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-

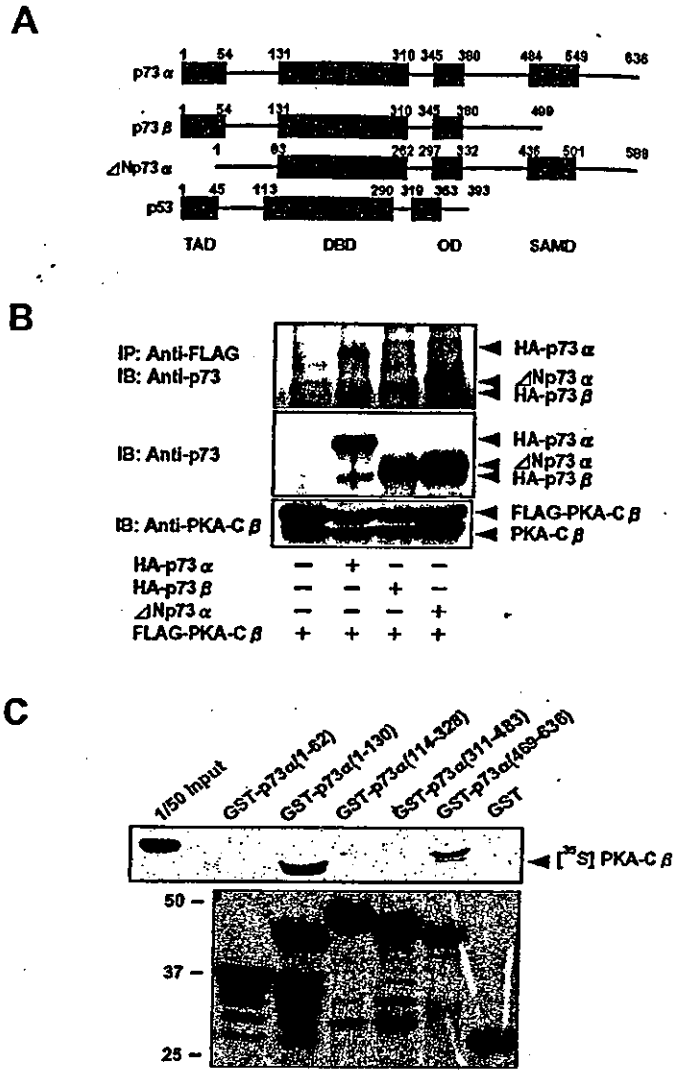

FIG. 2. **Interacting region within p73 for PKA-Cβ.** *A*, domain structures of p73 and p53. The transactivation domain (*TAD*), DNA-binding domain (*DBD*), oligomerization domain (*OD*), and sterile α motif domain (*SAMD*) are indicated. The *numbers* above the p73 variants and p53 indicate amino acid 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.

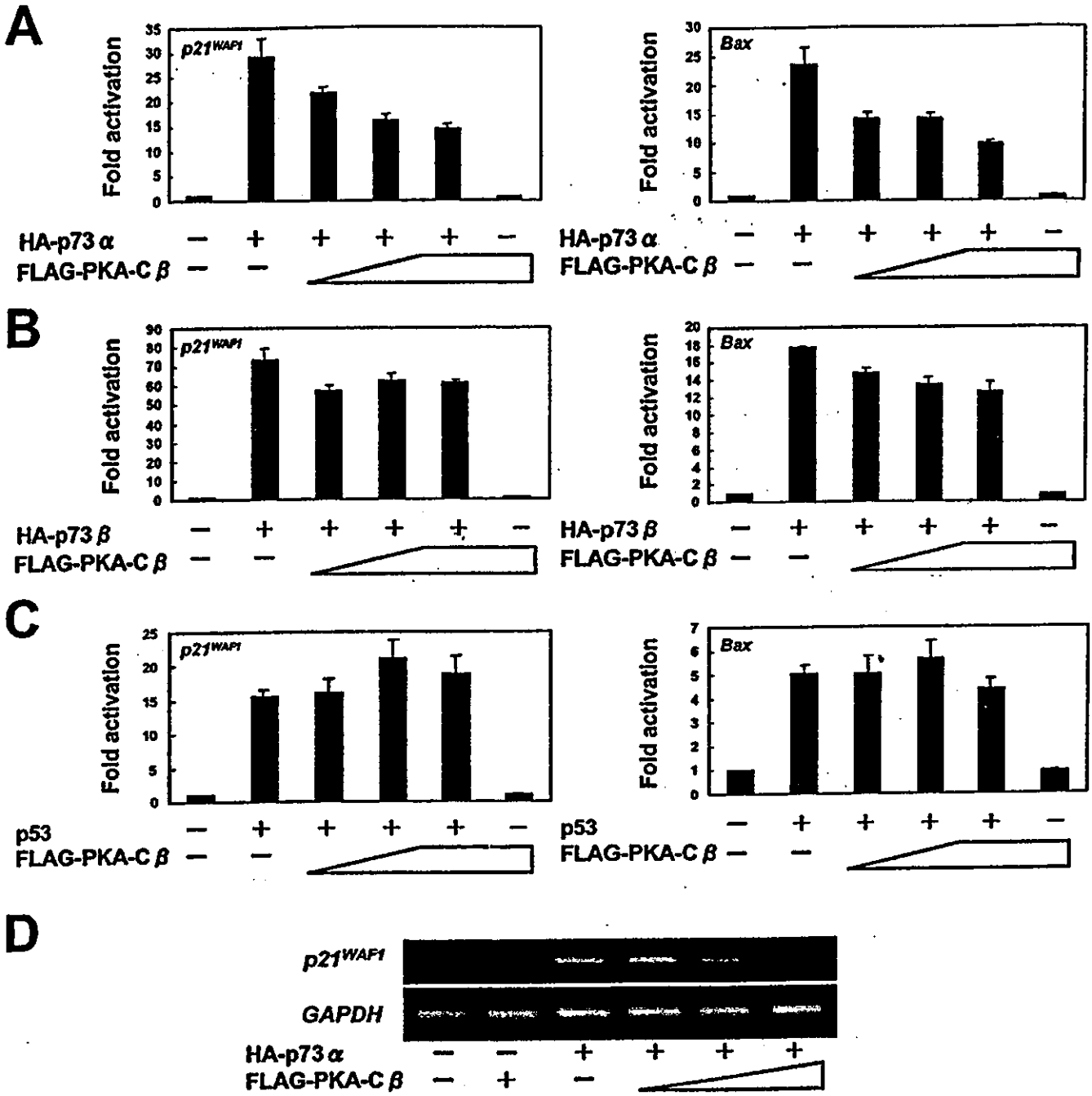

FIG. 3. **PKA-Cβ inhibits p73α-mediated transcriptional activation.** *A–C*, luciferase reporter assays. H1299 cells (5×10^4 cells/12-well plates) were transiently cotransfected with 25 ng of the expression plasmid for HA-p73α (*A*), HA-p73β (*B*), or p53 (*C*); 100 ng of the luciferase reporter construct 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

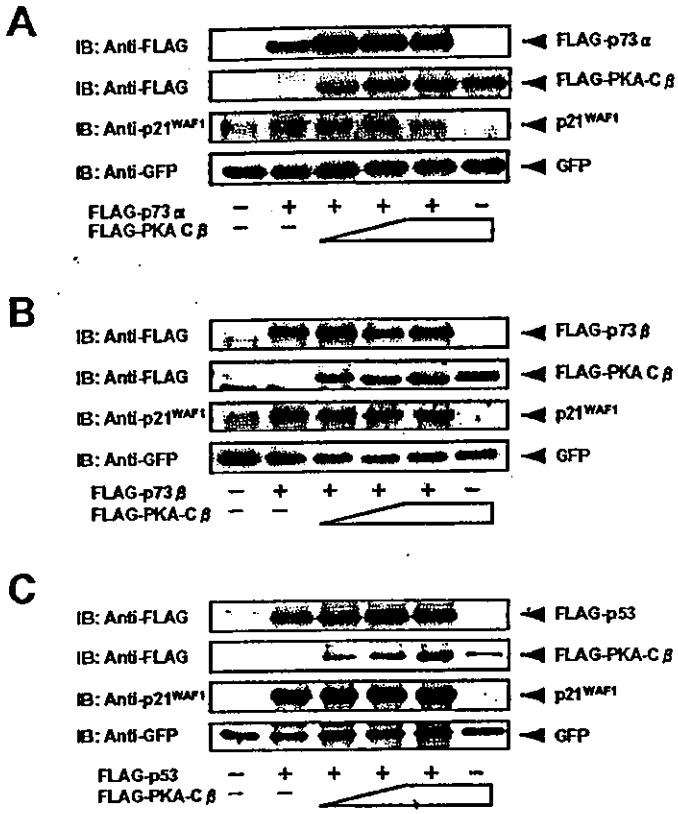

FIG. 4. **PKA-Cβ inhibits the p73α-dependent accumulation of endogenous p21^{WAF1}.** H1299 cells were transiently cotransfected with 200 ng of the expression plasmid for FLAG-p73α (*A*), FLAG-p73β (*B*), or FLAG-p53 (*C*) and 50 ng of the GFP expression plasmid with or without increasing amounts of 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.

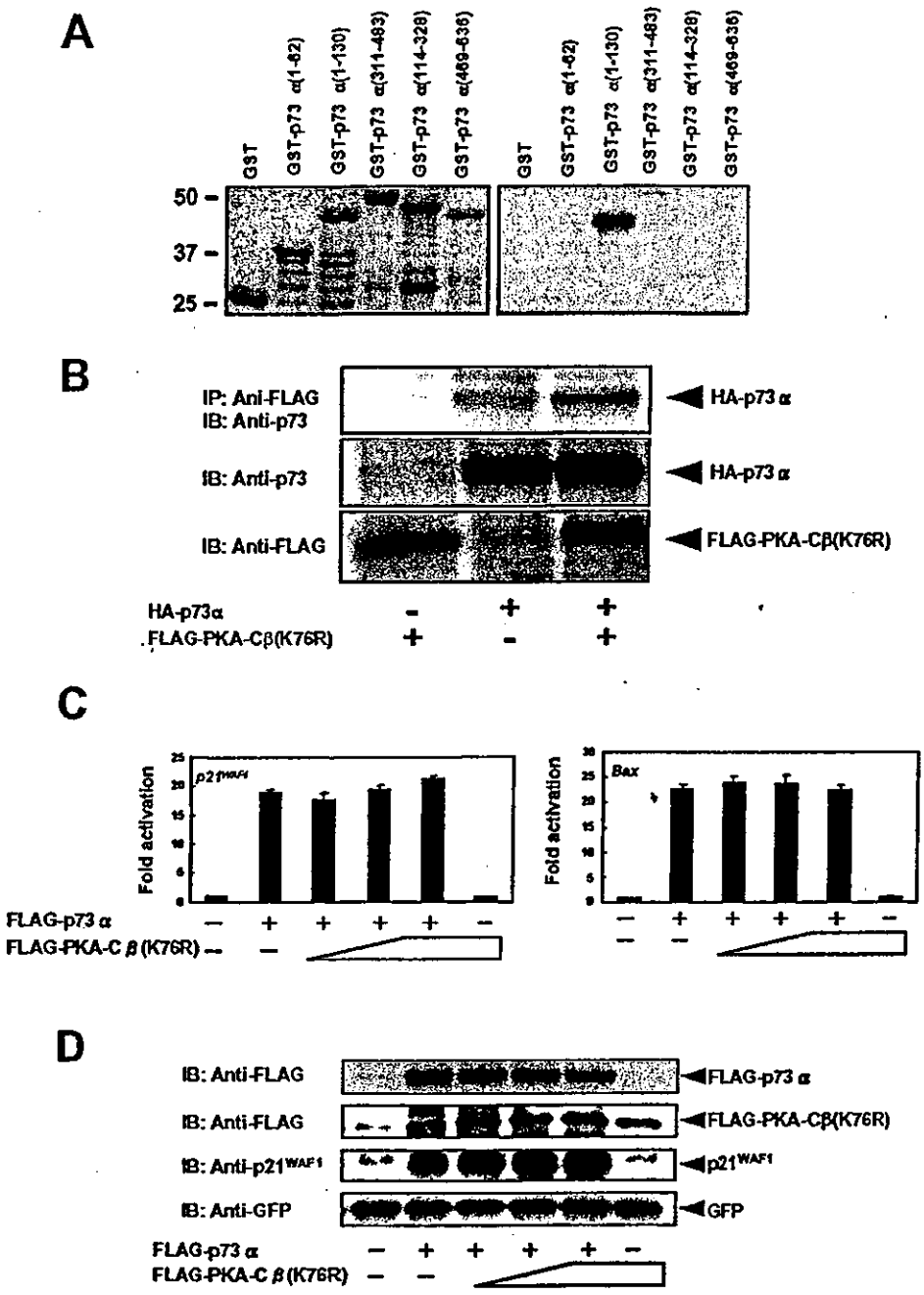

FIG. 5. **PKA-Cβ phosphorylates p73α, and the kinase-deficient mutant of PKA-Cβ fails to inhibit the transcriptional activity of p73α.** *A*, PKA-Cβ can phosphorylate p73α *in vitro*. GST or GST-p73α fusion proteins bound to glutathione-Sepharose beads were incubated with the purified catalytic subunit 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.

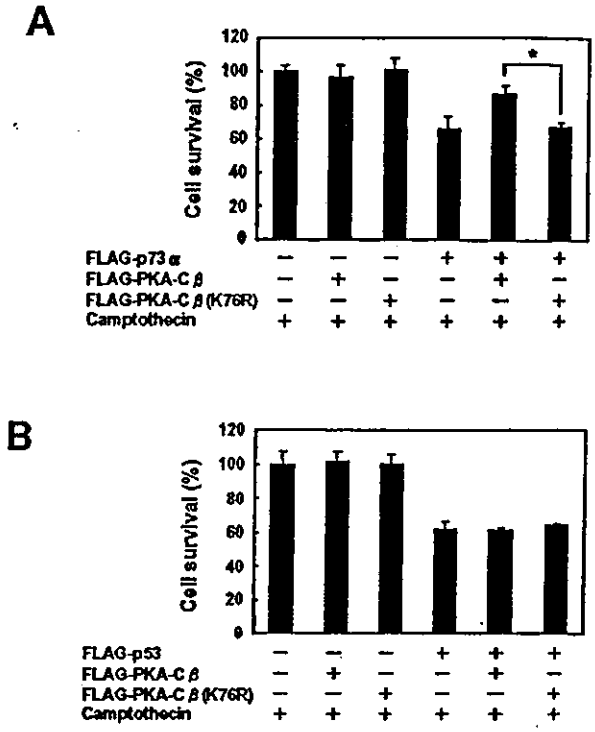

FIG. 6. **p73α-mediated increase in sensitivity to camptothecin is suppressed by wild-type PKA-Cβ, but not by kinase-deficient PKA-Cβ.** H1299 cells were transiently cotransfected with the expression plasmid for FLAG-p73α (*A*) or FLAG-p53 (*B*) with or without the expression plasmid encoding FLAG-PKA-Cβ 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

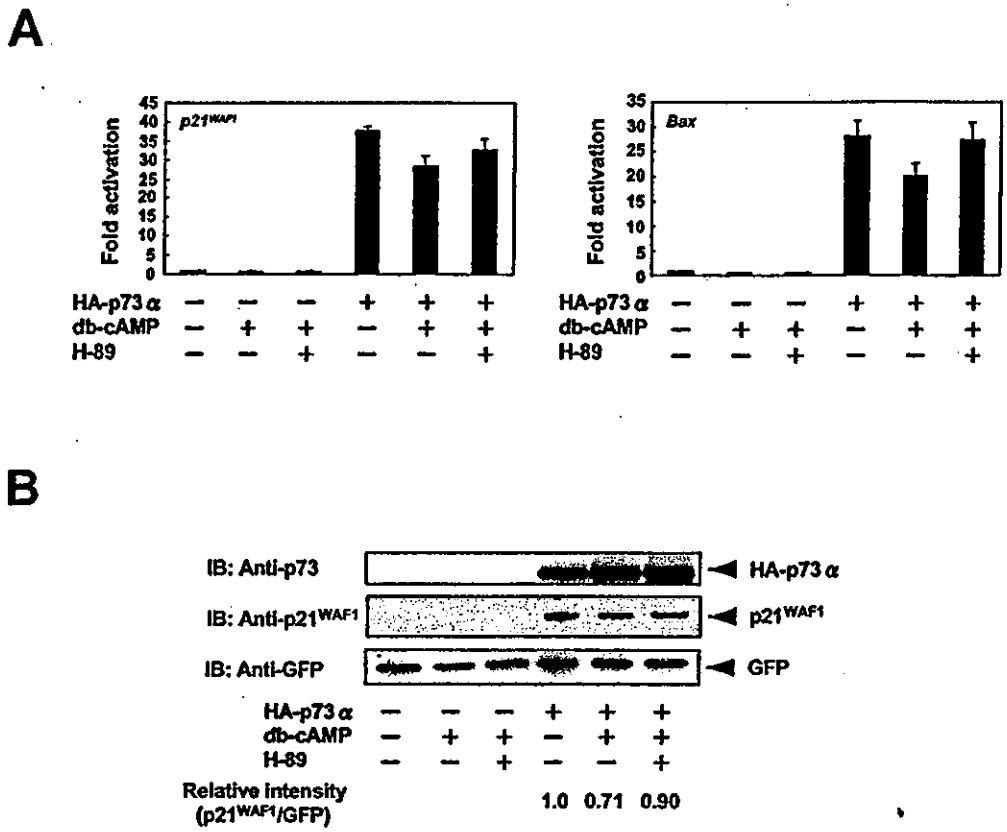

FIG. 7. **Effects of Bt_2cAMP on p73α-mediated transcriptional activation.** *A*, luciferase reporter analysis. H1299 cells were transiently cotransfected with 25 ng of the expression plasmid for HA-p73α, 100 ng of the luciferase reporter construct containing the p53/p73-responsive element derived from 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

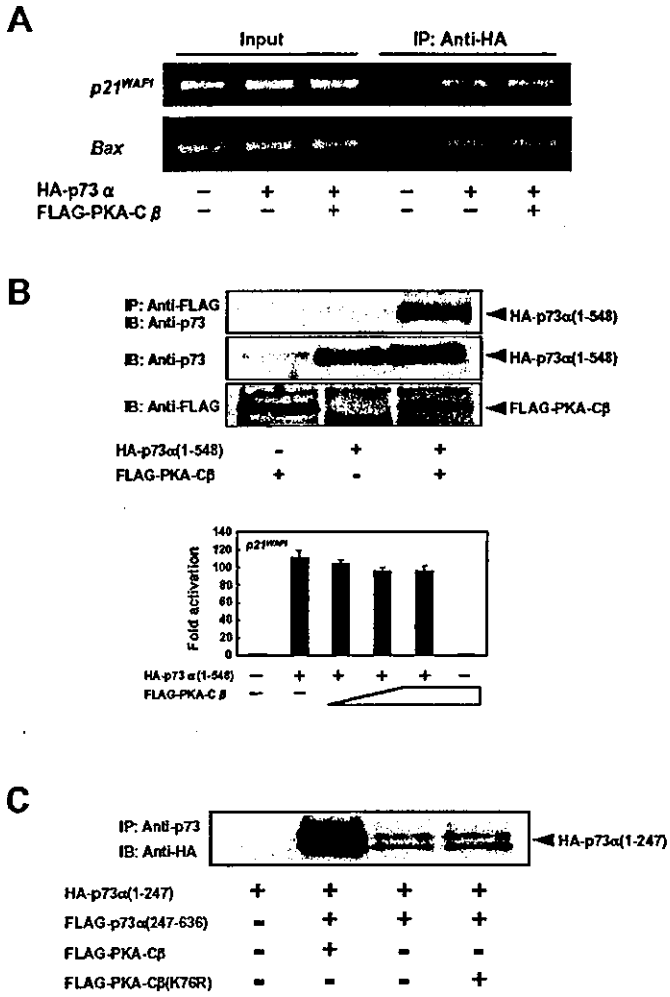

FIG. 8. **PKA-Cβ-mediated intramolecular interaction of p73α contributes to the down-regulation of the transcriptional activity of p73α.** *A*, ChIP assay. H1299 cells were transiently cotransfected with the expression plasmid for HA-p73α or HA-p73α plus FLAG-PKA-Cβ. Thirty-six hours after transfection, 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.

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