p73 and p63 Are Homotetramers Capable of Weak Heterotypic Interactions with Each Other but Not with p53*

(Received for publication, January 14, 1999, and in revised form, April 7, 1999)

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Mutations in the p53 tumor suppressor gene are the most frequent genetic alterations found in human cancers. Recent identification of two human homologues of p53 has raised the prospect of functional interactions between family members via a conserved oligomerization domain. Here we report in vitro and in vivo analysis of homo- and hetero-oligomerization of p53 and its homologues, p63 and p73. The oligomerization domains of p63 and p73 can independently fold into stable homotetramers, as previously observed for p53. However, the oligomerization domain of p53 does not associate with that of either p73 or p63, even when p53 is in 15-fold excess. On the other hand, the oligomerization domains of p63 and p73 are able to weakly associate with one another in vitro. In vivo co-transfection assays of the ability of p53 and its homologues to activate reporter genes showed that a DNA-binding mutant of p53 was not able to act in a dominant negative manner over wild-type p73 or p63 but that a p73 mutant could inhibit the activity of wild-type p63. These data suggest that mutant p53 in cancer cells will not interact with endogenous or exogenous p63 or p73 via their respective oligomerization domains. It also establishes that the multiple isoforms of p63 as well as those of p73 are capable of interacting via their common oligomerization domain.

Tumor suppressor p53 is a transcriptional regulator of genes involved in control of the cell cycle and/or apoptosis (reviewed in Refs. 1–3). In response to cellular stress, particularly DNA damage, p53 protein levels rise, leading to changes in expression of p53 responsive genes and subsequent cell cycle arrest and/or apoptosis. Growth arrest or cell death prevents damaged DNA from being replicated and suggests a role for p53 in maintaining the integrity of the genome (4). This DNA damage checkpoint activity is central to its role as a tumor suppressor and also of major importance in the response of many cancers to conventional therapies that trigger apoptosis by damaging DNA. Inactivation of p53 through deletion, mutation, or interaction with cellular or viral proteins is a key step in over half of all human cancers (5, 6). Reactivation of the p53 pathway leading to apoptosis in cancer cells containing inactive p53 could greatly improve current modes of treatment (7, 8) and could potentially be a treatment modality in itself (9, 10).

Recently, two p53 homologues have been identified that can activate some of the same target genes as p53 and can induce apoptosis. The existence of these new proteins adds new complexity to our understanding of the p53 pathway and offers new potential for its reactivation in cancer cells. Human p73 (11, 12) and p63 (13–15) share regions of homology with the activation, DNA-binding, and oligomerization domains of p53. This raises the possibility of physical, and/or genetic interactions between p53 family members (13), as is often the case with other families of transcription factors such as the homeodomain proteins (16) or the Myc superfamily (17).

Both p63 and p73 exist in multiple isoforms with different biological activities, designated p73α and p73β (11) or p63α-γ and ΔNp63α-γ (13). To address the question of whether p53 family members and their isoforms can homo- and/or hetero-oligomerize and thereby affect the transcriptional activities of one another, we have assessed the ability of each oligomerization domain to homo- and hetero-oligomerize in vitro, as well as the in vivo effects of coexpressing pairs of proteins.

MATERIALS AND METHODS

Protein Purification—The sequences encoding residues 310–360 of human p53, 355–404 of human p63, and 345–383 of human p73 were subcloned into the pET15b vector (Novagen Inc.), and residues 359–399 of p63 and residues 350–383 of p73 were subcloned into pET19b (Novagen Inc.) using standard techniques (18). Residue numbers for p63 correspond to those of the p63 isoform containing the full N-terminal 1450 M (13). The resulting plasmids express 6 histidine (pET15b) or 10 histidine (pET19b) residues followed by a linker containing a thrombin (pET15b) or enterokinase (pET19b) cleavage site and a His-Met dipeptide immediately N-terminal to the protein of interest. Recombinant proteins were expressed in Escherichia coli BL21(DE3)-[pLys-S] harboring the desired plasmid and purified by nickel affinity chromatography as described previously (19). The yield of purified protein was 10–20 mg/liter of culture. When desired the polyhistidine tags were cleaved with the appropriate enzyme according to the manufacturer’s recommendations. The proteins separated from the His tag peptide by nickel affinity chromatography. Prior to experiments, samples were dialyzed for 24 h at 4 °C three times against 2 liters of buffer (25 mM sodium phosphate, 150 mM NaCl, pH 7.0) using Spectra/por 3500 molecular weight cut-off dialysis membranes. Protein concentration was determined spectrophotometrically using the extinction coefficient ε = 1450 M -1 cm -1 (at 275 nm) per solvent exposed tyrosine residue in 6.5 M guanidine HCl.

Circular Dichroism—Far UV CD spectra were recorded on an Aviv 62A DS circular dichroism spectrometer equipped with a thermoelectric temperature controller. Measurements were made using a 0.1-cm cuvette at a sample concentration of 75 μM (monomer). Thermal unfolding was followed by monitoring ellipticity at 222 nm as a function of temperature every 3 °C allowing 4 min for sample equilibration and a signal average of 99 s for each data point.

Hetero-oligomerization—Equimolar samples of His10-tagged and untagged proteins were mixed at ratios of 1:1, 1:10, and 1:15 and incubated in one of three conditions for assessment of hetero-oligomerization: 1) Samples were incubated at room temperature overnight in a buffer of 150 mM NaCl, 25 mM sodium phosphate, pH 7.0. 2) Samples were incubated at 95 °C for 20 min and cooled to 37 °C and then to 25 °C over 2 h at each step. 3) Samples were dialyzed against 2 liters of 8 M
urea, 150 mM NaCl, 25 mM sodium phosphate, pH 7.0, in Spectra/por 3500 molecular weight cut-off dialysis membrane for 8 h at 25 °C, followed by step-wise dialysis against 1 liter of similar buffers containing 6 and 4 M urea at 25 °C and then 2 M urea and two steps against 2 liters of 0 M urea, 150 mM NaCl, 25 mM sodium phosphate, pH 7.5, at 4 °C. Samples were subsequently loaded onto a 300-μl bed of Qiagen nickel-nitriotriacetic acid-agarose beads and washed with 10–15 ml of 35 mM imidazole, 500 mM NaCl, 40 mM Tris-HCl, pH 7.5, and eluted in 1-ml fractions with 4 ml of 500 mM imidazole, 500 mM NaCl, 40 mM Tris-HCl, pH 7.5. Aliquots (20 μl) from each fraction were run on 15% SDS-polyacrylamide gel electrophoresis and subsequently in 10 μl of distilled water before running on a 15% SDS-polyacrylamide gel to visualize the protein.

Glutaraldehyde Cross-linking—Samples of 75 μM His6-p73(345–383), p73(345–383), His6-p63(355–404), or p63(355–404) in 250 mM NaCl, 25 mM sodium phosphate, pH 7.5, were mixed with glutaraldehyde (with final glutaraldehyde concentrations ranging from 0.0 to 0.28%) in a total volume of 15 μl. Samples were incubated at 25 °C for 30 min, and the reaction was stopped by the addition of 5 μl of standard SDS-denaturing gel loading buffer. The resulting 20-μl samples were then loaded and run on a 15% SDS-polyacrylamide gel.

Size Exclusion Chromatography—A Superdex 75 (Amersham Pharmacia Biotech) column pre-equilibrated with 25 mM sodium phosphate, pH 7.2, 250 mM NaCl, via a Biologic FPLC system (Bio-Rad) was loaded with 200 μl of protein and chromatographed with 1 column volume (about 40 ml) of buffer at 0.4 ml/min. Fractions of 0.5 ml were collected for further analysis. Eluted protein was detected by UV at 280 (molecular weight standards) or 254 nm (tetramerization domains); the oligomerization domains absorb UV light poorly at 280 nm, and high protein concentrations were not possible with p63 and p73, necessitating the use of a lower wavelength for detection. The following proteins (and their corresponding masses) were used as molecular weight standards to calibrate the column: bovine serum albumin (66,000), carbonic anhydrase (29,000), lysozyme (14,400), cytochrome c (12,400), aprotinin (6,000), and insulin chain B (3,500). Dextran blue (200,000) was used to determine the void volume. A plot of V/V0 (elution volume for a given protein as a fraction of the void volume divided by the void volume of the column) versus log molecular weight was used to determine the molecular weights and thus the oligomeric states of the oligomerization domains.

DNA Transfection and Luciferase Assay—Cells were seeded into 6-well plates at a concentration of 2–5 × 104 cells/well, 16–24 h prior to transfection. DNA transfection was achieved using LipofectAMINE Plus System (Life Technologies, Inc.) according to the manufacturer’s instructions. In a typical experiment, 1 μg of reporter plasmid PSRC-Luc was transfected with 70 ng of either pcDNA3 vector containing p53 or p73α and 70, 350, or 700 ng of pcDNA3 containing either p53 R273H or p73 R293H. The final DNA concentration was adjusted to 1,770 μg/well using pcDNA3. 24 h after transfection, cells were lysed in 500 μl of passive lysis buffer (Promega), and luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega). Western Blot Analysis—SK-NAS cell extracts prepared in SDS sample buffer were fractionated on a 10% polyacrylamide gel and transferred to Immobilon membranes by electrophoresis. Membranes were probed with p53 and p73α antibodies using a standard protocol and developed by chemiluminescence (Pierce).

RESULTS

p63 and p73 Are Tetramers—The tetramerization domain of p53 (residues 325–355) is the primary determinant of its oligomeric state (20–22). To determine whether p73 and p63 form homotetramers similar to those of p53, we expressed recombinant constructs containing the homologous domains of each protein as shown in Fig. 1. Fig. 2 shows SDS-polyacrylamide gels of p63(355–404) and p73(345–383) in the presence of increasing amounts of glutaraldehyde. As seen previously for p53, both p63 and p73 are clearly able to form homo-oligomers, with tetramers predominating at the highest glutaraldehyde concentrations. Size exclusion chromatography (data not shown) also indicate that p73(345–383) elutes with a molecular weight of 23,000, corresponding to that of a tetramer.

We then used CD spectroscopy to compare the amount of secondary structure present in p73(345–383) to that of a similar domain of p53 (Fig. 3A). The shapes of the CD spectra for both protein domains are consistent with a similar secondary structure, taking into account the different lengths of each protein construct. We used the CD signal at 222 nm, which is indicative of α-helix, to monitor the stability of p73(345–383) as a function of temperature. As shown in Fig. 3B, the tetramerization domain of p73 is very stable and does not start to unfold until temperatures of 60 °C or higher. Upon cooling the sample back to 25 °C, the original CD spectrum is obtained, showing that the thermal unfolding of this domain is reversible. This behavior is very similar to that of the p53 tetramerization domain (19).

Similar CD spectra of p63(355–404) could not be obtained because of precipitation of the protein under CD conditions. However, heating of p63(355–404) to 85 °C followed by slow cooling and subsequent incubation with glutaraldehyde, re-
dependence of the a for histidine-tagged p53 and p73 (data not shown). This domain is reversible. Similarly, reversible unfolding was observed and the original spectrum was reproduced, indicating that unfolding of following thermal unfolding of p73, the sample was cooled to 25 °C (75 m') showing that p53 and p73 oligomerization domains remain folded over a large temperature range.

The slightly different shape of the p53 spectrum between 210–220 nm -helical content. The biological significance of this result is unclear because it remains to be determined whether and to what extent p73 and p73 via their tetramerization domains under a wide variety of conditions.

p73 and p63 Tetramerization Domains Can Form Hetero-oligomers—When the same refolding experiments were performed with histidine-tagged p63 and untagged p73 (Fig. 5), we found evidence of weak interactions between these two proteins. These were only observable when the experiments were performed with an excess of the untagged protein and with silver staining to visualize the protein bands. However, in contrast to the residual interaction of p53 with p73 shown in Fig. 4C, the ratio of co-eluted untagged protein to His-tagged protein was much more significant in the case of p63/p73 (compare relative intensities in lane 10 of Fig. 4C with those in lane 11 of Fig. 5). Nevertheless, the extent of hetero-oligomerization between p63 and p73 appears to be less efficient than the homo-oligomerization of either protein. The biological significance of this result is unclear because it remains to be determined whether and to what extent p73 and p63 are co-expressed in the same cellular environment.

p53 (R273H) Mutant Does Not Interfere with Transcriptional Activation by p73—The absence of hetero-oligomerization between p53 and p73 suggests that dominant negative mutants such as p53 R273H (23) or p73 R293H (11), which are defective for DNA binding and transcriptional activity, will not interfere with transcriptional activation by heterologous wild-type factors. Therefore, to further establish that p53 and p73 do not interact in vivo, p53 or p73a were co-expressed in SK-NA-S cells with various amounts of either p53 R273H or p73 R293H. Fig. 6A, shows that, as previously reported (23), excess of p53 R273H completely abolished the transcriptional activation of a p53-responsive luciferase gene by p53, whereas increasing

FIG. 3. p73(345–383) is a stable, independently folded domain. A, far UV CD spectra of recombinant p53(310–360) (●) and p73(345–383) (▲), both lacking the polyhistidine tag, at 25 °C (75 μm protein monomer) shows that both domains contain similar amounts of secondary structure. The strong minimum at 222 nm reflects α-helical content. The slightly different shape of the p53 spectrum between 210–220 nm is due to an additional 12 residues in this construct that are outside the conserved tetramerization domain and that are unstructured (24). Following thermal unfolding of p73, the sample was cooled to 25 °C (△), and the original spectrum was reproduced, indicating that unfolding of this domain is reversible. Similarly, reversible unfolding was observed for histidine-tagged p53 and p73 (data not shown). B, temperature dependence of the α-helical signal (222 nm) of p53(310–360) (●) and p73(345–383) (△) showing that p53 and p73 oligomerization domains remain folded over a large temperature range.

p53 Oligomerization Domain Does Not Interact with That of p63 and p73—To determine whether the tetramerization domain of p53 can associate with that of p63 and p73 to form hetero-oligomers, we performed protein–protein interaction assays using a polyhistidine-tagged p53 tetramerization domain and untagged p73(345–383) or p63(355–404). Mixing purified tagged and untagged proteins together followed by Ni²⁺ affinity chromatography consistently showed that only the histidine-tagged p53 protein was retained by the resin and that all of the untagged protein eluted in the wash (data not shown). However, it is possible that heterotetramers could not form under the conditions used above because of the high stability of the preformed homotetramers. Therefore, we used two methods (thermal and chemical denaturation) to unfold binary mixtures of homotetramers and dissociate them into monomeric subunits with subsequent refolding under native conditions. This process of simultaneous unfolding mimics the process of co-expression of the two proteins in vivo. As shown in Fig. 4, refolding of His-tagged p53 with either p63(355–404) or p73(345–383) followed by Ni²⁺ chromatography again indicated no formation of hetero-oligomers. Because the unfolding of all three proteins is fully reversible, both in the presence and absence of the histidine tags (as shown above (Fig. 3) and in Ref. 19), a negative result because of the inability of each protein to refold under these conditions can be ruled out.

In cancer cells, inactive mutant p53 is often overexpressed and could potentially hetero-oligomerize with and inactivate endogenous p73 or p63. Because the above experiments were performed at roughly equimolar ratios, we repeated the experiments using a 15-fold excess of p53 tetramerization domain. Again no interaction of p53 with p73 or p63 was observed using Coomassie Blue staining of the gel (data not shown). Alternatively, if a 10-fold excess of p73 was used relative to p53 and silver stain was used to visualize the protein bands, then we were able to see a very weak band for untagged p73 co-eluting with His-tagged p53 (Fig. 4C). However, the ratio of co-eluted p73 was much less than that of p53. Taken together these results demonstrate that p53 does not hetero-oligomerize to a significant extent with either p63 or p73 via their tetramerization domains under a wide variety of conditions.

resulted in cross-linking of the protein primarily to tetramers, indicating that p63 also refolds to a native-like state after thermal unfolding. Taken together these data show that the oligomerization domains of p63 and p73 form independently folded tetramers with a secondary structure and probably tertiary structure very similar to that of the homologous region of p53.
p73 and p63 Oligomerization

FIG. 4. p53 does not hetero-oligomerize with p73 or p63. A, a representative 15% SDS-polyacrylamide Tricine gel of hetero-oligomerization assays stained with Coomassie Blue. Equimolar samples of His10-tagged p53(310–360) were incubated at 95 °C for 20 min, cooled to 25 °C, and then loaded onto a bed of Qiagen nickel-nitrioltriacetic acid-agarose beads (lane 3) and subsequently washed (lanes 5–8) and eluted (lanes 9–12) as described in “Materials and Methods.” B, same procedure as in A. Lane 1, His10-tagged p53(310–360); lane 2, p63(355–404); lane 3, mixture; lanes 4 and 6–8, wash; lanes 9–12, eluate. p53(310–360) appears as a double band because of a small amount of C-terminal proteolytic degradation. C, silver-stained gel of a similar experiment using His10-tagged p73(350–383) (lane 1) and a 10-fold excess of untagged p63(355–404) (lane 3) were allowed to interacted as described in the legend to Fig. 4 and under “Materials and Methods.” Lane 4, mixture after incubation; lanes 6–9, wash; lanes 10–13, eluate. The presence of untagged p63 in lane 11 indicates that p63 and p73 are able to hetero-oligomerize when folded in the presence of each other.

amounts of the analogous mutant p73 R293H had almost no effect on p53 transactivation activity. The slight and constant inhibition observed with ×1, ×5, and ×10 excess of p73 R293H (Fig. 6A) was not reproducible and was correlated with a lower level of p53 expression than in the control experiment. When p73α was co-expressed with either p53 R273H or p73 R293H, the same results were obtained (Fig. 6B); increasing amounts of p73 R293H efficiently blocked p73α-mediated transactiva-

FIG. 5. p63 hetero-oligomerizes with p73. His10-tagged p73(350–383) (lane 1) and a 10-fold excess of untagged p63(355–404) (lane 3) were allowed to interacted as described in the legend to Fig. 4 and under “Materials and Methods.” Lane 4, mixture after incubation; lanes 6–9, wash; lanes 10–13, eluate. The presence of untagged p63 in lane 11 indicates that p63 and p73 are able to hetero-oligomerize when folded in the presence of each other.

p73 (R293H) but Not p53 (R273H) Interferes with p63 Transcriptional Activity—In light of our results showing that p73 and p63 oligomerization domains can form heterotetramers, it was of interest to test for possible interaction of the two proteins when produced in the same cellular context. We therefore performed co-transfection experiments in which p53 R273H or p73 R293H were alternatively co-expressed with p63β in SK-NA-S cells, and the resulting p63-mediated transactivation of a RGC-luciferase reporter gene was measured. Fig. 7 shows that the dominant negative p53 mutant does not interfere with p63 transcriptional activity, in good agreement with the observed lack of hetero-oligomerization of their respective tetramerization domains. p73 R293H on the other hand, clearly inhibits transactivation by p63 in this assay, also in agreement with our in vitro data. However, the degree of inhibition appears to be less efficient compared with that of either the p73 R293H inhibition of p73 or the p53 R273H inhibition of p53. This is also in agreement with our protein-protein interaction assays, which show a weak but significant interaction between p63 and p73 oligomerization domains.

DISCUSSION

Both p63 and p73 are expressed in multiple isoforms with different transcriptional and biochemical properties that have yet to be fully understood. Although these isoforms differ in the number of residues at the N or C termini, all isoforms of a given gene product share an identical oligomerization domain. We have shown that the oligomerization domains of p63 and p73 form homotetramers, suggesting that proteins formed in vivo will likely be mixed oligomers composed of a statistical distribution of those isoforms available in a given cell type. This is consistent with the report by Yang et al. (13) in which a glutathione S-transferase-tagged p63γ was able to interact with several other p63 isoforms. Kaghad et al. (11) also found weak interactions between p73α and p73β using the yeast two-hybrid assay. Both of these observations likely reflect interactions mediated by the oligomerization domain of p63 and p73, respectively, and likely have important implications for the activity of p63 or p73 in cells expressing multiple isoforms.

We have also shown that p63 and p73 can potentially hetero-oligomerize via their tetramerization domains. This interaction appears to be weak because it is only observable with an excess of one of the two proteins, and dominant negative p73 mutants were not fully able to inhibit p63 transactivation even in 10-
fold excess. Nevertheless, these data raise the possibility of cooperative or antagonistic interactions between p63 and p73 if they are co-expressed or incorrectly expressed in the same tissue. However, co-expression of both proteins has yet to be demonstrated, and further data on the expression patterns of both p63 and p73 will be needed to better understand the functional relationship, if any, between these two proteins.

Many families of transcription factors exhibit combinatorial homotypic and heterotypic interactions via conserved oligomerization or protein-protein interaction domains. Our in vitro and in vivo experiments clearly support the notion that p53 does not hetero-oligomerize with p63 or p73, even though all three proteins likely homo-oligomerize via a similar three-dimensional structure. These facts can be rationalized if one considers the symmetry of the tetrameric form of the p53 oligomerization domain. The p53 tetramerization domain has three 2-fold axes of symmetry (24, 25). If we consider a single 2-fold axis (illustrated schematically in Fig. 8), the symmetric nature of the oligomers may favor homotypic interactions at the expense of heterotypic interactions. In this two-dimensional example, the overall shape of each subunit (the rectangles) and oligomer (the squares) would correspond to the global three-dimensional fold of each subunit and tetramer, respectively. The smaller surface details of each subunit (pointed or rounded) correspond to the different amino acid side chains of each subunit. Here the complementary fit between subunits favors homotypic interactions. The fact that the p63 and p73 oligomerization domains are more closely related to each other (58% identity) than either is to p53 (35 and 42% identity, respectively) may explain why p63 and p73 are able to interact to some extent. A recent mutagenesis study in which the oligomerization domains of p53 and chimeric p53/p63 and p53/p73 mutants were assayed for interaction also supports a symmetry-related explanation for the selectivity of hetero-oligomerization (26).

Our results suggest that the accumulation of mutant p53, as is frequently observed in human cancers, will likely not interfere with p63 or p73 transcriptional activity. However, while this manuscript was under review, it was reported that mutant p53 (R175H and R248W) but not wild-type p53 could co-immunoprecipitate with p73 and that these mutants but not wild-type p53 could reduce the transcriptional and apoptotic activities of p73 in H1299 cells (27). Because different p53 mutants and cell lines were used in this study, it is difficult to directly compare the results with ours. Nevertheless, the data presented here show that putative interactions between mutant p53 and p73 are unlikely to involve the oligomerization domains of each protein. Given that 1) only mutant p53 was observed to interact with p73, 2) the interaction was weak (5–10% of p73 associated with mutant p53 (27)), and 3) the interaction appears to be cell type-dependent (no interaction was observed in yeast (27) or SK-NA-S (this study)) a more likely explanation is that the p73–mutant p53 interaction may be mediated by an as yet unidentified molecule specific to mutant p53 and only present in certain cell types. Our results also suggest that transactivation-deficient isoforms of p63 or p73 (such as N-terminally truncated proteins (13)) will not hetero-oligomerize with wild-type p53, thereby affecting p53 transcriptional activity.

Dominant negative inhibition via hetero-oligomerization of
DNA-binding inactive and active p53 family members is a distinct mechanism from that involving competitive DNA binding between two or more family members (or isoforms thereof) with differing transcriptional activation properties. We have shown that the former mechanism is unlikely. However, this does not rule out a role for the latter mechanism in normal or tumorigenic cells, as recently suggested for p63 (13).

Acknowledgment—We thank Peter Yin for excellent technical support.

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