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Citation for published version:

Digital Object Identifier (DOI):

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular and Cellular Biology

Publisher Rights Statement:
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The Proline Repeat Domain of p53 Binds Directly to the Transcriptional Coactivator p300 and Allosterically Controls DNA-Dependent Acetylation of p53

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Received 24 March 2003/Returned for modification 5 May 2003/Accepted 29 July 2003

The transcription coactivator p300 cannot acetylate native p53 tetramers, thus revealing intrinsic conformational constraints on p300-catalyzed acetylation. Consensus site DNA is an allosteric effector that promotes acetylation of p53, suggesting that p300 has an undefined conformationally flexible interface within the p53 tetramer. To identify such conformationally responsive p300-binding sites, p300 was subjected to peptide selection from a phage-peptide display library, a technique that can define novel protein-protein interfaces. The enriched p300-binding peptides contained a proline repeat (PXXP/PXXP) motif, and five proline repeat motifs actually reside within the p53 transactivation domain, suggesting that this region of p53 may harbor the second p300 contact site. p300 binds in vitro to PXXP-containing peptides derived from the proline repeat domain, and PXXP-containing peptides inhibit sequence-specific DNA-dependent acetylation of p53, indicating that p300 docking to both the LXXLL and contiguous PXXP motif in p53 is required for p53 acetylation. Deletion of the proline repeat motif of p53 prevents DNA-dependent acetylation of p53 by occluding p300 from the p53-DNA complex. Sequence-specific DNA places an absolute requirement for the proline repeat domain to drive p53 acetylation in vivo. Chromatin immunoprecipitation was used to show that the proline repeat deletion mutant p53 is bound to the p21 promoter in vivo, but it is not acetylated, indicating that proline-directed acetylation of p53 is a post-DNA binding event. The PXXP repeat expands the basic interface of a p300-targeted transactivation domain, and proline-directed acetylation of p53 at promoters indicates that p300-mediated acetylation can be highly constrained by substrate conformation in vivo.

The tumor suppressor protein p53 is one of the most well-studied stress-responsive eukaryotic transcription factors that function in a damage-induced cell cycle checkpoint pathway. The biochemical activity of p53 linked to its tumor suppression function is a sequence-specific DNA binding and transactivation function that controls the expression of gene products implicated in cell cycle arrest and apoptosis (39).

p53 has been dissected into functional domains that contribute to its transactivation activity. The central domain of p53 contains the sequence-specific DNA binding domain that is often mutated in human cancers (31). Regulatory domains at the amino and carboxyl terminal of p53 modulate protein-protein interactions and DNA-protein interactions that affect the rate of p53-dependent transcription. The C terminus of p53 contains a domain whose phosphorylation at Ser15 in vivo by cyclin-dependent kinases (7) or at Ser392 by CK2/FACt stimulates the DNA-binding activity of p53 (23). The N-terminal domain of p53 contains the highly conserved BOX-I transactivation domain that directs the binding of p53 to the transcriptional adapter protein p300 (2). Phosphorylation of p53 in the transactivation domain at Ser15 activates p53 by an ATM-dependent pathway (37). Adjacent phosphorylation of the p53 activation domain at Thr18 or Ser20 by CHK2 activates p53 (36) by stabilizing the binding of p300 to p53 (11). Docking of p300 to the Thr18/Ser20 phosphorylated-LXXLL transactivation domain of p53 in turn promotes sequence-specific DNA-dependent acetylation of p53 in the C-terminal domain of p53, thus stabilizing the p300-p53Ac complex (12). These data highlight the complementary role of phosphorylation and acetylation in assembling a p53-p300 transcription complex.

The coactivator p300 plays a central role in signal integration with transcriptional components allowing for gene expression changes in response to a variety of stimuli (8). Tumor suppressor proteins like E2F and p53 recruit p300/CBP as their main coactivators, thus revealing these adapter polypeptides as key partners in transcription-dependent cancer control. In addition to the scaffolding role of p300/CBP, a role for the coactivator family in chromatin remodeling has been identified via an intrinsic acetyltransferase activity (24). The steady-state levels of histone acetylation mediated by p300/CBP and antagonizing histone deacetylases modulates chromatin remodeling and the rates of gene expression. Further, since the discovery that p300/CBP also acetylates nonhistone transcription factors like p53, E2F, and MyoD (18, 29, 35), most studies have demonstrated that the general role for acetylation appears to be in the stimulation of sequence-specific DNA binding.

The complex regulation and role of p53 acetylation is beginning to be unraveled (33). The original study using p53 showed that acetylation stimulates the latent DNA-binding function of p53 (18), while a later study did not show an effect of acetylation on activating the latent DNA-binding activity of p53 (14). We have started to reconstitute the stages in the assembly of...
the p300-p53-DNA transactivation complex in order to further clarify the regulation and function of p53 acetylation. Such studies have identified three key stages in the assembly reaction. First, phosphorylation by CHK2 at Thr18 or Ser20 in the p53 activation domain stabilizes p30 docked to the p53 activation domain (11) via the IBiD and IHD phosphopeptide binding domains of p30 (12). Second, this docking of p300 is essential for sequence-specific DNA-dependent acetylation of p53, indicating that p53 tetramer acetylation has intrinsic conformational constraints in the absence of DNA (12). Third, the function of acetylation as a post-DNA-binding event is to clamp the p300-p53AC complex into a very stable state (12). This clamping of p300-p53 after acetylation is consistent with cellular data showing that acetylation may function to recruit coactivator complexes at a promoter (4), presumably through the Bromo homology domain of p300/CBP which has the potential to bind to acetylated residues (28, 32).

One notable feature of the p300-p53 assembly reaction is the sequence-specific DNA dependence in p53 acetylation (12), which is consistent with recent ideas that DNA can function as an allosteric effector to regulate protein-protein interactions at a promoter (26). The DNA dependence in p53 acetylation indicates that conformational restraints are placed on the p300-catalyzed acetylation reaction through conformationally flexible motifs on p53. To identify such flexible p300-docking motifs in p53, combinatorial approaches were used to show that a second, conformationally flexible p300-binding motif exists within the proline repeat domain of p53. We show here that the proline repeat domain can bind directly to p300, that the proline repeat domain responds to conformational changes mediated by DNA binding, and that p53 binding to promoter sites in vivo places an absolute requirement on the proline repeat domain for acetylation to occur. The identification of a relatively ubiquitous proline repeat p300-binding transactivation motif complementing the classic hydrophobic LXXLL motif highlights an additional layer of combinatorial regulation of core p300 protein-protein interactions at a promoter.

MATERIALS AND METHODS

Plasmids and constructs. EGFP-PRO was constructed by ligating double-stranded oligonucleotides encoding amino acids (aa) 64 to 92 of human p53 (EGFP-PRO) into XhoI/Xbal-digested EGFP-C3 plasmid (Clontech). EGFP-NS, EGFP-BOX-I, EGFP-S20D, p32-Luc, Bac-Luc, pGIL3-Basic, PG13-CAT, MG13-CAT, and CMV-p300 have been described previously (12). Individual PXXP deletion mutants of p53 were constructed using PCR, and the integrity of the entire reading frame was confirmed by DNA sequencing. The pCMVp33APROAE plasmid (p33APXXP) and baculovirus were gifts from Arnold Levine (Rockefeller University, New York, N.Y.). pcDNA3.1-p53-6KR was obtained from Ron Hay (University of St. Andrews, St. Andrews, United Kingdom).

RESULTS

P300 binds proline repeat polypeptides. The phage-peptide display technique is a relatively powerful method to identify...
novel protein-binding domains on a polypeptide and to select for high-affinity peptide ligands under carefully controlled in vitro conditions. For example, using this approach Shimizu et al. previously identified a novel MDM2-binding site in the core domain of p53 that is required for p53 ubiquitination (38), and an independent study selected peptides from a phage-peptide library that only bind to the activated conformation of protein kinase C (1). The enrichment procedure we used involves presentation of approximately 10 ng of native protein to a phage-peptide library, and the selection for high-affinity peptides is usually extended to three rounds of binding and elution in order to develop a peptide consensus site. Two rounds of selection do not enrich enough to acquire peptides of a similar sequence, and four rounds of selection give only one sequence of the highest affinity.

To define the flexible contact site in p53 that mediates DNA-dependent acetylation by p300, peptides that bind to full-length p300 protein were selected from a phage-peptide library. Selection of high-affinity binding phage yielded four types of related proline repeat peptide consensus motifs: PXX PXXP, PXXP, PXXP, and PRLP (where P is proline, R is arginine, L is leucine, and X is any amino acid) (representative peptides are shown in Fig. 1A). The sequence ITFLYPKP PYPH was selected three times, the sequence NFMESSL PYPH was selected twice, and the PRLP motif was enriched in four peptides. The remaining peptides were enriched only once, and this proportion of duplicate to unique peptides with a common consensus motif indicates that the three rounds of selection were optimal for this enrichment of p300-binding peptides.

As a control to define peptide library integrity, purified MDM2 was used as bait, since MDM2 and p300 bind to the same N-terminal transactivation domain of the tumor suppressor protein p53. After three rounds of selection, the MDM2 consensus site FXXXXWWXXL was isolated (Fig. 1B). This consensus motif matches the amino acid residues involved in contacting MDM2 in the crystal structure of the MDM2-p53 complex (25). The sequence TSFAEYWNLLSGL was selected three times (two are depicted in Fig. 1B), and the remaining 20 sequences (data not shown) were unique apart from the core FXXXXWWXXL motif. As the majority of peptides were enriched only once, this indicates that the three rounds of selection were also optimal for acquiring a consensus peptide motif.

It is relatively surprising that we did not find LXXLL peptides enriched in this selection when using full-length p300, since many LXXLL motif-binding domains exist in p300, including C/H1, C/H3, IHD, and IBiD (Fig. 2C). The former two LXXLL-binding domains of p300 are histidine-cysteine rich and have a metal ion stabilizing the structure, while the latter two homologous LXXLL-binding domains are not necessarily stabilized by a cofactor. Since the numbers of duplicate PXXP peptides and unique PXXP peptides are relatively equivalent (Fig. 1A), the lack of LXXLL peptides enriched cannot be explained by overrepresentation of proline-rich peptides. This appears to suggest that (i) LXXLL peptides are not stable in *Escherichia coli* or that the phage containing these peptides grow very slowly; (ii) all four LXXLL-binding domains of p300 are denatured under these conditions; or (iii) the PXXP peptide-binding activity of p300 is dominant in this assay. The lack of enrichment of LXXLL peptides cannot be due to the denaturing of the LXXLL-binding domains of p300, since the pure protein is active in the LXXLL peptide-binding assay and in LXXLL-dependent acetylation of p53 (see below). Further, our preliminary data indicate that, using the minimal IHD domain of p300 (12) in the phage-peptide selection procedure, we do in fact acquire peptides with a very high degree of homology to the LXXLL domain of p53 (L. Finlan and T. R. Hupp, unpublished data). This indicates that the LXXLL-containing phage-peptides are not toxic to *E. coli* and/or prevent phage propagation. Thus, the reason for PXXP enrichment when using phage-peptide display appears to stem from the fact that this may be a dominant activity of full-length p300 or that the p300-PXXP peptide complex is relatively more stable than LXXLL peptide complexes.

The PXXPXPX, PXXP, PXXP, and PRLP motifs that were bound by p300 are actually present in a variety of LXXLL-containing transcription factors (Fig. 1C) and have the classic LXXLL motif flanking the PXXP repeat motif (data not shown). Two of these transcription factors stand out as relevant to this study: (i) SMAD-4 does not have a LXXLL activation domain but has an atypical activation domain that contains a proline-rich activation motif (10), and (ii) p53 has a proline repeat domain that is required for its transcription activity (40). However, the function of the polyproline domain in driving p53 activity is not defined. An in vitro peptide-binding assay was utilized to demonstrate that p300 could bind directly to these PXXP repeat domains. P300 bound both SMAD-4 PXXP repeat domains when increasing levels of the PXXP motif peptides were added to the binding assay (from 0.01 to 1 ng of peptide) (Fig. 1D, rows 1 and 2 versus the background of no peptide). All three PXXP-containing domains derived from p53 bound p300 protein (Fig. 1D, rows 3 to 5 versus background of no peptide). Phosphorylation of one PXXP repeat peptide at Thr<sup>31</sup> (JNK site) blocked the stable binding of p300 (Fig. 1E, row 3 versus rows 1 and 2). This contrasts with the stabilizing effect of phosphorylation at Ser<sup>20</sup> on the LXXLL motif binding to p300 (Fig. 1D, row 6 [BOX-I-S20P] versus row 7 [unphosphorylated BOX-I]) and highlights a difference in the sensitivities of the LXXLL- and PXXP-binding domains of p300 to phosphate addition.

The two phospho-LXXLL-binding domains of p300 map to an N-terminal domain named IHD (12) and a C-terminal domain named IBiD (27), and mapping of the proline repeat-binding domain of p300 was carried out by using an identical assay. In vitro PXXP peptide pull-down assay was employed that used p300 miniprotein domains obtained from HCT116 (p53<sup>-/-</sup>) cells transfected with the indicated GAL4-p300 fusion constructs (Fig. 2A). The p300-Gal4 fusion fragments were incubated with an anti-GAL4 antibody in microtiter wells to capture the p300 fragments onto the solid phase, the corresponding biotinylated peptides (nonphosphorylated LXXLL peptide from the BOX-I domain of p53, PXXP peptide, or no peptide) were added, and p300-peptide complex stability was quantitated using streptavidin-horseradish peroxidase. As a positive control, GAL4 fused to full-length p300 protein bound specifically to the PXXP-containing peptide relative to the unphosphorylated BOX-I domain of p53 (LXXLL) or no-peptide controls (Fig. 2A, row 2 versus row 1). The minimal PXXP-binding domains in p300 were fine-mapped to two distinct regions, one in the N terminus and one
The Gal4 fusion p300 miniproteins GAL4-2-337 (Fig. 2A, row 3) and Gal4-192-504 (Fig. 2A, row 4) bound specifically to the PXXP-containing peptide. This minimal PXXP-binding domain (SPC-1) in p300 flanks the N-terminal side of the peptide recognition domain in the C/H1 and IHD domains (Fig. 2C). The C-terminal site for PXXP contact was also mapped using fragments of p300. The Gal4 fusion p300 miniprotein lacking the IBiD domain, GAL4-1709-1913, bound significantly to the PXXP peptide (Fig. 2A, row 9). An immunoblot of the GAL4 fusion proteins demonstrates equivalent levels of each p300 miniprotein in each PXXP-binding reaction (Fig. 2B, lanes 1 to 7). This minimal PXXP-binding domain (SPC-2) resides between aa 1737 and 1913 (Fig. 2C).

In summary, the PXXP-binding domains of p300 (SPC-1 and SPC-2) flank the phospho-LXXLL-binding domains (IBiD and IHD) and the classic C/H1 and C/H3 domains, suggesting that p300 can embrace the p53 substrate over a relatively large interface.

Although the regions of p300 that bind p53 were originally reported to be within the C/H1 and C/H3 domains (15), more

FIG. 1. Proline repeat peptides bind p300. (A) Identification of novel p300-binding peptide motifs by phage-peptide display. The clones isolated using full-length recombinant p300 protein as a bait for phage-peptide display are highlighted, with the consensus motifs in grey, and include four representative subsets: PXXPXXP, PXXP, PRLP, and PXPXP. (B) Control reactions using MDM2. Using MDM2 as bait for phage-peptide display, clones containing the canonical MDM2-binding motif, FXXXWXXL, were isolated. (C) Sequence alignments of the four proline repeat motifs found in commonly studied transcription factors. (D and E) p300 binds directly to proline repeat motif peptides. Biotinylated peptides were used as a target for p300 in a microtiter well pull-down assay containing proline repeat peptide sequences from the aligned Smad4 (PXXPXXP, rows 1 and 2) and p53 (PXXP, rows 3 to 5) regions (D). As a control, p300 binds to the Ser^{39} phosphorylated LXXLL motif from the known BOX-I transactivation domain of p53 (row 6) versus unphosphorylated LXXLL motif (row 7). Phosphovariants of the PXXP domain at Thr_{81} (JNK site, rows 1 and 2) or the unphosphorylated PXXP domain of p53 (row 3) were used as ligands (E). The amounts of peptide captured are indicated (0, 0.01, 0.1, and 1 ng), and the amount of p300 protein bound to the indicated peptide domain was quantitated as RLU by using a peroxidase-linked secondary-antibody coupled to an anti-p300 antibody.
FIG. 2. Mapping of the proline repeat binding domain of p300 (SPC-1/2). (A) HCT116 p53−/− cells were transfected with 5 μg of GAL4 control (row 1), GAL4-p300 (row 2), or the indicated GAL4-p300 miniproteins derived from the N and C termini of p300 (rows 3 to 9). Lysates were captured onto the solid phase with an anti-GAL4 antibody, and the indicated biotinylated peptide (1 ng) (PXXP peptide [from p53 aa 55 to 74], unphosphorylated LXXLL motif [from p53], or no peptide) was added and the amount of peptide bound to p300 protein was quantitated as RLU by using peroxidase linked to streptavidin. (B) Quantitation of GAL4 fusion protein levels. HCT116 p53−/− cells were transfected with 5 μg of GAL4 control (lane 1), GAL4-p300 (lane 2), or the indicated GAL4-p300 miniproteins derived from the N and C termini of p300 (lanes 3 to 9). Lysates were immunoblotted with an anti-GAL4 antibody. (C) Schematic diagram illustrating the two minimal sites for proline contact (SPC-1 and SPC-2) relative to other p300 subdomains including CH1, IHD, CH3, KIX, Bromo, CH2, and IBiD. The minimal N-terminal SPC-1 domain (aa 192 to 337) is distinct from the phospho-LXXLL p53-binding domain IHD. The minimal C-terminal SPC-2 domain (aa 1737 to 1913) is distinct from the IBiD domain and partially overlaps with the CH3 domain (aa 1653 to 1817).
recent reconstitution studies have identified two new phospho-LXXLL-binding domains named IHD (12) and IBiD (27). With these added to the PXXP-binding domains in p300, there are at least six domains of p300 that can contact p53 under various conditions. Although the IHD and IBiD domains may be higher-affinity binding regions for p53, this does not exclude a contribution of the C/H1 and C/H3 domains of p300 since it is not clear how p300 embraces the tetravalent p53 substrate. For example, for p53 tetramer acetylation to occur there may be a requirement for one C/H1 domain to bind to one monomer, IHD to bind to another monomer, IBiD to bind to a third subunit, and a PXXP-binding domain to contact a fourth monomer. Further, it is possible that the in vitro assay we used to measure p300 activity unfolds the C/H1 and C/H3 domains, thus releasing IHD and/or IBiD domains to compensate. The main assay used to access p300 protein integrity is the docking-dependent acetyltransferase activity of p300. This purified fraction of p300 is active in histone acetylation, p53-phospho-LXXLL binding, PXXP-peptide binding, p53 protein binding, and LXXLL-dependent (docking-dependent) p53 acetylation. To maintain p300 protein activity and conformation from the outset, EDTA was not included in the lysis buffers nor was EDTA included in the GAL4-p300 fusion protein-binding assay. However, p53 is purified in buffers containing 0.1 mM EDTA (22). This amount of EDTA does not disrupt folding of the p53 tetramer nor does it dissociate zinc from the protein. When diluted into reaction buffers with purified p300, the final concentration of EDTA ranges from 1 to 3 μM, which is not known to disrupt the very stable Cys/His structure. Further, p300-coactivated transcription by p53 in vivo is phospho-LXXLL dependent (11) and is inhibited by transfected IBiD or IHD domains (12), suggesting that the in vitro character of p300 reflects its in vivo activity. Thus, it appears that in vitro and in vivo, the C/H1 or C/H3 domain of p300 contributes to p53 binding to a lesser extent than does IBiD or IHD. We are presently developing single, double, triple, and quadruple mutants of C/H1, IHD, C/H3, and IBiD in p300 to determine which are the major docking domains on p300 for the LXXLL motif of p53.

Proline repeat peptides inhibit p53 acetylation. The addition of consensus site DNA to reactions containing p53, p300, and acetyl-coenzyme A (CoA) stimulates p53 acetylation (12). Further, docking of p300 to the LXXLL motif of p53 is essential for DNA-dependent acetylation of p53, thus indicating two possibly interrelated conformational constraints to p53 tetramer acetylation (12). These two conformational constraints are released by consensus site DNA and presumably by a second p300-docking interface. If the proline repeat motif were in fact the second conformationally sensitive interface driving p53 acetylation, then there should be a connection between p300 binding to the proline repeat domain and p53 acetylation. We investigated whether the adjacent PXXP repeat motif of p53 was also required for mediating acetylation.

Using the p300 acetylation reaction where acetyl-CoA, the PXXP repeat peptide reduced the stable binding of p300 to p53 (Fig. 3E). Furthermore, the PXXP repeat peptide attenuated acetylation of p53 in the complete acetylation reaction when p53 was captured in ELISA format (Fig. 3F), similar to that seen in the immunoblot (Fig. 3B). The PXXP peptide, at concentrations ranging from 50 to 200 μM, is a more potent destabilizer of the p53-p300 protein complex than is the phospho-LXXLL peptide (Fig. 3E), and the extent of destabilization mirrors the extent of inhibition of p53 acetylation (Fig. 3F). These data suggest that the proline repeat motif is the second interaction site we were looking for and indicate that p300 contacts two contiguous peptide domains on p53 (LXXLL and PXXP).

There is some apparent inconsistency between the assays we used to assess p300 binding to PXXP peptides. First, the PXXP peptides in solution were more effective inhibitors of p300-catalyzed acetylation than the phospho-LXXLL peptides (Fig. 3F). Consistent with this, PXXP peptides were enriched in phage-peptide display (Fig. 1A), suggesting that proline repeat rather than leucine repeat peptides bind better in solution to p300. Second, both PXXP and phospho-LXXLL peptides disrupt the p53 tetramer-p300 complex (Fig. 3E), indicating that disruption of one docking interaction prevents the other from compensating. However, the PXXP peptides bind more poorly to p300 in the solid-phase ELISA than the phospho-LXXLL peptide (Fig. 1D), and this assay may underestimate the avidity of p300 for PXXP peptides. It is possible that the PXXP peptides are hindered sterically by attachment to the solid phase and that this attenuates p300-PXXP peptide complex stability. For example, we found that a phosphospecific monoclonal antibody binding to a phosphopeptide can be hindered by attachment of the peptide to the solid phase but the monoclonal antibody can bind when the phosphopeptide is cross-linked to bovine serum albumin and attached indirectly to the solid phase (data not shown). Thus, according to the results of three different assays, the affinities of the PXXP and phospho-LXXLL peptides for p300 are different: phage-peptide display suggested that the PXXP peptide-p300 complex is much more stable than the LXXLL-p300 complex, the ELISA suggested that the phospho-LXXLL peptide binds better to p300 than does the PXXP peptide, and the acetylation assay showed that the PXXP peptides are more effective inhibitors.

To begin to address the significance of the PXXP repeat domain to p53-dependent transcription, we fused a 30-aa PXXP repeat peptide (aa 64 to 93) to EGFP for in vivo transfection. The cotransfection of either phosphomimetic LXXLL (S20D) or PXXP peptide-GFP fusions with the p53 gene attenuated p53 activity from the p21 or bax promoters (Fig. 4A and B). p300 binding to p53 is required for p53 stabilization after DNA damage (42), and we investigated whether the EGFP-LXXLL phosphomimetic peptide or the EGFP-PXXP peptide could neutralize p300 and destabilize p53 protein in vivo. Notably, the cotransfection of either LXXLL or PXXP-EGFP fusions alone did not affect basal p53 protein levels (Fig. 4C, lanes 6 and 8 versus lane 2). However, the cotransfection of the LXXLL and PXXP-EGFP fusions together induced a striking decrease in steady-state p53 protein levels (Fig. 4C, lane 10 versus lane 2). The levels of the EGFP fusions remain equivalent in each transfection (Fig. 4D, lanes 2, 4, 6, 8, and 10). The cotransfection of LXXLL and PXXP-EGFP fusions
alone or together was sufficient to attenuate endogenous p21 protein levels (Fig. 4E, lanes 6, 8, and 10 versus lanes 2 and 4), which is consistent with the data showing that p21-Luc reporter activity is reduced by the peptides (Fig. 4A). This decrease in p53 protein steady-state levels by cotransfection of both LXXLL and PXXP-EGFP fusions is due to proteosome-dependent degradation (Fig. 4F, lane 2 versus lane 1). These data indicate that although disrupting either of the two p300-docking sites attenuates p53 activity in cells, disrupting both p300-binding motifs is required to promote p53 protein degradation.

These in vivo data are consistent with in vitro data showing that the phospho-LXXLL or PXXP peptides inhibit p53 acetylation (Fig. 3F).

The effects of the LXXLL and PXXP-EGFP fusion proteins on MDM2-dependent ubiquitination of p53 were also examined (Fig. 4G). The cotransfection of the indicated EGFP constructs with His-Ub, MDM2, and p53 demonstrates that MDM2-dependent ubiquitination of p53 is not affected by the p300-binding peptides (Fig. 4G, lanes 6 to 8 versus lane 4). However, there was a marginal increase in p53 ubiquitination when using the MDM2 peptide inhibitor (BOX-I EGFP fusion) (Fig. 4G, lane 5 versus lane 4). The cotransfection of the LXXLL and PXXP-EGFP fusion proteins together resulted in p53 protein destabilization (Fig. 4H, lane 8 versus lanes 6 and 7). The transfected EGFP peptides were expressed at similar levels (Fig. 4I). Thus, two independent p300-binding domains (LXXLL and PXXP) are required to stabilize p53 protein.

It is curious that total p53 protein can be destabilized by the GFP fusion peptides that bind p300 without affecting MDM2-dependent p53 ubiquitination. Since the PXXP and DLXXLL GFP fusion peptides do not bind MDM2 in vivo, we expected few changes in MDM2-dependent monoubiquitination of p53. The monoubiquitinated p53 was pulled out by using a HIS pull-down assay, and this pool represents a very small pool of the total p53 protein. Therefore, the depletion of p53 protein levels (Fig. 4H) by the p300 binding peptides is more representative of the total pool of p53 than the minor proportion of p53 that is HIS monoubiquitinated. Presumably, therefore, in this cotransfection system, p53 ubiquitination is uncoupled from protein degradation.

Proline repeat transactivation motif promotes DNA-dependent acetylation of p53 by p300 in vitro and in vivo. The mechanism of proline-directed acetylation of p53 was determined by examining the effects of deleting the proline repeat domain on p53 acetylation. Sequence-specific DNA-dependent acetylation was observed with wild-type p53 (Fig. 5B, lane 2...
FIG. 4. p300-binding peptides attenuate p53 activity and destabilize p53 protein steady-state levels in vivo. (A and B) Competitive inhibition of p53-dependent transcription by p300-binding peptides. pCMV-p53 (1 μg) was cotransfected with the luciferase reporters (1 μg of p21-Luc [A] or bax-Luc [B] and the transfection control [pCMVβ-Gal] and either the GFP-NS [1 μg; control], GFP-LXXLL phosphomimetic [1 μg], or GFP-PXXP [1 μg] peptide). The RLU is expressed as a ratio of Luc to the internal transfection control (pCMVβ-Gal). (C to E) p53 and p21 protein levels are destabilized by cotransfection of GFP fusion p300-binding peptides. pCMV-p53 (1 μg) and the indicated GFP fusion construct (1 μg; NS, nonspecific; BOX-I, the MDM2-binding peptide; S20D, the phosphomimetic LXXLL; PRO, proline repeat) and the levels of the indicated proteins were determined by immunoblotting. (F) Destabilization of p53 by p300 targeting is mediated by the proteosome. Acetyl-Leu-Leu-norleucine (ALLN) was added for 2 h after transfection of the indicated plasmids for 24 h, and the levels of p53 protein are as indicated. (G to I) p53 ubiquitination by MDM2 is not altered after cotransfection of GFP fusion p300-binding peptides. pCMV-p53 (1 μg), the indicated GFP fusion construct (1 μg), pCMV-MDM2, or pCMV-His-ubiquitin was transfected for 24 h, and the levels of the indicated proteins were determined by immunoblotting. MDM2-dependent ubiquitinated products were purified using a His pull-down assay, blotted with p53 antibodies, and are shown in panel G.
versus lane 1), while the p53<sup>APXXP</sup> tetramer displayed no acetylation (Fig. 5B, lane 4 versus lane 2). The mixed wild-type p53/p53<sup>APXXP</sup> tetramer was also found to be defective in acetylation (Fig. 5B, lane 6 versus lane 2). Thus, despite the fact that this mixed tetramer had four LXXLL motifs and two PXXP motifs (six of eight docking motifs), p300 cannot acetylate wild-type p53/p53<sup>APXXP</sup>, suggesting a dominant negative effect of proline repeat deletion on p53 conformation. After incubation of the acetylation reaction mixture in the presence of nonspecific DNA for 5 h instead of 10 min (12), we could begin to see p53<sup>APXXP</sup> tetramer and wild-type p53/p53<sup>APXXP</sup> tetramer acetylation in the presence of nonspecific DNA (Fig. 5D, lanes 3 and 5 versus lane 1). The inclusion of consensus site DNA prevented acetylation of the proline-deleted p53 tetramer and the mixed wild-type p53/p53<sup>APXXP</sup> tetramer (Fig. 5D, lanes 4 and 6 versus lanes 3 and 5), further indicating that the DNA-bound conformation of p53 requires the PXXP motif to mediate acetylation.

The acetylation of p53 in the presence of nonspecific DNA after 5 h of incubation reflects a reaction with a very low specific activity and may also reflect changes in p53 conformation over this time frame. The original acetylation study on p53 (18) demonstrated that bacterially expressed p53 can be acetylated, though the stoichiometry of acetylation on p53 protein was not determined nor was the percentage of p53 in the folded conformation determined. The folded and unfolded ratios of recombinant p53 protein can be quantified with monoclonal antibodies specific for the denatured or unfolded forms of the protein (20). The p53 protein used in our experiments is derived from insect cells, where the majority of the protein is folded (22). The enhanced 5-h incubation that resulted in some basal p53 acetylation in the absence of consensus site DNA may be due to the unfolding of the p53 tetramer, resulting in loss of the conformational constraint on acetylation (see Fig. 10). The native p53 tetramer is known to “breathe” in an equilibrium between unfolded and folded states (9), and this breasting presumably permits weak docking-independent and DNA-independent acetylation to occur.

How does consensus site DNA prevent p53<sup>APXXP</sup> tetramer acetylation? Two potential mechanisms could account for this: (i) after DNA binding, the p53<sup>APXXP</sup> tetramer binds p300, but the acetyltransferase activity cannot engage; or (ii) after DNA binding, the p53<sup>APXXP</sup> tetramer changes conformation to preclude p300 binding. To determine whether DNA binding by
the p53APXXP tetramer prevented p300 binding, we quantitated p300-p53APXXP complex stability in the absence and presence of consensus site DNA (from 0 to 40 ng) (Fig. 5E to G). In the absence of consensus site DNA, wild-type p53 bound significantly to p300 (Fig. 5E, column 1) and the p300-p53 complex was marginally stabilized further by addition of increasing amounts of consensus site DNA oligonucleotide (from 10 to 40 ng) (Fig. 5E, columns 2 to 4 versus column 1). Deletion of the PXXP motif did not prevent p300 from complexing with p53 (Fig. 5F, column 1), presumably due to the LXXLL activation domain in the tetramer. However, p53APXXP binding to p300 was completely destabilized by the addition of consensus site DNA (Fig. 5F, columns 2 to 4 versus column 1). Further, a mixed p53/p53APXXP tetramer was similarly destabilized from p300 by DNA (Fig. 5G, columns 2 to 4 versus column 1), indicating that the PXXP domain on wild-type p53 could not rescue the p300-p53APXXP complex. This explains why DNA-complexed p53APXXP cannot be acetylated by p53 (Fig. 5B and D), as p300 cannot form an interface with a DNA-bound p53 tetramer lacking PXXP repeat motifs. Thus, it is the proline repeat motif that places conformational constraints on p53 acetylation.

It was important to evaluate the contribution of the PXXP motif to DNA-dependent acetylation in vivo. We previously developed an in vivo consensus site-DNA-dependent acetylation assay for p53 (12). After cotransfection of genes encoding p53 and p53APXXP (Fig. 6A, lanes 1 to 8) along with either nonspecific or consensus site plasmid DNA, cells were lysed after 24 h and the transfected p53 protein was assayed for both in vivo acetylation and binding to endogenous p300 protein. After immunoprecipitation of transfected p53 protein and immunoblotting with acetylation-specific p53 antibodies, consensus site plasmid DNA-dependent acetylation was observed (Fig. 6B, lane 2 versus lane 1). After immunoprecipitation of endogenous p300 and immunoblotting with an anti-p53 antibody, the amounts of transfected wild-type p53 bound to p300 were similar after the cotransfection of either consensus site or nonspecific plasmid DNA (Fig. 6C, lane 2 versus lane 1). However, after immunoprecipitation of endogenous p300 and immunoblotting with an anti-acetyl-p53 antibody, only wild-type p53 cotransfected with consensus site plasmid DNA was acetylated (Fig. 6D, lane 2 versus lane 1).

Notably, the converse was observed when the proline deletion mutant, p53ΔPXXP, was used. After immunoprecipitation of endogenous p300 and immunoblotting with an anti-p53 antibody, the amounts of p53APXXP-p300 complexes were similar after the cotransfection of either consensus site or nonspecific plasmid DNA (Fig. 6C, lane 4 versus lane 3). Furthermore, after cotransfection of p53APXXP with consensus site plasmid DNA, the p53APXXP complex in complex with p300 was not acetylated (Fig. 6D, lane 4 versus lane 2). However, transfection of p53APXXP with the mutant consensus site supercoiled plasmid DNA did not appear to acetylate p53APXXP (Fig. 6C, lane 2 versus lane 1). After immunoprecipitation of transfected p53APXXP protein and immunoblotting with acetylation-specific p53 antibodies, acetylation was also observed in the presence of consensu site DNA. This suggests that in vivo, consensus site DNA places an absolute requirement for the proline repeat motif to drive p53 acetylation.
There is an apparent contradiction in data showing the effects of consensus site DNA on the stability of the p300-
PXXP mutant. While in vitro studies using purified proteins indicated that p53-PXXP cannot bind to p300 in the presence of consensus site DNA, after transfection the p300 still binds the p53-PXXP mutant in vivo, though the mutant p53 is not acetylated. It is possible that in vivo cotransfection would force the interactions of p53-PXXP and p300 even in the presence of transfected consensus site plasmid DNA, since it is known that p300 can recruit cotransfected proteins into nuclear inclusions or particles (16). Further, using purified proteins in vitro, a dose-dependent titration can be better controlled under conditions where the intrinsic stability of the complex can be assessed. Alternatively, differences in complex stability and the assay conditions could account for the differences. The solid-phase ELISA used to demonstrate that DNA prevents formation of a stable complex between p300 and p53-PXXP involves incubations with primary and secondary antibodies with washings over a 150-min time period, while the immunoprecipitation from cells involves quick washing times of only 15 min. The longer washing times in the ELISA would ensure that weakly bound p300-p53-PXXP complexes were not detected. This may be the most likely explanation for the discrepancy, since basal p53 acetylation by p300 in the presence of nonspecific DNA results in destabilization of the p300-p53 complex, while p53 acetylation by p300 in the presence of consensus site DNA results in stabilization of the p300-p53 complex (12). Thus, deletion of the PXXP domain may prevent p53 acetylation when the tetramer is DNA bound, most likely by increasing the off-rate rather than decreasing the on-rate for p300.

Deletion of a single PXXP motif attenuates p53 activity in vivo. Presumably, since p53-PXXP cannot be acetylated by p300 when bound to consensus site DNA in vitro or in vivo, the proline deletion mutant should be inert as a transcription factor in vivo. Saos-2 (p53−/−) cells were cotransfected with p53 or p53-PXXP genes with or without p300 and the corresponding p53-dependent luciferase reporter constructs (p21 or bax). p53-dependent transactivation of the p21 and bax promoters was attenuated by deletion of the PXXP domain of p53 (Fig. 7A and C, lanes 4 versus lanes 3) despite the similarity in expression levels of the wild-type and mutant p53 proteins (Fig. 7B and D, lanes 4 versus lanes 3). The p53-PXXP mutant failed to synergize with p300 protein, and its activity was inhibited on the p21 and Bax promoters relative to that of wild-type p53 (Fig. 7A and C, lanes 6 versus lanes 5). Since the nature of this transcription assay depends on reporter constructs, which have not necessarily been processed with eukaryotic chromatin assembly proteins and subsequent chromatin remodeling enzymes, the response from the endogenous p21 and bax promoters was normalized by Western blotting of endogenous proteins. Induction of p21 and Bax proteins was reduced after transfection of the p53-PXXP mutant relative to what was seen with wild-type p53 (Fig. 7B and D, lanes 3 and 5 versus lanes 4 and 6).

There are three PXXP repeats and one PXXP motif in the p53 activation domain from amino acids 71 to 90 (Fig. 7E), and we evaluated whether deletion of any proline repeat motif alters p53 activity and steady-state levels. Deletion mutants of each PXXP motif produced the recombinant proteins p53Δ71-75, p53Δ76-80, p53Δ81-85, and p53Δ86-90, and any one of these PXXP motif mutants attenuated p53-dependent transcription from the p21 reporter (Fig. 7F) and failed to induce endogenous p21 protein (Fig. 7I). Further, although the p53Δ72-75, p53Δ81-85, and p53Δ86-90 proteins were expressed at levels similar to that of wild-type p53 protein, p53Δ76-80 was expressed at very low levels (Fig. 7G). p53Δ76-80 protein was degraded by a proteosome-dependent pathway (Fig. 7H, lane 7 versus lane 6). A titration of lower amounts of p53 DNA in the transfection also revealed that p53Δ81-85 was expressed at very low levels (data not shown), indicating that the core proline repeat sequence, 76-APAAPTPAAP-85 containing the JNK phosphorylation site, is a major determinant of p53 protein steady-state levels. However, sites outside this core (aa 76 to 85) proline repeat region are likely to be important under differing conditions, since the Pro72-Arg72 polymorphism also has reduced specific activity under the conditions described above (data not shown).

p53 acetylation in vivo is a post-DNA-binding event. The role of the C-terminal domain of p53 is twofold. It functions as a negative regulatory domain whose phosphorylation stimulates in vitro and in vivo the specific DNA-binding function of p53 (7, 23). The molecular basis for p53 protein latency requires the C-terminal domain to destabilize and unfold the core DNA-binding domain by allosteric effects (5), suggesting that p53 is part of a growing group of regulatory proteins that are native and unfolded in the absence of posttranslational modification (13). Phosphorylation of p53 in the C-terminal domain neutralizes the destabilizing effect of the C terminus on the core domain of p53 and maintains p53 conformation and activity (30).

The C-terminal domain of p53 also functions as a positive regulatory domain that is acetylated and that recruits coactivator complexes (4, 14). A molecular explanation for why acetylation recruits coactivators may be found in the ability of acetyl-CoA to stabilize the p300-p53AC complex in the presence of consensus site DNA (12). Thus, although the role for acetylation of p53 remains relatively unclear (33), two complementary models are emerging. First, when “latent” fractions of p53 are used, acetylation can stimulate the DNA-binding activity of p53 by neutralizing basic residues implicated in nonspecific DNA binding (18). Second, when “kinase-activated” p53 protein is used, acetylation is a post-DNA-binding event and its role is to stabilize the p300-p53 AC complex (12). The proline repeat deletion mutant of p53 can be used to investigate which of these two models is favored in vivo. This relies on our observation that p53-PXXP is acetylated in vivo in the absence of consensus site DNA, but p53-PXXP cannot be acetylated in vivo in the presence of consensus site DNA (Fig. 6). If p53-PXXP was acetylated in vivo before p21 promoter binding, then acetylated p53-PXXP should be detected at the p21 promoter by ChIP. However, if p53-PXXP is acetylated only after DNA binding, then because the p53-PXXP-DNA complex cannot bind stably to p300 (Fig. 5), there should be no acetylated p53-PXXP cross-linked to the p21 promoter.

Transfection of p53 and two p53 mutants (PXXP deletion and the acetylation-defective mutant 6KR) into cells followed by formaldehyde cross-linking of protein-DNA complexes, immunoprecipitation of sonicated protein-DNA complexes, and PCR amplification with p21- (Fig. 8, bottom panel) and
FIG. 7. Deletion of a single PXXP motif attenuates p53 activity in vivo. (A to D) Deletion of the entire proline repeat domain inhibits p53. The transactivation activity of p53 and p53^ΔPXXP on the p21 (A) and bax (C) luciferase reporter promoters (RLU) is expressed as the ratio of p21-Luc or bax-Luc to the internal transfection control (pCMVβ-Gal). Expression levels of transfected p53 forms and endogenous target gene products are shown in panels B and D as follows: (B) transfected p53 protein (lanes 3 and 5) or p53^ΔPXXP protein (lanes 4 and 6) and endogenous p21 protein and (D) transfected p53 protein (lanes 3 and 5) or p53^ΔPXXP protein (lanes 4 and 6) and endogenous Bax protein were quantitated by Western blotting with an anti-p53, anti-p21, or anti-Bax protein antibody and enhanced chemiluminescence. In each transfection, 1 μg of pCMV-p53 or pCMV-p53^ΔPXXP alone or with 5 μg of pCMVβ-p300 was added as indicated. (E) Map of the clustered PXXP repeat motifs in the N-terminal activation domain of p53 and site of the PXXP deletions used in this study. (F) The transactivation activity of p53 and individual p53^ΔPXXP deletion mutants on the p21 luciferase reporter promoter (RLU) is expressed as a ratio of p21-Luc to the internal transfection control (pCMVβ-Gal). (G and I) Expression levels of transfected p53^ΔPXXP deletion mutant p53 proteins (G) and endogenous p21 protein (I) were quantified by immunoblotting. (H) The unstable PXXP deletion mutant (76-80) was stabilized by ALLN. Twenty-four hours after transfection of p53^Δ76-80, ALLN was added for 2 h prior to harvesting of the cells and p53 was blotted with DO-1 monoclonal antibody.
GAPDH-specific primers after reversing the chemical cross-link demonstrated a unique requirement for PXXP in promoting p53 acetylation at the p21 promoter. Using cell lysates transfected with the wild-type p53 gene (Fig. 8, lanes 6) or vector-only control (Fig. 8, lanes 5), immunoprecipitation of p300 (Fig. 8A) resulted in the coprecipitation and PCR amplification of the p21 promoter (as indicated in the bottom panel) or GAPDH promoter. The immunoprecipitation was carried out with antibodies specific for p300 (A), p53 (B), and acetylated p53 (C). The data are plotted as input DNA or as immunoprecipitated DNA. Quantitation of the bioluminescence is depicted below each lane. Controls include DNA amplified in reactions processed from cells without antibody in the immunoprecipitation reaction (lane 9) or with vector control only (lanes 1 and 5). The diagram at top depicts the region of the p21 promoter that was focused onto isolate p53-bound transcription complexes.

FIG. 8. The PXXP motif is required to acetylate p53 at the p21 promoter in vivo. HCT116 p53+/−/− cells were transfected with various p53 pCMV DNAs encoding p53 (lanes 2 and 6), p53^{APXXP} (lanes 3 and 7), and the nonacetylable mutant, p53-6KR (lanes 4 and 8). Following transfection of the indicated construct, cross-linking of protein-DNA complexes with formaldehyde, immunoprecipitation, and processing of the samples as described in Materials and Methods, the released DNA was PCR amplified using primers to the p21 promoter (as indicated in the bottom panel) or GAPDH promoter. The immunoprecipitation was carried out with antibodies specific for p300 (A), p53 (B), and acetylated p53 (C). The data are plotted as input DNA or as immunoprecipitated DNA. Quantitation of the bioluminescence is depicted below each lane. Controls include DNA amplified in reactions processed from cells without antibody in the immunoprecipitation reaction (lane 9) or with vector control only (lanes 1 and 5). The diagram at top depicts the region of the p21 promoter that was focused onto isolate p53-bound transcription complexes.
immunoprecipitation was carried out with antibodies specific for promoter (Fig. 8C, lane 7 [0.8 RLU] versus lane 5 [0.5 RLU]).

That a relatively large amount of p53 (Fig. 8B, lane 7 [55 RLU] versus lane 5 [0.5 RLU]), indicating coprecipitation and PCR amplification of p21 promoter sites in vivo. Using cell lysates transfected with the p53 gene (Fig. 9, lanes 7) or vector-only control (Fig. 9, lanes 6) or acetylhistone failed to immunoprecipitate the p21 promoter. The data are plotted as input DNA or as immunoprecipitated DNA. Quantitation of the bioluminescence is depicted below each lane. Controls include DNA amplified in reactions processed from cells without antibody in the immunoprecipitation reaction (lane 9) or with vector control only (lanes 1 and 5).

FIG. 9. The PXXP motif is required to recruit TRRAP and BRG-1 to the p21 promoter in vivo. HCT116 p53−/− cells were transfected with various p53 pCMV DNAs encoding p53 (lanes 2 and 6), p53APXXP (lanes 3 and 7), and the nonacetylatable mutant, p53-6KR (lanes 4 and 8). Following transfection of the indicated construct, cross-linking of protein-DNA complexes with formaldehyde, immunoprecipitation, and processing of the samples as described in Materials and Methods, the released DNA was PCR amplified using primers to the p21 promoter (as indicated in the bottom panel) or GAPDH promoter. The immunoprecipitation was carried out with antibodies specific for TRRAP (A), BRG-1 (B), and acetylated histone H4 (C). The data are plotted as input DNA or as immunoprecipitated DNA. Quantitation of the bioluminescence is depicted below each lane. Controls include DNA amplified in reactions processed from cells without antibody in the immunoprecipitation reaction (lane 9) or with vector control only (lanes 1 and 5).

p53’s PXXP motif prevents it from being acetylated at promoter sites in vivo. Using cell lysates transfected with the p53APXXP gene (Fig. 8, lanes 7) or vector-only control (Fig. 8, lanes 5), immunoprecipitation of p53APXXP resulted in the coprecipitation and PCR amplification of the p21 promoter (Fig. 8B, lane 7 [55 RLU] versus lane 5 [0.5 RLU]), indicating that a relatively large amount of p53APXXP protein is chromatin bound. However, the p53APXXP bound was not acetylated, since immunoprecipitation with acetyl-p53 antibodies did not result in the coprecipitation and PCR amplification of the p21 promoter (Fig. 8C, lane 7 [0.8 RLU] versus lane 5 [0.5 RLU]). This absence of acetylation can be explained by the inability of p53APXXP in complex with DNA to bind to p300 (Fig. 5). Similar data were obtained using ChIP from the bax promoter (data not shown). Thus, p53APXXP is not acetylated before DNA binding in vivo, confining acetylation of p53 in vivo to a proline-driven post-DNA-binding event. These data indicate that the proline deletion mutant behaves like an acetylation mutant, consistent with the sequence-specific DNA dependency in proline-directed acetylation.

We evaluated whether the proline repeat domain can influence the recruitment of additional transcription factors implicated in chromatin remodeling and activation of gene expression (Fig. 9). Using cell lysates transfected with the wild-type p53 gene (Fig. 9, lanes 6) or vector-only control (Fig. 9, lanes 5), immunoprecipitation of TRRAP (Fig. 9A) resulted in the coprecipitation and PCR amplification of the p21 promoter (Fig. 9A, lane 6 [35 RLU] versus lane 5 [8 RLU]). Furthermore, immunoprecipitation of BRG-1 resulted in the coprecipitation and PCR amplification of the p21 promoter (Fig. 9B, lane 6 [59 RLU] versus lane 5 [8 RLU]). Also, enhancement of histone acetylation was p53 dependent, since immunoprecipitation with acetylhistone antibodies resulted in the coprecipitation and PCR amplification of the p21 promoter (Fig. 9C, lane 6 [210 RLU] versus lane 5 [60 RLU]). In contrast to TRRAP and BRG-1 recruitment when wild-type p53 was used, p53APXXP was unable to recruit a significant amount of BRG-1 or TRRAP. Using cell lysates transfected with the p53APXXP gene (Fig. 9, lanes 7), the use of antibodies to TRRAP, BRG-1, or acetylhistone failed to immunoprecipitate the p21 promoter (Fig. 9A to C, lanes 7 versus lanes 5 and 6).

DISCUSSION
Regulation of eukaryotic gene expression is a dynamic process regulated by signal transduction networks that assemble and disassemble a variety of protein-protein machines. Characterizing these fundamental protein-protein interactions and their regulation will provide a molecular basis for understanding how the control of gene expression is linked to biological pathways such as differentiation, development, and cell cycle control. The coactivator p300 plays a central role in signal integration with transcriptional components, allowing for gene expression changes in response to a variety of stimuli (8). Understanding the mechanism whereby p300 and p53 cooperate as tumor suppressors will shed light on mechanisms that modulate cancer progression.

One key molecular stage in the cooperation between these two proteins links sequence-specific DNA binding by p53 and acetyltransferase activity of p300 (12). Conformational restraints on p53 acetylation by p300 are overcome by p53 binding to its consensus site DNA (Fig. 10). DNA-dependent acetylation of p53 requires p300 docking to the LXLL domain of p53 through at least two subdomains in p53, named IHD and IBiD (12). Importantly, however, the ability of consensus site DNA to override conformational constraints on p53 acetylation by p300 suggested to us that p300 contacts an undefined conformationally sensitive interaction site on p53. Such a model is supported by nuclear magnetic resonance studies showing that the surface of p53 changes conformation when in complex with consensus site DNA (34). This has led to our search for a second, conformationally sensitive domain on p53 that binds p300 and led to the identification of the PXXP repeat domain as the flexible motif driving sequence-specific DNA-dependent acetylation of p53. The direct interaction of the proline repeat motif of p53 with p300 is consistent with the importance of the proline repeat domain for p53 activity (3, 40). However, we show an absolute requirement for the proline repeat motif on p53 activity from transfected p21 or bax reporter templates, while other studies have shown that p53APXXP can be active from transfected p21 reporter templates (3).

There have been other molecular studies on proteins that interact with the proline repeat domain of p53, but none of these proteins explains the direct contribution of the PXXP domain to stress-activated transcription. Deletion of the proline repeat domain of p53 sensitizes p53 to MDM2-dependent degradation (6), presumably because this deletion releases p53...
from a “stable” partner protein. The key PXXP-binding protein that stabilizes p53 appears to be p300 based on four criteria. First, p300 is essential for stabilizing p53 in response to DNA damage (42). Second, p300 has an intrinsic proline repeat-binding activity (Fig. 1). Third, proline repeat peptides inhibit sequence-specific DNA-dependent p53 acetylation (Fig. 3). Fourth, the LXXLL and PXXP-GFP fusion peptides can together destabilize p53 in vivo (Fig. 4). These latter data are inconsistent with a role for p300 in degrading p53 (17), unless the sequestration of the PXXP- and LXXLL-binding domains of p300 by the LXXLL and PXXP-GFP fusion peptides frees up alternate domains in p300 to degrade p53 protein. Other proteins can bind to the PXXP domain, including histone deacetylases and proline isomerases. Histone deacetylase binding to the PXXP domain switches on the transrepression function of p53 (43), which is activated in response to hypoxia, thus indicating a fundamental difference between classic DNA damage and oxygen deprivation. It may be that the energy limitations in cells orchestrating a p53 response in cells undergoing anaerobic metabolism induce a more passive switch-off of gene expression, rather than the more energetically demanding DNA damage response. The ability of proline isomerase to stimulate p53 identifies another proline-related modifier of p53 (41). It will be interesting in the future to examine whether proline isomerases or hydroxylases antagonize or synergize with p300 and affect DNA-dependent acetylation of p53.

In summary, a direct function for the proline domain in mediating the transactivation function of p53 was identified: the PXXP motif binds p300. There are therefore two distinct motifs (LXXLL and PXXP) in p53 that bind directly to p300 and that are required for p300-catalyzed acetylation. The ubiquity of the PXXP domain in many transcription factors (Fig. 1) flanking the classic LXXLL motif expands on the core p300-activation motifs. Our preliminary data indicate that proteins known to bind p300 and having both the LXXLL and PXXP motifs, including IRF-3 and E-KLF, do in fact require both motifs for maximal transcription activity (data not shown). The intrinsic conformational constraints on native, folded, p53 tetramer acetylation by p300 can be overcome after p53 DNA binding via p300 contacting two transactivation domains in p53 (Fig. 10). This built-in restraint presumably ensures that acetylation of p53 only occurs at promoters in vivo and reveals an allosteric role for DNA in controlling protein-protein interac-

FIG. 10. DNA induces a conformational change that mediates proline-directed p53 acetylation by p300. (A) In the absence of DNA, p300 can dock to the p53 tetramer via LXXLL and PXXP binding, but conformational constraints in the native p53 tetramer prevent acetylation. (B) Sequence-specific DNA binding changes the conformation of p53 (34), thus permitting acetylation to occur in a PXXP-dependent manner. Thus, DNA binding does not change p300 binding as much as it activates p53 acetylation, suggesting that in the DNA-free state the C terminus of p53 is cryptic with respect to acetylation. (C) When the PXXP domain is deleted, p300 cannot acetylate DNA-bound p53APXXP due to the inability of p300 to form stable contacts with the p53AxxP tetramer. However, the p53AxxP tetramer can be acetylated by p300 in the absence of DNA, with the proline deletion essentially converting the p53APXXP tetramer to a histone-like substrate which can be acetylated in a docking-independent and DNA-independent manner. These latter data also suggest that DNA binding creates a specific interface in p53 for p300 and that the PXXP domain forms an integral part of this interface. Thus, both the PXXP/LXXLL domains and the C-terminal acetylation motif act concertedly after DNA binding to permit p300-catalyzed acetylation. There is a precedent for the C-terminal acetylation domain of p53 being cryptic in the DNA-free state. The p300 acetylation sites in p53 are within the epitope for monoclonal antibody PAb421. When p53 is DNA free, the PAb421 epitope is cryptic and DNA binding exposes the epitope. This led to the postulation that long-range allosteric effects mediate DNA binding by p53 (19). The cryptic nature of the p53 acetylation motif in the DNA-free conformation of p53 builds into the tetramer an intrinsic negative regulatory mechanism to prevent acetylation until the tetramer is promoter bound.
tions at a promoter (26). The fact that protein kinases and proline isomerases modify the core LXXLL and PXXP transactivation domains of p53 identifies signal transduction pathways, like CHK2 and proline isomerase, that stimulate p300 coactivated p53-dependent transcription. Furthermore, the realization that many transcription factors have LXXLL motifs contiguous to PXXP motifs expands our understanding of the basal p300 activation domain interactions at a promoter that may scaffold, bridge, or mediate the recruitment of multiprotein complexes at promoters in vivo.

ACKNOWLEDGMENTS

D.D. was supported by a PhD studentship from the BBSRC. T.R.H. is supported by a program grant from Cancer Research UK, an MRC Career Establishment grant, and the Association for International Cancer Research.

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