Activation of p53 Function by Human Transcriptional Coactivator PC4: Role of Protein-Protein Interaction, DNA Bending, and Posttranslational Modifications

Kiran Batta and Tapas K. Kundu*

Transcription and Disease Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, P.O. Bangalore-560064, India

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Tumor suppressor p53 controls cell cycle checkpoints and apoptosis via the transactivation of several genes that are involved in these processes. The functions of p53 are regulated by a wide variety of proteins, which interact with it either directly or indirectly. The multifunctional human transcriptional coactivator PC4 interacts with p53 in vivo and in vitro and regulates its function. Here we report the molecular mechanisms of the PC4-mediated activation of p53 function. PC4 interacts with the DNA binding and C-terminal domains of p53 through its DNA binding domain, which is essential for the stimulation of p53 DNA binding. Remarkably, ligation-mediated circularization assays reveal that PC4 induces significant bending in the DNA double helix. Deletion mutants defective in DNA bending are found to be impaired in activating p53-mediated DNA binding and apoptosis. Furthermore, acetylation of PC4 enhances, while phosphorylation abolishes, its ability to bend DNA, activate p53 DNA binding, and, thereby, regulate p53 functions. In conclusion, PC4 activates p53 recruitment to p53-responsive promoters (Bax and p21) in vivo through its interaction with p53 and by providing bent substrate for p53 recruitment. These results elucidate the general molecular mechanisms of activation of p53 function, mediated by its coactivators.

p53, a well-known tumor suppressor, maintains genomic integrity and cellular homeostasis. The tumor suppressor function of p53 is a contribution of its ability to exert DNA repair, cell cycle arrest, and apoptosis (11, 21, 31). Under normal conditions, p53 is a short-lived protein and is maintained at very low levels; only upon genotoxic insult is p53 stabilized and activated by several factors. Therefore, the stabilization and activation of p53 play pivotal roles in modulating p53 function (2). The cellular functions of p53 depend on its protein levels (32), posttranslational modifications, and functional interacting partners (24). Posttranslational modifications differentially regulate p53 function, depending on the types and sites of modification. For example, phosphorylation of residue S15 in p53 releases MDM2 from p53 and enhances the interaction with acetyltransferases p300/CBP (39). p300/CBP-mediated acetylation of residue K373 in p53 leads to apoptosis, whereas acetylation of residue K320 by PCAF leads to cell cycle arrest (23). Acetylation of p53 is also reported to augment its DNA binding ability (14). While ubiquitination of p53 leads to its proteosomal degradation (7), methylation by Set9 restricts it to the nucleus and stabilizes the protein (8).

Several p53-interacting proteins regulate its stability and function either positively or negatively. MDM2, COP1, and PirH1 act as negative regulators of p53 by promoting its ubiquitination (10, 15, 25, 28, 34). Proteins p19ARF and HAUSP positively regulate p53 function by preventing MDM2-p53 inter-
gene Bax (4). However, the mechanism of PC4-mediated activation of p53 function remains elusive.

The present study investigated the molecular mechanisms of the PC4-mediated activation of p53 function. We have identified the p53-interacting region in PC4, which is important for the activation of p53 DNA binding. Posttranslational modifications of PC4, such as acetylation, induce its ability to activate p53 DNA binding, whereas phosphorylation dramatically abolishes the enhancement of p53 DNA binding. Interestingly, we have found a new activity of PC4, an intrinsic DNA-bending ability, which is also regulated by posttranslational modifications. The DNA-bending ability of PC4 significantly contributes to the activation of p53 recruitment to p53-responsive promoters in vivo. Presumably PC4, through its interaction with p53 and its DNA-bending ability, induces the recruitment of p53 to p53-responsive gene promoters and thereby activates the physiological functions of p53.

MATERIALS AND METHODS

Plasmid construction. Full-length recombinant PC4 and its truncated mutants PC4 1-40, PC4 1-62, PC4 1-67, PC4 1-72, PC4 1-77, PC4 1-82, PC4 1-87, PC4 22-127, PC4 40-127, and PC4 62-127 were constructed by PCR amplification of genes corresponding to amino acids from a PC4 full-length cDNA clone. The amplified products were cloned into the pET28b vector at the NcoI and XhoI genes corresponding to amino acids from a PC4 full-length cDNA clone. The interacting proteins were detected by immunoblotting with PC4 antibodies. α, anti. (C) Three nanograms of a γ-32P-labeled oligonucleotide containing a p53 binding site was incubated with 50 ng of p53 either in the absence of PC4 (lane 2) or with increasing concentrations of PC4 (lanes 3 and 4), PC4 1-62 (lanes 5 and 6), PC4 1-87 (lanes 7 and 8), PC4 22-127 (lanes 9 and 10), or PC4 62-127 (lanes 11 and 12). Lane 1 contains the γ-32P-labeled oligonucleotide alone. +, present; −, absent. (D) Quantitative representation of the effect of PC4 and its truncation mutants PC4 1-62, PC4 1-87, PC4 22-127, and PC4 62-127 on p53-mediated DNA binding. The levels of induction of p53-mediated binding are represented on the y axis, with increasing concentrations of PC4 and its truncation mutants on the x axis.

FIG. 1. PC4 domain involved in p53 interaction and activation of p53 DNA binding. (A) Schematic representation of PC4 truncation mutant constructs. Plus signs indicate strength of p53 interaction, and minus sign indicates no interaction. (B) GST-p53 (panel I), GST-p53 C-terminal domain (CTD) (panel II), GST-p53 DNA binding domain (DBD) (panel III), and GST (panel IV) proteins were incubated individually with PC4 or one of its truncation mutants, and the interacting proteins were detected by immunoblotting with PC4 antibodies. α, anti. (C) Three nanograms of a γ-32P-labeled oligonucleotide containing a p53 binding site was incubated with 50 ng of p53 either in the absence of PC4 (lane 2) or with increasing concentrations of PC4 (lanes 3 and 4), PC4 1-62 (lanes 5 and 6), PC4 1-87 (lanes 7 and 8), PC4 22-127 (lanes 9 and 10), or PC4 62-127 (lanes 11 and 12). Lane 1 contains the γ-32P-labeled oligonucleotide alone. +, present; −, absent. (D) Quantitative representation of the effect of PC4 and its truncation mutants PC4 1-62, PC4 1-87, PC4 22-127, and PC4 62-127 on p53-mediated DNA binding. The levels of induction of p53-mediated binding are represented on the y axis, with increasing concentrations of PC4 and its truncation mutants on the x axis.
the proteins as indicated in the figures for 30 min at 30°C. The samples were analyzed on 5% native PAGE gels containing 0.5× Tris-borate-EDTA (TBE) buffer and 0.05% NP-40 and electrophoresed at 200 V for 2 h at 4°C. The gels were dried, and the DNA-protein complexes were visualized with a phosphor-imager analyzer (Fuji) using Image Gauge software. The quantification of the level of induction in the formation of DNA-protein complexes was done as an average of the induction measured in three different independent experiments.

**Ligation-mediated circularization assay.** The ligation-mediated circularization assays were performed as described previously (40), with some minor modifications. A 1 nM concentration of γ-<sup>32</sup>P-labeled 123-bp DNA was incubated with the proteins indicated in Results in 30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM dithiothreitol, and 0.5 mM ATP (1× T4 DNA ligase buffer) in a reaction mixture volume of 10 μl on ice for 20 min. An amount of 0.05 U of T4 DNA ligase was then added, and the ligation reaction allowed to proceed for 30 min at 30°C, followed by heat inactivation at 65°C for 20 min. Some samples were treated with 50 U of exonuclease III for 30 min at 37°C. One microliter of stop solution (10% SDS, 10 μg/ml proteinase K) was added to all samples, followed by deproteination and precipitation of the DNA. The samples were loaded on 5% native PAGE (29:1 acrylamide:N,N’-methylene bisacrylamide) gels in 45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA, pH 8.3 to 8.4 (0.5× TBE buffer) and the gels were run for 2 h at room temperature. The gels were dried and analyzed with a phosphorimager (Fuji) using Image Gauge software.

**In vitro phosphorylation of PC4.** An amount of 1 μg of recombinant PC4 was incubated in phosphorylation buffer (50 mM HEPES-K<sup>+</sup> [pH 7.6], 125 mM NaCl, 10 mM MgCl₂, 6% [vol/vol] glycerol, 5 mM dithiothreitol, and 0.5 mM phenylmethylsulfon fluoride) containing 5 mM ATP with 40 mU of rat liver CKII in a total volume of 20 μl at 30°C. For complete phosphorylation, CKII and ATP were replenished three times in the reaction mixture, at 30-min intervals. Mock phosphorylation was performed in a similar way but in the absence of ATP. The reaction mixtures were then dialyzed against BC100 (20 mM Tris HCl, pH 8.3 to 8.4 (0.5× TBE buffer) and the gels were run for 2 h at 4°C. The gels were dried and analyzed with a phosphorimager (Fuji) using Image Gauge software.

**In vitro acetylation of PC4.** An amount of 1 μg of recombinant PC4 was incubated in a 30-μl final reaction mixture consisting of 50 mM Tris-HCl, pH 8.0, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfuoride, 0.1 mM EDTA, pH 8.0, 10 mM sodium butyrate, 0.5 μl 3.3 Ci/mmol [³H]acetyl-coenzyme A (acetyl-CoA), and p300 at 30°C. For complete acetylation, p300 and acetyl-CoA were replenished three times in the reaction mixture, at 30-min intervals. Mock acetylation was performed in a similar way but in the absence of ATP. The reaction mixtures were then dialyzed against BC100 (20 mM Tris HCl, pH 7.4), 20% glycerol, 0.2 mM EDTA, 0.1% NP-40, 100 mM KCl) and used for further experiments.

**Transfection assay.** The p53 null human lung carcinoma cell line H1299 was grown at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and appropriate antibiotics. Cells were seeded at 1 million cells per 60-mm dish in Dulbecco’s modified Eagle’s medium with antibiotics and supplemented with 10% fetal bovine serum 24 h before transfection. DNA was used for transfections in the amounts indicated in the figure legends, using Lipofectamine according to the manufacturer’s protocol. An equal amount of DNA was used in all transfections.

**Endogenous gene expression assay.** H1299 cells were transfected with PC4/PC4 mutants, p53, or both the mammalian expression constructs. After 24 h of transfection, total RNA was isolated from the transfected cells using Trizol reagent (Invitrogen) and cDNA was made using oligo(dT) (26-mer) and M-MLV reverse transcriptase (Invitrogen). This cDNA was used for real-time PCR analysis using iQ™ SYBR Green supermix (Bio-Rad) with gene-specific primers for Bax, p21, and Actin.

**ChIP.** H1299 cells transfected with PC4/PC4 mutants, p53, or both the mammalian expression constructs were used for the chromatin immunoprecipitation assay (ChIP) assay. The ChIP assay was performed as described previously (36). The pull-downs were done with p53 antibody or control immunoglobulin G antibody, and the immunoprecipitated samples were deproteinated, ethanol precipitated, and used for PCR analysis. Real-time PCR analysis was done using the primers specific to the p53-responsive site in the Bax promoter.

**Apoptosis assay.** H1299 cells were seeded at 0.6 million cells per 60-mm dish before 24 h of transfection. The amounts of DNA indicated in the figure legends were used for transfections, using Lipofectamine according to the manufacturer’s protocol. After 24 h of transfection, the cells were fixed with 4% formaldehyde and stained with Hoechst stain. The cells were visualized under a fluorescent microscope using a blind approach.

**RESULTS**

PC4-p53 interaction is essential for PC4-mediated activation of p53 binding to its cognate sites. The multifunctional transcriptional coactivator PC4 activates p53 function (4). Given that PC4 interacts with p53 in vitro and in vivo and that p53 can be regulated by its interacting proteins via different mechanisms, we investigated the functional significance of the PC4-p53 interaction in the activation of p53 function. In order to identify the domain of PC4 that interacts with p53, we generated a panel of PC4 truncation mutants (Fig. 1A) which were expressed, purified, and verified by immunoblotting with PC4 antibodies. These truncation mutant proteins were incubated with affinity-purified GST or GST-p53 proteins for the interaction studies. As shown in Fig. 1B (panels I and IV),
full-length PC4, PC4 1-87, and PC4 22-127 showed significant interaction with GST-p53, but not GST alone, whereas PC4 1-62 showed very weak binding and PC4 62-127 did not interact with GST-p53. Similar results were also found when pull-down assays were performed in the presence of DNase to exclude the possibility that the PC4-p53 interactions were merely via DNA (data not shown). PC4 interacts with p53 through its DNA binding domain and C-terminal domain. To further delineate the PC4 domain that interacts with the p53 DNA binding domain and C-terminal domain, pull-down assays were performed with GST-p53 DNA binding domain and C-terminal domain. The results (Fig. 1B, panels II and III) showed that the same domain of PC4 is required for its interaction with the DNA binding domain and the C-terminal domain of p53.

In order to verify the functional correlation of PC4-p53 interaction and the activation of p53 function, we examined the effects of PC4 truncation mutants on p53 DNA binding. EMSAs were performed with a 40-bp GADD45 promoter containing p53-responsive elements. FLAG-p53, expressed and purified in bacteria, formed a sequence-specific complex, and as expected, the addition of full-length PC4 enhanced p53-mediated DNA binding in a dose-dependent manner (Fig. 1C, lane 2 versus lanes 3 and 4). PC4 1-67, PC4 1-72, PC4 1-77, or PC4 1-82, and the interactions were monitored by immunoblotting with PC4 antibodies. 

Effects of PC4 truncation mutants on p53 DNA binding, EMSAs were carried out with increasing concentrations of PC4 or its truncation mutants in the presence of p53, and the induction of p53 DNA binding (n-fold) was plotted (Fig. 1D). The results showed that at a given concentration (0.54 μM), PC4 1-62 and PC4 62-127 were able to enhance p53 DNA binding 2-fold, whereas PC4, PC4 1-87, and PC4 22-127 enhanced p53 DNA binding 8-, 11.5-, and 10-fold, respectively.

The above results suggest that amino acids 62 to 87 of PC4 that are involved in the PC4-p53 interaction could be responsible for the activation of p53 DNA binding. To further substantiate this, an internal deletion mutant (PC4 62-87) was made (Fig. 2A). As expected, PC4 62-87 showed a weak interaction with p53 (Fig. 2B, lane 1 versus lane 2), and consequently, no significant increase in p53 DNA binding was detected (Fig. 2C, lane 2 versus lanes 3 to 5 and 6 to 8). Based on these data, a series of truncation mutants, PC4 1-67, PC4 1-72, PC4 1-77, and PC4 1-82 (Fig. 3A), covering the region of amino acid residues 62 to 87 were constructed to find out the precise amino acid stretch responsible for enhancing p53 DNA binding. These truncation mutants were expressed and purified using affinity chromatography and confirmed by immunoblotting with PC4 antibodies. Pull-down assays revealed that PC4 1-67 interacts with p53 very weakly, but the other truncation mutants showed similar strengths of interaction with p53 (Fig. 3B, panel I). Consistent with the interaction data, DNA binding assays with these truncation mutants also revealed that PC4 1-67 could enhance p53 DNA binding only very weakly (Fig. 3C, lane 2 versus lanes 3 and 4, 7 and 8, 9 and 10, and 11 and 12). However, at a higher concentration, PC4 62-127 activated p53 DNA binding (data not shown). In order to quantify the effects of PC4 truncation mutants on p53 DNA binding, EMSAs were carried out with increasing concentrations of PC4 or its truncation mutants in the presence of p53, and the induction of p53 DNA binding (n-fold) was plotted (Fig. 1D). The results showed that at a given concentration (0.54 μM), PC4 1-62 and PC4 62-127 were able to enhance p53 DNA binding 2-fold, whereas PC4, PC4 1-87, and PC4 22-127 enhanced p53 DNA binding 8-, 11.5-, and 10-fold, respectively.
tants PC4 1-67, PC4 1-72, PC4 1-77, and PC4 1-82 on p53-mediated DNA binding are represented in Fig. 3D. The results showed that at a concentration of 0.36 μM, PC4 1-67 could activate p53 binding 1.5-fold, whereas the other truncation mutants, PC4 1-72, PC4 1-77, and PC4 1-82, could enhance p53 binding 3- to 4-fold, suggesting that amino acids 68 to 72 are important for this function (Fig. 4A). To further elucidate the amino acid residues responsible for the activation of p53 DNA binding, K68 and M69 of PC4 were mutated to alanine (Fig. 4B), and the results showed that these point mutations in PC4 affect neither its binding with p53 (Fig. 4C, panel I) nor its ability to activate p53 function (Fig. 4E, lane 2 versus lanes 3 and 4, and lanes 9 and 10). However, PC4 R70AY71A, where R70 and Y71 of PC4 were mutated to alanine (Fig. 4B), showed a dramatic reduction in p53 interaction (Fig. 4C, panel I) and was not potent enough to activate p53 DNA binding, suggesting the importance of these amino acids in the PC4-mediated activation of p53 function (Fig. 4E, lane 2 versus lanes 3 and 4 and lanes 11 and 12). Further evidence that the PC4-p53 interaction is responsible for the PC4-mediated activation of p53 DNA binding comes from the PC4 truncation mutants PC4 1-40 and PC4 40-127 (Fig. 4B). PC4 1-40 did not show any interaction with p53 (Fig. 4D, panel I) and also could not enhance p53-mediated DNA binding (Fig. 4E, lane 2 versus lanes 3 and 4 and lanes 5 and 6), whereas PC4 40-127 interacted with p53 (Fig. 4D, panel I) and activated p53 DNA binding similarly to native PC4 (Fig. 4E, lane 2 versus lanes 3 and 4 and lanes 7 and 8). In the absence of p53, none of the truncation mutants of PC4 formed any sequence-specific DNA-protein complex (data not shown).

Glutaraldehyde cross-linking experiments and the crystal structure of the PC4 C-terminal domain gave us a clue that PC4 may exist as a dimer and a tetramer in vitro (data not shown). Furthermore, the results of immuno-pull-down of FLAG-PC4 from the transfected whole-cell lysates revealed that PC4 exists as a multimer in vivo (data not shown). Taken together, these results suggest that possibly a dimer of PC4 interacts with the DNA binding domain and C-terminal domain of p53 through the stretch of amino acid residues 62 to 87, and the interaction is essential to enhance the DNA binding of p53. Presumably, a dimer of PC4 interacts with a monomer of p53, forming a complex containing eight molecules of PC4 with a tetramer of p53.

Posttranslational modifications of PC4 regulate its ability to activate p53 binding to its cognate sites. The transcriptional coactivator PC4 undergoes posttranslational modifications like acetylation and phosphorylation (13, 26). Phosphorylation of PC4 abolishes its coactivator function, its ability to interact with dsDNA and different transcription factors, whereas acetylation is known to enhance its dsDNA binding ability (13, 26). In order to examine the effects of posttranslational modifications of PC4 on its ability to activate p53 function, PC4 was phosphorylated and acetylated by CKII and p300, respectively, in vitro. Upon complete phosphorylation, PC4 showed distinctly slower mobility than native PC4 on a 15% SDS-PAGE gel (Fig. 5A, panel I). The level of acetylation was determined...
Phosphorylation of PC4 abolishes its ability to activate p53 function. (A) PC4 was phosphorylated in vitro by CKII as described in Materials and Methods, and the native PC4 and phosphorylated PC4 were analyzed on a 15% SDS-PAGE gel (panel I). GST-p53 protein was incubated with PC4 or phosphorylated PC4 (panel II), and the interacting proteins were monitored by immunoblotting with PC4 antibodies (panel III). α, anti. (B) PC4 was phosphorylated in vitro by CKII, using ATP as the substrate, and mock phosphorylation was done in the absence of ATP. EMSA was performed with 50 ng of p53 either in the absence of PC4 (lane 2) or with increasing concentrations of PC4 (lanes 3 to 5), mock-phosphorylated PC4 (lanes 6 to 8), or phosphorylated PC4 (lanes 9 to 11). +, present; −, absent. (C) The levels of induction of p53-mediated binding are represented on the y axis, with increasing concentrations of PC4 or its phosphorylated form on the x axis. P, phosphorylated.

PC4 is a DNA-bending protein. p53 binds more efficiently to the bent DNA surface than to linear DNA (30, 33). Our data suggest that the protein-protein interaction may not be the only molecular event responsible for the activation of p53 DNA binding by PC4. We presumed that PC4 may also induce DNA bending to perform this function. The effect of PC4 on DNA structure was addressed by performing ligation-mediated circularization assays, which have been extensively used to determine the DNA-bending ability of proteins. This assay works on the principle that any dsDNA of less than 150 bp in length will not circularize itself in the presence of ligase because of inherent limitations in the flexibility of DNA. Therefore, circularization is seen only in the presence of a DNA-bending protein. In order to verify the DNA-bending properties of PC4, we have performed these assays with a 123-bp γ-32P-labeled oligonucleotide. A 1 nM concentration of the oligonucleotide was incubated with different concentrations of PC4 in ice, and the ligation reactions were performed by the addition of T4 DNA ligase. After the ligation reaction, exonuclease III treatment was done to verify the circularization of the DNA (Fig. 7A, lane 8). As shown in Fig. 7A, increasing the concentration of PC4 could result in the formation of circle monomer and circle dimer in a dose-dependent manner, whereas ligase alone could not form any circles (lane...
2 versus lanes 3 to 8). To further analyze the domain of PC4 responsible for DNA bending, we performed ligation-mediated circularization assays with PC4 truncation mutants, and the results showed that, except for PC4 1-62, all of the truncation mutants could efficiently bend the DNA (Fig. 7B, lanes 6, 10, 14, and 18). Significantly, PC4 62-127, which did not interact with p53 but could activate p53 DNA binding at higher concentrations, efficiently bent the DNA. However, PC4/62-87, which showed weak interaction with p53, could not bend the DNA (Fig. 7C, lane 6). Taken together, these results elucidate the intrinsic DNA-bending property of PC4 which is one of the important functional components in its enhancement of p53 function.

Posttranslational modifications of PC4 regulate its DNA-bending ability. Acetylation of PC4 enhances its ability to activate p53 function; however, acetylation of PC4 does not alter its ability to interact with p53. Presumably, this posttranslational modification might have a different mechanism to activate p53 function, which could be the regulation of PC4-mediated DNA bending. To address this possibility, we carried out ligation-mediated circularization assays with in vitro-acetylated PC4. The results showed that, indeed, acetylated PC4 could bend DNA and form circles in the presence of ligase much more efficiently than the unmodified form (Fig. 8A, lanes 3 to 6 versus lanes 7 to 10). In contrast, in vitro-phosphorylated PC4 could not bend DNA, while unmodified PC4 could efficiently bend the DNA (Fig. 8B, lanes 3 to 6 versus lanes 7 to 10). These data clearly establish the role of PC4 acetylation as an inducing factor for DNA bending and, thereby, the activation of p53 function.

PC4, through its interaction with p53 and by its inherent DNA-bending ability, activates p53 function. Promoter-specific DNA binding by p53 is important for p53-mediated transcriptional regulation, as well as its tumor suppressor function. PC4 activates p53 DNA binding and, thereby, its transcriptional regulatory functions. In order to investigate the in vivo mechanisms of PC4-mediated activation of p53 recruitment, p53-induced gene expression, and p53 downstream cellular processes like apoptosis, we generated several mammalian expression clones of PC4 mutants. To further investigate the effects of PC4 and its mutants in the recruitment of p53 to the p53-responsive site in the Bax promoter in vivo, ChIP assays...
were performed with H1299 cells transfected with p53 and PC4/PC4 mutant mammalian constructs. Real-time PCR analysis was performed with primers specific to the p53-responsive site in the \textit{Bax} promoter. The results showed that the recruitment of p53 to the \textit{Bax} promoter is pronounced in the presence of PC4 compared to recruitment with p53 alone (Fig. 9A and B). PC4 1-87, PC4 22-127, and PC4 62-127 could activate p53 recruitment minimally and PC4 1-40 could not activate p53 recruitment (Fig. 9A and B). The effects of PC4 truncation mutants on p53-mediated transactivation were observed by performing transient transfections of PG13 Luc (contains 13 p53-responsive sites), p53, and PC4 or its truncation mutants. The results showed that, in correlation with the ChIP data, the PC4 truncation mutants PC4 1-62, PC4 1-87, PC4 22-127, and PC4 62-127 could activate p53 transactivation, with distinct abilities (data not shown). Similar results were also seen when transfections were done with an MDM2 Luc construct (data not shown). In order to find out the correlation between p53 recruitment and p53-responsive gene expression, H1299 cells were transfected with p53 in combination with either PC4 or its mutants. Real-time PCR analysis showed high levels of \textit{Bax} mRNA and \textit{p21} mRNA when p53 was cotransfected with PC4, PC4 1-87, PC4 22-127, PC4 40-127, or PC4 R70AY71A in comparison to the levels with p53 transfection alone (Fig. 9C and D and data not shown). To investigate the downstream effect of p53-responsive proapoptotic gene expression, in a separate set of similar experiments, cells were stained with Hoechst stain to visualize the apoptotic nuclei and the percent apoptotic cells was quantitated as described in Materials and Methods. Consistent with the results of the real-time PCR analysis of \textit{Bax} gene expression, full-length PC4 could enhance p53-mediated apoptosis. Interestingly, PC4 1-87, PC4 22-127, and PC4 62-127 could also activate p53-mediated apoptosis at levels similar to the level with full-length PC4 (Fig. 9E and F). However, PC4 1-62 could activate p53-mediated \textit{Bax} gene expression and apoptosis only very minimally. In conclusion, the results suggest that PC4 activates p53 recruitment to the p53-responsive promoter through its interaction with p53 and by providing bent substrate for p53 recruitment (Fig. 10). The activation mechanism is further regulated by posttranslational modifications of PC4, as well as of p53.

**DISCUSSION**

The tumor suppressor p53 regulates the expression of several genes involved in various cellular processes (11, 21, 31). The activity of p53 is in turn regulated by several factors. A few transcriptional coactivators play significant roles in p53 function via regulating its stability and transcriptional activity (2). The highly abundant human transcriptional coactivator PC4 activates p53 function by enhancing p53 DNA binding and, thereby, p53-responsive gene expression (4). Here we have provided biochemical and cellular evidence revealing the molecular mechanisms of the PC4-mediated activation of p53 function. Our data suggest that three different molecular mechanisms, namely, protein-protein (p53-PC4) interaction, alteration of the secondary structure of DNA by PC4, and posttranslational modifications (acetylation/phosphorylation) of PC4, are involved in the activation of p53 function.
PC4 interacts with p53 at two distinct domains of p53, the DNA binding domain and the highly flexible C-terminal regulatory domain. Surprisingly, we have found that the same region in the DNA binding domain of PC4 mediates both the interactions. Presumably, a multimeric form of PC4 (mostly homodimer) could be interacting with p53. The detailed analysis of several truncation mutants of PC4 identified amino acids 68 to 72 in PC4 as being responsible for the PC4-p53 interaction (Fig. 3B). Recent analysis of the cocrystal structure of PC4 with 25-nucleotide single-strand DNA showed that this region is present in a loop-β sheet structure of the protein (42). Interestingly, we found that PC4 62-127 does not interact with p53 despite the presence of a p53-interacting region in it. Presumably, the deletion of the N-terminal region could be inhibiting the formation of the secondary structure that is responsible for p53 interaction (Fig. 1B, panel I). This assumption is supported by our observation that phosphorylation of PC4 abolishes the PC4-p53 interaction completely. PC4 is phosphorylated by CKII on at least 8 serine residues in the N-terminal region, which presumably alters its structure (19). Although the phosphorylation sites are not in the near vicinity of the p53 interaction region, a structural alteration in PC4 upon phosphorylation could be acting as an inhibitory factor for the PC4-p53 interaction. PC4 gets acetylated by p300 in vitro and in vivo (26; K. Batta and T. K. Kundu, unpublished data). Acetylation of PC4 does not alter the PC4-p53 interaction but could induce the DNA binding ability of p53, suggesting an alternate mechanism for the PC4-mediated activation of p53 function, such as a PC4-induced alteration in DNA conformation. The identification of PC4 as a DNA-bending protein is a significant finding of this study. The more-efficient binding of p53 to circular DNA than to linear DNA suggests a role for PC4-mediated DNA bending in the enhanced DNA binding of p53. As reported previously, HMGB1 also induces p53 DNA binding by providing a bent surface for p53 recruitment (30). DNA binding is known to be associated with transcriptional activation, and p53 requires a bent DNA substrate for its sequence-specific DNA binding (33, 43). The fact that PC4 is a coactivator, DNA binding protein, as well as a p53-interacting protein, indicates that PC4 binds and bends the p53-responsive site, thereby allowing it to form a configuration which shows high affinity for p53. The bending ability of PC4 is further potentiated by p300-mediated acetylation. Mechanistically, this could be due to the higher affinity of acetylated PC4 for dsDNA (26). More-efficient bending by acetylated PC4 leads to the enhanced binding affinity of p53 for its consensus sequence. Our study indicates that posttranslational modifications of the transcriptional coactivators also play a role in the activation of p53 function.

The significant implications of these in vitro interaction and DNA binding studies relate to the importance of transcriptional activation in the p53 response. The ChIP assays and real-time PCR analyses showed the in vivo recruitment of p53 to the Bax promoter and Bax mRNA levels in the presence of various PC4 mutant constructs and p53. The results revealed the importance of interaction (PC4 1-62) and DNA bending (PC4 62-127) in the PC4-mediated activation of p53 function. Although PC4 1-62 could not bend the DNA, it activated p53-mediated gene expression with much lower efficiency than the wild-type PC4 through its weak interaction with p53. This weak interaction of PC4 1-62 and p53 could be further assisted in vivo by endogenous PC4. It has been reported that inter- or intramolecular interaction of the PC4 N-terminal domain with the PC4 C-terminal domain regulates the interaction of PC4 with the transcriptional activator. Recent nuclear magnetic resonance studies mapped the site of PC4 interaction with VP16 to the C-terminal domain of PC4, but the N-terminal domain of PC4 assists and enhances the interaction of the C-terminal domain with VP16 (18). PC4 is a highly abundant nuclear protein (>10⁶ molecules per cell), so the overexpressed PC4 1-62 could interact with endogenous PC4 and enhance its ability to interact with p53, thereby bringing about the transcriptional activation mediated by p53. PC4 62-127,
which did not show any interaction with p53, activated p53-mediated gene expression, presumably by providing a bent substrate for p53 recruitment. Homodimerization of PC4 is linked to transcriptional activation. Overexpressed PC4 62-127 can dimerize with endogenous PC4 and, thus, can bring about the activation of p53 function. Similarly, although PC4 R70AY71A showed very weak interaction with p53, it activated p53 function significantly, possibly through its dimerization and DNA-bending abilities (data not shown).

However, PC4 is a very small multifunctional protein (127 amino acids) with several overlapping functional domains, so naturally if any of the mutants shares (partially/completely) any one of these functions, that could result in the activation of p53 function. Similarly, although PC4 R70AY71A showed very weak interaction with p53, it activated p53 function significantly, possibly through its dimerization and DNA-bending abilities (data not shown).

It would be interesting to find out the specificity of PC4-mediated coactivation in different cell types and classes of promoter. p53, a sequence-specific transcriptional activator (quite often acting as a repressor) binds to the cognate sites and recruits histone acetyltransferases p300/CREB. Possibly through protein-protein interaction, PC4 is also recruited to the cognate sites and is acetylated by p300/CREB. The enhanced binding of p53 in the presence of acetylated PC4 could lead to the activation of downstream processes, such as apoptosis. Therefore, the PC4-mediated activation of p53 function may be responsible for global regulation of cellular function.
Several reports postulate PC4 as a putative tumor suppressor, and our results strengthen this concept. According to the results of our study, PC4 gene expression is also regulated on AP-2 transcriptional interference. Mol. Cell. Biol. 27:513–523.

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