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Physical and Functional Interaction between p53 Mutants and Different Isoforms of p73*

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p53 is the most frequently inactivated tumor suppressor gene in human cancer, whereas its homologue, p73, is rarely mutated. Similarly to p53, p73 can promote growth arrest or apoptosis when overexpressed in certain p53-null tumor cells. It has previously been shown that some human tumor-derived p53 mutants can exert gain of function activity. The molecular mechanism underlying this activity remains to be elucidated. We show here that human tumor-derived p53 mutants (p53His175 and p53Gly281) associate *in vitro* and *in vivo* with p73α, β, γ, and δ. This association occurs under physiological conditions, as verified in T47D and SKBR3 breast cancer cell lines. The core domain of mutant p53 is sufficient for the association with p73, whereas both the specific DNA binding and the oligomerization domains of p73 are required for the association with mutant p53. Furthermore, p53His175 and p53Gly281 mutants markedly reduce the transcriptional activity of the various isoforms of p73. Thus, human tumor-derived p53 mutants can associate with p73 not only physically but also functionally. These findings define a network involving mutant p53 and the various spliced isoforms of p73 that may confer upon tumor cells a selective survival advantage.

The p53 tumor suppressor gene is the most frequent target for genetic alterations in human cancers (1). The wild type p53 protein can elicit a variety of biological effects, ranging from growth arrest to apoptosis and differentiation (2–4). These effects are mainly exerted by wild-type p53 through the activation of a growing plethora of p53-responsive target genes (5). In human cancers, the most prevalent type of p53 mutations consists of missense mutations, often within the highly conserved DNA binding core domain of the protein (2, 3). One certain outcome of those mutations may be the elimination of cellular wild-type p53 activity. However, at variance with other tumor suppressor genes, cells with p53 mutations typically maintain expression of full-length protein. This may suggest that at least certain mutant forms of p53 can contribute actively to cancer progression through gain of function activity (6–12).

A new gene, p73, sharing considerable sequence homology as well as structural homology with p53, has recently been identified (13, 14). Similarly to p53, the p73 protein can be roughly divided into three main domains: (*a*) the N-terminal transactivation domain, which shares 29% homology with the N-terminal part of p53; (*b*) the sequence-specific DNA binding domain, which shares 63% of homology with the corresponding p53 domain; and (*c*) the tetramerization domain, which shares 42% homology with the oligomerization domain of p53 (15, 16). Furthermore, ectopic expression of p73 can transactivate p53 responsive target genes, and also induces apoptosis in different cell lines (13, 14). As revealed by the comparison of the p73 and p53 sequences, the DNA binding domains share the highest homology. However, although this domain in p53 is the major site of the mutations, no mutations could be found so far in p73 despite extensive efforts (17–23). Unlike p53, p73 is not inactivated by viral oncoproteins, such as T-antigen, E6, and E1Bp55, well known inactivators of p53 (24–28). It was originally reported that p73, unlike p53, is not induced upon UV irradiation, highlighting an additional difference from p53 (14). More recently, however, it was found that p73 is induced and tyrosine-phosphorylated by exposure of cells to DNA-damaging agents, such cisplatin and γ-radiation, suggesting a differential behavior of p73 in response to different types of DNA damage (29–31). Of note, different p73 variants exist in the cell, giving rise to a family of proteins that adds a new level of complexity to the understanding of the p73 signaling in cancer cells (13, 32, 33). Interestingly, it was recently reported that human tumor-derived p53 mutants can associate with p73α and interfere with its transcriptional activity and ability to induce apoptosis when co-expressed in transient transfection assays (34).

Here we investigate *in vitro* and *in vivo* the interaction between human tumor-derived p53 mutants and the various p73 spliced isoforms. We report that (*a*) the association between mutant p53 and p73 occurs under physiological conditions; (*b*) two different human tumor-derived p53 mutants (His175 and Gly281) associate with p73 α, β, γ, and δ when co-transfected transiently in H1299 cells; (*c*) the DNA binding domain of mutant p53 is sufficient for the association with p73 isoforms; (*d*) a region of p73 that includes the sequence-specific DNA binding and the oligomerization domains is sufficient for the association with mutant p53; (*e*) p73-tyrosine phosphorylation is dispensable for the association with mutant p53; and (*f*) human tumor-derived p53 mutants interfere with the transcriptional activity of p73α, β, γ, and δ.

EXPERIMENTAL PROCEDURES

Cell Lines—The H1299 cell line is derived from a human large cell lung carcinoma (35). H1299 cells were maintained in RPMI medium,

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supplemented with 10% fetal calf serum (FCS)[1] (Life Technologies, Inc.). Before transfection, the culture medium was changed to Dulbecco's modified Eagle's medium supplemented with 10% FCS. H1299-pVgRXR cells were maintained in the same medium containing zeocin (100 μg/ml) (Invitrogen, San Diego, CA). H1299-pIND clone 1 and H1299-mp53His175 clone 41 cells were maintained in medium containing zeocin and G418 (400 μg/ml). To induce m-p53 expression, ponasterone A, a synthetic analog of ecdysone (Alexis Biochemicals, San Diego, CA) was added to the medium (final concentration, 2.5 μM). H1299-mp53His175 cells were transfected and maintained in culture as previously reported (11). T47D and SKBR3 breast cancer cell lines were maintained in RPMI containing 10% FCS. T47D-HA and T47D-HA-DD cell lines were maintained in the same medium containing G418 (400 μg/ml).

Plasmids and Stable Transfections—Plasmids pVgRXR and pIND were from Invitrogen. pIND/m-p53His175 was prepared by cloning the *Bam*HI/*Bam*HI fragment of human mutant p53 cDNA into pIND. H1299 cells were transfected with each plasmid using the calcium phosphate method (36). Clone selection was with zeocin (100 μg/ml) for pVgRXR and with G418 (400 μg/ml) for pIND or pIND/m-p53His175 (37).

T47D cells were transfected with the pcDNA3-HA-DD plasmid (kindly provided by Dr. G. Del Sal) or with pcDNA3-HA using the calcium phosphate method. The transfection was performed in Dulbecco's modified Eagle's medium +10% FCS, and then the medium was changed again to RPMI medium + 10% FCS. Stable transfected polyclonal populations were selected in medium containing G418 (400 μg/ml) for 3 weeks.

Plasmids and Transient Transfections—Overexpression of p73 was achieved by transfection of pcDNA3-HA-p73α and pcDNA3-HA-p73β (kindly provided by Dr. W. G. Kaelin, Jr.), pcDNA3-HA-p73γ and pcDNA3-HA-p73δ (kindly provided by Dr. G. Melino), pCDNA3-HA-p73α^{Y99F}, pCDNA3-HA-p73α^{Y121F}, and pCDNA3-HA-p73$\alpha^{Y99F/Y121F}$. Overexpression of mutant p53 was achieved by transfection of the following plasmids: pCDNA3-p53His175, pCDNA3-p53His175-(22,23), pCDNA3-p53His175-Δproline, pCDNA3-p53His175-(1–338), pCDNA3-p53His175-(1–355), and pCDNA3-p53Gly281, all driven by the CMV early promoter/enhancer. pCDNA3-p53His175-(22,23) was prepared by replacing a *Hin*dIII/*Sgr*aI fragment from pRVCMV-wtp53-(22,23) (38) into pCDNA3-p53His175. pcDNA3-p53His175-Δproline was prepared by cloning a *Hin*dIII/*Xba*I fragment (39) into pCDNA3-neo. pCDNA3-p53His175-(1–338) and pCMVp53His175-(1–355) were prepared by cloning the fragments *Eco*RI/*Not*I into pCDNA3-neo. Overexpression of the core domain of mutant p53 was achieved by transfection of pEGFP-p53His175(74–298) prepared by cloning a *Eco*RI/*Xho*I fragment into pEGFP-C2 expression vector (Invitrogen).The parental vector pCMV-neo was used to keep the amount of the transfected DNA constant among samples. Transient transfections were done by calcium phosphate method in the presence of BES (Sigma). The precipitates were left for 12 h, after which the medium was changed again to RPMI medium + 10% FCS. The cells were harvested 36 h posttransfection.

Immunoprecipitation and Western Blot Analysis—H1299 cells were transfected in 100-mm plates with 8 μg of DNA and harvested at 36 h after the transfection. Cells were lysed in 900 μl of lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 mM NaF, 1 mM MgCl$_2$, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors), and the extracts were sonicated for 10 s and centrifuged at 14000 × rpm for 10 min to remove cell debris. Protein concentrations were determined by a colorimetric assay (Bio-Rad). After preclearing for 60 min at 4 °C, immunoprecipitations were performed by incubating 1.5 mg of whole-cell extract with anti-p73 polyclonal-Abs C-17 and C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or with anti-HA antibody or with a mixture of anti-p53 mAbs DO1 and 1801, with rocking at 4 °C for 1 h. Immunocomplexes were precipitated with protein G-agarose (KPL, Guilford, CA). The immunoprecipitates were washed three times with 1 ml of wash Net-gel buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.1% Nonidet P-40). The excess liquid was aspirated, and 40 μl of 5× sample buffer was added. Immunoprecipitates as well as 1% of each extract were resolved by SDS-10% PAGE. Protein gels were transferred to nitrocellulose membranes (Bio-Rad). For p73 detection, a polyclonal p73 antibody was used at 1:3000 dilution; for p53 detection, a mixture of p53 monoclonal antibodies DO1 and 1801 was used at 1:40 dilution; for HA-p73, a supernatant containing mAb 12CA5 was used at a 1:5 dilution; for GFP-p53His175-(74–298) a polyclonal GFP antibody (Invitrogen) was used at 1:5000 dilution. Western blot analysis was performed with the aid of the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Inc.).

Production of Recombinant Proteins—GST fusion proteins containing large fragments of p73α were obtained by polymerase chain reaction amplification of the appropriate fragment from monkey p73α, followed by cloning in the pGEX-2TK expression vector (Invitrogen), in frame with the GST moiety. Sequences of the oligonucleotides and primers used are available on request. pGEX-2TK.1/p53His175-(74–298) was prepared by cloning *Eco*RI/*Xho*I fragment from pLex/p53 His175-(74–298) (kindly provided by Dr. G. Del Sal) vector in frame with the GST moiety. Purification of the GST fusion proteins by glutathione-Sepharose 4B (Sigma) was performed following standard procedures (40–42).

In Vivo Binding Assays—*In vivo* binding assays were performed using 20 μg of immobilized purified GST fusion proteins or wild type GST that were incubated with 2 mg of total cellular proteins prepared from H1299 cells transiently transfected with p73α or m-p53His175 or m-p53Gly281. The lysates were first precleared with glutathione-agarose beads and then incubated for 2 h at 4 °C. Following three consecutive washes with HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), excess liquid was aspirated, and 40 μl of 5× sample buffer was added. Immunoprecipitates, as well as 1% of each extract, were resolved by 10% SDS-PAGE. The immunoblots were probed with anti-p73 polyclonal antibody or with a mixture of the p53 monoclonal antibodies DO1 and 1801. Detection was performed with the aid of the ECL system (Amersham Pharmacia Biotech).

Luciferase Assays—H1299 cells were transfected with reporter plasmid together with the indicated expression plasmid combinations. 36 h later, cells were rinsed with cold phosphate-buffered saline, resuspended in cell lysis buffer (Promega Corp., Madison, WI), and incubated for 10 min at room temperature. Insoluble material was spun down, and luciferase activity was quantitated using a commercially available kit (Promega) with the aid of a TD-20E luminometer (Turner).

RESULTS

Generation of Stable Cell Lines Expressing Mutant p53His175 or DD Miniprotein—To generate inducible cell lines overexpressing mutant p53, the ecdysone-inducible system was used (43). The human lung cancer cell line H1299 was chosen for this aim because it is p53-null (35). Ecdysone-inducible cell lines were generated in two steps. First, H1299 cells were transfected with pVgRXR followed by zeocin selection. The isolated clones were transiently transfected with pIND/m-p53His175 and maintained in the presence of ponasterone A. The highest expressor of mutant p53 was chosen and subject to transfection with pIND/m-p53His175 as well as with pIND vector control followed by G418 selection. Western blot and immunostaining analyses with a mixture of the anti-p53 mAbs DO1 and 1801 were performed to screen for p53-inducible expression and intracellular localization. As seen in Fig. 1*A* (*lanes 5–8*), a representative clone for each cell type (H1299-mp53His175 clone 41 and H1299-pIND clone 1) was chosen for immunoprecipitation experiments. Immunostaining revealed a predominant nuclear localization of mutant p53 (data not shown).

T47D, a human breast carcinoma cell line carrying endogenous mutant p53Phe194, was stably transfected with pCDNA-HA-DD or with vector control followed by G418 selection for 3 weeks. A polyclonal population for each cell line (see Fig. 4*B*) was chosen to perform coimmunoprecipitation experiments.

Human Tumor-derived p53 Mutants Associate with Endogenous p73α—To determine whether human tumor-derived p53 mutants associate with endogenous p73α, we performed coimmunoprecipitation experiments from extracts of the H1299-pIND clone 1 and H1299-mp53His175 clone 41 cell lines. In order to induce mutant-p53 expression, the cell lines were grown in the presence of ponasterone A (2.5 μM/ml) for 24 h. Cell extracts were precleared with protein G-Sepharose, followed by immunoprecipitation with a mixture of anti-p73 poly-

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[1] The abbreviations used are: FCS, fetal calf serum; CMV, cytomegalovirus; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; mAb, monoclonal antibody; DD, oligomerization domain. PAGE, polyacrylamide gel electrophoresis.

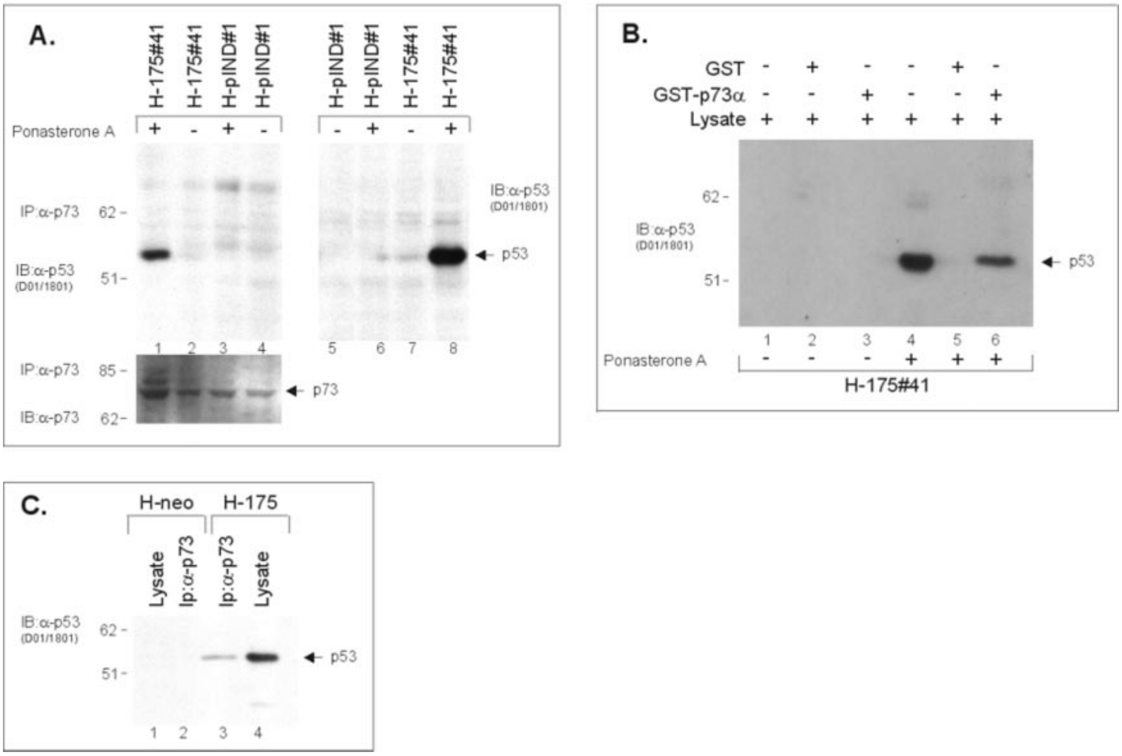


FIG. 1. ***In vivo* association between mutant p53 and endogenous p73α.** *A,* H1299-inducible cell lines were generated in two steps (see under "Results"). Cell extracts were prepared either from His-175 clone 41 and from H-pIND clone 1 cell clones 24 h after the exposure to 2.5 μM of ponasterone A. Identical cell lysates were prepared from untreated cells. Aliquots containing 2 mg of total protein were subjected to immunoprecipitation (*IP*) with a mixture of anti-p73 polyclonal-Abs C-17 and C-20. Immunoprecipitates were resolved by SDS-PAGE, followed by immunoblot (*IB*) with a mixture of anti-p53 mAbs DO1 and 1801 (*lanes 1–4*). The blot was reprobed with anti-p73 polyclonal serum (*bottom panel*). Aliquots containing 100 μg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801 (*lanes 5–8*). *B,* binding of p53His175 to GST-p73α or to GST alone. Total cellular lysates (2 mg) of His-175 clone 41 (*H-175#41*) prepared as reported in *A* were incubated with GST fusion proteins for 2 h at 4 °C. Specifically bound mutant p53 was detected by immunoblotting with a mixture of anti-p53 mAbs DO1 and 1801 (*lane 6*). *Lanes 1* and *4* contain aliquots of unprocessed lysates (100 μg/lane), loaded directly on the gel. *C,* p53-null H1299 cells and His-175 cells, carrying an exogenous mutant p53His175, were lysed and subjected to immunoprecipitation as described for *A*. *Lanes 2* and *3* represent immunoprecipitates with 2 mg of total cell protein. *Lanes 1* and *4* contain aliquots of unprocessed lysates (100 μg/lane), loaded directly on the gel. Positions of protein molecular size markers are indicated on the *left*.

clonal antibodies. Immunoprecipitates as well as 1% of each total extract were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. As seen in Fig. 1*A,* mutant p53His175 was brought down only when the H1299-m-p53His175 clone 41 cells were treated with ponasterone A (*lanes 1* and *2*). As expected no coprecipitated mutant p53 was brought down from H1299-pIND clone 1 cells with or without ponasterone A stimulation (*lanes 3* and *4*). The presence of endogenous p73 was checked by reprobing the blot with anti-p73 polyclonal antibody (Fig. 1*A, bottom panel, lanes 1–4*).

We have previously reported (11) that stable exogenous expression of mutant p53His175 in H1299 cells causes increased chemoresistance to etoposide and cisplatin treatment. To verify whether, in these cells, mutant p53 could associate with endogenous p73α, we performed a coimmunoprecipitation experiment as previously reported. As seen in Fig. 1*C,* coprecipitated mutant p53 was detected only in H1299 cells overexpressing m-p53His175 (*lane 3*) but not in the cells stably transfected with an empty vector (*lane 2*). An aliquot containing 1% of total cell lysate from unprocessed cells directly applied to the gel was concomitantly subjected to immunoblotting with a mixture of anti-p53 mAbs DO1 and 1801 (*lanes 1* and 4). These data raise the possibility that gain of function of mutant p53, evident as increased chemoresistance to etoposide or cisplatin, might involve inactivation of p73-induced apoptosis by association with mutant p53.

To further confirm the specificity of the association between p53His175 and p73α, we performed an *in vivo* binding assay. To this end, total cell lysates of H1299-m-p53His175 clone 41 with or without ponasterone A induction (Fig. 1*B*) as well as His-175 polyclonal populations (data not shown) were incubated with GST-p73α full-length or GST alone. Specifically bound m-p53 was detected by immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. In agreement with the coprecipitation data (Fig. 1*A*), bound p53His175 was detected only when expression of mutant p53 was induced by ponasterone A (Fig. 1*B, lanes 4* and *6*) and in polyclonal populations overexpressing m-p53His175 (data not shown). No specifically bound m-p53His175 was detected without ponasterone A induction (Fig. 1*B, lanes 1* and *3*) or in H1299 cells transfected with the empty vector (data not shown).

The Association between Mutant p53 and p73 Occurs under Physiological Conditions—In order to verify whether the association between mutant p53 and p73 occurs under physiological conditions, we performed coprecipitation experiments using T47D breast cancer cells carrying endogenous mutant p53Phe194 (43). Following a preclearing, equal portions of the cell extract were taken for immunoprecipitation with control anti-HA antibody (Fig. 2*A, lane 1*), with a mixture of anti-p53 mAbs DO1 and 1801 (*lane 2*), or with a mixture of anti-p73 polyclonal antibodies (*lane 3*). Immunoprecipitates were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. Aliquots of total cell lysate from unprocessed cells (Fig. 1*B, lanes 4* and *5*) were directly applied on the gel. As seen in Fig. 2*A*, a coprecipitated mutant p53 was detected only in the immunoprecipitates with p53 or with p73α (*lanes 2* and 3) and

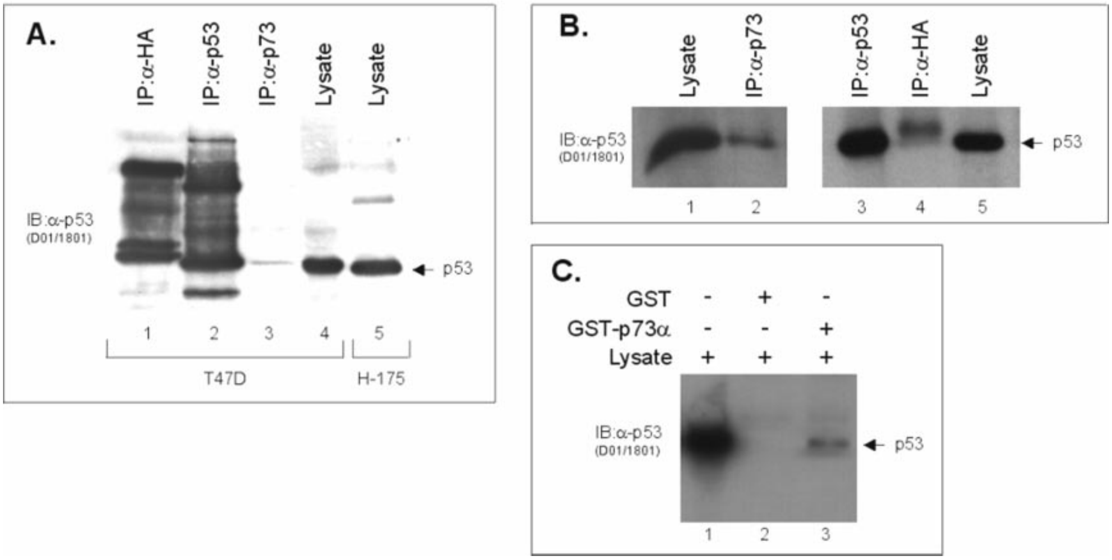


FIG. 2. **The association between mutant p53 and p73α occurs under physiological conditions.** *A,* T47D human breast cancer cells carrying endogenous mutant p53 Phe194 were extracted and subjected to immunoprecipitation (*IP*) analysis as described for Fig. 1*A*. *Lanes 1–3* represent immunoprecipitates corresponding to 5 mg of total cell protein. *Lanes 4* and *5* contain aliquots of unprocessed extracts from the indicated cells lines (100 μg/lane), applied directly to the gel. *IB,* immunoblot. *B,* SKBR3 human breast cancer cells carrying endogenous mutant p53His175 were extracted and subjected to immunoprecipitation analysis as described for Fig. 1*A*. *C,* binding of p53His175 to GST-p73α or to GST alone. Total cellular lysates (2 mg) of SKBR 3 cells, prepared as reported in Fig. 1*A,* were incubated with GST fusion proteins for 2 h at 4 °C. Specifically bound mutant p53 was detected by immunoblotting with a mixture of anti-p53 mAbs DO1 and 1801 (*lane 3*). *Lane 1* contains an aliquot of unprocessed lysate (100 μg/lane), loaded directly on the gel.

not in the anti-HA immunoprecipitates (*lane 1*).

We further confirmed these results by performing similar coprecipitation experiments (Fig. 2*B*), as well as an *in vivo* binding assay (Fig. 2*C*), on SKBR3 breast cancer cells that carry endogenous mutant p53His175 (43). Cell extracts were precleared with protein G-Sepharose, followed by immunoprecipitation with a mixture of anti-p73 polyclonal antibodies or with a mixture of anti-p53 mAbs DO1 and 1801 or with anti-HA mAb. Immunoprecipitates, as well as 1% of each total extract, were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. As seen in Fig. 2*B* (*lanes 2* and *3*), coprecipitated p53His175 was detected in the anti-p73 as well as in the anti-p53 immunoprecipitates. No specific coprecipitated p53His175 was detected in the anti-HA immunoprecipitate (Fig. 2*B, lane 4*). Specifically bound p53His175 was detected only when the cell lysate was incubated with GST-p73α but not with GST alone (Fig. 2*C, lanes 2* and *3*).

Taken together, these data indicate that the association between mutant p53 and p73α occurs under physiological conditions.

Human Tumor-derived p53His175 and p53Gly281 Mutants Associate in Vivo with Different p73 Isoforms—It has been reported that different spliced isoforms of p73 exist in the cells (13, 32, 33, 44, 45). We investigated whether human tumor-derived p53 mutants associate with distinct p73 isoforms. To this end, we first transiently co-transfected a vector encoding mutant p53His175 or p53Gly281, together with a vector encoding for p73α, into H1299 cells. Cell extracts were precleared with protein G-Sepharose, followed by immunoprecipitation with a mixture of anti-p53 mAbs DO1 and 1801. Immunoprecipitates were subjected to immunoblot with anti-p73 polyclonal antibody. As previously reported (34), p73α was brought down from extracts of cells co-transfected with mutant p53His175 but not with empty vector (Fig. 3*A, top panel, lanes 1* and *2*). Similar results were obtained with the mutant p53Gly281 (Fig. 3*A, top panel, lane 3*). A similar picture was revealed when identical extracts were first immunoprecipitated with anti-HA antibody and then subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. Again, coprecipitation of human tumor-derived p53His175 and p53Gly281 mutant with p73α was clearly seen when both proteins were present (Fig. 3*A, bottom panel, lanes 1* and *3*). Aliquots containing 1% of unprocessed cell lysates were loaded directly on the gel, followed by immunoblotting with anti-p73 polyclonal serum or with a mixture of anti-p53 mAbs DO1 and 1801 (Fig. 3*A, middle panels*). These data were further supported by *in vivo* binding assays in which total cell lysates of H1299 cells transiently transfected with p53His175 (data not shown) or p53Gly281 (Fig. 3*B*) were incubated with GST-p73α or GST alone. As shown in Fig. 3*B, lane 6* (and data not shown), both human tumor-derived p53 mutants bound specifically full-length GST-p73α.

Similar coprecipitation experiments were performed to verify whether both human tumor-derived p53 mutants associate with p73β, γ, and δ. As seen in Fig. 3*C,* m-p53His175 and m-p53Gly281 associate with p73β (Fig. 3*C, lanes 7* and *8*). Furthermore, both p53 mutants associated with p73γ and δ isoforms (Fig. 3, *C, lanes 9* and *10,* and *D, lanes 2* and *3*). A similar picture was revealed when identical extracts were first immunoprecipitated with anti-p53 mAb DO1 and 1801 and then subjected to immunoblot with anti-p73 antibody (data not shown).

The DNA Binding Domain of Mutant p53 Is Sufficient for Association with p73—In an attempt to further characterize the association of human tumor-derived p53 mutants with p73α, we determined the p53 domain(s) involved in that association. To this end, we first checked whether derivatives of m-p53His175 in which residues 22–23 were mutated, the proline-rich region was deleted, or a large segment of the C-terminal domain was truncated were still able to associate with p73α when transiently co-transfected in H1299 cells. As seen in Fig. 4*A* (*top panel*), the different mutants of m-p53His175, as well as the full-length protein, were brought down in the p73 immunoprecipitates. A similar picture was obtained when equal amounts of the same lysates were first immunoprecipitated with a mixture of anti-p53 mAbs DO1 and 1801 and then subjected to immunoblot with p73 antibody. Again, a coprecipitated p73 was detectable in all cases except for the cells trans-

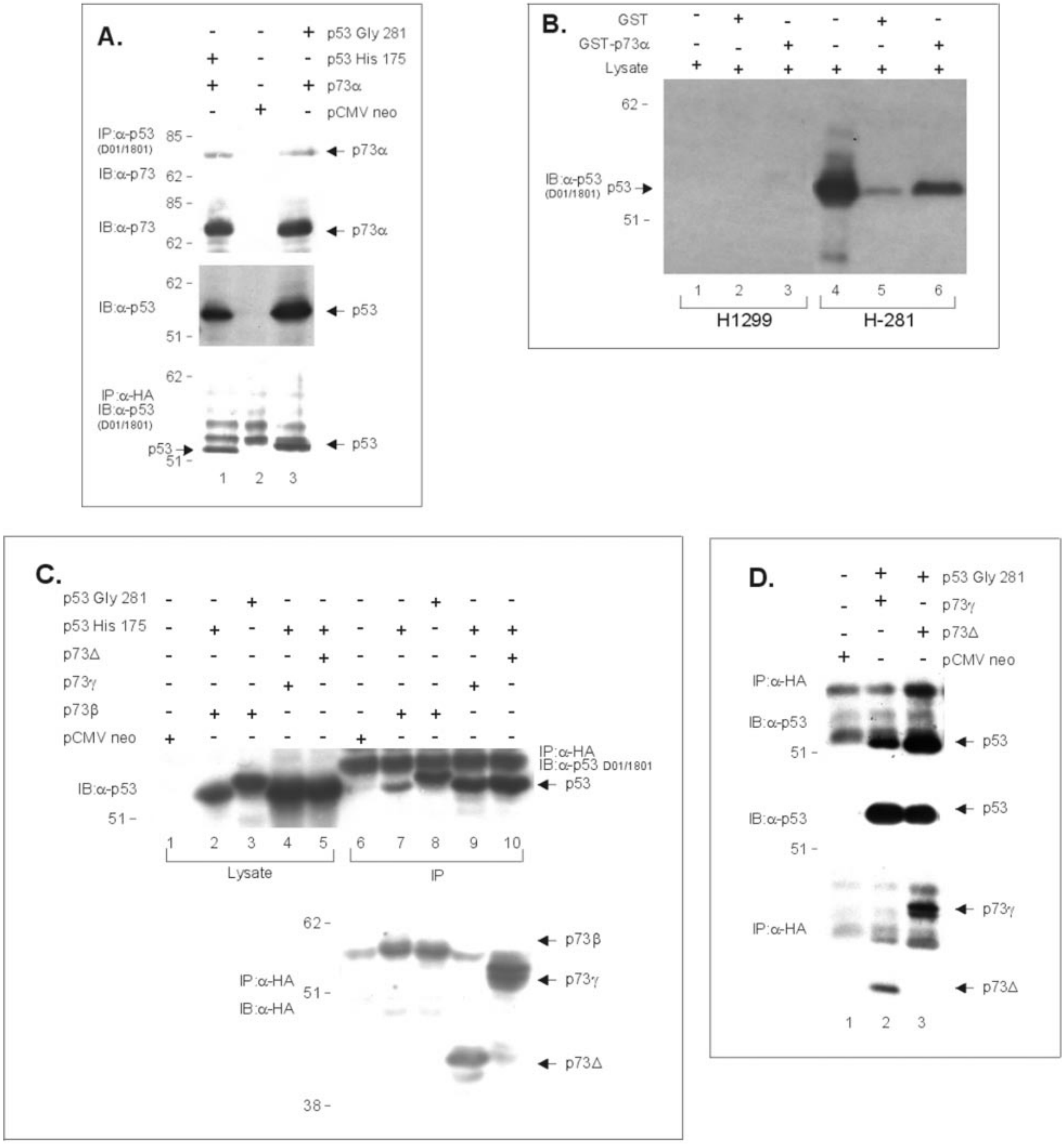
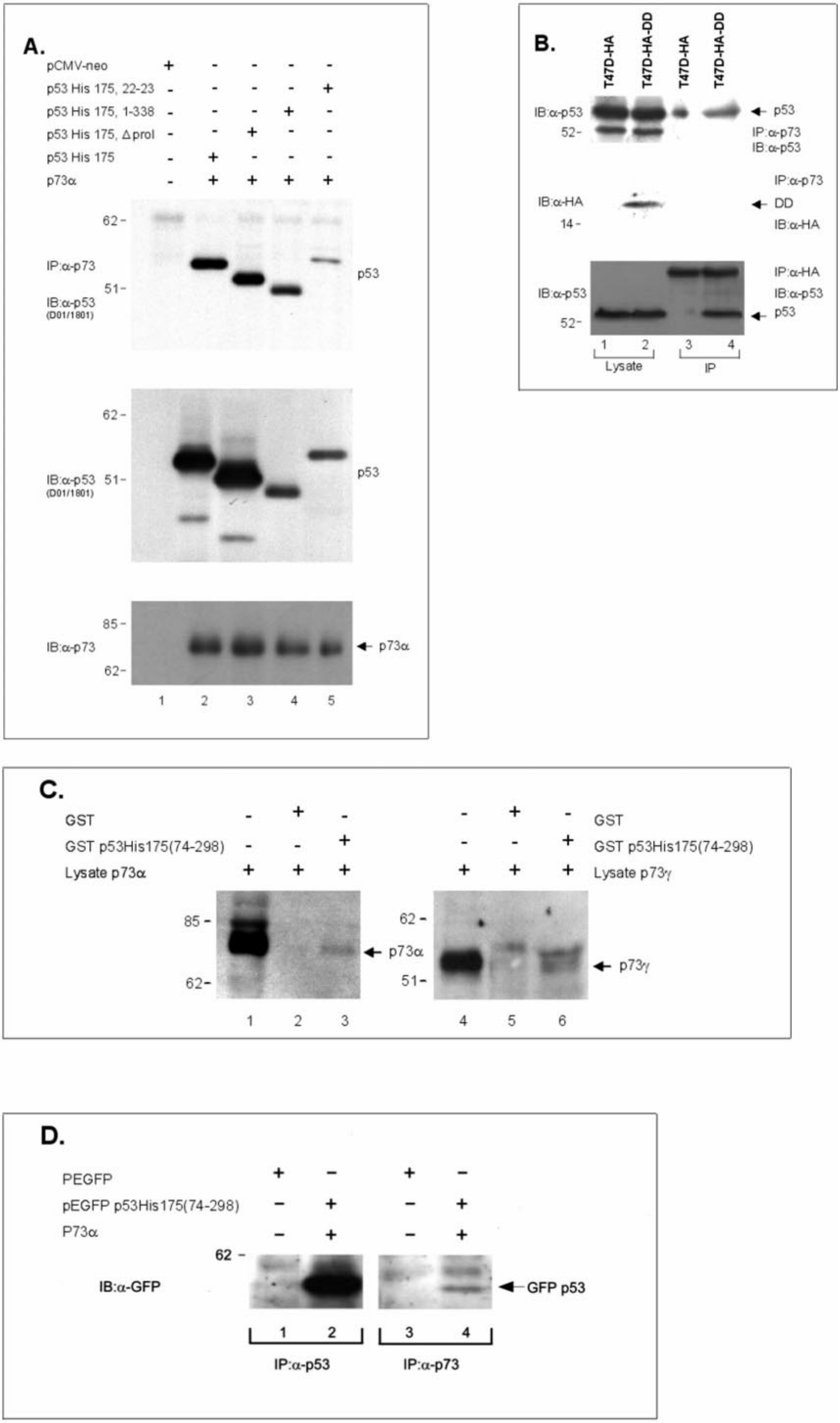


FIG. 3. **p53His175 and p53Gly281 associate *in vivo* with p73α, β, γ, and δ isoforms.** *A,* human tumor-derived p53 mutants and the various isoforms of p73 were overexpressed in H1299 cells by transient transfection. Cell extracts were precleared with protein G-Sepharose, followed by immunoprecipitation (*IP*) with a mixture of anti-p53 mAbs DO1 and 1801 (*top panel, lanes 1* and *3*) or with anti-HA mAb (*bottom panel, lanes 1* and *3*). Immunoprecipitates were subjected to immunoblot (*IB*) with anti-p73 polyclonal serum (*top panel*) or with a mixture of anti-p53 mAbs DO1 and 1801 (*bottom panel*). Aliquots of total cell extracts from unprocessed cells (100 μg/lane) were directly subjected to immunoblot analysis (*middle panels*). *B,* binding of p53Gly281 from transient transfected H1299 cells to GST-p73α (*lane 6*) and to GST alone (*lane 5*) was performed as reported in Fig. 1*B*. Specifically bound mutant p53 was detected by immunoblotting with a mixture of anti-p53 mAbs DO1 and 1801. *Lanes 1* and *4* contain aliquots of unprocessed cell lysates (100 μg/lane), loaded directly on the gel. *C,* the indicated plasmid combinations were co-transfected in H1299 cells and processed as reported in *A*. *Lanes 6–10* represent immunoprecipitations with anti-HA mAb followed by immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. The blot (*bottom panel, lanes 6–10*) was reprobed with anti-HA mAb to visualize the transfected isoforms of p73. *Lanes 1–5* contain aliquots of total cell lysates (100 μg/lane), loaded directly on the gel. *D,* the indicated plasmid combinations were co-transfected in H1299 cells and processed as reported in *A* and *C*. Positions of protein molecular size markers are indicated on the *left*.

fected with the empty vector (data not shown). Aliquots of unprocessed lysates (100 μg/lane) were directly applied on the gel, followed by immunoblotting with a mixture of anti-p53 mAbs DO1 and 1801 or with anti-p73 polyclonal antibody (Fig. 4*A*, *middle* and *bottom panels*).

It is well established that p53 is present in the cells as a tetramer, and that the association occurs through the oligomerization domain located in the C-terminal part of the protein. To investigate whether the oligomerization domain of mutant p53 is involved in the association with p73α, we employed T47D cells stably transfected with a vector encoding the DD miniprotein or with an empty vector. The DD miniprotein comprises residues 302–390 of mouse p53, including the entire oligomerization domain (45). Equal amounts of cell extracts from


A.
pCMV-neo
p53 His 175, 22-23
p53 His 175, 1-338
p53 His 175, Δprol
p53 His 175
p73α
62
IP:α-p73
IB:α-p53 (D01/1801)
51
p53
62
IB:α-p53 (D01/1801)
51
p53
85
IB:α-p73
62
p73α
1 2 3 4 5
B.
T47D-HA
T47D-HA-DD
T47D-HA
T47D-HA-DD
IB:α-p53
52
p53
IP:α-p73
IB:α-p53
IP:α-p73
IB:α-HA
14
DD
IB:α-HA
IP:α-HA
IB:α-p53
IB:α-p53
52
p53
1 2 3 4
Lysate
IP
C.
GST
GST p53His175(74-298)
Lysate p73α
85
62
p73α
1 2 3
GST
GST p53His175(74-298)
Lysate p73γ
62
51
p73γ
4 5 6
D.
PEGFP
pEGFP p53His175(74-298)
P73α
62
IB:α-GFP
GFP p53
1 2 3 4
IP:α-p53
IP:α-p73

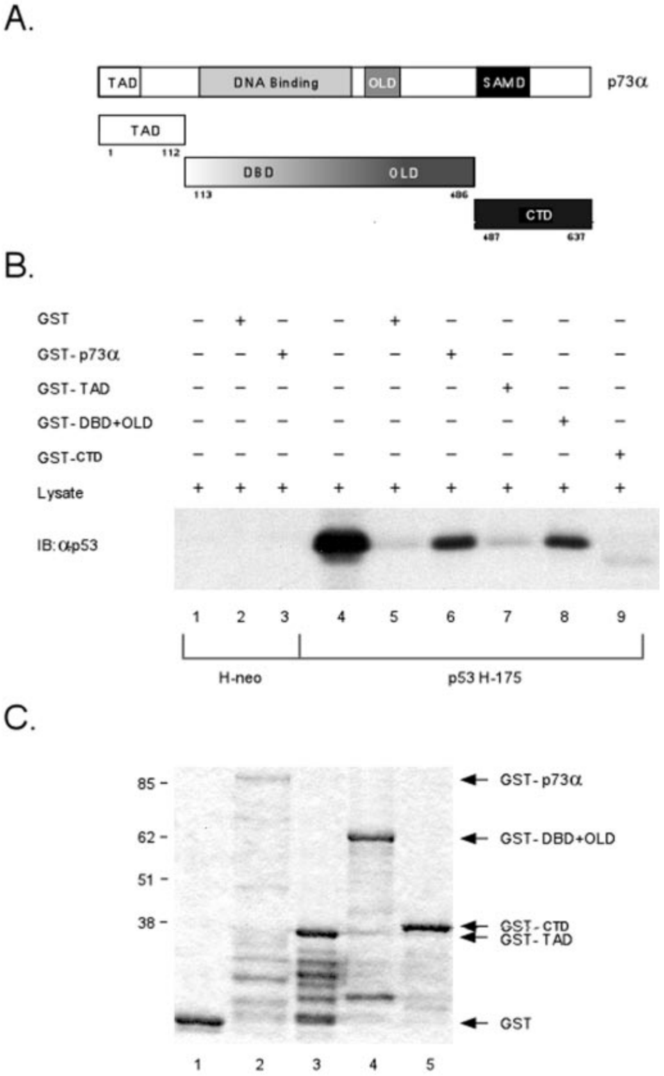


FIG. 5. **The specific DNA binding and the oligomerization domains of p73α are required for the association with mutant p53.** *A,* schematic representation of p73α segments present in each of the GST fusion proteins employed in *B*. Indicated are the positions of the transcriptional activation domain (*TAD*), the DNA binding domain and the oligomerization domain (*DBD+OLD*), and the C-terminal domain (*CTD*). *B, in vivo* binding of mutant p53 to p73α-derived GST fusion proteins. Total cellular lysates from H1299 cell transiently transfected with m-p53His175 plasmid were incubated with GST fusion proteins (25 μg) for 2 h at 4 °C. Specifically bound m-p53His175 was detected by immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. Aliquots containing 100 μg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801. *C,* Coomassie staining of a replica gel showing the p73α GST fusion protein segments. Positions of protein molecular size markers are indicated on the *left*.

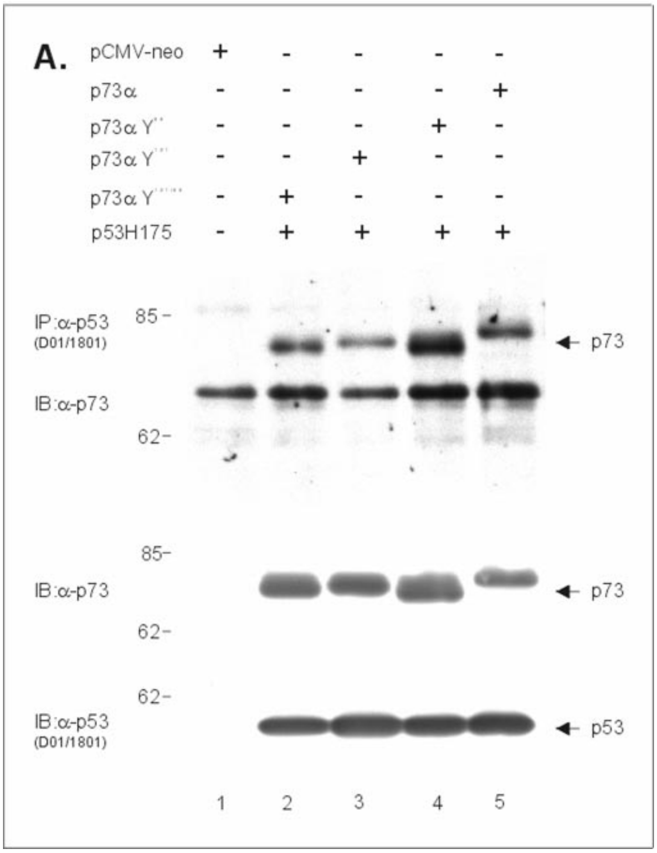


FIG. 6. **Tyrosine phosphorylation of p73α is dispensable for the association with mutant p53.** H1299 cells were transiently transfected with a plasmid encoding m-p53His175 in combination with p73α (*lane 5*), p73α^{Y99F} (*lane 4*), p73α^{Y121F} (*lane 3*), or p73α$^{Y99F/Y121F}$ (*lane 2*) plasmid. Cell extracts were prepared 36 h later. Aliquots of 1.5 mg of total protein from unprocessed lysates were subjected to immunoblot (*IB*) with a mixture of anti-p53 mAbs DO1 and 1801 or with an anti-p73 polyclonal serum. *IP,* immunoprecipitation. Positions of protein molecular size markers are indicated on the *left*.

T47D-HA and T47D-HA-DD were first immunoprecipitated with anti-p73 polyclonal antibodies (Fig. 4*A, top* and *middle panels*) or with anti-HA mAb (*bottom panel*) and then subjected to immunoblotting. As seen in Fig. 4*B* (*top panel, lanes 3* and 4), coprecipitated mutant p53 protein was detected in the p73 immunoprecipitates. Conversely, coprecipitated DD miniprotein was not detected by immunoblotting the identical p73 immunoprecipitates with anti-HA mAb (Fig. 4*B, middle panel, lanes 3* and *4*). Furthermore, coprecipitated DD miniprotein was detected by immunoblotting anti HA-DD immunoprecipitates with a mixture of anti-p53 mAbs DO1 and 1801 (Fig. 4*B, bottom panels, lanes 3* and *4*). Aliquots of unprocessed lysates (100 μg/lane) were directly subjected to immunoblotting with the indicated antibodies (Fig. 4*B, lanes 1* and *2*).

Taken together, these results strongly suggested an involve-

FIG. 4. **The specific DNA binding domain of mutant p53 is sufficient for the association with p73α and γ isoforms.** *A,* H1299 cells were transiently transfected with a plasmid encoding monkey p73α in combination with p53His175, p53His175Δproline, p53His175-(1–338), or p53His175-(22–23) plasmid. Cell extracts were prepared 36 h later. Aliquots containing 1.5 mg of total proteins were subjected to immunoprecipitation (*IP*) with a mixture of anti-p73 polyclonal-Abs C-17 and C-20. Immunoprecipitated proteins were resolved by SDS-PAGE, followed by immunoblot with a mixture of anti-p53 mAbs DO1 and 1801 (*top panel*). Aliquots containing 100 μg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 mAbs DO1 and 1801 (*middle panel*) or with anti-p73 polyclonal serum (*bottom panel*). *B,* cell lysates extracted from T47D-HA and T47D-HA-DD polyclonal populations were subjected to immunoprecipitation with anti-p73 polyclonal antibodies (*top* and *middle panels, lanes 3* and *4*) or with anti-HA mAb (*bottom panel, lanes 3* and *4*). Immunoprecipitations, as well as aliquots of 100 μg/lane, were immunoblotted with anti-p53 mAbs DO1 and 1801 (*top* and *bottom panels, lanes 1–4*) or with anti-HA mAb (*middle panel, lanes 1–4*). Positions of protein molecular size markers are indicated on the *left*. *C,* binding of p73α and p73γ to GSTp53His175-(74–298) fusion protein or to GST alone. Total cellular lysates from H1299 cells transiently transfected with p73α or p73γ (2 mg) were incubated with GST fusion proteins (25 μg) for 2 h at 4 °C. Specifically bound p73α or p73γ was detected by immunoblot with anti-p73 polyclonal serum. Aliquots containing 100 μg of total protein from unprocessed lysates were subjected to immunoblot with anti-p73 polyclonal serum (*lanes 1* and *4*).

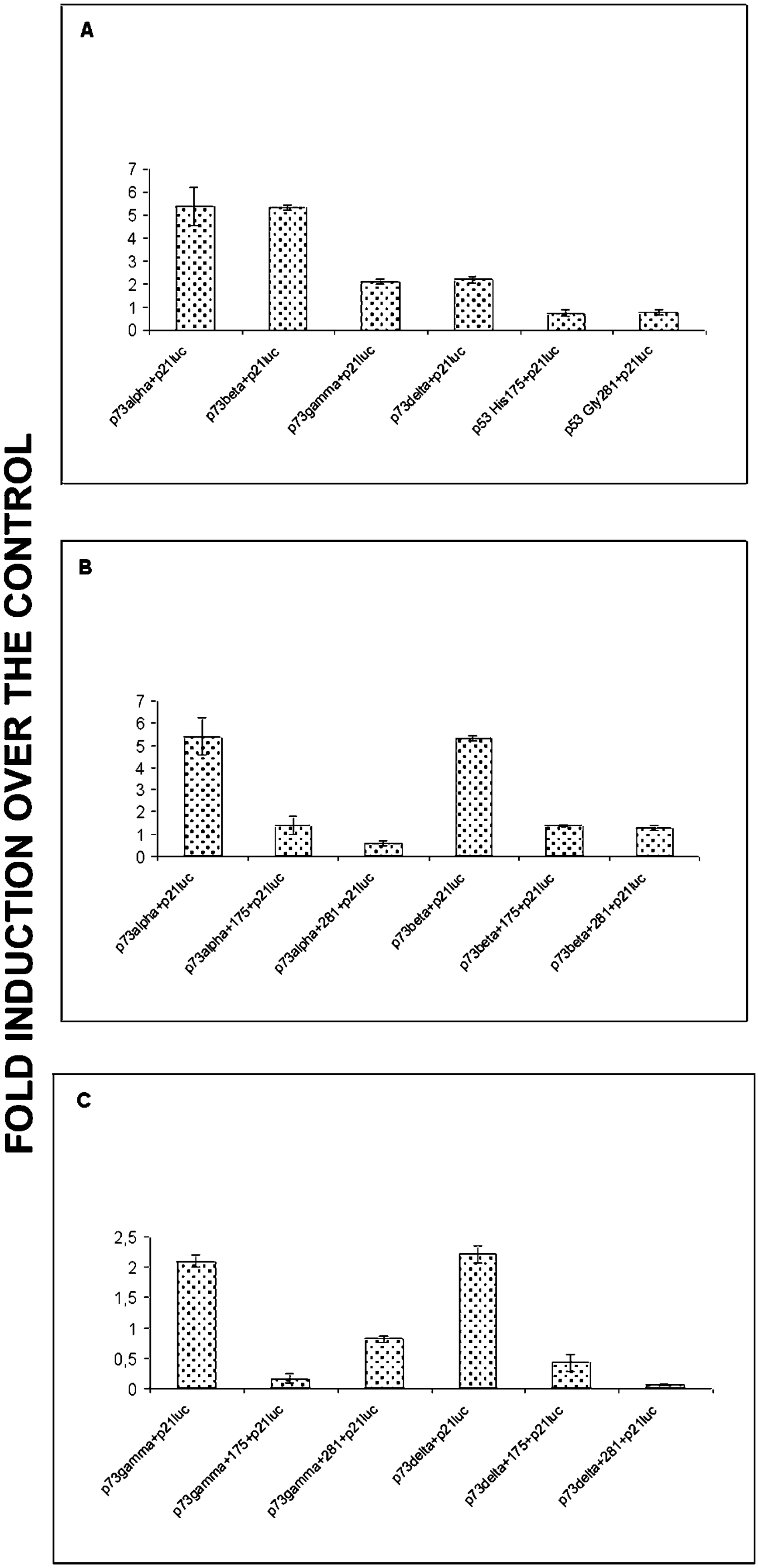


FIG. 7. **Human tumor-derived p53 mutants interfere with the transcriptional activity of p73α, β, δ, and γ.** H1299 cells (5 × 10^5/60 mm dish) were transiently cotransfected with the p73α (*A* and *B*), p73β (*A–C*), p73δ (*A–D*), or p73γ (*A–E*) expression plasmids (25 ng/dish) with or without expression plasmids encoding p53His175 or p53Gly281 (100 ng/dish), respectively, together with the $p21^{waf1}$ luciferase reporter plasmid (50 ng/dish). The total amount of transfected DNA in each dish was kept constant by addition of extra pcDNA-HA vector control DNA wherever necessary. Cell extracts were prepared 36 h later and subjected to determination of luciferase activity. Results are represented as fold induction of luciferase activity compared with control cells transfected with an empty CMV expression plasmid. Histograms show the mean of a typical experiment of three performed in triplicate; *bars* indicate S.D.

ment of the DNA binding domain of mutant p53 in the association with p73. To verify whether the DNA binding domain of mutant p53 is sufficient for that association, we employed an *in vivo* binding assay. Total cell lysates of H1299 cells transiently transfected with p73α or p73γ were incubated with GSTp53His175(74–298) or with GST alone. Specifically bound p73α or p73γ was detected by immunoblotting with anti-p73 polyclonal serum (Fig. 4*C, lanes 3* and *6*). *Lanes 1* and *4* of Fig. 4*C* represent aliquots from unprocessed cell lysates (100 μg/lane) directly applied on the gel. We next wished to determine whether the DNA binding core domain of mutant p53 interacts with p73 also *in vivo*. To this end, H1299 cells were transiently transfected with pEGFPp53His175(74–298) vector in combination with p73α expression plasmid. Equal amounts of cell extracts were subjected to immunoprecipitation with anti-p53 mAb 240 or with a mixture of anti-p73 polyclonal antibodies, followed by immunoblotting with anti-GFP serum. As shown in Fig. 4*D,* the GFP-p53His175(74–298) protein was brought down from extracts of co-transfected cells (*lanes 2* and 4) but not in cells transfected with an empty vector (*lanes 1* and 3). In conclusion, the DNA binding domain of mutant p53 is sufficient for the association with p73.

The Region of p73α Including the Sequence-specific DNA Binding and the Oligomerization Domains Is Sufficient for the Association with Mutant p53—To define the domain(s) of p73 involved in interaction with m-p53, an *in vivo* binding assay was performed. To this end, total cell lysates of H1299 cells transiently transfected with p53His175 expression plasmid were incubated with recombinant GST fusion proteins containing different segments of p73α or with GST alone (Fig. 5*A*). Specifically bound mutant p53 was detected either with GST-p73α full-length or with a fragment encompassing the DNA binding and the oligomerization domains (Fig. 5*B, lanes 6* and *8*) by immunoblotting with a mixture of p53 mAbs DO1 and 1801. No specifically bound m-p53 was detected when cell lysates of H1299 cells transfected with an empty vector were incubated with GST-p73α full-length or GST alone (*lanes 2* and 3). *Lanes 1* and *4* of Fig.. 5*B* represent aliquots of total cell lysates from unprocessed cells directly applied on the gel.

Tyrosine Phosphorylation of p73α Is Dispensable for Association with Human Tumor-derived p53 Mutants—It was previously reported (29, 31) that c-abl phosphorylates p73α and β on a tyrosine residue at position 99, both *in vitro* and in cells exposed to ionizing radiation. Conversely, it was also reported that p73 can be stabilized upon exposure to cisplatin without any detectable tyrosine phosphorylation (30). Moreover, exposure to UV radiation does not cause p73 stabilization (14). Therefore, we checked whether p73 tyrosine phosphorylation influences the association with human tumor-derived mutant p53. To this end, we transiently co-transfected a plasmid encoding mutant p53His175 or p53Gly281 with plasmids encoding the p73α mutants p73α^{Y99F}, p73α^{Y121F}, and p73α$^{Y99F/Y121F}$, into H1299 cells. Cell lysates were precleared with protein G-Sepharose and then subjected to immunoprecipitation with a mixture of anti-p53 mAbs DO1 and 1801. Immunoprecipitates were subjected to immunoblot with anti-p73 polyclonal antibody. As shown in Fig. 6, coprecipitated p73α was detected in cells co-expressing mutant p53His175 (*lanes 2–5*). That coprecipitation was not detected in the cells transfected with empty vector (Fig. 6, *lane 1*). Similar data were obtained with mutant p53Gly281 (data not shown). These data indicate that p73 phosphorylation, at least on Tyr-99, Tyr-121, or both, does not influence the association with mutant p53.

Human Tumor-derived p53 Mutants Interfere with the Transcriptional Activity of the Different p73 Isoforms—To investigate whether the association with mutant p53 interferes with the transcriptional activity of the various p73 isoforms, we cotransfected H1299 cells with p73α, β, δ, or γ, together with two human tumor-derived p53 mutants and a luciferase reporter gene driven by the p53-responsive p21^{waf1} promoter. As shown in Fig. 7*A,* the different p73 isoforms differ in their transcriptional potency. p73α and β are comparable transcriptional activators of p21 promoter (Fig. 7*A*), and are more potent than p73δ and γ (Fig. 7*A*). Of note, both human tumor-derived p53 mutants markedly reduced the transcriptional activity of p73 α, β, δ, and γ on the p21^{waf1} promoter (Fig. 7, *B* and *C*).

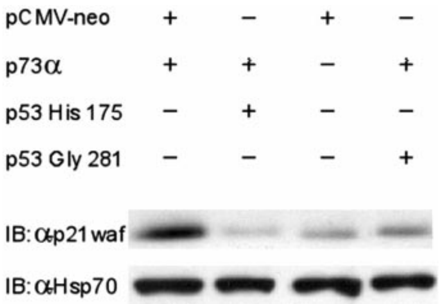


FIG. 8. **Overexpression of mutant p53 (p53His175 or p53Gly281) markedly reduces the amount of p73-inducible p21^{waf1} protein.** H1299 cells were transiently transfected with the indicated plasmid combinations. The total amount of transfected DNA was keep constant by addition of pcDNA3-neo vector control DNA. Cell extracts (50 μg/lane) were prepared 36 h later, subjected to SDS-PAGE, and immunoblotted with anti-p21^{waf1} polyclonal serum or with anti-α-Hsp70 antibody for equal loading.

To determine whether p53 mutants can also interfere with the activation of endogenous target genes by p73α, we analyzed the levels of p21^{waf1} protein in cells overexpressing p73α alone or together with a plasmid encoding mutant p53His175 as well as p53Gly281. As shown in Fig. 8, overexpression of p73α caused a clear accumulation of p21^{waf1} protein that is markedly reduced when mutant p53 is concomitantly overexpressed. Thus, human tumor-derived p53 mutants can interact with p73 not only physically but also functionally.

DISCUSSION

We report here that human tumor-derived p53 mutants can engage in a physical association with p73α, β, γ, and δ. Furthermore, this association occurs under physiological conditions. In agreement with the previously reported association between mutant p53 and p73α (34), our findings contribute to the definition of a network involving mutant p53 and the various p73 isoforms in cancer cells. These data do not exclude the possibility that other human tumor-derived p53 mutants, distinct from those used in our experiments, may be unable to associate with p73 (44). Several reports have clearly suggested that some human tumor-derived p53 mutants can exert gain of function activity (7, 9, 10, 12). This activity is dependent on the type of the p53 mutation, as well as on the cell context in which the biological gain of function is measured. We and others have previously reported that conformational mutants such as p53His175, but not DNA contact mutants, can increase the resistance to etoposide or contribute to genomic instability by abrogating the mitotic spindle checkpoint and consequently leading to polyploidy of human cells (8, 11). We are currently investigating whether association with p73 is a specific property of gain of function p53 mutants. Of note, the finding that the core domain of mutant p53 is sufficient for the association with p73 highlights the potential role of this domain as a module for protein-protein interaction. Further investigation employing yeast two-hybrid screening or an immunoaffinity approach is needed to verify whether the core domain of mutant p53 plays an important role in the gain of function activity.

Moreover, in view of the substantial similarity of the core domains between p73 and p63, another member of the p53 family, it is of interest to find out whether p63 can also participate in that network (46–49). It is conceivable that other proteins interacting either with mutant p53 or with p73 might also interfere with the biological outcome of the entire network (29–31, 50–54).

The studies performed to date suggest that p73 is rarely mutated in human cancers and that ectopic expression of p73 induces apoptosis in cancer cells. Thus, agents that increase p73 expression may provide new potential anticancer treatments. Conversely, proteins inactivating or interfering with p73 might have an impact upon cellular properties, clinical responses to therapy, and prognosis of a tumor. Our data, showing that human tumor-derived p53 mutants can interact with p73 not only physically but also functionally, might implicate mutant p53 as a likely candidate for such type of proteins. Along this line, an intriguing question can be raised: how does mutant p53 inactivate p73? In accordance with our novel finding that both the core and the oligomerization domains of p73 are involved in the association with mutant p53, we might depict two possible scenarios. On the one hand, mutant p53 binding the oligomerization domain of p73 can interfere with the formation of p73 homo-oligomers. On the other hand, mutant p53 binding the core domain of p73 can interfere with its binding to DNA.

The recent observation that p73-deficient mice do not exhibit an increase in spontaneous tumors suggests that the association between mutant p53 and p73 might have an impact mainly upon tumor chemoresistance rather than on tumor development (55). If this prediction is proven correct, one would expect that cells from p73-deficient mice will be less prone to killing by anticancer drugs than their wild type counterparts.

The establishment of a precise role for the association between mutant p53 and p73 might be very useful for anticancer treatment. To this end, further evidence needs to be collected.

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Physical and Functional Interaction between p53 Mutants and Different Isoforms of p73

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