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Structural Mechanism of the Bromodomain of the Coactivator CBP in p53 Transcriptional Activation

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Summary

Lysine acetylation of the tumor suppressor protein p53 in response to a wide variety of cellular stress signals is required for its activation as a transcription factor that regulates cell cycle arrest, senescence, or apoptosis. Here, we report that the conserved bromodomain of the transcriptional coactivator CBP (CREB binding protein) binds specifically to p53 at the C-terminal acetylated lysine 382. This bromodomain/acetyl-lysine binding is responsible for p53 acetylation-dependent coactivator recruitment after DNA damage, a step essential for p53-induced transcriptional activation of the cyclin-dependent kinase inhibitor p21 in G1 cell cycle arrest. We further present the three-dimensional nuclear magnetic resonance structure of the CBP bromodomain in complex with a lysine 382-acetylated p53 peptide. Using structural and biochemical analyses, we define the molecular determinants for the specificity of this molecular recognition.

Introduction

The human tumor suppressor protein p53 plays a pivotal role in cellular response to numerous stress signals in cell cycle arrest, senescence, DNA repair, or apoptosis (Ko and Prives, 1996; Levine, 1997; Prives and Hall, 1999; Vogelstein et al., 2000). The indispensable functions of p53 are underscored by the fact that *p53* is one of the most frequently mutated cellular genes and its mutations occur in nearly 50% of all human cancers (Vogelstein et al., 2000). p53 functions as a sequence-specific transcription factor that induces or represses transcription of a large number of genes, including p21/WAF1/CIP1, Mdm2, GADD45, Bax, cyclin G, and PAC-1 (Attardi et al., 1996; El-Deiry et al., 1993; Miyashita and Reed, 1995; Yin et al., 2003). Alteration of transcription of these genes has been directly linked to p53-mediated cell cycle arrest or apoptosis in response to DNA damage (Yin et al., 2003; Zhao et al., 2000). Interestingly, the biological functions of p53 itself are dependent upon the expression of the genes that it regulates.

p53 contains 393 amino acids, consisting of several functional domains, including an N-terminal activation domain, a PXXP region, a sequence-specific DNA binding domain, and C-terminal tetramerization and basic regions. The biological activity of p53 is tightly regulated by posttranslational modifications in its N- and C-terminal regions (Alarcon-Vargas and Ronai, 2002; Prives and Hall, 1999). Upon DNA damage, p53 is extensively phosphorylated within the N-terminal activation domain, which relieves it from association with the negative regulator Mdm2, resulting in p53 stabilization and activation as a transcription factor (Fuchs et al., 1998b; Haupt et al., 1997; Kubbutat et al., 1997; Momand and Zambetti, 1997). In addition, phosphorylation occurs in the C terminus of p53, which has been shown to enhance its DNA binding in vitro (Hupp and Lane, 1994; Wang and Prives, 1995).

In response to DNA damage, p53 becomes acetylated on multiple lysine residues in its C terminus (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Particularly, it has been reported that transcriptional coactivator histone acetyltransferases (HATs) p300/CBP (CREB binding protein) and p300/CBP-associated factor (PCAF) acetylate K373 and K382 (to a lesser extent K372 and K381) and K320, respectively. Lysine acetylation or deacetylation of p53 has been directly linked to its ability to regulate cell cycle arrest and apoptosis (Guo et al., 2000; Ito et al., 2001; Luo et al., 2000), as well as senescence (Pearson et al., 2000). It had been hypothesized on the basis of in vitro data that p53 acetylation enhances its DNA binding through the relief of negative regulation of DNA binding exerted by the C-terminal region (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). However, more recent in vivo studies show that lysine acetylation of p53 does not result in any enhancement of its DNA binding ability but, rather, promotes its recruitment of coactivators, leading to histone acetylation and transcriptional activation of target genes (Barlev et al., 2001).

While the molecular mechanisms of p53 acetylation-dependent coactivator recruitment are not known, notably, these p53-associating coactivators contain evolutionarily conserved bromodomains, which function as acetyl-lysine (AcK) binding domains (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Marmorstein and Berger, 2001; Owen et al., 2000; Winston and Allis, 1999; Zeng and Zhou, 2001). The bromodomain, first reported in the *Drosophila* protein brahma (Haynes et al., 1992; Tamkun et al., 1992), is present in a large number of chromatin-associated proteins and nuclear histone acetyltransferases (Jeanmougin et al., 1997). The biological importance of the bromodomain/AcK recognition has further been demonstrated by the role of the bromodomain in tethering transcriptional HATs to specific chromosomal sites (Brownell and Allis, 1996; Manning et al., 2001; Travers, 1999) and in the assembly and activity of multiprotein chromatin remodeling complexes, including SAGA and SWI/SNF (Agalioti

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et al., 2002; Brown et al., 2001; Hassan et al., 2002; Sterner et al., 1999), as well as in HIV-1 transcriptional activation (Dorr et al., 2002; Mujtaba et al., 2002).

To understand the role of lysine acetylation in p53 function, we examined possible involvement of the bromodomain in acetylation-mediated p53 association with the transcriptional coactivators. Our study, reported here, shows that the bromodomain of the coactivator CBP binds specifically to p53 at the C-terminal acetylated K382. This bromodomain-mediated action is responsible for p53 recruitment of CBP in vivo upon DNA damage, a molecular interaction that is crucial for p53-induced transcriptional activation of the cyclin-dependent kinase inhibitor p21 in cell cycle arrest. We further determined the three-dimensional structure of the CBP bromodomain in complex with a K382-acetylated p53 peptide by nuclear magnetic resonance (NMR) spectroscopy. Our structural and mutational analyses provide the detailed structural basis for this molecular mechanism of coactivator recruitment that is essential for p53 transcriptional regulation in DNA damage control.

Results

CBP Bromodomain Recognizes p53 Acetylated at K382 In Vitro

To test whether p53 acetylation-dependent recruitment of CBP involves the CBP bromodomain, we performed NMR titration of CBP bromodomain binding to synthetic p53 peptides derived from three lysine acetylation sites. As shown in 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra, addition of p53 peptides acetylated at K373 or K320 resulted in little, if any, chemical shift perturbations of any protein residue (Figure 1A). On the contrary, several protein residues exhibited major chemical shift perturbations as a function of concentration of a p53 peptide containing AcK382, indicating interactions between the protein and the peptide. This binding is dependent upon K382 acetylation of the peptide with a K_D of $\sim$50 μM, as estimated from NMR titration. None of the p53 peptides showed any significant binding to the homologous bromodomain from PCAF (Jeanmougin et al., 1997) (data not shown). Notably, the p53 AcK373 and AcK382 peptides have the same sequence except that the acetylated lysine is in a different position, underscoring the selective nature of CBP bromodomain/p53 AcK382 recognition.

To examine the specificity of CBP bromodomain/p53 binding, we performed an in vitro binding assay using GST-fusion bromodomains and a biotinylated p53 AcK382 peptide that was immobilized onto streptavidin-agarose beads. Only the CBP bromodomain but not those from PCAF or transcriptional intermediary factor 1β (TIF1β, also known as KAP-1) (Friedman et al., 1996) formed interactions with the p53 peptide (Figure 1B). These results confirm that the CBP bromodomain can specifically interact with p53 acetylated at K382.

CBP and p53 Association In Vivo Requires the Bromodomain Interaction

We next assessed the biological relevance of CBP bromodomain/p53 binding in cell transfection experiments. The CBP bromodomain transfected in 293T cells was properly expressed (Figure 2A). Expression of p53 transfected in p53 null 10.1 mouse embryo fibroblasts (MEF, $p53^{-/-}$) cells was markedly induced after UV-C irradiation (Figure 2B), agreeing with a similar effect reported previously (Barlev et al., 2001). Interestingly, we observed that p53 acetylation at K382 was dependent upon the degree of the UV exposure (data not shown) and detected as early as 2 hr and peaked at 8 hr after UV-C exposure at 50 J/m^2 (Figure 2B). Under the optimized condition, the bromodomain of CBP formed a stable complex with lysine-acetylated p53 (Figure 2C). Mutation of K373 to alanine did not alter K382 acetylation, nor did it affect p53 association with the CBP bromodomain. However, a single K382A or double K373A/K382A mutation completely abolished the p53/CBP bromodomain association (Figure 2C), confirming that the CBP bromodomain interacts with p53 by recognizing the acetylated K382 site in the cell.

It is reported that the N-terminal region of p53 interacts with effector proteins, including Mdm2 and CBP, and that stress-induced phosphorylation in the region frees p53 from association with these proteins (Avantaggiati et al., 1997; Gu and Roeder, 1997; Haupt et al., 1997; Kubbutat et al., 1997; Lill et al., 1997; Scolnick et al., 1997). To determine whether p53 N-terminal region association with CBP is a prerequisite for p53 acetylation, we examined N-terminal deletion effect on p53/CBP binding. Strikingly, a p53 deletion mutant (deleting residues 13–52) was well expressed in the transfected 10.1 cells and acetylated at K382 regardless of UV irradiation (Figure 2D). This is in a sharp contrast to the full-length p53, which was expressed at a very low level and underwent a marked induction upon UV-C irradiation (Figures 2B and 2D). Moreover, the expressed, K382-acetylated p53 deletion mutant formed a stable complex with GST-CBP bromodomain immobilized on glutathione-sepharose resin (Figure 2D). These results suggest that lysine acetylation of p53 is not likely dependent upon its N-terminal region interaction with CBP but may be controlled by regulatory events involving the N-terminal region that precede the C-terminal lysine acetylation after UV-C irradiation.

We further examined this CBP/p53 interaction in the context of the full-length proteins. In 10.1 cells, the transfected p53 formed a stable complex with the endogenous CBP upon UV-C treatment, peaking at 8 hr after the exposure (Figure 3A). The N-terminal deletion mutant of p53, acetylated at K382 with or without UV-C irradiation, also associated with the endogenous CBP (Figure 3B). The UV-dependent CBP/p53 association was further confirmed in COS-7 cells by coexpressing HA-tagged CBP and Flag-tagged p53 (Figure 3C). Although p53 expression was markedly increased upon UV treatment, the UV-induced p53 acetylation at K382 resulted in an at least 10-fold enhancement of the CBP/p53 complex formation. Mutation of K382 to alanine drastically reduced p53 binding to CBP to a minimal level similar to that observed without UV treatment. These results argue that despite possible other protein-protein interactions between CBP and p53, the bromodomain-acetyl-lysine interaction is the major force that promotes these two proteins to form a stable complex upon UV treatment.

It has been reported that p53 lysine acetylation in

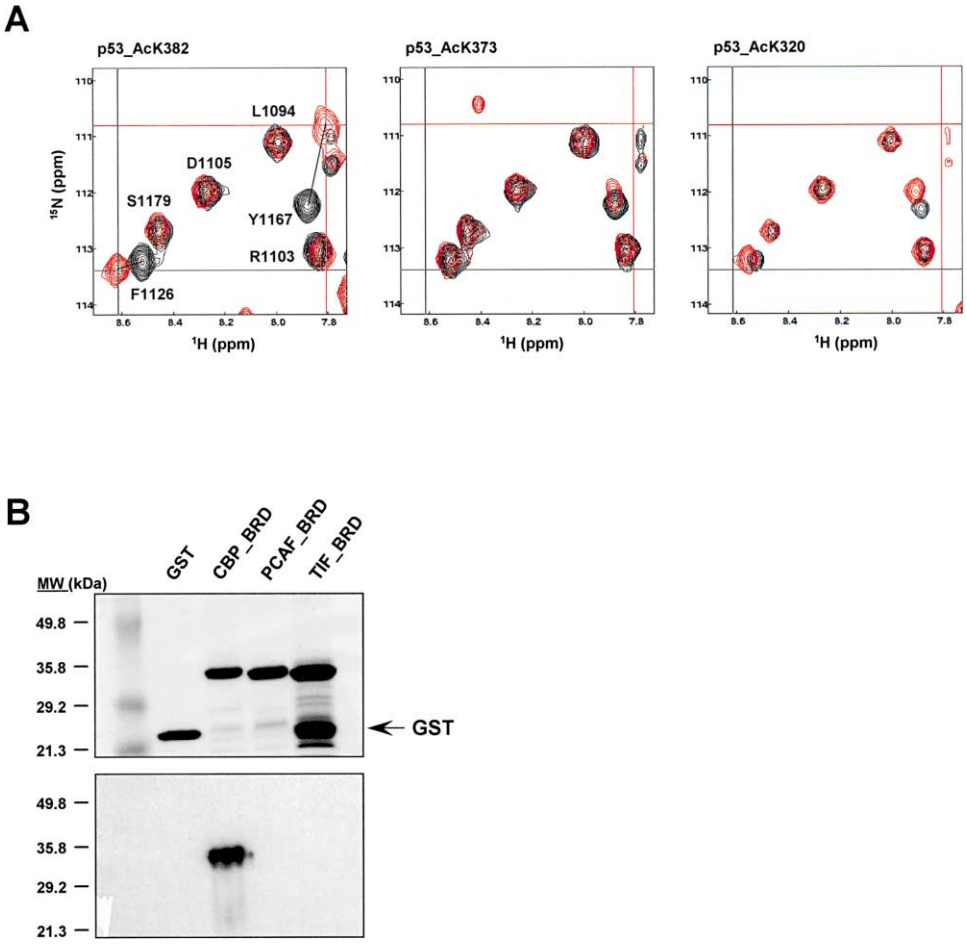


Figure 1. Recognition of Lysine-Acetylated p53 by the CBP Bromodomain

(A) CBP bromodomain binding to p53 peptides acetylated at K382 (SHLKSKKGQSTSRHK-AcK-LMFK) (left), K373 (SHLKSK-AcK-GQSTSRHK-K-LMFK) (middle), or K320 (SSPQPK-AcK-KPLDGE). Superimposition of the 2D ^{15}N HSQC spectra of the CBP bromodomin depicts differences of protein backbone resonances between the free form (black) or in the presence of a p53 peptide (red). Protein-to-peptide molar ratio was kept at 1:3.

(B) Binding of the purified GST-bromodomains of CBP, PCAF, and TIF1β (top) to a biotinylated p53 AcK382 peptide bound to streptavidin-agarose beads was determined by Western blotting using anti-GST-antibody (bottom).

response to stress signals is carried out by CBP/p300 (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998), two coactivator HATs that are highly homologous in sequence and size. Indeed, we showed that the recombinant and purified GST-fusion p53 or its N-terminal truncation mutant can be effectively acetylated at K382 by the HAT domain of CBP or p300 in vitro (Figure 3D). To address the question whether p300 also interacts with the acetylated p53 after UV-C treatment, we examined the endogenous p53 interactions with CBP or p300 in NIH/3T3 cells. As expected, the endogenous p53 formed a stable complex with the endogenous CBP in a K382 acetylation-dependent manner, which was effectively blocked by the transfected CBP bromodomain (Figure 3E). However, while p300 also weakly interacted with p53, this interaction was not dependent upon lysine acetylation, nor affected by the transfected CBP bromodomain. These data suggest that p53/p300 association likely does not involve a bromodomain-acetyl-lysine interaction, which seems consistent with the recent notion that these two homologous coactivators may function differently in cells (Giordano and Avantaggiati, 1999).

p53-Induced p21 Activation Requires the CBP Bromodomain/p53 AcK382 Interaction

To understand functional importance of the bromodomain-mediated CBP/p53 association, we investigated p53-induced p21 activation in 10.1 cells using a luciferase assay. UV-C treatment of the 10.1 cells transfected with p53 resulted in nearly a 7-fold enhancement of p21 luciferase activity (Figure 4A, lane 2). This p53-mediated p21 activation was dropped to a 5- or 2-fold increase in the cells transfected with a K373A or a K382A mutant, respectively (lanes 3 and 4), and almost completely disappeared with a K373A/K382A double mutant (lane 5). Notably, while expression of wild-type or mutant p53 upon UV-C treatment was enhanced to a similar level, the degree of p21-elevated expression agrees with that of its activation, which is dependent upon p53 acetylation at K382. The ratio of p21 expression level be-

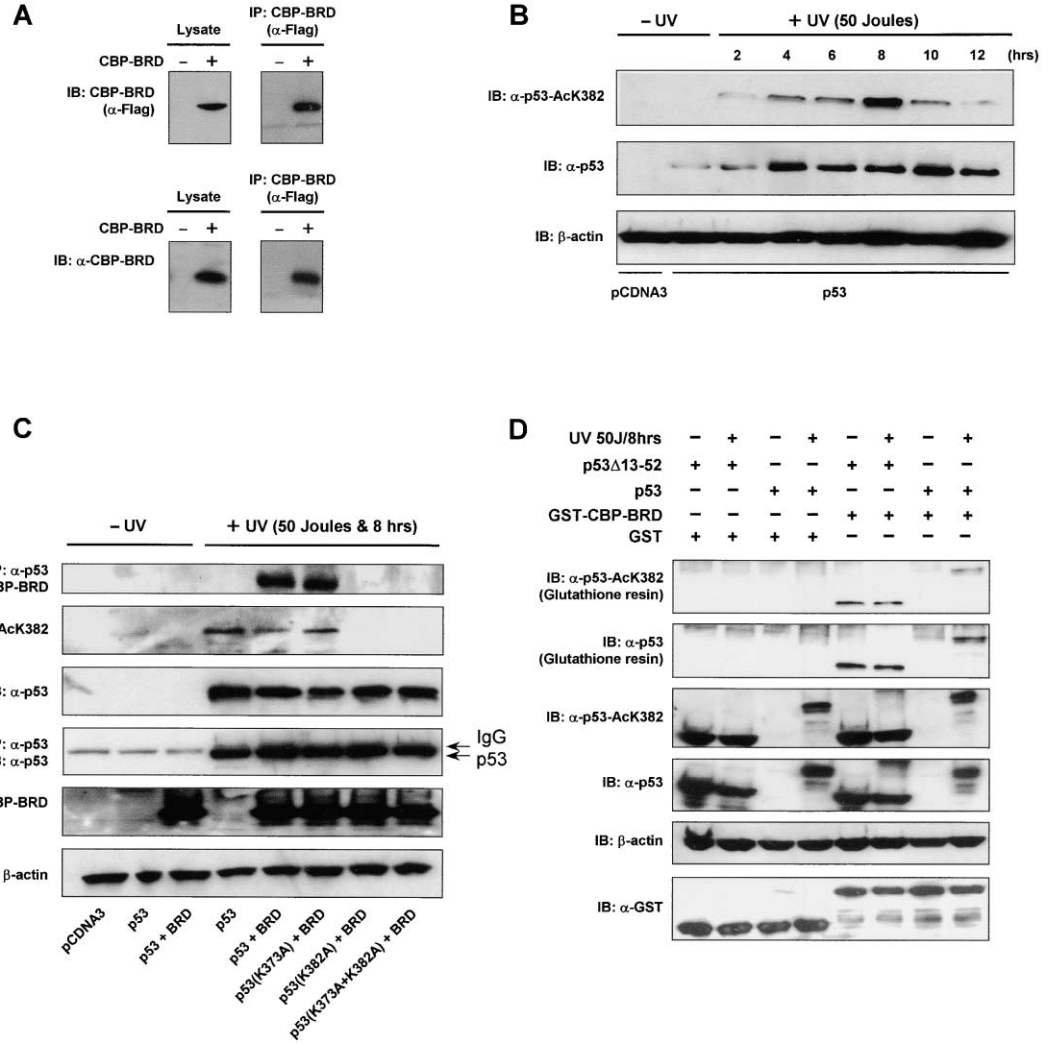


Figure 2. CBP Bromodomain Binding to Lysine-Acetylated p53 In Vivo

(A) Expression of the CBP bromodomain in 293T cells was assessed by Western analysis of cell lysates (left) or immunoprecipitate (right) using CBP bromodomain- or Flag-specific antibodies.

(B) Kinetics of K382 acetylation of p53 in the 10.1 cells following UV treatment was analyzed by Western blots using antibodies specific for p53 or p53 AcK382.

(C) Binding of CBP bromodomain to p53 or p53 mutants in 10.1 cells was examined by Western blotting analysis using antibodies specific to p53, p53-AcK382, and the CBP bromodomain.

(D) Effect of p53 N-terminal deletion on CBP bromodomain binding. p53 or the p53 deletion mutant in the transfected 10.1 cells treated with UV-C irradiation was assessed for their binding to GST-CBP bromodomain bound to glutathione-sepharose beads by Western blotting analysis.

tween UV-treated and nontreated 10.1 cells that were transfected with the wild-type, K373A, K382A, or K373A/K382A mutant is 3:1, 2.5:1, 1.6:1, or 1.1:1, respectively (Figure 4B). Remarkably, both the p53-induced p21 activation and the K382 acetylation dependence largely disappeared when the cells were cotransfected with the CBP bromodomain (Figures 4A and 4B, lanes 6–10). This inhibitory effect was not observed with the structurally homologous bromdomain of PCAF or TIF1β (Figure 4C), which was expressed at a comparable level (Figure 4D). Although we cannot rule out possible other functions of CBP, these data imply that the bromodomain from CBP but not that of PCAF or TIF1β selectively competes against CBP/p53 binding in cells, agreeing with our in vitro binding results (Figure 1C). Collectively, our data argue that p53-mediated p21 activation likely requires acetylation of p53 at K382 (to a lesser extent at K373) and the subsequent bromodomain-mediated p53/CBP association at AcK382.

Role of CBP Bromodomain/p53 AcK382 Association in Cell Cycle Arrest

To assess whether bromodomain-mediated CBP/p53 association is functionally important to p53-induced cell cycle arrest, we analyzed the cell cycle profile of the 10.1 cells transfected by p53 by flow cytometry analysis. As shown in Figure 4E, UV exposure of the 10.1 cells transfected with wild-type p53 resulted in a significant increase of cell population in G1 phase from ~20% to ~35%. Cells transfected with the K373A mutant showed

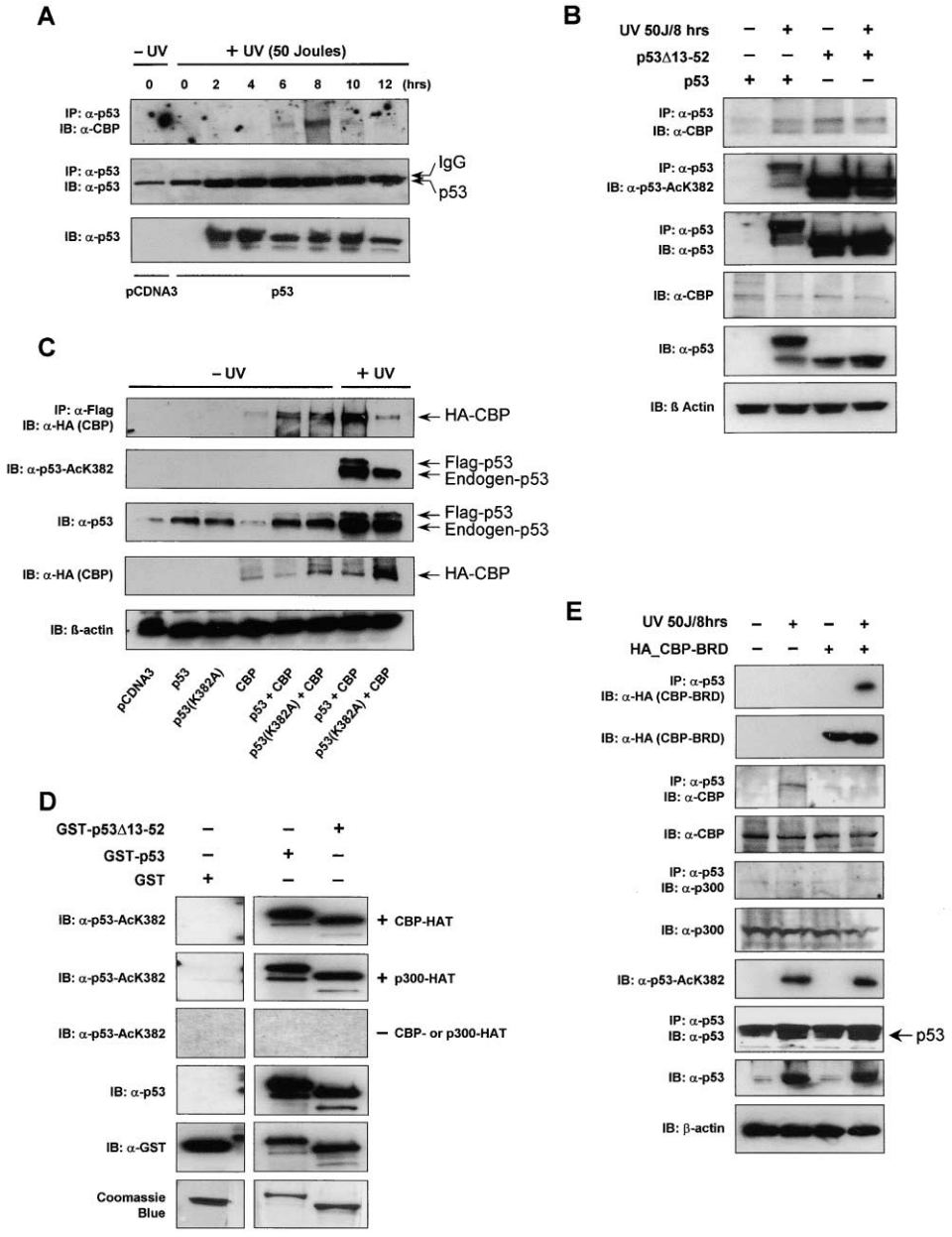


Figure 3. Association of the Endogenous CBP and p53 in 10.1 Cells

(A) Endogenous CBP binding to p53 in the 10.1 cells following UV-C irradiation. Expression of the transfected p53 and CBP/p53 binding were demonstrated by Western analysis.

(B) Effect of p53 N-terminal deletion on p53 binding to the endogenous CBP was assessed in the 10.1 cells transfected with p53 and p53 N-terminal deletion mutant and treated with UV-C p53 acetylation at K382.p53 binding to CBP was determined by Western blotting using antibodies specific for p53, p53-AcK382, or CBP bromodomain.

(C) Effects of K382A mutation on p53 association with the full-length CBP in COS-7 cells. The cells were transfected with the HA-tagged full-length CBP alone or with the Flag-tagged, wild-type p53 or mutant p53. Protein expression, p53 acetylation at K382, and CBP/p53 binding was examined by Western analysis using specific antibodies to p53, p53 AcK382, HA-tag, or Flag-tag.

(D) In vitro acetylation of K382 in p53 and p53 N-terminal deletion mutant by the HAT domain of CBP or p300 was assessed by Western blotting analysis using p53 AcK382 antibody.

(E) Acetylation-dependent endogenous p53 and CBP or p53 and p300 interactions in NIH/3T3 cells. Expression of p53, CBP, or p300, p53 acetylation at K382, and CBP/p53 binding upon UV-C irradiation was assessed in Western blotting by various specific antibodies.

a similar effect, whereas cells transfected with the K382A or K373A/K382A mutant exhibited no increase, if not a decrease. Notably, the 10.1 cells transfected with just the control vector pCDNA3 also showed a decreased G1 phase population upon UV-C exposure, similar to the effects with K382A and K373A/K382A mutants. Remarkably, this K382-dependent cell growth arrest by p53 was completely diminished when cotransfected

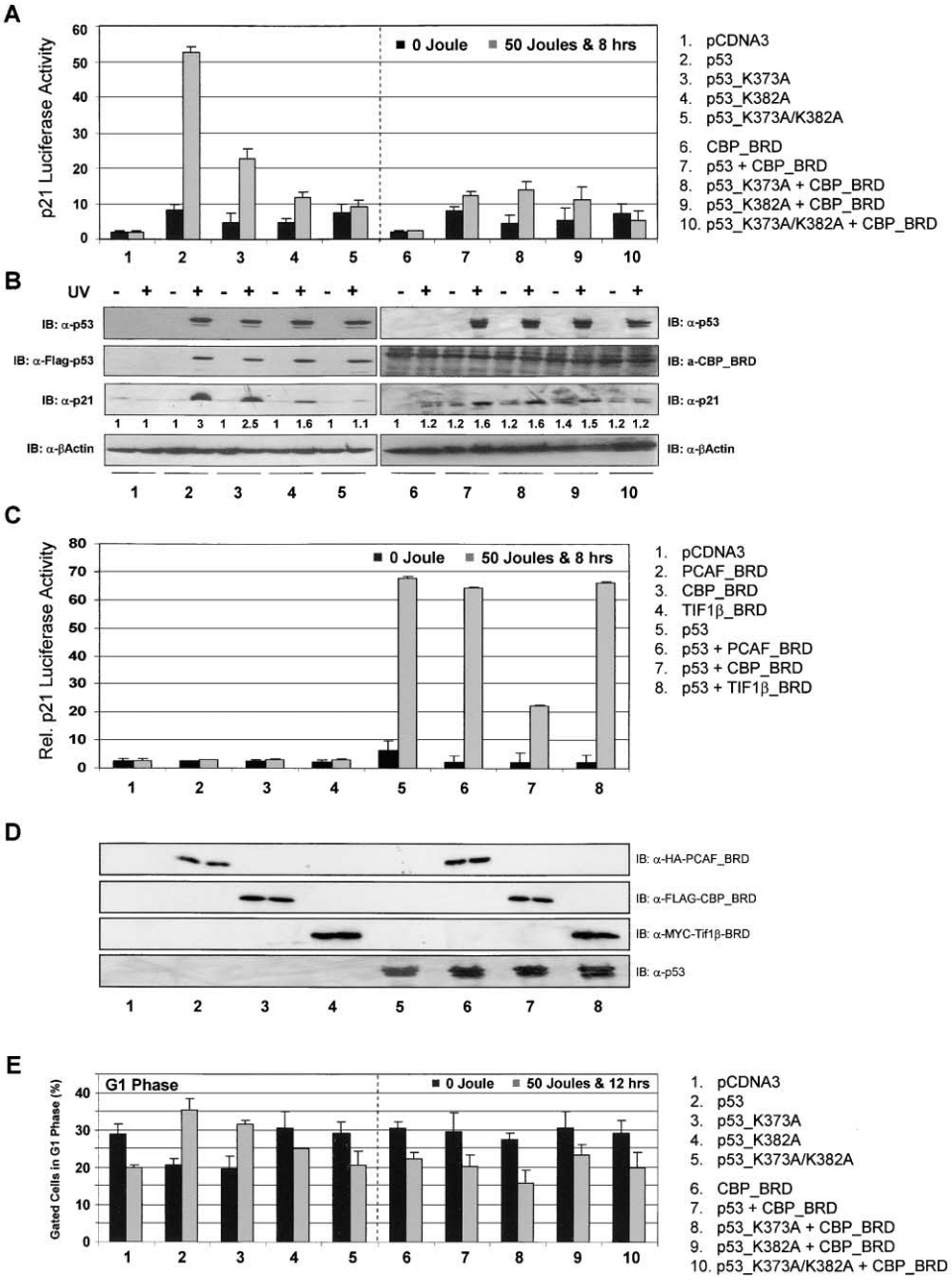


Figure 4. Functional Role of the CBP Bromodomain in CBP/p53 Association

(A) Effect of CBP bromodomain on p53-induced p21 activation in 10.1 cells after UV treatment. p21 activity of the 10.1 cells transfected with p53 or p53 mutant together with p21 luciferase and β-galactosidase, with or without CBP bromodomain, was measured in a luciferase-based assay. Mean values of the luciferase activities represent at least three independent cell transfections.

(B) Western blotting analysis assessing protein expression in the transfected 10.1 cells. Note that numerals below the p21 blot represent ratio of p21 expression in the cells transfected with p53, with or without CBP bromodomain, to that in the cells transfected with only the empty vector pCDNA3. The signals were quantitated using Kodak 1D Digital Image Analysis Software.

(C) Assessing effects of cotransfected bromodomains on p53-induced p21 activation in the 10.1 cells. The cell transfections and p21 luciferase activity analysis were same as described in (A).

(D) Western blots assessing expression of various bromodomains and p53 in the transfected 10.1 cells with or without UV-C treatment.

(E) Effect of the CBP bromodomain on cell cycle distribution induced by p53 in the 10.1 cells transfected with Us9-GFP, wild-type, or mutant p53, with or without the CBP bromodomains. The DNA content of the gated GFP-positive cells in G1 phase was determined by PI staining and FACS analysis. Average values of the DNA content of the cell cycle phases in each different experiment represent at least three independent transfection trials.

with the CBP bromodomain (Figure 4E, lanes 7–10). Transfection of the CBP bromodomain alone had an effect on cell cycle arrest similar to that of the control (Figure 4E, lanes 1 and 6). Finally, it appears that UV-C treatment of 10.1 cells transfected with the wild-type or mutant p53, with or without the CBP bromodomain, did not affect cell population in S or G2/M phase (data not shown). Taken together, our results demonstrate that p53 acetylation at K382 (to a much lesser extent, if any, at K373) in response to UV irradiation is responsible for p53-induced p21 activation and cell growth arrest and that the CBP bromodomain/p53 AcK382 binding is a key interaction for p53 acetylation-dependent coactivator recruitment essential for p53 transcriptional activity.

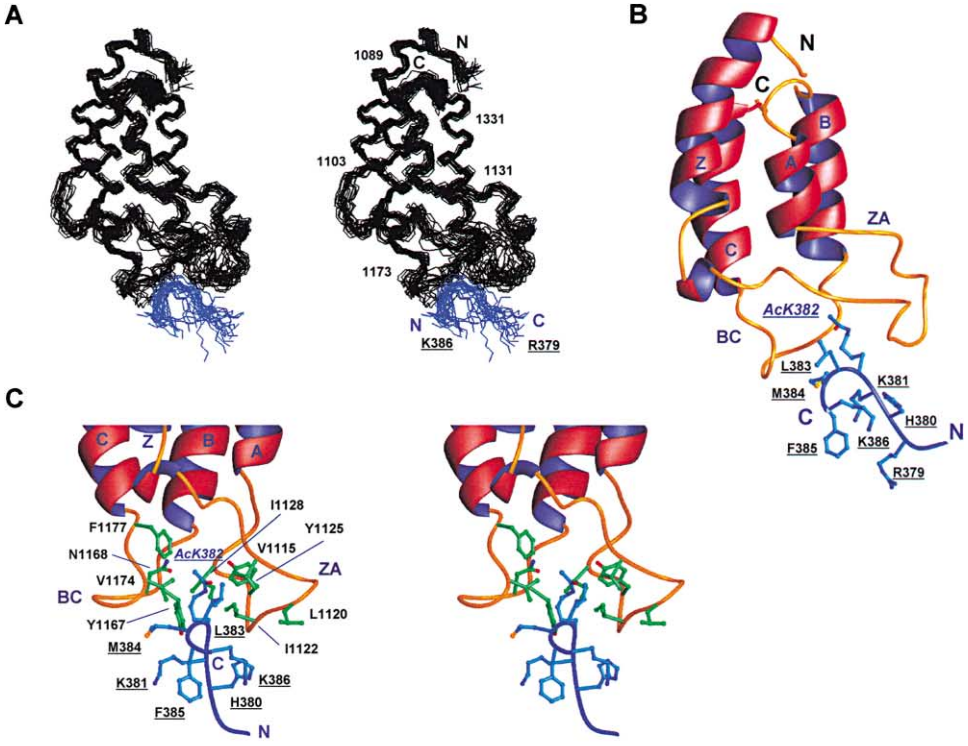
Structure of the CBP Bromodomain/p53 Peptide Complex

To understand the structural basis of CBP bromodomain/p53 recognition, we solved the three-dimensional structure of the CBP bromodomain in complex with a lysine-acetylated p53 peptide (residues 367–386) by NMR. The structure for the protein (residues 1081–1196) and the AcK382 peptide (residues 381–385) complex was well defined by the NMR data (Figure 5A, Table 1). The structure of the CBP bromodomain consists of a left-handed four-helix bundle (helices α_Z, α_A, α_B, and α_C) (Figure 5B) and is similar to those of the bromodomains from PCAF (Dhalluin et al., 1999), GCN5 (Hudson et al., 2000; Owen et al., 2000), and the double bromodomain module of $TAF_{II}250$ (Jacobson et al., 2000). The majority of the conformational differences among the bromodomains involve the ZA and BC loops that comprise the acetyl-lysine binding site. The corresponding regions in PCAF were shown to undergo conformational changes to accommodate peptide binding (Mujtaba et al., 2002). In contrast to the extended conformation of the Tat peptide in the PCAF bromodomain complex (Mujtaba et al., 2002), the p53 peptide, which lies across a pocket formed between the ZA and BC loops in the CBP bromodomain, adopts a β turn-like conformation with the acetylated K382 being at the beginning of the turn (Figure 5B). The side chain of the acetyl-lysine intercalates into the protein hydrophobic cavity and interacts with V1115, L1122, Y1125, and I1128 of the ZA loop and Y1167, V1174, and F1177 of the BC loop (Figure 5C). While K(AcK-1) contacts Y1167 of the protein, a large number of intermolecular NOEs were observed for two hydrophobic residues C-terminal to the acetyl-lysine. Particularly, L(AcK+1) shows numerous interactions with V1115, L1120, and I1122 in the ZA loop, V1174 and F1177 in the BC loop, whereas M(AcK+2) contacts largely Y1167 and V1174 of the BC loop. Most of these p53 binding residues are highly conserved in the bromodomain family except for L1120, which belongs to a two amino acid insertion in the ZA loop unique to CBP and a few closely related homologs in the bromodomain family (Figure 5D). Collectively, these specific interactions confer a selective association between the CBP bromodomain and p53.

Specificity of CBP Bromodomain and p53 Recognition

To determine functional importance of CBP bromodomain residues in p53 binding, we examined binding of bromodomain mutants to a biotinylated p53 AcK382 peptide immobilized onto streptavidin-agarose beads (Figure 6A). These residues are grouped according to their mutation effects on the complex formation: (1) point mutation of I1120, I1122, Y1125, I1128, or F1177 to alanine impaired up to ~50% of bromodomain/p53 peptide binding; (2) proteins containing a mutation of V1115A, P1123G, N1163A, or V1174A showed an 80% or greater reduction in the binding; (3) substitution of Y1167 or N1168 to alanine almost abolished the p53 peptide binding; (4) because of the relatively weak effect of I1120A or I1122A mutation, loss of p53 binding by the I1120-G1121-I1122 deletion mutant is likely due to local structural perturbations caused by the deletion in the ZA loop. This reasoning may explain an 80% reduction in p53 binding with P1123G (Figure 6A); and (5) a dramatic reduction in p53 binding by A1164V likely resulted from introduction of a bulky amino acid in the acetyl-lysine binding pocket, which interferes with the bromodomain/p53 interaction. These results agree with the intermolecular interactions observed in the structure of the bromodomain/p53 peptide complex (Figure 5C) and confirm that (1) both Y1167 and N1168 are essential for acetyl-lysine recognition; (2) V1115 is important for binding to both the acetyl-lysine and L(AcK+1); (3) V1174 is critical for recognition of possibly all three key amino acid residues AcK382, L(AcK+1) and M(AcK+2) in the p53 peptide; and (4) the residues at the two amino acid insertion of L1120-G1121, unique to the CBP bromodomain, make important contributions to the CBP/p53 association by direct interactions with AcK382 and L(AcK+1) and by defining the ZA loop conformation that is required for p53 binding.

To verify further the molecular determinants in the p53 sequence for CBP bromodomain binding, we tested mutant p53 peptides in a competition assay, in which a nonbiotinylated p53 peptide competed against a biotinylated p53 AcK382 peptide immobilized onto streptavidin-agarose beads for binding to the CBP bromodomain. As anticipated, the acetylated but not the nonacetylated K382 peptide of p53 effectively blocked the resin-immobilized p53 peptide from binding the bromodomain (Figure 6B, lanes 2 and 3). However, two p53 peptides acetylated at K373 or K320 showed almost no competition against p53 AcK382 peptide in bromodomain binding (Figure 6B, lanes 4 and 5 versus lane 3), confirming that the latter interaction is of higher affinity and specificity, consistent with the NMR binding data (Figure 1A). Alanine substitution of F(AcK+3) or M(AcK+2) in the p53 AcK382 peptide only slightly weakened its binding to the bromodomain (Figure 6C, lanes 6 and 7). Conversely, change of H(AcK−2) (lane 10), K(AcK−1) (lane 9), or L(AcK+1) (lane 8) to alanine caused a ~40%–70% reduction or a nearly complete loss of bromodomain binding. These mutation results confirm that AcK382 and L(AcK+1) are important in stabilizing the CBP bromodomain/p53 complex, agreeing with the extensive intermolecular interactions observed for these residues in the structure (Figure 5C) and the complementary mutational data of the protein (Figure 6A). This clear preference for a bulky hydrophobic residue at AcK+1 explains as to why the CBP bromodomain binds AcK382 but not AcK373 or AcK320 site in p53, the latter of which contains a Gly or Lys at AcK+1. Finally, the



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                1104          1114          1124          1134          1164          1174
                |             |  *          |  *          |             |  *  **      |  *
hsCBP           QD....PESLPFRQ.PVDPQLLGIPDYFDIVKNP...MD  ...  NAWLYNRK..TSRVYKFC
mmCBP           QD....PESLPFRQ.PVDPQLLGIPDYFDIVKNP...MD  ...  NAWLYNRK..TSRVYKYC
ceCBP           SE.....DAAPFRV.PVDAKLLNIPDYHEIIKRP...MD  ...  NAWLYNRK..NSKVYKYG
hsP300          QD....PESLPFRQ.PVDPQLLGIPDYFDIVKSP...MD  ...  NAWLYNRK..TSRVYKYC
scBDF1-1        .......DARPFLQ.PVDPVKLDIPFYFNYIKRP...MD  ...  NSIKFNGP..NAGISQMA
scBDF1-2        KH...ASYNYPFLE.PVDPVSMNLPTYFDYVKEP...MD  ...  NCYTFNPD..GTIVNMMG

hsPCAF          HQ.....SAWPFME.PVKRTE..APGYYEVIRSP...MD  ...  NCKEYNAP..ESEYYKCA
scGCN5          HA.....AAWPFLQ.PVNKEE..VPDYYDFIKEP...MD  ...  NCRMYNGE..NTSYYKYA
hsGCN5          HP.....SAWPFME.PVKKSE..APDYYEVIRFP...ID  ...  NCREYNPP..DSEYCRCA
mmGCN5          HP.....SAWPFME.PVKKSE..APDYYEVIRFP...ID  ...  NCREYNPP..NSEYCRCA
ttP55           HK....QS.WPFLD.PVNKDD..VPDYYDVITDP...ID  ...  NAKIYNQP..DTIYYKAA
sSNF2α          SS..GRQLSEVFIQLPSRK.E..LPEYYELIRKP...VD  ...  NAQTFNLE..GSQIYEDS
hsBRG1          SS.SGRQLSEVFIQLPSRK.E..LPEYYELIRKP...VD  ...  NAQTFNLE..GSLIYEDS

hsTAF2d1        L.....PNTYPFHT.PVNAKV..VKDYYKIITRP...MD  ...  NSATYNGP..KHSLTQIS
hsTAF2d2        AV....PDSWPFHH.PVNKKF..VPDYYKVIVNP...MD  ...  NSVKYNGP..ESQYTKTA
hsTIF1α         .......MSLAFQD.PVPLT...VPDYYKIIKNP...MD  ...  NCAEFNEP.DSEVANAGI
mmTIF1α         .......MSLAFQD.PVPLT...VPDYYKIIKNP...MD  ...  NCAEFNEP.DSEVANAGI
hsTIF1β         ............PCRPLHQLAT.DSTF.SLDQ.PGGTLD  ...  Q...FNKL.TEDKADVQS
mmTIF1β         ............PCRPLHQLAT.DSTF.SMEQ.PGGTLD  ...  Q...FNKL.TEDKADVQS
hsTIF1γ         .......LSIEFQE.PVPAS...IPNYYKIIKKP...MD  ...  NCERFNEMMKVVQVYADT
ggPB1-1         EQ..GRLLCELFIRAPKRRN...QPDYYEVVSQP...ID  ...  Q.LLFNNA...KAYYKPD
ggPB1-2         ..PSGRLISELFQKLPSKVQ...YPDYYAIIKEP...ID  ...  NAKTYNEP..GSQVFKDA
ggPB1-3         QG...QLISEPFFQLPSKKK...YPDYYQQIKTP...IS  ...  NAKRYNVP..NSAIYKRV
ggPB1-4         GT..QRRLCDLFMVKPSKKD...YPDYYKIILEP...MD  ...  NARHYNEE..GSQVYNDA
ggPB1-5         TDKRGRRLSAIFLRLPSRSE...LPDYYITIKKP...VD  ...  NACTYNEP..ESLIYKDA

                ZA loop                                      BC loop
```

Figure 5. NMR Structure of the CBP Bromodomain/p53 AcK382 Peptide Complex

(A) Stereoview of the backbone atoms (N, Cα, and C′) of 25 superimposed NMR structures of the CBP bromodomain (black) (showing residues 1085–1196) bound to the p53 AcK382 peptide (blue) (showing residues 379–386). For clarity, p53 residues are underlined.

(B) Ribbons (Carson, 1991) representation of the average minimized NMR structure of the CBP bromodomain/p53 peptide complex.

(C) Stereoview of the p53 binding site in the bromodomain showing side chains of the protein (green) and peptide (blue) residues that are directly involved intermolecular interactions.

(D) Sequence alignment of bromodomains highlighting amino acid variations in the ZA and BC loops. Bromodomains are grouped according to sequence similarities. Sequence numbers of CBP are shown above the sequence. Absolutely and highly conserved residues in bromodomains are colored in red and blue, respectively. Residues in the CBP or PCAF bromodomain that interact with p53 or HIV-1 Tat peptide, as shown by intermolecular NOEs, are underlined. Similarly, the residues of the yeast GCN5 bromodomain that directly contact the histone H4 peptide, as defined in the crystal structure, are also underlined. Residues in CBP bromodomain important for p53 binding, as determined by the mutational analysis, are highlighted by asterisk.

Table 1. Summary of NMR Structural Statistics

Total Experimental Restraints	2093	
Total NOE distance restraints[a]	2045	
Protein		
Total ambiguous	34	
Total unambiguous	1910	
Intraresidue	612	
Interresidue		
Sequential ($\|i - j\| = 1$)	475	
Medium ($2 \leq \|i - j\| \leq 4$)	435	
Long range ($\|i - j\| > 4$)	388	
Peptide	30	
Intermolecular	71	
Hydrogen bond restraints	48	
Final energies (kcal·mol^{-1})		
E_{Total}	456.0 ± 26.5	
E_{NOE}	68.1 ± 9.7	
$E_{Dihedral}$	0.6 ± 0.3	
E_{L-J}[b]	−550.6 ± 85.3	
	Protein/Peptide Complex[f]	Secondary Structure
Ramachandran plot (%)		
Most favorable region	67.9 ± 2.9	91.0 ± 2.7
Additionally allowed region	25.4 ± 2.8	8.2 ± 2.6
Generously allowed region	5.0 ± 2.0	0.8 ± 0.4
Disallowed region	1.7 ± 0.9	0.0 ± 0.0
Cartesian coordinate RMSDs (Å)[c]		
Backbone atoms (N, Cα, and C′)[d]	0.78 ± 0.12	0.39 ± 0.07
Heavy atoms[d]	1.35 ± 0.13	0.87 ± 0.06
Backbone atoms (N, Cα,and C′)[e]	0.55 ± 0.15	
Heavy atoms[e]	1.83 ± 0.50	
Backbone atoms (N, Cα, and C′)[f]	0.80 ± 0.12	0.52 ± 0.10
Heavy atoms[f]	1.39 ± 0.11	1.04 ± 0.06

[a] Of the total 2045 NOE-derived distance restraints, only 67 were obtained by using ARIA program, of which 34 are classified as ambiguous NOEs. The latter NOE signals in the NMR spectra match with more than one proton atom in both the chemical shift assignment and the final NMR structures.
[b] The Lennard-Jones potential was not used during any refinement stage.
[c] None of these final structures exhibit NOE-derived distance restraint violations greater than 0.3 Å or dihedral angle restraint violations greater than 5°.
[d] Protein residues 1085–1196.
[e] Peptide residues 381–385.
[f] Protein residues 1085–1196 and peptide residues 381–385.

mutational analysis data and few intermolecular NOEs observed for H(AcK−2) and K(AcK−1) in the NMR spectra suggest that while these residues N-terminal to AcK382 are functionally important for p53/CBP bromodomain binding, they are likely structurally dynamic in the complex.

Discussion

Growing evidence from in vivo studies demonstrates that stress-induced lysine acetylation of p53 play an important role in p53 stabilization and activation as a transcription factor (Barlev et al., 2001; Ito et al., 2001, 2002; Li et al., 2002). These studies show that acetylation-induced p53 activation in its transcriptional regulation in response to DNA damage does not result from an increase of its DNA binding activity as hypothesized previously (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998) but rather from its recruitment of coactivators and subsequent histone acetylation (Barlev et al., 2001). While multiple acetylation sites within the C-terminal tail of p53 have been reported, i.e., K320, K373, and K382 (to a lesser extent K372 and K381), specific effects of single or combined acetylation of these lysine residues on p53 activity remain elusive. Our study presented here provides the structural understanding of the molecular mechanism by which p53 recruits the coactivator CBP but not p300 upon UV-induced DNA damage via an interaction between its acetylated K382 and the bromodomain of CBP.

Our study provides several lines of evidence to support that the CBP bromodomain and p53 AcK382 interaction is functionally important for p53 function in response to cellular stress. First, our data suggest that the N-terminal region of p53 plays a negative role in its C-terminal acetylation by CBP upon DNA damage, agreeing with the notion that upon DNA damage, p53 becomes extensively phosphorylated within its N-terminal activation domain, which triggers its dissociation from the negative regulator Mdm2, resulting in p53 stabilization and activation as a transcription factor (Fuchs et al., 1998b; Haupt et al., 1997; Kubbutat et al., 1997; Momand and Zambetti, 1997). Second, our in vitro and in vivo binding results demonstrate that the specific CBP bromodomain/p53 AcK382 binding is a major force for

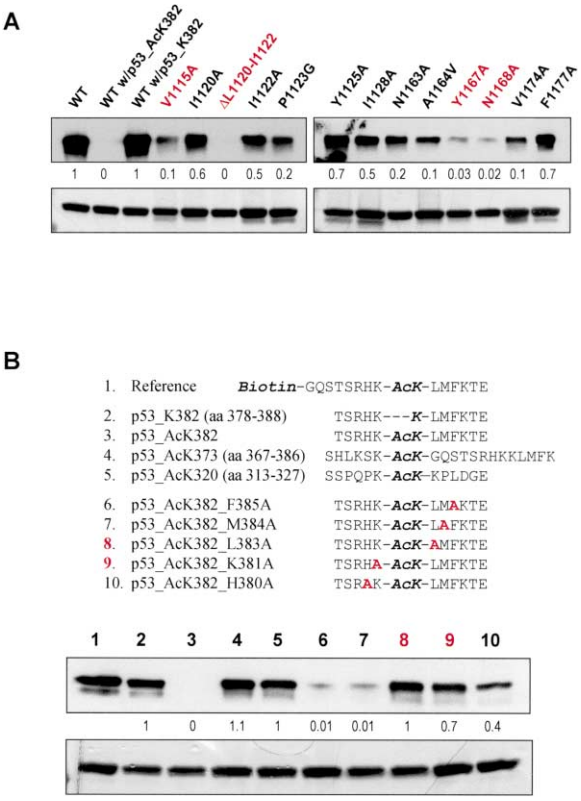

Figure 6. Mutational Analyses of CBP Bromodomain Binding to p53

(A) Effect of point mutation of CBP bromodomain residues on p53 peptide binding. Western blot with GST antibody shows GST-CBP bromodomain binding to the biotinylated p53 AcK382 peptide bound to streptavidin-agarose beads (top). Relatively equal amount of proteins was used in each binding experiment (bottom). Mutants highlighted in red exhibited markedly reduced binding to the p53 peptide. Mutational effects on the protein/peptide binding were quantitated.

(B) Mutational analysis of p53 peptide residues, assessed in a competition assay as described in Experimental Procedures. Mutant p53 peptides that showed a major reduction in binding to the bromodomain are indicated in red.

the endogenous p53 and CBP association after DNA damage. Third, our data confirm that acetylation at K382 (to a much less extent at K373) is responsible for p53-induced p21 activation and cell growth arrest in G1 phase but not S or G2/M phase (El-Deiry et al., 1995; Tang et al., 1998). This K382 acetylation effect is exerted through its interaction with the CBP bromodomain, which can be effectively blocked by the transfected CBP bromodomain but not by the homologous bromodomain of PCAF or TIF1β. Finally, our kinetics data demonstrate the dynamic nature of p53 acetylation and activation in response to stress signals and enabled us to systematically evaluate the biological importance of CBP bromodomain/p53 AcK382 interaction in p53 functions.

Our observation of acetylation-induced p53 expression, also reported previously (Barlev et al., 2001), agrees with the idea that acetylation promotes p53 stability by inhibiting its ubiquitnation by Mdm2 on the same C-terminal lysine residues p53 (Li et al., 2002) and that Mdm2/HDAC1-mediated deacetylation of p53 is required for its degradation (Ito et al., 2002). These observations that the single or double mutants of K382 and/or K373, which abrogates both acetylation and ubiquitnation at these sites, exhibit UV-induced increased stability similar to that of wild-type p53 suggest that this acetylation-induced stability likely results from collaboratively multiple C-terminal residues, i.e., K320, K373, and K382 (to a lesser extent K372 and K381).

Our finding of p53/CBP association via a bromodomain/AcK382 binding also provides new insights into p53 functions in cellular senescence or apoptosis in response to oncogenic transformation or DNA damage. It has been shown that in these processes the tumor suppressor PML controls the targeting of p53 and CBP into the PML nuclear bodies where a p53-PML-CBP complex is formed (Pearson et al., 2000). The formation of such a trimeric complex, which is required for p53-induced senescence or apoptosis, is dependent upon acetylation at K382 (Pearson et al., 2000). Our results presented here imply that the effect of K382 acetylation on p53 association with CBP and PML in the PML nuclear bodies is likely exerted through its specific interaction with the bromodomain of CBP.

In addition to p53, CBP interacts with or acetylates a large number of cellular proteins, thus playing an important role in a wide variety of cellular processes (Giordano and Avantaggiati, 1999; Vo and Goodman, 2001). Chromosomal translocation between the *MLL* (mixed-lineage leukemia) gene and *CBP* has been reported to cause acute myeloid leukemia (Ayton and Cleary, 2001; Lavau et al., 2000; Liedman and Zeleznik-Le, 2001; Sobulo et al., 1997). Notably, it has been recently shown that in the MLL-CBP fusion protein, which consists of the N-terminal portion of MLL fused in frame to parts of CBP, consisting of its bromodomain and HAT domain, are minimally necessary and sufficient for developing acute myeloid leukemia—deletion of either the BRD or HAT domain of CBP completely abolishes the transforming activity of MLL-CBP (Lavau et al., 2000; Sobulo et al., 1997). Although the biological ligand(s) of the CBP bromodomain in the MLL-CBP fusion protein may or may not be p53, the new structural knowledge of ligand specificity of the CBP bromodomain reported here should facilitate identification and investigation of its biological ligand(s) in MLL-CBP-induced leukemogenesis.

The new structure of the CBP bromodomain/p53 AcK382 peptide complex extends our knowledge on ligand selectivity of the bromodomain family. Structural comparison of the CBP bromodomain/p53 AcK382 peptide (SHLKSKKGQSTSRHK-AcK-LMFK), the PCAF bromodomain/HIV-1 Tat AcK50 peptide (SYGR-AcK-KRRQR) (Mujtaba et al., 2002), and the GCN5p bromodomain/histone H4 AcK16 peptide (A-AcK-RHRKILRNSIQGI) (Owen et al., 2000) complexes reveals that the mechanism of acetyl-lysine recognition is conserved in all three bromodomain/ligand complexes. The acetyl-lysine binding involves nearly identical sets of conserved residues in the different bromodomains, corresponding to V1115, Y1167, N1168, and V1174 in CBP. However, each bromodomain does recognize different sets of amino acid residues flanking the AcK to achieve their specificity. For instance, PCAF bromodomain makes specific interactions with Y(AcK−3), R(AcK+3), and Q(AcK+4) on both sides of AcK50 in Tat, whereas the yeast GCN5p bromodomain has a few contacts with H(AcK+2) and

(AcK+3) at the AcK16 site in histone H4. These intermolecular interactions are distinctly different from those of the CBP bromodomain, which recognizes L(AcK+1), K(AcK−1), and H(AcK−2) at the AcK382 site in p53. Specifically, V763 in PCAF interacts directly with Y(AcK−3) in HIV-1 Tat (Mujtaba et al., 2002), whereas mutation of the corresponding I1128 to alanine in CBP has only a partial reduction in p53 peptide binding. Moreover, E756 in PCAF, which is important for interactions with R(AcK+3) and Q(AcK+4) at the AcK50 in Tat, is changed to L1119 followed by the unique two amino acid insertion in the CBP bromodomain. The hydrophobic residues near the two amino acid insertion are directly involved in CBP bromodomain binding to the L(AcK+1) and H(AcK−2) at the AcK382 site in p53. These distinct intermolecular interactions confer CBP bromodomain's recognition of AcK382 over AcK373 or AcK320 site in p53. Finally, because of these different modes of ligand recognition, the conformation of the bound peptide in CBP and PCAF bromdomains is also different—the p53 peptide forms a β turn-like conformation, whereas the HIV-1 Tat peptide adapts an extended conformation (Mujtaba et al., 2002). Taken together, these structural features of bromodomain/ligand complexes reinforce the notion that differences in ligand selectivity attribute to a few but important differences in bromodomain sequences, mostly in the ZA loop.

In summary, we show in this study that the bromodomain of the coactivator CBP binds specifically to p53 at the C-terminal transiently acetylated K382 upon UV-C irradiation. This bromodomain action is responsible for acetylation-dependent p53/CBP association in vivo, which is required for activation of the cyclin-dependent kinase inhibitor p21 in p53-induced cell cycle arrest. Our three-dimensional structure of the CBP bromodomain in complex with a lysine 382-acetylated p53 peptide together with complementary mutational analyses provides the detailed structural understanding for this selective molecular recognition and suggests that evolutionary changes of amino acid compositions in the ZA loop contribute directly to differences in ligand specificity of different bromodomains. Our findings underscore a specific bromodomain/acetyl-lysine interaction as a key step in p53 acetylation-dependent coactivator recruitment that is essential for the activation of p53 transcriptional regulation in DNA damage control.

Experimental Procedures

Sample Preparation and Plasmid Constructs

The bromodomain of CBP (residues 1082–1197) was expressed in *Escherichia coli* BL21(DE3) cells in the pET15b vector (Novagen). Isotope-labeled proteins were prepared from cells grown on a minimal medium containing $^{15}NH_4Cl$ with or without $^{13}C_6$-glucose in H_2O or 75% ^2H_2O. The protein was purified by nickel-IDA affinity chromatography, followed by thrombin cleavage to remove an N-terminal poly-His-tag. GST-fusion bromodomains of CBP (residues 1082–1197), PCAF (residues 712–832), and TIF1β (residues 702–812) were expressed in *E. coli* in the pGEX4T3 vector (Pharmacia) and purified with a glutathione-sepharose column. NMR spectra of the recombinant CBP, PCAF, and TIF1β were acquired to ensure proper protein folding. The acetyl-lysine-containing peptides were ordered from BioSynthesis, Inc. (Lewisville, TX). The CBP bromodomain was also subcloned into HA- (Clontech) or Flag-tagged (Stratagene) vectors. HA-tagged p53, p21 minimal promoter-driven luciferase construct, and β-galactosidase (β-gal expression vector) were described previously (Fuchs et al., 1998a). The expression constructs for human Flag-tagged p53 and mutants and CBP (pRSV-HA-CBP) are same as those described previously (Kwok et al., 1996; Nakamura et al., 2000).

In Vitro Protein-Peptide Binding Assay and Mutational Analysis

Site-directed mutagenesis of CBP was performed with the QuikChange kit (Stratagene). DNA sequencing confirmed the desired mutations. GST-bromodomains of CBP, PCAF, or TIF1β (10 μM) were incubated with an N-terminal biotinylated p53 AcK382 peptide (50 μM) in a 50 mM Tris buffer (pH 7.5) containing 50 mM NaCl, 0.1% BSA, and 1 mM DTT at 22°C for 2 hr. Streptavidin-agarose beads were added to the mixture and washed in the Tris buffer containing 500 mM NaCl and 0.1% NP-40. Proteins eluted from the beads were separated by SDS-PAGE and visualized in Western blot by anti-GST antibody and horseradish-peroxidase-conjugated goat anti-rabbit IgG. Peptide competition assay was performed by incubating a nonbiotinylated peptide with the CBP bromodomain and the biotinylated p53 AcK382 peptide. The molar ratio of the biotinylated and nonbiotinylated peptides in the mixture was kept at 1:2.

In Vivo p53 and CBP/p300 Binding Analysis

Mouse embryonic fibroblast 10.1 cells were propagated in Dulbecco's modified Eagle medium with 10% fetal bovine serum and penicillin-streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2. COS-7, 293T, and NIH/3T3 cells were maintained in 10% fetal calf serum. Transfections were performed using lipofectamine-plus reagent (Life Technologies) or the calcium phosphate method (Fuchs et al., 1998a). Plasmid DNAs of 1.0–2.5 μg were used in cell transfections. The transfected 10.1 cells were subject to UV-C irradiation of 50 J/m^2 36 hr after transfection and harvested 2–12 hr later. Protein expression was examined by Western blotting using monoclonal antibodies to HA (Roche Diagnostics), Flag (Stratagene), or β actin (Sigma), as well as rabbit polyclonal antibodies to CBP bromodomain, p53, and AcK382 of p53 (Trevigen). Western blots for endogenous p53, CBP, and p300 in NIH/3T3 cells were performed by specific antibodies from Santa Cruz Biotech.

Transcriptional Activation Assay

The 10.1 cells, transfected with p53 (2.5 μg), p21 luciferase (0.5 μg), and β-galactosidase (0.5 μg) with and without the CBP bromodomain (1.0 μg) were irradiated by UV-C and harvested as described above. The cell extracts were assayed for luciferase activity using a luciferase assay (Promega). Luciferase activity derived from p53-induced p21 activation was normalized to a cotransfected vector expressing β-galactosidase.

Cell Cycle Analysis

Cell cycle analysis of the 10.1 cells, transfected with Us9-GFP, wild-type, or mutant p53 with or without the CBP bromodomain was performed on Calibur flow cytometer (Becton Dickinson) using GFP and PI staining. Cells were treated with the UV-C irradiation, harvested after 12 hr by trypsinization, washed with PBS, and fixed in chilled 70% ethanol in PBS buffer. One hour before acquiring the data, cells were washed again with PBS and stained with PI. The data were acquired and analyzed using CellQuest software.

In Vitro HAT Assay

In vitro HAT acetylation was done using a procedure described previously (Lavau et al., 2000). The purified GST, GST-p53, or GST-p53 mutant was incubated with the HAT domain of p300 (residues 1195–1707) (Upstate Biotech) or CBP (residues 1174–1850) in a 50 mM Tris buffer (pH 8.0) containing 2% glycerol, 0.1 mM EDTA, and 1 mM dithiotheritol and acetyl-CoA for 1 hr at 30°C. Acetylation of p53 was assessed by Western analysis using p53 AcK382 antibody.

NMR Spectroscopy

NMR samples contained a protein/peptide complex of ~0.5 mM in 100 mM phosphate buffer (pH 6.5) containing 5 mM perdeuterated DTT and 0.5 mM EDTA in $H_2O/^2H_2O$ (9/1) or ^2H_2O. All NMR spectra were acquired at 30°C on a Bruker 500 or 600 MHz NMR spectrometer. The ^1H, ^{13}C, and ^{15}N resonances of the protein were assigned

by triple-resonance NMR spectra collected with a $^{13}C/^{15}N$-labeled and 75% deuterated protein bound to an unlabeled peptide (Yamazaki et al., 1994). The distance restraints were obtained in 3D ^{13}C- or ^{15}N-NOESY spectra (Clore and Gronenborn, 1994). Slowly exchanging amides, identified in 2D ^{15}N-HSQC spectra recorded after a H_2O buffer was changed to a ^2H_2O buffer, were used with structures calculated with only NOE distance restraints to generate hydrogen-bond restraints for final structure calculations. The intermolecular NOEs were detected in ^{13}C-edited (F_1), $^{13}C/^{15}N$-filtered (F_3) 3D NOESY spectrum (Clore and Gronenborn, 1994).

Structure Calculations

Structures of the CBP bromodomain/p53 peptide complex were calculated with a distance geometry-simulated annealing protocol using the X-PLOR program (Brunger, 1993). Long-range NOE assignments were assisted by model structures generated by homology modeling with the program Modeller (Sali and Blundell, 1993), using the PCAF bromodomain structure as a template (Dhalluin et al., 1999; Mujtaba et al., 2002). Manually assigned NOE-derived distance restraints were used to calculate initial structures. ARIA (Nilges and O'Donoghue, 1998)-assigned distance restraints agree with structures calculated using only the manually determined NOE distance restraints. Ramachandran plot analysis of the final structures was performed with Procheck-NMR program (Laskowski et al., 1996).

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Accession Numbers

Coordinates for the three-dimensional structure of the CBP bromodomain/p53 peptide complex have been deposited in the Brookhaven Protein Data Bank under the accession code 1JSP.