THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 17, Issue of April 29, pp. 16665–16675, 2005
Printed in U.S.A.

Identification of Protein Kinase A Catalytic Subunit β as a Novel Binding Partner of p73 and Regulation of p73 Function*

Received for publication, December 20, 2004, and in revised form, January 31, 2005
Published, JBC Papers in Press, February 21, 2005, DOI 10.1074/jbc.M414323200

Takayuki Hanamoto‡§, Toshinori Ozaki‡, Kazushige Furuya‡, Mitsuchika Hosoda‡, Syunji Hayashi‡, Mitsuru Nakanishi‡, Hideki Yamamoto‡, Hironobu Kikuchi‡, Satoru Todo§, and Akira Nakagawara‡¶

From the ‡Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan and the §Department of General Surgery, Hokkaido University School of Medicine, Kita-ku, Sapporo 060-8638, Japan

Post-translational modifications play a crucial role in regulation of the protein stability and pro-apoptotic function of p53 as well as its close relative p73. Using a yeast two-hybrid screening based on the Sos recruitment system, we identified protein kinase A catalytic subunit β (PKA-Cβ) as a novel binding partner of p73. Co-immunoprecipitation and glutathione *S*-transferase pull-down assays revealed that p73α associated with PKA-Cβ in mammalian cells and that their interaction was mediated by both the N- and C-terminal regions of p73α. In contrast, p53 failed to bind to PKA-Cβ. *In vitro* phosphorylation assay demonstrated that glutathione *S*-transferase-p73α-(1–130), which has one putative PKA phosphorylation site, was phosphorylated by PKA. Enforced expression of PKA-Cβ resulted in significant inhibition of the transactivation function and pro-apoptotic activity of p73α, whereas a kinase-deficient mutant of PKA-Cβ had no detectable effect. Consistent with this notion, treatment with H-89 (an ATP analog that functions as a PKA inhibitor) reversed the dibutyryl cAMP-mediated inhibition of p73α. Of particular interest, PKA-Cβ facilitated the intramolecular interaction of p73α, thereby masking the N-terminal transactivation domain with the C-terminal inhibitory domain. Thus, our findings indicate a PKA-Cβ-mediated inhibitory mechanism of p73 function.

p73 has been identified as a structural and functional homolog of the tumor suppressor p53 (1). p53 and p73 share the same domain organization, consisting of an N-terminal transactivation domain, a central sequence-specific DNA-binding domain, and a C-terminal oligomerization domain. As expected, several pieces of evidence suggest that p73 can bind to the p53-responsive element and transactivate an overlapping set of p53 target genes, thus leading to induction of G_1/S cell cycle arrest and apoptosis (1–6). In marked contrast to p53, p73 is expressed as multiple isoforms arising from alternative splicing of the primary p73 transcript (p73α, p73β, p73γ, p73δ, p73ε, p73η, and p73ζ) termed the TA variant (1, 3, 7–9). These alternatively spliced isoforms vary in their C termini and display different transcriptional and biological properties. Additionally, the ΔN variant (ΔNp73α and ΔNp73β), which is generated by alternative promoter utilization, lacks the N-terminal transactivation domain and exhibits dominant-negative behavior toward wild-type p73 as well as p53 (10–12). Recently, we (14) and others (13, 15) demonstrated that p73 directly transactivates the expression of its own negative regulator (ΔNp73), creating an autoregulatory feedback loop in which both the activity of p73 and the expression of ΔNp73 are regulated. Thus, the pro-apoptotic activity of p73 is determined by the relative expression levels of its TAp73 and dominant-negative ΔNp73 variants in cells.

In sharp contrast to p53, it was initially reported that p73 was not induced by DNA damage (1). However, recent studies demonstrated that, in response to a subset of DNA-damaging agents, p73 is positively regulated by multiple post-translational modifications, including phosphorylation and acetylation. During cisplatin-mediated apoptosis, phosphorylation of p73 at Tyr-99 by the non-receptor tyrosine kinase c-Abl results in an increase in its stability and pro-apoptotic activity (16–18). In addition to c-Abl, the protein kinase Cδ catalytic fragment has the ability to phosphorylate p73 at Ser-289 and contributes to the accumulation of p73 during the apoptotic response to cisplatin treatment (19). It is worth noting that the physical and functional interaction between c-Abl and protein kinase Cδ leads to the cross-activation of their kinase functions (20, 21). Furthermore, the enzymatic activity of Chk1 (checkpoint kinase-1) is enhanced in response to DNA damage (22–24), and Chk1 has the ability to phosphorylate p73 at Ser-47 upon DNA damage, thereby enhancing its transactivation ability and pro-apoptotic activity without affecting the level of total p73 protein, whereas Chk2 has no detectable effect on p73 (25). Alternatively, Zeng *et al.* (26) found that the acetyltransferase p300/CBP (cAMP-responsive element-binding protein-binding protein) interacts with the N-terminal region of p73 and stimulates p73-mediated transcriptional activation and apoptosis. Recently, Costanzo *et al.* (27) reported that doxorubicin treatment induces the p300-mediated acetylation of p73 at Lys-321, Lys-327, and Lys-331 in a c-Abl-dependent manner, which is associated with the efficient recruitment of p73 to the promoter of the apoptotic target gene *p53AIP1*. Additionally, it has been shown that p300-mediated acetylation of p73 results in its significant stabilization in a prolyl isomerase Pin1-dependent manner (28).

To identify cellular protein(s) that could interact with full-length p73α and regulate its function, we screened a human fetal brain cDNA library using a yeast two-hybrid method

* This work was supported in part by a grant-in-aid from the Ministry of Health, Labor, and Welfare for Third Term Comprehensive Control Research for Cancer; a grant-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and a grant-in-aid for scientific research from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Div. of Biochemistry, Chiba Cancer Center Research Inst., 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan. Tel.: 81-43-264-5431; Fax: 81-43-265-4459; E-mail: akiranak@chiba-cc.jp.

based on the Sos recruitment system. We report here that protein kinase A catalytic subunit β (PKA-Cβ)[1] bound to p73α in cells, but not to p53, and that their interaction was mediated by the N- and C-terminal regions of p73α. *In vitro* kinase assays revealed that the catalytic subunit of PKA phosphorylated p73α. PKA-Cβ inhibited the p73α-mediated transcriptional activation of the $p21^{WAF1}$ and *Bax* promoters and p73α-dependent apoptosis in response to camptothecin. On the other hand, the kinase-deficient mutant of PKA-Cβ had little effect on p73α. Of note, we found that PKA-Cβ facilitated the intramolecular interaction of p73α. Our results strongly suggest the PKA-Cβ-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-incubated fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin. p53-deficient human lung carcinoma H1299 cells were maintained in RPMI 1640 medium supplemented with 10% heat-incubated fetal bovine serum and antibiotic mixture. The cells were cultured at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2.

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 p73α was amplified by PCR using pcDNA3-p73α 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-p73α. The resulting pSos-p73α"bait" plasmid was used to identify the cDNA encoding the p73α-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 (cdc25Hα) was cotransformed with pSos-p73α 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-Cα polyclonal antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-PKA-Cβ 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-p73α fusion proteins were expressed in *E. coli* strain DH5α and loaded onto glutathione-Sepharose 4B beads (Amersham Biosciences). PKA-Cβ was generated *in vitro* in the presence of [^{35}S]methionine using the TnT quick-coupled *in vitro* transcription/translation system (Promega Corp., Madison, WI) according to the manufacturer's instructions. ^{35}S-Labeled PKA-Cβ was incubated with GST or GST-p73α fusion proteins bound to glutathione-Sepharose beads for 2 h at 4 °C in a total volume of 400 μl 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× 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× 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)-p73α and FLAG-PKA-Cβ. 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 (5×10^4 cells in a 12-well plate) were transiently cotransfected with a constant amount of the indicated expression plasmid (HA-p73α, HA-p73β, 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-Cβ. 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′-ATGAAATTCACCCCCTTTCC-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; Bt_2cAMP, dibutyryl cAMP.

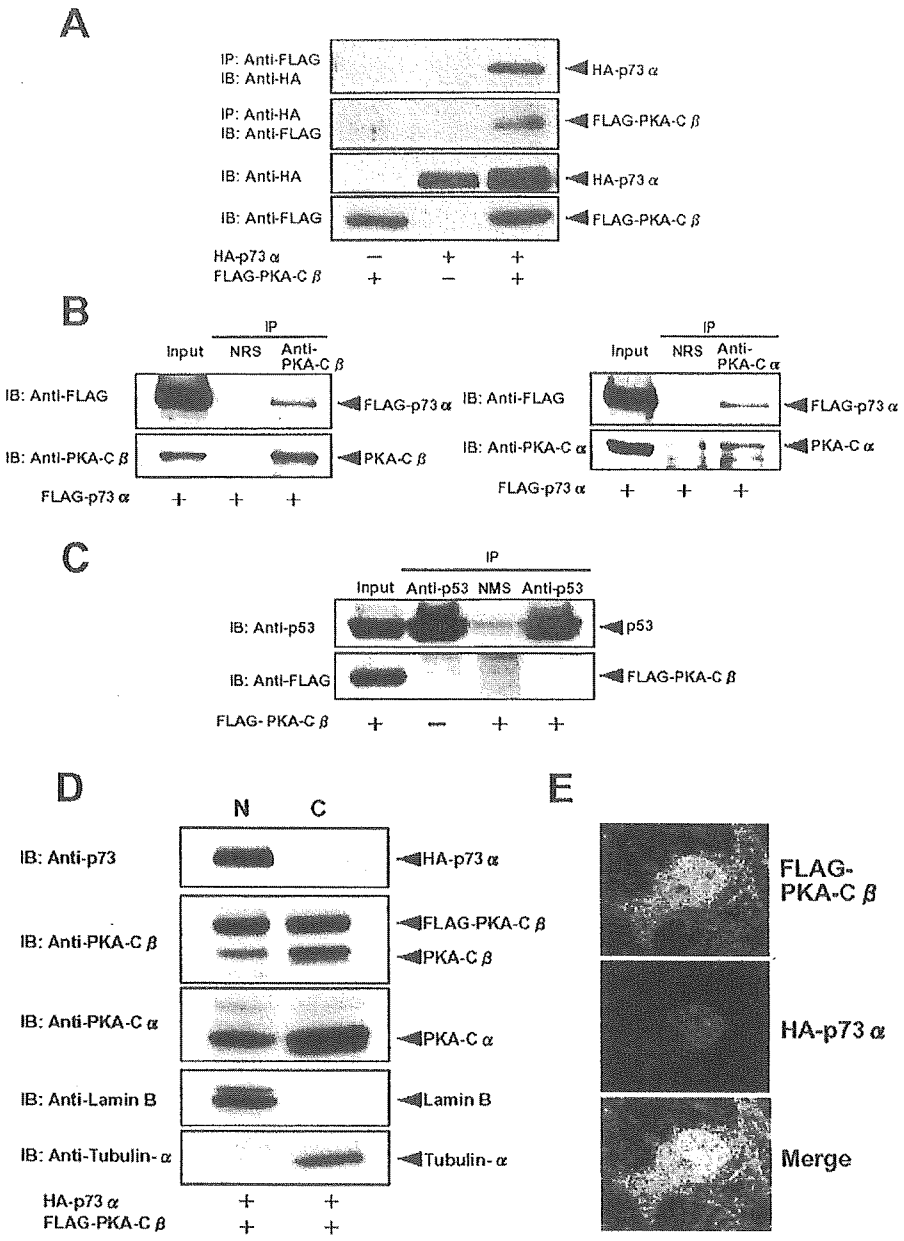

FIG. 1. **Interaction between p73 and PKA-Cβ in mammalian cultured cells.** *A*, p73α forms a complex with PKA-Cβ in COS-7 cells. Whole cell lysates prepared from COS-7 cells transiently cotransfected with the indicated combinations of expression plasmids were immunoprecipitated (*IP*) with anti-FLAG 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-p73α or FLAG-PKA-Cβ, respectively. *B*, p73α binds to endogenous PKA-C in COS-7 cells. COS-7 cells were transiently transfected with the expression plasmid for FLAG-p73α. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to immunoprecipitation with anti-PKA-Cβ (*left panels*) or anti-PKA-Cα (*right panels*) polyclonal antibody. Immunoprecipitation with normal rabbit serum (*NRS*) was used as a negative control. After immunoprecipitation, coprecipitating FLAG-p73α was detected by immunoblotting with anti-FLAG monoclonal antibody. *C*, PKA-Cβ 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-Cβ. 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-Cβ. p53-deficient H1299 cells were transiently cotransfected with the expression plasmids for HA-p73α and FLAG-PKA-Cβ (*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-Cβ. H1299 cells plated on coverslips were cotransfected with the expression plasmids for HA-p73α and FLAG-PKA-Cβ and processed for immunocytochemical detection using anti-HA and anti-FLAG antibodies. The merged image shows the nuclear co-localization of p73α and PKA-Cβ.

(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-p73α fusion proteins 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 $MgCl_2$). The washed beads were incubated with 30 μl of kinase buffer containing 2 units of purified PKA catalytic subunit (Sigma), 2 mM

dithiothreitol, and 10 μCi of [γ-^{32}P]ATP for 30 min at 4 °C. The reaction mixtures were boiled in 2× 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 μM) 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-Cβ—The K76R mutation was introduced into wild-type PKA-Cβ by PCR-based mutagenesis using *PfuUltra*™ 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′-CATGGCATAATACTGTTCAGTGGCT-3′. This yielded the expression plasmid pcDNA3-FLAG-PKA-Cβ(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-p73α (1.2 μg) and FLAG-PKA-Cβ (4.8 μg). Thirty-six hours after transfection, cells were cross-linked with 1% formaldehyde in medium for 15 min at 37 °C. Cells were then washed with ice-cold PBS and resuspended in 200 μl 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 μl of protein A-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 °C. The beads were removed, and the chromatin solution was immunoprecipitated overnight with anti-HA monoclonal antibody at 4 °C, followed by incubation with protein A-agarose beads for an additional 1 h at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$) and formaldehyde cross-links were reversed by heating at 65 °C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45 °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′-CACCTTTCACCATTCCCCTA-3′ and 5′-GCAGCCCAAGGACAAAATAG-3′ for p21^{WAF1} and 5′-AAAGCTCAGAGGCCCAAAAT-3′ and 5′-AGGCTGAGACGGGGTTATCT-3′ for *bax*.

RESULTS

Identification of PKA-Cβ 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 p73α as bait in a new CytoTrap yeast two-hybrid screen relying on the Sos recruitment system. A temperature-sensitive yeast strain (cdc25Hα) was cotransformed with a bait plasmid and a human fetal brain cDNA library. Of a total of 1 × 10^6 primary transformants grown on medium containing glucose at 30 °C, one clone (termed F115) exhibited galactose-dependent growth at 37 °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-Cβ. Of the PKA catalytic subunit isoforms (PKA-Cα, PKA-Cβ, and PKA-Cγ), PKA-Cβ is highly expressed in brain and reproductive tissues, whereas PKA-Cα is ubiquitously expressed in mammalian tissues (31, 32).

PKA-Cβ Associates with p73 in Mammalian Cultured Cells—To confirm the interaction between PKA-Cβ 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-Cβ and HA-p73α. As shown in Fig. 1*A*, the anti-FLAG immunoprecipitates contained HA-p73α. Used as a control, HA-p73α was not detectable in the anti-FLAG immunoprecipitates of COS-7 cells expressing FLAG-PKA-Cβ or HA-p73α alone. Analysis of the anti-HA immunoprecipitates also demonstrated that FLAG-PKA-Cβ co-immunoprecipitated with HA-p73α. Next, we examined whether endogenous PKA-Cβ could interact with p73α. To this end, whole cell lysates prepared from COS-7 cells transfected with the expression plasmid for FLAG-p73α were immunoprecipitated with normal rabbit serum or with the specific antibody against PKA-Cβ, followed by immunoblotting with anti-FLAG anti-

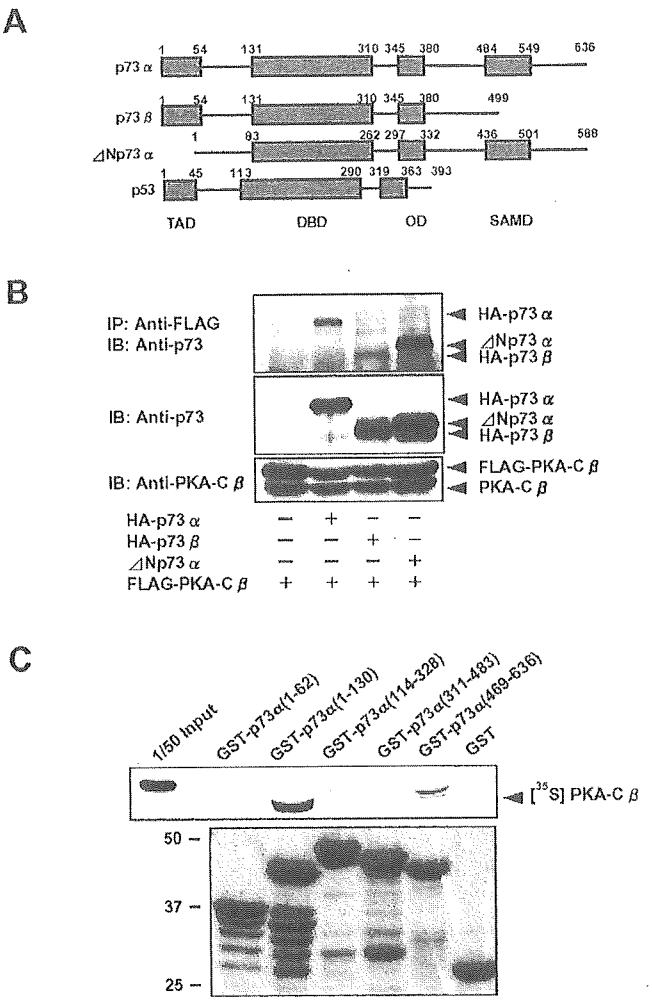

FIG. 2. **Interacting region within p73 for PKA-Cβ.** *A*, domain structures of p73 and p53. The transactivation domain (*TAD*), DNA-binding domain (*DBD*), oligomerization domain (*OD*), and sterile α motif domain (*SAMD*) are indicated. The *numbers* above the p73 variants and p53 indicate amino acid numbering. *B*, interaction of PKA-Cβ with various p73 variants. Whole cell lysates prepared from H1299 cells transiently cotransfected with the expression plasmid for HA-p73α, HA-p73β, or ΔNp73α and with the expression plasmid for FLAG-PKA-Cβ 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-Cβ (*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-p73α fusion proteins were incubated with *in vitro* translated ^{35}S-labeled FLAG-PKA-Cβ 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-Cβ used for *in vitro* pull-down assays that was directly loaded on the same gel as a control. GST and GST-p73α fusion proteins were stained with Coomassie Brilliant Blue (*lower panel*). The positions of molecular mass markers are indicated on the left in kilodaltons.

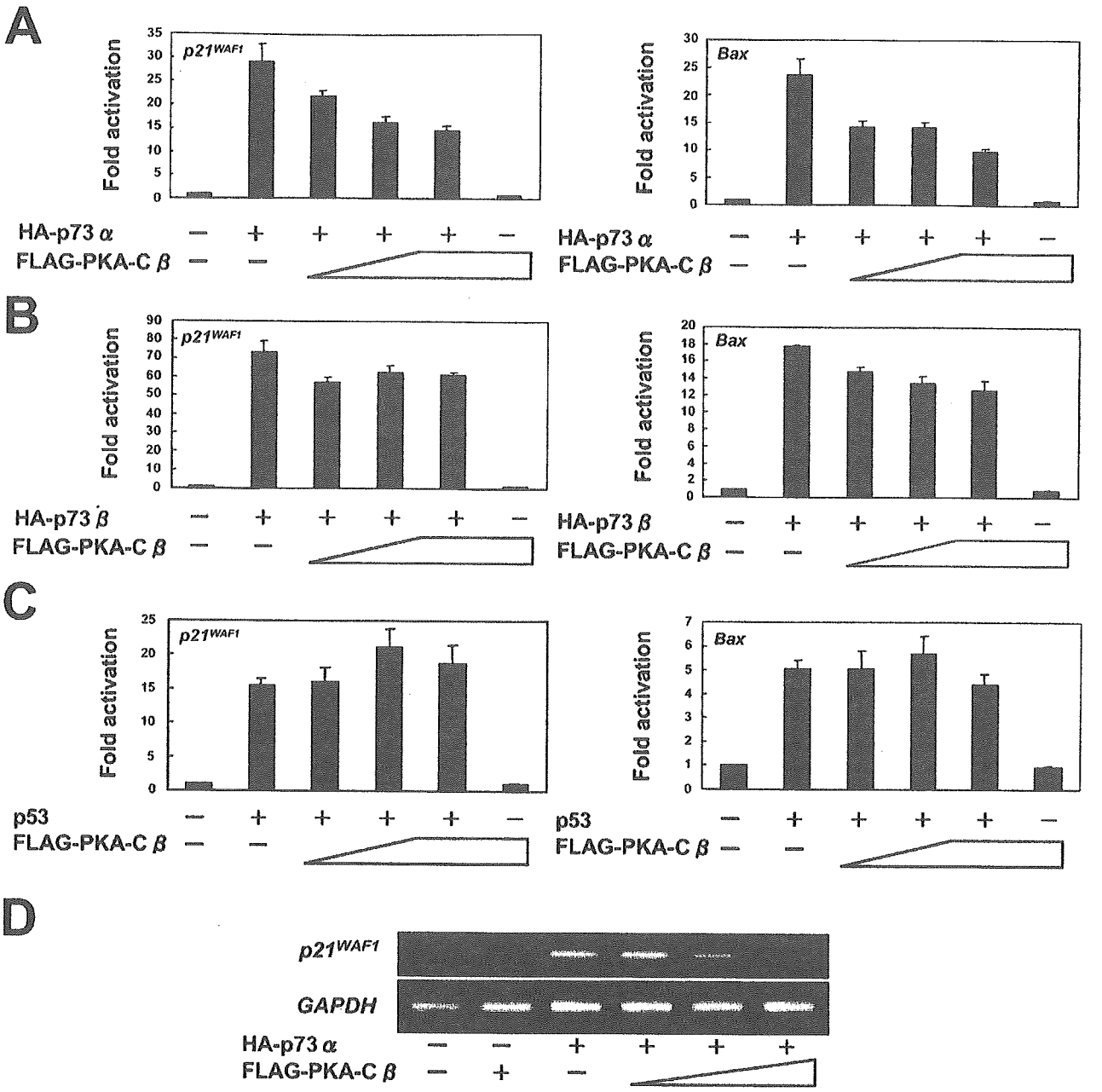

FIG. 3. **PKA-Cβ inhibits p73α-mediated transcriptional activation.** *A–C*, luciferase reporter assays. H1299 cells (5×10^4 cells/12-well plates) were transiently cotransfected with 25 ng of the expression plasmid for HA-p73α (*A*), HA-p73β (*B*), or p53 (*C*); 100 ng of the luciferase reporter construct containing the p53/p73-responsive element derived from the $p21^{WAF1}$ (*left panels*) or *bax* (*right panels*) promoter; and 10 ng of *Renilla* luciferase plasmid (pRL-TK) with or without increasing amounts of the expression plasmid for FLAG-PKA-Cβ (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-p73α (200 ng) with or without increasing amounts of the expression plasmid for FLAG-PKA-Cβ (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. 1*B* (*upper panels*), FLAG-p73α co-immunoprecipitated with endogenous PKA-Cβ. Because the amino acid sequences of PKA-Cα and PKA-Cβ are 91% identical (31), we examined whether endogenous PKA-Cα could bind to p73α. Co-immunoprecipitation experiments revealed that, like PKA-Cβ, endogenous PKA-Cα associated with FLAG-p73α (Fig. 1*B*, *lower panels*). In sharp contrast to p73α, p53 failed to interact with FLAG-PKA-Cβ under our experimental conditions (Fig. 1*C*).

To investigate the subcellular distribution of PKA-Cβ in the presence of exogenous p73α, we employed the biochemical fractionation of transfected H1299 cells. H1299 cells transiently cotransfected with the expression plasmids for HA-p73α and FLAG-PKA-Cβ 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. 1*D*, HA-p73α was detected exclusively in the nuclear fractions, whereas FLAG-PKA-Cβ and endogenous PKA-Cβ and PKA-Cα were present in both the cytoplasmic and nuclear fractions. As expected, confocal microscopy of immunostained H1299 cells expressing FLAG-PKA-Cβ and HA-p73α revealed that both proteins co-localized in the cell nucleus (Fig. 1*E*).

Identification of the Interacting Region within p73—To examine which region(s) of p73 could be engaged in the interaction with PKA-Cβ, we performed co-immunoprecipitation and GST pull-down experiments. Fig. 2*A* 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. 2*B*, HA-p73α and ΔNp73α co-purified with FLAG-PKA-Cβ, whereas the binding of HA-p73β to FLAG-PKA-Cβ was significantly weaker than seen with HA-p73α and ΔNp73α, suggesting that the C-terminal region of p73α might be required for the interaction with PKA-Cβ. To verify these results, *in vitro* GST pull-down assays were carried out using a series of GST-p73α fusion proteins. *In vitro* translated ^{35}S-labeled FLAG-PKA-Cβ was incubated with glutathione-Sepharose beads complexed either with GST alone or with GST-p73α. The autoradiogram in Fig. 2*C* (*upper panel*) shows that GST-p73α-(1–130) and GST-p73α-(469–636) were able to interact with FLAG-PKA-Cβ. The Coomassie Brilliant Blue staining shown in Fig. 2*C* (*lower panel*) revealed that the glutathione-Sepharose beads contained equal amounts of GST-p73α 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 p73α might be essential for the interaction with PKA-Cβ.

PKA-Cβ Inhibits p73α-mediated Transcriptional Activation—In view of the ability of PKA-Cβ to interact with p73α, we next examined whether PKA-Cβ could affect p73α 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-p73α, HA-p73β, 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-Cβ. All cotransfections included pRL-TK to monitor transfection efficiency, and controls included cotransfections with the empty control plasmid. As shown in Fig. 3*A*, coexpression of FLAG-PKA-Cβ and HA-p73α resulted in marked repression of the p21^{WAF1}- and *bax*-luciferase activities induced by HA-p73α in a dose-dependent manner, and FLAG-PKA-Cβ alone had no effect on the reporter gene activity. In contrast, FLAG-PKA-Cβ had no obvious effects on p73β- and p53-mediated transcriptional activation (Fig. 3, *B* and *C*). These results strongly suggest that there is a correlation between the capacity of PKA-Cβ to interact with p73 or p53 and its ability to inhibit their transactivation function. To determine whether PKA-Cβ could inhibit the p73α-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. 3*D*, ectopic expression of HA-p73α resulted in a remarkable up-regulation of endogenous p21^{WAF1} expression, and coexpression of FLAG-PKA-Cβ and HA-p73α inhibited the p73α-mediated induction of p21^{WAF1} in a dose-dependent manner.

To further confirm the inhibitory effect of PKA-Cβ on the transcriptional activity of p73α, H1299 cells were transiently

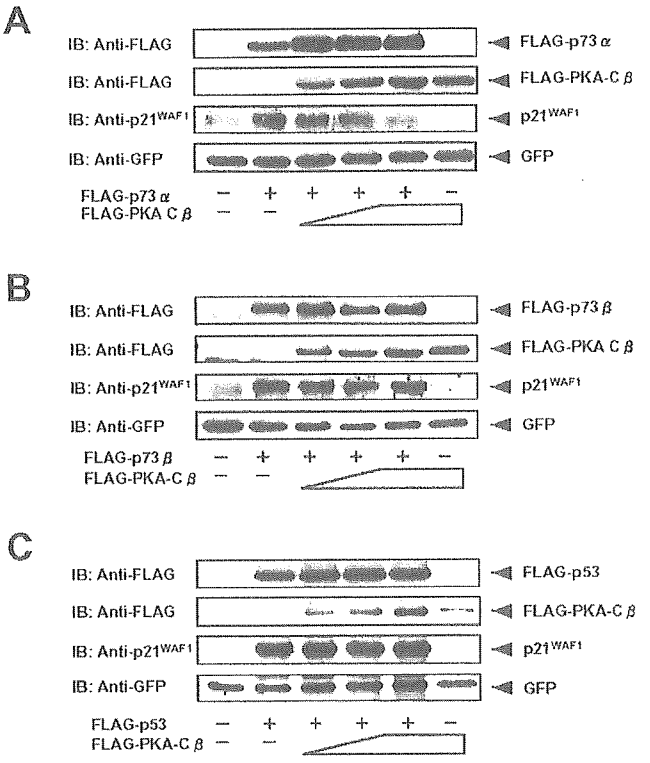

FIG. 4. **PKA-Cβ inhibits the p73α-dependent accumulation of endogenous p21^{WAF1}.** H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for FLAG-p73α (*A*), FLAG-p73β (*B*), or FLAG-p53 (*C*) and 50 ng of the GFP expression plasmid with or without increasing amounts of the expression plasmid for FLAG-PKA-Cβ (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-p73α, FLAG-p73β, or FLAG-p53 with or without increasing amounts of the expression plasmid for FLAG-PKA-Cβ, and the protein levels of endogenous p21^{WAF1} were determined by immunoblotting. As shown in Fig. 4*A*, endogenous p21^{WAF1} was increased by ectopic FLAG-p73α expression, whereas overexpression of FLAG-PKA-Cβ resulted in a reduction in the level of endogenous p21^{WAF1} induced by FLAG-p73α, supporting the notion that PKA-Cβ inhibits the transcriptional activity of p73α. In contrast, PKA-Cβ had no detectable effects on the p73β- 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-p73α and FLAG-PKA-Cβ resulted in a slight increase in the amounts of FLAG-p73α, whereas FLAG-PKA-Cβ had a negligible effect on the amounts of FLAG-p73β and FLAG-p53 (Fig. 4). FLAG-p73α decayed at slower rates in the presence of FLAG-PKA-Cβ than in its absence (data not shown); however, its physiological implications remain to be determined.

PKA-Cβ Phosphorylates p73—To determine whether p73 could be a substrate for PKA-Cβ, the GST-p73α 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-^{32}P]ATP. Of the GST-p73α fusion proteins tested, only GST-p73α-(1–130) was phosphorylated by the PKA catalytic subunit (Fig. 5*A*). The N-terminal region of p73α might be involved in phosphorylation by the PKA catalytic subunit.

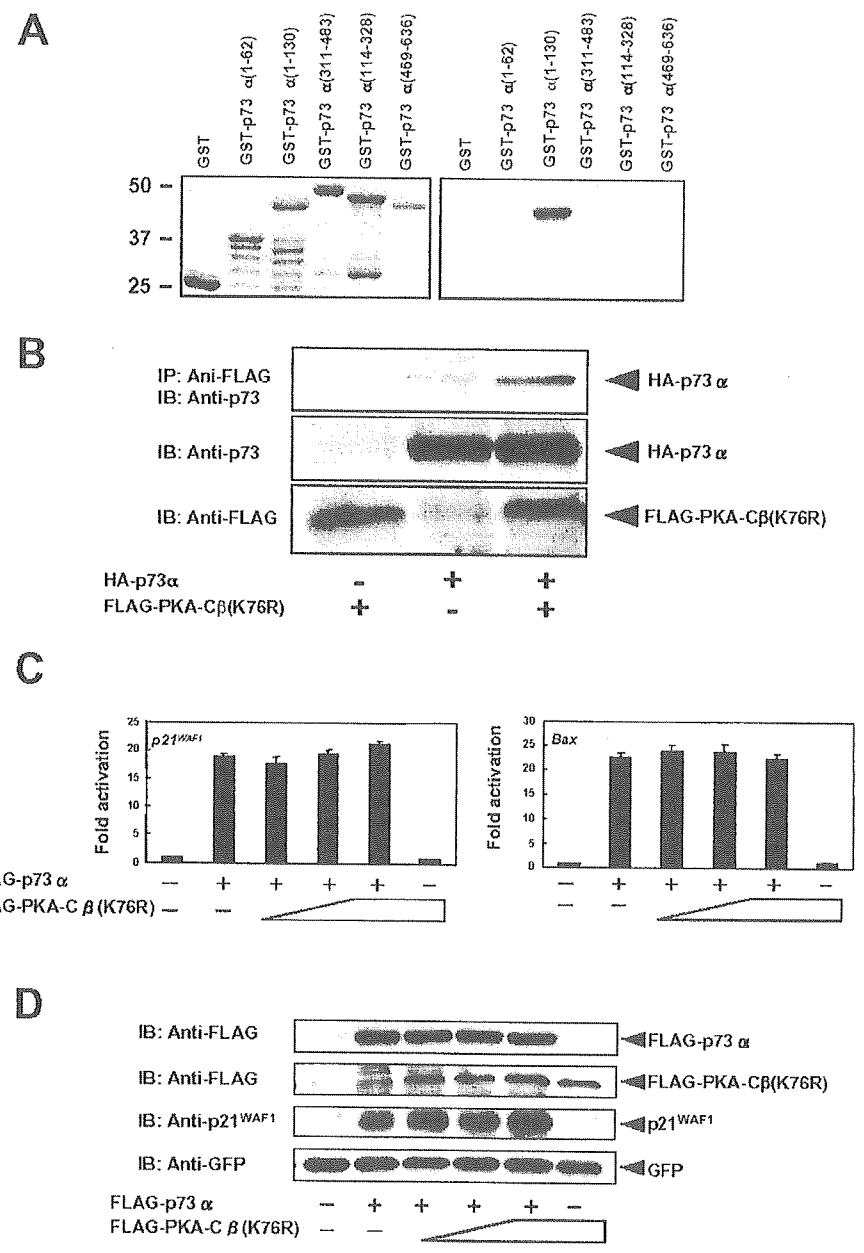

FIG. 5. **PKA-Cβ phosphorylates p73α, and the kinase-deficient mutant of PKA-Cβ fails to inhibit the transcriptional activity of p73α.** *A*, PKA-Cβ can phosphorylate p73α *in vitro*. GST or GST-p73α fusion proteins bound to glutathione-Sepharose beads were incubated with the purified catalytic subunit of PKA in the presence of [γ-^{32}P]ATP for 30 min at 30 °C. Samples were then directly boiled in 2× 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-p73α 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-Cβ retains the ability to interact with p73α. 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-p73α, 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-Cβ(K76R). Forty-eight hours after transfection, luciferase activity was determined as described in the legend to Fig. 4. *D*, PKA-Cβ(K76R) has no detectable effect on the p73α-dependent induction of endogenous p21^{WAF1}. H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for FLAG-p73α and 50 ng of the GFP expression plasmid with or without increasing amounts of the expression plasmid for FLAG-PKA-Cβ(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-Cβ on the transcriptional activity of p73α is dependent on its kinase activity. As described previously (33, 34), PKA-Cβ(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-Cβ(K76R) and tested whether PKA-Cβ(K76R) could bind to p73α and also repress p73α-mediated transcriptional activation. Co-immunoprecipitation experiments demonstrated that, like wild-type PKA-Cβ, the kinase-deficient form of PKA-Cβ bound to FLAG-p73α in cells (Fig. 5*B*). Notably, luciferase reporter analysis revealed that FLAG-PKA-Cβ(K76R) had little effect on the ability of p73α to drive transcription from the p21^{WAF1} and *bax* promoters (Fig. 5*C*). In accordance with the results from luciferase reporter analysis, FLAG-PKA-Cβ(K76R) failed to reduce the expression levels of endogenous p21^{WAF1} induced by FLAG-p73α as examined by immunoblotting (Fig. 5*D*). Taken together, these results strongly suggest that PKA-Cβ inhibits p73α-mediated transcriptional activation by a kinase activity-dependent mechanism.

Reduction in the Pro-apoptotic Activity of p73α by PKA-Cβ upon DNA Damage—To extend the functional consequences of the interaction between p73α and PKA-Cβ, we investigated whether PKA-Cβ could affect the pro-apoptotic function of p73α 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 p73α. H1299 cells were transiently cotransfected with the expression plasmid for FLAG-p73α or FLAG-p53 with or without the expression plasmid encoding FLAG-PKA-Cβ or FLAG-PKA-Cβ(K76R) and then treated with camptothecin at a final concentration of 1 μM for 24 h. After camptothecin action, cell viability was examined by cell survival assay. As shown in Fig. 6*A*, H1299 cells expressing FLAG-p73α alone exhibited an enhanced sensitivity to apoptosis following exposure to camptothecin, which was consistent with previous observations (35). Of note, coexpression of FLAG-PKA-Cβ and FLAG-p73α resulted in a reduction in the cellular

sensitivity to camptothecin, whereas kinase-deficient PKA-Cβ had no significant effect on cell viability. As was also observed in H1299 cells expressing FLAG-p73α, ectopic expression of FLAG-p53 enhanced camptothecin-induced apoptosis (Fig. 6*B*). In sharp contrast to p73α, wild-type or kinase-deficient PKA-Cβ had a negligible effect on p53.

cAMP Analog Inhibits p73α-mediated Transcriptional Activation—Given the inhibitory effect of exogenous PKA-Cβ on p73α in transfected cells, we sought to determine whether the activation of PKA attenuates p73α-mediated transcriptional activation. H1299 cells were transiently cotransfected with or without the expression plasmid for HA-p73α 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 (Bt_2cAMP) in the presence or absence of the PKA inhibitor H-89. As shown in Fig. 7*A*, Bt_2cAMP treatment inhibited p73α-induced $p21^{WAF1}$ and *bax* promoter activation. Intriguingly, the inhibitory effect of Bt_2cAMP was attenuated when cells were exposed to H-89. Under the identical experimental conditions, endogenous $p21^{WAF1}$ was significantly induced by exogenously expressed HA-p73α (Fig. 7*B*). Densitometric scanning of the immunoblot revealed that Bt_2cAMP treatment decreased the level of $p21^{WAF1}$ by 29% relative to that induced by HA-p73α, 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 p73α-mediated transcriptional activation.

PKA-Cβ Stimulates the Intramolecular Interaction of p73—To clarify the precise molecular mechanism by which PKA-Cβ impairs the transcriptional activity of p73α, we performed ChIP analysis. Cross-linked chromatin prepared from H1299 cells transiently co-transfected 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-p73α was efficiently recruited to the $p21^{WAF1}$ and *bax* promoters in the absence of exogenous PKA-Cβ (Fig. 8*A*). No significant decrease in chromatin binding was detected in cells expressing HA-p73α and FLAG-PKA-Cβ, suggesting that PKA-Cβ has little effect on the sequence-specific DNA binding activity of p73α.

It has been reported recently that the extreme C-terminal regions of p73α and p63α (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 p73α could be involved in the PKA-Cβ-mediated down-regulation of p73α, we performed additional luciferase reporter analyses in H1299 cells cotransfected with the expression plasmid for HA-p73α-(1–548) and FLAG-PKA-Cβ. As shown in Fig. 8*B* (*upper panel*), HA-p73α-(1–548), which lacks the extreme C-terminal extension of wild-type p73α, interacted with FLAG-PKA-Cβ as determined by co-immunoprecipitation experiments. It is worth noting that, in contrast to wild-type p73α, FLAG-PKA-Cβ had no detectable effect on the transcriptional activity of HA-p73α-(1–548) (Fig. 8*B*, *lower panel*), indicating that the extreme C-terminal region of p73α plays a critical role in the PKA-Cβ-mediated inhibition of p73α.

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-Cβ interacts with p73α through its N- and C-terminal domains, it is possible that PKA-Cβ could stimulate the intramolecular interaction between the two domains of p73α, thereby inhibiting its transcriptional activity. To test this possibility, 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-Cβ on the complex formation between HA-p73α-(1–247) and FLAG-p73α-(247–636) was examined. The anti-p73 antibody used for this assay recognizes the C-terminal portion of p73 and thus does not detect p73α-(1–247). As shown in Fig. 8*C*, HA-p73α-(1–247) efficiently co-immunoprecipitated with FLAG-p73α-(247–636) in the presence of FLAG-PKA-Cβ, whereas FLAG-PKA-Cβ(K76R) had a negligible effect on the complex formation between HA-p73α-(1–247) and FLAG-p73α-(247–636). The few complexes observed in the absence of FLAG-PKA-Cβ could be due to endogenous PKA-Cβ. These results strongly suggest that FLAG-PKA-Cβ contributes to the intramolecular interaction of p73α between the N-terminal transactivation and C-terminal inhibitory domains.

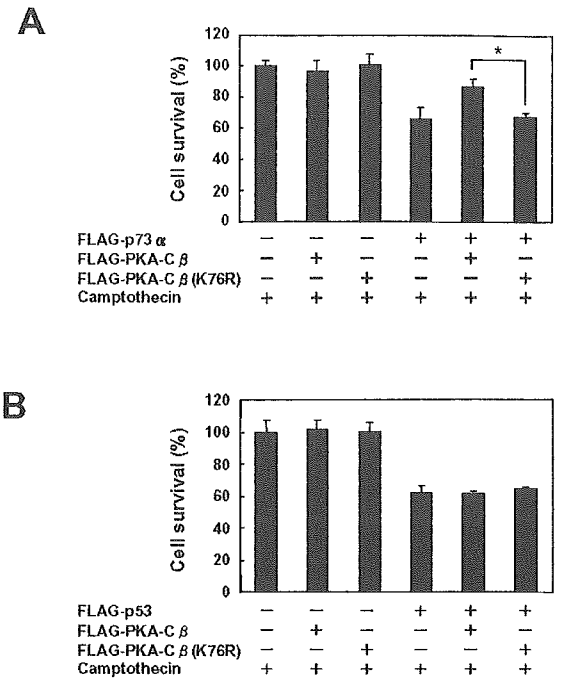

FIG. 6. **p73α-mediated increase in sensitivity to camptothecin is suppressed by wild-type PKA-Cβ, but not by kinase-deficient PKA-Cβ.** H1299 cells were transiently cotransfected with the expression plasmid for FLAG-p73α (*A*) or FLAG-p53 (*B*) with or without the expression plasmid encoding FLAG-PKA-Cβ or FLAG-PKA-Cβ(K76R). Twenty-four hours after transfection, cells were exposed to camptothecin (final concentration of 1 μM) 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-Cβ.

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-Cβ as a p73α-binding protein. PKA-Cβ associated with p73α through its N- and C-terminal regions in mammalian cultured cells and significantly inhibited its transactivation function. Under our experimental conditions, PKA-Cβ, which did not bind to p53, had a negligible effect on p53. Intriguingly, PKA-Cβ might bridge the N-terminal transactivation and C-terminal inhibitory domains of p73α, thereby rendering p73α a latent inactive form. *In vitro* kinase assay demonstrated that PKA can phosphorylate p73, and the kinase-deficient mutant of PKA-Cβ (PKA-Cβ(K76R)) failed to

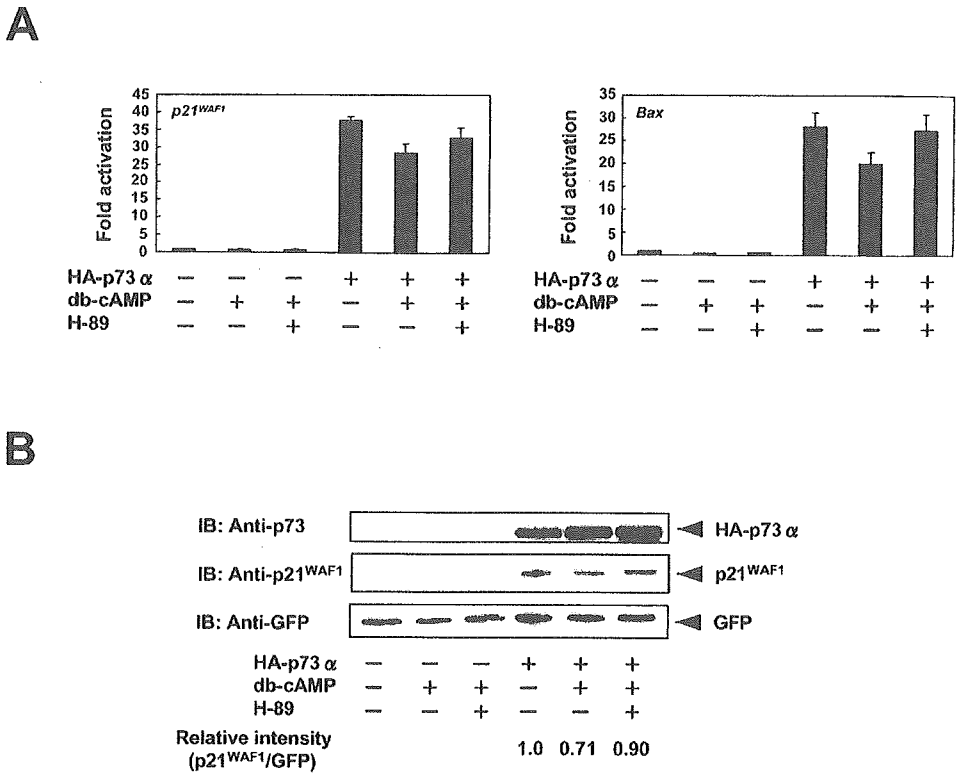

FIG. 7. **Effects of Bt_2cAMP on p73α-mediated transcriptional activation.** *A*, luciferase reporter analysis. H1299 cells were transiently cotransfected with 25 ng of the expression plasmid for HA-p73α, 100 ng of the luciferase reporter construct containing the p53/p73-responsive element derived from 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 Bt_2cAMP (*db-cAMP*; 1 mM) or with Bt_2cAMP (1 mM) plus the PKA inhibitor H-89 (10 μM) 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-p73α and 50 ng of the GFP expression plasmid. Twenty-four hours after transfection, cells were treated with or without Bt_2cAMP (1 mM) in the presence or absence of H-89 (10 μM) 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 p73α, suggesting that the kinase activity of PKA-Cβ is required for its inhibitory effect on p73α. In accordance with these results, the transient activation of the cAMP/PKA signaling pathway by Bt_2cAMP reduced p73α-mediated transcriptional activation, whereas the inhibitory effect of Bt_2cAMP 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 p73α might be a novel inhibitory mechanism of its activity.

As described previously (38), the PKA catalytic subunit family is composed of three isoforms: PKA-Cα, PKA-Cβ, and PKA-Cγ. PKA-Cα is expressed ubiquitously, whereas PKA-Cβ is expressed predominantly in brain and reproductive tissues (31, 32). PKA-Cβ is expressed as at least six variants (Cβ1, Cβ2, Cβ3, Cβ4, Cβ4ab, and Cβ4abc) 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-Cβ we identified in this study is PKA-Cβ4ab. According to our *in vitro* phosphorylation assay using various truncated forms of GST-p73α as substrates, the N-terminal region of p73α (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 p73α for a putative PKA recognition site(s) showed three related motifs (^{78}RAAS^{81}, ^{164}KVST^{167}, and ^{402}KLPS^{405}). Ser-81 exists in the N-terminal region of p73α. 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-Cβ(K76R), which retained the ability to bind to p73α, failed to inhibit p73α-mediated transcriptional activation, it is conceivable that the PKA-dependent phosphorylation of p73α 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 Cδ 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 p73α by PKA is necessary to confirm the functional significance of the PKA-mediated phosphorylation of p73α.

We (36) and others (7, 43) reported that p73α exhibits a low level of transactivation ability relative to that of p73β, suggesting that the C-terminal extension of p73α 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

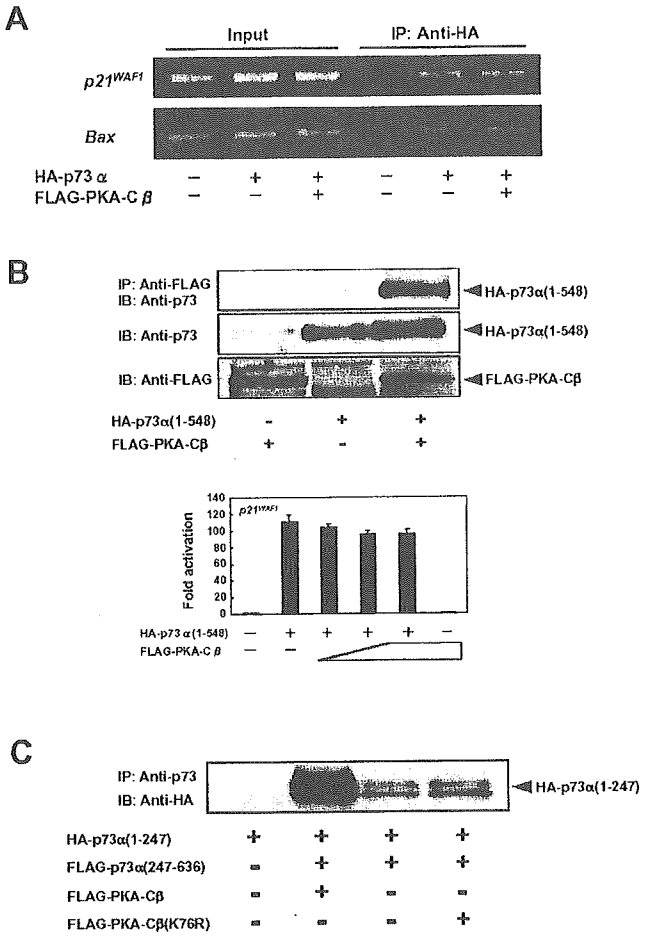

FIG. 8. **PKA-Cβ-mediated intramolecular interaction of p73α contributes to the down-regulation of the transcriptional activity of p73α.** *A*, ChIP assay. H1299 cells were transiently cotransfected with the expression plasmid for HA-p73α or HA-p73α plus FLAG-PKA-Cβ. Thirty-six hours after transfection, cells were fixed in formaldehyde and lysed, and DNA was sheared into 200–500-bp fragments by sonication. HA-p73α-bound DNA was immunoprecipitated (*IP*) with anti-HA monoclonal antibody. The amounts of HA-p73α 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-Cβ fails to inhibit p73α-(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-p73α-(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-Cβ (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 p73α is stimulated by PKA-Cβ. 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 p63α is significantly inhibited by an intramolecular interaction. In sharp contrast to p53, the extreme C-terminal region of p63α 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 p73α and p63α 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-Cβ bound to the N- and C-terminal regions of p73α. Furthermore, the co-immunoprecipitation experiments demonstrated that p73α-(1–247) efficiently coprecipitated with p73α-(247–636) in the presence of PKA-Cβ, suggesting that PKA-Cβ might promote the intramolecular interaction of p73α 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-Cβ had no significant effect on the DNA binding activity of p73α. Because the kinase-deficient mutant of PKA-Cβ failed to bridge p73α-(1–247) and p73α-(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 (xtracellular 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-Cβ. According to their results, constitutive expression of PKA-Cβ results in the promotion of colony formation in soft agar medium, and PKA-Cβ- 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-3β 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.

Acknowledgments—We thank members of our laboratory for stimulating discussions and Yuki Nakamura for excellent technical assistance.

REFERENCES

1. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell* **90,** 809–819
2. Jost, C. A., Marin, M. C., and Kaelin, W. G., Jr. (1997) *Nature* **389,** 191–194
3. De Laurenzi, V., Costanzo, A., Barcaroli, D., Torrinoni, A., Falclco, M., Annicchiarico-Petruzzelli, M., Levrero, M., and Melino, G. (1998) *J. Exp. Med.* **188,** 1763–1768
4. Di Como, C. J., Gaiddon, C., and Prives, C. (1999) *Mol. Cell. Biol.* **19,** 1438–1449
5. Steegenga, W. T., Shvarts, A., Riteco, N., Bos, J. L., and Jochemsen, A. G. (1999) *Mol. Cell. Biol.* **19,** 3885–3894
6. Zeng, X., Chen, L., Jost, C. A., Maya, R., Keller, D., Wang, X., Kaelin, W. G., Jr., Oren, M., Chen, J., and Lu, H. (1999) *Mol. Cell. Biol.* **19,** 3257–3266
7. Ueda, Y., Hijikata, M., Takagi, S., Chiba, T., and Shimotohno, K. (1999) *Oncogene* **18,** 4993–4998
8. De Laurenzi, V., Catanl, M. V., Teminoni, A., Corazzari, M., Melino, G., Costanzo, A., Levrero, M., and Knight, R. A. (1999) *Cell Death Differ.* **6,** 389–390
9. Ishimoto, O., Kawahara, C., Enjo, K., Obinata, M., Nukiwa, T., and Ikawa, S. (2002) *Cancer Res.* **62,** 636–641
10. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkens, P., Sharpe, A., McKeon, F., and Caput, D. (2000) *Nature* **404,** 99–103
11. Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R., and Miller, F. D. (2000) *Science* **289,** 304–306
12. Stiewe, T., Zimmermann, S., Frilling, A., Esche, H., and Putzer, B. M. (2002) *Cancer Res.* **62,** 3598–3602
13. Grob, T. J., Novak, U., Maisse, C., Barcaroli, D., Luthi, A. U., Pirnia, F., Hugli, B., Graber, H. U., De Laurenzi, V., Fey, M. F., Melino, G., and Tobler, A. (2001) *Cell Death Differ.* **8,** 1213–1223
14. Nakagawa, T., Takahashi, M., Ozaki, T., Watanabe, K., Todo, S., Mizuguchi, H., Hayakawa, T., and Nakagawara, A. (2002) *Mol. Cell. Biol.* **22,** 2575–2585

15. Zaika, A. I., Slade, N., Erster, S. H., Sansome, C., Joseph, T. W., Pearl, M., Chalas, E., and Moll, U. M. (2002) *J. Exp. Med.* **196,** 765–780
16. Gong, J., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G., Jr., Levrero, M., and Wang, J. Y. (1999) *Nature* **399,** 806–809
17. Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999) *Nature* **399,** 809–813
18. Yuan, Z.-M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y. Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999) *Nature* **399,** 814–817
19. Ren, J., Datta, R., Shioya, H., Li, Y., Oki, E., Biedermann, V., Bharti, A., and Kufe, D. (2002) *J. Biol. Chem.* **277,** 33758–33765
20. Yuan, Z.-M., Utsugisawa, T., Ishiko, T., Nakada, S., Huang, Y., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1998) *Oncogene* **16,** 1643–1648
21. Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000) *J. Biol. Chem.* **275,** 7470–7473
22. Sanchez, Y., Wong, S., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) *Science* **277,** 1497–1501
23. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMaya, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) *Genes Dev.* **14,** 1448–1459
24. Zhao, H., and Piwnica-Worms, H. (2001) *Mol. Cell. Biol.* **21,** 4129–4139
25. Gonzalez, S., Prives, C., and Cordon-Cardo, C. (2003) *Mol. Cell. Biol.* **23,** 8161–8171
26. Zeng, X., Li, X., Miller, A., Yuan, Z., Yuan, W., Kwok, R. P., Goodman, R., and Lu, H. (2000) *Mol. Cell. Biol.* **20,** 1299–1310
27. Costanzo, A., Melro, P., Pediconi, N., Fulco, M., Sartorelli, V., Cole, P. A., Fontemaggi, G., Fanciulli, M., Schiltz, L., Blandino, G., Balsano, C., and Levrero, M. (2002) *Mol. Cell* **9,** 175–186
28. Mantovani, F., Piazza, S., Gostissa, M., Strano, S., Zacchi, P., Mantovani, R., Blandino, G., and Del Sal, G. (2004) *Mol. Cell* **14,** 625–636
29. Watanabe, K., Ozaki, T., Nakagawa, T., Miyazaki, K., Takahashi, M., Hosoda, M., Hayashi, S., Todo, S., and Nakagawara, A. (2002) *J. Biol. Chem.* **277,** 15113–15123
30. Nakamura, Y., Ozaki, T., Nakagawara, A., and Sakiyama, S. (1997) *Eur. J. Cancer* **33,** 1986–1990
31. Uhler, M. D., Chrivia, J. C., and McKnight, G. S. (1986) *J. Biol. Chem.* **261,** 15360–15463
32. Cadd, G., and McKnight, G. S. (1989) *Neuron* **3,** 71–79
33. Taylor, S. S., Buechler, J. A., and Yenemoto, W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
34. Scott, J. D. (1991) *Pharmacol. Ther.* **50,** 123–145
35. Irwin, M. S., Kondo, K., Marin, M. C., Cheng, L. S., Hahn, W. C., and Kaelin, W. G., Jr. (2003) *Cancer Cells* **3,** 403–410
36. Ozaki, T., Naka, M., Takada, N., Tada, M., Sakiyama, S., and Nakagawara, A. (1999) *Cancer Res.* **59,** 5902–5907
37. Serber, Z., Lai, H. C., Yang, A., Ou, H. D., Sigal, M. S., Kelly, A. E., Darimont, B. D., Duijf, P. H. G., van Bokhoven, H., McKeon, F., and Dotsch, V. (2002) *Mol. Cell. Biol.* **22,** 8601–8611
38. Skalhegg, B. S., and Tasken, K. (2000) *Front. Biosci.* **5,** 678–693
39. Ørstavik, S., Reinton, N., Frengen, E., Langeland, B. T., Jahnsen, T., and Skalhegg, B. S. (2001) *Eur. J. Biochem.* **268,** 5066–5073
40. Kemp, B. E., Graves, D. J., Benjamin, E., and Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 4888–4894
41. Higuchi, H., Yamashita, T., Yoshikawa, H., and Tohyama, M. (2003) *EMBO J.* **22,** 1790–1800
42. Gaiddon, C., Lokshin, M., Gross, I., Levasseur, D., Taya, Y., Loeffler, J.-P., and Prives, C. (2003) *J. Biol. Chem.* **278,** 27421–27431
43. Lee, C.-W., and La Thangue, N. B. (1999) *Oncogene* **18,** 4171–4181
44. Yang, A., and McKeon, F. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 199–207
45. Friend, S. (1994) *Science* **265,** 334–335
46. Hupp, T. R., and Lane, D. P. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59,** 195–206
47. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83,** 237–245
48. Smets, L. A., and Van Rooy, H. (1987) *J. Cell. Physiol.* **133,** 395–399
49. Zachary, I., Masters, S. B., and Bourne, H. R. (1990) *Biochem. Biophys. Res. Commun.* **168,** 1184–1193
50. Chen, J., and Iyengar, R. (1994) *Science* **263,** 1278–1281
51. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1331
52. Anderson, C. N., and Tolkovsky, A. M. (1999) *J. Neurosci.* **19,** 664–673
53. Sable, C. L., Filippa, N., Hemmings, B., and Van Obberghen, E. (1997) *FEBS Lett.* **409,** 253–257
54. Filippa, M., Sable, C. L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999) *Mol. Cell. Biol.* **19,** 4989–5000
55. Wu, K.-J., Mattioli, M. M., Morse, H. C., III, and Dalla-Favera, R. (2002) *Oncogene* **21,** 7872–7882
56. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J.-S., Taylor, S. S., Scott, J. D., and Korsmeyer, S .J. (1999) *Mol. Cell* **3,** 413–422
57. Li, M., Wang, X., Meintzer, M. K., Laessig, T., Birnbaum, M. J., and Heidenreich, K. A. (2000) *Mol. Cell. Biol.* **20,** 9356–9363

American Journal of Pathology, Vol. 167, No. 1, July 2005

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, Sagamihara-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 proliferation, 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-

Supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan. Y.O-H. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan.

Accepted for publication March 23, 2005.

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 linage 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 activated[25] 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% CO_2 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.

Immunocytostaining

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 *ALK*‡	Amplification of N-*myc* (*n*)
		Location	Clinical stage†		
1	3y5m	Adrenal gland	IV	2.0 ± 0.2	+ (35)
2	5y0m	Peritoneum	IV	1.8 ± 0.1	+ (>150)
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 αALK 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 μg 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 μl of serum-free growth medium and 4 μl of jetSI per well were preincubated for 5 to 10 minutes at room temperature. While the incubation was being performed, 100 μl of serum-free growth medium was mixed with 5 μl of 20 μmol/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 μl of jetSI serum-free medium solution was added to the 100 μl of siRNA

duplex solution, gently mixed, and incubated for 30 minutes at room temperature. The growth medium on the cells was removed, and 800 μl of serum-free medium was added to each well. A total of 200 μl of the entire mixture was overlaid onto the cells, and cells were incubated for 4 hours at 37°C in a 5% CO_2 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× 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 αALK 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 μl of TE lysis buffer. The lysates were incubated with 5 μl of enzyme A (RNase A) at 37°C for 10 minutes and then at 55°C for 30 minutes after the addition of 5 μl of Enzyme B (Proteinase K). Afterward, 5 μl of ammonium acetate solution and 100 μl 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 μl 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 (αALK) (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 evaluation[31] 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 (αALK) 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

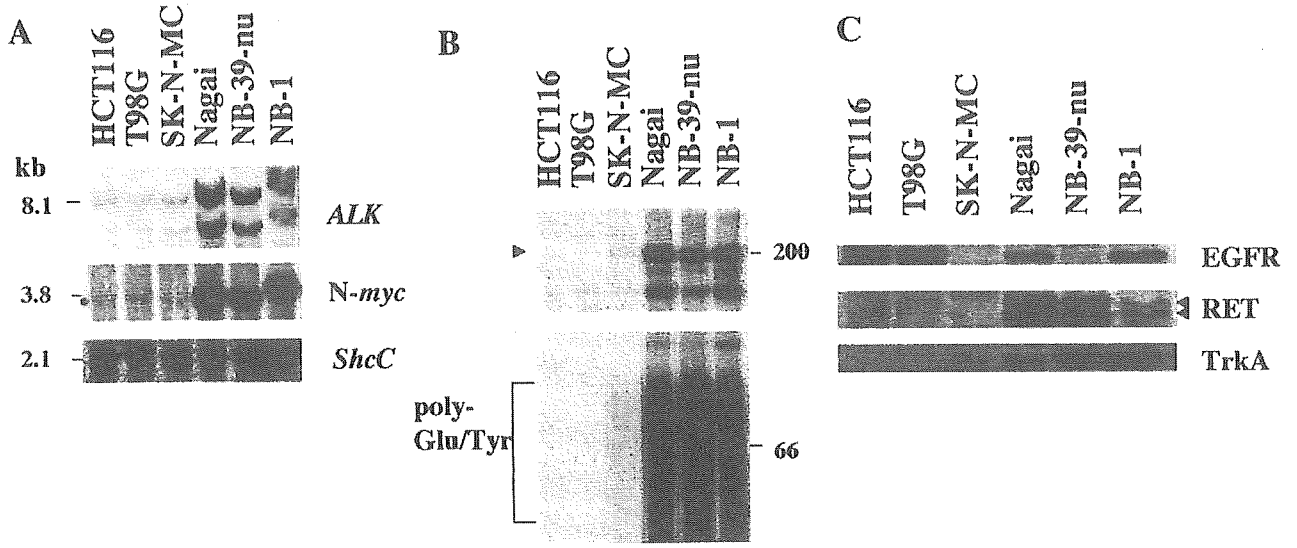

Figure 1. Marked gene amplification of the *ALK* locus and significant elevation of kinase activity of ALK in NB-39-nu, Nagai, and NB-1 cells. **A:** To detect ALK gene amplification, samples of 10 μg of DNA were digested with *Eco*RI. Fragments of about 2.5, 3.1, 6.1, and 8.1 kb were detected using the ^{32}P-labeled probe prepared as previously described.[27] Amplification of the N-*myc* gene was detected using the same filter re-hybridized with the probe for N-*myc*. As a control for the amounts of DNA, the same filter was re-hybridized with the probe for *ShcC*. **B:** *In vitro* kinase assay of ALK in neuroblastoma cells immunoprecipitated with αALK was performed as previously described.[27] Kinase reaction was performed without (**top panel**) or with (**bottom panel**) poly-Glu/Tyr (4:1) as exogenous substrates. Autophosphorylated ALK protein is marked by an **arrow**. Phosphorylated poly-Glu/Tyr is detected as smear indicated by the bracket. **C:** The expression patterns of other receptor tyrosine kinases in neuroblastoma cell lines. Each cell line was harvested, and about 30 μg of whole-cell lysates was subjected to Western blot analysis using the antibodies as indicated on the right. RET proteins are marked by **arrows**.

other types of solid tumor cell lines used as controls. *In vitro* kinase assay revealed outstanding ALK kinase activity in these three cell lines compared with other cells (Figure 1B), which is consistent with our previous study.[27] To examine whether overexpressed and activated ALK affects the expression of other RTKs in these cells, protein expression levels of RTKs, including EGFR, Ret, and TrkA, are compared with other cell lines. Significantly high levels of expression of EGFR and TrkA were observed in two of three cell lines overexpressing ALK (Figure 1C, top and bottom). Ret expression was commonly elevated in all three cell lines with activated ALK, especially in Nagai and NB-39-nu (Figure 1C, middle), consistent with previous study by Northern blotting.[32] Although it is unknown whether overexpression of these RTKs is related to overexpression of ALK, no obvious down-regulation of other RTKs was found in these *ALK*-amplified cell lines.

Inhibition of Activated ShcC, MAPKs, and Akt by Suppressing Activated ALK

To investigate the effect of suppressing the ALK expression level in *ALK*-amplified neuroblastoma cells using the RNAi technique, we synthesized two different RNA duplexes directed against nucleotide positions 153 to 171 and 399 to 417 within coding region *ALK* cDNA (ALK-siRNA1 and ALK-siRNA2, respectively). Because cotransfection of ALK-siRNA1 and ALK-siRNA2 was very effective in suppressing ALK expression, we performed all experiments presented here using a combination of two siRNAs, although similar results were obtained using only ALK-siRNA2. A sequence against the firefly luciferase gene (luc-siRNA) was used as a negative control. The expression of ALK protein is remarkably elevated in NB-39-nu and Nagai compared with other neuroblastoma cell lines, such as SK-M-MC (Figure 2A), caused by gene amplification.[27] The RNA duplexes were transfected into NB-39-nu cells with *ALK* gene amplification and SK-N-MC cells containing only a single copy of the *ALK* gene. We also tried to introduce ALK-siRNAs in several different neuroblastoma cell lines with or without *ALK* amplification in addition to NB-39-nu and SK-N-MC cells, resulting in partial or no reduction of ALK expression presumably due to the unsuccessful introduction in those cells. Therefore, we decided to use these two cell lines to perform further analysis of the effect of ALK knockdown by RNAi technique. RT-PCR analysis revealed that ALK mRNA level was reduced in both NB-39-nu cells and SK-N-MC cells treated with ALK-siRNAs, not in the cells treated with luc-siRNA and s-siRNA (Figure 2B). Both expression and phosphorylation of ALK kinase were significantly suppressed in the NB-39-nu cells treated with ALK-siRNAs compared with a mock-transfection control or cells treated with luc-siRNA (Figure 2C). In these cells, phosphorylation of ShcC was also suppressed despite the unchanged total amount of ShcC (Figure 2C), demonstrating that ShcC is a potent substrate of activated ALK kinase and that activation of ALK is actually responsible for the hyperphosphorylation of ShcC in these cancer cells. While the expression of downstream molecules, such as p44/42 MAPKs and Akt, was not affected by ALK-siRNAs, phosphorylation of these molecules was markedly reduced (Figure 2C). These results suggest that the Ras-MAPK pathway and the phosphatidylinositol 3-kinase/Akt pathway are dominantly regulated by activated ALK kinase in these cells. Interestingly, in SK-N-MC cells treated with ALK-siRNAs, phosphorylation levels of ShcC, p44/42 MAPKs, and Akt were not affected by

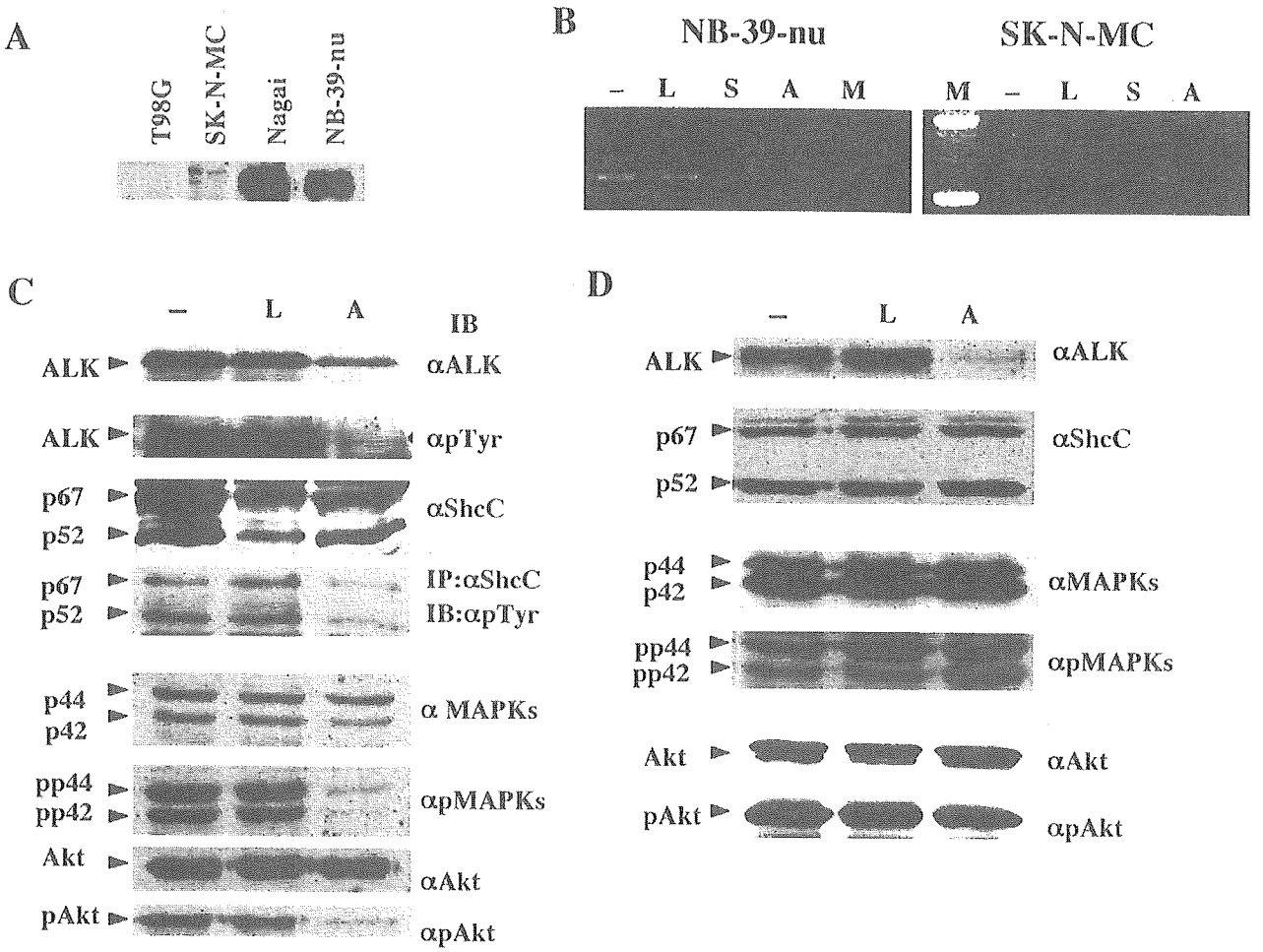

Figure 2. Suppression of ALK expression by siRNAs and changes in downstream molecules NB-39-nu cells and SK-N-MC cells. **A:** Expression levels of ALK protein in neuroblastoma cell lines including NB-39-nu and SK-N-MC. Each cell line was harvested, and about 30 μg of whole-cell lysates was subjected to Western blot analysis using αALK. **B:** mRNA levels of *Alk* in NB-39-nu cells. The cells were lysed at 84 hours after transfection and analyzed by RT-PCR. −, mock transfection; L, cells treated with luc-siRNA; S, cells treated with s-siRNA; A, cells treated with ALK-siRNAs; M, marker. **C:** NB-39-nu cells were harvested 84 hours after transfection. About 10 μg of whole-cell lysates or 250 μg of lysates immunoprecipitated with αShcC was subjected to Western blot analysis using the antibodies as indicated on the right. −, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs. **D:** SK-N-MC cells were harvested 48 hours after transfection. About 10 μg of whole-cell lysates was subjected to Western blot analysis using the antibodies as indicated on the right. Bands of ShcC are marked by **arrows**. −, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs.

ALK-siRNAs despite further suppression of the basal ALK expression level (Figure 2D), indicating that these pathways are not under the control of ALK in SK-N-MC cells.

Induction of Apoptosis by Suppression of Activated ALK

At 84 hours after transfection, apoptotic morphological changes, such as cell rounding, cytoplasmic blebbing, and irregularities of shape, were observed in NB-39-nu cells treated with ALK-siRNAs, whereas no significant changes were seen in the mock-transfected cells or in the luc-siRNA and the s-siRNA treated cells (Figure 3A). These morphological changes were not observed in SK-N-MC cells treated with ALK-siRNAs (data not shown). At 90 hours after transfection, NB-39-nu cells treated with ALK-siRNAs started to detach from the dish due to cell death.

To examine the localization of expression of ALK kinase, we performed double staining by anti-ALK antibody and TOTO-3, which stains the nucleus, in several neuroblastic cell lines. As shown in Figure 1D, unexpectedly, ALK protein overexpressed in NB-39-nu cells is localized in both membrane and cytoplasm. ALK staining was very weak in cell lines such as YT-nu and SK-N-MC with one copy of the *ALK* gene, however, its localization appeared to be the same as in NB-39-nu (data not shown). It was observed that the expression of ALK was completely lost after the RNAi-induced suppression of ALK (Figure 3C, top). To confirm whether the cell death resulted from apoptosis, cells were also analyzed by immunofluorescent TUNEL staining in NB-39-nu cells. TUNEL staining was clearly positive in these cells at 84 hours after transfection (Figure 3C, middle), indicating that apoptosis was induced in NB-39-nu cells treated with ALK-siRNAs. No significant TUNEL staining was observed in the mock-transfected cells or the luc-siRNA treated cells. Finally, DNA fragmentation assay was performed to measure the endonuclease activity accompa-

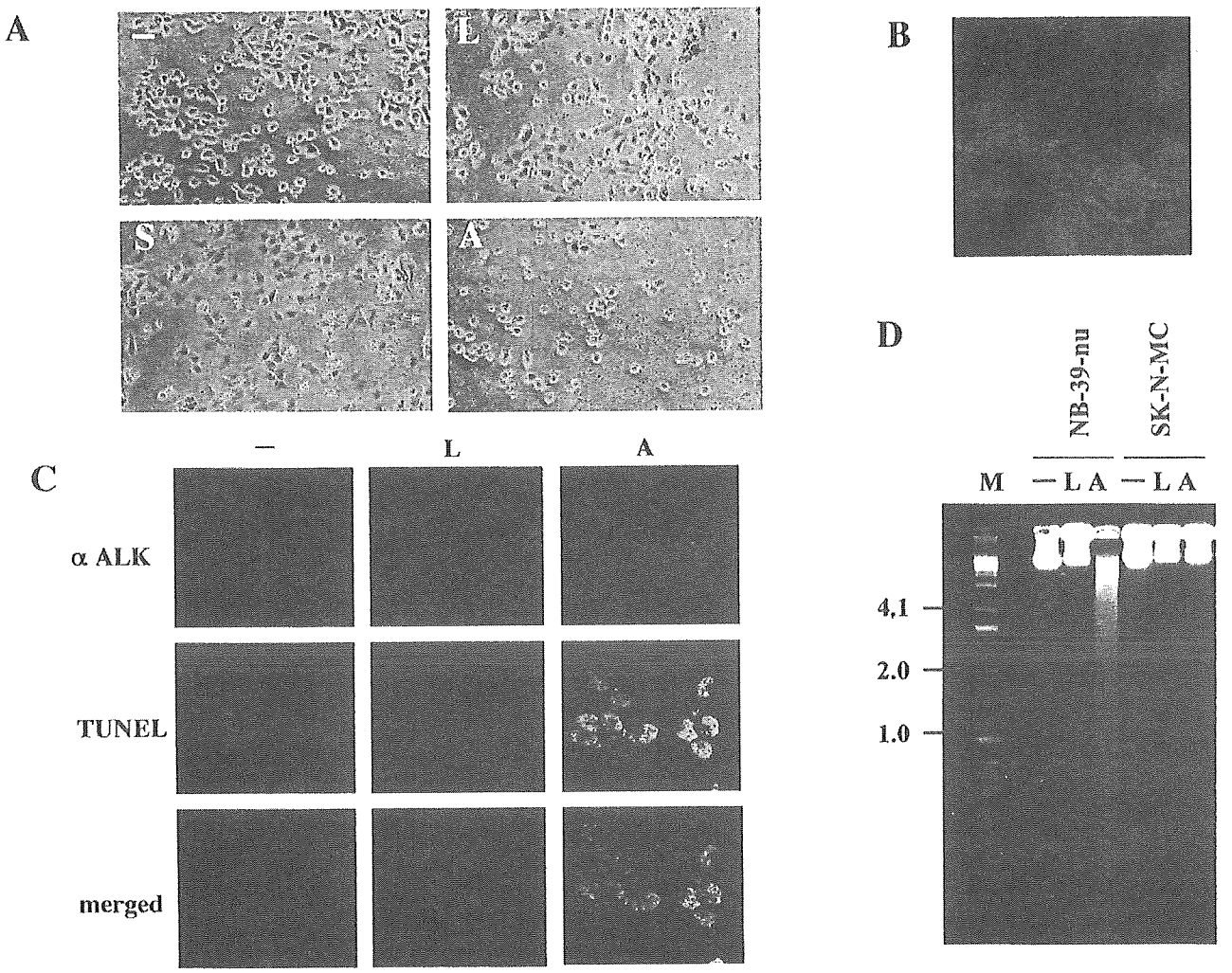

Figure 3. Induction of apoptosis in NB-39-nu cells treated with ALK-siRNAs. **A:** NB-39-nu cells on the dish were observed 84 hours after transfection under a light microscope. −, mock transfection; L, cells treated with luc-siRNA; S, cells treated with s-siRNA; A, cells treated with ALK-siRNAs. **B:** Cytoplasmic expression of ALK by immunocytostaining. The cells were stained for the expression of ALK (red) and apoptotic cells by TOTO-3 (blue). **C:** Cells on 24-well plates were fixed, and TUNEL assay was followed by staining with αALK (GST). The cells were stained for the expression of ALK (red) and apoptotic cells by TUNEL (green). −, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs. **D:** DNA fragmentation assay in NB-39-nu cells and SK-N-MC cells treated with siRNAs. Genomic DNA was extracted 84 hours and 48 hours after transfection in NB-39-nu and in SK-N-MC, respectively. They were analyzed using electrophoresis. −, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs; M, marker.

nied by apoptosis. The formation of significant DNA fragmentation was observed in the NB-39-nu cells but not in SK-N-MC cells treated with ALK-siRNAs (Figure 3D), indicating that cell apoptosis was induced through the suppression of ALK only in the NB-39-nu cells. This suggests that signaling pathways downstream of activated ALK dominantly regulate the survival of neuroblastoma cells with amplified ALK; therefore, the loss of ALK protein results in apoptotic changes to these cells.

Expression of ALK in Primary Neuroblastoma Tissues

Immunohistochemically, ALK was positively detected both in the cytoplasm of the neuroblastic cells and in the fine meshwork of neuropil of seven of nine tumors with favorable histology cases with nonamplified *N-myc* (FH&NA) (Figure 4, B and C). All seven unfavorable histology tumors (two UH&A tumors and five UH&NA tumors) were positive in the cytoplasm and/or in the fine meshwork of neuropil for ALK (Figure 4A). There was no correlation between the frequency or intensity of ALK-staining and histology of neuroblastoma tissues, showing majority of neuroblastoma samples showed a detectable amount of ALK. There was no significant staining using preimmune serum from the same rabbit as that for anti-ALK antibody (data not shown). Essentially the same results were obtained using a mouse monoclonal antibody against human ALK (ALK1: DAKO) (data not shown).

Amplification of the ALK Gene in Primary Neuroblastoma Tissues

It is essential to show whether ALK overexpression or gene amplification occurs in actual human neuroblastoma tissues in addition to neuroblastoma cell lines.

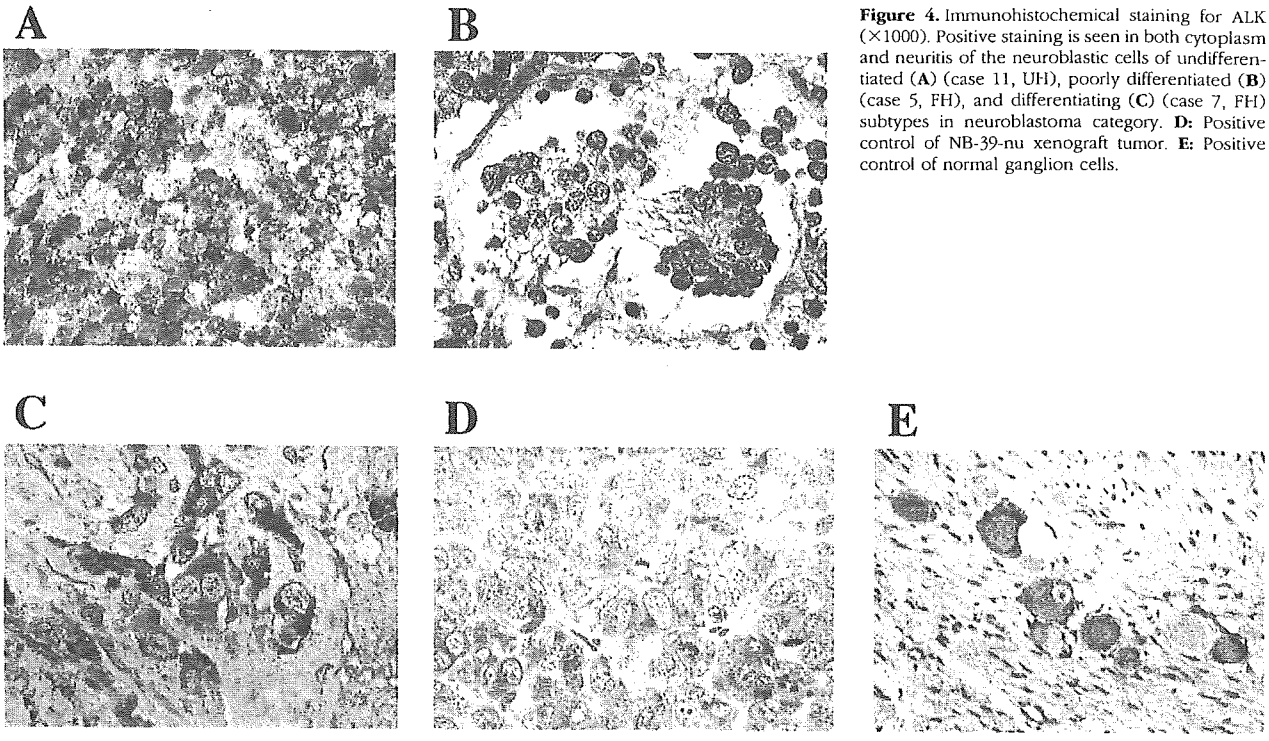

Figure 4. Immunohistochemical staining for ALK (×1000). Positive staining is seen in both cytoplasm and neuritis of the neuroblastic cells of undifferentiated (**A**) (case 11, UH), poorly differentiated (**B**) (case 5, FH), and differentiating (**C**) (case 7, FH) subtypes in neuroblastoma category. **D:** Positive control of NB-39-nu xenograft tumor. **E:** Positive control of normal ganglion cells.

Therefore, the mRNA amount of ALK kinase was first examined by RT-PCR on 32 primary neuroblastoma tissues (16 tissues with N-*myc* amplification and 16 tissues without N-*myc* amplification). Two of 32 cases showed slight elevation of *ALK* mRNA expression using several primer sets beyond the average expression level (data not shown).

To obtain more precise information about the copy numbers of *ALK*, we next analyzed the genomic DNAs of primary neuroblastoma tissues using Southern blot analysis. Whole purified DNA samples of tumors from 85 patients were examined. About the same number of N-*myc*-positive and N-*myc*-negative samples were collected to examine the relation between *Alk* and N-*myc* amplification. The intensities of signals on Southern blot membranes corresponding to the *ALK* gene and control *ShcC* gene, which is located on 9q22, were measured using a Molecular Imager FxPro (Bio-Rad), and the ratio of *ALK* signals to *ShcC* signals was calculated for each sample. Because more than 80% (70 samples) showed consistent ratios with each other in each experiment, these samples are treated as putative "single copy" controls. As several other samples showed apparently elevated intensity ratios, suggesting *ALK* amplification, relative copy numbers of *ALK* were calculated in comparison with average intensity ratios of putative single copy controls in each experiment. The results showed that there was significant *ALK* gene amplification in 8 of 85 patients (9.4%) (Figure 5). Seven of these eight cases, however, had only 1.8 to 3.0 copies of the *ALK* gene, suggesting a moderate gain of chromosomal focus rather than severe amplification. There was only one case that had outstanding amplification of ALK with approximately 10 copies. N-*myc* gene amplification was also detected in this case. The characteristics of the eight patients with *ALK* gain or amplification are shown in Table 1. Whereas seven of eight patients were classified as Stage III or IV (one as Stage III and six as Stage IV), the rest was classified as Stage I. The case with *ALK* amplification had N-*myc* amplification and was classified as Stage IV. Seven of eight patients were more than 1 year of age.

Discussion

Studies on ALK kinase demonstrate that activated ALK is involved in malignant tumor formation as forms of fusion proteins that force oligomerization of this kinase. We recently showed that the intact form of ALK protein is con-

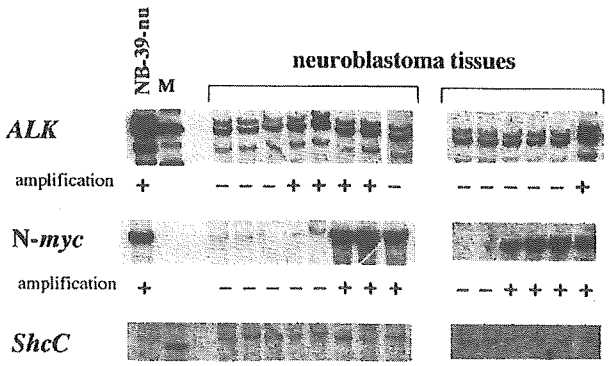

Figure 5. Detection of gene amplification of *ALK* and N-*myc* in primary neuroblastoma tissues. *ALK* was amplified in eight cases, and five of these eight cases are shown. The probe for *ALK* was removed from the filters, and the filters were re-hybridized in turn with other probes. Of eight cases with *ALK* amplification, N-*myc* amplification was detected in six cases and not detected in two cases. The probe for *ShcC* was used as a control for the amounts of DNA. M, marker.

stitutively activated by *ALK* gene amplification in three neuroblastoma cell lines, indicating a novel mechanism of activation of ALK kinase in malignancies.[27] In this study, amplification of the *ALK* gene was detected in primary neuroblastoma tissues for the first time. This suggests that activated ALK kinase plays a real role in the pathophysiology of neuroblastoma, such as giving a more malignant phenotype to the tumors by perturbing signal transduction. Recently, Motegi et al[33] showed that ALK transmits both mitogenic and differentiation signals, and that the MAPK pathway plays an important role in these effects in SK-N-SH cells without *ALK* gene amplification. Together with the fact that activated ALK surpasses regulation by other RTKs in cell lines with *ALK* gene amplification,[27] our new results showing apoptotic changes caused by the suppression of activated ALK protein clearly demonstrate the dominant role of ALK kinase in the survival of the *ALK*-amplified type of neuroblastoma.

The frequency and copy numbers of gene amplification of ALK were significantly lower in neuroblastic tumors compared with neuroblastic cell lines. Remarkable amplification of the *ALK* gene was detected in 1 tumor tissue of 85 tumor samples examined. Three neuroblastoma cell lines with *ALK* amplification had more than 30 copies of *ALK*, whereas primary neuroblastoma containing *ALK* gene amplification had within a range of 2 to 10 copies. This may be due to underestimation of the copy number in the tumor cells because of contamination of stromal cells and lymphocytes into the tumor tissues.[34,35] There may also be a mechanism in which cells with a higher copy number of *ALK* become the major population during the establishment of cell lines because of their growth advantage. Immunohistochemical analysis demonstrated, however, universal cytoplasmic expression of ALK in a wide range of neuroblastoma tumor samples, suggesting some transcriptional or posttranslational regulation of the ALK amount might exist in neuroblastoma cells. Although, due to the condition of the samples, we were unable to obtain information on the copy numbers of the *ALK* gene as for the samples used in the immunohistochemical analysis, further immunohistochemical screening may reveal neuroblastoma tissues with an outstanding amount of ALK protein because of gene amplification.

The N-*myc* gene was also amplified in this tumor and in all three cell lines with *ALK* amplification (NB-39-nu, Nagai, and NB-1). N-*myc* is located on 2p24.3 and *ALK* is on 2p23.2, suggesting that there is a tendency to synchronic amplification between N-*myc* and *ALK*. We were unable to conclude that there was an association between ALK amplification and prognosis mainly due to the limited number of positive samples and the short-term follow-up. Moreover, the *ALK* gene locus appears too far from the N-*myc* gene locus to be within a single amplicon. Further analysis in a greater number of samples with longer follow-up is necessary.

The activation of ALK results in hyperphosphorylation of ShcC in neuroblastoma cells, and NB-39-nu cells treated with ALK-siRNAs show suppressed tyrosine phosphorylation of ShcC, followed by apoptotic changes to these cells, suggesting that ShcC is a physiological substrate of the activated ALK kinase and that the ALK-ShcC pathway dominantly controls the survival of NB-39-nu cells even with the existence of other RTKs, such as EGFR, TrkA, and Ret. In neuronal cells, both ShcB (Sli/SCK) and ShcC (Rai/N-Shc) can bind activated RTKs, including the EGFR and Trk receptor.[36–39] Mice lacking both ShcB and ShcC exhibit a significant loss of sympathetic neurons, suggesting that ShcB and ShcC act in supporting sympathetic development and survival.[28] A recent study also showed that ShcC is a physiological substrate of Ret kinase and that it exerts a prosurvival function in neuronal cells.[40] Although high levels of TrkA expression correlate with a favorable outcome of neuroblastoma patients,[20] TrkA expression was significantly high in NB-39-nu and Nagai, which derive from tumors with a poor prognosis. This discrepancy may also be explained by the overwhelming control of cell survival by ALK kinase in these cell lines. Neuronal apoptosis is regulated through the action of critical protein kinase cascades, such as the phosphatidylinositol 3-kinase/Akt pathway and the Ras-MAPK pathway.[41,42] Apparently, neither pathway is properly controlled by EGF or nerve growth factor in NB-39-nu cells or Nagai cells.[27] Here, we also demonstrated that the suppression of activated ALK blocks MAPKs and Akt in these cells, resulting in apoptosis. On the other hand, the activity of MAPKs and Akt was not reduced by the suppression of a single copy of *ALK* in SK-N-MC cells. These results suggest that activation of ALK kinase completely remodeled the cellular signaling transduction pathways through ShcC so that cell survival entirely depended on signals originating from ALK kinase.

In conclusion, phosphorylation of several signaling molecules and cancer survival might be under the control of activated ALK kinase when gene amplification of ALK is as significant as in NB-39-nu cells, although the frequency of gene amplification in neuroblastoma tissues is not high. Cytoplasmic expression of ALK in neuroblastoma cells may suggest distinct function of this kinase in cell proliferation and survival. These findings further suggest that activated ALK kinase will be indispensable information for prognosis and treatment of neuroblastoma although the frequency is low.

References

1. Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell 1990, 61:203–212
2. Heldin CH: Dimerization of cell surface receptors in signal transduction. Cell 1995, 80:213–223
3. Blume-Jensen P, Hunter T: Oncogenic kinase signalling. Nature 2001, 411:355–365
4. Pawson T: Protein modules and signalling networks. Nature 1995, 373:573–580
5. Kozlowski M, Larose L, Lee F, Le DM, Rottapel R, Siminovitch KA: SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. Mol Cell Biol 1998, 18:2089–2099
6. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, Look AT: Fusion of a kinase gene ALK, to a nucleolar protein gene NPM, in non-Hodgkin's lymphoma. Science 1994, 263:1281–1284