

## Cyclin-dependent Kinases Phosphorylate p73 at Threonine 86 in a Cell Cycle-dependent Manner and Negatively Regulate p73\*

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p73 transcription factors are members of the p53 family and participate in developmental processes and DNA damage response. p73 expression was shown to be regulated during the cell cycle, suggesting that p73 might play a role in cell growth and might be a target for cyclin-dependent kinases. Consistent with this hypothesis, we discovered that p73 interacts physically with various cyclins (A, B, D, and E). Furthermore, cyclin A/CDK1/2, cyclin B/CDK1/2, and cyclin E/CDK2 complexes can phosphorylate multiple p73 isoforms *in vitro* at threonine 86. A specific antibody directed against phosphorylated Thr<sup>86</sup> showed that this site is phosphorylated *in vivo* and that such phosphorylation is regulated in a cell cycle-dependent manner. Thr<sup>86</sup> phosphorylation is induced during S phase and is maximal in the G<sub>2</sub>/M phase. Accordingly inhibitors of cell growth, such as p16 and serum starvation, reduce Thr<sup>86</sup> phosphorylation. Finally, we found that cyclin-dependent kinase (CDK)-dependent Thr<sup>86</sup> phosphorylation represses the ability of p73 to induce endogenous p21 expression. Our results demonstrate that p73 proteins are targets of CDK complexes and that phosphorylation on Thr<sup>86</sup> by CDKs regulates p73 functions.

p73 proteins are members of the p53 family that consists of three distinct genes (p53, p63, and p73) sharing significant homology and whose products can function as sequence-specific transcriptional activators (1–6). Proteins encoded by these three genes share the same modular organization consisting of an N-terminal transactivation domain, central sequence-specific DNA-binding domain, and a C-terminal tetramerization domain. However, the p73 gene encodes multiple isoforms varying in their N and C termini. In some cases, the use of a cryptic promoter generates isoforms lacking the transactivation domain located in the N terminus of p73 ( $\Delta$ Np73 $\alpha$  and  $\Delta$ Np73 $\beta$ ). The p73 gene also generates several forms with varying C-terminal extensions (p73 $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ) that are produced by alternative splicing (7, 8). At a functional level, it has been shown that ectopic expression of p73 can transactivate endogenous targets of p53, such as the cell cycle inhibitor p21

(1–3), as well as p21 promoter-containing reporters (1, 3, 6). We have compared the ability of p73 and p53 to activate a number of p53-responsive reporter constructs (9), whereas in a more physiological context Zhu *et al.* (10) found significant differences in the abilities of p53 and p73 proteins to activate several targets.

The precise functions of p73 proteins in the organism and the signaling pathways that regulate their activity are still not well established. However, various *in vitro* and *in vivo* experiments have shown or suggested that, like p53, p73 proteins may play a role both in developmental processes and DNA damage response. Several lines of evidence show that p73 may play a role in nervous system and immune system development. Indeed, p73<sup>–/–</sup> mice harbor defects in the development of the hippocampus and in the inflammatory response (11). In lymphoid cells, p73 is involved in T cell receptor-induced apoptosis (12). Supporting role(s) of p73 in neurons, *in vitro* experiments have shown that p73 protein levels are up-regulated during neuronal differentiation and that p73 overexpression is sufficient to induce neuronal differentiation (13). Furthermore,  $\Delta$ N isoforms of p73 can protect neurons from apoptosis (14).

Several reports have attributed to p73 a role in the response to cellular stress, as has been well described for p53. p73 overexpression can induce apoptosis (1, 3, 6, 10). Moreover, up-regulation of p73 protein levels and c-Abl-dependent activation of p73 in response to  $\gamma$  radiation or cisplatin treatment leads to apoptosis (15–18). c-Abl can also activate p73 activity through the p38 kinase that phosphorylates p73 at a still unidentified threonine phosphorylation site adjacent to a proline (TP site)<sup>1</sup> (19). Other oncogenes can also increase p73 protein levels (20). An interesting recent report shows that p73 is required for p53-dependent apoptosis induced by DNA damage (21). These observations and the fact that p73 expression is affected in certain tumors suggest that p73 may function as a tumor suppressor gene. However, p73<sup>–/–</sup> mice are not particularly prone to cancer (11), and only rarely have mutation or inactivation of p73 expression been found in human tumors. Further, contradictory results have been reported concerning either overexpression or repression of the p73 gene in some cancers.

Various proteins have been described to interact or regulate

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<sup>1</sup> The abbreviations used are: TP site, threonine phosphorylation site adjacent to a proline; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; PIPES, 1,4-piperazinediethanesulfonic acid; CRM, cyclin recognition motif; phospho-TP, phosphorylated TP site; wt, wild type; dn, dominant negative; min luc, minimal luciferase.

p73 protein activity. Among negative regulators, Hdm2 inhibits p73 but does not affect its stability (22–25) and a subset of p53 tumor-derived mutants down-regulate p73 functions (9, 27–29). WW proteins were reported to interact and stimulate p73 as well as Ik3-1/Cable (30, 31). However, the physiological relevance of these interactions and regulation have not yet been clearly established.

Irvin *et al.* reported that p73 protein levels are regulated through the cell cycle in experiments showing that cells released from serum starvation produce increasing levels of p73 starting at the end of G<sub>1</sub> (32). p73 expression in this context is mediated by E2F protein(s) that bind to the p73 promoter. In fact, E2F proteins are transcriptional regulators of p73 expression, and it has been shown that p73 is involved in E2F-dependent apoptosis (12, 33). Because p73 expression is regulated through the cell cycle, we hypothesized that p73 activity might be modulated by cyclin-dependent kinases at a post-translational level.

In this study, we analyzed the physical and functional interactions between p73 proteins and cyclin-dependent kinases (CDK). We found that p73 can interact physically with cyclins and can be phosphorylated by S/G<sub>2</sub>/M CDK complexes. We identified Thr<sup>86</sup> as a phosphorylation site for CDK complexes and showed that Thr<sup>86</sup> is phosphorylated in a cell cycle-dependent manner *in vivo*. Finally, we found that mutation of Thr<sup>86</sup> significantly affects p73 transcriptional activity, suggesting a regulatory role for the CDK complexes through this site.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—H1299, Cos1, and T98G cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in the presence of 5% CO<sub>2</sub>, 95% air at 37 °C. Sf9 insect cells were grown in F100 medium at 30 °C.

**Expression Vectors**—Expression of human HA-p73 $\alpha$ , HA-p73 $\beta$ , and HA-p73 $\gamma$  cDNA from the CMV promoter are as described (7). pCMV cyclin A, pCMV cyclin B, and pCMV p16 expression vector were gifts from Dr. K. Okamoto (Cancer Center, Tokyo, Japan). PCDNA constructs encoding CDK1 or CDK2 proteins are a gift from Dr. S. van den Heuvel. pBacp73 $\beta$  were obtained by subcloning a HindIII/XhoI fragment of the CMVp73 expression constructs into the FastBac vector (Invitrogen).

**Expression and Purification of p73 Proteins and CDK Complexes**—Infection, expression, and purification of CDK complexes from insect cells were done as previously described (34). Generation and amplification of the p73 baculoviruses using the FastBac system were done as indicated by the manufacturer (Invitrogen). Purification of p73 proteins was performed as described previously (27). Briefly, Sf9 cells freshly seeded (1 h) at 90% confluence in 20-cm plates (20–40 plates) were infected for 1 h with 500  $\mu$ l of virus diluted in 2 ml of medium for each plate. After 48 h, the cells were removed from the plates in their medium and washed twice in phosphate-buffered saline. The cells were then lysed for 30 min in buffer A (50 mM Tris-HCl, pH 8, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, and protease inhibitors, 0.1% aprotinin). The soluble fraction was isolated by centrifugation at 4 °C for 30 min at 20,000 rpm and added to protein A-Sepharose beads (1–2 ml) cross-linked to HA monoclonal antibody (12CA5) that were rocked overnight at 4 °C. The beads were then washed two times in buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40, 100 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF) and two times in buffer C (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF) and then poured into a 5-ml syringe. After washing with three column volumes of buffer D (20 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 250 mM NaCl, 1 mM DTT), p73 proteins were eluted with a HA peptide (final concentration, 10  $\mu$ g/ml) in a buffer containing 20 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 500 mM NaCl, and 1 mM DTT. Fractions containing p73 were assessed by SDS-PAGE and pooled according to their similarity in concentration and purity.

**Reporter Vectors**—p21 min luc contains a duplex oligonucleotide encoding the p53-responsive *cis*-acting element from p21, cloned upstream of the minimal *c-fos* promoter (–53 to +42) in pGL3-OFLUC (kindly provided by N. Clarke). The synthesized oligonucleotides (Operon Tech-

nologies, Inc.) encoding the p21 *cis*-acting p53-responsive element were as follows: 5'-GATCCTCGAGGAACATGTCCCAACATGTTGCTCGAG-3' and 5'-GATCCTCGAGCAACATGTTGGGACATGTTTCCTCGAG-3'. The resulting plasmid was sequenced to determine the orientation and number of inserts of the oligoduplex.

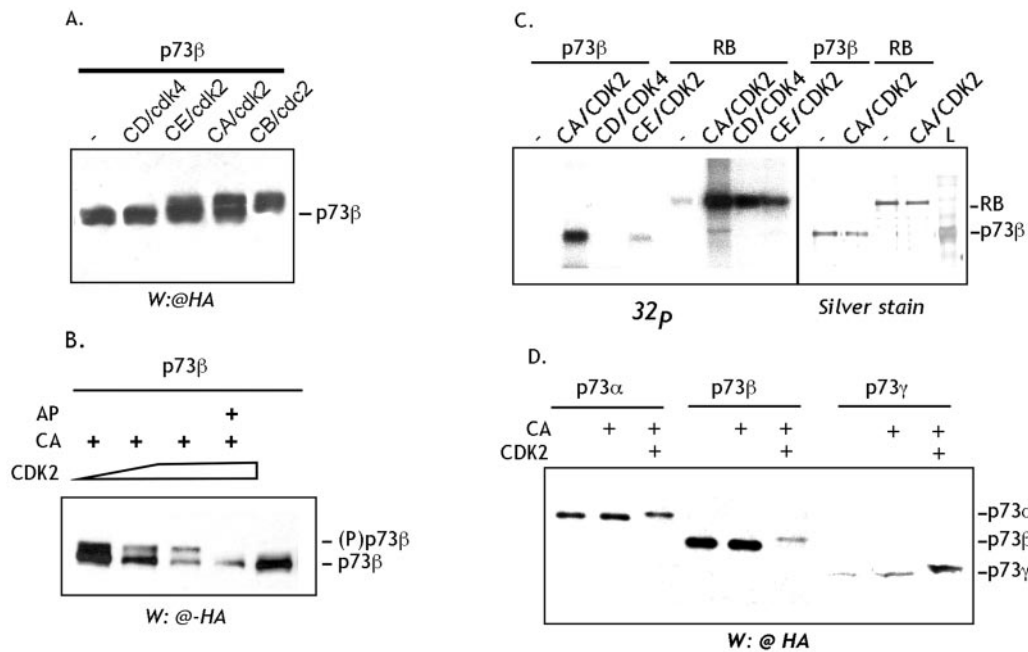
**Transfection and Luciferase Assays**—H1299 and Cos1 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS in 5% CO<sub>2</sub> at 37 °C. The cells were transfected by a lipopolyamine-based (Transfectam<sup>TM</sup>) protocol as described previously (35). Briefly, cells were grown in DMEM, 10% FBS and transfected with various amounts of DNA. The precipitate was left on the cells for 6 h, after which fresh DMEM, 10% FBS was added. For luciferase assays, the cells were seeded in 12-well, 3.8-cm<sup>2</sup> plates and transfected at 90% confluency with one of the expression vectors (200 ng of each) and two different reporter constructs (250 ng of each), a CMV-expressed luciferase cDNA from *Renilla*, and a p53-responsive luciferase cDNA from firefly. Luciferase activity was measured in each well 24 h later by a dual luciferase reporter gene assay (Promega).

**Preparation of Whole Cell Extracts, Immunoblotting, and Immunoprecipitation Analysis**—H1299 and Cos1 cells in 6-cm plates were transfected with the indicated plasmids (8  $\mu$ g) and harvested 48 h later. Sf9 cells were infected with 50  $\mu$ l of virus in six-well plates. The cells were lysed in 300  $\mu$ l of TEGN buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM DTT, 10% glycerol, 5 mM PMSF, 100  $\mu$ M benzamide, 300  $\mu$ g/ml leupeptine, 10  $\mu$ g/ml bacitracin, 100  $\mu$ g/ml,  $\alpha$ -macroglobulin and phosphatase inhibitors) (Cocktails I and II; Sigma), and the extracts were centrifuged at 13,000 rpm for 12 min to remove cell debris. The protein concentrations were determined using a colorimetric assay (Bio-Rad). p73 proteins (400–750  $\mu$ g of whole cell extract) were incubated with 30  $\mu$ l of 50% slurry protein A-Sepharose beads cross-linked to an anti-HA antibody (12CA5) followed by rocking at 4 °C for 3 h. After incubation, the samples were washed four times with 1 ml of wash buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 10% glycerol). Excess liquid was aspirated, 35  $\mu$ l of sample buffer (36) was added, and samples were heated to 95 °C for 3 min and centrifuged for 3 min at 13,000 rpm, followed by electrophoresis through a 10% SDS-polyacrylamide gel followed by transfer to nitrocellulose membranes (Schleicher & Schuell). For HA detection, the 16B12 monoclonal HA antibody (Babco; 1 mg/ml) was used at 1:1000. TP phosphorylation was observed using a phospho-specific TP antibody (New England Biolabs; 1:5000). Thr<sup>86</sup> phosphorylation was followed with a Thr(P)<sup>86</sup> antibody, antigen-purified, and used at a dilution of 1:1000. To assess p73-regulated p21 expression, the cells were transfected in 6-well plates with 1  $\mu$ g of p73 expression vector (or control vector), 3  $\mu$ g of dnCDK1 (or control vector), and 0.2  $\mu$ g of EGFP expressing vector. The cells were lysed 20 h after transfection, and the proteins were separated on a 10% SDS gel. For each point, two wells were transfected, and lysates were mixed. p21 protein was detected using a monoclonal p21 antibody (Pharmingen, clone SX118, 1/500). Cyclin immunoprecipitation and immunoblotting were performed with a monoclonal anti-cyclin A antibody (1:50 and 1:1000, respectively; Sigma). The proteins were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences).

**GST Pull Down**—Interactions with purified proteins were assessed in a buffer containing 2  $\mu$ g/ $\mu$ l bovine serum albumin, 20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% Nonidet P-40, 250 mM NaCl, 1 mM DTT, 10% glycerol, and protease inhibitor. Immunopurified GST-cyclin A (300 ng) was incubated for 30 min with anti-GST beads (glutathione-Sepharose 4B; Pharmacia Corp.; 20  $\mu$ l). After two washes, HA-p73 $\beta$  proteins (100 ng) were added and incubated for another 30 min. Then beads were washed five times with a TEGN buffer containing 250 mM NaCl. The samples were heated to 95 °C for 5 min, centrifuged for 3 min at 13,000  $\times$  rpm, electrophoresed through a 10% SDS-polyacrylamide gel, and transferred to nitrocellulose for immunoblotting. GST and GST-cyclin A were detected with a GST antibody (Ab3; Oncogene Research Products; 1:1000).

**In Vitro Phosphorylation Assays**—Immunopurified p73 $\beta$  or RB proteins (100 ng) were incubated in the presence of immunopurified cyclin/CDK complexes (100 ng) in 20  $\mu$ l of a kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 100  $\mu$ M ATP, 15  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP) for 30 min at 37 °C. After the addition of 10  $\mu$ l of 3 $\times$  sample buffer, the proteins were separated on a 10% SDS-PAGE gel. The gels were dried and analyzed by autoradiography. The purity and the quantity of the proteins used in the assay were verified simultaneously by SDS-PAGE gel separation and silver staining.

**FACS Analysis**—FACS analysis was performed as described by Lamm *et al.* (37). Briefly, the cells were fixed in 2% paraformaldehyde in a fixative buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 1 mM



**FIG. 1. p73 proteins are phosphorylated by S/G<sub>2</sub>/M CDK complexes *in vitro* and in insect cells.** A, cyclin A/CDK2, cyclin B/cdc2, and cyclin E/CDK2 induce a mobility shift of p73 migration on a SDS-PAGE gel. Baculoviruses expressing p73β (25 μl) and CDK complexes (30 μl of cyclin and 30 μl of CDK, as indicated) were used to co-infect Sf9 insect cells in 6-well plates. The cells were lysed 48 h after infection in 100 μl of lyses buffer. The proteins were separated on a 7.5% SDS-PAGE gel and immunoblotted with anti-HA antibody. B, cyclin A/CDK2-induced p73 mobility shift on SDS-PAGE is due to phosphorylation. p73β (25 μl), cyclin A (30 μl), and CDK2 (10, 25, and 50 μl) were co-expressed in insect cells as in A. After extraction, the proteins were incubated at 37 °C for 15 min with 200 units of alkaline phosphatase (AP) and then separated on a 7.5% SDS-PAGE gel followed by transfer to nitrocellulose. p73 proteins were detected with HA antibody. C, *in vitro* phosphorylation of p73β by cyclin A/CDK2, cyclin E/CDK2, or cyclin D/CDK4 complexes (100 ng) for 30 min at 37 °C in presence of <sup>32</sup>P-labeled ATP. The proteins were separated on a SDS-PAGE gel and subjected to autoradiography (left panel). The right panel shows another gel silver-stained gel of the p73β and RB proteins used in the experiment shown on the left. D, cyclin A/CDK2 phosphorylates multiple p73 family members. p73 proteins (HA-tagged p73α, HA p73β, and HA p73γ) were co-expressed in insect cells with cyclin A and CDK2 as in A. The proteins in cell extracts were separated on an SDS-PAGE gel. p73 proteins were detected with a HA antibody. CA, cyclin A; W, Western blot.

EGTA, 10 mM PIPES, pH 6.8) for 20 min and then in 95% methanol for 1 h. The fixed cells were washed three times with phosphate-buffered saline and exposed to propidium iodide (60 μg/ml), and RNase A (50 μg/ml) for 30 min before analysis (FACScalibur; Becton Dickinson) for DNA content (propidium iodide) with a 610-nm long pass filter. An excitation wavelength of 488 nm was used for propidium iodide. The data were analyzed using CELLQuest software (Becton Dickinson).

## RESULTS

**p73 Proteins Are Phosphorylated by Cyclin-dependent Kinases**—We identified in p73 a consensus phosphorylation site for CDK complexes (\*T<sup>86</sup>PEH). To support the likelihood that p73 may be phosphorylated by CDKs, insect cells (Sf9 cells) were co-infected with HA-tagged p73β expressing baculovirus (27) and a combination of cyclin and CDK expressing baculoviruses (Fig. 1A). The cells were lysed 48 h after infection, the proteins were separated by SDS-PAGE, and p73 expression was detected by immunoblotting using anti-HA antibody (HA.11). As shown in panel A, p73 gel migration was retarded when it was co-expressed with cyclin A/CDK2, cyclin B/CDK1/cdc2, or cyclin E/CDK2 but not when co-expressed with cyclin D/CDK4, suggesting that a subset of CDK complexes can indeed induce post-translational modification of p73 polypeptide. Infection with increasing amounts of CDK2 baculovirus reduced overall p73 expression (shifted plus nonshifted polypeptides), which might be due to competition or a squelching effect between the co-infecting recombinant baculoviruses (Fig. 1B). However, we observed that at the highest quantity of CDK2, ~50% of the total amount of p73 polypeptide was shifted (Fig. 1B). To confirm that the retarded migration was due to phosphorylation of p73, the extracts were treated with alkaline phosphatase (AP) (Fig. 1B). This treatment prevented

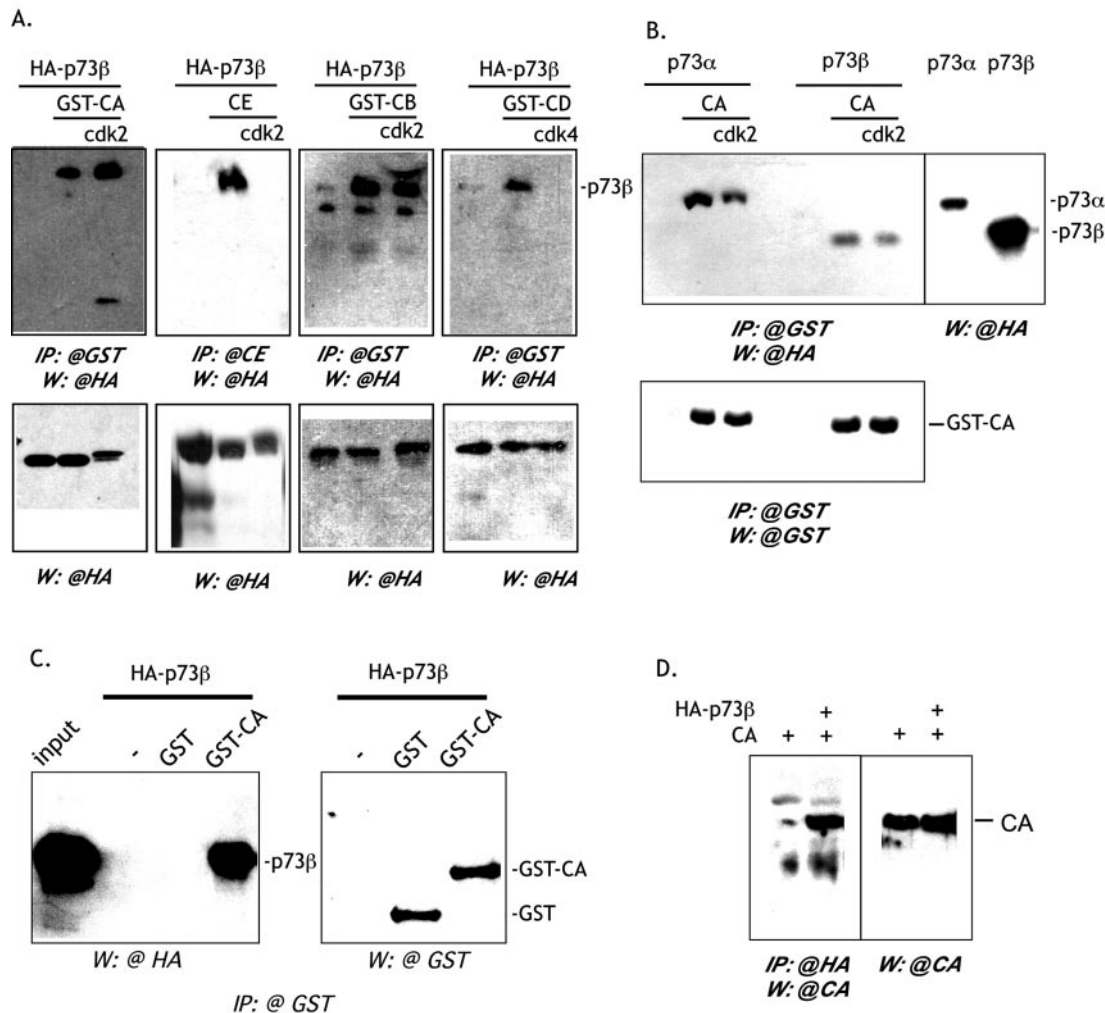
the shift in p73 migration, indicating that the shift was caused by CDK-induced phosphorylation of p73 in insect cells.

To further investigate whether p73 is a direct target of CDK complexes, we performed *in vitro* protein kinase assays using immunopurified proteins. Rb, which is a well known substrate for all of the common CDK complexes, was used for comparison in these experiments (38). Similar amounts of purified p73 and Rb proteins were used as substrates for purified cyclin A/CDK2, cyclin D/CDK4, and cyclin E/CDK2 (Fig. 1C, right panel, silver-stained gel). As expected, all three CDK complexes phosphorylated Rb protein *in vitro* (Fig. 1C, left panel). In the same experimental conditions, p73 was phosphorylated far more efficiently by cyclin A/CDK2 than by cyclin E/CDK2. No phosphorylation of p73 by cyclin D/CDK4 was observed under conditions that allowed Rb phosphorylation by this kinase. Thus, p73 proteins can serve as substrates for cyclin A/CDK2 and cyclin E/CDK2 but not for cyclin D/CDK4.

Because the p73 gene encodes multiple isoforms varying in their C or N termini, we compared the ability of the different isoforms to be phosphorylated by the CDK using the same approach. Upon co-infection of insect cells with cyclin A and CDK2 expressing baculoviruses, we found that at least three isoforms (p73α, p73β, and p73γ) displayed a mobility shift (Fig. 1D). This suggests that the CDK phosphorylation site(s) is located in the common portion of the various p73 isoforms.

Our experiments indicate that p73 proteins are direct targets for a subset of CDK complexes. The fact that they phosphorylate p73 *in vitro* with a similar efficiency to Rb, leading to a gel mobility shift in p73 migration, suggests that p73 is a good substrate for S/G<sub>2</sub>/M CDK complexes.





**FIG. 2. p73 proteins interact with cyclins.** A and B, p73 co-precipitates with cyclin A, cyclin B, cyclin E, and cyclin D. p73α, p73β, cyclin A, cyclin B, cyclin E, cyclin D, CDK2, and CDK4 were co-expressed as indicated in insect cells for 48 h. The proteins were extracted in TEGN buffer, and GST-cyclin A (A and B), GST-cyclin B (A), or GST-cyclin D (A) were immunoprecipitated (IP) in presence of anti-GST beads (Pharmacia Corp.) and cyclin E with a cyclin E antibody (Santa Cruz). The precipitates were then separated on a 10% SDS-PAGE gel, and p73 proteins were detected with an anti-HA antibody (upper panels). The lower panels in A and the right upper panel in B show p73 expression levels in the respective extracts. In B, the lower panel shows GST-cyclin A expression in the corresponding extracts used for immunoprecipitations. C, p73 and cyclin A interact physically *in vitro*. Immunopurified GST cyclin A (300 ng) was incubated for 30 min with anti-GST beads (20 μl). After two washes, HA-p73β proteins (100 ng) were added and incubated for another 30 min. The beads and the complexes were washed five times with TEGN buffer containing 250 mM NaCl. The complexes were then separated on a 10% SDS-PAGE gel and transferred on nitrocellulose and immunoblotted. The proteins were detected as described for A. D, p73 and cyclin A interact in mammalian cells. HA-p73β and cyclin A expression vectors were co-transfected into H1299 cells grown in 10-cm plates. After 36 h, the proteins were extracted in TEGN buffer (400 μl/plate), and p73 proteins were immunoprecipitated with protein A-Sepharose beads cross-linked to anti-HA antibody (left panel). After four washes with the TEGN buffer, electrophoresis sample buffer was added, and the beads and complexes were boiled and loaded on a 10% SDS-PAGE gel. After transfer to nitrocellulose, cyclin A was detected with an anti-cyclin A antibody (monoclonal antibody 160, dilution, 1:2). The right panel shows cyclin A levels detected in the extracts. CA, cyclin A; CB, cyclin B; CD, cyclin D; CE, cyclin E; W, Western blot.

**p73 Proteins Interact with Cyclins**—In some cases, phosphorylation by CDK complexes is associated with a direct interaction between the cyclin and the substrate through a cyclin recognition motif (CRM): RXL or KXL. The p73 protein sequence exhibits two potential CRM sites (position 149, KKL; position 515, RAL), suggesting that p73 may interact with cyclins. The first CRM is located in the core domain of the protein (position 149, KKL) and is common to all p73 isoforms (see Fig. 3A). The second CRM is located in the extreme C-terminal part of the p73α isoform at position 515 (RAL; Fig. 3A). We first examined the interaction between p73 and cyclins in insect cells co-infected with baculoviruses expressing HA-tagged p73 and GST-tagged cyclin A, cyclin B, cyclin E, or cyclin D (Fig. 2A). Infected cells were lysed 48 h after infection, GST-tagged cyclins were pulled down with GST beads, and HA-p73 proteins were detected by immunoblotting using an

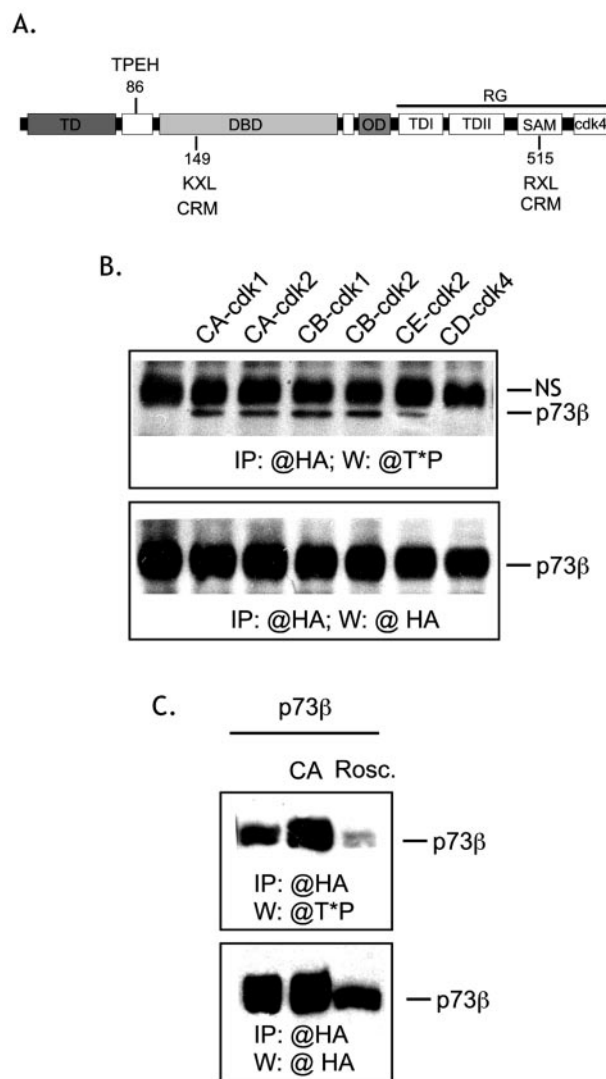
anti-HA antibody. As shown in Fig. 2A, p73β was present in the complex with cyclin A, cyclin B, cyclin E, and cyclin D. Co-expression of cyclin A and cyclin B with CDK2 and CDK1, respectively, did not significantly interfere with the ability of p73 to co-immunoprecipitate with these cyclins. Interestingly, co-expression of cyclin E and cyclin D with CDK2 and CDK4 reduced the amount of p73 proteins in the complex. Moreover, when we compared the ability of p73α and p73β to co-precipitate with cyclins, we observed better co-precipitation between p73α and cyclin A, although p73β is expressed at higher levels than p73α (Fig. 2B). This suggests that both CRM sequences participate in the interaction between cyclins and p73α. To confirm that the physical interaction between p73 and the cyclins is direct, co-immunoprecipitation experiments using immunopurified proteins were performed (Fig. 2C). Purified p73β proteins were pulled down with GST-tagged cyclin A protein but not with

GST alone. We estimate that ~5% of each protein is associated with each other (Input = 10% of the purified protein used for the immunoprecipitation; Fig. 2C, left panel).

To examine whether an interaction between p73 and cyclin A could be demonstrated in mammalian cells, H1299 cells were co-transfected with HA-p73 $\beta$  and cyclin A expression vectors. After immunoprecipitation of HA-p73 proteins with HA beads, we observed the presence of cyclin A proteins in the complex (Fig. 2D). In absence of p73 protein, only a very small amount of cyclin A was detected after immunoprecipitation, even though cyclin A protein was expressed at a similar level (Fig. 2D, left panel). Thus, p73 proteins can interact physically and directly *in vivo* with at least one cyclin (cyclin A).

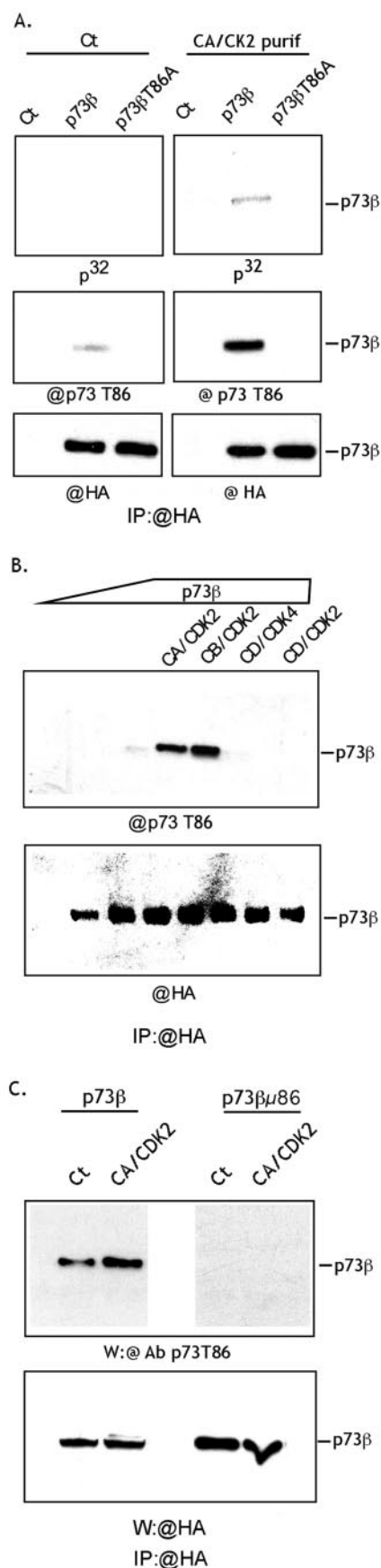
**Expression of CDKs Leads to Phosphorylation of p73 on a TP Site *in Vivo***—As mentioned above, Thr<sup>86</sup> is a potential CDK phosphorylation site ((S/T)PB, where B indicates a basic residue) in the N terminus of p73 (Fig. 3A; T\*PEH). To test the possibility that this site can be phosphorylated by CDK, we first used a commercially available antibody that recognizes phosphorylated TP sites (phospho-TP). Insect cells were co-infected with baculoviruses expressing various CDK complexes. After immunoprecipitation of p73 proteins, the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the phospho-TP antibody (Fig. 3B). When p73 was co-expressed with cyclin A/CDK1, cyclin A/CDK2, cyclin B/CDK1, cyclin B/CDK2, or cyclin E/CDK2, the phospho-TP antibody was able to detect p73 proteins (Fig. 3B, upper panel). Consistent with the results shown in Fig. 1, the antibody did not recognize p73 when cyclin D/CDK4 (Fig. 3B) or cyclin D/CDK2 (data not shown) was co-expressed. The lower panel in Fig. 3B indicates that similar amounts of p73 protein were immunoprecipitated in the presence or absence of CDK. To support the possibility that CDKs can phosphorylate p73 protein in human cells, H1299 cells were transfected with constructs expressing p73 with or without cyclin A. p73 proteins were detected with the phospho-TP antibody even without overexpression of cyclin A (Fig. 3C, upper panel). However, upon cyclin A overexpression, p73 phosphorylation was increased as detected by the phospho-TP antibody. Conversely, when the cells were treated with an inhibitor of CDKs, roscovitine for 3 h, p73 phosphorylation on TP was strongly reduced (Fig. 3C, lower panel). Further evidence that reduction of p73 phosphorylation had occurred was obtained using an anti-p73 antibody, because the upper band of the doublet disappeared under roscovitine treatment (Fig. 3C, lower panel). Thus, CDK complexes phosphorylate p73 proteins on one or more TP sites, and this phosphorylation occurs in mammalian as well as insect cells.

**p73 Threonine 86 Is Phosphorylated *in Vitro* and *in Vivo***—To confirm that Thr<sup>86</sup> is a target for CDK, an antibody was raised that recognizes p73 when Thr<sup>86</sup> is phosphorylated. To validate the anti-Thr(P)<sup>86</sup> p73 antibody, a mutant form of p73 $\beta$  was generated in which threonine 86 was changed to alanine. Constructs expressing p73 $\beta$  wild type and p73 $\beta$ T86A proteins were transfected into H1299 cells and then immunoprecipitated from H1299 cell extracts with HA antibody cross-linked to protein A-Sepharose beads. After washes, the pellets of p73 proteins and beads were resuspended in a kinase buffer containing 100 ng of purified cyclin A/CDK2 complex or no kinase, and [ $\gamma$ -<sup>32</sup>P]ATP (Fig. 4A). Half of the proteins were run on an SDS-PAGE gel that was subsequently dried and autoradiographed (Fig. 4A, upper panels). The other half was also resolved on an SDS-PAGE gel and then transferred to nitrocellulose for immunoblotting using the p73 anti-Thr(P)<sup>86</sup> antibody (Fig. 4A, middle panels). In the absence of cyclin A/CDK2 complex, we did not observe any incorporation of radioactivity

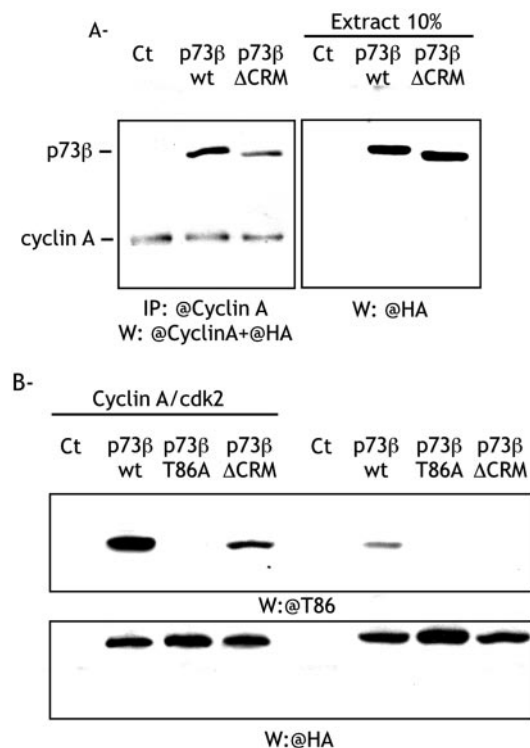


**FIG. 3. S/G<sub>2</sub>/M CDK complexes phosphorylate p73 at a TP site.** A, schematic representation of p73 $\alpha$  protein structure. TD, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain; TDI, transactivation domain I; TDII, transactivation domain II; SAM, sterile alpha motif; cdk4, sequence homology with CDK4; RG, regulatory region; TPXH, threonine-proline-Xaa-histidine = potential CDK phosphorylation site. B, baculoviruses expressing p73 $\beta$ , cyclin A, cyclin B, cyclin E, cyclin D, CDK1, CDK2, or CDK4 were used as indicated to infect insect cells. The cells were lysed in TEGN buffer 48 h after infection, and p73 proteins were immunoprecipitated (IP) in the presence of protein A-Sepharose beads cross-linked to anti-HA antibody. After four washes with the TEGN buffer, the sample buffer was added, and the beads and complexes were boiled and loaded on a 10% SDS-PAGE gel. After transfer to nitrocellulose, the blot was probed with an anti-phospho-TP antibody (Biolabs; dilution, 1:5000) (upper panel). The lower panel shows the level of immunoprecipitated p73 proteins present in the upper panel after having stripped the blot and reprobed it with an anti-HA antibody. C, cyclin A overexpression induces phosphorylation of p73 at a TP site in H1299 cells. H1299 cells were co-transfected with p73 $\beta$  and cyclin A expression vector. The cells were treated with roscovitine for 3 h before lysis. After immunoprecipitation of p73 proteins by A-Sepharose beads cross-linked to an HA antibody, the precipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with anti-phospho-TP antibody (upper panel). The blot was then stripped and reprobed with an anti-HA antibody to show the levels of p73 proteins immunoprecipitated (lower panel). Rosc., roscovitine; W, Western blot.

(upper left panel). The addition of purified cyclin A/CDK2 led to readily detectable phosphorylation of wild type p73 but not p73 $\beta$ T86A (only barely detectable upon long exposure). This observation suggested that Thr<sup>86</sup> is the main phosphorylation site for cyclin A/CDK2 phosphorylation on p73. The Thr<sup>86</sup> phos-



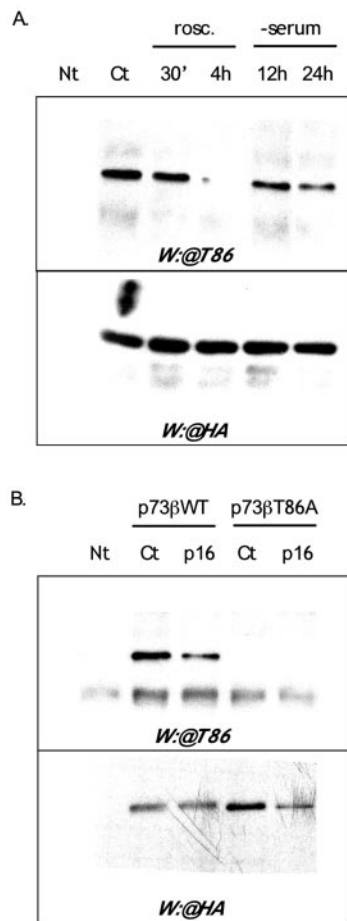
**FIG. 4. S/G<sub>2</sub>/M CDK complexes phosphorylate p73 at Thr<sup>86</sup>.** A, p73 Thr<sup>86</sup> is a major phosphorylation site for the cyclin A/CDK2 complex. p73β wt and p73β mutated in the Thr<sup>86</sup> site (p73βT86A) were expressed in H1299 cells. The proteins were extracted in a TEGN buffer containing no phosphatase inhibitor, and p73 proteins were immuno-



**FIG. 5. Role of the CRM located at position 149 in Thr<sup>86</sup> phosphorylation by CDKs.** A, p73β wt and p73β ΔCRM were expressed in H1299 cells. The cells were lysed 24 h after transfection, and cyclin A protein was immunoprecipitated (IP) with anti-cyclin A antibody and 50% slurry protein A-Sepharose beads. After four washes, the proteins were separated on a 10% SDS-PAGE gel and immunoblotted. HA-p73 proteins and cyclin A were detected with mouse monoclonal HA antibody and mouse monoclonal cyclin A antibody, respectively. The secondary antibody used for cyclin A detection was directed against the mouse Ig light chain (Pharmingen; 1:5000). The left panel shows p73 proteins expression in 10% of the extracts. B, p73β wt, p73β mutated in the Thr<sup>86</sup> site (p73βT86A), and p73β with the CRM deleted were expressed in H1299 cells. The proteins were extracted in TEGN buffer containing no phosphatase inhibitor, and p73 proteins were immunoprecipitated as described before. After four washes, the kinase reaction was performed as described in the legend to Fig. 4. p73 phosphorylation was then detected with the phospho-specific Thr<sup>86</sup> antibody by immunoblotting (upper panel). The same blot was also reprobed with an anti-HA antibody to detect the level of p73 proteins immunoprecipitated (lower panel). Ct, control; W, Western blot.

precipitated (IP) as described for C. The beads were resuspended in 30 μl of a kinase buffer; after four washes, 100 ng of purified cyclin A/CDK2 complex were added; and the mixtures were incubated at 37 °C for 30 min. Two-thirds of the mixtures were run on a SDS-PAGE gel that was subsequently dried and subjected to autoradiography (top panels). One-third of the proteins were run on a SDS-PAGE, transferred on nitrocellulose, and probed with phospho-specific Thr<sup>86</sup> antibody (middle panels). The same blot was also reprobed with an anti-HA antibody to detect the level of p73 proteins immunoprecipitated (bottom panels). B, G<sub>2</sub>/M CDK complexes phosphorylate p73 on Thr<sup>86</sup> in insect cells. p73β, cyclin A (CA), cyclin B (CB), cyclin D (CD), CDK2, and CDK4 were co-expressed in insect cells grown in 6-well plates. The proteins were extracted 48 h after infection, run on a 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and probed with an antibody directed against Thr<sup>86</sup> when phosphorylated (upper panel). In the lower panel, the same blot was stripped and reprobed with an anti-HA antibody. C, cyclin A/CDK2 overexpression increases p73 phosphorylation at Thr<sup>86</sup>. H1299 cells were co-transfected with p73β, cyclin A, and CDK2 expression vectors. After immunoprecipitation of p73 proteins with protein A-Sepharose beads cross-linked to an HA antibody, the precipitates were separated on a SDS-PAGE gel and transferred onto nitrocellulose. p73 phosphorylation was assessed by using the anti-phospho-Thr<sup>86</sup> antibody (upper panel; dilution, 1:1000). The lower panel shows the levels of immunoprecipitated p73 proteins. Ct, control; W, Western blot.



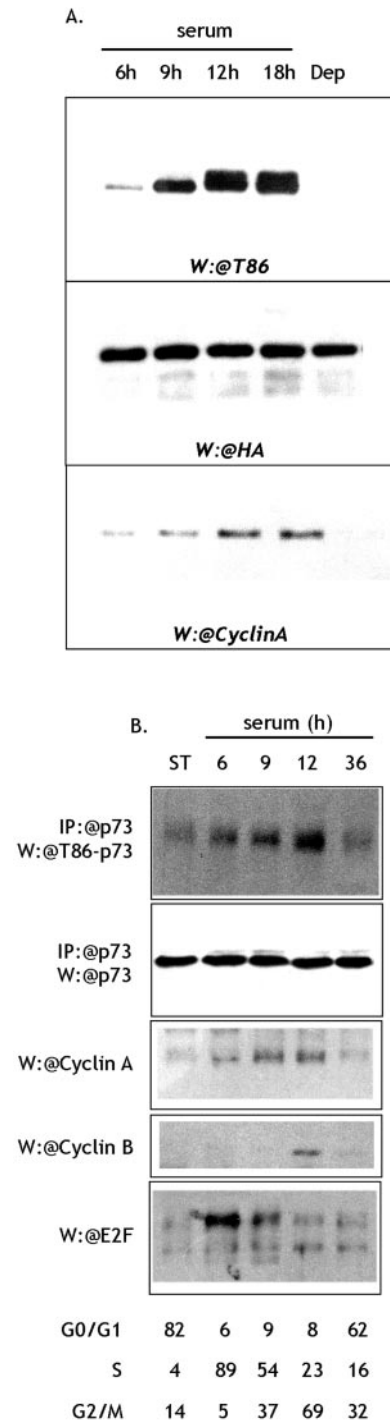


**FIG. 6. Phosphorylation of p73 at Thr<sup>86</sup> is reduced by CDK inhibitors.** A, Thr<sup>86</sup> phosphorylation is reduced by roscovitine and serum deprivation. p73β was expressed in Cos-1 cells for 36 h. As indicated, the cells were treated with roscovitine (*rosc.*, for 30 min or 4 h, 70 nM) or deprived in serum (*-serum*, for 12 or 24 h) before harvesting and protein extraction. B, overexpression of p16 reduces Thr<sup>86</sup> phosphorylation. p73β wt or p73βT86A were co-expressed with p16 in Cos1 cells. The cells were lysed 36 h after transfection, and the proteins were separated on a 10% SDS-PAGE gel, transferred onto nitrocellulose, and probed with the anti-Thr(P)<sup>86</sup> antibody. The lower panel shows p73 expression levels when the blot was stripped and reprobed with HA antibody. *Ct*, control; *Nt*, not transfected; *W*, Western blot.

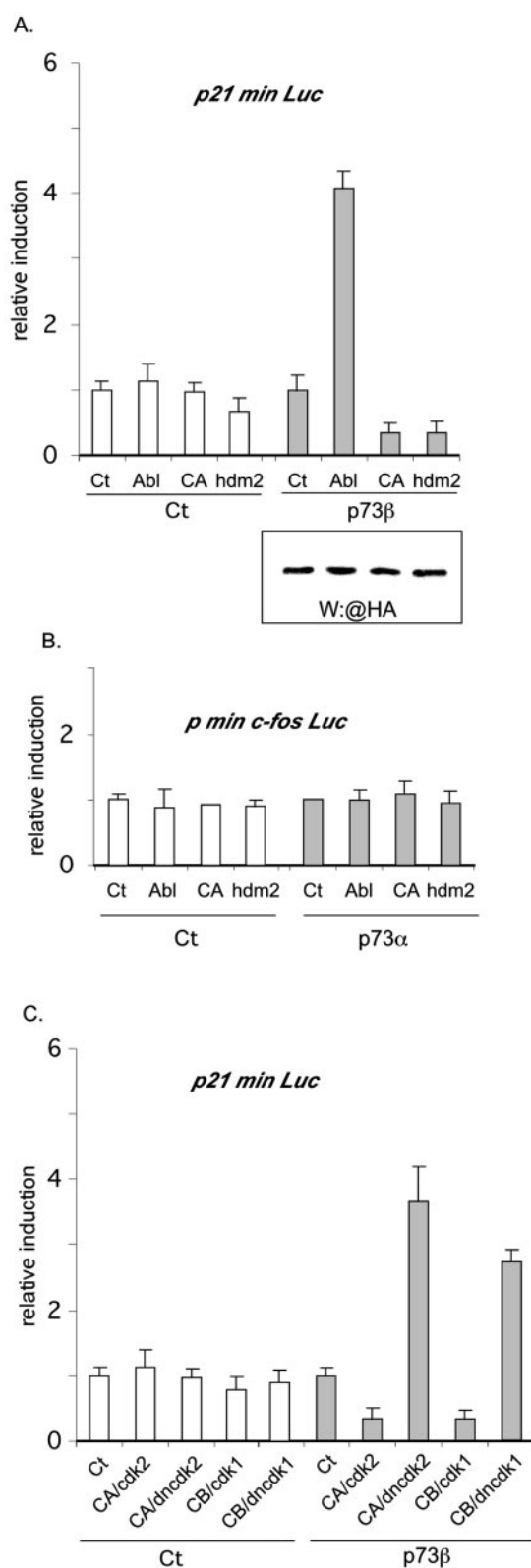
pho-specific antibody weakly recognized wild type p73β but not p73βT86A, and after incubation with cyclin A/CDK2, recognition of p73 wild type by the anti-phospho-Thr<sup>86</sup> antibody was markedly increased. Thus, the Thr<sup>86</sup> phospho-specific antibody specifically recognizes the Thr<sup>86</sup> site of p73 when phosphorylated. The weak reactivity observed in the absence of phosphorylation by the CDK complex suggests that some p73 is normally phosphorylated in H1299 cells at Thr<sup>86</sup>.

To confirm that Thr<sup>86</sup> can be phosphorylated by CDK complexes *in vivo*, insect cells were co-infected with p73 and CDK expressing baculoviruses as described in the legend to Fig. 1. Immunoprecipitated p73 was subsequently probed with the Thr<sup>86</sup> phospho-specific antibody. Cyclin A/CDK2 and cyclin B/CDK2 induced phosphorylation of p73 at Thr<sup>86</sup>, but cyclin D/CDK2 or cyclin D/CDK4 complexes failed to do so (Fig. 4B).

These observations were then extended to examine H1299 cells in which p73β was co-expressed with cyclin A and CDK2. p73 proteins were immunoprecipitated, and the phosphorylation of p73 was detected by immunoblotting using the Thr<sup>86</sup> phospho-specific antibody. As we had observed *in vitro*, Thr<sup>86</sup> phosphorylation was detected even in the absence of overexpressed cyclin A/CDK2 complex. However, cyclin A/CDK2 co-



**FIG. 7. Phosphorylation at Thr<sup>86</sup> is regulated through the cell cycle.** A, Thr<sup>86</sup> phosphorylation increases with passage through cell cycle. Cos-1 cells serum-deprived for 36 h (*Dep*) were transfected with a p73β expression vector, then serum (10%) was added, and the cells were harvested at the indicated time points. Thr<sup>86</sup> phosphorylation was tested as described in the legend to Fig. 6A. The bottom panel shows endogenous cyclin A protein levels following serum stimulation. B, regulation of Thr<sup>86</sup> phosphorylation on endogenous p73 protein during the cell cycle. T98G cells were starved from serum during 48 h, then serum was added, and the cells were harvested at the indicated time points (6, 9, 12, and 36 h). After treatment, the cells were lysed, and p73 proteins were immunoprecipitated with a p73 antibody (ER15; Oncogene; 1:50). The proteins were then separated on a 10% SDS-PAGE gel, transferred on nitrocellulose, and probed with the Thr<sup>86</sup> phospho-specific antibody. Cyclin A, cyclin B, and E2F expression was analyzed by immunoblotting the same extracts. At all time points, a fraction of the cells were fixed with methanol and analyzed by FACS. Percentages of cells in the three different states (G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M) are indicated below the blots. *W*, Western blot.



**FIG. 8. Overexpression of CDK regulates p73 transcriptional activity.** A and B, H1299 cells were co-transfected with p73, cyclin A (CA), Abl, and hdm2 expression vectors and a p53 reporter construct p21 min luc that contains a p53 binding site from the p21 promoter upstream the minimal c-fos promoter and the luciferase cDNA (A). The control reporter p min c-fos luc is the same as p21 min luc except that it does not contain the p53 binding site (B). The luciferase activity was assessed 36 h after transfection and is represented as induction relative to the control (Ct). The histograms represent the means of triplicate samples in a representative experiment, and the bars show standard deviations. The autoradiograms indicate proteins levels of overexpressed HA-p73 (right panel). In this experiment, p73β overexpression

expression significantly increased phosphorylation at this site (Fig. 4C, left panel). As expected, p73βT86A was not detected by the anti-phospho-antibody (Fig. 4C, right panel).

**The p73 Cyclin Recognition Motif Facilitates Thr<sup>86</sup> Phosphorylation by Cyclin-dependent Kinases**—As shown above, p73 proteins possess at least one CRM sequence and can interact with cyclins. We wished to determine the importance of the CRM in p73 for both p73 phosphorylation and p73 activity. To this end, a variant of p73β lacking the three amino acids KKL (position 149) was generated (p73ΔCRM). We tested the ability of cyclin A/CDK2 to phosphorylate and interact with this mutant. Wild type p73 and p73ΔCRM proteins were expressed in H1299 cells, and endogenous cyclin A was immunoprecipitated from cell extracts. After four washes, the proteins were separated on a 10% SDS gel and immunoblotted, and p73 proteins were detected with HA antibody (Fig. 5A). Although interactions with both wild type p73 and p73ΔCRM were observed, the CRM-deleted form of p73 interacted less efficiently than wild type p73. Next, we examined phosphorylation of p73βΔCRM using the Thr(P)<sup>86</sup> antibody (Fig. 5B). Interestingly, p73βΔCRM was much less efficiently phosphorylated by cyclin A/CDK2 than was wild type p73β, suggesting that the CRM is important for Thr<sup>86</sup> CDK-dependent phosphorylation (Fig. 5B). Finally, we tested the activity of p73βΔCRM on the p21 min luc reporter gene, and we observed that this mutant was not able or barely able to induce p21 min luc expression (data not shown). Because the CRM lies within the DNA-binding region of p73, this result was not unexpected. Nevertheless, our results identify this region as being important for facilitating phosphorylation of p73 at Thr<sup>86</sup>.

**Phosphorylation of p73 at Thr<sup>86</sup> Is Regulated through the Cell Cycle**—Because we showed that several CDK complexes can phosphorylate p73 on Thr<sup>86</sup> *in vitro* and p73 can be phosphorylated at Thr<sup>86</sup> *in vivo*, it was of interest to determine whether phosphorylation at this site is regulated through the cell cycle in human cells. In the first set of experiments, HA-p73β was overexpressed in Cos-1 cells, and the state of the cells or the activity of the CDK were modulated experimentally. Either the cells were treated with roscovitine (Fig. 6A, *rosc.*) or serum-starved (–*serum*) or p73 was co-expressed with p16 an inhibitor of CDK in G<sub>1</sub> phase (Fig. 6B). The phosphorylation status of Thr<sup>86</sup> was then monitored by immunoblotting with the Thr<sup>86</sup> phospho-specific antibody. We observed that all treatments known to reduce CDK activity and cell cycle progression (p16, roscovitine, serum starvation) significantly reduced Thr<sup>86</sup> phosphorylation (Fig. 6). Note that in all conditions, the variation in Thr<sup>86</sup> phosphorylation was not due to a decrease in the level of immunoprecipitated p73 proteins as shown by reblotting with an anti-HA antibody (Fig. 6, lower panels in both A and B).

We next examined phosphorylation of p73 at Thr<sup>86</sup> upon cell cycle re-entry. The cells were first depleted of serum for 48 h and then were transfected with the p73 expression vector for 14 h before induction with serum (Fig. 7A). The cells were then harvested at various times after serum treatment, and both Thr<sup>86</sup> phosphorylation and cyclin A expression (an indicator of the progression of the cell cycle) were monitored. Serum starvation abolished almost all Thr<sup>86</sup> phosphorylation (seen with a longer exposition). In contrast, serum treatment led to phosphorylation of p73 at Thr<sup>86</sup>. Remarkably, this phosphorylation correlated closely with cyclin A expression levels (Fig. 7A, top

induced the activity of the reporter gene by 18-fold (not shown). C, H1299 cells were co-transfected with p73β and p21 min luc and the indicated CDK complexes. dnCDK2 and dnCDK1 are the dominant negative mutant forms of CDK2 and CDK1, respectively. The luciferase activity was tested as described for A. W, Western blot.



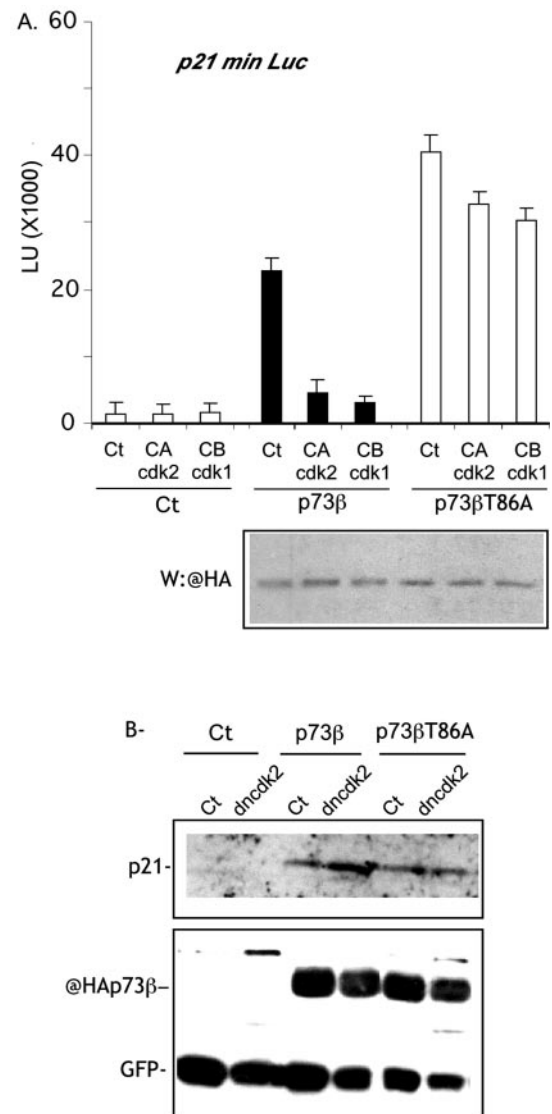
and bottom panels). In all conditions, similar levels of p73 $\beta$  were immunoprecipitated (Fig. 7A, middle panel).

To analyze in more detail the phosphorylation status of endogenously expressed p73 at Thr<sup>86</sup> through the cell cycle, we used T98G cells in which it was previously shown that p73 expression is regulated through the cell cycle (32). T98G cells were induced with serum after starvation, and progression of their cell cycle was monitored by FACS analysis (not shown) and expression of specific markers (cyclin A, cyclin B, and E2F; Fig. 7B). Endogenous p73 protein was immunoprecipitated with a p73 antibody and after SDS-PAGE was immunoblotted with the anti-Thr(P)<sup>86</sup> antibody. We normalized immunoprecipitation conditions to have comparable levels of p73 proteins on the blot. When cells were starved for 48 h, only weak phosphorylation of p73 was observed (Fig. 7B, ST). In this condition, no markers of the cell cycle were significantly expressed, and the cell cycle profile obtained by FACS analysis indicated that 82% of the cells were in G<sub>1</sub>/G<sub>0</sub>. Six hours after serum treatment, the majority of the cells were in S phase, and E2F, as well as cyclin A, was detected. Thr<sup>86</sup> phosphorylation was clearly increased at this stage and continued to rise as the cells progressed through the cell cycle (Fig. 7B). Twelve hours after serum treatment, the cells were mainly in G<sub>2</sub>/M phase (69%), and expression of both cyclin A and cyclin B was observed. It was also at this stage that p73 phosphorylation on Thr<sup>86</sup> was maximal. These results strongly support the physiological relevance of our observations that CDK complexes containing cyclins A or B preferentially phosphorylate p73.

**Cyclin A/CDK2 or Cyclin B/CDK1 Overexpression Regulates p73 Transcriptional Activity**—To evaluate the impact of phosphorylation by CDK on p73 transcriptional activity, a luciferase reporter gene containing p53-binding sites from the p21 promoter upstream a minimal *c-fos* promoter was transfected into H1299 cells (39) (p21 min luc; Fig. 8A). H1299 cells do not express endogenous p53 but express low levels of p73. As controls for the experiment, we also overexpressed c-Abl and HDM2, which were previously shown to be positive and negative regulators of p73, respectively (15–18, 22–25). As expected, we observed that c-Abl expression increased the activity of p73 on the p21 min luc reporter gene (Fig. 8A). Note that results are shown as fold induction relative to the control in the absence of Abl, cyclin A, or MDM2 overexpression. Overexpression of p73 alone induced the reporter gene activity by a factor of 18 (data not shown). In contrast, HDM2 expression decreased the activity of p73 by ~50%. When cyclin A was overexpressed in similar conditions, p21 min luc reporter activity was decreased to a similar extent as observed with HDM2 (Fig. 8A, upper panel). In all of the conditions described, p73 protein levels remained the same (Fig. 8A, lower panel). We also used the parental reporter gene p min *c-fos* luc as a control, and no change in its activity was observed in any conditions, indicating the specificity of the effects obtained (Fig. 8B).

To extend our analysis of the regulation of p73 activity by CDKs, we co-expressed p73 proteins along with cyclin A/CDK2 or cyclin B/CDK1 complexes. Both CDK complexes reduced p73 transcriptional activity as was observed when cyclin A was expressed alone (Fig. 8C). Strikingly, when we co-expressed p73 with dominant negative forms of CDK1 (dnCDK1) or CDK2 (dnCDK2), p73 activity was markedly increased (Fig. 8C).

We then tested whether phosphorylation at Thr<sup>86</sup> is important for the functions of p73 by comparing activity of wild type p73 $\beta$  and p73 $\beta$ T86A on the p21 min luc reporter gene in H1299 cells in the presence or absence of CDK complexes (Fig. 9A). Both p73 $\beta$  wt and p73 $\beta$ T86A activated p21 min luc expression. However, CDK complexes were far less effective in reducing p73 $\beta$ T86A transcriptional activity than wild type p73 $\beta$  (Fig. 9A). This series



**FIG. 9. Mutation in Thr<sup>86</sup> site affects p73 $\beta$  transcriptional activity.** A, p73 $\beta$  wt or p73 $\beta$  T86A were co-transfected with the p21 min luc reporter gene in H1299 cells. Luciferase activity was assessed 36 h after transfection and is indicated as light units (LU). The data represent the means of triplicate samples from a representative experiment. The bars show standard deviations. The autoradiograms indicate protein levels of overexpressed HA-p73 $\beta$  wt or p73 $\beta$ T86A proteins. B, regulation of p21 protein levels by wild type p73 and p73 $\beta$ T86A. H1299 cells were co-transfected with expression vectors coding for wild type p73 $\beta$ , p73 $\beta$ T86A, or control vector and a dominant negative form of CDK1 as indicated. The cells were lysed 20 h after transfection, and the proteins were separated on a 10% SDS gel and immunoblotted. p21, p73, and green fluorescent protein expression were detected with respectively a p21 antibody (Pharmingen; 1:500), a HA antibody (Babco; 1:1000), and a green fluorescent protein antibody (Clontech; 1:4000). CA, cyclin A; CB, cyclin B; Ct, control; W, Western blot.

of experiments suggests that phosphorylation of p73 by CDK negatively regulates p73 transcriptional activity.

To confirm that CDKs control p73 function in a more physiological context, we tested the ability of p73 to regulate endogenous p21 protein expression in H1299 cells in the presence or absence of the dominant negative form of CDK1 (dnCDK1). As with the p21 min luc reporter gene, p73 $\beta$  overexpression in H1299 cells led to increased p21 protein levels, and the capacity of p73 to induce p21 expression was significantly enhanced by dnCDK1 (Fig. 9B). Importantly, although p73 $\beta$ T86A induced p21 to the same extent than wild type p73 $\beta$ , it did not show similar stimulation by dnCDK2 (Fig. 9B, see Ct lane in

p73 $\beta$  lanes). Altogether our results support the likelihood that p73 phosphorylation at Thr<sup>86</sup> by CDK1 leads to decreased p73 transcriptional activity.

#### DISCUSSION

In this study, we addressed the possibility that p73 proteins can be phosphorylated by cyclin-dependent kinases and that this post-translational modification regulates p73 protein activity. We found that several CDK complexes phosphorylate p73 proteins with various degrees of efficacy, and we identified Thr<sup>86</sup> as an important site for CDK phosphorylation. We also observed that cyclins interact directly with p73. At a functional level, we demonstrated that phosphorylation of p73 by CDK decreases p73 transcriptional activity on the p21 gene and that this regulation is impaired when Thr<sup>86</sup> is mutated.

**p73 Is a Relevant Target for S/G<sub>2</sub>/M CDK Complexes**—To assess whether p73 proteins are targets of cyclin-dependent kinases, we tested various CDK complexes and found that S/G<sub>2</sub>/M CDK complexes are able to phosphorylate p73 proteins. The phosphorylation was observed both *in vitro* and in various cell lines (mammalian and insect), either with exogenously expressed protein or endogenous p73 proteins, indicating that this process occurs normally *in vivo*. Although the significance of phosphorylation of p73 by CDKs is not yet established, our results suggest that the proportion of p73 protein that is phosphorylated is fairly substantial. First, we observed that both p73 and pRb can be phosphorylated to approximately similar extents *in vitro*, and pRb is a well known target of CDK complexes (Fig. 1). Second, CDK phosphorylation of p73 results in a significant gel mobility shift of the p73 polypeptide, in some cases leading to its complete retardation. Importantly, we found that modulation of p73 phosphorylation at Thr<sup>86</sup> occurs during the cell cycle, suggesting strongly that CDK complexes can affect p73 status at this site in a physiologically relevant manner. However, our data do not exclude the possibility that in mammalian cells Thr<sup>86</sup> phosphorylation could also be regulated by other cell cycle-regulated kinases in addition to CDKs. It is also possible that under other physiological conditions, protein kinases whose activity is unrelated to the cell cycle might phosphorylate Thr<sup>86</sup>.

The fact that p73 is most highly phosphorylated at Thr<sup>86</sup> in G<sub>2</sub> and M (Fig. 5) is consistent with our observation that it is phosphorylated most efficiently by cyclin A and cyclin B containing complexes (Fig. 1). By contrast cyclin E containing CDK complex phosphorylates p73 quite inefficiently, and cyclin D containing CDK complexes appeared to be virtually inert in this regard. We cannot exclude the possibility that under specific conditions cyclin E or cyclin D complexes phosphorylate p73, but the cell cycle specificity of p73 phosphorylation suggests that this is not likely to be the case.

**Thr<sup>86</sup> Is an Important Site for CDK Phosphorylation in p73 Protein**—Cyclin-dependent kinases phosphorylate proteins at TP or SP sites one residue from a basic residue on the C-terminal side of the site. In p73 proteins, we identified Thr<sup>86</sup> as the most likely candidate (sequence: TPEH). Using a Thr<sup>86</sup>-specific phospho-antibody, we confirmed that different CDK complexes indeed phosphorylate this site. We cannot exclude that CDKs can phosphorylate p73 on other residues. Indeed, we were still able to observe residual phosphorylation of the p73 $\beta$ T86A mutant by cyclin A/CDK2 (data not shown), and we found that transcriptional activity of p73 $\beta$ T86A is still partially repressed by CDK overexpression (Fig. 7). Nevertheless, both phosphorylation and the repression by CDKs of p73 $\beta$ T86A were drastically reduced when compared with wild type p73, strongly suggesting that Thr<sup>86</sup> is the principal site phosphorylated by CDK in p73.

**Role of a CRM Sequence in p73 Phosphorylation by CDK Complexes**—Phosphorylation by CDK complexes is sometimes dependent on the interaction between cyclins and the target through cyclin recognition motifs (40). All p73 proteins have a CRM located within the N-terminal portion of the DNA-binding domain (KKL; position 149). We identified a second CRM specific to p73 $\alpha$  located at position 515 (RAL). From our studies, it appears that both sequences are involved in the ability of cyclins to interact with p73 and to allow phosphorylation of p73 by CDK. Although we showed that deletion of the CRM located at position 149 reduced strongly the interaction with cyclin A or the phosphorylation by cyclin A/CDK2, the interaction between cyclin A and p73 $\alpha$  is stronger than with p73 $\beta$  (Figs. 2 and 5). The exact role of CRMs in the regulation of p73 activity and in particular whether the interaction with the cyclin might regulate p73 function(s) without phosphorylation is difficult to assess because one of CRM sequence is located in the DNA-binding domain of p73. The fact that CRM(s) are present in p73 and can impact its ability to be phosphorylated by CDKs further support the likelihood that CDKs, particularly those containing S and G<sub>2</sub> type cyclins, play a significant role in the regulation of p73.

**How Is the Function of p73 Regulated by CDKs?**—We observed that overexpression of two CDK complexes (cyclin A/CDK2 and cyclin B/CDK1) reduces p73 transcriptional activity in our conditions. Importantly, the p73 $\beta$ T86A mutant is markedly less sensitive to CDK repression. This conclusion was based on transient co-transfection experiments with a p53/p73-responsive reporter (Fig. 8) and perhaps more convincingly by examining directly the expression levels of endogenous p21 protein in H1299 cells (Fig. 9). Therefore, under our conditions, CDK phosphorylation leads to the repression of at least some of the functions of p73. At this stage, we do not know the mechanism involved. When the effect of *in vitro* phosphorylation by CDK on the ability of purified p73 to bind DNA was tested, we observed a modest (2-fold) increase under those conditions (data not shown). Although this is not consistent with our observations, it should be noted there that when experiments were performed with overly confluent cells, we observed that overexpression of CDKs actually stimulated transcriptional activity of wild type p73 but not the p73 $\beta$ T86A mutant (data not shown). This observation may be related to our observation of modest stimulation of p73 DNA binding by CDKs *in vitro*.

It is possible that the repression mechanism seen under the culture conditions used in our experiments (*i.e.* use of subconfluent freshly plated cells) reflects modulation of the interaction of p73 with other molecules that would be affected by Thr<sup>86</sup> phosphorylation. Indeed, we observed that the mutant p73 $\beta$ T86A is more strongly stabilized by MDM2 than wt p73 $\beta$  (data not shown). The physiological relevance of this observation is still under investigation. It is also interesting to note that the location of Thr<sup>86</sup> within p73 is similar to threonine 81 in p53, which is also adjacent to a proline. Recently, Thr<sup>81</sup> in p53 was shown to be required for interaction with and stimulation by the prolyl isomerase PIN1 (41, 42). It is attractive to speculate that CDK-dependent phosphorylation at Thr<sup>86</sup> may also control the activity of p73 by modulating the interaction of p73 with PIN1.

**What Is the Function of CDK-dependent Phosphorylation of p73 during the Cell Cycle?**—p73 phosphorylation of Thr<sup>86</sup> increases during the cell cycle such that it is greatest at G<sub>2</sub>/M. Because p73 harbors pro-apoptotic and growth arrest properties, these functions require a tight control by CDKs to allow a cell cycle to be completed, which is consistent with our finding that CDKs down-regulate p73 activity. However, it is intriguing that the maximum of Thr<sup>86</sup> phosphorylation is at G<sub>2</sub>/M

where the mitotic checkpoint can take place when microtubules are disrupted. Interestingly, it has recently been shown that cyclin B/CDK1 is involved in this mitotic checkpoint by up-regulating the activity of Survivin (43), a gene that can be repressed by p53 (26). Although p53 is not involved in the mitotic checkpoint (43), it is possible that p73 and its phosphorylation by CDK might play a role at this key stage of the cell cycle.

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**Cyclin-dependent Kinases Phosphorylate p73 at Threonine 86 in a Cell Cycle-dependent Manner and Negatively Regulate p73**

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