Phosphorylation of p53 Serine 15 Increases Interaction with CBP*

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p53 exerts its cell cycle regulatory effects through its ability to function as a sequence-specific DNA binding transcription factor. CREB-binding protein (CBP)/p300, through its interaction with the N terminus of p53, acts as a coactivator for p53 and increases the sequence-specific DNA-binding activity of p53 by acetylating its C terminus. The same N-terminal domain of p53 has recently been shown to be phosphorylated at Ser15 in response to γ-irradiation. Remarkably, we now demonstrate that phosphorylation of p53 at Ser15 increases its ability to recruit CBP/p300. The increase in CBP/p300 binding was followed by an increase in the overall level of acetylation of the C terminus of p53. These results provide a mechanism for the activation of p53-regulated genes following DNA damage, through a signaling pathway linking p53 N-terminal kinase and C-terminal acetyltransferase activities.

The N-terminal region interacts with a number of proteins, including MDM2, TBP, dTAF10, and hTAF10. hTAF10, CBP1, the p62 subunit of TFIIH, and the endogenous E1B 55-kDa protein (12). It also contains a threonine/serine/threonine/threonine (13, 14), DNA-dependent protein kinase (DNA-PK) (15), and c-Jun N-terminal kinase (16). Recently, the cyclin-dependent kinase-activating kinase, which is a cyclin-dependent kinase 7/cyclinH/p36MAT1 complex, was also shown to phosphorylate human p53 (17). Importantly, Ser15 has been shown to be phosphorylated in vitro in response to ionizing radiation (18, 19) and is hyperphosphorylated in human T-cell lymphotropic virus I-transformed cells (20). Phosphorylation of Ser15 has been shown to inhibit binding of TFII D in vitro (20), and Ser15/Thr18 phosphorylation has been shown to correlate inversely with MDM2 binding (19).

The coactivator proteins CBP and p300 mediate transcriptional activation through a number of transcription activators, including CREB, NF-κB, c-Myb, and nuclear hormone receptors (21–25). Recently, p300 and CBP have also been shown to associate with p53 in vitro and in vivo and to cooperate with p53 in transactivation of a cotransfected reporter plasmid or the endogenous p21 gene. The interaction of the N terminus of p53 with the C-terminal region of CBP is thought to be important for this cooperative activity (9, 10, 26, 27). Both p300 and CBP have histone acetyltransferase activity (28, 29). Significantly, the acetyltransferase activity of p300 has been shown to recognize the C-terminal region of p53 as a substrate, and the acetylation of this regulatory region by p300 increases the sequence-specific binding of p53 in vitro (30).

We present evidence that phosphorylation of p53 in its N-terminal domain increases the association of p53 and CBP/p300 in vitro and that there is a corresponding increase in the acetylation of p53. Ser15 phosphorylation appears to be critical for the interaction of p53 and CBP/p300. These results provide a possible mechanism linking phosphorylation and acetylation signals in the amplification of the p53 response to ionizing radiation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Protein Preparations—**For oligonucleotide-mediated, site-directed mutagenesis of p53, the full-length sequence was subcloned into pBluescript SK(−), and single-stranded DNA was prepared using M13K07 helper phage. Mutations were introduced using the Amersham Pharmacia Biotech Sculptor kit according to the manufacturer's instructions. The mutated p53 sequences were then cloned back into either pOEX-2T or pOEX-6P-1 (Amersham Pharmacia Biotech). The fusion proteins were prepared from Escherichia coli HB101 grown to an A600 of approximately 0.5, induced for 4 h at 37 °C with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (200–ml culture). The bacteria were pelleted, resuspended in ice-cold phosphate-buffered saline/1% Triton X-100, and lysed by sonication. After removal of the debris by centrifugation, the fusion protein was adsorbed onto glutathione-Sepharose beads (200 μl of a 50% slurry), and the beads were washed three times with the cold lysis buffer and resuspended in the same buffer to give a 50% slurry. For most purposes, the proteins were used while still adsorbed to the glutathione-Sepharose beads. Free p53 was obtained by preparing the fusion protein from the pOEX-6P-1 vector, as above, and then cleaving from the GST moiety with PreScission Protease (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Phosphorylation by DNA-PK (Promega) was carried out on small (2.5 μl) samples of GST-p53 on beads, by adding 19 μl of reaction mix composed according to the manufacturer's instructions. Specifically, the reaction mixture included 11 μl of sample buffer (25 mM HEPES-KOH, pH 7.5, 12.5 mM MgCl2, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 50 mM KCl), 2 μl of reaction premix (15 mM spermidine, 4 mM MgCl2), 4 μl 0.1 mM ATP, and 2 μl 100 μg/ml double-stranded DNA (plasmid DNA cut into fragments with HaeIII, extracted with phenol/CHCl3, and ethanol-precipitated). 20 units DNA-PK was added, and incubation was carried out for 1 h at 30 °C. Following this, a further 20 units of enzyme was added and the incubation was continued for the...
same duration. Control samples were treated with reaction buffer alone. Where labeling of the p53 was required, 5 μCi of [γ-32P]ATP was included in the reaction mix, and only a single 30-min incubation was carried out. Following phosphorylation, the beads were washed three times with 1 ml of CBP binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 20% glycerol, 100 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40 (9)). As much supernatant as possible was removed, and 90 μl of CBP binding buffer, composed as above but with 60 mM KCl, was added. CBP/p300 Binding and p53 Acetylation Assays—HeLa whole cell extract was prepared by the method of Manley et al. (31). Whole cell extract was chromatographed over a phosphocellulose column, and the 0.3 and 0.5 mM KCl fractions, which were enriched for CBP/p300, were concentrated by spin dialysis and used for CBP binding studies. Ten microliters of the phosphocellulose fraction (typically a total of 5–20 μg of protein) or a control aliquot of extract buffer was added to the GST-p53 samples in CBP binding buffer described above. For CBP/p300 binding assays, the samples were then rocked at 4°C overnight before being washed three times with 100 mM KCl CBP binding buffer. The pellets were then taken up in SDS loading buffer, resolved on 4–20% SDS-PAGE gels, and electroblotted onto Immobilon membranes. Immunoblotting was carried out with antibodies to CBP (kindly provided by Dr. R. Goodman), p300 (Oncogene Science), or p53 (pAb421, Oncogene Science), and detection was achieved by ECL (Amersham Pharmacia Biotech).

Acetylation assays were carried out using GSTp53 on glutathione-Sepharose by binding CBP/p300 as above, but for only 2 h. Sodium butyrate was added to 10 mM along with 0.05–0.15 μM of [1-14C]acetyl-CoA (Amersham Pharmacia Biotech), and samples were rocked at 30°C for 4 h. The pellets were washed three times with phosphate-buffered saline/1% Triton X-100 and analyzed by SDS-PAGE as described above. For the acetylation of p53 in solution, 1 μg of the protein was first treated with DNA-PK in 30 μl of the kinase buffer (volumes were increased proportionally). CBP binding buffer (65 μl, 60 mM KCl) incorporating wortmannin (5 μM), sodium butyrate (10 mM), and 0.05–0.15 μM of [1-14C]acetyl-CoA was then added, followed by 5 μl of the 0.5 mM phosphocellulose fraction, and the reactions were allowed to proceed at 30°C for 4 h. Radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) (450 μl) was added, and the p53 was immunoprecipitated with 1 μg each of the antibodies DO-1 and pAb1801 (Oncogene Science) and protein A/G-Sepharose. The acetylated proteins were analyzed by SDS-PAGE and autoradiography on phosphorimager screens.

Preparation of Purified CBP/p300 Fraction—CBP/p300 was highly purified by a procedure based on that of Shiekhattar et al. (32). Approximately 3 g of crude HeLa nuclear extract was fractionated on a P11 phosphocellulose column (1-liter column volume) with a KCl gradient in a buffer containing 20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. A fraction (400 mg) that eluted at 0.3 μM KCl was loaded onto a DEAE-Sephacel column (100 ml column volume); material eluted with 0.5 μM KCl (280 mg) was further fractionated on Q-Sepharose (100 ml column volume) with a linear gradient of 100 to 600 mM KCl. Those fractions (57 mg) containing CBP/p300 (at 200 mM KCl) were dialyzed to 10 mM potassium phosphate in 5 mM HEPES, pH 7.5, and loaded onto a hydroxypatite column (20 ml column volume), which was eluted with a gradient of 10 to 500 mM potassium phosphate. The CBP eluted at 400 mM potassium phosphate. The Q-Sepharose and hydroxypatite columns were resolved with 10 column volumes of buffer.

RESULTS

Phosphorylation of p53 by DNA-PK Increases CBP/p300 Binding—Phosphorylation of p53 at Ser15 and Ser37 regulates the interaction of p53 with TFIID and MDM2 (19, 20). Because the N terminus of p53 interacts with the coactivator CBP/p300, we were interested to see what effect phosphorylation might have on this association. DNA-PK, which phosphorylates p53 on Ser15 and Ser37, was used to introduce phosphate groups on these amino acids. Wild-type and mutant p53 proteins were expressed in E. coli as 80-kDa GST fusion proteins (Fig. 1A). The proteins were purified as described under “Experimental Procedures” and checked for purity on an SDS-PAGE gel (Fig. 1B). CBP/p300 was partially purified from HeLa whole cell extracts by chromatography over a phosphocellulose column. GST-p53 or GST protein on glutathione-Sepharose was treated with DNA-PK or mock-treated with buffer alone, washed, and then incubated with a phosphocellulose fraction containing partially purified CBP/p300. DNA-PK treatment caused a 5–8-fold increase in the binding of CBP to GST-p53, as judged by Western blot analysis with anti-CBP antibody (Fig. 2A, lanes 2 and 4). No association of control GST with control GST was seen either with or without DNA-PK treatment (lanes 5 and 7). The difference in the Western blot pattern for the input material and the GST-p53-bound CBP is likely in part due to the selection of full-length CBP, rather than breakdown products, in the binding reaction. There is also a decrease in the mobility of the major CBP band following binding. The reason for the change in migration is currently under investigation.

An important point in the execution of these experiments was that the cell fraction used contained sufficient levels of CBP/p300 to allow the increased recruitment by phosphorylated GST-p53. In experiments in which levels of CBP/p300 were low, or in which too much GST-p53 was added to the assay, the level of binding to unphosphorylated p53 was enough to deplete the reaction mixture of CBP/p300, making it impossible for any increase in overall binding to the phosphorylated protein to occur. In the experiments that gave the clearest results, at least 10–20% of the input CBP remained in the supernatant of the binding reactions containing phosphorylated p53.

p53 Ser15 Is Important for Interaction with CBP—Varying combinations of phosphorylation on Ser15 and Ser37 have distinct effects with respect to different protein interactions (19, 20). In this study, the serine residues phosphorylated by DNA-PK are indicated. B, Coomassie-stained SDS-PAGE showing wild-type (wt), S15A, S37A, S1537A, and 1–300 GST-p53s.
Phosphorylation of p53 at Serine 15 and 37 increases its ability to bind CBP. GST-p53 or GST bound to glutathione-Sepharose beads was treated with DNA-PK, washed thoroughly, and then incubated with a HeLa cell fraction containing high levels of CBP/p300. Samples of the supernatant were taken to examine the free (B) fraction of CBP, after washing, the beads were taken up in SDS loading buffer, and the bound (F) fraction was eluted. The samples were run on a 4–20% gel in SDS buffer and transferred to Immobilon membranes before being probed with an antibody to CBP.

DNA-PK failed to increase CBP binding significantly (Fig. 2B, lanes 6 and 8). Mutation of Ser37 had little effect on the ability of DNA-PK treatment to increase binding of CBP (Fig. 2B, lanes 10 and 12). We also show in this experiment that CBP binding to a GST-p53 deletion mutant, GST-p53 1–300 (Fig. 1A), which lacks the C-terminal 93 amino acids, is induced approximately 5-fold by DNA-PK treatment (Fig. 2B, lanes 14 and 16). In contrast to the results seen with DNA-PK, casein kinase I and c-Jun N-terminal kinase had no effect on the binding of CBP to p53 (data not shown). These results indicate that although Ser37 phosphorylation may be capable of making some contribution to CBP binding, as evident from the slight increase in binding on phosphorylation of the S15A mutant, the phosphorylation of Ser15 alone is sufficient to account for the levels of inducible binding seen with the wild-type p53. Stripping of the blot and reprobing with an antibody to p300 revealed a binding pattern identical to that of CBP (data not shown).

It was conceivable that the increased CBP binding was due to phosphorylation of GST-p53 at sites other than Ser15 and Ser37. To examine this possibility, we analyzed the phosphorylation of GST-p53 mutants containing serine to alanine substitutions at amino acids 15, 37, or 15 and 37. As seen in Fig. 3A, GST-p53 wild-type (Fig. 3, lane 1), GST-p53 S15A (lane 2), and GST-p53 S37A (lane 3), but did not significantly phosphorylate GST-p53 S15A/S37A or the GST backbone protein (lanes 4 and 5). This experiment demonstrated that phosphorylation of the GST-p53 protein by DNA-PK is confined to Ser15 and Ser37.

Association of CBP with Phospho-p53 Increases p53 Acetylation—It has previously been demonstrated that p300 acetylates the C terminus of p53. Given the increased recruitment of CBP/p300 to phosphorylated p53, it was of interest to determine whether the interaction stimulated the acetylation of p53. Following binding of CBP/p300 to GST-p53, [1-14C]acetyl-CoA was added to the mixture, which was then incubated for 4 h at 30 °C. In earlier experiments, excess CBP/p300 was washed out before acetyl-CoA addition, but in subsequent reactions, these washes were omitted in an attempt to more nearly mimic the conditions in the cell nucleus. We observed little or no difference in the results obtained with these two methods, indicating that CBP/p300 stably bound to the GST-p53 was likely responsible for most, if not all, of the acetylation. Consistent with the increase in p53-CBP/p300 binding observed above, a 7-fold increase in the acetylation of p53 was observed in the DNA-PK-treated p53 sample, relative to a mock-treated sample (Fig. 4A, lanes 1 and 2). No acetylation of the GST control protein was observed (Fig. 4A, lanes 3 and 4), demonstrating the specificity of the reaction for p53. Importantly, when Ser15 was mutated to alanine, the increase in p53 acetylation was strongly diminished (Fig. 4B, lanes 3 and 4). In contrast, when Ser37 was mutated to alanine, there was no significant decrease in p53 acetylation (Fig. 4B, lanes 5 and 6). To demonstrate the specificity of the p53 acetylation for the p53 C terminus, we utilized the C-terminal deletion mutant GST-p53 1–300. As shown in Fig. 4B, lanes 7 and 8, there was no significant acetylation of this protein despite the fact that the p53-CBP complex is formed (Fig. 2B, lane 16). The increase in acetylation was abolished by the omission of ATP from the kinase reaction and was inhibited by the inclusion of wortmannin, an inhibitor of DNA-PK (data not shown). In addition, no
Acetylation was seen if the CBP/p300-containing fraction was omitted from the acetylation reaction (data not shown). Similar to the results for CBP binding, the ratio of overall acetylation of the phosphorylated to the unphosphorylated form of p53 depended on the level of CBP in the cell fraction used; those batches with higher levels showed greater proportional increases.

It was of importance to demonstrate that the enhanced acetylation of p53 was not due to the presence of the GST moiety. Wild-type p53 protein was released from the GST fusion protein as described under “Experimental Procedures” and treated with DNA-PK. Phosphorylated p53 was then incubated with CBP/p300, and the acetylation reaction was carried out as described above. Wortmannin was included in the acetylation reaction to ensure that the DNA-PK did not phosphorylate proteins in the CBP/p300 extract. Following incubation, Radioimmune precipitation buffer was added, the p53 protein was immunoprecipitated, and the resulting material was subjected to SDS-PAGE and autoradiographed. DNA-PK treatment led to a 6-fold increase in the level of p53 acetylation, similar to that seen with the GST-p53 fusion protein (Fig. 4C). Importantly, similar to the experiments presented above, mutation of Ser15 to alanine diminished the CBP/p300-dependent acetylation of p53 (data not shown).

**p53 Acetylation Is Due to CBP/p300**—To demonstrate that p53 acetylation was due to CBP/p300, the CBP/p300 fraction was precleared with antibodies specific for CBP/p300 and treated with DNA-PK. Phosphorylated p53 was then incubated with CBP/p300, and the acetylation reaction was carried out as described above. Wortmannin was included in the acetylation reaction to ensure that the DNA-PK did not phosphorylate proteins in the CBP/p300 extract. Following incubation, Radioimmune precipitation buffer was added, the p53 protein was immunoprecipitated, and the resulting material was subjected to SDS-PAGE and autoradiographed. DNA-PK treatment led to a 6-fold increase in the level of p53 acetylation, similar to that seen with the GST-p53 fusion protein (Fig. 4C). Importantly, similar to the experiments presented above, mutation of Ser15 to alanine diminished the CBP/p300-dependent acetylation of p53 (data not shown).

**FIG. 4.** The increased binding of CBP/p300 is paralleled by an increase in the acetylation of p53. A, glutathione-Sepharose-bound GST-p53 and GST were treated with DNA-PK and allowed to bind to CBP/p300 before the addition of [1-14C]acetyl-CoA and incubation at 30 °C for 4 h. The excess label was then removed by washing and the labeled proteins resolved by SDS-PAGE. B, acetylation of mutant p53s and the (1–300) truncated protein under the same conditions. wt, wild-type. C, GST-p53 was cleaved to release the p53, which was then treated with DNA-PK. Acetylation was carried out as described under “Experimental Procedures,” and the products were immunoprecipitated with antibodies to p53, resolved by SDS-PAGE, and autoradiographed.

**FIG. 5.** CBP/p300 is responsible for the increased acetylation. A, acetylation of p53 was carried out using the HeLa CBP/p300 fraction untreated (lanes 1 and 2), or immunodepleted with either antibodies to CBP and p300 (lanes 3 and 4) or with preimmune IgG (lanes 5 and 6). B, a highly purified preparation of CBP was used in an acetylation experiment.
Phosphorylation Controls p53-CBP Interaction

p53 is a critical protein in the response of a cell to DNA damage. It is involved both in the cell cycle arrest that allows DNA repair to take place and in the induction of apoptosis should the damage be too severe to allow recovery. The transcriptional activation activity of the N-terminal domain of p53 is important for both these functions (3, 4, 33). Moreover, it is becoming increasingly apparent that phosphorylation of specific amino acids in the N terminus regulates the activity of the protein. For example, phosphorylation of Ser15 and Ser37 by DNA-PK in vitro decreases the binding of MDM2 (19) and phosphorylation of Ser15 alone impairs TFIIID binding (20). Importantly, γ-radiation-induced Ser15 phosphorylation correlates with a decrease in MDM2 binding by p53 in cell extracts (19), allowing the escape of p53 from the inhibitory effects of MDM2 (34–36). At the same time, the increase in the ability of p53 to recruit CBP/p300 following phosphorylation on these residues, as shown in this report, provides a positive mechanism for increasing its transcriptional activity. It will be of interest to determine whether the increase in p53-CBP/p300 interaction represents an increase in binding affinity or binding stoichiometry.

CBP/p300 associates with p53 in vivo and in vitro (9, 10, 26, 27) and, in transfection experiments, cooperates with p53 in activation of a cotransfected reporter plasmid (9, 26, 27) or the endogenous p21/waf1 gene (10). Further, p300 is specifically required for p53 transactivation of the mdm2 promoter (37). Increased binding of CBP/p300 by p53 could activate transcription by two means: first, by increasing recruitment of the coactivator into the transcriptional complex, and second, by increasing the specific DNA binding activity of p53 following acetylation of the C terminus (30). It will be of interest, therefore, to determine the effect of CBP/p300 activation domain and acetyltransferase mutants on p53-CBP/p300 activation of independent genes.

Phosphorylation on Ser15 occurs following DNA damage induced by γ-radiation or chemicals, and mutation of this residue to alanine interferes with the cell cycle-arresting properties of p53 (38). γ-Irradiation induces double strand breaks in DNA; the ends of DNA so generated can activate DNA-PK through its Ku DNA-binding subunits, and so DNA-PK might be thought an attractive candidate for transmitting the DNA damage signal, via p53, to the cell cycle arrest pathway (39–41). The observation that murine scid cells, which are deficient in the catalytic subunit of DNA-PK, show normal apoptotic responses or arrest in G1 and G2 following γ-irradiation (42, 43) appeared inconsistent with this hypothesis, but it has been reported that these cells constitutively phosphorylate p53 on Ser15 (19), and low but detectable levels of DNA-PK activity have been detected in scid MEF cells (44). Interestingly, Woo et al. (44) have recently reported that DNA-PK is necessary for the induction of p53-specific DNA binding in response to DNA damage. However, it is not yet established whether DNA-PK acts on p53 directly or indirectly in vivo, and it is important to note that Woo et al. (44) found that in addition to DNA-PK, a radiation-inducible factor in nuclear extracts was required for the induction of the specific DNA binding activity of p53; possibly, other constitutive factors in these extracts are also required. These nuclear extracts would also contain CBP/p300, but these proteins are not known to have intrinsic radiation-inducible activities, and other factors are likely to be involved. The ATM kinase (which, like DNA-PK, is a member of the phosphatidylinositol 3-kinase (PI 3-kinase) family and which appears to be involved in the normal response of the cell to ionizing radiation (reviewed in Ref. 45)) can also phosphorylate p53 at Ser15 (46, 47) and could therefore also be responsible for increased CBP/p300 recruitment to p53. ATM kinase activity is induced following exposure of cells to ionizing radiation or radiomimetic drugs (46, 47), and it has been shown that cells deficient for the ATM gene exhibit delayed and reduced phosphorylation of p53 Ser15 following exposure to ionizing radiation (18). Although we have used DNA-PK as a reagent for the site-specific phosphorylation of these regulatory sites, it is entirely possible that a different kinase is responsible for the phosphorylation seen in vivo. In fact, it is quite possible that redundant phosphorylation pathways operate. Alternatively, different kinases could be involved according to the cell type and DNA-damaging agent.

The question arises of why phosphorylation is utilized for regulation of the p53-CBP/p300 interaction. The answer may lie in the increasingly apparent complexity of the p53 N terminus and its interactions. Not only is the phosphorylation state known to vary with respect to stimulus (13, 14, 18, 48, 49), but recent work has identified separate activation subdomains, one existing within the first 42 amino acids and the second lying just C-terminally in amino acids 43–63 (33, 50). Furthermore, it appears that these separate regions mediate the activation of different subsets of p53 target genes. The second domain has been implicated in directing apoptosis (33). Phosphorylation of the p53 N terminus might therefore be used to control protein interactions, regulating transcription of downstream genes and thereby providing a subtle and powerful means for determining the response of the cell to DNA damage.

Given the variety of p53 phosphorylation patterns seen in response to different stimuli (13, 14, 18, 48, 49), it will be of importance to determine which p53 response pathway(s) stimulates the association of p53 with CBP/p300. Such a response might be restricted to certain types of damage. Certainly, recent reports that UV, but not ionizing radiation, induces phosphorylation of murine p53 on Ser389 suggest that different DNA-damaging agents induce different phosphorylation pathways (49). This observation raises the interesting possibility that the response to ionizing radiation, including double-strand break repair, activates p53 binding by acetylation, but the response to UV, including nucleotide excision repair, utilizes phosphorylation of Ser389 or its apparent human equivalent, Ser392. These various pathways would not only allow differentiation between alternative damage responses but also the temporal regulation of the activities of p53, presumably concluding with the restoration of MDM2 inhibition, p53 degradation, and resumption of the cell cycle.

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