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Acetylation of the C Terminus of Ku70 by CBP and PCAF Controls Bax-Mediated Apoptosis

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Summary

Apoptosis is a key tumor suppression mechanism that can be initiated by activation of the proapoptotic factor Bax. The Ku70 DNA end-joining protein has recently been shown to suppress apoptosis by sequestering Bax from mitochondria. The mechanism by which Bax is regulated remains unknown. Here, we identify eight lysines in Ku70 that are targets for acetylation in vivo. Five of these, K539, K542, K544, K533, and K556, lie in the C-terminal linker domain of Ku70 adjacent to the Bax interaction domain. We show that CBP and PCAF efficiently acetylate K542 in vitro and associate with Ku70 in vivo. Mimicking acetylation of K539 or K542 or treating cells with deacetylase inhibitors abolishes the ability of Ku70 to suppress Bax-mediated apoptosis. We demonstrate that increased acetylation of Ku70 disrupts the Ku70-Bax interaction and coincides with cytoplasmic accumulation of CBP. These results shed light on the role of acetyltransferases as tumor suppressors.

Introduction

Histone acetyltransferases (HATs) were first characterized as proteins involved in the regulation of chromatin structure through modification of specific lysines in histone tails. Recently, these enzymes have also been recognized as critical regulators of nonhistone proteins that can coordinate critical cellular processes, such as cell growth, differentiation, and apoptosis (Chan and La Thangue, 2001). In humans, there are five families of acetyltransferases: PCAF/GCN5, p300/CBP, TAF250, SRC1, and MOZ (Kouzarides, 2000). Of these, PCAF/GCN5 and p300/CBP are the most well characterized. p300 and CBP were originally identified as transcriptional adaptors that respond to signals from other activators and were subsequently shown to possess HAT activity (Ogryzko et al., 1996). Although p300, CBP, and PCAF are found to associate in vivo and can target the same residue, they typically exhibit a high degree of specificity, even targeting different residues within the same polypeptide (Giordano and Avantaggiati, 1999; Kouzarides, 2000; Liu et al., 1999). Despite this specificity, no clear consensus for acetylation has been found.

There is a growing body of evidence that acetyltransferases, such as p300, CBP, and PCAF, play an important role in tumor suppression (Giordano and Avantaggiati, 1999; Phillips and Vousden, 2000; Schiltz and Nakatani, 2000). For example, deletions, translocations, and point mutations within p300, CBP, and PCAF have been found in numerous human tumors (Giles et al., 1997; Ida et al., 1997; Muraoka et al., 1996; Ozdag et al., 2002; Sugita et al., 2000; Tillinghast et al., 2003), and mice lacking CBP or p300 are predisposed to hematologic malignancies (Blobel, 2000; Kung et al., 2000; Rebel et al., 2002). In addition, the tumor suppressors p53 and Rb have recently been shown to be regulated by acetylation (Chan et al., 2001; Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Although the mechanisms by which acetyltransferases suppress tumorigenesis remain unclear, their role in regulating the activity of these tumor suppressors is likely to be a major factor (Giordano and Avantaggiati, 1999).

A key mechanism of tumor suppression is cell death by apoptosis (reviewed by Evan and Vousden, 2001). A key regulatory step in this process is activation of the proapoptotic factor Bax. Although the mechanisms by which Bax becomes activated by cellular damage have remained unclear, several downstream events have been elucidated. Following its activation, Bax translocates to the outer mitochondrial membrane where it oligomerizes, renders the membrane permeable, and releases several death-promoting factors, including cytochrome c (Scorrano and Korsmeyer, 2003).

A recent study has shed light on a mechanism by which Bax is rendered inactive. In normal, undamaged cells, Bax interacts with the C terminus of the Ku70 protein, sequestering it from mitochondria (Sawada et al., 2003b). Overexpression of Ku70 blocks Bax-mediated apoptosis, whereas depletion of Ku70 renders cells more sensitive to a variety of apoptotic stimuli (Kim et al., 1999; Sawada et al., 2003b). Furthermore, the interaction between Ku70 and Bax is abolished following UV damage. Together, these results demonstrated that Ku70 is a physiologically relevant inhibitor of Bax-mediated apoptosis (Sawada et al., 2003b).

Ku70 was first characterized as part of the Ku70/Ku80 heterodimer that is essential for the repair of DNA double-strand breaks by nonhomologous end joining (NHEJ) and the rearrangement of antibody and T cell receptor genes via V(D)J recombination (Featherstone and Jackson, 1999). The Ku70/80 heterodimer also has important roles in telomere maintenance and transcriptional regulation (Tuteja and Tuteja, 2000). Ku70$^{-/-}$ knockout mice are hypersensitive to ionizing radiation (Ouyang et al., 1997), are immune compromised (Manis et al., 1998), and have increased apoptotic neuronal death during embryonic development (Gu et al., 2000).

Interestingly, cells from Ku70 knockout mice are also hypersensitive to agents, such as staurosporine (STS), that promote apoptosis in the absence of DNA damage (Chechlacz et al., 2001). This is consistent with a physio-

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logical role for Ku70 in suppressing apoptosis, independent of its role in DNA repair. Although Ku70 is a predominately nuclear protein, it is suspected that the less abundant cytoplasmic pool is responsible for Bax sequestration (Sawada et al., 2003b). Given Ku70's dual role in DNA end joining and suppressing apoptosis, it could conceivably be a central player in coordinating DNA repair with the decision between cell survival and programmed cell death.

Apart from a single previous study showing that Ku70 can be phosphorylated by DNA-PK in vitro (Chan et al., 1999), no posttranslational modifications of Ku70 have been reported and the means by which this protein is regulated are poorly understood. In this paper, we identify eight lysines in Ku70 that are modified by acetylation in vivo. Five of these residues lie in the C-terminal linker of Ku70 adjacent to the Bax-interaction domain, in a region that aligns with two clusters of acetylated residues in p53 (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). We present multiple lines of evidence that acetylation of either K539 or K542 in the C-terminal linker is sufficient to completely block the ability of Ku70 to suppress Bax-mediated apoptosis. Further, we show that CBP and PCAF associate with Ku70 in vivo, target K542 for acetylation, and inhibit the ability of Ku70 to suppress Bax-mediated apoptosis. Similarly, treatment of cells with histone deacetylase (HDAC) inhibitors blocks Ku70's ability to suppress apoptosis, and we show that this is due to disruption of the Ku70-Bax interaction. These findings demonstrate that acetylation negatively regulates the antiapoptotic function of Ku70 in vivo, thus providing a critical link between acetyltransferases and tumor suppression.

Results

Ku70/80 Is Acetylated In Vivo by CBP and PCAF

Acetylation is emerging as an important mechanism by which many nonhistone proteins are regulated (Chan et al., 2001; Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). For example, acetylation of three lysines in the C terminus of p53 (i.e., K373, K382, and K320) by CBP, PCAF, or p300 increases the stability of the protein and increases p53-dependent transcription, thus promoting growth arrest and apoptosis (reviewed in Grossman, 2001). To identify additional factors that might be acetylated following a DNA damage signal, we searched for proteins with homology to the two clusters of acetylation sites in the C terminus of p53 (aa 302–326 and 367–392). One of the closest matches was to the C-terminal linker region of Ku70, which has been difficult to define structurally due to its apparent flexibility (Zhang et al., 2001) (Figure 1A). The Ku70/p53 alignment suggested a potential consensus sequence [(T)KRKX_{3-5}SGSX_2KK] that also aligned with known acetylated domains in the flap endonuclease FEN1, the transcription factor GATA1, and the transcription initiation factor EFIIEβ (Figure 1B). Based on this alignment, we predicted that lysines within the C-terminal linker domain of Ku70 would be likely targets for acetylation in vivo.

To test this prediction, we generated a rabbit polyclonal antibody against pan-acetyl-lysines (panAc-K). By Western blot analysis, this antibody specifically recognized acetylated proteins and did not recognize unacetylated recombinant Ku70 (data not shown). Cell extracts from HeLa cells were immunoprecipitated with an anti-Ku70 monoclonal antibody (mAb) or an antihemagglutinin (HA) mAb as a negative control and probed with the panAc-K antibody. As shown in Figure 1C, two bands were recognized by the panAc-K in the anti-Ku70 immunoprecipitation (IP) lane but not in the control (left panel). Reprobing the blot with anti-Ku70 or anti-Ku80 monoclonal antibodies confirmed that the acetylated bands corresponded to the positions of Ku70 and Ku80 (Figure 1C, middle and right panels). In a reverse experiment, immunoprecipitation with the panAc-K antiserum but not preimmune serum precipitated Ku70 and Ku80 (Figure 1D). These results provide strong evidence that Ku70 and Ku80 are acetylated in vivo.

The three histone acetyltransferases CBP, p300, and PCAF are known to target nonhistone proteins for acetylation (Brown et al., 2000). To test whether Ku70 interacts with these acetyltransferases in vivo, we immunoprecipitated Ku70 from HeLa or 293 cells and the immunocomplex was probed for CBP, p300, or PCAF. In both cell lines, we could detect an interaction between native CBP and Ku70 but not Ku80 (Figure 1E and data not shown). The CBP-Ku70 interaction was not disrupted by the DNA intercalating agent ethidium bromide (50 μg/ml), indicating that the protein interaction was not bridged by DNA. A weaker interaction between PCAF and Ku70 was also observed by IP, and no interaction could be detected between p300 and Ku70 (data not shown).

Mapping Regions of Ku70 Acetylation In Vitro

Next, we tested whether the Ku70/80 complex could serve as a substrate for CBP, PCAF, or p300 using an in vitro acetylation assay. Recombinant Ku70/80 complex was purified from insect cells and incubated with [^{3}H]-acetyl-CoA and the histone acetyltransferase (HAT) domains of CBP, PCAF, or p300. The reaction products were then resolved by SDS-PAGE and analyzed by autoradiography. As shown in Figure 2A, a strongly labeled band corresponding to the size of Ku70 was observed in each of the complete acetyltransferase reactions (lanes 4–6) but not in reactions lacking recombinant Ku70/80 (lane 1–3) or an acetyltransferase (lane 7). A weak band corresponding to Ku80 was also observed (lanes 4–6). Under these conditions, p53 control peptides known to act as substrates of these enzymes were labeled to a similar extent by CBP, PCAF, and p300 (Liu et al., 1999) (data not shown). These results demonstrate that Ku70 can serve as an efficient substrate for all three acetyltransferases. Based on the intensity of the bands, CBP has the strongest preference for Ku70, which is consistent with the robust interaction between Ku70 and CBP in vivo.

Due to the strong interaction between Ku70 and CBP and the efficient acetylation of Ku70 in vitro, we sought to define the regions of Ku70 that are targeted for acetylation. A library of 31 peptides was synthesized to cover the entire Ku70 sequence (Figure 2B). Each of these peptides was incubated in an acetylation reaction as above, with either PCAF or CBP. Again, a p53 peptide

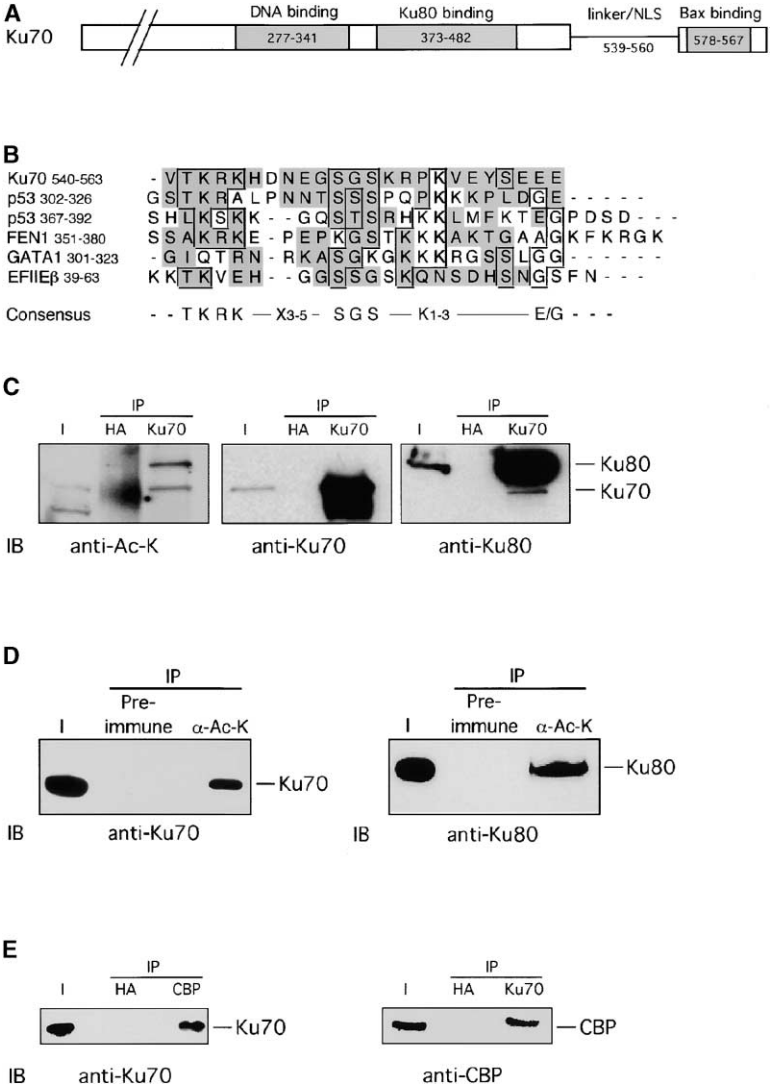

Figure 1. The Ku70/80 Heterodimer Is Acetylated In Vivo

(A) Schematic representation of Ku70 showing the C-terminal linker relative to the known functional domains.

(B) A multiple sequence alignment of the Ku70 linker region with known acetylation sites of other proteins. A putative consensus sequence is shown below the alignment.

(C) The Ku70/80 complex was immunoprecipitated from HeLa cell extracts with anti-Ku70 antibody and immunoblotted using a polyclonal antibody against pan-acetylated lysines (anti-panAc-K). The cell extract input lane (I) was loaded as 1/15 dilution of the pre-IP extract and an anti-HA mAb served as a negative control. Reprobing of the membrane with anti-Ku70 and anti-Ku80 mAb showed that the two acetylated bands corresponded to the position of Ku70 and Ku80.

(D) Immunocomplexes were precipitated from HeLa extracts with the anti-panAc-K antibody and immunoblotted with an anti-Ku70 or anti-Ku80 mAb. The input lane was loaded as 1/15 dilution of the pre-IP extract, and preimmune serum served as a negative control.

(E) CBP was immunoprecipitated from HeLa extracts with an anti-CBP monoclonal antibody and the immunocomplex was probed with an anti-Ku70 mAb (left panel). Ku70 was immunoprecipitated with an anti-Ku70 mAb, and the immunocomplex was blotted using anti-CBP polyclonal antibody (right panel). An anti-HA mAb served as a negative control for both experiments.

served as a positive control. As shown in Table 1, five of the peptides (3, 8, 15, 16, and 29) were acetylated by PCAF but only two (16 and 29) were strongly acetylated by both PCAF and CBP (Figure 2C). Interestingly, peptide 16 (RQIILEKEETEELKRFD$_{325–341}$), which contains two lysines (K331 and K338), is located within the region of Ku70 that forms a ring structure that threads onto broken DNA (Walker et al., 2001) (see Figure 3C). Peptide 29 (TKRKHDNEGSGSKRPKVEYSEE$_{541–562}$), which contains four lysines (K542, K544, K553, and K556), is located within the C-terminal flexible linker region that we had previously identified as a potential target for acetylation (see Figure 1B).

To determine which lysines in peptide 29 were being acetylated in the reaction, a series of substitutions were made in which three out of the four lysines were replaced with arginine, a residue that cannot be acetylated. Each peptide was then incubated with either PCAF or CBP and analyzed by autoradiography as above. As shown in Figure 2D, the peptide that retained K542 (KRRR) was the preferred target of both PCAF and CBP and was acetylated to almost the same extent as the original peptide 29 (KKKK). K553 (RRKR) was also weakly acetylated by PCAF and CBP. These results suggest that K542 and K553 might be targets of CBP and PCAF in vivo.

Identifying Residues in Ku70 that Are Acetylated In Vivo

To test whether the C-terminal linker of Ku70 could be acetylated in vivo, amino acids 537–557 of Ku70 were expressed as a fusion to GFP (pEGFP-Ku70$_{537–557}$) (Bertinato et al., 2001). The fusion peptide was immunoprecipitated from HeLa cells using an anti-GFP antibody, and acetylation was assessed by Western analysis using the panAc-K polyclonal antibody. As shown in Figure 2E, the panAc-K antibody strongly recognized the GFP-Ku70$_{537–557}$ fusion but not the untagged GFP control, suggesting that the Ku70 linker region is targeted for acetylation in vivo.

Next, we sought to provide more conclusive evidence that this region and others in Ku70 are subject to acetylation in vivo. We purified Ku70 either from 293 cells stably expressing 6xHIS-Ku80 using a one-step purification

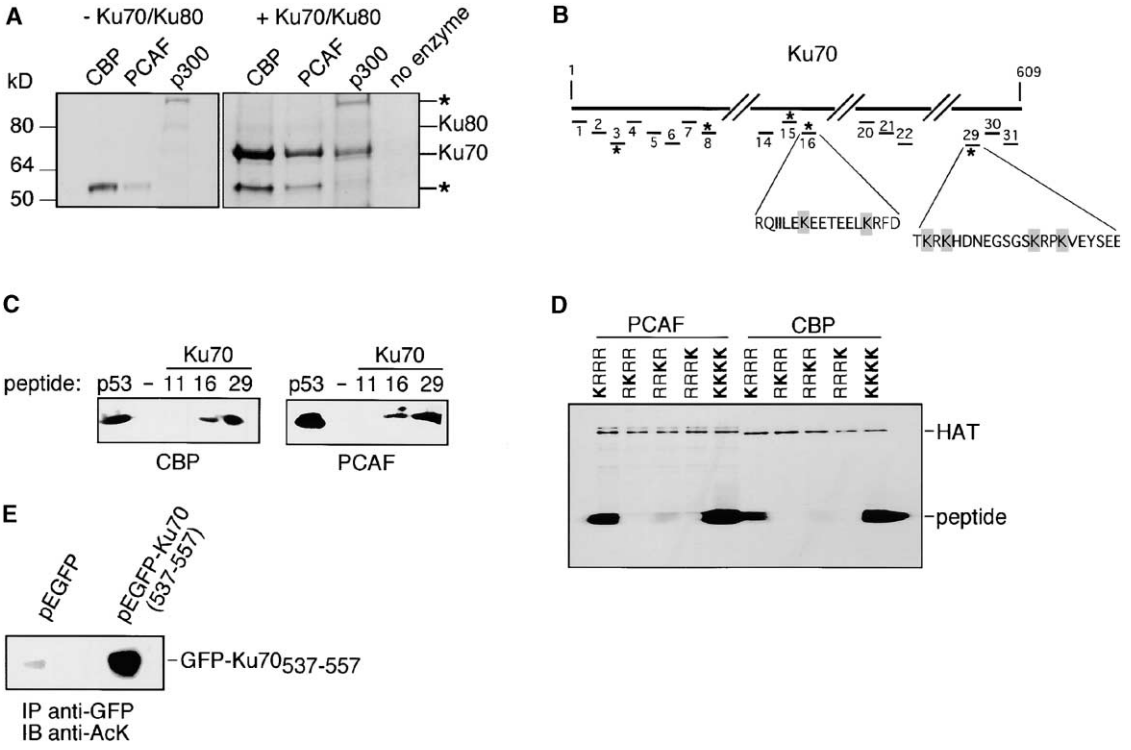

Figure 2. Mapping of Ku70 Acetylation Sites In Vitro

(A) Acetylation assays were performed by incubating the recombinant histone acetyltransferase (HAT) domains of CBP, PCAF, or p300 with recombinant Ku70/80 in the presence of ^{3}H-acetyl-CoA. The products of the reactions were separated by SDS-PAGE and analyzed by autoradiography. Reactions lacking Ku70/80 are shown in the left panel. Bands marked with asterisks at 55 kDa and 90 kDa correspond to autoacetylation products that have been described previously (Liu et al., 2000).

(B) Schematic representation of the synthetic peptide library spanning the entire length of Ku70. Each peptide was incubated with PCAF and ^{3}H-acetyl-CoA and analyzed as in (A). Peptides that were acetylated by PCAF in vitro are indicated by an asterisk.

(C) Representative acetylation products resolved by SDS-PAGE are shown for peptides 16 and 29 (PCAF reaction, left panel; CBP reaction, right panel). The acetylated domain of p53 (aa 315–325) served as a positive control for acetylation. Peptide 11, which was not a target for acetylation, served as a negative control.

(D) A series of scanning synthetic peptides of peptide 29 were synthesized, with three out of the four lysines (K) substituted for arginine (R), a residue that cannot be acetylated. Peptides were incubated in acetylation reactions with PCAF or CBP and resolved by SDS-PAGE as above.

(E) HeLa cells were transfected with vectors expressing GFP-Ku70$_{537–557}$ or GFP alone. GFP-containing immunocomplexes were precipitated with an anti-GFP mAb and immunoblotted with the anti-panAc-Lys antibody.

on a Ni-NTA agarose column or from HeLa cells by immunoprecipitation using an anti-Ku70 polyclonal antibody followed by SDS-PAGE separation. Isolated proteins were then digested with either trypsin, chymotrypsin, V8, or AspN and subjected to tandem mass spectrometry analysis (LC-MS/MS, see Experimental Procedures). Multiple proteases were used in order to maximize sequence coverage.

Ku70-derived peptides covering 80% of the sequence were analyzed, and eight acetylation sites were identified using the MASCOT search algorithm (Perkins et al., 1999). Six sites were located within the regions covered by peptides 16 and 29 (K331, K338, and K542, K544, K553, K556, respectively) (Figure 3A), the same two peptides that were strongly acetylated in vitro by PCAF and CBP (see Figure 2C). Evidence of in vivo acetylation was also obtained for K317 and K539. The latter residue is located proximal to the region of peptide 29 and may also be part of this apparent C-terminal acetylation domain. Most peptides appeared to be acetylated on more than one lysine and several were fully acetylated, indicating that there are multiple species of acetylated Ku70

Table 1. Ku70 Peptides Acetylated by PCAF In Vitro

Peptide Number	Amino Acid Position	Peptide Sequence	Relative Intensity of Acetylation[a]
3	44–58	ASKAMFESQSEDELT	+
8	157–173	VQFKMSHKRIMLFTNED	++
15	310–322	LLLPSDTKRSQIY	+++
16	325–341	RQIILEKEETEELKRFD	+++
29	541–562	TKRKHDNEGSGSKRPKVEYSEE	+++++

[a]Band intensity was measured using NIH ImageJ software and normalized to the intensity of peptide 3.

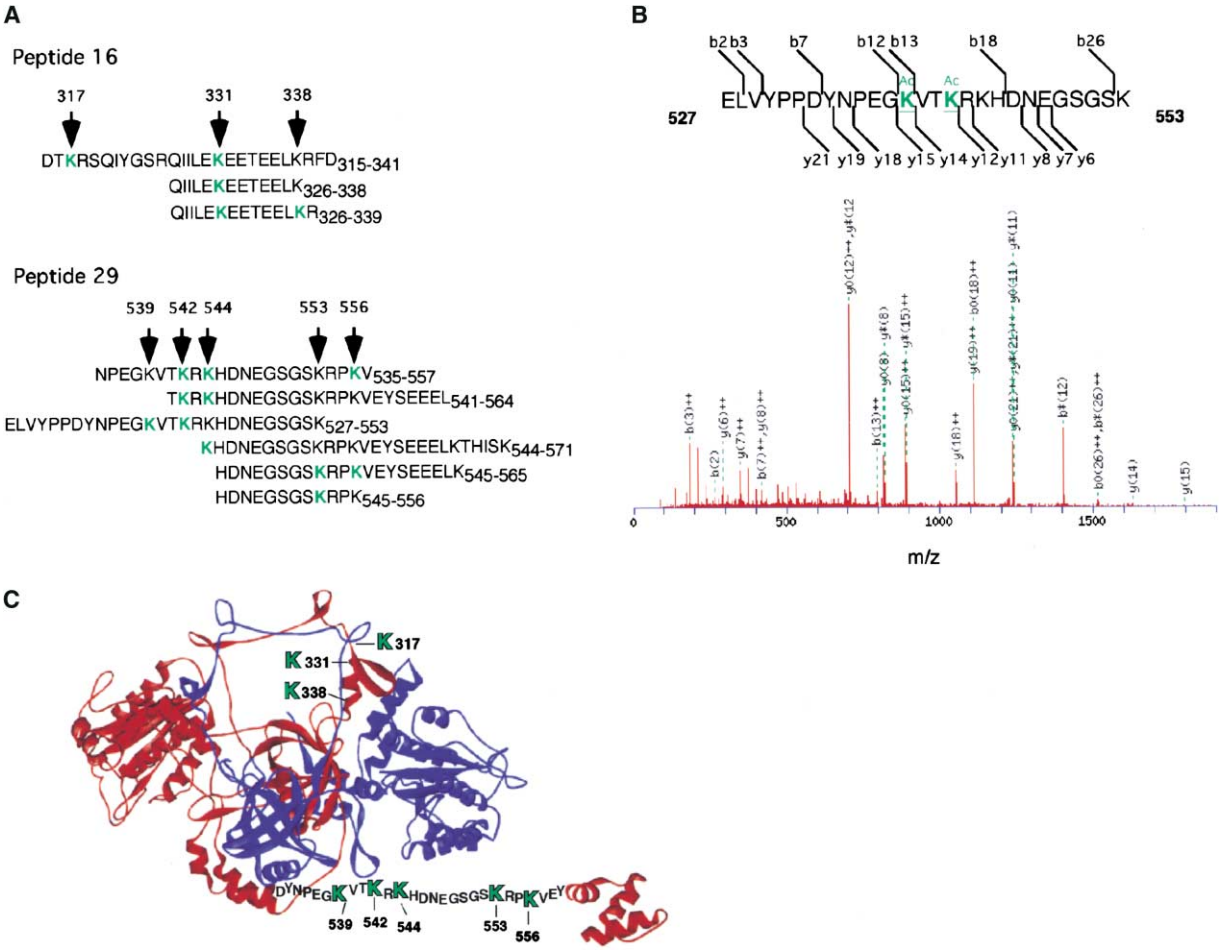

Figure 3. Identification of Residues in Ku70 that Are Acetylated In Vivo

(A) Endogenous Ku70 complexes were purifed on a large scale and subjected to tandem mass spectrometry (LC-MS/MS) analysis. The following acetyl-lysine residues were identified: 317, 331, 338, 539, 542, 544, 553, and 556 (green). These sites were typically identified multiple times on mono-, di-, or triacetylated peptides.

(B) Representative MS/MS spectrum of a Ku70-derived tryptic peptide (aa 527–553) as identified by MASCOT software (see Experimental Procedures). The $(M + H)^{4+}$ species of the peptide 527–553 (MW, 3215.45) contains modifications on Glu527 (sodium), Glu537 (sodium), Lys539 (acetyl), Lys542 (acetyl), and a sodiated C terminus. b and y ions are also indicated.

(C) Lysine residues in the C-terminal linker and DNA-contacting loop of Ku70 that are targeted for acetylation in vivo, superimposed on a ribbon diagram of Ku70/Ku80 based on a crystal structure (Walker et al., 2001). Acetylation sites confirmed by MS/MS are indicated (green).

in vivo (Figure 3A). Most of the acetylated lysine residues were detected in overlapping peptides derived from at least two independent protein preparations. The appearance of the 143 Da immonium ion for each peptide, as demonstrated for the peptide (aa 527–553), provided additional evidence of acetylation (Figure 3B). The position of the acetylated residues in peptides 16 and 29 are shown on a predicted Ku70 crystal structure (Walker et al., 2001) (Figure 3C).

Ku70 Is a Target for HDAC and Sirtuin Deacetylases

Protein acetylation levels in vivo are the result of a dynamic equilibrium between the activity of acetyltransferases and the opposing deacetylases. Histone deacetylases (HDACs) can be divided into three classes based on their homology, substrate requirements, and sensitivity to certain inhibitors. Class I/II deacetylases are sensitive to the inhibitor trichostatin A (TSA), whereas class III deacetylases of the NAD^+-dependent sirtuin family are specifically inhibited by nicotinamide (NAM) (Bitterman et al., 2002; Landry et al., 2000; Luo et al., 2001; Yoshida and Horinouchi, 1999).

To determine which class of deacetylase targets Ku70 in vivo, cells were treated with either TSA or NAM and the acetylation level of Ku70 was detected using the panAc-K antibody. Treatment with either NAM (5 mM) or TSA (1 μM) increased the total acetylation level of Ku70 by 1.8- and 2.4-fold, respectively (Figure 4A). The effect of combined treatment was additive, increasing total acetylation $\sim$4-fold (Figure 4A). These results suggest that Ku70 is targeted for deacetylation in vivo by both class I/II HDACs and class III/sirtuin deacetylases.

Ku70 Acetylation Regulates Bax-Mediated Apoptosis

Given that the C-terminal linker domain of Ku70 is a target for CBP and PCAF in vitro and that it lies adjacent

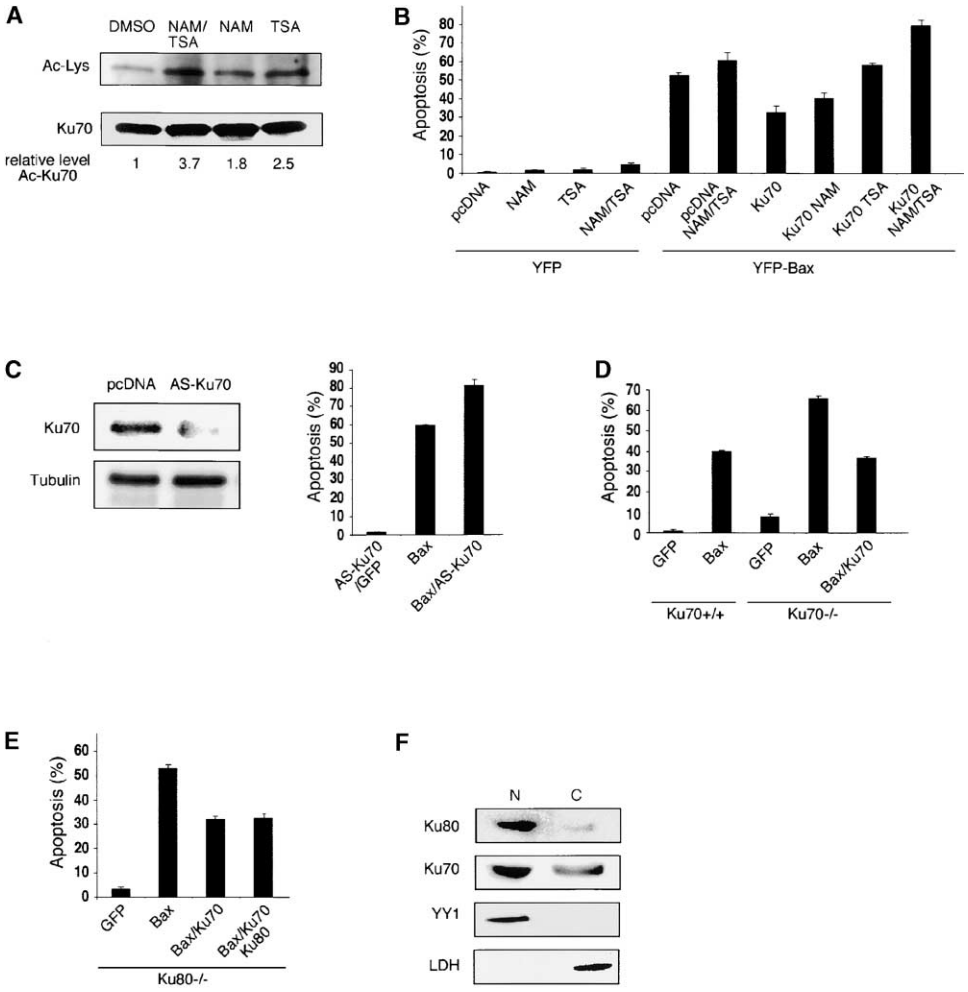

Figure 4. Endogenous Ku70 Suppresses Apoptosis Independently of Ku80, and This Function Is Blocked by Acetylation

(A) HeLa cells were grown under one of the following conditions: 0.1% DMSO, 1 μM TSA, 5 mM nicotinamide (NAM), or TSA and NAM. Ku70 was immunoprecipitated from whole-cell extracts and probed for lysine acetylation using a panAc-Lys Ab. The level of acetylated Ku70 (Ac-Ku70) normalized to the DMSO treatment is shown below the blot.

(B) 293T cells were cotransfected with YFP-Bax and pcDNA-Ku70 in the presence or absence of TSA/NAM. The percentage of cells with apoptotic nuclei were scored 24 hr posttransfection.

(C) An antisense Ku70 construct (AS-Ku70) was cotransfected with a Bax-GFP construct into 293T cells, and the percentage of cells undergoing apoptosis was quantified as above. Ku70 protein levels in the AS-Ku70 transfected cells was determined by Western blotting in which β-tubulin served as a loading control.

(D) Bax-GFP or GFP constructs were transfected into mouse embryonic fibroblasts (MEFs) derived from $Ku70^{+/+}$ or $Ku70^{-/-}$ littermates. To determine whether the apoptotic phenotype of $Ku70^{-/-}$ cells was due specifically to the absence of Ku70, the effect of Bax expression was also determined in $Ku70^{-/-}$ cells into which Ku70 was reintroduced.

(E) The ability of Ku70 and/or Ku80 to suppress Bax-mediated apoptosis was assessed in xrs6 ($Ku80^{-/-}$) MEFs, as described in (B).

(F) Nuclear (N) and cytosolic (C) fractions were isolated by differential centrifugation, and the relative levels of Ku70 and Ku80 were determined by Western blotting. The purity of each fraction was ascertained by reprobing the blot for nuclear and cytoplasmic markers (YY1 and LDH, respectively).

to the Bax interaction domain, we hypothesized that acetylation of this region might play a role in regulating the ability of Ku70 to suppress apoptosis. Human embryonic kidney cells (293T) were transfected with a Bax-YFP expression construct and YFP-positive cells were scored 24 hr later for a fragmented nucleus, a well-characterized apoptotic phenotype (Sawada et al., 2003b). Consistent with previous reports, overexpression of full-length Ku70 suppressed the induction of apoptosis by Bax (Figure 4B).

To test whether increased Ku70 acetylation affected Bax-mediated apoptosis, the same experiment was conducted in the presence of the HDAC inhibitors NAM and/or TSA. As shown in Figure 4B, treatment of cells with NAM or TSA abrogated the ability of Ku70 to suppress apoptosis. In the case of TSA, apoptosis suppression was completely blocked. Simultaneous treatment with both inhibitors had an additive effect on apoptosis (Figure 4B) such that cell death was slightly higher than untreated cells, raising the possibility that acetylated Ku70 plays an additional role in promoting apoptosis. Treatment of cells with HDAC inhibitors in the absence

of Bax transfection had no appreciable effect on apoptosis.

We wished to ensure that the results observed in the presence of ectopic Ku70 expression were representative of the role of the endogenous protein. First, expression of endogenous Ku70 was reduced 7-fold by introducing a Ku70 antisense (AS-Ku70) construct into 293T cells. Consistent with a previous report (Sawada et al., 2003b), this led to a marked increase in Bax-mediated apoptosis compared to an empty vector control (Figure 4C). Second, mouse embryonic fibroblasts (MEFs) lacking Ku70 ($Ku70^{-/-}$) were transfected with YFP Bax, and the level of apoptosis was determined as above (Figure 4D). Consistent with the antisense experiment, the $Ku70^{-/-}$ cells exhibited higher levels of Bax-mediated apoptosis compared to the $Ku70^{+/+}$ MEFs. Furthermore, reintroduction of Ku70 into $Ku70^{-/-}$ cells restored levels of apoptosis to that of wild-type $Ku70^{+/+}$ cells. Together, these results demonstrate that endogenous Ku70 suppresses Bax-mediated apoptosis.

Next, we addressed whether Ku70 suppresses Bax-mediated apoptosis as part of the Ku70/80 complex or whether Ku70 acts as a single polypeptide. As shown in Figure 4E, Ku70 suppressed Bax-mediated apoptosis in CHO cells lacking Ku80 (Bertinato et al., 2001), demonstrating that the ability of Ku70 to suppress apoptosis does not depend on an association with Ku80. Furthermore, comparison of the subcellular distributions of Ku70 and Ku80 showed that there is a significantly higher proportion of Ku70 than Ku80 in the cytosol, relative to the nuclear pool (Figure 4F). Together, these findings indicate that Ku70 sequesters Bax independently of Ku80 and that this association likely occurs in the cytosol.

Acetylation of K539 and K542 Promotes Bax-Mediated Apoptosis

To further test the possibility that acetylation of Ku70 regulates its ability to suppress Bax, we examined Bax-induced apoptosis in cells overexpressing CBP and PCAF. Consistent with the TSA/NAM results, overexpression of either CBP or PCAF eliminated the ability of Ku70 to suppress apoptosis, whereas overexpression of CBP or PCAF in the absence of Ku70 had no appreciable effect (Figures 5A and 5B). There was also no significant effect of overexpressing CBP or PCAF alone (data not shown).

Next, we examined whether this phenotype was specifically due to the acetylation of lysines within the flexible linker region of Ku70. We replaced each of these residues with either glutamine (K to Q) or arginine (K to R) to mimic constitutively acetylated and nonacetylated states, respectively (Li et al., 2002). 293T cells were then cotransfected with the YFP-Bax expression construct along with wild-type or each of the mutated Ku70 expression vectors, which we confirmed by Western analysis were expressed at similar levels to the wild-type construct (data not shown). The percentage of YFP-positive cells undergoing apoptosis was scored 24 hr later. Single substitution of any of the five lysine residues with arginine (K539R, K542R, K544R, K553R, or K556R) had no significant effect on the ability of Ku70 to suppress Bax-mediated apoptosis (Figure 5C). In contrast,

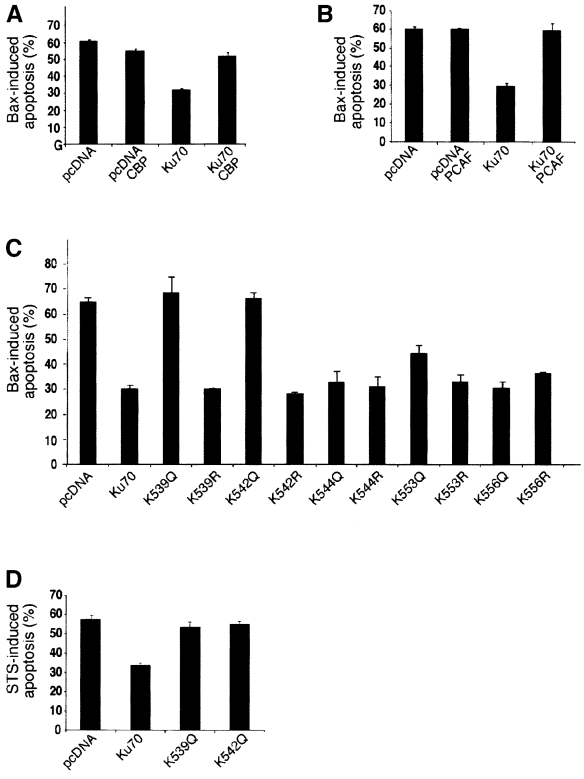

Figure 5. Identification of Lysines 539 and 542 in Ku70 as Critical Regulators of Bax-Mediated Apoptosis

(A) 293T cells were cotransfected with Bax and/or CBP with Ku70 or empty vector controls. Apoptosis was scored 24 hr later, as above.

(B) 293T cells were cotransfected with Bax and/or PCAF with Ku70 or empty vector controls.

(C) A YFP-Bax fusion construct was cotransfected with pcDNA, Ku70, or Ku70 mutants bearing K→Q or K→R substitutions for each acetylation site in the Ku70 linker region, as indicated. Levels of apoptosis were determined as above.

(D) The Ku70 wild-type and Ku70 mutants bearing K→Q substitutions at positions K539 and K542 were examined for their ability to suppress staurosporine (STS)-induced apoptosis.

the substitution of either lysine 539 or 542 with glutamine (K539Q and K542Q) completely blocked the ability of Ku70 to inhibit Bax, while the K553Q substitution had an intermediate effect (Figure 5C).

Because Ku70 is a DNA repair protein, we wanted to examine the effect of Ku70 on apoptosis induced in the absence of DNA damage. Staurosporine (STS) is an alkaloid that inhibits phospholipid/Ca^{2+}-dependent and cyclic nucleotide-dependent kinase and can induce apoptosis independent of DNA damage by activating proapoptotic Bcl2 family members, such as Bax and Bak (Rampino et al., 1997; Wei et al., 2001). In STS-treated cells, Ku70 is known to selectively inhibit Bax-mediated apoptosis (Sawada et al., 2003b). As shown in Figure 5D, overexpression of Ku70 blocked apoptosis in STS-treated cells whereas the mutants K539Q and K542Q did not. Together with the in vitro acetylation studies and the LC-MS/MS data, these results provide strong evidence that acetylation of residues K539 and K542 in Ku70 are critical for the regulation of Bax-mediated apoptosis.

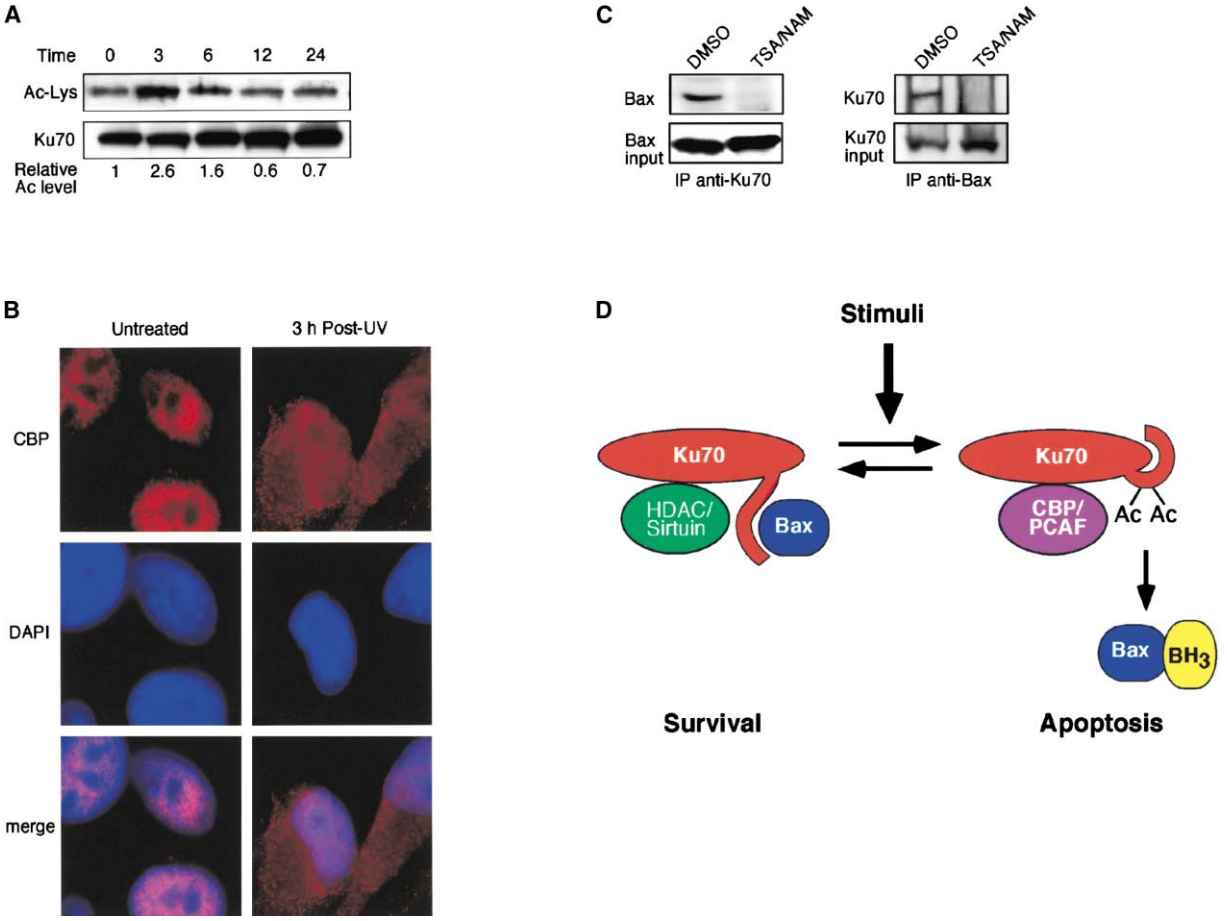

Figure 6. Acetylation of Ku70 Increases Following UV and Abrogates the Association between Ku70 and Bax

(A) 293T cells were treated with 200 J/cm^2 of UV and the levels of Ku70 acetylation were determined 3, 6, 12, and 24 hr posttreatment. Numbers represent band quantitation using NIH ImageJ software.

(B) 293T cells treated as in (A) and immunostained for CBP (red) and DAPI (blue). Staining pattern shown is representative of >90% of cells.

(C) 293T cells were grown in the presence of DMSO or deacetylase inhibitors TSA and NAM. Ku70 was immunoprecipitated and products were immunoblotted with an anti-Bax polyclonal Ab (left panel). The reverse IP is also shown (right panel).

(D) Model for the regulation of Bax-mediated apoptosis by Ku70 acetylation. Cytosolic Ku70 functions independently of Ku80 to sequester the proapoptotic protein Bax from mitochondria. Under normal growth conditions, Ku70's C-terminal α-helical domain is maintained in an unacetylated state by HDACs and/or sirtuin deacetylases, thus ensuring that the Bax-interaction domain is exposed. Cell stress causes CBP and/or PCAF to translocate to the cytosol where they target specific lysines in Ku70's flexible C-terminal linker region for acetylation. This results in a conformational change in Ku70 that releases Bax. Liberation of Bax allows it to initiate apoptosis by associating with BH3-only proteins and releasing cytochrome c from mitochondria.

Based on the above results, we predicted that the level of Ku70 acetylation would increase following cellular damage. To test this, we performed a time course analysis of Ku70 acetylation following UV treatment, a condition under which Ku70 is known to suppress apoptosis (Sawada et al., 2003b). 293T cells were exposed to 200 J/cm^2 of UV and the levels of Ku70 acetylation were then determined after 3, 6, 12, and 24 hr. The time course showed that Ku70 acetylation increased between 3 and 6 hr following exposure to UV (Figure 6A), which correlates with Bax activation (Sawada et al., 2003b). There are conflicting reports concerning the stability of Ku70 following DNA damage (Nothwehr and Martinou, 2003), and in our experiments we did not detect a decrease in overall Ku70 levels (Figure 6A). Interestingly, the increase in Ku70 acetylation correlated with migration of CBP to the cytosol (Figure 6B). This observation indicates that the relocalization of CBP from the nucleus to the cytosol following cellular damage might be a key regulatory step in Bax-mediated apoptosis.

HDAC Inhibitors Abolish the Endogenous Ku70-Bax Interaction

The simplest explanation of these results was that acetylation regulates Ku70s antiapoptotic function by interfering with its ability to sequester Bax from mitochondria. To test this model, we examined the endogenous Ku70-Bax interaction in 293T cells treated with TSA/NAM, a condition that we had previously shown to increase Ku70 acetylation (see Figure 4A). Cells were treated with the inhibitors for 12 hr, and the Ku70-Bax interaction was assessed by immunoprecipitating Ku70 and probing the immunocomplex for Bax. As shown in Figure 6C, treatment with TSA and NAM significantly

decreased the amount of Bax that was associated with Ku70. In a reverse-IP experiment, TSA and NAM completely abolished the ability of anti-Bax antibodies to immunoprecipitate Ku70. Based on these results, we conclude that acetylated Ku70 does not inhibit apoptosis because it is unable to bind and sequester Bax.

Discussion

A number of recent observations have linked acetyltransferases to tumor suppression, but their role in this process is not well understood (Giordano and Avantaggiati, 1999). In this study we show that (1) the Ku70 linker region aligns with clusters of known acetylation sites in other proteins; (2) Ku70 is acetylated at multiple sites in vitro and in vivo, including residues in the DNA binding domain and the flexible linker region; (3) CBP and PCAF associate with and target Ku70 for acetylation in vitro and in vivo; (4) the ability of endogenous Ku70 to suppress Bax-mediated apoptosis is independent of Ku80; (5) this function can be inhibited by treatments that increase Ku70 acetylation, either by treating cells with HDAC inhibitors or by overexpressing CBP or PCAF; (6) mutations that mimic acetylation of two critical lysines in the C-terminal linker region of Ku70 (K539 and K542) are sufficient to block the antiapoptotic function of Ku70; (7) increasing the level of Ku70 acetylation by treating cells with HDAC inhibitors abolishes the interaction between Ku70 and Bax; and (8) the acetylation level of Ku70 increases following UV treatment and this coincides with the relocalization of CBP from the nucleus to the cytoplasm. Together, these results show that acetylation of Ku70 by CBP and/or PCAF plays a pivotal role in determining a cell's fate following an apoptotic signal.

Model for the Regulation of Ku70 by Acetylation

We suggest the following model for the regulation of Bax-mediated apoptosis by Ku70. Under normal conditions, the majority of Ku70 is complexed with Ku80 in the nucleus but a subset exists independently of Ku80 in the cytosol. This pool of Ku70 serves to sequester Bax from mitochondria and maintain cell survival (Figure 6D). This model is consistent with our observations that there is an excess of Ku70 in the cytosol, relative to Ku80, and that Ku70 can suppress apoptosis independently of Ku80. Following an apoptotic stimulus, CBP and/or PCAF migrate to the cytosol where they acetylate Ku70, thereby releasing Bax. In support of this, increased levels of CBP are observed in the cytosol 3 hr after exposure to UV, in accordance with the time course of Ku70 acetylation. We do not exclude the possibility that the small fraction of Ku70 that has been shown to associate with mitochondria (Coffey et al., 1999) is also involved in this process.

The Ku70 C-terminal region is comprised of two domains: a flexible linker region and a well-defined structure consisting of three α helices (see Figure 3C) (Zhang et al., 2001). Previous work has shown that Ku70 inhibits apoptosis by interacting with Bax through the structured C-terminal domain (Sawada et al., 2003a). Acetylation of K539 or K542 by CBP or PCAF may lead to a conformational change in the Ku70 linker region, rendering the Bax-interaction domain inaccessible, thus releasing Bax and promoting apoptosis. If acetylation is required to provide order to this region, this would explain why structural studies using recombinant (i.e., nonacetylated) Ku70 have failed to detect structure in this region. Our model for Ku70 regulation is reminiscent of that proposed for p53, in which acetylation alters an intramolecular interaction that exposes the DNA binding domain of p53 (reviewed in Giordano and Avantaggiati, 1999).

Defining Sites of Acetylation

Although lysine acetylation has become recognized as an important regulatory mechanism for nonhistone proteins, the number of proteins found to be regulated by acetylation remains relatively small. This is due, in part, to the limited number of tools that are currently available for studying acetylation. In this paper, we demonstrate a powerful combination of complimentary techniques for identifying acetylation sites. We show that sequence alignments and scanning peptide libraries can be used successfully to identify potential in vivo targets of acetylation and their corresponding acetyltransferases. The validity of this approach is exemplified by the recent confirmation of our prediction that K305 of p53 is acetylated in vivo (Wang et al., 2003) (see Figure 1). We observed a high degree of specificity in the in vitro acetyltransferase reaction, and the sites identified in vitro were good predictors of in vivo targets.

Regulation of Ku70 Acetylation

In addition to Ku70, three other proteins involved in apoptosis are known to be regulated by acetylation, namely p53, p73, and Rb (Abraham et al., 2000; Chan et al., 2001; Costanzo et al., 2002; Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Among these proteins, Ku70 is unique because it is also a DNA repair protein. The dual role of Ku70 in DNA repair and the regulation of apoptosis places it in a key position to coordinate these two processes in response to cellular damage. Detailed studies are underway to elucidate the role of acetylation in regulating other Ku70 functions. In particular, it will be of interest to determine whether acetylation of K317, K331, and K338, which lie within the DNA binding ring (see Figure 3C), affect DNA repair and V(D)J recombination. Additional experiments will also be needed to determine what signals and proteins promote the activity of CBP and PCAF under proapopototic conditions. Perhaps CBP and PCAF interact with the apoptosis regulator PKCδ, which has been shown to inhibit the HAT activity of p300 (Yuan et al., 2002).

Links between HATs and Cancer

It is becoming increasingly apparent that acetyltransferases, such as p300, CBP, and PCAF, act as mediators of environmental signals that can dictate the commitment to cell growth, differentiation, or apoptosis. Their importance in these pathways is underscored by the finding that deletions, translocations, and point mutations within these acetyltransferase genes have been found in a number of tumors and are linked to the cancer predisposition disease Rubenstein-Taybi syndrome (Rebel et al., 2002). Our results indicate that a primary mechanism by which acetyltransferases might suppress tumorigenesis is by regulating Bax-mediated

apoptosis. In this study, we used 293T cells, which lack functional p53. Therefore the effects we observed were presumably independent of p53 activity. Interestingly, acetylation of p53 following UV treatment occurs within the same time frame as Ku70 acetylation and Bax activation (Liu et al., 1999). This raises the possibility that CBP and PCAF promote apoptosis via two parallel pathways, one involving acetylation of Ku70 leading to Bax activation and the other involving the acetylation and activation of p53.

Histone deacetylase inhibitors are now being tested for the treatment of leukemia and solid tumors (Johnstone and Licht, 2003). Why cancer cells but not normal cells are sensitive to HDAC inhibitors is unclear. To explain this, it has been suggested that the primary target for HDAC inhibitors in cancer therapy may not be transcription (Johnstone and Licht, 2003). Our findings suggest that the efficacy of such compounds may be due to inhibition of the activity of Ku70 and identify this protein as an attractive target for anticancer therapy. Many studies using inhibitors, such as TSA, TPX, and sodium butyrate, as anticancer drugs have been reported in the literature (Rahman et al., 2003; Yoshida et al., 2001). Based on our result that the combination of nicotinamide and TSA completely blocks Ku70-dependent inhibition of Bax, we propose that combining a class I/II HDAC inhibitors with a class III inhibitor, such as nicotinamide, should augment the efficacy of HDAC inhibitors as chemotherapeutic agents.

Experimental Procedures

Cells and Media

Cells were grown in the presence of 20% O_2 and 5% CO_2 at 37°C in humidified chambers. Human epithelial carcinoma (HeLa), human embryonic kidney (HEK 293), 293T, mouse Ku70$^{+/+}$ fibroblasts (Sawada et al., 2003b), mouse Ku70$^{-/-}$ fibroblasts (Sawada et al., 2003b), and hamster Ku80$^{-/-}$ fibroblast (V15B) (Bertinato et al., 2001) were grown in DME with FBS (10%), glutamine (1%), and penicillin/streptomycin (1%).

In Vitro Acetylation Assays

Protein acetyltransferase assays were performed in 30 μl of reaction buffer containing 50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM Na-butyrate, 1 μl [^{3}H]-acetyl-CoA, 1 μg recombinant Ku70/80 complex (gift of M. Oettinger, MGH) or Ku70 peptide, and 100 ng of recombinant HAT domains of p300, PCAF, or CBP. Reactions were incubated at 30°C for 1 hr and separated by SDS-PAGE (10%), stained with Coomassie blue, treated with EN^{3}HANCE autoradiography enhancer (NEN), dried, and exposed to film for 3–7 days. p53 peptides used as positive controls were $p53_{315-3235}$ and $p53_{377-389}$.

Immunoprecipitation and Western Blotting

For immunoprecipitation (IP) of Ku70, 1 mg of protein was precleared by incubation with protein A/G Sepharose beads (Santa Cruz). The supernatant was incubated with agarose-conjugated goat polyclonal anti-Ku70 antibody (Santa Cruz), followed by three washes in 1% triton in PBS. The immunocomplex was separated by SDS-PAGE and proteins were detected with a rabbit polyclonal anti-pan-acetyl-lysine (panAc-K) antibody raised against acetylated rabbit's serum. Co-IP of endogenous Ku70 and CBP from HeLa cells was performed in the presence of 50 μg/ml EtBr (Lai and Herr, 1992). Co-IP of endogenous Ku70 and Bax from 293T cells was performed in Chaps buffer (Sawada et al., 2003b)

Apoptosis Assays

Apoptosis was induced as previously described (Sawada et al., 2003b). In all apoptosis experiments, full-length Ku70 was expressed. Values represent the average of three experiments in which at least 200 cells were counted. Error bars represent the standard error of the mean.

Large-Scale Purification of Native Ku70

293 cells were stably transfected with a 6xHIS-Ku80 vector. Cell extracts from 10 liter of cells (180 mg protein) were applied to a Ni-NTA Sepharose column and Ku70/Ku80 was eluted with imidazole (600 mM imidazole). Alternatively, a large-scale IP was performed on cell extracts from 20 liter of HeLa MC118 cells grown in suspension using 500 μg of an agarose-conjugated goat polyclonal anti-Ku70 antibody (Santa Cruz). Purified proteins from both methods were separated by SDS-PAGE, and the band corresponding to Ku70 was excised and analyzed by MS/MS.

Tandem Mass Spectrometry

In-gel proteolytic digestion was performed essentially as described (Kinter and Sherman, 2000). For the analysis of posttranslational modifications, trypsin, chymotrypsin, AspN, and GluC (V8) were used (Roche). Samples were subjected to a nanoflow liquid (LC) chromatography system (Waters CapLC) equipped with a picofrit column (75 μm ID, 10 cm, NewObjective) at a flow rate of approximately 150 nl/min using a nanotee (Waters) 16/1 split (initial flow rate 5.5 μl/min). The LC system was directly coupled to a QTOF micro tandem mass spectrometer (MS) (Micromass, UK). Analysis was performed in survey scan mode and parent ions with intensities greater than seven were sequenced in MS/MS mode using MassLynx 4.0 Software (Micromass, UK). MS/MS data were processed and subjected to database searches using ProteinLynx Global Server 1.1 Software (Micromass, UK) against Swissprot, TREMBL/New (www.expasy.ch), or Mascot (Matrixscience) (Perkins et al., 1999) against the NCBI nonredundant database (NCBInr) or the Ku70 sequence alone. Acetylation was identified by the additional mass of 42 on Lys residues and the presence of 126 and 143 MW immonium ions.

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