Physical and functional interaction between p53 mutants and different isoforms of p73.

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SUMMARY

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 p73 α, 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.
INTRODUCTION

The p53 tumor suppressor gene is the most frequent target for genetic alterations in human cancers (1). The wild type p53 (wt-p53) protein can elicit a variety of biological effects ranging from growth arrest to apoptosis and differentiation (2, 3, 4). These effects are mainly exerted by wt-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 wt-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, 7, 8, 9, 10, 11, 12).

A new gene, p73, sharing considerable sequence 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 amino terminal transactivation domain that shares 29% homology with p53's N-terminal part; (b) the sequence-specific DNA binding domain that shares 63% of homology with the corresponding p53 domain; (c) the tetramerization domain, that shares 42% homology with p53's oligomerization domain (15, 16). Furthermore, ectopic expression of p73 can transactivate p53 responsive target genes as well as induce 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, while this domain in p53 is the major site of the mutations, no mutations could be found so far in p73 despite extensive efforts (17, 18, 19, 20, 21, 22, 23). Unlike p53, p73 is not inactivated by viral oncoproteins such as T-antigen, E6 and E1Bp55, well known inactivators of p53 (24,
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 as cis-platin or γ-radiation, suggesting a differential behaviour of p73 in response to different types of DNA damage (29, 30, 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, Gly281) associate with p73α, β, γ, 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 including 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; (f) human tumor-derived p53 mutants interfere with the transcriptional activity of p73α, β, γ, 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, supplemented with 10% fetal calf serum (FCS, GIBCO, BRL, Gaithersburgh, MD). Before transfection, the culture medium was changed to DMEM supplemented with 10% FCS. H1299-pVgRXR cells were maintained in the same medium containing zeocin (100µg/ml) (InVitrogen, San Diego, USA). H1299-pIND#1, H1299-mp53His175#41 cells were maintained in medium containing zeocin and G418 (400µg/ml). To induce m-p53 expression, ponasterone A, a synthetic analog of ecdysone (Alexis Biochemicals, San Diego, CA, USA) 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 (400µg/ml).

Plasmids and Stable Transfections. Plasmids pVgRXR, pIND were from Invitrogen pIND/m-p53His175 was prepared by cloning the BamHI/BamHI 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 (100µg/ml) for pVgRXR and G418 (400µg/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 DMEM +10% FCS and then the medium was changed again to RPMI+ 10% FCS. Stable transfected polyclonal populations were selected in medium containing G418 (400µg/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. jr. Kaelin), pcDNA3-HA-p73γ and pcDNA3-HA-p73δ (kindly provided by Dr. G. Melino), pCDNA3-HA-p73α,Y99-F, pCDNA3-HA-p73α,Y121-F, pCDNA3-HA-p73α,Y99/121-F. Overexpression of mutant p53 was achieved by transfection of the following plasmids: pCDNA3-p53His175, pCDNA3-p53His175-22,23, pCDNA3-p53His175-Delta proline, pCDNA3-p53His175 (1-338), pCDNA3-p53His175 (1-355), pCDNA3-p53Gly281, all driven by the CMV early promoter/ehnancer. pCDNA3-p53His175-22,23 was prepared by replacing an HindIII/SgraI fragment from pRVCMV-wtp53/22,23 (38) into pCDNA3-p53His175. pcDNA3-p53His175-Delta proline was prepared by cloning a HindIII/XbaI fragment (39) into pCDNA3-neo. pCDNA3-p53His175 (1-338) and pCMVp53His175 (1-355) were prepared by cloning the fragments EcoRI/NotI into pCDNA3-neo. Overexpression of the core domain of mutant p53 was achieved by transfection of pEGFP-p53His175(74-298) prepared by cloning a EcoRI/XhoI fragment into pEGFP-C2 expression vector (InVitrogen). The parental vector pCMVneo 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 Chemical. St. Louis, MO, USA). The precipitates were left for 12 h after which the medium was changed again to RPMI+10% FCS. The cells were harvested 36 h post-transfection.

**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, 1mM EDTA, 100 mM NaF, 1 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride and protease inhibitors) and the extracts were sonicated for 10 seconds and centrifuged at 14000 x rpm for 10 min to remove cell
debris. Protein concentrations were determined by a colorimetric assay (Bio-Rad Laboratories, Hercules, CA, USA). After pre-clearing 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, USA) or with anti-HA antibody or with a mixture of anti-p53 MAbs DO1 and PAb1801, with rocking at 4°C for 1h. 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% NP40). The excess liquid was aspirated, and 40 µl of 5x 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 PAb1801 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. Piscataway, NJ).

Production of Recombinant Proteins. GST fusion proteins containing large fragments of p73α were obtained by PCR 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 EcoRI/XhoI 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, 41, 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 5x sample buffer was added. Immunoprecipitates, as well as 1% of each extract, were resolved by SDS-10% PAGE. The immunoblots were probed with anti-p73 polyclonal antibody or with a mixture of the p53 monoclonal antibodies DO1 and PAb1801. Detection was performed with the aid of the enhanced chemiluminescence (ECL) system (Amersham).

**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, USA), and incubated for 10 min at room temperature. Insoluble material was spun down, and luciferase activity was quantitated using a commercially available kit (Promega, USA) 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 since 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 PAb1801 were performed to screen for p53 inducible expression and intracellular localization. As seen in Fig. 1A (lanes 5-8) a representative clone for each cell type (H1299-mp53His175#41 and H1299-pIND#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 p53 Phe194, was stably transfected with pCDNA-HA-DD or with vector control followed by G418 selection for three weeks. A polyclonal population for each cell line (Fig. 4B) 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#1 and H1299-mp53His175#41 cell lines. In order to induce mutant-p53 expression the cell lines were grown in the presence of ponasterone A (2.5µM/ml) for 24 h. Cell extracts were precleared with Protein G-sepharose, followed by
immunoprecipitation with a mixture of anti-p73 polyclonal antibodies. Immunoprecipitates as well as 1% of each total extract were subjected to immunoblot with a mixture of anti-p53 MAbs DO1 and PAb1801. As seen in Fig. 1A mutant p53His175 was brought down only when the H1299-m-p53His175#41 cells were treated with ponasterone A (lanes 1 and 2). As expected no coprecipitated mutant p53 was brought down from H1299-pIND#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 (lower 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 cis-platin 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 1C, 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 PAb1801 (lanes 1 and 4). These data raise the possibility that gain of function of mutant p53, evident as increased chemoresistance to etoposide or cis-platin, 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#41 plus or minus ponasterone A induction (Fig. 1B) as well as H-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 PAb1801. In agreement with the coprecipitation data (Fig
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1 A-C) bound p53His175 was detected only when expression of mutant p53 was induced by ponasterone A (Fig. 1B lanes 4 and 6) as well as in polyclonal populations overexpressing m-p53His175 (data not shown). No specifically bound m-p53His175 was detected without ponasterone A induction (Fig. 1B 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 (lane 1), or with a mixture of anti-p53 MAbs DO1 and PAb1801 (lane 2) or with a mixture of anti-p73 polyclonal antibodies (lane 3), respectively. Immunoprecipitates were subjected to immunoblot with a mixture of anti-p53 MAbs DO1 and PAb1801. Aliquots of total cell lysate from unprocessed cells (lanes 4 and 5) were directly applied on the gel. As seen in Fig. 2A a coprecipitated mutant p53 was detected only in the immunoprecipitates with p53 or with p73α (lanes 2 and 3) but not in the anti-HA immunoprecipitates (lane 1). We further confirmed these results by performing similar coprecipitation experiments (Fig. 2B), as well as an in vivo binding assay (Fig. 2C), 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 on anti-p53 MAbs DO1 and PAb1801 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 PAb1801. As seen in Fig. 2B (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. 2B, lane 4). Specifically bound p53His175 was detected only when the cell lysate was incubated with GST-p73α but not with GST-alone (Fig. 2C, 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 Gly281 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 PAb1801. 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. 3A-upper panel, lanes 1 and 2). Similar results were obtained with the mutant p53Gly281 (Fig. 3A, upper 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 PAb1801. Again, coprecipitation of human tumor-derived p53His175 and p53Gly281 mutant with p73α was clearly seen when both proteins were present (Fig. 3A, lower 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 PAb1801 (Fig. 3A, 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
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(Fig. 3B) were incubated with GST-p73α or GST alone. As shown in Fig. 3B (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. 3C, m-p53His175 as well as Gly281 associate with p73β (Fig. 3C-lanes 7 and 8). Furthermore, both p53 mutants associated with p73γ and δ isoforms (Fig. 3C, lanes 9 and 10; Fig. 3D, lanes 2 and 3). A similar picture was revealed when identical extracts were first immunoprecipitated with anti-p53 MAb DO1 and PAb1801 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, or 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 4A (upper 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 PAb1801 and then subjected to immunoblot with p73 antibody. Again a coprecipitated p73 was detectable in all cases except for the cells transfected 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 PAb1801 or with anti-p73 polyclonal antibody (Fig. 4A, middle and lower 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 (OLD) 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 OLD (45). Equal amount of cell extracts from T47D-HA and T47D-HA-DD were first immunoprecipitated with anti-p73 polyclonal antibodies (upper and middle panels), or with anti-HA MAb (lower panel), and then subjected to immunoblotting. As seen in Fig. 4B (upper 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. 4B, 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 PAb1801 (Fig. 4B, lower panels, lanes 3 and 4). Aliquots of unprocessed lysates (100 µg/lane) were directly subjected to immunoblotting with the indicated antibodies (Fig. 4B lanes 1 and 2).

Taken together, these results strongly suggested an involvement 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. 4C, lanes 3 and 6). Lanes 1 and 4 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) or with an empty vector. The DD miniprotein comprises residues 302-390 of mouse p53, including the entire OLD (45). Equal amount of cell extracts from H1299-HA and H1299-HA-DD were first immunoprecipitated with anti-p73 polyclonal antibodies (upper and middle panels), or with anti-HA MAb (lower panel), and then subjected to immunoblotting. As seen in Fig. 4C (upper 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. 4C, 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 PAb1801 (Fig. 4C, lower panels, lanes 3 and 4). Aliquots of unprocessed lysates (100 µg/lane) were directly subjected to immunoblotting with the indicated antibodies (Fig. 4C lanes 1 and 2).

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vector in combination with p73α expression plasmid. Equal amounts of cell extracts were subjected to immunoprecipitation with anti-p53 antibody PAb240 or with a mixture of anti-p73 polyclonal antibodies, followed by immunoblotting with anti-GFP serum. As shown in Fig. 4D 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.5A). Specifically bound mutant p53 was detected either with GST-p73α full length as well as with a fragment encompassing the DNA binding and the oligomerization domains (Fig. 5B, lanes 6 and 8) by immunoblotting with a mixture of p53 MAbs DO1 and PAb1801. 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 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 cotransfected a plasmid encoding mutant p53His175 or Gly281 with plasmids encoding the p73α mutants p73α,Y99-F, p73α,Y121-F, p73α,Y99/121-F, 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 PAb1801. 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 Y-99 or Y-121 or on both residues, 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<sub>waf1</sub> promoter. As shown in Fig 7A, the different p73 isoforms differ in their transcriptional potency. p73α and β are comparable transcriptional activators of p21 promoter (Fig. 7A), and are more potent than p73δ and γ (Fig. 7A). Of note, both human tumor-derived p53 mutants markedly reduced the transcriptional activity of p73 α, β, δ, and γ on the p21<sub>waf1</sub> promoter (Fig. 7 B, C).

To determine whether p53 mutants can also interfere with the activation of endogenous target genes by p73α, we analyzed the levels of p21<sub>waf1</sub> 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 p21waf1 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 previous 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, 47, 48, 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, 30, 31, 50, 51, 52, 53, 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 anti-cancer treatments. Conversely, proteins inactivating or interfering with p73 might 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. On this line an intriguing question can be raised: how does mutant p53 inactivate p73? Accordingly to 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 homooligomers. 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 mouse do not exhibit an increase in spontaneous tumors suggests that the association between mutant p53 and p73 might mainly impact upon tumor chemoresistance rather than on tumor development (50). 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.
Acknowledgements

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Fig. 1. **In vivo association between mutant p53 and endogenous p73α.** A, H1299 inducible cell lines were generated in two steps (see Results). Cell extracts were prepared either from H-175#41 and H-pIND#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 PAb1801 (lanes 1-4). The blot was re-probed with anti-p73 polyclonal serum (lower panel). Aliquots containing 100 µg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 MAbs DO1 and PAb1801 (lanes 5-8). B, Binding of p53His175 to GST-p73α or to GST alone. Total cellular lysates (2 mg) of 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 PAb1801 (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 H-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.

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 analysis as described for Fig. 1A. Lanes 1-3 represent immunoprecipitates corresponding to 5 mg of total
cell protein. Lanes 4 and 5 contain aliquot of unprocessed extracts from the indicated cells lines (100 µg/lane), applied directly to the gel. B, SKBR3 human breast cancer cells carrying endogenous mutant p53His175 were extracted and subjected to immunoprecipitation analysis as described for Fig 1A. 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. 1A 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 MAb DO1 and PAb1801 (lane 3). Lane 1 contains an aliquot of unprocessed lysate (100 µg/lane), loaded directly on the gel.

Fig. 3. **p53His175 and p53Gly281 associate in vivo with p73α, β, γ, 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 with a mixture of anti-p53 MAb DO1 and PAb1801 (upper panel-lanes 1and 3) or with anti-HA MAb (lower panel-lanes 1 and 3). Immunoprecipitates were subjected to immunoblot with anti-p73 polyclonal serum (upper panel) or with a mixture of anti-p53 MAb DO1 and PAb1801 (lower panel). Aliquot 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. 1B. Specifically bound mutant p53 was detected by immunoblotting with a mixture of anti-p53 MAb DO1 and PAb1801. 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 MAb DO1 and PAb1801. The blot (lower 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.

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, or p53His175∆proline, or 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 PAb1801 (upper panel). Aliquots containing 100 µg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 MAbs DO1 and PAb1801 (middle panel) or with anti-p73 polyclonal serum (lower panel). B, Cell lysates extracted from T47D-HA and T47D-HA-DD polyclonal populations were subjected to immunoprecipitation with anti-p73 polyclonal antibodies (upper and middle panels-lanes 3 and 4) or with anti-HA MAb (lower panel-lanes 3 and 4). Immunoprecipitations as well as aliquots of 100 µg/lane were immunoblotted with anti-p53 MAbs DO1 and PAb1801 (upper panel and lower 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 γ to GSTp53His175(74-298) fusion protein or to GST alone. Total cellular lysates from H1299 cells transiently transfected with p73α or γ (2 mg) were incubated with GST fusion proteins (25 µg) for 2 h at 4 °C. Specifically bound p73α or γ were 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, 4).

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 PAb1801. Aliquots containing 100 µg of total protein from unprocessed lysates were subjected to immunoblot with a mixture of anti-p53 MAbs DO1 and PAb1801. 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), or p73αY99-F (lane 4), or p73αY121-F (lane 3) or p73αY99/121-F (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 with a mixture of anti-p53 MAbs DO1 and PAb1801 or with an anti-p73 polyclonal serum. Positions of protein molecular size markers are indicated on the left.

Fig. 7. Human tumor derived p53 mutants interfere with the transcriptional activity of p73α, β, δ, γ. H1299 cells (5 x 10⁵/60mm dish) were transiently
cotransfected with the p73α (A–B), or p73β (A–C), or p73δ (A–D), or p73γ (A–E) expression plasmids (25 ng/dish) with or without expression plasmids encoding p53H175 or p53Gly281 (100 ng/dish) respectively, together with the p21<sub>waf1</sub> luciferase reporter plasmid (50 ng/dish). The total amount of transfected DNA in each dish was keep 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 to control cells transfected with an empty CMV expression plasmid. Histograms show the mean of a typical experiment of three performed in triplicate; bars indicate the standard deviation of the mean.

Fig. 8. Overexpression of mutant p53 (His175 or Gly281) markedly reduces the amount of p73-inducible p21<sub>wa1f</sub> 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 and subjected to SDS-PAGE and immunoblotted with anti-p21<sub>waf1</sub> polyclonal serum or with anti-α-Hsp70 antibody for equal loading.
FIGURE 2

A.

B.

C.

GST
GST-p73
Lysate

T47D H-175
### FIGURE 4 C-D

#### C.

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<tr>
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<th>GST</th>
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<tr>
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**Blot:**
- Lane 1: GST
- Lane 2: GST p53-His175(74-298)
- Lane 3: Lysate p73α

#### D.

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**IB:**
- Lane 1: α-GFP

**IP:**
- Lane 2: α-p53
- Lane 3: α-p73

**Blot:**
- Lane 4: GFP p53
### FIGURE 6

**A.**

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**Top** panel shows the results of IP×p53 (doxin801) and IB×p73.

**Bottom** panel shows the results of IB×p53 (doxin801).
**FIGURE 8**

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<td>p53 Gly 281</td>
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IB: αp21 waf  
IB: αHsp70
Physical and functional interaction between p53 mutants and different isoforms of p73
Sabrina Strano, Eliana Munarriz, Mario Rossi, Barbara Cristofanelli, Yosef Shaul, Luisa Castagnoli, Arnold J. Levine, Ada Sacchi, Gianni Cesareni, Moshe Oren and Giovanni Blandino

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