DNA-dependent Acetylation of p53 by the Transcription Coactivator p300*

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Reconstitution of the stages in the assembly of the p300-p53 transcription complex has identified a novel type of DNA-dependent regulation of p300-catalyzed acetylation. Phosphorylation at the CHK2 site (Ser20) in the N-terminal activation domain of p53 stabilized p300 binding. The phosphopeptide binding activity of p300 was mapped in vitro to two domains: the C-terminal IBiD domain and the N-terminal IHD domain (IBiD homology domain). The IHD or IBiD minidomains can bind to the p53 activation domain in vivo as determined using the mammalian two-hybrid VP16-GAL4 luciferase reporter assay. The IHD and IBiD minidomains of p300 also functioned as dominant negative inhibitors of p53-dependent transcription in vivo. Upon examining the effects of p300 binding on substrate acetylation, we found that the p53 consensus site DNA promotes a striking increase in p53 acetylation in vitro. Co-transfection into cells of the p53 gene and plasmid DNA containing the consensus DNA binding site of p53 activated DNA-dependent acetylation of p53 in vivo. The phosphopeptide binding activity of p300 is critical for DNA-dependent acetylation, as p53 acetylation was inhibited by phospho-Ser20 peptides. Consensus site DNA-dependent acetylation of p53 stabilized the p300-p53 protein complex, whereas basal acetylation of p53 by p300 in the presence of nonspecific DNA resulted in p300 dissociation. These data identify at least three distinct stages in the assembly of the p300-p53 complex: 1) p300 docking to the activation domain of p53 via the IBiD and/or IHD domains; 2) DNA-dependent acetylation of p53; and 3) stabilization of the p300-p53 complex after acetylation. The ability of DNA to act as an allosteric ligand to activate substrate acetylation identifies a conformational constraint that can be placed on the p300-acetylation reaction that is likely to be an amplification signal and influence protein-protein contacts at a promoter.

Characterizing the protein-protein interactions that assemble transcription complexes will provide a molecular basis for combinatorial specificity at the promoter level and provide an understanding of how transcriptomes affect disease progression. One of the most studied regulators of eukaryotic gene transcriptional activation is the stress-activated tumor suppressor protein p53 (1). The vast array of genes that p53 induces and represses in response to cell injury provides an excellent model system with which to identify the complex and transient associations with co-activators/repressors and the core promoter transcriptional apparatus that drive promoter activation.

The tumor suppressor activity of p53 is linked to its activity as a stress-activated transcription factor. p53 is subject to multiple post-translational modifications such as phosphorylation, acetylation, and ubiquitination that modulate the function of p53 as a transcription factor. p53 protein is tetrameric (2) and the oligomeric nature of p53 provides the basis for complex intra/interdomain control mechanisms that modulate its activity (3, 4). The most widely studied post-translational modification of p53 is phosphorylation via protein kinases that target specific domains on the assembled tetramer that can activate its function by a two-step process (5). The first identified involves phosphorylation of the C-terminal regulatory domain of p53 by CK2/FAC-T at Ser392 and cyclin-dependent protein kinases at Ser15, which play a role in activating its latent sequence-specific DNA binding activity in vitro (6, 7) and in vivo (8, 9). The second step involves phosphorylation of specific N-terminal residues of p53 that reside within the activation domain by an ATM-dependent signal transduction cascade at Ser15 (10), which stimulates p300 binding to p53 in vitro (11) and in vivo (12). Additionally, an adjacent Ser20 phosphorylation event by a CHK2-dependent pathway activates p53 (13) by stabilizing the transcription coactivator p300-p53 complex formation (14). The specific interaction of p300 in the p53-dependent transactivation pathway became apparent when it was demonstrated that ectopically expressed p300 stimulated p53-dependent gene expression and that adenoviral E1A protein inhibited p53-dependent transcription by virtue of binding to p300 (15–17). Although this initial p300-p53 interaction was mapped to the N-terminal BOX-I domain of p53, an additional role for p300 in the control of p53 activity came from the observation that acetylation of p53 by p300 in the C-terminal negative regulatory domain adjacent to the CK2/FAC-T phosphorylation site activated the specific DNA binding function of p53 to short oligonucleotides containing the consensus binding site for p53. Together, these data suggest the existence of a phosphorylation-acetylation cascade that targets p53 in response to genotoxic stress (19).

The acetyltransferase activity of p300 and its homolog CBP on histone and nonhistone substrates has been well documented and is important for co-activator function (20, 21). The

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role for acetylation on the N-terminal tails of histones is somewhat more widely appreciated, with the acetylation of lysine residues potentially weakening the nucleosome-DNA interaction, thereby allowing promoter access for the core transcriptional machinery such as TFID and RNA polymerase II. Acetylation of transcription factors is now a widely observed phenomenon (22–25) but the role and mechanism of acetylation of transcription factors remains relatively elusive (26). However, more recent studies have also suggested that acetylation may function to stabilize coactivator complexes at a promoter (27, 28), presumably in part through the Bromo-homology domain of p300/CBP that has the potential to bind to acetylated residues (29).

The molecular mechanism underlying how p53 tetramers interact with p300 and how this modulates acetylation has not been defined biochemically. Such analysis will likely give novel insights into how p300 protein interacts with an oligomeric substrate as well as information into how p300 links transcription to cell cycle control. We have started to reconstitute the p300-p53 protein complex in vitro by first examining how full-length p300 interacts with various phosphorylated regions in the N-terminal BOX-I activation domain of p53 (14). This study has indicated that of three N-terminal phosphorylation sites in Ser20 has the most stabilizing affect on p300 (13432).

Materials and Methods

Plasmids and Constructs—p21-Luc, Bax-Luc, pGL3-Basic, pCMV-β-gal, pCMV-p53, and pCMVβ-p300 have been previously described (14). pCMV-p53–6KR (32) was a gift of Dr. Ron Hay, St. Andrews University, Scotland, UK. Vectors used for the p300 minidomain two-hybrid studies (Fig. 3A), pACT, pG55uc, and pBIND, were obtained from Promega and used according to the manufacturers instructions and as described previously (41). PACT-IID encoded amino acids 2050–2094 of human p300, pact-IID encoded amino acids 406–566 of human p53, and pACT-CH3 encoded a region of the standard C/H3 domain from amino acids 1709 to 1913. GAL4, GAL4-p300, GAL-p300–(1–504), GAL-p300–(1–703), GAL-p300–(192–504), GAL-p300–(192–800), GAL-p300–(192–703), GAL-p300–(192–1004), GAL-p300–(504–1238), GAL-p300–(852–1071), GAL-p300–(636–2414), GAL-p300–(1064–2414), and GAL-p300–(1757–2414) have been previously described (33). GAL4-N1, GAL4-N2, GAL4-N3, GAL4-C1, GAL4-C2, and GAL4-C3 were a gift from Y. Shi (Harvard Medical School, Boston, MA). Described (33). GAL4-N1, GAL4-N2, GAL4-N3, GAL4-C1, GAL4-C2, and GAL4-C3 were a gift from Y. Shi (Harvard Medical School, Boston, MA). Described (33). GAL4, GAL4-p300, GAL4-p300–(1–504), GAL-p300–(1–703), GAL-p300–(192–504), GAL-p300–(192–800), GAL-p300–(192–703), GAL-p300–(192–1004), GAL-p300–(504–1238), GAL-p300–(852–1071), GAL-p300–(636–2414), GAL-p300–(1064–2414), and GAL-p300–(1757–2414) have been previously described (33). GAL4-N1, GAL4-N2, GAL4-N3, GAL4-C1, GAL4-C2, and GAL4-C3 were a gift from Y. Shi (Harvard Medical School, Boston, MA).

Immunological Assays—The peptide or p53 tetramer binding activity of p300 was examined by enzyme-linked immunosorbent assay (ELISA), as described previously (3, 14). Essentially, 96-well plates (Dyne Microlite) were first coated with p53, antibody-captured p530, or steptavidin and the indicated biotinylated peptide for 1 h as described previously (14). Nonreactive sites were blocked in 3% bovine serum albumin in PBS/Tween 20 (0.02% v/v) to reduce the nonspecific binding. This was followed by titrating increasing amounts of p53, p500, MDM2, peptides, or with DNA (poly(G)) (data now shown) (as in Ref. 34) in 3% bovine serum albumin in PBS/Tween 20 (0.02% v/v) for 1 h, followed by an extensive wash, and incubation with the indicated IgG. All reactions were carried out at 4°C and detected by the appropriate secondary antibody linked to horseradish peroxidase from DAKO. The signal detection by enhanced chemiluminescence was developed using Fluoroscopic Ascent FL. The BOX-I transactivation domain of p53 or its Ser20–phosphorylated derivative contains amino acids 14–27, as described previously (14). All synthetic peptides were obtained from Chiron Minitopes.

Cell Culture, Transfections, ELISAs, and Western Blots—A375 and Saos-2 cells were maintained in Dulbeco’s modified Eagle’s medium (Invitrogen), whereas HTC116 cells were in McCoy’s 5A medium, both supplemented with 10% fetal bovine serum and incubated at 37°C with an atmosphere of 10% CO2. Transient transfections and ELISAs were carried out as previously described (14). Full-length p300 and His-p300-infected SF9 cells were harvested 72 h postinfection and purified as described previously (14). SF9-expressed wt53 tetramers were purified by heparin-Sepharose chromatography as described previously (3). Transfected lysates were run on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an affinity-purified monoclonal to mouse p300, MDM2, peptides, or with RNA (poly(G); data now shown) (as in Ref. 34) in 3% bovine serum albumin in PBS/Tween 20 (0.02% v/v) for 1 h, followed by an extensive wash, and incubation with the indicated IgG. All reactions were carried out at 4°C and detected by the appropriate secondary antibody linked to horseradish peroxidase from DAKO. The signal detection by enhanced chemiluminescence was developed using Fluoroscopic Ascent FL. The BOX-I transactivation domain of p53 or its Ser20–phosphorylated derivative contains amino acids 14–27, as described previously (14). All synthetic peptides were obtained from Chiron Minitopes.
then immunoprecipitated using a p53-specific antibody (DO-1 or ICA-9) or p300 antibody (N-15) and then probed for acetyl-p53. The specific acetylation was normalized to the quantity of total p53 captured assessed by probing with anti-p53 (DO-1 or CM1/5).

RESULTS

Developing a Consensus p300-binding Motif by Mapping Its Contact Site in the Ser20-phosphorylated BOX-I Activation Domain of p53—The overlapping docking site for MDM2 and p300 on p53 (Fig. 1E) creates a negative and positive effect, respectively, on its ability to function as a tumor suppressor protein. The notable differences in the affinity of p300 and MDM2 toward p53-derived Thr18 and Ser20 phosphopeptides suggested an intrinsic difference in their binding contacts in the BOX-I activation domain (14). To define essential amino acid contacts, a series of alanine-substituted Ser20-phosphorylated peptides were synthesized and the protein-ligand binding assay was employed using full-length p300 and full-length MDM2 proteins (Fig. 1, A and B, respectively). Ser20 phosphorylation of p53 is required for maximal full-length p300 binding to this domain, whereas MDM2 is not inhibited (14) thus allowing the same phosphodomain of p53 to be used for consensus site mapping of MDM2 and p300. If Gln16, Trp23, Lys24, Leu25, or Leu26 are individually mutated to alanine, then this abrogates the ability of the p300 protein to bind to the Ser20 phosphodomain (Fig. 1A). Certain alanine substitutions in the Ser20 phosphodomain including Ser15, Glu17, Thr18, Phe19, and Asp21 stabilized the binding of p300 to the phospho-motif (Fig. 1A). In contrast to p300, a Thr18, Phe19, Leu22, Trp23, or Leu26 substitution to alanine on the BOX-I-derivated Ser20-phosphorylated domain inhibits MDM2-binding (Fig. 1B), correlating with the essential residues required for MDM2 protein binding to p53 identified by the crystal structure of the MDM2-p53 peptide complex (37). This evidence that MDM2 and p300 have distinct binding contacts explains, in part, their differential binding to phospho-Thr18 or phospho-Ser20 p53 (14).

Interestingly, a search for proteins in the data base that have significant homology to the consensus BOX-I p300-binding motif on p53, QXXXSPXXXWKKL (Fig. 1C), identified the HMG box architectural factor, UBF1 (upstream binding factor 1; Fig. 1C). UBF necessitates major chromatin remodeling and the SXXWKL region of UBF is within a domain that is already known to interact with the p300 homologue, CBP (CREB-binding protein) (38). To determine whether p300 can bind to this region of UBF1 we employed the protein-ligand binding assay containing two putative SPXXXWKKL consensus peptides from UBF1 (amino acids 231–246 and 321–336) with or without a phosphate moiety at the predicted serine residue. Notably, when a phosphate group was attached to Ser239 in UBF1-(321–336), which contains strict homology to the SPXXXWKKL motif, p300 bound with an affinity slightly less than the p53 BOX-I-Ser20 phosphopeptide (Fig. 1D), suggesting that this may indeed be a consensus contact region for p300. In contrast, UBF1-(231–246) did not bind p300 with or without a phosphate substitution at Ser239 (Fig. 1D) nor did p300 bind the BOX-I domain of p53 without a phosphate (Fig. 1D). These differences may be because of the absence of a tryptophan residue at position 242 (or the presence of a destabilizing lysine) (Fig. 1C), which is required for p300 binding to the p53 BOX-I-Ser20 phosphopeptide alanine scan (Fig. 1A).

Identification of Two p53-activation Motif Binding Domains on p300—Various techniques have been utilized to originally define the p53 activation domain-binding sites in p300 to within the C/H1, KIX, and C/H3 minidomains of p300 (Fig. 2). However, the most recent mapping of the p53-binding domain of p300 was localized to the C-terminal interferon-binding domain (IBID, Fig. 2A), which is known to bind to peptides of apparently unrelated primary amino acid homology including phosphorylated peptides derived from interferon regulatory domains (IBID, Fig. 2A).
factor-3 (30). This motif on IRF-3 has homology to the phosphorylated p53-activation domain (data not shown) and in a recent study, a p53-activation domain-binding region in p300 was mapped to the IBiD domain and independently called SRC-1 (Fig. 2A) (31). An in vitro assay was employed to localize the p53 peptide-binding domains of full-length p300 to either previously known or unmapped regions of p300 protein by assaying fragments of p300 for its binding activity (RLU) using a peroxidase-linked secondary antibody coupled to an anti-p300 antibody.

Identification of two phosphopeptide-binding domains on p300 using an in vitro p300-miniprotein binding assay. A, a summary of the well-characterized peptide-binding domains of p300: C/H1, KIX, CRD1, Bromo, C/H3, IBiD, and (see Fig. 3) the N-terminal phosphopeptide-binding domain with homology to IBiD (IHD). B, mapping of the phosphopeptide-binding domain to two large regions of p300. HCT116 p53−/− cells were transfected with 5 μg of GAL4, GAL4-p300, or the indicated GAL4-p300 miniproteins and the p300 from lysates was captured on ELISA wells with an anti-GAL4 antibody to purify the indicated miniproteins. The indicated biotinylated peptide (BOX-I, Ser20-, phospho-BOX-I, or no peptide) was added and the amount of p300 protein bound is quantitated as luciferase activity (RLU) using a peroxidase-linked secondary antibody coupled to an anti-p300 antibody. The indicated biotinylated peptide (BOX-I, Ser20-phospho-BOX-I, or no peptide) was added and the amount of p300 protein bound is quantitated as luciferase activity (RLU) using a peroxidase-linked secondary antibody coupled to an anti-p300 antibody.
IBiD domain of p300, we used the VP16-GAL4 two-hybrid system that was used originally to fine map the C-terminal IBiD domain on p300 that binds to the activation domain of p53 when fused to GAL4 (30, 31). The co-transfection of pACT-VP16-IBiD or pACT-VP16-IHD along with p53-TAD:GAL4 both supported GAL4-dependent transcription (Fig. 3A, right 5 panels; 1063 RLUs for IBiD and 877 RLUs for IHD), relative to the negative control using pACT-VP16 only (180 RLUs). The activity is summarized as relative light units normalized to β-galactosidase activity. B, the IBiD and IHD domains attenuate p53-dependent transcription in cells. HCT116 p53−/− cells were co-transfected with the indicated constructs (1 μg; pCMV-p53, pCMV-p53 + pCMV-p300, alone, or with the indicated pACT fusion constructs), pCMV-β-gal (1 μg), and the p53-responsive p21-luc reporter vector. The activity is summarized as relative light units normalized to β-galactosidase activity. C, the identity between the C-terminal IBiD and N-terminal IHD domains is summarized.

As an independent test of the ability of IHD and IBiD as important effectors of p300-coactivated p53-dependent transcription, the ability of pACT-IBiD and pACT-IHD were tested as dominant negative inhibitors of p53-dependent transcription from the p21-Luc reporter (Fig. 3B). The maximal p53-dependent transcription upon co-transfection with p300 (Fig. 3B, 2483 RLUs) was significantly attenuated using either pACT-IBiD (1002 RLUs) or pACT-IHD (1146 RLUs), relative to pACT-C/H35 (1918 RLUs) or pACT only (2538 RLUs). Together, these data identify IBiD as the major p53-binding domain of p300 and the novel N-terminal domain IHD as an equally effective p53-binding domain. We cannot rule out the previously identified p53-binding domains including the C/H3, the KIX, or the C/H1 domains as effective p53-binding domains of p300, as the folding and assembly of the relatively large p300 protein with its various repressor and activator motifs complicates a clear understanding of how p300 really binds to p53. For example, there is some weak, but significant in vivo p53-binding activity associated with the region of the C/H3 domain used in this study (Fig. 3, A and B). However, IBiD and IHD are the predominate p53-binding domains with similar specific activities as defined in: (i) in vitro p53-binding assays (Fig. 2B); (ii) in vivo p53-binding assays (Fig. 3A); (iii) dominant negative inhibitors in p53-dependent transcription assays (Fig. 3B).

Consensus Site DNA-dependent Acetylation of p53—To establish a function for the IBiD and IHD domains of p300, the contribution of the phosphopeptide-binding activity of p300 to p53 acetylation was examined. Specifically, does binding of p300 to the BOX-I domain of p53 affect acetylation in the C-terminal NRD1 domain of p53 (see p53 domain structure in Fig. 1E). Before setting up an in vitro p53 acetylation assay, the purified p300 protein was first characterized enzymatically using a well known substrate, histone H4. The kinetics of the acetylation reaction between p300 and histone H4 were determined (Fig. 4, A and B). The data indicate that p300 is behaving like a classical histone acetyltransferase by displaying monophasic kinetics on a histone substrate (39, 40) and this preparation of p300 was tested for the ability to acetylate native p53 tetramers expressed in Sf9 cells (41). Using an antibody against acetylated Lys373/382 of human p53 that has been previously described as being specific for acetyl-p53 (27) and normalizing to levels of p53 protein with an anti-p53 antibody, acetyl-CoA-dependent acetylation of p53 was observed (Fig. 4C, lane 3 versus lane 2).

In a further characterization of p53 acetylation, we found that including consensus site DNA in the acetylation buffer.
resulted in an unexpected stimulation in p53 acetylation (see below). Using the basal p300-acetylation reaction where acetyl-CoA promotes weak p53 acetylation (Fig. 4C, lane 3 versus lane 2), experiments demonstrated a stimulation of p53 acetylation by p300 upon titration of an excess of consensus site oligonucleotide DNA (pG DNA; Fig. 5A, lanes 5–8 versus lane 4). Nonspecific oligonucleotides could not stimulate the acetylation under the same condition (NS-DNA; Fig. 5B, lanes 5–8 versus lanes 3 and 9). A titration of consensus site DNA demonstrated that at a ratio of DNA to p53 of −1:1, the acetylation of p53 approached saturation (Fig. 5C, lanes 4 and 5 versus lane 1). If consensus site DNA were stimulating general p300 activity by an allosteric mechanism via a sequence-specific DNA-binding domain on p300, then basal histone acetylation (Fig. 4, A and B) would be expected to be stimulated by DNA. However, histone acetylation by p300 was neither inhibited nor stimulated by the addition of consensus site DNA (Fig. 5D, lanes 1–6). These data suggest that the acetylation of p53 was unique to a change in the conformation of p53 when bound to consensus site DNA.

The kinetics of p300 acetylation on histones has been published recently (40) and displays a ping-pong mechanism, via an ordered reaction involving first the formation of a stable p300-acetyl intermediate followed by binding of the histone substrate and transfer of the acetyl moiety to a lysine residue. As a final control to define the integrity of the acetyltransferase reaction, we determined whether the acetylation of DNA-bound p53 by p300 displays similar kinetics to that of histone substrates. The linearity of the p53-acetylation reaction over time (Fig. 6A) and as a function of DNA concentration (Fig. 6B) was optimized using bioluminescent quantification of reaction products. By changing the concentration of acetyl-CoA at a fixed p300 concentration and consensus site DNA concentration, varying the p53 levels resulted in linear changes in the levels of acetylation. After quantification using bioluminescence, a double reciprocal plot of the initial velocities was drawn using Prism kinetics software (Fig. 6C). p300 acts in a similar manner to p53-DNA complexes as a substrate compared with histones suggesting that the p53-p300 acetylation reaction similarly displays a ping-pong mechanism as opposed to a sequential (ternary complex) mechanism. Kinetics could not be performed on p53 acetylation without DNA as the stoichiometry of acetylation was too low to acquire statistically significant data.²

Although acetylation can stimulate latent p53 binding to small consensus site DNA oligonucleotides (18), acetylation does not change the affinity of p53 for its consensus site in large fragments of DNA (28). As such, the role of acetylation in the control of p53 function has remained elusive (26). However, it is very difficult to acquire pure isoforms of recombinant latent phosphorylated p53, latent unphosphorylated p53, or kinase-activated p53 (41), especially in insect cell expression systems where dozens of p53 isoforms exist (42). This heterogeneity in p53 modifications possibly explains, in part, the apparent controversy on the role of acetylation in modulating the specific activity of p53 using in vitro DNA binding assays. It therefore is important to determine whether consensus site DNA stimulation of p53 acetylation also could be promoted in large plasmids of the DNA rather than small oligonucleotides.

Using a supercoiled plasmid DNA containing multiple copies of the p53 consensus site (pG13) (43), a DNA-dependent stimulation of p53 acetylation by p300 was observed, compared with parallel reactions containing pMG13 nonconsensus site control DNA (Fig. 7A, lane 4 versus lane 3). These data indicate that DNA-dependent acetylation was not confined to small oligonucleotides and indicate that although acetylation can stimulate unphosphorylated and latent p53-DNA binding (18), our use of in vivo kinase-activated, purified p53 protein isoforms (as in Ref. 41) demonstrates that acetylation is a post-DNA binding event.

It was next important to determine whether sequence-specific DNA-dependent acetylation could occur in vivo. Two p53 constructs (p53 and p53–6KR (the nonacetylatable p53 (56))) were co-transfected with consensus site plasmid DNA (or plasmid control containing the mutated consensus site) and the expressed p53 protein was assayed for both binding to endogenous p300 protein and in vivo acetylation (Fig. 7, B–E). The transfection of the p53 gene with either pMG13 DNA or pG13 DNA, followed by immunoprecipitation with a p300 antibody resulted in the same level of p53 protein being co-precipitated with p300 as detected by blotting with a p53 antibody (Fig. 7C, lanes 1 and 2). However, only consensus site pG13 DNA promoted a substantial acetylation of p53 in the p300 immune complex as detected by blotting with a p53-acetylation site antibody (Fig. 7B, lane 2 versus lane 1). This is in contrast to the control transfection of the p53 gene with the mutant consensus site supercoiled plasmid pMG13, which did not produce a stable complex between p300 and acetylated p53 (Fig. 7B, lane 1). Thus, because significant amounts of nonacetylated p53 protein bound to p300 upon co-transfection with the p53 gene and pMG13 plasmid DNA (Fig. 7, B and C, lanes 1), these data indicate that acetylation of p53, rather than p53 binding to p300, is promoted by consensus site plasmid DNA. As a further control for total p53 acetylation, co-transfection of the

²D. Dornan and T. R. Hupp, unpublished data.
stimulate histone acetylation by p300. Acetylation reactions were carried out as described in the legend to Fig. 5 to define parameters (time and DNA concentration) for linearity. By varying the concentrations of acetyl-CoA (500 to 2000 μM) at different concentrations of p53 (0.3, 0.6, and 1.2 μM), as indicated, acetylation was quantitated using bioluminescence with a Genegnome-Syngene Bioimaging System and a resultant double-reciprocal plot was drawn (C).

p53 gene with pG13 plasmid (versus the pMG13 plasmid) stimulated total acetylation of p53 after precipitation of protein complexes with an anti-p53 antibody and immunoblotting with an acetylation-specific p53 antibody (Fig. 7D, lane 2 versus lane 1). This is similar to the in vitro stimulation of p53 acetylation by p300 with the inclusion of pG13 DNA (Fig. 7A, lane 4 versus lane 3). Together, these data indicate that consensus site plasmid DNA-dependent acetylation can be observed in vitro or vivo.

Acetyl-CoA Stabilizes the p300-p53AC Complex—With the DNA-dependent p53 acetylation assay biochemically characterized, we could finally access the role of the phospho-Ser20 peptide-binding domains of p300 to both DNA-dependent p53 acetylation and DNA-independent histone acetylation (Fig. 8). In the absence of peptide, acetyl-CoA was required for DNA-dependent acetylation of p53 by p300 (Fig. 8A, from left, lane 2 versus lane 1). A titration of BOX-I peptide displayed no effect on the p53 acetylation reaction (Fig. 8A, from left, lanes 3–6 versus lane 2). However, a titration of the BOX-I phospho-Ser20 peptide inhibited p53 acetylation with an IC50 of ~150 μM in this assay (Fig. 8A, from left, lanes 7–10 versus lane 2). These data indicate that the phosphopeptide-binding domain(s) of p300 interacts with the N-terminal domain of p53 to facilitate acetylation in the C-terminal domain of p53. As a control, histone acetylation was not affected by the BOX-I phospho-Ser20 peptide (Fig. 8B) indicating that the acetylation reaction is docking- and DNA-independent with respect to histone acetylation. Using the p53 acetylation assay in an ELISA format where monoclonal-captured p53 could be probed with an anti-acetylation polyclonal antibody, the phospho-Ser20 peptide similarly prevented DNA-dependent acetylation of full-length p53 (Fig. 8C).

In summary, our reconstitution of the p300-p53 complex has indicated that acetylation is DNA-dependent and requires p300 docking to the N terminus of p53 presumably via the IBiD and/or IHD domains of p300. This data provides an alternate
DNA Is an Allosteric Effector of p53 Acetylation

Fig. 7. Consensus site DNA-dependent acetylation of p53 using supercoiled plasmid DNA in vitro and in vivo. A, in vitro p53 acetylation. Human recombinant p53 (lanes 2–4) was purified from insect cells and incubated with p300 and supercoiled plasmid containing the p53-consensus site (pG13, lanes 4) or nonspecific DNA (pMG13, lanes 3). Relative acetylation was quantitated by Western blot using an anti-p53 acetylation antibody and normalized to total p53 protein using the monoclonal antibody DO-1. B–E, in vivo DNA-dependent acetylation of p53 by p300. pCMV-P53 (lanes 1 and 2) or pCMV-p53–6KR (lanes 3 and 4; mutated acetylation sites) were co-transfected into p53−/− cells in the presence of consensus site plasmid DNA (pG13-CAT, lanes 2 and 4) or plasmid DNA without the consensus site (pMG13-CAT, lanes 1 and 3). The amount of total p53 in each transfection was quantitated by direct immunoblotting with the monoclonal antibody DO-1. B and C, IP p300 panel: endogenous p300 protein was immunoprecipitated with an anti-p300 antibody and levels of total p53 protein (C) or acetylated p53 bound to p300 (B) were quantitated by immunoblotting, as indicated, with either the acetylation-specific antibody or a p53 protein antibody to normalize to the total p53 protein. D and E, IP p53 panel: the total p53 protein was immunoprecipitated with a mixture of ICA-9 and DO-1 monoclonal antibodies and the total levels of acetylated p53 (D) or p53 protein (E) were quantitated by immunoblotting, as indicated, with either the acetylation-specific antibody or a p53 protein antibody to normalize to the total p53 protein.

model for acetylation compared with other studies showing that acetylation can either stimulate the latent DNA binding activity of p53 (18), have no effect on p53 DNA binding (28), or

block MDM2-dependent ubiquitination of p53 (32). What then is the role of the acetylation of DNA-bound p53? One previous study has suggested a clue, as acetylation of MyoD by CBP (in the absence of DNA) can promote a high salt-resistant stable complex between the two proteins presumably through the bromo-homology domain (29). The stability of the p300-p53 complex was therefore quantitated without or with acetyl-CoA using the ELISA solid phase assay to determine whether p300 dissociates from p53 after acetylation in the absence or presence of consensus site DNA. In contrast to the immunoprecipitation of p300-p53 complexes that is a very sensitive, but rapid assay not allowing significant time for dissociation of weak protein-protein complexes (Fig. 7), the ELISA measures very stable complex formation because 20–30-fold longer wash times permit weakly bound proteins to dissociate. Using purified p300 and p53 proteins, we show that small oligonucleotides containing the p53 consensus site (Fig. 9A) or supercoiled plasmid DNA containing the p53 consensus site (Fig. 9B), can promote a striking stabilization of the p300-p53 complex after the addition of acetyl-CoA (Fig. 9, A and B, lane 12), relative to nonspecific DNA (Fig. 9, A and B, lane 11 versus 12). As controls for this quantitation, acetyl-CoA-de-
that one role of acetylation is to allow p300 to nucleate interactions with nonspecific DNA (Fig. 9, A when p53 is pre-bound to consensus site DNA (Fig. 9, B p53 protein complex stabilization by consensus site supercoiled DNA.-lanes 1–3 versus 8–9). The amount of p300 protein stably bound to p53 protein: (∼+/+)–acetyl-CoA, (∼/+−)–NS-DNA, or (∼+/+)–pG-DNA was determined using an antibody to p300 and quantitated as p300 bound/total p300 using enhanced chemiluminescence expressed as relative light units (RLU). lane 2 versus lane 11, lane 7 versus 9). The amount of p300 protein stably bound to p53 protein: (∼+/+)–acetyl-CoA, (∼+/+)–NS-DNA, or (∼+/+)–pG-DNA was determined using an antibody to p300 and quantitated as p300 bound/total p300 using enhanced chemiluminescence expressed as relative light units. The notable effects include the dissociation of p300 from p53 after acetylation in reactions with nonconsensus site DNA (lanes 11 versus 5), whereas a stabilization of the p300–p53 complex occurs after acetylation in reactions with consensus site DNA (lanes 12 versus 11).

The acetylation of histone and nonhistone chromosomal proteins is critical for the fine-control of gene expression and the characterization of how p300 recognizes and acetylates these classes of proteins will help to further define how gene expression is controlled. Histone acetylation reduces the extent of nucleosome assembly presumably by minimizing electrostatic DNA-protein interactions thus assisting in stabilizing the RNA polymerase preinitiation and transcription complex. By contrast, acetylation of sequence-specific transcription factors including E2F, p53, and MyoD have been shown to enhance their affinity for specific sequences in promoter regions (44). The molecular mechanism of p300 binding to and acetylation of sequence-specific transcription factors has not been defined. The relatively large size of p300 suggests that an intrinsically complex intradomain and/or interdomain communication exists between itself and the variety of target proteins at a promoter. The interferon-responsive enhancerome is one such example of multiprotein nucleation at a promoter (45). Given that molecular reactions in vivo likely involve large protein complexes, or protein machines (46), it is likely that p300 will be regulated and function in a complex fashion. Furthermore, because acetylation may prove to be a covalent modification as important as phosphorylation mechanistic insight into acetylation function and regulation is of fundamental importance. In this report, we have assembled in vitro the p300–p53 complex, defined co-factors that modulate the acetylation of p53, and reconstituted a docking-dependent and DNA-dependent acetylation reaction required to clamp the p300–p53 complex.

The oligomeric nature of p53 provides a unique model with which to define conformational elements that modulate the binding and acetylation of a target protein by the transcriptional co-activator p300. The N-terminal BOX-I domain of p53 was originally shown to contain a p300-binding site, as mutation of this region produces a transcriptionally inert protein. The first evidence for a multidomain component for the interaction between p53 and p300 came from data showing that phosphorylation of p53 at Ser15 by DNA-PK stimulates acetylation in the C terminus (11). Subsequent studies have shown that phosphorylation of p53 in the BOX-I domain at the CHK2 Thr18 and Ser20 phosphorylation sites can stabilize the p300–p53 protein complex (14). As different class of p53-activating kinases target the Ser15, Thr18, or Ser20 residues (ATM, CK1, and CHK2, respectively), these phosphorylation events provide a method for kinase signaling networks to regulate gene expression by altering the stability of the p300–p53 complex (14). Developing a consensus phosphate-binding motif for p300 that is distinct from the contact sites involved in the MDM2–p53 complex (Fig. 1) explains why kinase phosphorylation can differentially affect p300–p53 and MDM2–p53 complex stability and lead to the identification of phosphorylated-UBF as a p300-binding sites (Fig. 1, C and D).

Our mapping of one of the p53-binding domains of p300 to IBiD is consistent with recent studies (30), whereas our identification of a second domain with homology to IBiD (IHD) was previously undefined. The original studies on mapping the p53–p300 interactions have focused on the use of glutathione S-transferase fused N- and C-terminal fragments of p53 in pull-down and immunoprecipitation experiments. For example, the C-terminal region of p300 (amino acids 1990–2141) containing the Gln-rich domain and IBiD/SRC-1 binds p53 (amino acids 7–72) in vitro; consistent with our findings that p300–GAL4-C2 binds p53 (amino acids 1945–2141). Other groups have shown interactions between p53 and the KIX domain and CH1 or CH3 domain exclusively (47), which are not observed in vitro using our p300-phospho-ligand binding assays. However, weak binding of the CH3δ domain of p300 to the N-terminal activation domain of p53 was observed in cells

**DISCUSSION**

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In summary, the use of biochemical techniques to reconstitute the p300/p53 complex using active forms of full-length p300 has identified specific stages in the assembly reaction that include a docking-dependent and consensus site DNA-dependent acetylation of p53. The docking dependence in the DNA-dependent acetylation of p53 identifies a conformational constraint imposed on the p500-acetylation reaction at a promoter. Identification of the mechanism of conformational regulation of p53 acetylation may reveal novel factors that modulate acetylation of the DNA-bound transcription factor.

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