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Targeting of p300/CREB Binding Protein Coactivators by Simian Virus 40 Is Mediated through p53

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The primary transforming functions of simian virus 40 large T antigen (SV40 LT) are conferred primarily through the binding and inactivation of p53 and the retinoblastoma family members. Normal p53 function requires an association with the CREB binding protein (CBP)/p300 coactivators, and a ternary complex containing SV40 LT, p53, and CBP/p300 has been identified previously. In this report, we have evaluated a secondary function of p53 bound to the SV40 LT complex in mediating the binding of human CBP/p300. We demonstrate that p53 associated with SV40 LT was posttranslationally modified in a manner consistent with the binding of CBP/p300. Furthermore, expression of SV40 LT induced the proportion of p53 phosphorylated on S15. An essential function for p53 in bridging the interaction between SV40 LT and CBP/p300 was identified through the reconstitution of the SV40 LT-CBP/p300 complex upon p53 reexpression in p53-null cells. In addition, the SV40 LT-CBP/p300 complex was disrupted through RNA interference-mediated depletion of endogenous p53. We also demonstrate that SV40 LT was acetylated in a p300- and p53-dependent manner, at least in part through the CH3 domain of p300. Therefore, the binding of p53 serves to modify SV40 LT by targeting CBP and p300 binding to direct the acetylation of SV40 LT.

Simian virus 40 (SV40) large T antigen (LT) is a multifunctional viral oncoprotein that can modulate a number of diverse cellular processes including gene transcription, differentiation, apoptosis, and cell cycle entry. These functions are conferred through the ability to associate with and disable key regulatory proteins involved in growth regulation. In a multistep model of human cell transformation, the activities of SV40 LT have recently been used to promote the early stages of carcinogenic conversion (34, 35). SV40 LT continues to provide important insight into distinct cellular pathways and proteins that are directly involved in malignant transformation.

The transforming functions of SV40 LT reside at multiple domains, which serve to bind CUL7 and to inactivate the tumor suppressor p53 and the retinoblastoma (Rb) family members RB1/pRb, RBL1/p107, and RBL2/p130. SV40 LT targets the binding of Rb through an N-terminal LXCXE domain (amino acids [aa] 103 to 107). In cooperation with the DnaJ domain (aa 1 to 82) of SV40 LT, this interaction leads to the dephosphorylation and thus the inactivation of Rb family function (76, 77, 90). A concomitant destabilization of the inhibitory complex between dephosphorylated Rb and the E2F family of transcription factors facilitates the transcription of target genes to promote cell cycle entry. An adjacent SV40 LT domain binds the CUL7 SCF complex to promote efficient transformation (2, 40). The C-terminal bipartite region that maps between amino acids 350 to 450 and 525 to 625 of SV40 LT (44) targets association to the p53 DNA binding domain (70). This association sequesters the gene transactivation function of p53 that normally promotes cell cycle arrest or apoptosis in response to abnormal cell signaling and DNA damage. The inactivation of p53 is critical for the transforming functions of SV40 LT. Paradoxically, this leads to a striking accumulation of p53 as part of an inactive complex with SV40 LT (68). Our laboratory has previously demonstrated that the LXCXE binding domain, the bipartite p53 binding domain, the CUL7 binding domain, and the DnaJ domain, although required for efficient transformation of rodent cells, functioned beyond their ability to simply disrupt Rb and p53 pathways (15). An additional role for p300 (EP300) and CREB binding protein (CBP) (CREBBP) targeting is suggested by the ability of SV40 LT, independent of Rb binding, to fully rescue the lost transforming function of a p300 binding mutant of E1A (87). Therefore, it has been of interest that SV40 LT can associate with p300 and CBP and disrupt their coactivator function (4, 24, 52).

p300 and CBP are highly homologous transcriptional coactivators that possess both distinct and overlapping functions. Their association with a wide array of transcription factors implicates their broad involvement in pathways that control cell growth and differentiation. Association with CBP/p300 promotes gene transcription by providing a linkage to TFIIB (27, 46), TATA box-binding protein (1, 20, 78), and RNA polymerase II (41) of the basal transcriptional machinery. In addition, the intrinsic acetyltransferase activity of CBP/p300 functions to acetylate the N-terminal tails of local core histones (7, 56, 63) to promote a transcriptionally favorable structure of the nucleosome (38, 56).

A direct interaction between p53 and CBP/p300 (5, 51) promotes p53 transcriptional activity by localizing CBP/p300 histone acetyltransferase activity to the promoter of target genes (26). This p53-CBP/p300 complex is facilitated through the phosphorylation of N-terminal serine residues of p53 through a kinase cascade involving ATM, ATR, Chk1, and DNA-PK. Phosphorylation of p53 at S20 can contribute to increased stability of p53 through dissociation of the Mdm2 E3 ligase

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from the N terminus of p53 (16, 23, 81). Additionally, phosphorylation of p53 on serine residue 15 has been shown to enhance the interaction between p53 and CBP/p300 (22, 48). p53 is also a target of p300 acetyltransferase activity both in vitro and in vivo. At least six lysine residues in the C terminus of p53 have been reported to be acetylated by p300 and CBP, with K373 and K382 serving as the primary acetylation sites (33, 54, 71, 84). Acetylation of p53 is normally detected at very low levels but is transiently induced upon exposure to cellular insults such as DNA damage (37, 54, 71). The effects of acetylation on p53 function are unclear (67) but have recently been implicated in promoting p53 stability, enhancing CBP interaction, and promoting sequential cofactor recruitment (9, 37).

Several small DNA tumor virus oncoproteins, including SV40 LT (24), human papillomavirus E6 (65, 93), polyomavirus LT (18, 61), and adenovirus E1A (3, 4, 8, 25, 36, 69, 73, 88), share the ability to bind the third cysteine/histidine-rich region (CH3) of CBP/p300. This region is also known as the TAZ2 domain (21). A requirement for CBP/p300 targeting in E1A-mediated carcinogenic conversion supports the model that CBP/p300 provides a tumor suppressor function (30, 31). Consistent with this model, p300 missense mutations and truncations have been identified in a subset of human cancers (29, 59). Furthermore, increased hematological malignancies have been observed in CBP heterozygous animals (45), and haploinsufficiency has been implicated as the causal factor in the tumor-prone Rubinstein-Taybi syndrome (58). A feature unique among the viral oncoproteins is that the association between SV40 LT and CBP/p300 is dependent on the p53 binding domain of SV40 LT (52). This raises speculation that p53 stabilized in the SV40 LT complex is not simply latent but serves as an adapter to bind CBP/p300. Previous reports of a ternary complex containing SV40 LT, p53, and CBP (24) and a requirement for p53 in promoting SV40 LT acetylation (66) provide indirect support for such a p53 bridging function.

While both p300 and CBP reportedly interact with SV40 LT in a variety of nonhuman cell types (4, 17, 24, 52, 82), we have recently reported that the acetylation of SV40 LT on a C-terminal K697 residue occurs largely through CBP (66). However, the loss of CBP expression through homozygous deletion did not completely eliminate SV40 LT acetylation, suggesting a contribution from an additional acetyltransferase protein. In this report, we have identified that SV40 LT can interact with endogenous CBP and p300 in both transformed and immortalized human cells. Acetylation of SV40 LT could be induced through overexpression of wild-type human p300 but not acetyltransferase p300 mutants. In addition, the CH3 domain of p300 was both necessary and sufficient for directing the acetylation of SV40 LT in human cells. We have employed small interfering RNA (siRNA)-directed depletion of endogenous p53 and the reintroduction of p53 into p53-null cells to directly establish an adapter function of p53 in the SV40 LT complex. Bound p53 was required for facilitating the interaction between SV40 LT and CBP/p300 and for promoting the acetylation of SV40 LT in both mouse and human cells. Inactivated p53 in complex with SV40 LT was phosphorylated and acetylated in a manner that is consistent with a mechanism facilitating CBP/p300 binding. We conclude that SV40 LT requires p53 to form a complex with both CBP and p300 and that p300 acetyltransferase activity can contribute to the acetylation of SV40.

MATERIALS AND METHODS

Plasmids. SV40 LT mammalian expression vectors pSG5-T, K1 (E107K), and D44N have been previously described (75, 76, 89). Retroviral expression vector pWZL-blast-LT contained a BamHI ligation of wild-type SV40 LT cDNA from pSG5-T. The mutant human p53 construct pBABE-hygro-p53 R175H was a generous gift from W. Hahn and was converted to a wild-type sequence using the mutagenic primer 5′-GCACATGACGGAGGTTGTGAGGCGCTGCCCCCA CCATGAGC-3′ with the QuickChange Multi site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The resulting wild-type pBABE-hygro-p53 vector was subsequently mutated to p53 L22Q/W23S (22/23) using the mutagenic primer 5′-GGAAACATTTTCAGACCAATCGAAACTA CTTCCTGAAAACAACGTTCTGTCCCC-3′.

The pLB(N)CX retroviral expression vector was constructed by excising the neomycin resistance gene from pLNCX (Clontech) using BsaBI and BstBI restriction digestion and replacing it with a PCR-generated blasticidin resistance gene. Annealed oligonucleotides were subsequently ligated into the HpaI site of pLB(N)CX to add an ApaI site to the multiple cloning region and a C-terminal dual FLAG-hemagglutinin (HA) epitope containing a double-glycine flexible hinge [pLB(N)CX C-FLAG-HA].

Mammalian expression constructs containing full-length human p300 (CMVβ-p300-CHA), a full-length p300 CH3 domain mutant (deletion of aa 1737 to 1809) (CMVβ-p300-CHA-Del33), and full-length acetyltransferase p300 mutants (CMVβ-p300-CHA-WY and CMVβ-p300-CHA-FPY) containing a C-terminal HA epitope were a generous gift from R. Eckner and have been previously characterized (10). A region of human p300 that spanned the entire acetyltransferase (AT) domain through the adjacent CH3 domain (aa 1195 to 1921) (AT-CH3) was PCR cloned using CMVβ-p300-CHA as the template and ligated in frame into the HindIII and ApaI restriction sites of pLB(N)CX C-FLAG-HA [pLB(N)CX-AT-CH3-FLAG-HA]. Corresponding FPY, WY, and Del33 mutants were produced by releasing the AT-CH3 domain from pLB(N)CX-AT-CH3-FLAG-HA using the internal restriction sites BglII and ApaI and then replacing them with BglII-ApaI mutant AT-CH3 fragments obtained from the appropriate CMVβ-p300-CHA vectors. The human p300 CH3 domain (aa 1709 to 1913), with an N-terminal FLAG epitope separated by a double-glycine flexible hinge, was PCR generated and cloned into the HindIII site of pLB(N)CX to produce pLB(N)CX-FLAG-CH3. DNA sequencing was used to verify all constructs.

Cell lines and transfection. All cells were cultured in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% FetalClone 1 serum (HyClone), 100 U penicillin per ml, and 100 μg streptomycin per ml (Gibco) at 37°C in a humidified incubator with 10% CO_2–air. The human osteosarcoma U-2 OS cell line was obtained from the American Type Culture Collection. For stable expression of wild-type and mutant SV40 LT, subconfluent 100-mm plates of U-2 OS cells were cotransfected with plasmids pSG5-T (or pSG5-K1 or pSG5-D44N) and pEpuro in a 5:1 ratio using Plus/Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Polyclonal cell lines stably expressing SV40 LT, K1, or D44N were selected in medium containing 2 μg/ml puromycin over a period of 2 to 3 weeks. Human BJ fibroblast cells stably expressing h*TERT* and SV40 LT using retrovirus were a generous gift from W. Hahn and have been previously described (34, 35).

Mouse embryonic fibroblasts (MEFs) that are null for p53 expression ($p53^{-/-}$) were isolated from 14.5-day-old embryos produced by the mating of heterozygous mice, strain B6.129S2-*Trp53*^{tm1Tyj}/J (Jackson Laboratory), as described previously (89). MEF $p53^{-/-}$ cell lines stably expressing SV40 LT and human p53 were produced using retrovirus. The appropriate retroviral expression vector (5 μg) was transfected into the Bosc 23 packaging cell line using Plus/Lipofectamine (Invitrogen). Retrovirus-containing supernatants were collected over two 24-h periods, filtered, supplemented with 5 μg/ml polybrene (Sigma), and used to infect subconfluent $p53^{-/-}$ MEFs. Cells were serially infected with retroviral pWZL-blast-LT and the appropriate pBABE-hygro-p53 expression vectors. Retroviral vectors containing only the drug resistance gene were used as controls. Stably expressing polyclonal cell populations were subsequently purified at each step through selection using blasticidin S (10 μg/ml) or hygromycin B (125 μg/ml).

Transient transfection of p300 and p300-CH3 constructs were carried out using Plus/Lipofectamine according to the manufacturer's recommendations (Invitrogen). Near-confluent 100-mm cultures were incubated in transfection medium for 3 h, replaced with standard media, and harvested 48 h posttransfection. p53 was silenced by siRNA. U-2 OS cells were transfected with a pool of

p53-specific siRNA (SMARTpool kit; Dharmacon) at 100 nM using Lipofectamine 2000 (Invitrogen). As a control, U-2 OS cells were transfected with a nonspecific luciferase siRNA duplex. Nuclear fractions were obtained using NE-PER extraction reagents (Pierce) at 72 h posttransfection.

Antibodies. The following antibodies were used for Western blotting and immunoprecipitations: mouse monoclonal antibody to p53 (DO-1 or pAb122) (Lab Vision); rabbit polyclonal antibody to p53 (Cell Signaling Technology); rabbit polyclonal antibody to acetyl-p53-K373 (Upstate); rabbit polyclonal antibody to phospho-p53-S15, phospho-p53-S20, and phospho-p53-S37 (Cell Signaling Technology); mouse monoclonal antibody to CBP/p300 (AC26); mouse monoclonal antibody to CBP (AC238); mouse monoclonal antibody to p300 (RW128) (Lab Vision); mouse monoclonal antibody to HA epitope (12CA5); mouse monoclonal antibody to FLAG epitope (M2) (Sigma); mouse monoclonal antibodies to SV40 LT (pAb419, pAb430, and pAb901); rabbit polyclonal antibody to acetylated lysine (Cell Signaling Technology); rabbit polyclonal antibody to acetylated LT-K697 (66); and mouse monoclonal antibody to vinculin (Sigma).

Cellular fractionation and immunoprecipitation. NE-PER extraction reagents (Pierce) were used to obtain cytoplasmic and nuclear fractions using modifications of the manufacturer's recommendations. Pelleted cells washed in phosphate-buffered saline (PBS) were resuspended in chilled CERI buffer supplemented with 1× Calbiochem protease inhibitor set I by gentle pipetting with a pipette tip (200 μl CERI per 20-μl cell pellet). Cells were incubated on ice for 10 min. Chilled CERII buffer (1.25 μl) was added, mixed by pipetting, incubated on ice for 1 min, and then quickly vortexed vigorously. Nuclei were pelleted at 14,000 × *g* for 5 min at 4°C, and the cytoplasmic extract supernatant was removed and saved on ice. The nuclear pellet was resuspended in chilled NER reagent (100 μl per 20-μl original cell pellet volume) supplemented with 1× Calbiochem protease inhibitor set I by gentle pipetting until nuclei were thoroughly dispersed. Nuclear extraction was performed on ice for 40 min with gentle pipetting every 10 min. Extracted nuclei were pelleted at 14,000 × *g* at 4°C for 10 min, and the nuclear extract supernatant was transferred into a fresh tube on ice.

For immunoprecipitation of endogenous CBP and p300, equal amounts of cellular fractions obtained from a 100-mm plate were adjusted to a 500-μl final volume with chilled NETN (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA [pH 8.0], 0.5% Nonidet P-40) containing 1× Calbiochem protease inhibitor set I. Twenty microliters of antibody was added to each sample and incubated for 2 h at 4°C. Immunocomplexes were collected with 20 μl of protein A-Sepharose beads during a 2-h incubation at 4°C. Pulse-pelleted beads were washed four times with chilled NETN. For SV40 LT immunoprecipitation, similar procedures were followed, except that lysis buffer 3 (25 mM Tris-HCl [pH 7.4], 1 mM $CaCl_2$, 1% Triton X-100) was substituted for NETN during the immunoprecipitation and wash steps.

Total cell lysates were obtained by washing cells in PBS and lysing cells in either ice-cold EBC buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.5% Nonidet P-40) or ice-cold NETN supplemented with 1× Calbiochem protease inhibitor set I and Sigma phosphatase inhibitor cocktail for 30 min on ice. Extracts were cleared by centrifugation at 14,000 × *g* for 5 min, and the protein concentration was quantified by the Bradford assay (Bio-Rad). For immunoprecipitation of the SV40 LT-p53 complex, 1 mg of EBC total cell lysate was adjusted to 1 ml with NETN buffer. Antibodies cross-linked to protein A-Sepharose using dimethyl pimelimidate (Sigma) were immunoreacted for 2 h at 4°C. Immunocomplexes collected by brief centrifugation were washed four times with NETN. To immunoprecipitate the SV40 LT-p300 fragment and SV40 LT LT-full-length p300-CHA complexes, 1 mg NETN total cell lysate was adjusted to 500 μl with NETN. Lysates were incubated with anti-SV40 LT antibody (pAb419 or pAb430) for 1 h at 4°C and then captured on protein A-Sepharose for 1 h at 4°C. Immunocomplexes were washed three times with NETN.

Western blotting. Immunoprecipitated proteins, total cell lysates, or cell fractions were denatured in loading buffer (2.23% sodium dodecyl sulfate, 10% glycerol, 0.0033% bromophenol blue, 100 mM dithiothreitol), separated in sodium dodecyl sulfate-polyacrylamide gels (6% for CBP and p300, 7.5% for SV40 LT, 10% for p53, and 15% for CH3 and CH3-FLAG) and then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% nonfat dry milk–TBS-T (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) for 1 h and then immunoreacted for 2 h in primary antibody diluted in 1% bovine serum albumin (BSA)–TBS-T. Membranes were washed three times for 10 min in TBS-T. Detection of proteins was performed with the appropriate horseradish peroxidase-conjugated secondary goat antibody (Pierce) at a 1:5,000 dilution for 1 h in TBS-T containing 5% nonfat dry milk. Membranes were washed four times for 10 min each, and proteins were visualized by enhanced chemiluminescence using SuperSignal West Pico solution (Pierce). Chemiluminescent signals were directly quantified using a Bio-Rad Fluor-S Multi-Imager.

Immunofluorescence. Cells were cultured on coverslips and fixed in 10% paraformaldehyde for 60 min. Fixed cells were permeabilized and blocked in PBS containing 0.5% Triton X-100 and 3% BSA for 30 min. Cells were immunoreacted with the appropriate primary antibody for 2 h and then with fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h in PBS containing 3% BSA. Washed cells were mounted with Vectashield mounting medium containing DAPI (4′,6′-diamidino-2-pheylindole) (Vector) and observed using a ×60 oil objective.

RESULTS

SV40 LT associates with human CBP and p300. An interaction between SV40 LT and CBP/p300 has been identified in rodent and primate cells (4, 17, 24, 52, 82). To investigate whether SV40 LT can associate with the human homologue of CBP and p300, we performed immunoprecipitation experiments in U-2 OS cells stably expressing SV40 LT or empty vector. To identify the cellular compartment of this interaction, cytoplasmic and nuclear protein fractions were isolated. Western blot analysis of these fractions (15% or 25% input) demonstrated that SV40 LT and endogenous CBP and p300 were present in the nucleus, although a small subset of SV40 LT could be detected in the cytoplasmic fraction (Fig. 1A and C). Furthermore, Western blot analysis demonstrated that stable expression of SV40 LT did not alter basal expression levels or nuclear localization of endogenous CBP and p300.

Endogenous CBP was immunoprecipitated from extracts obtained from wild-type U-2 OS cells and U-2 OS cells stably expressing SV40 LT. Western blot analysis of immunoprecipitated CBP identified a complex containing SV40 LT (Fig. 1A). This complex was identified specifically in the nuclear fraction of U-2 OS cells expressing SV40 LT. No such interaction was demonstrated in the cytoplasmic fraction or in control U-2 OS cells not expressing SV40 LT. The specificity of this interaction was further demonstrated by the inability of a nonspecific antibody (anti-HA) to coimmunoprecipitate SV40 LT under identical conditions. Immunoprecipitation of endogenous CBP followed by anti-SV40 LT Western blotting identified a similar association of CBP with SV40 LT in h*TERT*-immortalized human BJ fibroblast cells (Fig. 1B). A reciprocal interaction was demonstrated through the ability of an anti-SV40 LT antibody to coimmunoprecipitate a complex containing CBP only in the nuclear protein fraction of cells stably expressing SV40 LT. Similar experiments identified an interaction between SV40 LT and p300 (Fig. 1C). The ability of SV40 LT to coimmunoprecipitate endogenous human p300 was demonstrated using monoclonal antibodies directed against the N terminus (pAb430) or C terminus (pAb901) of SV40 LT. These results indicate that SV40 LT can form a specific complex with endogenous CBP and p300 in human cells.

p53 in complex with SV40 LT is phosphorylated and acetylated. CBP and p300 can directly associate with p53, and this interaction is partly facilitated through posttranslational modifications on specific p53 residues (22, 48). Since the binding of p53 by SV40 LT results in a dramatic accumulation of p53 in a highly stable SV40 LT complex, we wanted to further investigate a possible interrelationship between CBP/p300 and p53 in the SV40 LT complex. Stably expressed SV40 LT was immunoprecipitated from U-2 OS whole-cell lysates, and p53 bound to SV40 LT was characterized by Western blot analysis using a panel of residue-specific anti-phospho-p53 antibodies (Fig.

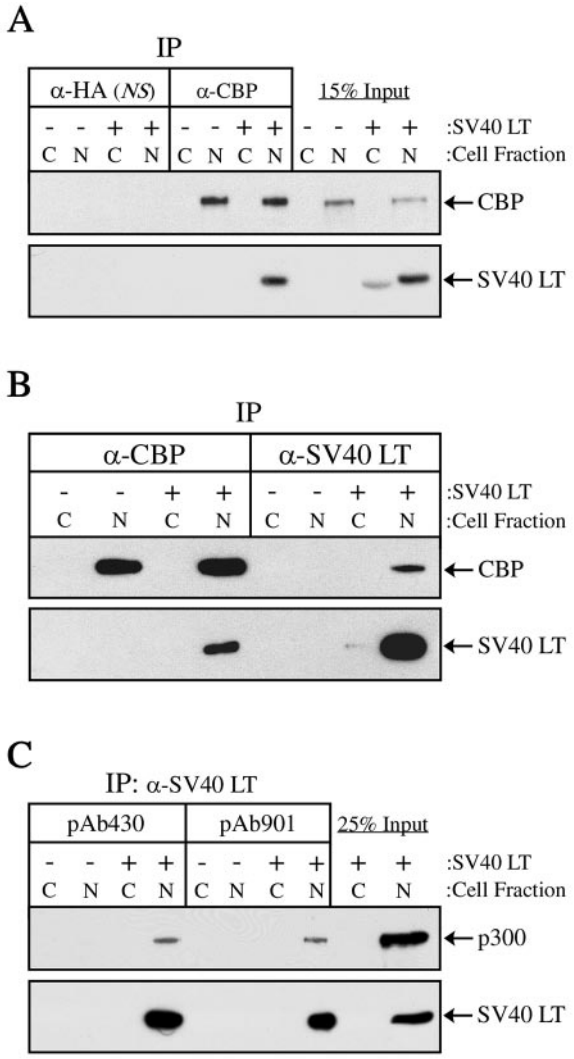

FIG. 1. SV40 LT associates with endogenous human CBP and p300. (A) A complex between SV40 LT and endogenous CBP was investigated. Nuclear (N) and cytoplasmic (C) protein fractions were obtained from wild-type U-2 OS cells (−) or U-2 OS cells stably expressing SV40 LT (+). Endogenous CBP was immunoprecipitated (IP) using anti-CBP (α-CBP) AC238, and coimmunoprecipitated SV40 LT was identified through Western blotting using anti-SV40 LT pAb901. Immunoprecipitation using anti-HA 12CA5 was used as a control for nonspecific (*NS*) interactions. Fractions corresponding to 15% of the input were loaded to identify relative CBP and SV40 LT levels. An interaction between SV40 LT and CBP (B) or p300 (C) was further investigated in h*TERT*-immortalized BJ fibroblast cells stably expressing SV40 LT (+) or vector control (−). Nuclear (N) and cytoplasmic (C) fractions were immunoprecipitated using anti-CBP AC238 or anti-SV40 LT pAb419, pAb430, or pAb901 antibody. Coimmunoprecipitated complexes were identified through Western blot analysis.

2A). The p53 in complex with SV40 LT was found to be phosphorylated on a number of N-terminal serine residues, including S15, S20, and S37. A similar level of phosphorylation on p53 associated with the K1 mutant (E107K) of SV40 LT that cannot bind retinoblastoma family members was noted. When accounting for the reduced levels of total p53 bound by the D44N DnaJ domain mutant of SV40 LT (−25% of wild-type SV40 LT), a reduction in levels of phosphorylated p53 associated with D44N was noted (S15, −13%; S20, −53%; S37, −50%) compared to levels in wild-type SV40 LT. Therefore, SV40 LT binds to phosphorylated forms of p53, which is facilitated in part by the J domain of SV40 LT.

To investigate whether SV40 LT activity can enhance the relative proportion of p53 that is phosphorylated, total cell lysates obtained from control U-2 OS cells (in the absence of SV40 LT expression) and U-2 OS cells stably expressing SV40 LT were adjusted to yield equivalent levels of total p53 (Fig. 2B, bottom panel). Use of the anti-phospho-p53 S15 antibody in Western blot analysis demonstrated that SV40 LT activity enhanced the relative proportion of total p53 that was phosphorylated at S15 to levels ~5-fold above those in the absence of SV40 LT expression, based on direct chemiluminescent quantification. This activity of SV40 LT was not dependent on retinoblastoma binding or intact DnaJ domain activity, since the SV40 LT K1 and D44N mutants promoted a relatively similar increase in levels of phospho-p53-S15 (87% and 89% of wild-type SV40 LT levels, respectively). When used in Western blotting of total cell lysates, the anti-phospho-p53 S20 and anti-phospho-p53 S37 antibodies produced too many nonspecific bands to be informative (data not shown).

ATR and ATM are known to phosphorylate p53 at the S15 residue (6, 11, 42, 47, 60, 80), and their kinase activities can be inhibited by caffeine treatment (72). We found that treatment of U-2 OS cells stably expressing SV40 LT with caffeine resulted in a time- and dose-dependent reduction in phosphorylated p53 at S15 when Western blot analysis was used for detection (Fig. 2C). Caffeine treatment at 5 mM for 2 h maximally inhibited p53 phosphorylation at S15 but had no effect on total p53 expression levels. In support of this, caffeine treatment similarly reduced levels of phospho-p53 at S15 that could be visualized through immunofluorescence imaging of U-2 OS cells stably expressing SV40 LT (Fig. 2D). Levels of nuclear ATM and ATR were unaltered by SV40 LT expression (data not shown). These results indicate that SV40 LT can enhance the phosphorylation status of associated p53 in an ATM- and/or ATR-dependent manner.

The acetyltransferase activities of CBP and p300 directly target the acetylation of p53 at discrete C-terminal lysine residues (33, 54, 71, 84). Since the acetylation of p53 is dependent on CBP/p300 binding, we investigated the acetylation status of p53 in complex with SV40 LT. SV40 LT complexes were immunoprecipitated from U-2 OS cells, and associated p53 was subsequently analyzed using a site-specific anti-acetyl-p53 antibody (Fig. 2E). Western blot analysis identified acetylation of SV40 LT-bound p53 at a C-terminal residue (K373) known to be targeted by CBP/p300 acetyltransferase activity. Reciprocal immunoprecipitations using the anti-acetyl-p53 K373 antibody were able to coimmunoprecipitate a complex containing SV40 LT (data not shown). These observations indicate that p53 bound to SV40 LT is posttranslationally modified in a manner consistent with that of CBP/p300 binding and further support indirect evidence that p53 serves as a scaffold to bridge the interaction between SV40 LT and CBP (24, 66).

p53 is required for interaction between SV40 LT and CBP/p300. A role for inactivated p53 in bridging CBP/p300 binding to the SV40 LT complex was directly assessed through siRNA-mediated disruption of endogenous p53 expression. siRNA oligonucleotides directed against human p53 or a control

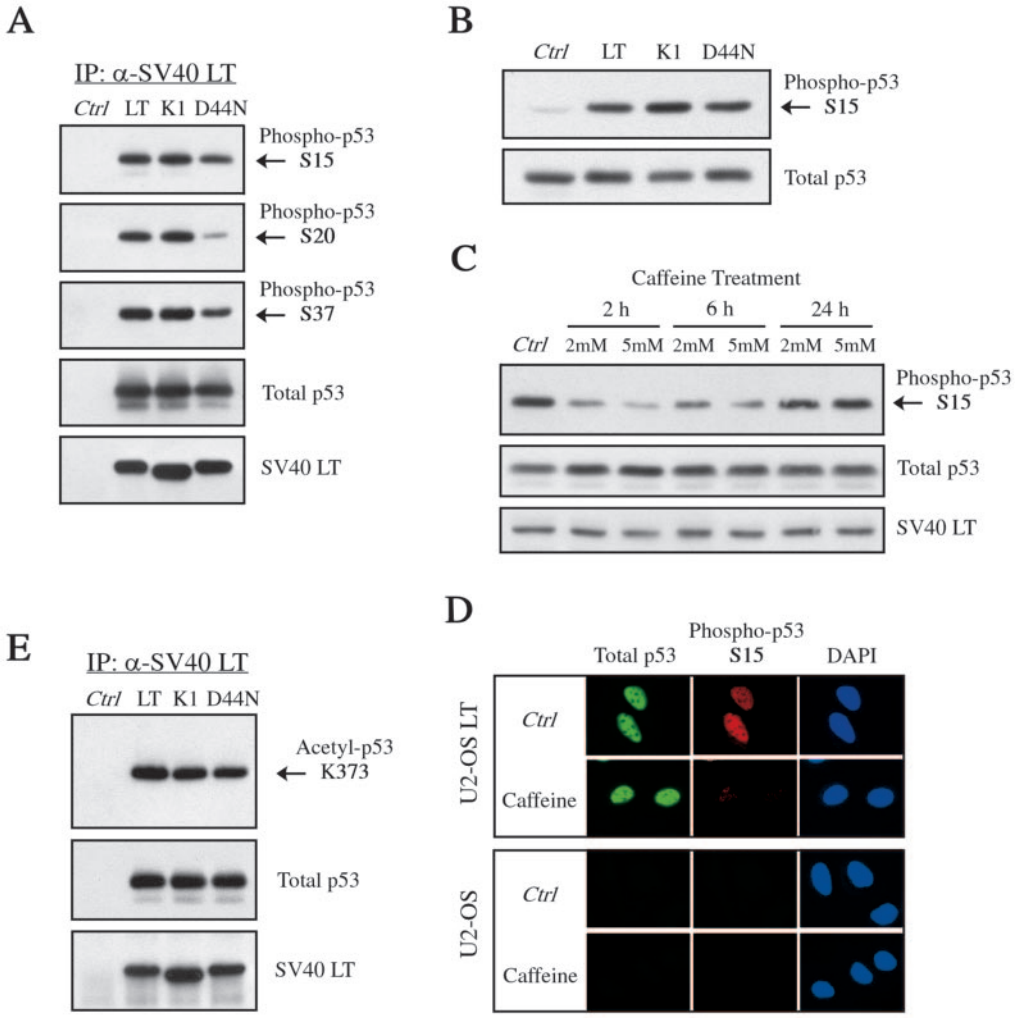

FIG. 2. p53 in complex with SV40 LT is phosphorylated and acetylated. (A) SV40 LT was immunoprecipitated (IP) from whole-cell lysates obtained from U-2 OS cells stably expressing either wild-type SV40 LT (LT), an Rb-binding mutant of SV40 LT (K1), a DnaJ domain mutant of SV40 LT (D44N), or empty vector (*Ctrl*). Western blots of the SV40 LT immunoprecipitates were probed to characterize the phosphorylation status of immunocomplexed p53 using a panel of anti-phospho-p53 antibodies. (B) The ability of SV40 LT to induce relative levels of phosphorylated p53 was explored in U-2 OS cells stably expressing either wild-type or mutant SV40 LT. Whole-cell lysates were adjusted to yield equivalent levels of total p53 protein across samples and confirmed through anti-p53 DO-1 Western blot analysis. Western blotting with an anti-phospho-p53 S15 antibody demonstrated the ability of SV40 LT to induce the relative magnitude of p53 phosphorylation. (C) The ability of caffeine treatment to diminish SV40 LT-mediated induction of p53 phosphorylation was investigated in U-2 OS cells stably expressing SV40 LT. Cells were treated with either 2 mM or 5 mM caffeine for the indicated times. Whole-cell lysates were analyzed through Western blot analysis using anti-phospho-p53 S15, anti-p53 DO-1, and anti-SV40 LT pAb419 antibodies. (D) Representative immunofluorescence image showing caffeine treatment diminishing the SV40 LT-mediated induction of p53 phosphorylation. Wild-type U-2 OS cells (U-2 OS) or U-2 OS cells stably expressing SV40 LT (U-2 OS LT) were plated onto coverslips and treated with either 5 mM caffeine (Caffeine) or vehicle (*Ctrl*) for 2 h. Cells were subsequently coimmunostained with monoclonal anti-p53 DO-1 (green) and polyclonal anti-phospho-p53 S15 (red) antibodies and counterstained with DAPI. (E) The acetylation status of p53 in complex with SV40 LT was investigated in U-2 OS cells stably expressing either wild-type SV40 LT (LT), an Rb-binding mutant of SV40 LT (K1), a DnaJ domain mutant of SV40 LT (D44N), or empty vector (*Ctrl*). SV40 LT was immunoprecipitated from whole-cell lysates, and Western blotting was used to characterize p53 in the SV40 complex using an anti-acetyl-p53 K373 antibody versus an anti-total p53 DO-1 antibody.

siRNA luciferase oligonucleotide was transfected into U-2 OS cells stably expressing SV40 LT (Fig. 3A to C). At 72 h post-transfection, nuclear fractions were collected, and endogenous p53 expression was assessed by Western blot analysis (15% input). Direct chemiluminescence quantification revealed that levels of p53 were reduced by 80 to 90% in cells that received the p53 siRNA oligonucleotides (p53 sample, 15% input) relative to cells that received the control luciferase oligonucleotide (Luc sample, 15% input). Basal expression levels of SV40 LT and endogenous CBP/p300 were unaffected by siRNA transfections. Endogenous CBP immunoprecipitated from nuclear extracts was analyzed by Western blot analysis to identify its association with SV40 LT and p53 (Fig. 3A). A trimeric complex containing CBP, SV40 LT, and p53 was identified in the control luciferase siRNA sample. However, the ability of CBP to form an immunocomplex with SV40 LT was almost

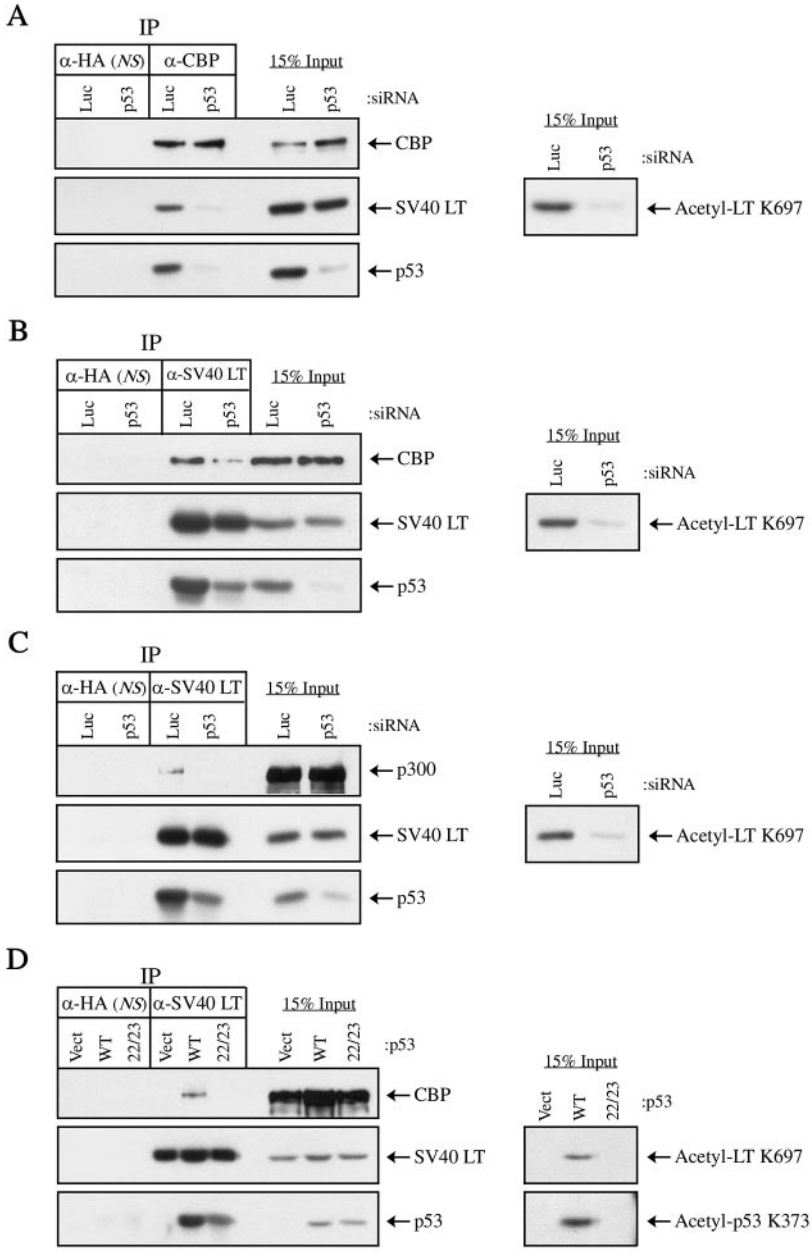

FIG. 3. p53 is required for SV40 LT binding to CBP and p300. p53 siRNA (p53) or control luciferase siRNA (Luc) oligonucleotides were transfected into U-2 OS cells stably expressing SV40 LT. At 72 h posttransfection, nuclear fractions were used to immunoprecipitate either endogenous CBP using monoclonal anti-CBP (α-CBP) AC238 or SV40 LT using monoclonal anti-SV40 LT pAb419. siRNA-mediated knockdown of p53 disrupted the ability of CBP to coimmunoprecipitate SV40 LT (A) and the reciprocal ability of SV40 LT to coimmunoprecipitate either endogenous CBP (B) or p300 (C), as determined through Western blot analysis using anti-CBP AC238 and anti-p300 RW128 antibodies, respectively. The effectiveness of p53 siRNA was evaluated through Western blot analysis using a polyclonal anti-p53 antibody against 15% of the nuclear extract input. Western blot analysis of duplicate nuclear fraction samples demonstrated reduced SV40 LT acetylation that paralleled the reduced total p53 expression (right panel). (D) Reintroduction of wild-type p53 restored SV40 LT-CBP complex formation in MEF $p53^{-/-}$ cells. MEF $p53^{-/-}$ cells stably expressing SV40 LT were infected with adenovirus to express wild-type human p53 (WT), the N-terminal mutant $p53^{22/23}$ (22/23), or the empty vector (Vect). Stable cell lines were established, and nuclear fractions were obtained. Immunoprecipitation of SV40 LT was carried out using the anti-SV40 LT pAb901 antibody, and protein complex formation was evaluated through Western blot analysis using anti-SV40 LT pAb419, anti-CBP AC238, and polyclonal anti-p53 antibodies. Western blot analysis of the duplicate 15% nuclear fraction input indicated a requirement for wild-type p53 for promoting SV40 LT acetylation using the site-specific anti-SV40 LT K697 antibody (right panel). The absence of $p53^{22/23}$ acetylation at K373 confirms the inability to associate with endogenous CBP/p300. Immunoprecipitations using an anti-HA antibody were used to ensure the absence of nonspecific interactions (*NS*).

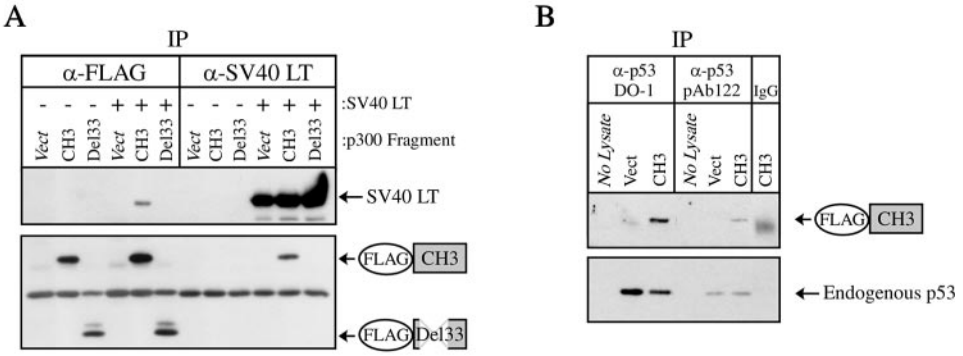

FIG. 4. SV40 LT and p53 interact with the CH3 domain of p300. (A) An N-terminally FLAG-tagged CH3 domain of p300 (CH3), an N-terminally FLAG-tagged Del33 mutant of p300 (Del33), or empty vector (Vect) was transiently expressed in wild-type U-2 OS cells (−) or U-2 OS cells stably expressing SV40 LT (+). Immunoprecipitation of whole-cell lysates with anti-FLAG (α-FLAG) M2 or anti-SV40 LT pAb419 antibody was performed, and coimmunoprecipitation was assessed through Western blot analysis. (B) Wild-type U-2 OS cells transiently expressed the N-terminally FLAG-tagged CH3 domain of p300 (CH3). Immunoprecipitation (IP) of endogenous p53 using the anti-p53 DO-1 or pAb122 antibody was subjected to Western blot analysis using anti-FLAG M2 or anti-p53 DO-1 antibody to identify an association of endogenous p53 with p300 CH3. Immunoprecipitation using immunoglobulin G was used as a negative control.

completely abolished following p53 siRNA transfection. The reduction in SV40 LT that could be coimmunoprecipitated with CBP was directly proportional to the reduction in coassociated p53. Reciprocally, the amount of endogenous CBP that was coimmunoprecipitated from nuclear fractions using an anti-SV40 LT antibody was similarly diminished following p53 siRNA transfection in proportion to reduced p53 expression levels (Fig. 3B).

Immunoprecipitation of SV40 LT demonstrated an association with endogenous p300 in a similar p53-dependent manner (Fig. 3C). However, the complex between SV40 LT and endogenous p300 in the control sample was considerably less evident than the complex between SV40 LT and endogenous CBP (Fig. 3, compare B and C). We have previously reported that SV40 LT is acetylated specifically at K697 by CBP in a p53-dependent manner in mouse cells (66). Western blot analysis of nuclear SV40 LT using an anti-acetyl-SV40 LT K697 antibody demonstrated that the disruption of the complex between SV40 LT and endogenous CBP/p300 through p53 siRNA was accompanied by a proportional reduction in acetylated SV40 LT (Fig. 3A to C).

Although we have previously demonstrated that homologous deletion of p53 reduced acetylation of SV40 LT, a requirement for p53 in promoting the complex between SV40 LT and CBP or p300 was not directly determined. Therefore, MEFs with a homozygous deletion of p53 (MEF $p53^{-/-}$) were used to further establish a requirement for p53 in bridging the interaction between SV40 LT and endogenous CBP/p300. Western blot analysis of anti-SV40 LT immunoprecipitations obtained from nuclear fractions could not identify a complex between SV40 LT and endogenous CBP in MEF $p53^{-/-}$ cells (Fig. 3D). Thus, wild-type human p53, mutant $p53^{22/23}$ that fails to bind CBP/p300 (83), or empty vector was expressed in MEF $p53^{-/-}$ cells to evaluate their ability to restore SV40 LT-CBP/p300 complex formation. Expression of mutant $p53^{22/23}$ failed to promote an interaction between SV40 LT and CBP, despite the ability of $p53^{22/23}$ to associate with SV40 LT. However, the reintroduction of wild-type p53 expression restored the ability of SV40 LT to target endogenous CBP for binding. Western blot analysis of nuclear fractions demonstrated that the acetylation of SV40 LT at the K697 residue could be restored upon expression of wild-type p53 and not mutant $p53^{22/23}$. Despite being expressed at equivalent levels, only wild-type p53 was acetylated at residue K373. The inability of mutant $p53^{22/23}$ to be similarly acetylated is further evidence of its inability to interact with endogenous CBP/p300. Therefore, p53 is required to promote complex formation between SV40 LT and endogenous CBP/p300 and direct the acetylation of SV40 LT at K697.

SV40 LT can bind p300 through the CH3 domain. It has been previously reported that a purified CH3 region of human p300 can interact with SV40 LT in vitro when incubated with mouse cell extracts (24). This region shares considerable sequence identity with the CH3 domain of CBP (80% similarity). To test this interaction in vivo, the p300 CH3 fragment (aa 1709 to 1913) was transiently expressed in wild-type U-2 OS cells and compared to U-2 OS cells stably expressing SV40 LT (Fig. 4A). Immunoprecipitations using an antibody directed against the N-terminal FLAG epitope of the CH3 fragment identified an interaction with SV40 LT. Conversely, a p300 CH3 fragment containing an in-frame deletion of residues 1737 to 1809 (Del33) could not similarly associate with SV40 LT. A reciprocal interaction was identified through the ability of an SV40 LT antibody to coimmunoprecipitate a specific complex with CH3, but not Del33, detected through anti-FLAG Western blot analysis (Fig. 4A).

The ability of the CH3 domain to independently interact with p53 (in the absence SV40 LT) was investigated in wild-type U-2 OS cells. The N-terminal FLAG-tagged CH3 domain or empty vector was transiently expressed, and endogenous p53 was immunoprecipitated using either anti-p53 DO-1 (N-terminal epitope) or pAb122 (C-terminal epitope) monoclonal antibody (Fig. 4B). Western blot analysis using an anti-FLAG antibody demonstrated that immunoprecipitated endogenous p53 can associate with the CH3 domain of p300 independent of SV40 LT binding. Specificity was demonstrated by the inability of cross-linked mouse immunoglobulin G alone to coimmunoprecipitate CH3. Therefore, SV40 LT and p53 can specifically

interact with the CH3 domain of p300 when expressed in human cells.

p300 promotes the acetylation of SV40 LT. We have recently reported that SV40 LT is acetylated on K697 primarily through CBP in mouse cells (66). However, CBP-null MEFs were not completely deficient in SV40 LT acetylation, and a role for p300 acetyltransferase activity could not be excluded. Given the ability of the CH3 domain from human p300 to interact with SV40 LT, we investigated the ability of p300 CH3 to specifically direct the acetylation of SV40 LT in human cells. A fragment of human p300 that encompassed the AT domain through the adjacent CH3 domain (aa 1195 to 1921) was cloned. Residues previously identified as essential for p300 acetyltransferase activity were substituted to WY(1466–1467)AS and FPY(1353–1355)AAA (10). These mutations inactivate the acetyltransferase function of the AT-CH3 fragment. Western blot analysis of the FLAG epitope demonstrated that these fragments were expressed at similar levels in U-2 OS cells in the presence or absence of SV40 LT (Fig. 5A). The deletion of 72 residues from the CH3 domain (AT-Del33) resulted in a faster-migrating protein relative to wild-type AT-CH3. Mutation of the acetyltransferase domain (FPY-CH3 and WY-CH3) also resulted in a slight increase in

typedi5romdi5romdi5ro3788ti--354.2(e8stern)-354.2(b8ot)-354-356.4(pnterafied)-234.5(pnterJT*[(p300)act)-288352.9(o8-27betwee2.9(o8-27s

endogenous CBP/p300. Although we could not find a stable complex between the Del33 mutant of full-length p300 and SV40 LT, this mutant could partially enhance the acetylation of SV40 LT. p53 has been shown to bind to the CH1 (32), KIX (83), acetyltransferase (5), and CR2 (55) domains of CBP and p300 as well as the CH3 domain (21, 62). It is not certain whether these domains provide a cooperative binding interface for a stable SV40 LT-p53 interaction or whether these additional domains provide a degree of redundancy that can partially overcome the CH3 deletion to a level that is below the resolution of our immunoprecipitation protocol. The complete absence of acetylated SV40 LT in MEF $p53^{-/-}$ cells indicates that these interactions are still conferred indirectly through p53 (Fig. 3D). Since acetylation of p53 at K373 was similarly increased by expression of the AT-CH3 fragment and full-length p300, but not by the corresponding acetyltransferase mutants (Fig. 5B), this further supports the ability of p53 to associate with the CH3 domain of p300. We therefore conclude that the acetyltransferase activity of p300 can contribute to the acetylation of SV40 LT.

DISCUSSION

We have demonstrated that SV40 LT can target endogenous CBP and p300 for binding not only in mouse cells but in human cells as well. In a recent report from our laboratory, we identified the acetylation of SV40 LT on a discrete K697 residue (66). This posttranslational modification was reduced by 15-fold through homologous disruption of CBP gene expression. The residual SV40 LT acetylation was not attributed to the PCAF acetyltransferase, but p300 acetyltransferase activity could not be ruled out. We have demonstrated in this report that SV40 LT can be acetylated directly through the acetyltransferase activity of p300, directed in part through the CH3 domain. While our previous report identified factors that promote LT acetylation, we now demonstrate that p53 is an integral bridging factor that promotes the interaction with CBP/p300 in the SV40 LT complex.

SV40 LT binding has been shown to repress the transactivation function of CBP and p300 (24). The potential impact of SV40 LT in reducing the amount active CBP is suggested by the increased incidence of tumors in patients with the CBP haploinsufficiency disorder Rubinstein-Taybi syndrome (58). While an increased incidence of hematological malignancies has been identified in mice that are heterozygous for CBP expression, no increased incidence of tumor formation was noted in matched p300 heterozygous mice (45). However, somatic missense mutations and truncations in p300 have been identified in a small subset of human epithelial cancers, usually associated with an inactivation of the second allele (29, 59). Although it is feasible that SV40 LT targeting of CBP/p300 could be required for efficient promotion of the transformed phenotype, additional experimentation is required.

Through p53 siRNA experiments and the reintroduction of p53 into p53-null cells, we have demonstrated that SV40 LT requires p53 to intermediate the SV40 LT and CBP/p300 complex. These observations are consistent with reports of SV40 LT existing in a ternary complex with p53 and CBP (24). While the human papillomavirus viral oncoprotein E6 promotes the degradation of p53 through E6-AP (79), E6 has the ability to target CBP/p300 in residues that are independent of p53 binding (65, 93). Polyoma LT does not associate with p53 but targets the binding of p300 and CBP (18, 61). E1A binds to p300 and CBP directly through the E1A N-terminal sequences and the CR1 domain, and these regions are essential for its transforming functions (39). Therefore, our observations that SV40 LT expression can promote the accumulation of p53 to levels 20- to 70-fold above those of the control, which is consistent with data from previous reports (68), suggest a mechanism for binding of CBP/p300 that is unique to SV40 LT. It also raises the question of whether previous studies that have inferred functions of SV40 LT through a mutation of the p53 domain actually implicated p53 inactivation as well as CBP/p300 targeting.

We have demonstrated that inactivated p53 in the SV40 LT complex is posttranslationally modified on discrete residues (S15, S20, and S37) known to be targeted by the DNA damage checkpoint kinases ATM, ATR, Chk1, and DNA-PK. The ability of SV40 LT to induce the relative level of S15 phosphorylation was of particular interest, as phosphorylation at this residue can stabilize the interaction of p53 with CBP/p300 (22, 48). The mechanisms by which SV40 LT induces these posttranslational modifications on p53 is unknown. However, SV40 LT activity is known to promote aneuploidy and other chromosomal abnormalities (74). Genomic instability may be manifested through the ability of SV40 LT to form a physical complex with checkpoint proteins involved in DNA repair and mitotic spindle checkpoint regulation. SV40 LT can enhance endoreduplication manifested during nocodazole treatment through binding and sequestration of the mitotic spindle checkpoint function of Bub1 (19). Endoreduplication of cellular and viral DNA imposed by SV40 LT has also been attributed to the binding of NBS1 (85). The ability of SV40 LT to promote chromosomal instability may lead to the activation of the DNA damage checkpoint kinases, with the subsequent phosphorylation of p53.

NBS1 associates with MRE11 and RAD50 to form a sensing or repair complex at the sites of DNA damage (12). While the NBS1/MRE11/RAD50 complex is required to recruit and activate ATM (13, 14, 49, 50), phosphorylation of NBS1 by activated ATM is subsequently required for promoting S-phase arrest and survival following ionizing radiation (28, 53, 86, 92). The inability of SV40 LT to interfere with NBS1 phosphorylation following DNA damage (85) suggests that SV40 LT may interfere with NBS1 function downstream of ATM activation. This is consistent with the ability of SV40 LT expression to induce the phosphorylation of p53 on residues targeted by ATM, ATR, Chk1, and DNA-PK. By targeting the inactivation of p53, SV40 LT association allows cells to bypass p53-dependent apoptosis and cell cycle arrest induced by DNA damage checkpoint control mechanisms while enhancing a p53 bridging function. The resulting complex with CBP and p300 could then direct the acetylation of SV40 LT and associated p53, as we have observed.

For p300, the domain possessing acetyltransferase activity has been localized to a region encompassing amino acids 1284 to 1669. This region is sufficient to acetylate core histones (H2A, H2B, H3, and H4) as well as a p53 peptide (10). We have demonstrated that this acetyltransferase domain of human p300 linked to the CH3 domain was sufficient to bind and

induce the basal acetylation of SV40 LT. Interestingly, although similar fragments containing mutated acetyltransferase activity could bind SV40 LT with a higher affinity, the mutant fragment did not diminish basal acetylation of SV40 LT. Furthermore, SV40 LT acetylation was induced by the overexpression of full-length p300. We initially interpreted these results as indicative of a transient association, as the ability to acetylate SV40 LT on K697 may have subsequently destabilized the association between SV40 LT and p300. However, immunoprecipitation experiments have not demonstrated an enhanced ability of acetylation-deficient SV40 LT K697R and K697A mutants to associate with endogenous human p300 (data not shown). Since we can increase the levels of basally acetylated SV40 LT by transient overexpression of p300, we postulate that only a subset of SV40 LT binds to p300 and CBP to become acetylated. Furthermore, these data suggest that SV40 LT binding does not inhibit the acetyltransferase activity of CBP and p300, consistent with data previously reported by Valls et al. (82).

Acetylation is a well-described posttranslational modification that can modulate protein interactions, DNA binding, and subcellular localization of a number of transcription factors. Acetylation of adenoviral E1A by p300 and PCAF on K239 disrupts the ability of E1A to repress CREB-mediated transcriptional activation. This has been attributed to either a reduced affinity for CtBP corepressor binding (91) or reduced nuclear translocation through an attenuated interaction with importin-α3 (57). In addition to its effects on promoting cell cycle entry, a number of SV40 LT functions are required for viral replication, including SV40 origin-specific DNA binding, helicase, and ATPase activities. Acetylation of the human immunodeficiency virus Tat transactivator by p300 has been shown to be required for transactivation of the human immunodeficiency virus promoter during viral replication (64) and for stimulating transcriptional elongation by dissociating Tat from TAR RNA (43). Therefore, acetylation of SV40 LT likely modulates the components within the SV40 LT protein complex, but further work is required to identify the specific function of SV40 LT that is impacted.

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