Evidence for a Distinct Inhibitory Factor in the Regulation of p53 Functional Activity*

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Under normal conditions, tumor suppressor protein p53 exists in the cell in its latent form and is unable to function as a transcription factor. The allosteric model of p53 regulation postulates that the extreme portion of p53 carboxyl terminus (aa 364–393) binds to the core domain of the protein, thereby abrogating specific DNA binding in that region. In this study we propose an alternative mechanism of p53 functional regulation, which involves a separate molecule acting in trans to inhibit p53 transcriptional activity. Through the use of chimeric proteins of p53, p63γ and p73β, we show that the extreme COOH-terminal domain of p53 exerts a powerful and specific inhibitory effect on the p73- and p63-driven expression of a reporter gene. Moreover, fusion of p53 extreme COOH terminus to a completely unrelated transcriptional activator Gal4-VP16 also results in significant inhibition of transactivation activity. Since p73, p63, or Gal4-VP16 cannot associate with any part of the p53 molecule, we conclude that p53(aa 364–393) represses transcriptional activity of chimeric proteins and p53 itself through the binding of external negative modulator(s) in that region and not by the allosteric mechanism of regulation. In accordance with the “distinct inhibitor” hypothesis, the activity of wild type p53 is substantially increased by overexpression of chimeric proteins bearing p53(aa 364–393), which might be due to the competitive removal of transcriptional inhibitor(s). Our findings provide the basis for the identification of such negative modulators of p53 transcriptional activity.

The product of tumor suppressor gene p53 regulates a multitude of cellular processes, most notably inducing cell cycle arrest and apoptosis in response to various forms of genotoxic stress such as DNA damage, hypoxia, and low intracellular concentration of ribonucleosides (1). Functioning as a transcription factor, p53 activates the expression of a number of its downstream target molecules, including cyclin-dependent kinase inhibitor p21 (WAF1, Cip-1), DNA repair protein GADD45, and a host of apoptotic mediators such as Bax, AIP, PIG, and others (for review, see Ref. 2).

Human p53 protein has been extensively characterized both structurally and functionally (3, 4). The NH₂-terminus of p53 harbors the transactivation region (aa¹ 1–42) that interacts with basal transcriptional machinery in inducing various gene expression, but also contains binding sites for negative regulators of p53 transcriptional activity (Mdm2, E1B). The core domain of p53(aa 113–290) is critical to the p53 function as a transcription factor and encompasses residues involved in sequence-specific DNA binding. The DNA-binding domain is the single most frequent site of missense mutations in the p53 gene that contribute to the process of malignant transformation in the cell (5). The carboxyl terminus of the p53 protein contains the oligomerization domain (aa 319–364), which is essential for tetramer formation, as well as regulatory sequences in the extreme COOH-terminal end (aa 364–393).

In the last few years, the extreme COOH-terminal end of p53(aa 364–393) has been the subject of intense scrutiny because of its role in the regulation of p53 functional activity. Under basal conditions, p53 exists in the cell at low levels and in its latent form. According to the current allosteric model of regulation, such latency is achieved through the association of extreme COOH-terminal region with the core domain of the protein, which effectively blocks sequence-specific DNA-binding sites and disables p53 as a transcription factor (6). It has been shown that deletion of aa 364–393, phosphorylation or acetylation of several residues in that region, as well as specific antibody binding, all dramatically induce p53 transactivation activity (7, 8, 9). In the cell, the disassociation of the COOH terminus from the core domain and subsequent increase in the p53 transactivation activity is believed to be triggered by binding of single-strand DNA or DNA ends to the carboxyl terminus (10). The latter observation supports the notion that p53 functions as a sensor of DNA damage in the cell and is activated by the presence of DNA breaks.

However, the experimental evidence that supports the allosteric model of p53 regulation can also be interpreted in favor of an alternative mechanism, including binding of transactivation inhibitors at the extreme COOH terminus of p53. In our laboratory, we have constructed a number of chimeric proteins by systematically exchanging various domains between p53 and its close homologue p73β (11). During the course of screening such chimeric proteins for their ability to transactivate p53-responsive elements in a reporter gene, we have observed a dramatic inhibition of p73 functional activity, compared with wild type, when the extreme COOH-terminal portion of p73 was replaced with aa 364–393 of p53. Since previously published in vitro binding data demonstrate that wild type p53 and p73 do not physically interact (11, 12), the current allosteric

¹ The abbreviations used are: aa, amino acid(s); PCR, polymerase chain reaction; GFP, green fluorescent protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TBP, TATA-binding protein.
Alternative Model of p53 Functional Latency

To gather further evidence to support our "distinct repressor" mechanism, we have created additional chimeric constructs and confirmed that the inhibitory effect of p53(aa 364–393) on other proteins is specific and not restricted to the p53 family members. In addition, we demonstrate here that p53 transcriptionsal activity cannot be explained by the model of p53 regulation that cannot explain the inhibitory effect conferred on p73 by aa 364–393 of p53.

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**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric Proteins—**CDNA clones of human p73α, p53, and p63γ have been described previously (11, 13). Fusion chimeras were generated using two-step polymerase chain reaction (PCR) and primers that encoded 18-nucleotide regions of complementarity between the sequences to be fused (11). Single amino acid substitutions (R273H for p53 and R292H for p73) were introduced into the wild type and chimeric constructs using PCR as described by us (11). PCR was carried out in PerkinElmer thermocycler using 1× Pfu PCR reaction buffer with MgSO4, 1× GC melt solution (to facilitate dissociation of double-stranded template DNA), 2.5 mM dNTPs, 10 pmol each of 5′ and 3′ primers, 1 unit of Pfu DNA polymerase, and 50 ng of template DNA. All constructs were cloned into FLAG-tagged pcDNA3.0 vector using BamHI and XbaI restriction sites and expressed in Escherichia coli following established procedures.

The pcDNA3.0-Gal4 DNA-binding domain (aa 1–147), pcDNA-3.0-VP16 activation domain (aa 413–491), and pGL3-GSSV plasmids were a kind gift of Dr. Y. Shi (Harvard Medical School, Boston, MA). Gal4 DNA-binding domain and VP16 activation domain were PCR-amplified separately and subcloned into FLAG-tagged pcDNA3.0 resulting in Gal4-VP16 fusion construct. Fragments of p53β(aa 364–393 and 291–319) were subsequently fused in frame to the COOH terminus of Gal4-VP16 using EcoRI and XbaI sites. Large scale plasmid DNA preparation was obtained using a Qiagen Maxi kit. Final plasmid yield and purity were determined spectrophotometrically at 260 and 280 nm and by restriction digest. Construct identities were further verified by DNA sequencing (Harvard Cancer Center, Boston, MA).

**Cell Culture—**p53−/− mouse embryonic fibroblasts, 293T cells, human small cell lung carcinoma H1299 cells, and human osteosarcoma Saos-2 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) in the presence of 10% bovine serum, 2 mM t-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO2 humidified atmosphere.

**Transient Transfections and Luciferase Assays—**For luciferase assays, 1–2 × 105 H1299, mouse embryonic fibroblasts, Saos-2, or 293T cells were plated onto 30-mm tissue culture dishes and allowed to reach approximately 60% confluence by incubating overnight. Transient cell transfections were carried out using the calcium phosphate precipitation method as described previously (14). Cells were co-transfected with either PG13-Luc (Promega) (0.5 μg/plate) or with pGL3-GSSV (0.5 μg/plate) and with various chimeric constructs (1–3 μg of DNA/plate). For competition experiments, 50 ng of wild type p53 plasmid was used either alone or with 2 μg of mutant chimeric proteins. Empty vectors were used to standardize for total DNA amount. Additionally, prL-TK vector (Promega) was included to provide a low level of Renilla luciferase expression and serve as transfection efficiency control. Luciferase activity was quantified at 36 h post-transfection using the Dual Luciferase detection system (Promega) following the manufacturer's instructions and a Lumat9507 luminometer (EG&G Berthold). Relative luciferase activity was determined as a ratio of Firefly (PG13, pGL3-GSSV) to Renilla (prL-TK) luciferase expression. All experiments were repeated at least three times in duplicate.

To determine protein expression levels, H1299 or 293T cells were plated onto 60-mm dishes and transfected with 2–5 μg of either wild-type, chimeric, or mutant construct DNA and with green fluorescent protein (GFP) pEGFP-C1 plasmid (CLONTECH) (0.5 μg/plate) and with various chimeric constructs (1–3 μg of DNA/plate). For competition experiments, 50 ng of wild type p53 plasmid was used either alone or with 2 μg of mutant chimeric proteins. Empty vectors were used to standardize for total DNA amount. Additionally, prL-TK vector (Promega) was included to provide a low level of Renilla luciferase expression and serve as transfection efficiency control. Luciferase activity was quantified at 36 h post-transfection using the Dual Luciferase detection system (Promega) following the manufacturer's instructions and a Lumat9507 luminometer (EG&G Berthold). Relative luciferase activity was determined as a ratio of Firefly (PG13, pGL3-GSSV) to Renilla (prL-TK) luciferase expression. All experiments were repeated at least three times in duplicate.

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model of p53 regulation cannot explain the inhibitory effect conferred on p73 by aa 364–393 of p53.
FIG. 2. p53, but not p73, binds p53 carboxyl terminus. 293T cells were transfected with either FLAG-pcDNA3.0 (Vector), FLAG-p53(R273H), FLAG-p73(R293H), or with pCMV-Mdm2 (5 μg/plate each), and cell lysates were subsequently incubated with GST proteins of p53 carboxyl or amino termini. Bound proteins were analyzed by Western blot with anti-FLAG or anti-Mdm2.

FIG. 3. A, mutant chimeric proteins. The p53M construct represents full-length p53 with a single amino acid substitution (R273H), which renders it deficient in DNA binding. Similar mutation was introduced into p53 protein carrying the p73 oligomerization domain (p53M [aa345–380]). Substitution R293H was generated in p73 chimeras containing either the entire p53 COOH terminus (p73M [aa320–393]) or the extreme carboxyl-terminal sequence of p53 (p73M [aa364–393]). B, equal expression levels of mutant chimeric proteins. 3 μg of FLAG-tagged mutant chimeric p73/p53 proteins, as well as 0.5 μg of pEGFP-C1, were transfected into H1299 cells and analyzed using anti-FLAG and anti-GFP Western blot 36 h post-transfection. C, co-expression of mutant chimeras bearing p53(aa364–393) "de-represses" wild type p53 activity. H1299 cells were co-transfected with 50 ng of wild type p53 and with 2 μg each of various mutant chimeric proteins. 0.5 μg of PG13-Luc reporter plasmid along with 0.05 μg of pRL-TK construct were used to record luciferase activity. Empty vector was also included where appropriate to maintain constant amount of DNA per plate. Luciferase activity was determined in the cell lysates 36 h later as described in the legend to Fig. 1C. The result is shown as fold induction of luciferase activity relative to vector control alone. Mean ± S.D. was derived from three separate experiments performed in duplicate.

Aprotinin) for 1 h at 4 °C. Cell lysates were further diluted to 0.5% Nonidet P-40 and incubated with 15 μl of GST beads containing p53 COOH or NH2 terminus for 3 h at 4 °C. Beads were then washed three times in lysis buffer, 0.05% Nonidet P-40, and immunocomplexes were liberated from the beads by boiling in SDS-PAGE sample buffer for 5 min. Samples were then electrophoresed through 10% acrylamide SDS-PAGE gel and transferred onto nitrocellulose filters. Membranes were incubated with anti-FLAG antibody solution and developed using enhanced chemiluminescence system (PerkinElmer Life Sciences). Nitrocellulose membranes were then stripped of antibodies and probed with anti-Mdm2 following a similar protocol. GST proteins were visualized using Ponceau S staining.

RESULTS

Chimeric Protein Constructs—It has been demonstrated that p63 and p73, two close structural homologues of p53, can transactivate p53-responsive promoters (13, 15) but do not form
heterooligomers with p53 (11, 12). Therefore, it seemed feasible for us to create chimeric constructs utilizing these proteins to test whether the inhibitory effect of the p53 COOH terminus (p53CT) is accomplished through the allosteric mode of regulation or through a "distinct repressor" mechanism. Should a separate repressor be involved in the p53 regulation, then p73- or p63-mediated transcriptional activity would be down-regulated by the introduction of p53CT. Otherwise, the allosteric model would hold true if the negative regulatory effect of p53CT is accomplished through the allosteric mode of regulation.

Alternative Model of p53 Functional Latency

In addition to several chimeric constructs of p53 and p73 that had been generated previously by us (Fig. 1A), the following chimeras were created during the course of this study: fusion of p73(aa 1–391) and p53(aa 291–319) and fusion of p63(aa 1–397) and p53(aa 364–393) (Fig. 4A). To confirm that the inhibitory effect of the p53 extreme COOH terminus is not exclusive to the p53 family members, sequences of p53(aa 364–393 and aa 291–319) were also fused to a completely unrelated transcriptional activator Gal4-VP16 (Fig. 5A).

The identity of chimeric proteins was confirmed by transfecting H1299 or 293T cells with 2–5 μg of purified plasmid DNA and conducting anti-FLAG Western blot of cell lysates. Abundant immunosignal was detected for all constructs, with molecular weights corresponding to the expected size of chimeric products (Figs. 1B, 3B, 4B, and 5B). Notably, while protein expression levels were comparable for all chimeras, they possessed different transcriptional activities.

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p53CT Inhibits Transcriptional Activity of p73—Transcriptional activity of p53-p73 chimeric proteins was assessed using luciferase reporter gene containing p53-responsive elements. 1 μg of each construct DNA and reporter plasmid (0.5 μg/plate) was co-transfected into p53-null H1299 cells. Analysis of the luciferase gene expression (Fig. 1C) revealed that constructs containing p53-p73 oligomerization domain "swap" (constructs p53(73aa345–390) and p73(53aa319–390)) induced luciferase gene expression to the same degree as wild type p53 and p73, respectively. On the contrary, a dramatic inhibition of luciferase activity was detected when either the entire COOH terminus of p53 or only its last 30 residues were fused to p73 (constructs p53(73aa319–390) and p73(53aa364–393)), defining the residues from 364 to 393 as the inhibitory domain. If the last 30 residues of p53 down-regulate its transcriptional activity, then substitution of this domain would relieve the inhibitory effect. Indeed, fusion of the corresponding portions of p53 into the p53 sequence (constructs p53(73aa345–499) and p53(73aa391–499)) was associated with the complete abrogation of functional repression when compared with wild type p53. Identical transcriptional activity profiles were also obtained in Saos-2 cells and p53−/− mouse embryonic fibroblasts (data not shown), thereby eliminating the possibility of cell type-specific effects.

p53 Does Not Bind the p53 Carboxyl Terminus—To determine whether p53CT inhibited p73 transcriptional activity via the association with the p73 DNA-binding domain, as predicted by the allosteric model, we set out to study the ability of
full-length p73 to bind p53CT in vitro. 293T cells were transfected with either vector alone, mutant p53 or p73 (mutant isoforms were used to ensure high level of protein expression), or with Mdm2, which served as positive control because of its well-documented ability to bind to the p53 NH₂ terminus. Following cell lyse incubation with GST fusion proteins of p53 carboxyl (GST-p53CT) or amino (GST-p53NT) termini and thorough washing to minimize nonspecific binding, proteins were released from glutathione beads, resolved on SDS-PAGE gel, blotted onto nitrocellulose membranes, and studied using anti-FLAG and anti-Mdm2. As expected, p53 readily associated with its carboxyl terminus that contained the oligomerization domain (Fig. 2, lane 3). In contrast, GST-p53NT, which formed a complex with Mdm2 (Fig. 2, lane 11), did not exhibit any detectable interaction with p53 (Fig. 2, lane 4), demonstrating the specificity of our binding assay. Under the same conditions, neither GST-p53CT nor GST-p53NT displayed any apparent binding to p73 (Fig. 2, lanes 7 and 8). Taken together, these results indicate that p53CT, when fused into the corresponding region of p73, potently inhibits the transcriptional activity of p73 in the absence of any physical association with the p73 molecule, an observation that is inconsistent with the allosteric model.

Our findings strongly implicated the last 30 residues of p53 in the inhibition of p73-mediated transactivation. However, it is also possible that the inhibitory effect rendered by the p53CT was due to an alteration of the p73 conformation caused by the domain swap rather than the inhibitory nature of the p53CT. To test this possibility, an additional chimera, as

chimeric proteins that harbored both the p53(aa 364–393) sequence and an appropriate point mutation in their DNA-binding domain (Fig. 3A). We intentionally utilized chimeric proteins as a delivery vehicle for the extreme carboxyl terminus of p53, since overexpression of short COOH-terminal peptides alone results in their diffused subcellular distribution due to the lack of nuclear localization sequence. Analysis of the transcriptional activity of mutant constructs revealed their complete functional incompetence due to their inability to bind DNA. As expected, both mutant p53 and mutant p73(33aa319–393) completely abolished p53 activity through oligomerization with the wild type protein. In sharp contrast, co-expression of mutant p73(53aa364–393) chimera resulted in the increase of p53-driven luciferase expression by more than 3-fold. A similar “de-repression” effect on p53 was observed when mutant p73(33aa345–390) was used (Fig. 3C). Both of these chimeric proteins were unable to form tetramers with wild type p53 because of their p73 oligodomains and contained last 30 amino acids of p53. These data indicate that p53 can be functionally activated by the introduction of the exogenous COOH-terminal domain as part of chimeric proteins. The above results lend further support to our model of p53 functional regulation by a distinct molecule bound at the p53 COOH terminus.

*p53(364–393) Inhibitory Effects Are Specific and Transferable to Other Proteins*—Our findings strongly implicated the last 30 residues of p53 in the inhibition of p73-mediated transactivation through a mechanism distinct from the allosteric model. However, it is also possible that the inhibitory effect rendered by the p53CT was due to an alteration of the p73 conformation caused by the domain swap rather than the inhibitory nature of the p53CT. To test this possibility, an additional chimera, as

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2 Z.-M. Yuan, unpublished observations.
shown in Fig. 4A, was created to determine whether the inhibitory effect of the p53CT was indeed specific. Fusion of the p53 sequence from outside of the COOH terminus (53aa291–319) to truncated p73(aa 1–391) had no inhibitory effect on the luciferase gene expression in H1299 cells (Fig. 4C).

To demonstrate that the inhibitory effect of p53CT is not restricted to p53 or p73 only, we introduced this sequence into p63γ, another structural homologue of p53 (Fig. 4A). Transcriptional activity of the p63(53aa364–393) chimeric protein in H1299 cells was reduced by approximately 70% compared with wild type p63 (Fig. 4D). In addition, we fused p53(aa 364–393), as well as the p53 sequence from outside the COOH terminus (aa 291–319), to Gal4-VP16 (Fig. 5A). The luciferase reporter that contained five Gal4-binding sites upstream of the SV40 promoter (pGL3-G5SV) was used to record transcriptional activity of these constructs in 293T cells. Gal4-VP16(53aa364–393) fusion had significantly lower transcriptional activity than either Gal4-VP16 alone or Gal4-VP16(53aa291–319) (Fig. 5C). Similar results were observed in H1299 cells. Taken together, these data clearly demonstrate that the last 30 amino acid residues of p53, in addition to repressing p53 functional activity, also potently and specifically inhibit p63, p73, and Gal4-VP16 transcriptional activity.

**DISCUSSION**

Induction of p53 often determines cellular fate and is therefore subject to complex and strict control through several pathways. p53 protein stability, for example, is tightly regulated via p53-Mdm2 autoregulatory negative feedback loop, where Mdm-2 acts as an E3 ubiquitin ligase and targets p53 for rapid proteosomal degradation (16). Restriction of p53 functional activity is yet another mechanism of controlling ilicit p53 signaling. The currently used allosteric model of p53 functional regulation stipulates that the extreme COOH-terminal region of p53(aa 364–393) is able to associate with the core domain of the protein, thereby blocking sequence-specific DNA binding sites and preventing p53 from exerting its transactivation effects (6). In this study, we provide first experimental evidence to support an alternative model of p53 functional latency, which might be preserved by binding of a distinct inhibitory factor at the extreme carboxyl terminus of p53.

Using chimeric constructs of p53 and either p63, p73, or Gal4-VP16, we demonstrate that the extreme COOH terminus of p53(aa 364–393) potently and specifically inhibits p73, p63, and Gal4-VP16 transcriptional activity. Since our own in vitro protein binding data presented herein, as well as published reports of other investigators (12), show that wild type p53 does not associate with p63, p73, or Gal4-VP16, allosteric model of regulation cannot explain the inhibitory effect of the p53 extreme COOH terminus. Therefore, it is plausible that a separate negative modulator, which is yet to be identified, could bind at the extreme COOH terminus of p53 and repress the transcriptional activity of chimeric proteins and, more importantly, preserve the latent status of endogenous p53 in the cell.

It was suggested that one mechanism by which p53CT can down-regulate transcription is through its association with basal transcriptional machinery. For instance, suppression of Gal4 DNA-binding domain basal transcriptional activity by the COOH terminus of p53 has been reported previously by Horikoshi et al. (17) in a finding that is consistent with our observations. Such inhibitory effect of p53CT was proposed to be due to its sequestration of the TATA-binding protein (TBP), which in turn supposedly leads to the disruption of transcription initiation complex. However, subsequent findings by Farmer et al. (18) showed that overexpression of TBP in the same experimental context fails to alleviate p53-mediated transcriptional repression. Therefore, there might be an alternative mechanism of transcriptional repression by p53CT. Results of our competition experiments support this notion and further exclude p53CT association with TBP as a single mode of transcriptional inhibition. Overexpression of p53(aa 364–393) as part of chimeric proteins along with wild type p53 results in the significant up-regulation of p53 transcriptional activity, contrary to the expected inhibition if only TBP were involved (presumably, overexpressed p53CT would bind TBP and abrogate transcription).

We suggest that an increase in the p53-driven transcription in the presence of chimeric constructs bearing p53(aa 364–393) could be explained by the competitive withdrawal of putative inhibitor(s) of p53 functional activity. The de-repression effect of p53CT-containing chimeras that we observed is similar to that previously described for p53 short COOH-terminal peptides. When microinjected into the nucleus, these peptides can dramatically increase p53 DNA binding and functional activity (19, 20). Selivanova et al. (20) detected direct interactions of the p53 COOH-terminal peptide with the DNA-binding domain of the protein in vitro, while others reported similar binding of COOH-terminal peptides to the full-length p53 (21). Therefore, it was argued that activation of p53 by the peptide is accomplished through competitive displacement of the native COOH terminus from the core domain and enhanced stability of the DNA-peptide-p53 complex. We, however, did not observe direct physical interactions of GST-p53(aa 364–393) with full-length p53 under our experimental conditions (data not shown). Moreover, it seems unlikely that in our competition experiments, large chimeric proteins bearing p53(aa 364–393) will bind to and remain associated with wild type p53, thus stabilizing p53-DNA complex, as proposed for COOH-terminal peptides. It seems more plausible that chimeras containing extreme carboxyl-terminal sequences of p53 will compete with full-length p53 for inhibitor binding thereby allowing de-repression of p53 functional activity.

Certainly in the absence of conclusive crystallographic data on the full-length p53 and its correct conformational folding, we cannot completely rule out the allosteric model of regulation. However, our results clearly demonstrate that p53 extreme carboxyl terminus inhibits p73-, p63-, and Gal4-VP16-driven transcription, and none of these findings can be explained by the allosteric model due to the well documented lack of physical interactions between p53 and p63, p73, or Gal4-VP16. Our study therefore opens the possibility of identifying distinct molecule(s) responsible for preserving p53 transcriptional latency via binding at its extreme COOH terminus. Interference with the activation of latent p53 is believed to contribute to the process of malignant transformation in some human tumor cells (22). Thus, further elucidation of mechanisms governing p53 repression and activation in the cell might prove useful in designing effective anti-cancer therapeutics.

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