Identification of Protein Kinase A Catalytic Subunit \(\beta\) as a Novel Binding Partner of \(\text{p73}\) and Regulation of \(\text{p73}\) 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

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Post-translational modifications play a crucial role in regulation of the protein stability and pro-apoptotic function of \(\text{p53}\) as well as its close relative \(\text{p73}\). Using a yeast two-hybrid screening based on the Sos recruitment system, we identified protein kinase \(\text{A catalytic subunit} \ \beta\) (PKA-\(\text{C}\beta\)) as a novel binding partner of \(\text{p73}\). Co-immunoprecipitation and glutathione S-transferase pull-down assays revealed that \(\text{p73}\alpha\) associated with PKA-\(\text{C}\beta\) in mammalian cells and that their interaction was mediated by both the \(\text{N-}\) and \(\text{C-terminal}\) regions of \(\text{p73}\alpha\). In contrast, \(\text{p53}\) failed to bind to PKA-\(\text{C}\beta\). In vitro phosphorylation assay demonstrated that glutathione S-transferase-\(\text{p73}\alpha\)-(1–130), which has one putative PKA phosphorylation site, was phosphorylated by PKA. Enforced expression of PKA-\(\text{C}\beta\) resulted in significant inhibition of the transactivation function and pro-apoptotic activity of \(\text{p73}\alpha\), whereas a kinase-deficient mutant of PKA-\(\text{C}\beta\) had no detectable effect. Consistent with this notion, treatment with \(\text{H}-89\) (an ATP analog that functions as a PKA inhibitor) reversed the dibutyryl cAMP-mediated inhibition of \(\text{p73}3\alpha\). Of particular interest, PKA-\(\text{C}\beta\) facilitated the intramolecular interaction of \(\text{p73}\alpha\), thereby masking the \(\text{N-terminal}\) transactivation domain with the \(\text{C-terminal}\) inhibitory domain. Thus, our findings indicate a PKA-\(\text{C}\beta\)-mediated inhibitory mechanism of \(\text{p73}\) function.

\(\text{p73}\) has been identified as a structural and functional homolog of the tumor suppressor \(\text{p53}\) (1). \(\text{p53}\) and \(\text{p73}\) share the same domain organization, consisting of an \(\text{N-terminal}\) transactivation domain, a central sequence-specific DNA-binding domain, and a \(\text{C-terminal}\) oligomerization domain. \(\text{p73}\) is characterized by several features, including the presence of \(\text{p53}\)-responsive elements, and the potential for alternative splicing of the primary \(\text{p73}\) transcript (\(\text{p73}3\alpha\), \(\text{p73}3\beta\), \(\text{p73}3\gamma\), \(\text{p73}3\delta\), \(\text{p73}3\eta\), and \(\text{p73}3\zeta\)) termed the TA variant (1, 3, 7–9). These alternatively spliced isoforms vary in their \(\text{C}\)-termini and display different transcriptional and biological properties. Additionally, the \(\Delta\text{N}\) variant (\(\Delta\text{NP73}\alpha\) and \(\Delta\text{NP73}\beta\)), which is generated by alternative promoter utilization, lacks the \(\text{N-terminal}\) transactivation domain and exhibits dominant-negative behavior toward wild-type \(\text{p73}\) as well as \(\text{p53}\) (10–12). Recently, we (14) and others (13, 15) demonstrated that \(\text{p73}\) directly transactivates the expression of its own negative regulator (\(\Delta\text{NP73}\)), creating an autoregulatory feedback loop in which both the activity of \(\text{p73}\) and the expression of \(\Delta\text{NP73}\) are regulated. Thus, the pro-apoptotic activity of \(\text{p73}\) is determined by the relative expression levels of its \(\text{TAp73}\) and dominant-negative \(\Delta\text{NP73}\) variants in cells.

In sharp contrast to \(\text{p53}\), it was initially reported that \(\text{p73}\) was not induced by DNA damage (1). However, recent studies demonstrated that, in response to a subset of DNA-damaging agents, \(\text{p73}\) is positively regulated by multiple post-translational modifications, including phosphorylation and acetylation. During cisplatin-mediated apoptosis, phosphorylation of \(\text{p73}\) at \(\text{Tyr-99}\) by the non-receptor tyrosine kinase \(\text{c-Abl}\) results in an increase in its stability and pro-apoptotic activity (16–18). In addition to \(\text{c-Abl}\), the protein kinase \(\text{C}6\) catalytic fragment has the ability to phosphorylate \(\text{p73}\) at Ser-289 and contributes to the accumulation of \(\text{p73}\) during the apoptotic response to cisplatin treatment (19). It is worth noting that the physical and functional interaction between \(\text{c-Abl}\) and protein kinase \(\text{C}6\) leads to the cross-activation of their kinase functions (20, 21). Furthermore, the enzymatic activity of \(\text{Chk1}\) (checkpoint \(\text{kinase-1}\)) is enhanced in response to DNA damage (22–24), and \(\text{Chk1}\) has the ability to phosphorylate \(\text{p73}\) at Ser-47 upon DNA damage, thereby enhancing its transactivation ability and pro-apoptotic activity without affecting the level of total \(\text{p73}\) protein, whereas \(\text{Chk2}\) has no detectable effect on \(\text{p73}\) (25).

Alternatively, \(\text{Zeng et al. (26)}\) found that the acetyltransferase \(\text{p300/\text{CBP}}\) (\(\text{cAMP-responsive element-binding protein-binding protein}\)) interacts with the \(\text{N-terminal}\) region of \(\text{p73}\) and stimulates \(\text{p73}\)-mediated transcriptional activation and apoptosis. Recently, \(\text{Costanzo et al. (27)}\) reported that doxorubicin treatment induces the \(\text{p500}\)-mediated acetylation of \(\text{p73}\) at \(\text{Lys-321}\), \(\text{Lys-327}\), and \(\text{Lys-331}\) in a \(\text{c-Abl}\)-dependent manner, which is associated with the efficient recruitment of \(\text{p73}\) to the promoter of the apoptotic target gene \(\text{p53AIP1}\). Additionally, it has been shown that \(\text{p300}\)-mediated acetylation of \(\text{p73}\) results in its significant stabilization in a prolyl isomerase \(\text{Pin1}\)-dependent manner (28).

To identify cellular protein(s) that could interact with full-length \(\text{p73}\) and regulate its function, we screened a human fetal brain \(\text{cDNA}\) 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 1794 solely to indicate this fact.

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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 p21WAF1 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% CO2.

Plasmids—The pCMV-p53 plasmid was used to identify the cDNA encoding the p73 full-length open reading frame of human p73 (Invitrogen) according to the manufacturer's instructions. The cDNA encoding the p73α-binding protein from a human fetal brain cDNA library cloned into the pMyr 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 (cdc25HΔ) was cotransformed with pSos-p73α and the cDNA library plasmid containing yeast prokaryotic and eukaryotic promoters 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 dyeode 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 hr, 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 hr with anti-FLAG monoclonal antibody (1:5000, Sigma), anti-green fluorescence protein (GFP) monoclonal antibody (1:4000, 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-p21WAF1 monoclonal antibody (Ab-1, Oncogene Research Products), or anti-FLAG-Cα monoclonal 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 hr at 4°C, and the precleared lysates were incubated with the indicated antibodies for 2 hr at 4°C, followed by incubation with protein G-Sepharose beads for an additional 1 hr 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 Escherichia coli strain DH5α and loaded onto glutathione-Sepharose 4B beads (Amersham Biosciences). PKA-Cβ was generated in vitro in the presence of [35S]methionine using the TNT quick-coupled in vitro transcription/translation system (Promega Corp., Madison, WI) according to the manufacturer’s instructions.35S-Labeled PKA-Cβ was incubated with GST or GST-p73α fusion proteins bound to glutathione-Sepharose beads for 2 hr 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 radiola beled 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.

Immunofluorescence Microscopy—H1299 cells were grown on coverslips and transiently cotransfected with the expression plasmids for hemagglutinin (HA-p73α, HA-p73β, or p53), a p21WAF1-responsive luciferase reporter construct (p21WAF1 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 (p21WAF1): p21WAF1, 5’-ATGAATTCACCCCTTCCC-3’ (sense) and 5’-CCCTAGGCTGTGCTCACTTC-3’ (antisense); and glycyralde-hyde-5-phosphate dehydrogenase, 5’-ACCTGACCTCGCTGCTGAC-3’.
**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.

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 MgCl₂). The washed beads were incubated with 30 μl of kinase buffer containing 2 units of purified PKA catalytic subunit (Sigma), 2 mM

\( 5'\text{-TCCACCACCCCTGTTGCTGTA-3'} \) (antisense). PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.
dithiothreitol, and 10 μCi of [γ-32P]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.

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 proteins(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 (edc25Hα) 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 P115) exhibited galactose-dependent growth at 37 °C. The plasmid DNA derived from the 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, PKA-Cβ, or ΔNp73α and with the expression plasmid for FLAG-PKA-Cβ were immunoprecipitated (IP) with anti-Full-length monoclonal antibody. The immune complexes were analyzed 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 [35S]-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). 150 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. 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, PKA-Cβ, or ΔNp73α and with the expression plasmid for FLAG-PKA-Cβ were immunoprecipitated (IP) with anti-Full-length monoclonal antibody. The immune complexes were analyzed 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 [35S]-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). 150 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.

[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. 1A, the anti-FLAG immunoprecipitates contained HA-p73α. Used as a control, HA-p73α was not detectable in the anti-FLAG immuno-
body. As shown in Fig. 1B (upper panels), FLAG-p73α co-immunoprecipitated with endogenous PKA-Cβ. Because the amino acid sequences of PKA-Co and PKA-Cβ are 91% identical (31), we examined whether endogenous PKA-Co could bind to p73α. Co-immunoprecipitation experiments revealed that, like PKA-Cβ, endogenous PKA-Co associated with FLAG-p73α (Fig. 1B, lower panels). In sharp contrast to p73α, p53 failed to interact with FLAG-PKA-Cβ under our experimental conditions (Fig. 1C).

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-
Functional Interaction between PKA and p73

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

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

To further confirm the inhibitory effect of PKA-C\(\beta\) on the transcriptional activity of p73\(\alpha\), H1299 cells were transiently cotransfected with a constant amount of the expression plasmid for FLAG-p73\(\alpha\), FLAG-p73\(\beta\), or FLAG-p53 with or without increasing amounts of the expression plasmid for FLAG-PKA-C\(\beta\), and the protein levels of endogenous p21\(^{WAPF}\) were determined by immunoblotting. As shown in Fig. 4A, endogenous p21\(^{WAPF}\) was increased by ectopic FLAG-p73\(\alpha\) expression, whereas overexpression of FLAG-PKA-C\(\beta\) resulted in a reduction in the level of endogenous p21\(^{WAPF}\) induced by FLAG-p73\(\alpha\), supporting the notion that PKA-C\(\beta\) inhibits the transcriptional activity of p73\(\alpha\). In contrast, PKA-C\(\beta\) had no detectable effects on the p73\(\beta\)- or p53-dependent induction of endogenous p21\(^{WAPF}\) (Fig. 4, B and C), consistent with the results obtained by luciferase reporter analysis. In addition, coexpression of FLAG-p73\(\alpha\) and FLAG-PKA-C\(\beta\) resulted in a slight increase in the amounts of FLAG-p73\(\alpha\), whereas FLAG-PKA-C\(\beta\) had a negligible effect on the amounts of FLAG-p73\(\beta\) and FLAG-p53 (Fig. 4). FLAG-p73\(\alpha\) decayed at slower rates in the presence of FLAG-PKA-C\(\beta\) than in its absence (data not shown); however, its physiological implications remain to be determined.

PKA-C\(\beta\) Phosphorylates p73—To determine whether p73 could be a substrate for PKA-C\(\beta\), the GST-p73\(\alpha\) fusion proteins used for the in vitro pull-down assay were incubated with the commercially available PKA catalytic subunit purified from bovine heart and \([\gamma\]\(^{32}\)P]ATP. Of the GST-p73\(\alpha\) fusion proteins tested, only GST-p73\(\alpha\)-(1–130) was phosphorylated by the PKA catalytic subunit (Fig. 5A). The N-terminal region of p73\(\alpha\) might be involved in phosphorylation by the PKA catalytic subunit.
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-PKA-Cβ(K76R) fusion proteins bound to glutathione-Sepharose beads were incubated with the purified catalytic subunit of PKA in the presence of γ-32P-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 p21WAF1 (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. 4D. PKA-Cβ(K76R) has no detectable effect on the p73α-dependent induction of endogenous p21WAF1. 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 p21WAF1. 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. 5B). Notably, luciferase reporter analysis revealed that FLAG-PKA-Cβ(K76R) had little effect on the ability of p73α to drive transcription from the p21WAF1 and bax promoters (Fig. 5C). In accordance with the results from luciferase reporter analysis, FLAG-PKA-Cβ(K76R) failed to reduce the expression levels of endogenous p21WAF1 induced by FLAG-p73α as examined by immunoblotting (Fig. 5D). 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. 6A, 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. 6B). 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 p21WAF1 or bax promoter. Twenty-four hours after transfection, cells were either left untreated or treated with the PKA-activating agent dibutyryl cAMP (Bt2cAMP) in the presence or absence of the PKA inhibitor H-89. As shown in Fig. 7A, Bt2cAMP treatment inhibited p73α-induced p21WAF1 and bax promoter activation. Intriguingly, the inhibitory effect of Bt2cAMP was attenuated when cells were exposed to H-89. Under the identical experimental conditions, endogenous p21WAF1 was significantly induced by exogenously expressed HA-p73α (Fig. 7B). Densitometric scanning of the immunoblot revealed that Bt2cAMP treatment decreased the level of p21WAF1 by 29% relative to that induced by HA-p73α, and the p21WAF1 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 cotransfected with the indicated combinations of expression plasmids and the subsequent PKA activation contribute to the reduction in p73α-mediated transcriptional activation. PKA-Cβ-mediated inhibition of p73α was determined by co-immunoprecipitation experiments. Whole cell lysates prepared from COS-7 cells transiently transfected with the indicated combinations of expression plasmids were 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 p21WAF1 and bax promoters in the absence of exogenous PKA-Cβ (Fig. 8A). 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. 8B (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. 8B, 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-HA antibody, followed by immunoblotting with anti-HA antibody, and the possible effect of FLAG-PKA-Cβ was determined by immunoblotting with anti-HA antibody, and the possible effect of FLAG-PKA-Cβ was determined by immunoblotting with anti-HA antibody, and the possible effect of FLAG-PKA-Cβ was determined 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 p73α and thus does not detect p73α-(1–247). As shown in Fig. 8C, 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.

**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. 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.
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 Bt2cAMP reduced p73α-mediated transcriptional activation, whereas the inhibitory effect of Bt2cAMP was attenuated when cells were exposed to H-89, a specific pharmacological inhibitor of PKA. Collectively, our present findings indicate that the PKA-mediated phosphorylation and conformational alteration of 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, Cβλ, Cβλab, and Cβλabc) 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-Cβλab. 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/KXXXS/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 (78RAAS81, 164KVST167, and 402KLPS405). 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, Fin1 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
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 (extracellular signal-regulated kinase) (51, 52) and phosphatidylinositol 3-kinase/Akt (53, 54) pathways. Recently, Wu et al. (55) found that c-Myc enhances the activity of PKA by transactivating the expression of PKA-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.

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