Regulation by phosphorylation of the relative affinities of the N-terminal transactivation domains of p53 for p300 domains and Mdm2

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Abstract

The transcriptional activity of the tumor suppressor p53 requires direct binding between its transactivation domain (TAD, 1-57) and the transcriptional coactivator p300. We systematically assessed the role of TAD phosphorylation on binding of the p300 domains CH3, Taz1, Kix and IBiD. Thr18 phosphorylation increased the affinity up to 7-fold for CH3 and Taz1, with smaller increases from phosphorylation of Ser20, Ser15, Ser37, Ser33, Ser46 and Thr55. Binding of Kix and IBiD was less sensitive to phosphorylation. Strikingly, hepta-phosphorylation of all Ser and Thr residues increased binding 40- and 80-fold with CH3 and Taz1, respectively, but not with Kix or Ibid. Substitution of all phospho-sites with aspartates partially mimicked the effects of hepta-phosphorylation. Mdm2, the main negative regulator of p53, competes with p300 for binding TAD. Binding of Mdm2 to TAD was reduced significantly only on phosphorylation of Thr18 (7-fold) or by hepta-phosphorylation (24-fold). The relative affinities of Mdm2 and p300 for p53 TAD can thus be changed by up to three orders of magnitude by phosphorylation. Accordingly, phosphorylation of Thr18 and hepta-phosphorylation dramatically shifts the balance to favouring binding of p300 with p53 and is thus likely to be an important factor in its regulation.

Keywords

CREB-binding protein; CBP; IHD; Hdm2; Mdmx; Mdm4; phosphorylation; fluorescence anisotropy; SRC-1; AD1; AD2; phospho-peptides; intrinsically disordered proteins

p53 is a homotetrameric transcription factor, which is frequently mutated in human cancers. In response to DNA damage and other stresses, p53 is stabilised and activates diverse genes involved in cell cycle arrest, apoptosis, senescence and metabolism. p53 upregulation occurs through a variety of DNA damage-induced post-translational modifications that include phosphorylation, acetylation, methylation, neddylation and sumoylation. (for reviews see (Lavin & Gueven, 2006; Toledo & Wahl, 2006)).

The intrinsically disordered p53 transactivation domain (TAD) is essential for p53 transcriptional activity, as its direct interactions with the acetyltransferase activity-containing coactivator proteins p300/CBP (as well as components of the basal transcriptional machinery) link p53 target gene binding to p53 target gene expression. (Figure 1a, b) (Lill et al., 1997; Thut et al., 1995). TAD also binds to the negative regulators Mdm2 and Mdmx, which are competitive inhibitors of p53 binding, and Mdm2 facilitates C-terminal ubiquitination and subsequent p53 degradation (Marine & Jochemsen, 2004; Toledo & Wahl, 2006). Concurrent phosphorylation of p53 TAD with DNA damage

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prompted numerous studies using mutational approaches to define the roles of individual TAD phosphorylations *in vivo*. Collectively, each phosphorylation site has apoptotic and/or cell cycle arrest-inducing functions ascribed (Lavin & Gueven, 2006). There are also modest cell cycle arrest and apoptotic defects in mouse knock-ins with mutations of Ser18/23, which are both conserved in humans (Ser15/Ser20) (Johnson & Attardi, 2006). Phosphorylation of short peptides of p53 TAD1 (residues 15-29, 1-39) increases binding to p300/CBP (Dornan & Hupp, 2001; Polley et al., 2008), and Ser15 phosphorylation increases p300 binding *in vivo* (Lambert et al., 1998). But, several studies have questioned the requirement of p53 phosphorylation in p53 activation altogether, as mutation of all Ser/Thr in p53 TAD in stable cell lines does not impair p53's stability and transactivation potential (Ashcroft et al., 1999; Blattner et al., 1999).

To address the question of the role of TAD phosphorylation at a molecular level, we dissected the effects of its phosphorylations on binding to its partner proteins: p300, being an essential positive effector for p53 activity, interacts with the full TAD (1-57) through four domains, Taz2/CH3, Taz1/CH1, Kix, and IBiD (Figure 1b) (Liu et al., 2003; Teufel et al., 2007). Conversely, the negative regulator Mdm2 interacts with a shorter segment of TAD (15-29) (Sakaguchi et al., 2000; Schon et al., 2002). We systematically assessed the role of each phosphorylation by comparing the affinities of different TAD (phospho-)peptides for those p300 domains and Mdm2. Although all experiments were run in physiological ionic strength buffer, absolute affinities between the proteins may be different within cells. But, the relative affinities between WT and phospho-peptides should be transferable to the full-length protein interactions within cells, regardless whether interactions occur elsewhere on the proteins, and regardless whether other factors contribute. Further, TAD has a similarly disordered structure to that in full length p53 (Wells et al., 2008). As the affinity of acidic transactivation domains from transcription factors to transcriptional machinery proteins correlates with transcriptional activity (Melcher, 2000), the use of peptides to assess the role of TAD phosphorylation presents an appropriate system for the evaluation of p53 phosphorylation in the context of p53 transactivation.

Seven TAD phospho-derivatives were generated (denoted as 1-57 S15(P), T18(P), S20(P), S33(P), S37(P), S46(P) and T55(P)), where all phospho-sites reside within the p300 domain binding interfaces. Using a C-terminal fluorophore (Lys-methoxycoumarin) as a probe on all peptides, their affinities for the isolated p300 and Mdm2 domains were determined by fluorescence anisotropy titrations as in our previous study (Teufel et al., 2007).

CH3 binds 1-57 WT p53 the tightest (K_D = 18 nM). S15 and T18 phosphorylation induced the greatest increase in affinity for CH3 (K_D = 2.8 and 3.6 nM, respectively), which was 6.3- and 5-fold tighter than for WT p53. Phosphorylation at S20, S33 or S37 caused a 3- to 5-fold increase in affinity. S46 or T55 phosphorylation gave a modest, less than 2 -fold increase (Figure 2a, Table 1).

Taz1, of similar sequence to Taz2, also interacts strongly with 1-57 p53 (K_D = 770 nM). Here, T18 or S20 phosphorylation increased binding most significantly (K_D = 110 and 130 nM, respectively), with smaller increases for the remaining phospho-variants of p53 (1.5- to 4-fold) (Figure 2b, Table 1). Phosphorylations at S46 or T55 again had the least effect on Taz1 binding.

Both Kix and IBiD bind more weakly to TAD (K_D = 3700 and 4400 nM, respectively). There were up to 3-fold increases in affinity for Kix on phosphorylation at S15, T18, S20, S33 or S37. The same phosphorylations affected IBiD binding up to 1.6-fold. T18 or S20 phosphorylation increased Kix-binding the most, while binding of IBiD was affected equally

on any of these phosphorylations. Interestingly, phosphorylation at S46 or T55 mildly reduced the affinity to both Kix and IBiD (Figure 2c, d, Table 1).

A less conserved region located between Taz1 and Kix (termed IHD, Figure 1b) is reported to bind to 1-39 p53 (phospho-)peptides (Dornan et al., 2003; Polley et al., 2008). Using NMR, we were unable to detect folded structure in IHD, and anisotropy titration showed no detectable binding with any of the p53 (phospho-)peptides.

p53 S15/S20 double phosphorylation is believed to enhance transcriptional activation (Chao et al., 2006; Xu, 2003). The doubly phosphorylated peptide, p53 10-57 S15(P)/S20(P), bound no more tightly than either of the singly phosphorylated peptides (Table 1).

In response to DNA damage, multiple phosphorylation occurs in p53 TAD, often in patterns depending on the nature of stress inflicted (Lavin & Gueven, 2006; Saito et al., 2003). It was not feasible for us to test all possible permutations and combinations of phosphorylations of the seven sites, which would generate 127 different peptides. As most single phosphorylations increase the affinity of TAD for p300 (apart from S46/T55), we reasoned that binding of the full hepta-phosphorylated TAD would approximate to the highest possible affinity to p300 domains. We made the hepta-phosphorylated peptide of 10-57 p53 (denoted as 10-57 Hepta-(P)) by a combined chemical and enzymatic synthesis (Figure 3). The hepta-phosphorylated peptide bound CH3 ~40-fold tighter than WT p53 (K_D = 530 pM) (Figure 2a). Taz1 binding dramatically increased ~80-fold (K_D = 11 nM); binding was >10-fold stronger than any single-phosphorylated peptide (Figure 2b). Interestingly, p53 hepta-phosphorylation did not increase Kix and IBiD binding significantly (3.8- and 1.4-fold, respectively); affinities were roughly equivalent to those with the singly phosphorylated peptides (i.e. T18(P)) (Table 1, Figure 2c, d). Consequently, Kix and IBiD are relatively insensitive to p53 phosphorylation. In contrast, the transcription factor CREB binds Kix 150 times more tightly upon phosphorylation of a single residue (Ser133) (K_D = 0.7 μM) (Zor et al., 2002). Thus, the phospho-serine selectivity observed with the Kix/CREB interaction is unique and cannot be generalised to p53.

Mutation of Ser or Thr to Asp generates "phospho-mimics" of p53 (Dornan & Hupp, 2001; Knights et al., 2003). We made the hepta-substituted aspartate variant of p53 TAD (10-57 Hepta-(Asp)). It bound to CH3 and Taz1 11- and 23-fold more tightly than did WT TAD, respectively. Although binding of Hepta-(Asp) was stronger than that of WT or any singly phosphorylated peptide, it was still ~4-fold weaker than that of Hepta-(P), and so the Hepta-(Asp) derivative is a real, but partial, mimic.

We also tested a Ser/Thr→Ala-substituted derivative of p53 TAD (1-57 Hepta-(Ala)), which are used in cell-based and knock-in studies to assess the requirement of Ser/Thr phosphorylation in p53 (Blattner et al., 1999; Johnson & Attardi, 2006). 1-57 Hepta-(Ala) bound to all domains at an affinity comparable to 1-57 WT p53, indicating that unphosphorylated Ser/Thr side chains of 1-57 p53 do not significantly contribute to the affinity for p300 (Table 1).

Mdm2, the main negative regulator of p53, interacts through its N-terminal domain (1-125) with 15-29 p53 TAD (Figure 1a). The interaction is strongly weakened by Thr18 phosphorylation (Brown et al., 2008; Lai et al., 2000; Sakaguchi et al., 2000; Schon et al., 2002). However, regions C-terminal to TAD1 (30-57) weakly contribute to the p53:Mdm2 interaction (Chi et al., 2005; Teufel et al., 2007). Consequently we tested all peptides from this study to allow consistent comparison. As with the shorter 15-29 peptides, Thr18 phosphorylation in 1-57 peptides weakened the interaction with Mdm2 significantly (Table 1, Figure 2e), while the other phosphorylations, 1-57 Hepta-(Ala) and 10-57 Ser15(P)/Ser20(P) had only small effects. Interestingly, hepta-phosphorylation reduced the affinity for

Mdm2 ~24-fold compared with the 7-fold reduction seen with Thr18 phosphorylation alone (K_D = 4700 nM, compared with K_D = 1300 nM for 1-57 T18(P)) (Table 1). Consistent with this, hepta-aspartylation strongly reduced binding (10-fold), but not as efficiently as hepta-phosphorylation.

For ease of comparison, we plotted the change in binding energy in response to phosphorylation against the p53 phosphorylated residue and protein domain investigated (Figure 4). The order of preference for any single TAD phosphorylation is roughly equivalent for all p300 domains. Strikingly, the tighter p53 interactors CH3 and Taz1 domains were more sensitive to p53 single phosphorylation, particularly of S15, T18, S20, S33 and S37.

Assuming that the effect of a given phosphorylation on binding each of the four p300 domains is additive and applicable to the whole p300 protein, p53 T18 phosphorylation stands out as the greatest enhancer of p300 binding (Figure 5). The greater effect of single phosphorylations on S15/T18/S20 is interesting as these residues are 100% conserved in all mammals (Figure 1a). The smaller effects of S46/T55 phosphorylation correlate with little sequence conservation outside the primate species, suggesting that S46/T55 and their phosphorylation have not evolved to enhance specifically p300 binding (Figures 1a, 5).

Full hepta-phosphorylation of p53 TAD drastically increased binding to CH3 and Taz1, but not Kix and IBiD. The magnitude of binding enhancement (40-/80-fold) is comparable to the Ser133 phosphorylation-enhanced CREB-Kix interaction, where affinity increases ~150-fold. Thus, p300 is a transcriptional adaptor protein that responds to transcription factor phosphorylation through at least three domains (Taz2/CH3, Taz1, Kix).

The experimentally determined change in binding energy ($\Delta\Delta G$) for seven phosphorylations of the same peptide (Figure 4) is smaller than the sum of $\Delta\Delta G$ for the seven individually phosphorylated peptides ($\Delta\Delta G_{\text{Hepta-(P)}} \approx$ -2.5 kcal/mol << $\Sigma[\Delta\Delta G_{\text{S15(P)}, \text{T18(P)}, \text{S20(P)}, \text{S33(P)}, \text{S37(P)}, \text{S46(P)}, \text{T55(P)}}] \approx$ -5 kcal/mol for both CH3 and Taz1), indicating that the effects of single phosphorylations are not additive with any of the p300 domains. Therefore, p300 domains are unlikely to have seven independent p53 phospho-group recognition sites. We suggest a loosely defined p300 domain - p53 scaffold where the hydrophobic residues L22/W23/W53/F54 of p53 are central to the interaction (Teufel et al., 2007), and where neighbouring phosphate groups irrespective of their positions on p53 TAD are flexibly accommodated (unpublished NMR data).

Our data suggest modulatory effects of 15, 20, 33 and 37 phosphorylations on p300 binding, while the binding of the p53 negative regulator Mdm2 remains unaffected. It is possible, however, that other proteins from the transcriptional machinery (Tfb1, Taf9, etc) mediate specific effects of these phosphorylations (Di Lello et al., 2006; Lu & Levine, 1995). Thr18 phosphorylation stands out as uniquely disrupting the interaction with Mdm2, while strongly increasing binding to p300 (Figure 4). The T18(P)-induced difference in binding between Mdm2 and CH3/Taz1 was exacerbated by factors of ~40, as opposed to less than 7- fold for any of the other single phosphorylations that do not affect Mdm2 binding (Table 1). Notably, the mutant T18A p53 in p53 null cells is stabilised 5-10 times less efficiently than WT p53, and transcriptional activation of p53 target genes is severely impaired (Bean & Stark, 2002). Conversely, the phospho-mimic T18E p53 recovers p300 binding, reduced Mdm2 binding, and reactivated p21 transcription in cancerous cells (Knights et al., 2003). Thus, the quantitatively determined *in vitro* effects of p53 T18 phosphorylation on p300/ Mdm2 binding correlate with the effects of T18 phosphorylation *in vivo*. Given the importance of T18 phosphorylation in binding p300, Mdm2 and Mdmx (Bottger et al.,

1999), it will be of interest to generate the corresponding knock-in mice (T21A and T21E) to assess their susceptibility to cancer development.

While hepta-phosphorylation has not been specifically described in the literature, it is known that multiple phosphorylations can occur simultaneously on many residues of the p53 N- and C-termini (Lavin & Gueven, 2006; Saito et al., 2003). Our data demonstrate that p53 TAD at the maximal possible phosphorylation state dramatically exacerbates the difference in affinity between Mdm2 and p300 CH3 (~900-fold) and Taz1 (~1900-fold), respectively (Table 1). Given the competition of p300 and Mdm2 for p53 TAD (Oliner et al., 1993; Teufel et al., 2007), the far tighter binding of hepta-phosphorylated p53 (or even the aspartylated derivative) to p300 should significantly increase the fraction of p53 bound to p300. Within the cellular context, T18 or hyper-phosphorylation of p53 may thus lead to upregulation of p53-dependent transcription of cell-cycle arrest and apoptotic genes.

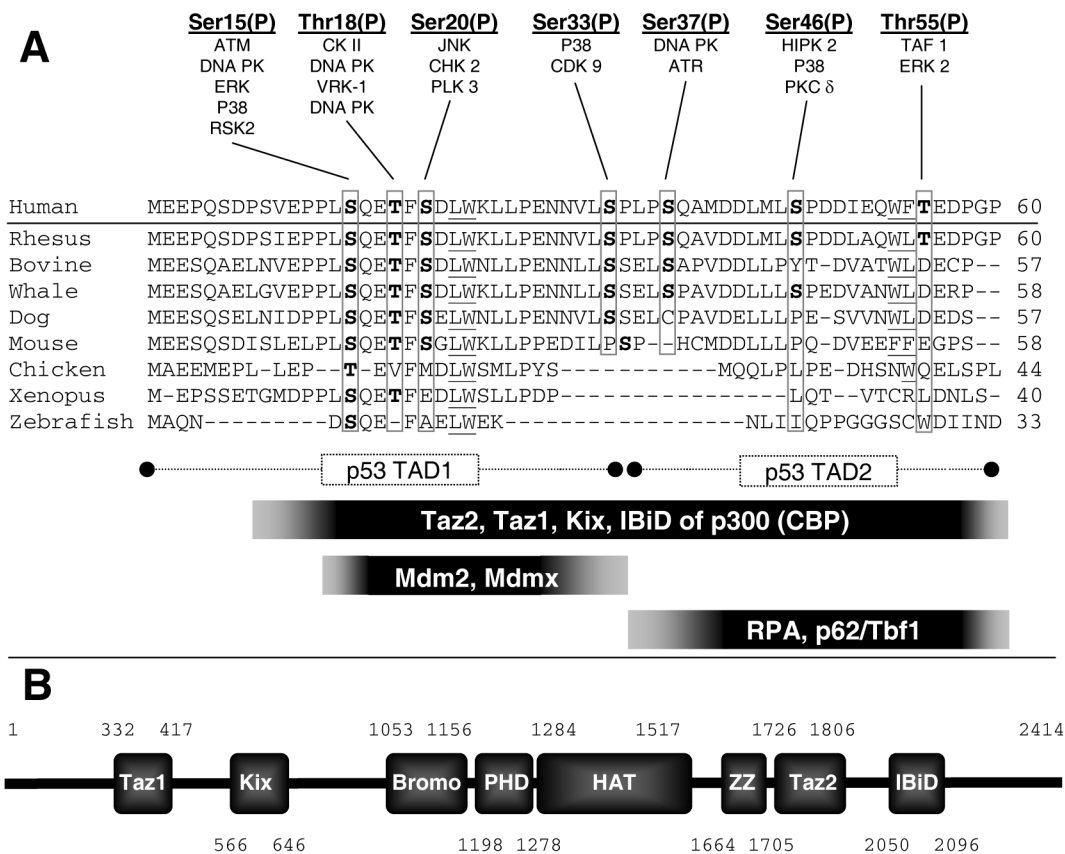## Acknowledgments

ABBREVIATIONS

TAD	p53 transactivation domain
CH1/CH3	cysteine-histidine rich regions 1 and 3

REFERENCES

Ashcroft M, Kubbutat MH, Vousden KH. Mol Cell Biol. 1999; 19:1751–8. [PubMed: 10022862]

Bean LJ, Stark GR. J Biol Chem. 2002; 277:1864–71. [PubMed: 11707453]

Blattner C, Tobiasch E, Litfen M, Rahmsdorf HJ, Herrlich P. Oncogene. 1999; 18:1723–32. [PubMed: 10208433]

Bottger V, Bottger A, Garcia-Echeverria C, Ramos YF, van der Eb AJ, Jochemsen AG, Lane DP. Oncogene. 1999; 18:189–99. [PubMed: 9926934]

Brown CJ, Srinivasan D, Jun LH, Coomber D, Verma CS, Lane DP. Cell Cycle. 2008; 7:608–10. [PubMed: 18256546]

Chao C, Herr D, Chun J, Xu Y. Embo J. 2006; 25:2615–22. [PubMed: 16757976]

Chi SW, Lee SH, Kim DH, Ahn MJ, Kim JS, Woo JY, Torizawa T, Kainosho M, Han KH. J Biol Chem. 2005; 280:38795–802. [PubMed: 16159876]

Di Lello P, Jenkins LM, Jones TN, Nguyen BD, Hara T, Yamaguchi H, Dikeakos JD, Appella E, Legault P, Omichinski JG. Mol Cell. 2006; 22:731–40. [PubMed: 16793543]

Dornan D, Hupp TR. EMBO Rep. 2001; 2:139–44. [PubMed: 11258706]

Dornan D, Shimizu H, Perkins ND, Hupp TR. J Biol Chem. 2003; 278:13431–41. [PubMed: 12499368]

Johnson TM, Attardi LD. Cell Death Differ. 2006; 13:902–8. [PubMed: 16557272]

Knights CD, Liu Y, Appella E, Kulesz-Martin M. J Biol Chem. 2003; 278:52890–900. [PubMed: 14555661]

Lai Z, Auger KR, Manubay CM, Copeland RA. Arch Biochem Biophys. 2000; 381:278–84. [PubMed: 11032416]

Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R, Brady JN. J Biol Chem. 1998; 273:33048–53. [PubMed: 9830059]

Lavin MF, Gueven N. Cell Death Differ. 2006; 13:941–50. [PubMed: 16601750]

Legge GB, Martinez-Yamout MA, Hambly DM, Trinh T, Lee BM, Dyson HJ, Wright PE. J Mol Biol. 2004; 343:1081–93. [PubMed: 15476823]

Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Nature. 1997; 387:823–827. [PubMed: 9194565]

Liu G, Xia T, Chen X. J Biol Chem. 2003; 278:17557–65. [PubMed: 12609999]

Lu H, Levine AJ. Proc Natl Acad Sci U S A. 1995; 92:5154–8. [PubMed: 7761466]

Marine JC, Jochemsen AG. Cell Cycle. 2004; 3:900–4. [PubMed: 15254433]

Melcher K. J Mol Biol. 2000; 301:1097–112. [PubMed: 10966808]

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Nature. 1993; 362:857–60. [PubMed: 8479525]

Polley S, Guha S, Roy NS, Kar S, Sakaguchi K, Chuman Y, Swaminathan V, Kundu T, Roy S. J Mol Biol. 2008; 376:8–12. [PubMed: 18155245]

Saito S, Yamaguchi H, Higashimoto Y, Chao C, Xu Y, Fornace AJ Jr. Appella E, Anderson CW. J Biol Chem. 2003; 278:37536–44. [PubMed: 12860987]

Sakaguchi K, Saito S, Higashimoto Y, Roy S, Anderson CW, Appella E. J Biol Chem. 2000; 275:9278–83. [PubMed: 10734067]

Schon O, Friedler A, Bycroft M, Freund SM, Fersht AR. J Mol Biol. 2002; 323:491–501. [PubMed: 12381304]

Teufel DP, Freund SM, Bycroft M, Fersht AR. Proc Natl Acad Sci U S A. 2007; 104:7009–14. [PubMed: 17438265]

Teufel DP, Kao RY, Acharya KR, Shapiro R. Biochemistry. 2003; 42:1451–9. [PubMed: 12578357]

Thut CJ, Chen JL, Klemm R, Tjian R. Science. 1995; 267:100–4. [PubMed: 7809597]

Toledo F, Wahl GM. Nat Rev Cancer. 2006; 6:909–23. [PubMed: 17128209]

Wells M, Tidow H, Rutherford TJ, Markwick P, Jensen MR, Mylonas E, Svergun DI, Blackledge M, Fersht AR. Proc Natl Acad Sci U S A. 2008; 105:5762–7. [PubMed: 18391200]

Xu Y. Cell Death Differ. 2003; 10:400–3. [PubMed: 12719715]

Zor T, Mayr BM, Dyson HJ, Montminy MR, Wright PE. J Biol Chem. 2002; 277:42241–8. [PubMed: 12196545]

A

	Ser15(P)	Thr18(P)	Ser20(P)	Ser33(P)	Ser37(P)	Ser46(P)	Thr55(P)
	ATM	CK II	JNK	P38	DNA PK	HIPK 2	TAF 1
	DNA PK	DNA PK	CHK 2	CDK 9	ATR	P38	ERK 2
	ERK	VRK-1	PLK 3			PKC δ	
	P38	DNA PK					
	RSK2						

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Human     MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWFTEDPGP  60
Rhesus    MEEPQSDPSIEPPLSQETFSDLWKLLPENNVLSPLPSQAVDDLMLSPDDLAQWLTEDPGP  60
Bovine    MEESQAELNVEPPLSQETFSDLWNLLPENNLLSSELSAPVDDLLPYT-DVATWLDECP--  57
Whale     MEESQAELGVEPPLSQETFSDLWKLLPENNLLSSELSPAVDDLLLSPEDVANWLDERP--  58
Dog       MEESQSELNIDPPLSQETFSELWNLLPENNVLSSELCPAVDELLLPE-SVVNWLDEDS--  57
Mouse     MEESQSDISLELPLSQETFSGLWKLLPPEDILPSP--HCMDDLLLPQ-DVEEFFEGPS--  58
Chicken   MAEEMEPL-LEP--T-EVFMDLWSMLPYS-----------MQQLPLPE-DHSNWQELSPL  44
Xenopus   M-EPSSETGMDPPLSQETFEDLWSLLPDP---------------LQT--VTCRLDNLS-  40
Zebrafish MAQN--------DSQE-FAELWEK----------------NLIIQPPGGGSCWDIIND  33
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p53 TAD1   p53 TAD2

Taz2, Taz1, Kix, IBiD of p300 (CBP)

Mdm2, Mdmx

RPA, p62/Tbf1

B

1   332   417   1053   1156   1284   1517   1726   1806   2414

Taz1   Kix   Bromo   PHD   HAT   ZZ   Taz2   IBiD

566   646   1198   1278   1664   1705   2050   2096

Figure 1.

(a): Sequence alignment of vertebrate p53 N-termini. Serines and threonines that are within the segment of p53 TAD contacted by p300 domains are in bold, and encircled by grey boxes. Kinases involved in their phosphorylation are shown above (Lavin & Gueven, 2006; Toledo & Wahl, 2006; Xu, 2003). p53 L22/W23 and W53/F54, which are required for p53-p300 binding and transcriptional activity, are underlined. The approximate boundaries of p53 transactivation subdomains TAD1 and TAD2 are shown below (dotted boxes), which are compared to the approximate extent of the p300/Mdm2/Mdmx/p62 contact regions (black boxes). (b) Domain structure of p300. Taz1 is often referred to as CH1, PHD as CH2 and ZZ-Taz2 as CH3. Taz2 alone is responsible for the interaction with p53 TAD (Legge et al., 2004; Teufel et al., 2007).
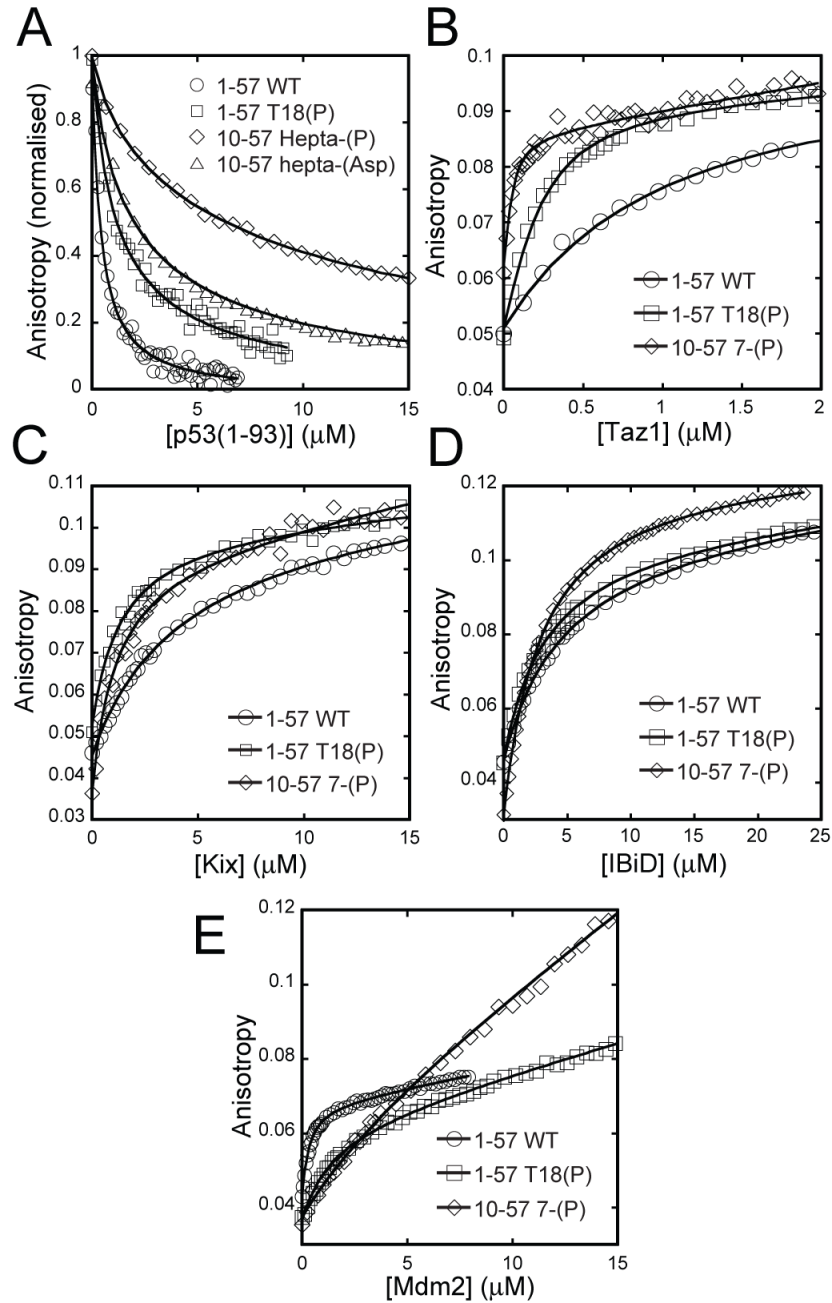
Figure 2.
Binding of p53 (phospho-)peptides and derivatives to p300 domains and Mdm2, using fluorescence anisotropy. (a) The very high affinities of phospho-peptide for CH3 had to be determined indirectly by competition. A complex of CH3 (500 nM) and p53 phospho-derivative (400 nM) was dissociated with increasing concentrations of recombinant unlabelled WT p53 (1-93). Data were fit numerically to a 1:1 binding model using software developed by Dmitry Veprintsev (MRC Centre for Protein Engineering), and normalised for the bound fraction of phospho-derivative. The K_D of unlabelled 1-93 p53 was 14 nM (personal communication, Jenifer Lum, MRC Centre for Protein Engineering). 7-(P), 7-(Asp) and 7-(Ala) refer to 10-57 Hepta-(P), 10-57 Hepta-(Asp) and 1-57 Hepta-(Ala), respectively. (b), (c), (d), (e): Direct anisotropy titrations, where p300 domains (Taz1, Kix,

IBiD) or Mdm2 (296 µL), respectively, are titrated into 1200 µL of 50-200 nM labelled phospho-peptide. All proteins were expressed and purified as previously described. Peptides were synthesised on a CEM Microwave Peptide Synthesiser (Matthews, N.C., USA) and purified by a combination of size exclusion, preparative and analytical HPLC; using acetonitrile/H_2O as solvents, and 0.1% trifluoroacetic or phosphoric acid as pH regulators. Titrations were run on a Horiba Jobin Yvon fluorimeter (Longjumeau Cedex, France), using the same physiological ionic strength buffer and extinction coefficients (Teufel et al., 2007).
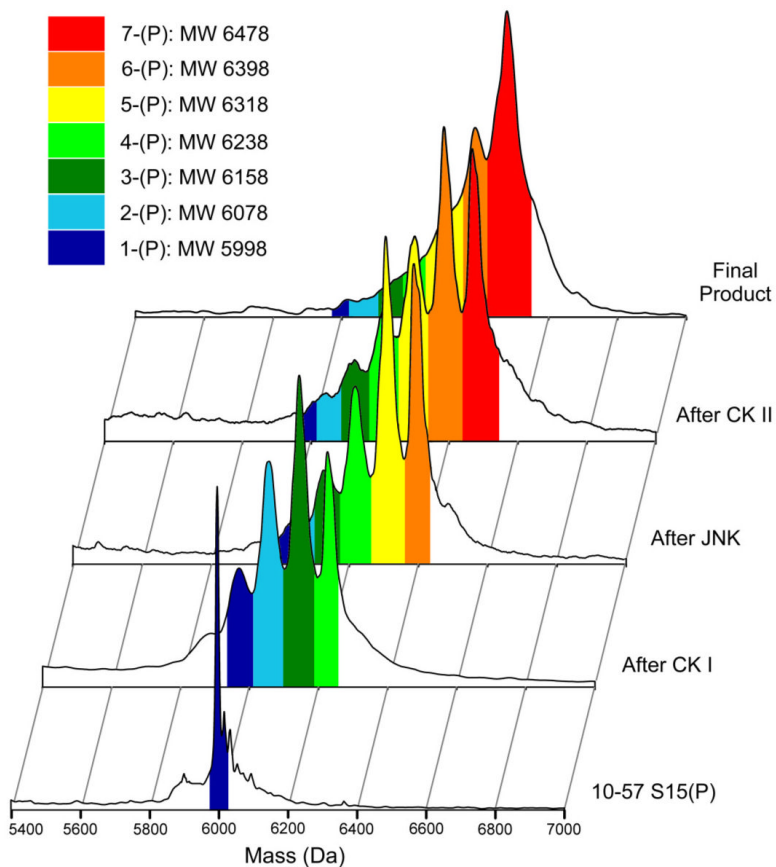
Figure 3.
MALDI TOF mass spectra of kinase-phosphorylated 10-57 S15(P). p53 10-57 S15(P) peptide was successively phosphorylated by casein kinase I, casein kinase II (New England Biolabs, MA, USA) and c-jun N-terminal kinase JNK (Millipore, MA, USA). Each enzyme phosphorylates three, two and one serine or threonine residue, respectively. Repeated rounds of kinase phosphorylations produced the final product, which is phosphorylated on all available seven Ser/Thr on p53 TAD. The presence of the respective mono- to hepta-phosphorylated peptides are colour-coded onto the mass spectra, with molecular weights shown top left. Mass peaks are broad due to the presence of multiple ionic salt species. Kinase reactions were performed for 24 hours at 30 °C according to the manufacturer's instructions, using (per mL) 200 µM peptide, 100 µL of 100 mM Mg-ATP, 10 µL enzyme, 2 µL EDTA-free protease inhibitor (Roche, Switzerland) and 1 µL β-mercaptoethanol. The final product was purified as above and consisted mostly of hepta-phosphorylated product as confirmed by MALDI-TOF, with small fractions of lower phospho-species.
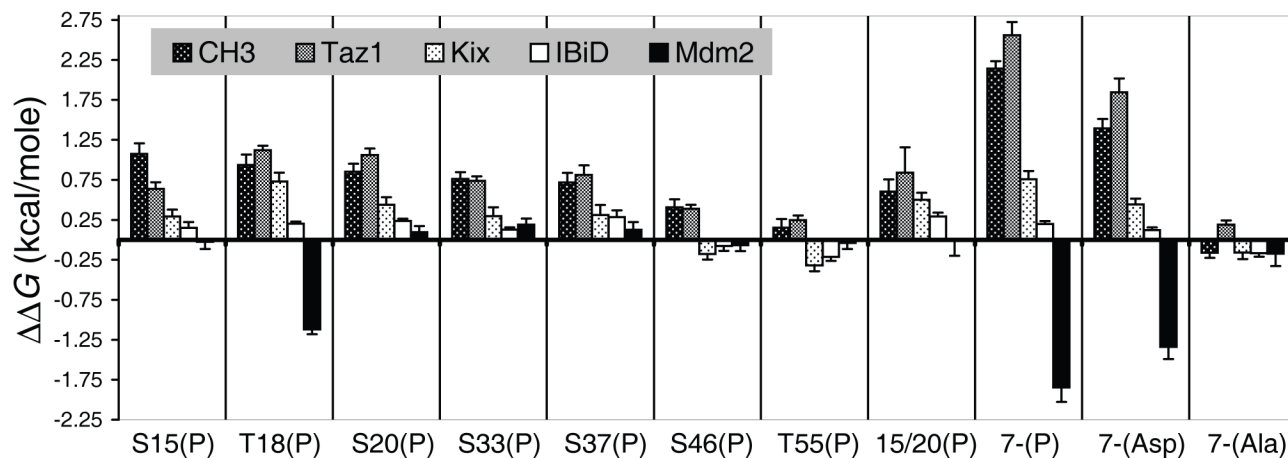
Figure 4.
Comparison of the effect of p53 phospho-derivatives against WT p53 for p300/Mdm2 domain binding. ΔΔG refers to the difference in free binding energy between p53 phospho-derivative and WT p53 peptide for any of the binding proteins. The bars shown therefore represent the free binding energy contributed by a phosphorylation (or Asp, Ala substitution as with 7-(Asp) and 7-(Ala)). ΔG was calculated by $-RT\ln K_D$, and ΔΔG was calculated by $-RT\ln(K_{D,WT}/K_{D,variant})$. Standard errors were determined as described previously (Teufel et al., 2003).

Figure 5.
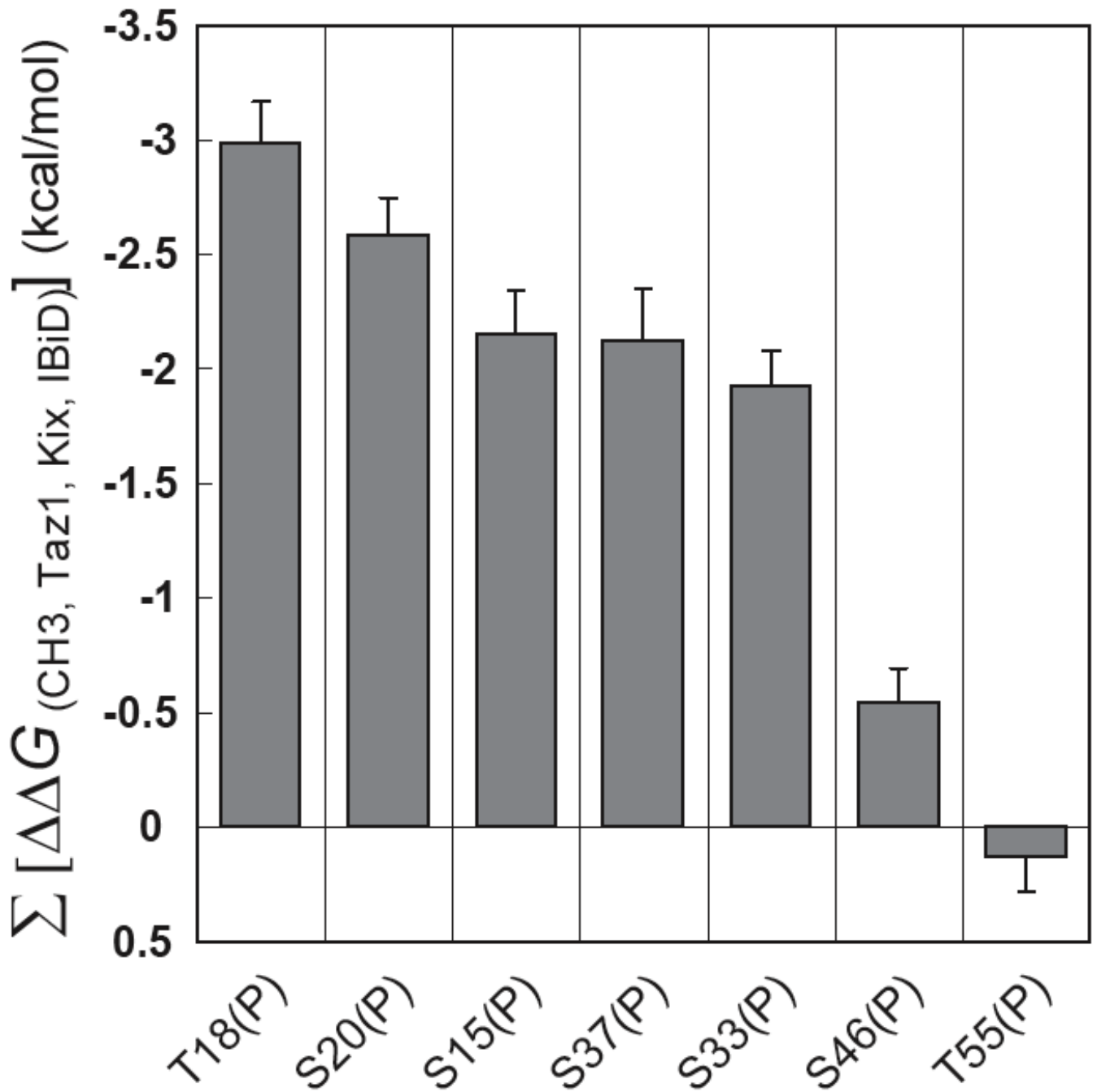Overall importance of single phosphorylations on p300 binding. The sum of ΔΔG of a
given phospho-peptide for all p300 domains shows the overall importance of a
phosphorylation on p300 binding.

Table 1

Dissociation constants (K_D) of p53 TAD phospho-peptides binding to CH3, Taz1, Kix and IBiD domains of p300, and the N-terminal p53-binding domain of Mdm2

p53 peptide	CH3		Taz1		Kix		IBiD		Mdm2	
	K_D (nM)	F^a	K_D (nM)	F^a	K_D (nM)	F^a	K_D (nM)	F^a	K_D (nM)	F^a
1-57 WT	18 ± 1.8	-	770 ± 50	-	3700 ± 400	-	4400 ± 180	-	200 ± 20	-
1-57 S15(P)	2.8 ± 0.6	6.3	260 ± 30	3.0	2200 ± 200	1.7	3400 ± 400	1.3	200 ± 25	1.0
1-57 T18(P)	3.6 ± 0.8	4.9	110 ± 10	7.0	1100 ± 170	3.3	3100 ± 80	1.4	1300 ± 30	0.15
1-57 S20(P)	4.2 ± 0.6	4.3	130 ± 16	6.0	1700 ± 200	2.1	2900 ± 90	1.5	170 ± 15	1.2
1-57 S33(P)	4.9 ± 0.6	3.7	220 ± 20	3.5	2200 ± 350	1.7	3500 ± 120	1.3	140 ± 15	1.4
1-57 S37(P)	5.2 ± 1.0	3.4	200 ± 40	3.9	2200 ± 400	1.7	2700 ± 360	1.6	160 ± 20	1.2
1-57 S46(P)	8.9 ± 1.2	2.0	400 ± 25	1.9	4900 ± 220	0.7	5000 ± 500	0.9	220 ± 20	0.9
1-57 T55(P)	14 ± 2.0	1.3	510 ± 10	1.5	6300 ± 390	0.6	6300 ± 520	0.7	210 ± 18	0.9
10-57 WT	21 ± 3.0	-	880 ± 180	-	3800 ± 460	-	3900 ± 170	-	200 ± 16	-
10-57 S15(P)/S20(P)	7.3 ± 1.6	2.8	210 ± 100	4.2	1600 ± 150	2.4	2400 ± 160	1.6	210 ± 60	1.0
10-57 Hepta-(P)	0.53 ± 0.04	38	11 ± 2	79	1000 ± 130	3.8	2800 ± 120	1.4	4700 ± 1400	0.04
10-57 Hepta-(Asp)	1.9 ± 0.3	11	38 ± 8	23	1800 ± 40	2.1	3200 ± 110	1.2	2000 ± 500	0.1
1-57 Hepta-(Ala)	23 ± 2.4	0.8	560 ± 45	1.4	4800 ± 400	0.8	5800 ± 400	0.8	270 ± 65	0.7

[a] $F = K_{D,WT}/K_D$, variant.