The N-terminal Interferon-binding Domain (IBiD) Homology Domain of p300 Binds to Peptides with Homology to the p53 Transactivation Domain*

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Two high affinity Ser-20-phospho-LXXLL p53-binding domains of p300 map to the C-terminal interferon-binding domain (IBiD) and N-terminal IBiD homology domain (IHD) regions. Purified fractions of a recombinant IHD miniprotein are active in a set of in vitro assays highlighting its affinity to the N-terminal LXXLL domain of p53 including (i) dose-dependent binding to Ser-20-phosphorylated p53 tetrRAMs; (ii) DNA-stimulated binding to p53 tetrRAMs; and (iii) inhibition of MDM2-mediated p53 ubiquitination. The active component of the IHD miniprotein was localized to a 75-amino-acid fragment corresponding to amino acids 401–475 on human p300. This minimal IHD miniprotein can function in vivo as a p53-binding polypeptide in assays including: (i) complex formation with VP16-LXXLL peptide motifs in the two-hybrid assay; (ii) action as a dominant negative inhibitor of p53 from p21 luciferase templates; and (iii) attenuation of endogenous p21 protein levels. Further, we show here that the IRF-1-dependent stabilization and reactivation of p53ΔPRO protein (LXXLL/PXXP) can be neutralized by the minimal IHD miniprotein, suggesting that IHD can bind to the p53 LXXLL domain in vivo. Phage-peptide display to the IHD miniprotein gave rise to an LSQXTFSXLXXL consensus binding site that displays significant homology to the LXXLL transactivation domain of p53. These data validate the IHD scaffold as an independent LXXLL peptide-binding domain within the p300 protein, complementing the known peptide-binding domains including IBiD, C/H1, and C/H3.

Characterizing the protein-protein interactions that assemble transcription complexes will provide a molecular basis for combinatorial specificity at the promoter level and give an insight into how transcriptosomes affect tumorigenesis. The tumor suppressor p53 is a central regulatory component of an evolutionary conserved mechanism that responds to certain stresses that impinge upon genomic integrity by inducing regulated responses such as cell cycle arrest, DNA repair, and/or programmed cell death (1). The stress-activated tumor suppressor protein p53 has been well characterized as a transcription factor that associates with basal transcription machinery. The vast array of genes that p53 induces and/or 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 (2). Central to the stress-activated transcription program of p53 is the interaction with the transcriptional co-activator p300 (3).

The p300/CREB-binding protein family of proteins participate in many physiological processes, including proliferation and differentiation and apoptosis (4). p300 functions as a transcriptional co-activator that is involved in multiple signal-dependent transcription events (5). Viral onco-proteins, such as the adenoviral E1A and SV40 T antigen, possess high affinity for specific binding sites with p300 (6), which results in the loss of cell growth control, enhancement of DNA synthesis, and blocks in cellular differentiation (7). There are data implicating altered p300 gene expression in an array of human tumors (5). p300 contains three broad functional domains including: (i) an acetyltransferase domain that mediates substrate acetylation; (ii) a bromodomain, which is implicated in binding to acetylated amino acids; and (iii) a variety of LXXLL, PXXP, and other transactivation peptide-binding domains. The coordinated function of these domains is thought to mediate the role of p300 as a bridge, scaffold, and/or catalyst for binding to transcriptional components and the activation of gene expression.

The tetrameric nature of p53, its allosteric properties, the enzymatic activity of p300 involved in its activation of p53, and the multiple transactivation peptide-binding domains of p300 make the p300:p53 complex an attractive model to understand the dynamic nature of a biological machine, an important goal in the dissection of the proteome. The molecular mechanism by which p53 tetrarams interact with p300 and how this modulates acetylation are beginning to be defined biochemically. Reconstitution of purified forms of the p300:p53 protein complex in vitro followed by validation in vivo have identified sequential stages in p300:p53 complex dynamics and include: (i) docking-dependent phosphorylation of the LXXLL motif at Thr-18 or Ser-20 by enzymes of the calcium-calmodulin superfamily such as CHK2 and CHK1 (8); (ii) stabilization of p300 binding to the p53 LXXLL domain in vitro by phosphorylation at Thr-18 or Ser-20 (9); (iii) anchoring of p300 to the contiguous phospho-LXXLL and PXXP activation domains in p53 (10); (iv) sequence-specific DNA binding by p53, which induces a conformational change in the tetramer, forcing acetylation to be DNA-dependent and PXXP-dependent (10); and (v) acetylation of DNA-bound p53 that in turn stabilizes the p300:acetylated p53:LXXL complex (11). How this multidomain scaffold arranges itself onto an “octavalent” substrate such as p53 is not known, nor is it known how acetylation of the native p53 oligomer can be constrained in the absence of consensus site DNA. The ability of IF-1 protein to bind to p300 and stimulate p53 acetylation by a novel allosteric mechanism (see also Ref.

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IHD can contact the L\_LL-p53 scaffold that promotes the formation of transcriptionally active complex between p53 and the co-activator p300.

22) further highlights the multifaceted and dynamic nature of the p300:p53 complex assembly. To continue dissecting out how p300 can bind to p53, we have mapped the various Ser-20-phospho-LXXLL and PXXP peptide-binding domains of p300 and have found them to map to regions distinct from the classic C/H1 and C/H3 p53 activation-binding domains (10). These peptide-binding regions in p300, in turn, are distinct from the binding of IRF-1 activation domain interfaces on p300 (see also Ref. 22). Together with mapping data from other approaches, these data identify at least eight peptide-binding minidomains of p300 that can contact p53 including C/H1, C/H3, IBD, IHD, SPC-1, SPC-2, KIX, and Bromo.

The major Ser-20-phospho-LXXLL p53-binding domains of p300 have been mapped to the C-terminal IBD region (12) and N-terminal IHD scaffolds (11). Both of these minipeptides can function as dominant negative inhibitors of p300-coactivated stimulation of p53-dependent gene expression in cells (11). However, only the C-terminal IBD domain has been examined structurally (12). The IBD scaffold was defined by mapping regions of p300 that bind to phosphorylated activation domain fragments from IRF-3, although the minipeptide can also bind to non-phosphorylated activation domain fragments of p53. This apparent lack of “sequence specificity” in IBD minipeptide binding to activation domain peptides is rationalized by the ability of unstructured activation domain peptides to acquire structure by an induced fit mechanism upon scaffold binding. Thus, it may be the capacity of a peptide to form a helix that makes it a good IBD-binding motif. The structure and minimal LXXLL peptide binding activity of the N-terminal IHD minipeptide are not known, nor is it known whether the IHD scaffold has intrinsic peptide consensus site for selected polypeptides. Characterization of IHD and determination of whether it has a stringent peptide-docking site consensus will advance dissection of the p300:p53 complex assembly. In this report, we define the minimal IHD scaffold to a 75-amino-acid fragment with homology to the minimal IBD region of p300, demonstrate that IHD can contact the LXXLL region of p53 by a variety of assays including inhibition of IRF-1 reactivation of p53Apro, and demonstrate by phage-peptide display that the IHD minipeptide prefers a consensus peptide motif with significant homology to the LXXLL motif of p53. These data indicate that IHD is a bona fide LXXLL-p53 scaffold that promotes the formation of transcriptionally active complex between p53 and the co-activator p300.

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**EXPERIMENTAL PROCEDURES**

Reagents and Enzymes—Recombinant human p53 was purified from insect cells and bacterial expression systems using heparin-Sepharose, as described previously (13). The IBD domain of human p300 (amino acids 401–575) was subcloned into the Invitrogen pDONR201 entry vector and shuttled by recombination into the His-tagged destination vector for selected polypeptides. Characterization of IHD and determination of whether it has a stringent peptide-docking site consensus will advance dissection of the p300:p53 complex assembly. In this report, we define the minimal IHD scaffold to a 75-amino-acid fragment with homology to the minimal IBD region of p300, demonstrate that IHD can contact the LXXLL region of p53 by a variety of assays including inhibition of IRF-1 reactivation of p53Apro, and demonstrate by phage-peptide display that the IHD minipeptide prefers a consensus peptide motif with significant homology to the LXXLL motif of p53. These data indicate that IHD is a bona fide LXXLL-p53 scaffold that promotes the formation of transcriptionally active complex between p53 and the co-activator p300.

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**TABLE I**

| Mutagenesis primers |
|---------------------|
| **POD-1 C-terminal** |
| 401–428: | Fwd 5-gaatataacagctgaatttacgttagcacec-3 |
| 401–475: | Rev 5-gggtctacagaattacattgcagttac-3 |
| 401–502: | Fwd 5-gttgtaacctctctagaatfaa-3 |
| 401–502: | Rev 5-attacattcaggagtaacag-3 |
| 401–502: | Fwd 5-caagctctctgtcagtagtctag-3 |
| 401–502: | Rev 5-ggctggaagtgagggagactg-3 |
| 401–502: | Fwd 5-gaagtgtctcctgtgctctag-3 |
| 401–502: | Rev 5-gctggagaagggagactg-3 |

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**Development of a IHD Consensus Binding Site**

Johns Hopkins University, H1299, SAOS-2, and A375, as indicated, and cells were propagated in either McCoy’s (HCT116) or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The mammalian two-hybrid system for evaluating p300 minidomain binding to the LXXLL activation domain of p53 was described previously (11). In vitro ubiquitination assays using purified MDM2 and p53 were described previously (14).

**Phage-peptide Display**—Phage-peptide display using purified IHD minidomain was done as described previously for MDM2 (14) and p300 (10). Briefly, a PhD-12 phage display library kit (New England Biolabs) was used, which contains random 12-mer peptides fused to the coat protein (pII) of M13 phage. The displayed 12-mer peptides are expressed at the N terminus of pII and followed by a short spacer (Gly-Gly-Gly-Ser) and then the coding region of wild-type pHIII protein. The complexity of the library was defined at 1.9 × 10^{10} different peptide sequences, and the titer was 2 × 10^{12} plaque-forming units/ml. A Microlite 2 96-well flat bottom plate (Dynatech Laboratories) was coated with 1 μg/ml of the anti-His monoclonal antibody by diluting in 100 μl of coating buffer (0.1 M NaHCO_{3}, pH 8.6) and incubating overnight at 4 °C. The wells were then blocked with blocking buffer (3% bovine serum albumin in PBS, 0.1% Tween 20) for 1 h at room temperature. The wells were then washed extensively with PBS containing 0.1% Tween 20 followed by incubation of IHD protein (100 ng) in 100 μl of blocking buffer. After 1 h, followed by extensive washing with PBS containing 0.1% Tween 20, the wells were then incubated for 1 h with 1 × 10^{13} phage particles in PBS containing 0.1% Tween 20 followed by extensive washing in PBS containing 0.1% Tween 20. The phage particles were eluted by incubation with 0.2 μg/ml glycine-HCl (pH 2.2), 1 mg/ml bovine serum albumin with gentle rocking for 10 min and then neutralized with 15 μl of 1 M Tris-HCl (pH 9.1). Eluted phage particles were then amplified by infection of ER2378 cells for 4.5 h, and the phage were polyethylene glycol-precipitated, according to the manufacturer’s instructions. The biopanning procedure was repeated three times, and ~1 × 10^{11} plaque-forming units of the second or third round amplified eluate were used as input phage. In addition, the Tween 20 concentration in the binding and wash buffers was increased stepwise (from 0.1 to 0.5%), with each successive round of biopanning to reduce nonspecific binding of the amplified phage-peptide particles. The third round polyethylene glycol-precipitated phage were titrated, individual plaques were tested for IHD-specific binding activity, and the DNA was amplified and prepared for transformation into the manufacturer’s (New England Biolabs) protocol. The Applied Biosystems Prism 377 automated DNA sequencer was used to sequence the DNA with the -96gIII primer. The sequence data from the 12-mer peptides obtained were then analyzed using the s-motif, blast NCBI databases, and ClustalW to identify consensus peptide motifs with homology to known or uncharacterized proteins (see Fig. 8 for summarized examples).

**ELISA Quantitation of the Binding of IHD to p53**—A Microlite 2 96-flat bottom plate (Dynatech Laboratories) was coated with purified p53 fractions, as indicated, in 0.1 M sodium borate (pH 9) and incubated at 4 °C overnight. The wells were blocked with 5% bovine serum albumin in phosphate-buffered saline plus 0.1% Tween 20 and then incubated for 1 h at room temperature with the following: IHD protein (increasing amounts or fixed levels, as indicated) diluted in a total volume of 50 μl in buffer (containing 1.5% glycerol, 2.5 mM HEPES (pH 7.5), 0.02% Triton X-100, 0.5 mM dithiothreitol, and 0.1 mM benzamidine). After incubation for 1 h at room temperature with a 1:500 dilution of anti-His tagged antibody in blocking buffer, the reactions were then detected with rabbit-anti mouse horseradish peroxidase monoclonal antibody.
(DAKO) and developed as detailed above. When p53 consensus site DNA was included in the binding reactions, the consensus site PG DNA was added prior to p53 addition to the wells.

RESULTS

The effects of MDM2 and p300 binding to serine 20 phosphorylated p53 have been defined; MDM2 binding is not affected by the serine 20 phosphorylation (15), whereas p300 full-length p300 protein binding is stabilized by a phosphate addition at the serine 20 residue (9). The major serine 20 LXXLL phospho-binding domains of p300 were mapped to two regions: the C-terminal IBiD (interferon-binding domain) miniprotein and an N-terminal 17-kDa miniprotein with homology to IBiD (Fig. 1, A and B) that we have named IHD (IBiD homology domain).

To fine-map IHD further and evaluate its specificity for LXXLL-containing peptides, the His6-tagged 17-kDa miniprotein was chromatographically separated from serine 20 unphosphorylated p53 (i.e. fraction 16; Fig. 2A) can be chromatographically separated from serine 20 unphosphorylated p53 (i.e. fraction 16; Fig. 2A). When equivalent amounts of total p53 protein (2.5–100 ng; fraction 16 or fraction 20) were adsorbed onto ELISA wells, and fixed amounts of IHD were added, p53-dependent binding to the IHD miniprotein was detected with an anti-His monoclonal antibody (Fig. 2, B and C). The serine 20 phosphorylated pool of p53 (fraction 20) bound more stably to the IHD miniprotein (from 5 to 7 RLU; Fig. 1C) when compared with unphosphorylated p53 (fraction 16), which bound weaker to IHD (from 0.7 to 1 RLU; Fig. 1B).

A titration of the IHD miniprotein into ELISA wells containing fixed amounts of p53 in the solid phase also demonstrated more stable complex binding to serine 20 phosphorylated p53 (fraction 20 versus fraction 16, ~10 versus ~3 RLU, respectively; Fig. 2D). As a control for p53 protein fractions 16 and 20 normalization, the antibody DO-1 (which contacts p53 within the MDM2-binding site (Fig. 3A)) bound to p53 (fractions 16 and 20) to similar extents (~800 RLU; Fig. 2E). Further, the antibody DO-1 bound to p53 with a relative affinity ~50-fold higher than the IHD miniprotein (compare the RLU at saturating protein levels (>40 ng)), which is consistent with the known high affinity of the mAb DO-1 for its epitope. Together, these data indicate that purified forms of the IHD miniprotein show binding to p53 that can in turn be stabilized further by the phospho-serine 20 residue.

One notable feature of p53 acetylation is the DNA dependence in the acetylation reaction (11). These data suggest that DNA binding changes the conformation of p53 to permit a more stable docking of p300 to p53. In fact, sequence-specific DNA can stabilize full-length p300 binding to p53 in the absence of acetyl-CoA (11). We tested whether the purified IHD miniprotein bound more stably to p53 in the presence of consensus site DNA. A titration of consensus site DNA into an ELISA reaction containing fixed amounts of p53 in the solid phase stimulated IHD-p53 protein complex formation (Fig. 2, F and G). These data suggest that the reason DNA stabilizes the p300:p53 complex can be attributed, at least in part, to an enhanced affinity of the IHD miniprotein for the LXXLL activation domain in p53. The ability of DNA to change the conformation of p53 in the LXXLL domain identifies an allosteric effect in p53, in particular identifying an interaction between the LXXLL-containing transactivation domain and the core DNA-binding domain.

We next evaluated whether IHD can bind to the transactivation domain of p53 as defined by its ability to interfere with MDM2-mediated ubiquitination, which binds to overlapping amino acids in the activation domain (Fig. 3A). MDM2-mediated ubiquitination of p53 (Fig. 3B, lane 2 versus lane 1) is

FIG. 1. Domain structure of p300. A, minidomains of p300 that interact with the LXXLL motif of p53. The major functional domains with respect to p53 binding are as indicated: C/H1, IHD, KIX, C/H2, bromo, acetyltransferase active site (AT), C/H3, and IBiD. The minimal IBiD domain is expanded to show the helix-turn-helix-turn-helix secondary structural motifs, a1-turn1-a2-turn2-a3 (12). B, comparison of the amino acid sequences of IBiD and IHD regions of p300. The amino acid sequences of the IBiD and IHD domains are aligned with conserved amino acids shown in purple. The minimal amino acid sequence of the C-terminal IBiD domain (starting from amino acid 2058) and homologous amino acids of the N-terminal IHD region (starting from amino acid 438), are highlighted by the boxes labeled a1, a2, and a3.
FIG. 2. IHD preferentially binds to serine 20 phosphorylated p53 tetramers. A, separation of serine 20 phospho and serine 20 non-phospho isoforms of p53. Human p53 expressed in Sf9 cells was fractionated using heparin-Sepharose (13) into serine 20 phospho and serine 20 non-phospho isoforms, as defined by immunoblotting with an anti-serine 20 phospho-monoclonal antibody, as reported previously (8). The fraction containing p53 with a relatively large amount of serine 20 phosphorylation was fraction 20, whereas that with relatively low levels was fraction 16. B and C, IHD binding into reactions with differing amounts of p53. Increasing amounts of serine 20 phospho p53 (0–100 ng; fraction 20 (C)) and serine 20 non-phospho p53 (0–100 ng; fraction 16 (B)) were adsorbed onto ELISA wells, and a fixed amount of His-tagged IHD miniprotein was added. To detect IHD binding, anti-His mAb was added followed by peroxidase-linked secondary antibody and IHD binding to p53 is quantified as relative light units (R.L.U.). D and E, IHD titration (D) and DO-1 titration (E) into reactions with fixed p53. Fixed amounts of serine 20 phospho p53 (100 ng; fraction 20, triangle) and serine 20 non-phospho p53 (0–100 ng; fraction 16, square) were adsorbed onto ELISA wells, and increasing amounts of His-tagged IHD miniprotein (D) or monoclonal antibody DO-1 (E) were added. To detect IHD binding (after anti-His mAb addition) or DO-1 mAb binding, peroxidase-linked secondary antibody was added, and binding to p53 is quantified using...
added. Ubiquitination reactions were stopped after 10 min, and reaction products were blotted with DO-1 to detect the p53 ubiquitination ladder.

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FIG. 3. The IHD miniprotein inhibits MDM2-mediated ubiquitination (Ub) of p53. A, contact sites for MDM2 and p300 in the activation domain of p53. The N-terminal activation domain of human p53 (Hp53) along with the homologous region in fish p53 (Fp53) are indicated. The upper arrows highlight key contacts for MDM2, whereas the lower arrows highlight the contacts for p300 (Ref. 9). The underlined amino acids are conserved amino acids in the p300 consensus binding site. B, IHD inhibits wild type p53 ubiquitination by MDM2 in vitro. Ubiquitination reactions were assembled without MDM2 (lane 1) or with MDM2 (lanes 2–12). A control antibody (lanes 4–8) or increasing amounts of IHD (lanes 9–12) was added. Ubiquitination reactions were stopped after 10 min, and reaction products were blotted with DO-1 to detect the p53 ubiquitination ladder. C, in vivo p53 ubiquitination. P53 wild-type (lane 2) or mutant p53 encoded by the R175H allele (lane 3) genes (0.5 μg of DNA) were transfected alone or with MDM2 into H1299 cells (2 μg of DNA; lanes 4 and 5). After 24 h, the cell lysates were immunoblotted to examine for changes in their amount of ubiquitination products by immunoblotting with antibodies to p53 (DO-1), as described previously (Shimizu et al. (14)). D, IHD inhibits mutant p53G175H ubiquitination in vivo. Co-transfected mutant p53G175H and MDM2 genes (lanes 1–4) were co-transfected with increasing amounts of VP16-tagged IHD (0.1, 0.25, 0.5, and 1 μg of DNA, lanes 2–4). After 24 h, the cell lysates were immunoblotted to look for changes in their amount of ubiquitination products by immunoblotting with antibodies to p53 (DO-1). VP-16 antibody (lower panel) was used to show the dose-dependent increases in VP16-tagged IHD miniprotein levels that perturb MDM2-mediated ubiquitination of p53.

unaffected by a control antibody that binds to denatured p53 (Fig. 3B, lanes 4–8). By contrast, a titration of IHD results in inhibition of MDM2-mediated ubiquitination of p53 (Fig. 3B, lanes 9–12). In vivo ubiquitination of p53 catalyzed by MDM2 (Fig. 3C, lanes 4 and 5 versus lanes 2 and 3) can also be attenuated by IHD in a dose-dependent manner (Fig. 3D, lanes 2–4 versus lane 1). Together, these data identify three biochemical properties of the IHD miniprotein (amino acids 401–566), suggestive of its ability to interact with the LXXLL transactivation domain of p53 tetramers: (i) enhanced affinity for serine 20 phosphorylated p53 tetramers; (ii) DNA-stimulated IHD-p53 tetramer complex stabilization; and (iii) sterical occlusion of MDM2 from the p53 transactivation domain and inhibition of MDM2-catalyzed ubiquitination.

The LXXLL binding activity associated with IHD miniprotein was fine mapped by constructing progressive deletions of the IHD coding region (Fig. 4C). This was required to determine which region of the 17-kDa IHD miniprotein was primarily responsible for LXXLL binding activity, especially in comparison with its homology domain in the C terminus, IBiD. The IBiD domain of p300 was localized to a 46-amino-acid fragment that has homology to the extreme N terminus of IHD (Fig. 1B). The GAL4-VP16 in vivo two-hybrid assay was used to evaluate the specificity of the IHD miniprotein fragments, rather than in vitro-specific activity, since expression of the IHD deletion fragments from Escherichia coli proved relatively difficult (data not shown). In comparing the in vivo-specific activity of the IHD miniprotein and its deletion derivatives for LXXLL-containing peptides, two other peptide-binding domains were evaluated: (i) IBiD, which binds to phosphorylated activation domain fragments from IRF-3 as well as non-phosphorylated fragments of other activation domains (12) and (ii) SPC-1 (site for proline contact (10), a C-terminal minidomain of p300 containing only half of the C/H3 domain and amino acid sequences without the classic C/H3 domain) that was identified previously as having LXXLL peptide binding activity despite lacking the N-terminal 56 amino acids of the standard C/H3 domain (164 amino acids in length). As a control for the two-hybrid assay, MyoD fused to VP16 displayed affinity for its peptide sequence in inhibitor of DNA binding when fused to GAL4 (Fig. 4B), whereas the LXXLL peptide from p53 bound weakly to MyoD (Fig. 4B). Under the conditions used in the in vivo two-hybrid assay, the IHD miniprotein (401–566) was the most active under these conditions toward the LXXLL peptide from the entire p53 activation domain (Fig. 4C) and lower activity to the LXXLL peptide in a very short sequence from the LXXLL activation domain of p53 (Fig. 4C).

Alterations in growth conditions can affect the binding activity of SPC-1(PAN) (pan-reactive peptide binding region of p300) and IBiD for peptides containing the LXXLL motifs so that the IHD domain does not always display the highest activity for LXXLL-containing peptides (as in Fig. 4E; data not shown), suggesting that competition for endogenous LXXLL-binding proteins can effect the GAL4-VP16 interaction. The most extreme deletion of the IHD miniprotein (amino acids 401–427) well into the motif with homology to IBiD (Fig. 1B) inactivates the LXXLL binding activity associated with the miniprotein (Fig. 4F). Interestingly, this region of IHD maps to the first helix in the IBiD domain required for IBiD activity (12) and suggests that the minimal IHD domain directly overlaps...
with a similar primary structure in IBiD, despite the fact that the entire 165-amino-acid fragment within IHD and IBiD share homology. The IHD miniprotein that retained the most significant activity was IHD-(401–475) (Fig. 4), thus localizing the major LX{LL} peptide binding activity to the N-terminal half rather than the C-terminal region of the 17-kDa IHD miniprotein.

The specific activity of the IHD miniprotein in the direct LX{LL} binding assay was compared with its ability to function as a dominant negative p300 inhibitor by attenuating p53-dependent transcription from the p21-luciferase promoter. In this system, p300 can stimulate maximal p53-dependent transcription (Fig. 5A), which can be attenuated by transfection of increasing amounts of the IHD miniprotein encoding vector (amino acids 401–566) (Fig. 5B), consistent with its ability to bind to the p53 transactivation domain. Since IHD-(401–566) cannot bind to the proline-repeat transactivation domain (Fig. 4C), the ability of IHD to inhibit p53 activity is presumably not due to its binding to the proline-repeat transactivation domain, which can also contact p300 directly. Progressive deletions of IHD (401–533, 401–501, and 401–475) do not neutralize IHD activity as a p53 inhibitor (Fig. 5, C–E). However, deletions of IHD into the region with homology to the minimal IBiD fragment (IHD-(401–427)) lost activity as a p53 inhibitor (Fig. 5, G versus H). There were some differences in the specific activity of IHD miniprotein deletion variants as p53 inhibitors; for example, the IHD derivative (amino acids 401–501) was not as effective as a dominant negative inhibitor of p53 as other IHD derivatives (amino acids 401–553), especially at intermediate levels of gene transfected (Fig. 5D versus C and E). These data suggest that polypeptide fragments encoded from amino acids 476–501 can affect the specific activity of IHD as a p53-binding domain and/or that factors in vivo are binding to it in trans to the region and competing it away from p53.

The activity of IHD deletion fragments as dominant negative inhibitors of p53 in the p21-luciferase assay were compared with its ability to attenuate endogenous p53-dependent activity.
induction of p21 protein (Fig. 6). Transfection of p300 and p53 together gave rise to increases in p21 levels (Fig. 6, lane 4), relative to empty vector controls (Fig. 6, lane 1). The co-transfection of IHD (amino acids 401–566) attenuated p21 induction by co-transfected p300 and p53 (Fig. 6, lane 5 versus lane 4), whereas the IHD deletion derivative (IHD amino acids 401–427) that was inactive as a p53 inhibitor when p53 activity was measured from the p21-luciferase promoter (Fig. 5), was also unable to attenuate p21 protein induction by p300 and p53 co-transfection (Fig. 6, lane 9). The other IHD derivatives attenuated p21 protein induction to different extents (Fig. 6, lanes 6–8), with the IHD fragment (amino acids 401–501) being the least effective inhibitor (Fig. 6, lane 7). These latter data are consistent with the lower specific activity of the IHD fragment (amino acids 401–501) as a p53 inhibitor from the p21-luciferase promoter.

A second activation domain in p53 is proline-rich and is contiguous to the LXXLL activation domain (10). Both of these domains contact p300 at different regions, and we evaluated the affinity of the IHD miniprotein toward the p53 LXXLL activation domain in a p53 variant that lacks the second trans-activation domain within the proline-rich region (p53ΔPRO). Transfection of p53ΔPRO into cells results in very little activity from the p21-luciferase reporter (Fig. 7A, lane 2) and very little stimulation by co-transfected p300 (Fig. 7A, lane 6).

We have shown independently that the genetic basis for IRF-1 cooperation with p53 as an inducer of the p21 promoter (16) is due to the ability of IRF-1 to bind to p300 and allosterically stimulate DNA-dependent acetylation of p53. Although p53ΔPRO is inactive on its own due to removal of the proline-rich activation domain and inability to be acetylated by p300 when bound to DNA (10), transfected IRF-1 is effectively able to re activate p53ΔPRO in its ability to induce p21 expression (Fig. 7B, lane 4 versus lanes 5 and 2). Interestingly, transfection of increasing amounts of IRF-1 protein is also able to stabilize p53ΔPRO (Fig. 7B, lanes 2–4 versus lane 1). These data indicate that p53 activity can be uncoupled from the proline-repeat activation domain, presumably by the ability of IRF-1 to enhance p300 contact with the LXXLL activation domain in p53, which in turn stabilizes and activates p53ΔPRO. This hypothesis can be tested using the IHD miniprotein. If IRF-1 effects on activating p53ΔPRO are due to the stabilization of the p300:LXXLL motif, then we would predict that IHD would block IRF-1 stimulatory effects. Maximal p53ΔPRO activity from the p21-luciferase reporter induced by IRF-1 transfection (Fig. 7C, lane 3 versus lanes 2 and 1) can be attenuated by the 17-kDa IHD miniprotein (Fig. 7C, lanes 4–7 versus lane 3) as well as the 8-kDa IHD miniprotein (Fig. 7C, lanes 8–11 versus lane 3). The attenuation by IHD of IRF-1-de-
Development of a IHD Consensus Binding Site

p53\textsuperscript{ΔPRO} activity (Fig. 7C) can also be linked to the destabilization by IHD of p53\textsuperscript{ΔPRO} protein levels in the presence of IRF-1 protein (Fig. 7D). Using fixed amounts of p53\textsuperscript{ΔPRO} and inhibitory levels of the IHD miniprotein (Fig. 7D, lanes 1–3), a titration of IRF-1 protein actually destabilizes p53\textsuperscript{ΔPRO} (Fig. 7D, lanes 2 and 3 versus lane 1). Using fixed amounts of p53\textsuperscript{ΔPRO} and the small IHD derivative (amino acids 401–475) (Fig. 7D, lanes 4–6), a titration of IRF-1 protein similarly destabilizes p53\textsuperscript{ΔPRO} (Fig. 7D, lanes 5 and 6 versus lane 3). These data suggest that the LXXLL activation domain is indeed the target of IRF-1 stabilization of p53. These data suggest that the IHD miniprotein can contact the LXXLL domain of p53 in vivo.

Structural studies on the IBiD, C/H1, and C/H3 domains of p300 have demonstrated an interaction of the minimal peptide-binding domains with activation domain peptides of no apparent sequence similarity. It is generally held that such small peptides acquire helical structure when bound to the peptide-binding scaffold. Although our characterization of IHD indicates that it can bind to LXXLL-containing polypeptides, it is not yet clear whether the IHD miniprotein displays any sequence preference for the p53 sequence, LXXLL-containing peptides, or other alternate consensus sequences. We have previously used phage-peptide display as an in vitro method for identifying novel protein-protein interfaces in MDM2-p53 and p300-p300 complexes (10, 14). This technique has the advantage of fixing bait protein in a solid phase in a native conformation and incubating with a library containing random 12-mer peptides. The 17-kDa His-tagged IHD miniprotein was captured in the solid phase with an anti-His monoclonal antibody and incubated with phage libraries through three rounds of selection. Identical His-tagged bait capture methods identified proline repeat-containing peptides as preferentially enriched using full-length p300 (10). In this latter study, it was relatively surprising that LXXLL-containing peptides were not selected given the number of LXXLL-binding domains present in p300. When high affinity peptides were enriched after three rounds of selection, three general classes of peptides were identified. In the first class of peptides (Fig. 8A), a striking similarity in the peptides isolated to the p53 activation domain identified critical consensus site contacts for IHD into three small motifs across the activation domain: PLSQ, T(F/Y)S, and SXXLXXL. These data suggest that the IHD miniprotein can display some relative specificity for p53 activation domain fragments, although other peptides with a match to this consensus can be identified using an e-motif search engine (Fig. 8D). A second class of peptides (Fig. 8B) showed less of a similarity than class I peptides but did display the retention of a Gln residue (which is critical for full-length p300 to contact the serine 20 phosphorylated p53 activation domain), a core (T/S)(F/W)(S/T)(D/E) motif, and an LXXL motif. The third class of peptides displayed less resemblance to p53 but maintained a Gln residue and a relatively loose consensus of (S/T)QXXXXXXL. A general consensus site developed for IHD was PLSQFXSX-LXXLL and suggests that either the miniprotein has a very high affinity for the p53 activation domain fragment or that such consensus site peptide fragments have the capacity to adopt a helical conformation upon IHD scaffold binding, giving rise to an apparent consensus site.

**DISCUSSION**

p53 has been shown to be regulated by two distinct pathways that direct the biochemical actions of the tumor suppressor. MDM2 forms a component of a negative regulatory feedback pathway that promotes the ubiquitination of p53 and subsequent degradation of p53 through the proteasome (17). p300 competes for the same binding region as MDM2 present within the N-terminal LXXLL transactivation domain of p53. This co-activator protein forms part of a positive regulatory component that is essential for p53 required for p53 stabilization after DNA damage and induces its transcriptional responses. p300-catalyzed acetylation of native p53 tetramers requires two associated events: p300 docking to the two contiguous LXXLL and PXXP/PXP activation domains of p53 and sequence-specific DNA to function as an allosteric activator by removing conformational constraints on p53 acetylation. It is not known how p300 docks to the p53 tetramer, whether it involves one or more of the peptide-binding domains of p300.
including C/H1, C/H3, IBiD, IHD, SPC-1, or SPC-2, and whether a rearrangement of the p300:p53 complex occurs after acetylation to stabilize the p300-bromo domain binding to the acetylated p53 residue. Such a rearrangement or an allosteric mechanism appears to operate since a small peptide derived from IRF-1:p300 interface can bind p300 and stimulate in trans the enzymatic activity of p300 (22).

As a means to begin answering these questions, in this report, we have fine-mapped one p53 docking site on p300, named IHD, and provided evidence that this minidomain of p300 is a bona fide polypeptide-binding domain that possesses an intrinsic ability to bind to peptides with homology to the p53 LXXLL activation domain. One key feature of the IHD mini- protein is that it mimics full-length p300 in its stabilization by phosphorylation at the serine 20 residue (Fig. 2). Although serine 20 phosphorylation function was originally thought to be in the reduction of MDM2-binding to p53, biophysical studies have demonstrated that serine 20 phosphorylation has no impact on MDM2 binding (15). Rather, this phosphorylation stabilizes p300-binding to p53, and as an indirect consequence, prevents MDM2 binding to p53 (23). Genetic studies have shown that mutation of the serine 20 residue in cell lines or in mice models attenuates p53 apoptotic activity (24, 25), suggesting that phosphorylation at this residue is important for p53 function. Further mutation of the equivalent serine 20 residue in murine p53 or knockout of the known serine 20 kinase, CHK2, have the same phenotype in mice; p53 protein can still be stabilized by irradiation but has a lower specific activity as transcription factor or apoptotic inducer (21, 25). These genetic data can be reinterpreted to suggest that the role of serine 20 phosphorylation is to stabilize p300 binding based on the following data. (i) p53 activity is attenuated by serine 20 phosphopeptide mimetics that bind to and neutralize p300 (9); (ii) full-length p300 is stabilized by phosphorylation of the p53 activation domain at serine 20 (9); and (iii) serine 20 phosphopeptides can block DNA-dependent acetylation of p53 by p300 (11). In this report, we show that the minimal IHD miniprotein can bind preferentially to full-length p53 tetramers that are phosphorylated at serine 20 and that IHD binding can be further stimulated in binding to p53 by consensus site DNA.
(Fig. 2). This mimics full-length p300 properties and suggests that the IHD domain may be an important component of p53 stabilization to p300 after DNA damage via CHK2 phosphorylation at serine 20.

Based on previous data, it was suggested that p300 minodomains such as IBiD do not necessarily prefer a specific polypeptide primary amino acid sequence. Our work using phage-peptide display suggests that IHD can prefer a specific peptide sequence. To find novel high affinity ligands for IHD domain, we screened phage display peptide libraries for IHD-binding phage and sequenced a series of clones encoding 12-mer peptides. The peptide sequences showed surprisingly good homology with the previously established p300-binding domain on p53 (14–25 amino acids), confirming that the peptide PLSQETFSDLWKLLP region is crucial for the interaction between p300 and p53 and that IHD can be responsible for stabilizing p300:p53 complexes that are transcriptionally competent. Underlined residues represent the match of the phage-peptides to the p53 activation domain. The selection for the three submotif-containing peptides (LSQ...F S...L...L ) is not common in our experience of this technique and therefore not due to the overenrichment or “stickiness” of this peptide class in the phage library. For example, using full-length p300, we enriched proline-repeat peptides (PXXP), using DAPK3, we enriched p21-like peptides (RKK...) and using ligand-complexed MDM2, we enriched a variety of peptides with little homology to the p53 activation domain (10, 14, 18). Further, using ligand-free MDM2 as a control, we did acquire peptides with homology to the p53 activation domain but with the consensus TFXXWXXL that fits with the known contacts in the MDM2-p53 activation domain structure (14).

Another advantage of phage-peptide display is that peptide consensus sequences developed can be used to identify novel binding partners. We have used this to identify HSP90 as a novel MDM2-binding protein that controls p53 unfolding and have identified novel cell cycle substrates of the interferon-induced kinase DAPK3 (14, 18). Further, the proline-repeat peptides pulled out using p300 as a bait (10) identified a large panel of LXLL containing transcription factors that, like p53, have flanking LXXLL motifs of a contiguous PXXP motif in their transactivation domain. In examining the IHD consensus peptides for homology to other proteins in the databases, there are several other interesting transcription factors and viral onco-proteins that possess IHD consensus sites that could potentially interact also with p300 through this docking site domain. These proteins include oncogenic Ras-related Rab 721, cyclin G, CHK2, SV40 large T antigen, DNA and RNA polymerases, Epstein-Barr nuclear antigen 6 (EBNA-6), and more (Fig. 8). The result that large T-antigen could potentially bind p300 via IHD is of great interest since p53 responses are abrogated in the presence of T-antigen and SV40 infection culminates in the degradation of p53 and loss of proliferative control (19), thereby supporting the notion that T-antigen has another mechanism of disrupting the critical activities of tumor suppressor proteins. In fact, T-antigen was reported to bind to p300 (20), and this may be mediated in part through the IHD domain.

In summary, we have characterized the minimal IHD LXXLL peptide-binding region of p300 and determined that it can display similar characteristics of full-length p300 including affinity for LXXLL-containing peptides, DNA-stimulated binding to serine 20 phosphorylated p53 tetramers, and in vivo

**Fig. 8.** Phage-peptide display identifies a consensus sequence for the IHD miniprotein. A–C, purified bacterial expressed histidine-tagged IHD was captured onto ELISA wells with monoclonal anti-His6 antibody. Following an incubation with naive phage-peptide library, elution of the phage with acid, and propagation of eluted phage, three rounds of amplification were carried out to acquire phage that bound with a high affinity to the p300 minidomain IHD. The peptide inserts in each phage were sequenced to determine the insert peptide sequence. Thirty clones were tested, revealing consensus sequences with over 50% homology to the conserved BOX-I motif within p300, and three representative classes of peptides are as depicted. D, an overview of the consensus match identified by phage display when directly compared with the evolutionary conserved transactivation domain of p53. The minimal core LSQXXF(S/T) consensus was screened using the e-Motif search engine database for domains with homology. The results from this screen suggest that a number of transcription-associated factors and viral onco-proteins possess domains with homology to the IHD consensus and thus have the potential to associate with p300 via its IHD region.
interaction with the LXXLL activation domain of p53. Future work will involve determining the dominance in the known LXXLL-binding domains in p300 including C/H1, C/H3, IBiD, and IHD and how these domains effect DNA-dependent acetylation of p53.

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