	Abdomen	IV	2.1 ± 0.8	(150)
4	8m	Adrenal gland	I	3.0 ± 1.0	—
5	4y9m	Abdomen	IV	2.0 ± 0.2	—
6	3y9m	Adrenal gland	III	2.7 ± 0.2	(150)
7	1y4m	Adrenal gland	IV	2.8 ± 1.0	(150)
8	1y7m	Adrenal gland	IV	9.5 ± 2.2	(100)

*Age of onset: year (y), month (m).

[†]The staging criterion was based on the International Neuroblastoma Staging System.

[‡]The averages of the calculated copy numbers from three independent blottings are shown.

30 minutes at room temperature. The cells were washed three times with PBS and mounted in glycerol-based 2.5% 1,4-diazabicyclo[2,2,2] octan. Confocal laser scanning analysis was carried out. For *ALK*/TUNEL, we first carried out TUNEL and then proceeded to standard immunocytochemistry using anti-*ALK* antibody. TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) with the following modifications. The NB-39-nu cells seeded on the 24-well plates that were treated with siRNAs were washed with PBS twice and fixed with 4% paraformaldehyde (methanol free) for 25 minutes at 4°C. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature. The cells were washed with PBS twice and covered with an equilibration buffer (from the kit) for 10 minutes at room temperature. The equilibration buffer was drained off, and a reaction buffer containing the equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the cells and incubated at 37°C for 1 hour, avoiding exposure to light. The cells were incubated for 15 minutes at room temperature with 2× standard saline citrate to stop the reaction. The cells were washed with PBS three times and then stained for *ALK* using immunofluorescence as follows. The cells were blocked with 2% bovine serum albumin (Boehringer Mannheim, Germany) for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of α *ALK* for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:40 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 30 minutes at room temperature. The cells were washed three times with PBS and then mounted and observed in the same manner as that for *ALK*/TOTO-3.

DNA Extraction and Southern Blotting

Genomic DNAs derived from neuroblastoma cell lines were obtained from cultured cells as described using the procedure of Perucho et al.²⁹ Samples of 85 neuroblastoma tissues were collected at the Chiba Cancer Center and stored as forms of genomic DNA. The characteristics of some of these patients are shown in Table 1. The stage

criterion was based on the International Neuroblastoma Staging System.³⁰ Samples of 5 μ g of DNA digested by *Eco*RI were electrophoresed in 0.8% agarose gel and blotted onto nitrocellulose filters (Hybond-N+; Amersham, Piscataway, NJ). The probes for detecting the *ALK* gene, *N-myc* gene, and *ShcC* gene were used in our previous study.²⁷ The intensities of these signals were measured using a Molecular Imager FxPro (Bio-Rad). This study was approved by the ethical judging committee of the National Cancer Center and the Chiba Cancer Center of Japan.

RNA Interference Technique

Twenty-one-nucleotide double-stranded RNAs were synthesized and purified using Dharmacon Research (Lafayette, CO). To suppress the expression of *ALK* protein, two different pairs of *ALK* siRNAs, *ALK*-siRNA1 and *ALK*-siRNA2, were obtained. The sequences were 5'-GAGUCUGGCAGUUGACUUCdTdT-3' for *ALK*-siRNA1 and 5'-GCUCCGGCGUGCCAAGCAGdTdT-3' for *ALK*-siRNA2, corresponding to coding region 153 to 171 and 399 to 417 relative to the first nucleotide of the start codon, respectively. Entire sequences were derived from the sequence of human *ALK* mRNA (accession no. HSU62540). An siRNA, targeting a sequence in the firefly (*Photinus pyralis*) luciferase mRNA, was used as a negative control (Dharmacon) (luc-siRNA). We also used a scramble siRNA, Scramble Duplex II (Dharmacon) (s-siRNA) as a mismatch siRNA control in addition to luc-siRNA.

NB-39-nu cells were trypsinized, diluted with growth medium containing 10% fetal calf serum, and transferred to 12-well plates at 6×10^4 cells per well for 24 hours before transfection. The transfection of siRNA was carried out using jetSI (Poly plus transfection). A total of 100 μ l of serum-free growth medium and 4 μ l of jetSI per well were preincubated for 5 to 10 minutes at room temperature. While the incubation was being performed, 100 μ l of serum-free growth medium was mixed with 5 μ l of 20 μ mol/L siRNA duplex (100 pmol). Total siRNA amounts of 50, 100, and 200 pmol were checked in preliminary experiments to find out 100 pmol is the minimal and optimal amount in this scale of RNAi. The 100 μ l of jetSI serum-free medium solution was added to the 100 μ l of siRNA

duplex solution, gently mixed, and incubated for 30 minutes at room temperature. The growth medium on the cells was removed, and 800 μ l of serum-free medium was added to each well. A total of 200 μ l of the entire mixture was overlaid onto the cells, and cells were incubated for 4 hours at 37°C in a 5% CO₂ incubator. After incubation, 1 ml of medium containing 4% fetal calf serum was added without removing the transfection mixture (final concentration 2%). The cells were assayed 84 hours after transfection. SK-N-MC cells were seeded in 12-well plates at a concentration of 1.3×10^5 cells per well. These were treated with siRNAs in the same way as NB-39-nu and assayed 48 hours after transfection. In the 24-well plate, the cells were seeded at the same concentration as the 12-well plate, and siRNAs and all other reagents were used at half volume. After transfection, the cells were examined under a light microscope every day.

Double Staining for ALK and TUNEL

For double staining, we first carried out TUNEL and then proceeded to standard immunocytochemistry using anti-ALK antibody. TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega) with the following modifications. The NB-39-nu cells seeded on the 24-well plates that were treated with siRNAs were washed with PBS twice and fixed with 4% paraformaldehyde (methanol free) for 25 minutes at 4°C. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature. The cells were washed with PBS twice and covered with an equilibration buffer (from the kit) for 10 minutes at room temperature. The equilibration buffer was drained off, and a reaction buffer containing the equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the cells and incubated at 37°C for 1 hour, avoiding exposure to light. The cells were incubated for 15 minutes at room temperature with 2 \times standard saline citrate to stop the reaction. The cells were washed with PBS three times and then stained for ALK using immunofluorescence as follows. The cells were blocked with 2% bovine serum albumin (Boehringer Mannheim) for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of α ALK for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:40 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 30 minutes at room temperature. The cells were washed three times with PBS and mounted in glycerol-based 2.5% 1,4-diazabicyclo[2,2,2]octan. Confocal laser scanning analysis was carried out.

DNA Fragmentation Assay

To detect apoptotic DNA cleavage, DNA fragmentation assay was performed using an Apoptotic DNA Ladder kit (Chemicon International, Inc., Temecula, CA). The cells seeded on the 12-well plates that were treated with siRNAs as previously mentioned were collected in 1.5-ml

microcentrifuge tubes. The cells were washed with PBS, centrifuged, and lysed with 20 μ l of TE lysis buffer. The lysates were incubated with 5 μ l of enzyme A (RNase A) at 37°C for 10 minutes and then at 55°C for 30 minutes after the addition of 5 μ l of Enzyme B (Proteinase K). Afterward, 5 μ l of ammonium acetate solution and 100 μ l of absolute ethanol were added, and the samples were kept at -20°C for 10 minutes. The samples were centrifuged, and the pellets were washed with 70% ethanol. Then the DNA pellets were dissolved in 30 μ l of DNA suspension buffer. DNA fragmentations were visualized by electrophoresis on 2% agarose gel containing ethidium bromide.

Immunohistochemistry

As for positive control, tumor xenograft was made by injection of NB-39-nu cells subcutaneously in 5-week-old SCID mice. Immunohistochemical staining with ALK antibody (α ALK) (1:1000), was performed on 16 human neuroblastoma tumors selected from the surgical pathology file at the Department of Pathology, Aichi Medical University based on the results of histopathology evaluation³¹ and N-myc status. All of those tumor samples were obtained before chemotherapy and irradiation therapy and included nine favorable histology cases with nonamplified N-myc (FH&NA), two unfavorable histology cases with amplified N-myc (UH&A), and five unfavorable histology cases with nonamplified N-myc (UH&NA).

Four-micrometer-thick sections from the formalin-fixed and paraffin-embedded tissue samples were deparaffinized and microwaved for three times for 5 minutes in Na-citrate buffer (pH 6.0) for antigen retrieval. The slides were first immersed in 0.3% hydrogen peroxide in methanol for 20 minutes and then in 10% normal goat serum for 30 minutes. The primary antibody (α ALK) was then applied at 4°C overnight, followed by a standard staining procedure using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin for light microscopic review and evaluation. ALK was always positively detected in the cytoplasm of NB-39-nu tumor xenograft and in the cytoplasm and neuritic processes of normal ganglion cells in the separate positive control sections as well as in the test sections as built-in control, whenever available. As for the negative controls, normal rabbit immunoglobulins (1:500 dilution; Vector Laboratories) or preimmune serum for α ALK (1:1000 dilution) was applied as the primary antibody.

Results

Significant Amplification of the ALK Gene and Constitutive Activation of ALK Kinase in Three Neuroblastoma Cell Lines

As shown in Figure 1A, NB-39-nu, Nagai, and NB-1 cells have significant levels of amplification of the ALK gene (30–40 copies per cell) among 25 neuroblastoma and neuroepithelioma cell lines examined. Other cell lines such as SK-N-MC have only one copy of the ALK gene just like the