Repression of p53-mediated Transcription by Adenovirus E1B 55-kDa Does Not Require Corepressor mSin3A and Histone Deacetylases

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The Ad E1B 55-kDa protein (E1B) is a potent transcriptional repressor. In vitro biochemical studies revealed that direct p53-E1B interaction is essential for E1B to block p53-activated transcription and a corepressor may be involved. To understand how E1B represses p53-mediated transcription in vivo, we expressed E1B in several tumor cell lines that express wild type p53. Here we show that E1B strongly suppresses the expression of p53 target genes such as p21 and Puma-α in normal growth conditions or after cells were treated with p53-activating chemotherapeutic agents, suggesting that E1B-mediated gene repression is dominant and cannot be reversed via p53 activation. Interestingly, we found that E1B binds to corepressor mSin3A. Mutagenesis analysis indicated that the sequence motif “LHLLA” near the NH2 terminus of E1B is responsible for mSin3A binding, and this motif is conserved among E1B proteins from different Ad serotypes. The conserved paired amphipathic helix domain 1 of mSin3A is critical for mSin3A-E1B interaction. Surprisingly, E1B mutants that cannot bind to mSin3A can still repress p53 target genes, indicating that it is not the corepressor required for E1B-mediated gene repression. In support of this notion, repression of p53 target genes by E1B is insensitive to HDAC inhibitor trichostatin A. We further show that both the NH2- and COOH-terminal domains of E1B are required for the repression function. Therefore, E1B employs a unique repression mechanism to block p53-mediated transcription.

The p53 and pRb tumor suppressor pathways are inactivated in virtually all human cancers regardless of their etiology (1, 2). Understanding the precise molecular mechanisms of these pathways is at the center stage of current research efforts in cancer biology. Small DNA tumor viruses such as adenoviruses (Ad),3 human papillomaviruses, and SV40 can transform cells and cause cancer (3, 4). Each of these viruses produces several proteins that disable both p53 and pRb tumor suppressor pathways in infected cells. Ad E1A proteins physically associate with pRb and release it from E2F transcription factors that activate expression of genes required for DNA replication and cell cycle progression. The Ad JB region encodes two major proteins in two overlapping ORFs: E1B 55- and 19-kDa, both of which are required for efficient cell transformation. The E1B 19-kDa functions as an inhibitor of apoptosis by binding to proapoptotic proteins Bax and Bak (5), and E1B 55-kDa (hereafter called E1B) participates in transformation by inactivating the p53 pathway (6). Several functions of E1B contribute to the inhibition of p53. Apart from sequestration of p53 in the cytoplasm that blocks p53-mediated apoptosis (7), E1B can inhibit acetylation of p53 and disrupt the interaction between p53 and coactivator p300/CBP-associated protein (8). Early studies clearly showed that the transcriptional repression function of E1B, but not p53-E1B interaction per se, is critical for Ad-mediated cell transformation (9, 10). These studies demonstrated that (i) direct physical interaction between p53 and E1B is required for E1B to repress p53, (ii) E1B stabilizes the p53-DNA complex, and (iii) a corepressor may be involved (9, 11–13). Thus, E1B associates with DNA-bound p53 and converts p53 from an activator to a repressor (6). It is well documented that E1B binds to the NH2-terminal TAD of p53. Several hydrophobic residues including Leu22 and Trp23 in the TAD are required for E1B to bind to p53 (14). Therefore, binding of E1B to the TAD of p53 might occlude the sites that are required for binding of factors and transcription by p53, (i) E1B associates with HDAC proteins and disrupts the interaction between p53 and HDACs, and (ii) E1B also disrupts the interaction between p53 and coactivator proteins. These results indicate that E1B binds to p53 and disrupts the interaction between p53 and coactivator proteins, thereby inactivating p53.

3 The abbreviations used are: Ad, adenovirus; 5-FU, 5-fluorouracil; aa, amino acid; Ad12, adenovirus type 12; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; GFP, green fluorescent protein; HDAC, histone deacetylases; IP, immunoprecipitation; ORF, open reading frame; PAH, paired amphipathic helix domain; PIC, transcriptional preinitiation complex; SD, synthetic dropout medium; TAD, transcription domain; TBP, TATA-binding protein; WT, wild type; PIPES, 1,4-piperazinediethanesulfonic acid; TSA, trichostatin A.
cofactors that are involved in p53-dependent transcription. Consistent with this, CBP binds to the TAD of p53 and the L22Q/W23S mutation also abolishes CBP-p53 interaction (15). Thus, it is conceivable that E1B might block the association of CBP with p53. Recently, An et al. (16) demonstrated that p300, a paralog of CBP, and histone arginine methyltransferases PRMT1 and CARM1 are activators of p53. They bind directly to p53; p300 and PRMT1 binds to p53 TAD, and CARM1 binds to the COOH-terminal regulatory domain of p53. Sequential modifications of histones 3 and 4 by these three coactivators, rather than p53 per se, depend strictly on the presence of p53, and such p53-directed histone modifications are critical for transactivation of p53 target genes (16). Together, these three coactivators cooperatively stimulate p53-dependent transcription in vitro and in transfected cells, and such activation directly correlates with the recruitment of these coactivators by chromatin-bound p53 and subsequent histone lysine acetylation and arginine methylation at specific residues (16). Because PRMT1 binds to aa 1–43 of p53, association of E1B with the TAD of p53 might also prevent p53-PRMT1 interaction. Thus, E1B-p53 interaction can potentially block the recruitment of multiple coactivators by p53 to its target promoters.

Besides interactions with the aforementioned coactivators, the TAD of p53 also interacts with components of basal transcriptional machinery or the Mediator complex (for a recent review see Ref. 17). These contacts stimulate the recruitments of TFIIID and TFIIA, and stabilize the preinitiation complex (PIC) assembled on the DNA template containing p53-binding sites (17), which contributes to p53-mediated transactivation. It has been shown that binding of MDM2 to the TAD of p53 masks the binding sites for these basal components, thereby canceling the effects of TAD (18, 19). Additionally, MDM2 can directly inhibit transcription when tethered to the promoter in the absence of p53. Thus, it was proposed that MDM2 inhibits p53-mediated transcription by dual mechanisms: masking the TAD and inhibiting the functions of other components of the basal machinery, as MDM2 can also bind to the p34 subunit of TFIIE (18). Although a corepressor is not needed for MDM2 to repress transcription in vitro using highly purified basal transcription factors (18), it can recruit corepressor CtBP2 to suppress p53-dependent transcription (20). Additionally, DNA damage-induced phosphorylation of p53 may hinder binding of MDM2 to the TAD of p53, and thus alleviating inhibition of p53 by MDM2 (21).

E1B and MDM2 bind to an overlapping but not identical sequence of the p53 TAD. Thus, E1B could exert similar TAD-masking effects on p53-dependent transcription (9, 11–13). Nonetheless, a corepressor is probably critical for E1B to repress transcription, as an Ad2 E1B mutant (R443) that can still bind to p53 is unable to repress transcription (9, 13). Indeed, an unknown corepressor that copurifies with RNA polymerase II seems to be required for E1B-mediated repression (11). The identity of this corepressor remains unknown.

Our current understanding of the mechanism underlying E1B-mediated repression of p53 is largely based on pioneering biochemical studies or reporter gene assays in cell culture (11, 13). How E1B blocks transcription of p53 target genes from their endogenous loci has not been examined. We have expressed Ad12 E1B in several tumor cell lines that express WT p53 via lentiviral vector. We report here that E1B strongly suppresses the expression of p21 and Puma-a, two prototypical p53 target genes. This repression is not affected by p53-activating agents, which contrasts with the observed alleviation of MDM2-mediated inhibition of p53 upon DNA damage-induced phosphorylation (21). Surprisingly, despite a highly specific E1B-mSin3A interaction, corepressor mSin3A is not involved in E1B-mediated repression of p53. Consistently, HDAC inhibitor trichostatin A (TSA) does not alleviate repression by E1B. These findings suggest a unique mechanism of gene repression by E1B.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The human tumor cell lines HCT116, LN-229, Saos2, G401 (a rhabdoid kidney tumor cell line), and its derivative G401-CC3, which was stably transfected with a vector expressing Ad12 E1B (7, 22), were cultured with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected with various DNA plasmids using the Effectene transfection reagent kit from Qiagen.

**Construction of Lentiviral Vector for E1B Expression**—The GFP-E1B fusion was cloned into a lentiviral vector under the control of the cytomegalovirus promoter. The resulting plasmid was cotransfected along with pCMV-VSV-G and pCMV8R8.2 plasmids into 293T cells using the calcium phosphate precipitation method. The viral supernatant was collected 48 h after transfection, and used for transducing various cell lines.

**Yeast Two-hybrid Assay**—The DNA fragments spanning different regions of the ORF for the mouse mSin3A or Ad12 E1B were fused either to the DNA sequence encoding the yeast Gal4 TAD in plasmid pGAD-C(x) or to that for the Gal4 DBD in plasmid pGBKDU-C(x). Various combinations of these plasmids were introduced into yeast strain P69-4A (23). The transformed yeast colonies were replica plated on synthetic dropout (SD) medium lacking histidine but containing 5 mM 3-aminoimidazole and SD medium lacking adenine, essentially as described previously (8, 24).

**Luciferase Reporter Gene Assays**—The luciferase reporter construct p21-Luc contains 2.4 kb of the p21 promoter with two upstream p53-binding sites, as described previously (25). Cells were seeded in 48-well plates. The reporter plasmids were transiently transfected into cells alone or with other plasmids as indicated in the legend to Fig. 7. Twenty-four hours after transfection, doxorubicin alone or together with TSA was added to the transfected cells to the final concentration of 1 (doxorubicin) and 0.4 μg/ml (TSA). The cells were then processed for dual-luciferase assays (Promega) 48 h after transfection. Firefly luciferase activity was normalized against sea pansy luciferase activity as described previously (26).

**Immunoprecipitation (IP)**—HEK293 cells were infected with a recombinant Ad vector carrying the coding sequence for the GFP-Ad12 E1B fusion. Cells were harvested 24 h after infection and fractioned into cytoplasmic and nuclear fractions using the Nuclear Complex Co-IP kit from Active Motif. IP was carried out according to the manufacturer’s protocol. In each IP, 200
µg of nuclear or cytoplasmic extracts were mixed with 1.5 µg of antibody. The antibodies used for IP were: the rabbit polyclonal anti-mSin3A antibody AK11 from Santa Cruz Biotechnology, the rabbit polyclonal anti-NusG antibody as the matched control IgG (27), the mouse monoclonal anti-GFP antibody from Babco, and the mouse monoclonal anti-β-galactosidase antibody as the matched control IgG (clone 40–1A, Developmental Studies Hybridoma Bank).

Chromatin Immunoprecipitation (ChIP)—We followed a typical ChIP protocol as described (25). Glioblastoma LN-229 cells and flow-sorted LN-229–E1B cells that stably express GFP–E1B were grown on 10-cm dishes, and the cells were fixed with 1% formaldehyde for 10 min at room temperature. Formaldehyde was neutralized by the addition of glycine to 125 mM (final concentration). Cells were scraped off the dishes and washed twice with ice-cold phosphate-buffered saline. Cells were resuspended in 1 ml of ice-cold swelling buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) supplemented with 100-fold diluted protease inhibitor mixture (Sigma P8340) and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 2000 × g at 4 °C for 5 min. The pellet was dissolved in 1 ml of SDS-lysis buffer (10 mM Tris-HCl, pH 8.0, 1% SDS) containing protease inhibitor mixture. The chromatin-protein complexes were aliquoted and one part is used as input. Each aliquot was subjected to ChIP using 5 µl of rabbit serum for anti-Ad12 E1B (28) at 4 °C overnight with rotation. The immune complexes were captured using 50 µl of Protein G-agarose beads (50% slurry, Roche Applied Science) with rotation at 4 °C for 2 h. The agarose beads were collected and washed sequentially with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 100 mM Tris-HCl, pH 8.1), and twice with TE (pH 7.4). The chromatin-protein complexes were eluted with 500 µl of elution buffer (1% SDS, 0.1 M NaHCO₃) at 65 °C for 15 min with shaking. The supernatant was heated at 65 °C for 4 h to reverse cross-linking. Samples were treated with RNase A at 37 °C for 30 min and then digested with proteinase K (40 µg/ml) at 37 °C for 1 h. Samples were diluted and passed through a Qiagen mini-prep column. After the wash, DNA was eluted with 100 µl of TE (pH 7.4). PCR was performed with one step at 95 °C for 5 min and 31–35 cycles of 95, 60, and 72 °C each for 95 s and a final step at 72 °C for 2 min. The PCR primers used were (from 5′ to 3′): p21-p53BS1: forward, CATGTTCCAGACCTTCTCTCC and reverse, ATCCGCTTTCTCACTGAAAC; p21-p53BS2: forward, ATCCGCTTGTGAGGAGAGA and reverse, AAAAAAGGCGCAGCTG; p21-proximal: forward, GGTTAATCTTGTGCTGGAGGAGAAGGAG and reverse, ACTCCCTCCTCCTCCAGCT; Bax: forward, AAAGAGACAAAGAGAGAGAGAGAGAGG and reverse, CAATGAGCAGCAGCTCCT; Chromosome 4 centromere: forward, CTGTCCCTATAATATATGCAGAATACCTCA and reverse, GTAAGTTATGATAATACATATTTGGGC. The PCR products were separated by agarose gel electrophoresis.

RESULTS

E1B Represses p21 Expression in Glioblastoma LN-229 Cells—To understand the precise mechanism underlying E1B-mediated transcriptional repression, we have sought to establish a cell-based system that permits us to analyze the detailed molecular events associated with gene repression due to E1B expression. Because a recent study suggests that Ad preferentially transforms cells of neuronal origin (29), we reason that E1B should elicit robust gene repression in cells descended from neural stem cells. Glioblastoma LN-229 cells have normal p53 pathway, although a proline to leucine substitution occurs at position 98 (30). Despite this, p53 is phenotypically considered as WT in this cell line (31, 32). We have transduced LN-229 cells with lentiviral vector for GFP (LV-GFP) or GFP–Ad12 E1B (LV-GFP-E1B). The cells were either mock-treated with Me2SO (lanes 1–3), or treated with TSA (0.4 µg/ml, lanes 4–6), 5-FU (0.4 mM, lanes 7–9), or TSA plus 5-FU (lanes 10–12) for 24 h. The cells were harvested for Western blotting with the indicated antibodies. Thirty micrograms of total cell extracts were loaded in each lane, except for the GFP blot, in which 3 µg per lane was loaded. DMSO, dimethyl sulfoxide.
E1B expression reduced the p21 levels in mock-treated cells (lane 3, top), despite increased p53 levels, indicating that E1B suppresses p53-mediated activation of p21. Consistent with reports in the literature (33, 34), TSA markedly induced p21 expression (lanes 4-6 in the p21 panel). The p53 levels were also increased in TSA-treated cells. However, despite increased levels of p53, the levels of p21 in LN-229-E1B cells were noticeably lower (lane 6), suggesting that E1B can still repress p21 in the presence of TSA. This provided the first evidence that E1B-mediated repression of p21 is independent of HDACs. It is worth noting that the levels of p21 were still much higher in cells treated with TSA than that in the control (cf. lanes 3 and 6), despite E1B expression. This is likely due to p53-independent activation of p21 by TSA and such activation could not be suppressed by E1B. We then wished to assess whether p53-activating chemotherapeutical agents could relieve E1B-mediated repression. In keeping with published reports (26, 35), 5-FU activated p53 and induced p21 expression in LN-229 or LN-229-GFP cells, but p21 expression was dramatically suppressed in LN-299-E1B cells (cf. lane 9 with lanes 7 and 8). A similar pattern of p21 expression was also observed in cells treated with both TSA and 5-FU (lanes 10-12 in Fig. 1). E1B can similarly repress p21 expression in LN-229 cells treated with doxorubicin (data not shown, but see Fig. 2). The expression of GFP-E1B from an Ad vector also resulted in similar p21 repression (see supplemental Fig. S2), indicating that vectors themselves had no effects on p21 expression. Collectively, these results demonstrate that E1B represses p53-activated p21 expression and this repression cannot be reversed by TSA. Furthermore, p53-activating drugs could not alleviate E1B-exerted gene repression.

Repression of p53 Target Genes by E1B in Colon Cancer HCT116 Cells—To assess whether E1B can also represses p21 and other p53 target genes in other cell types, we expressed GFP or GFP-E1B via lentiviral vector in colon cancer HCT116 cells, which has WT p53 and a normal p53 pathway (36). The parental and transduced cells were either non-treated or treated with doxorubicin, and the cells were then analyzed for the expression of various p53 target genes. As shown in Fig. 2, in untreated cells, E1B substantially repressed the expression of p21 and Puma-α. In doxorubicin-treated cells, the expression of Puma-α was also obviously repressed by E1B. p21 expression was again inhibited, although to a lesser extent than Puma-α. Intriguingly, Mdm2 expression was only slightly affected. Thus, it appears likely that E1B may repress p53 in a gene-specific manner. Taken together, our data demonstrated that E1B can effectively repress p53 target genes in different cell types.

Ad12 E1B Binds to the PAH1 Domain of mSin3A—Because mSin3A corepressor was shown to be present in the immunoprecipitates of an antibody against Ad2 E1B (37), we tested whether there is specific interaction between E1B and mSin3A using the yeast two-hybrid assay. Fusion of full-length ORF of the Ad12 E1B with Gal4 DBD was tested for interactions with various mSin3A ORF fragments fused with the coding sequence for Gal4 TAD. Data presented in Fig. 3 showed that Ad12 E1B only interacts with mSin3A fragments containing the conserved PAH1 (Fig. 3, A and B, sectors 2, 3, and 18). To further test the specificity of the interaction between Ad12 E1B and PAH1 of mSin3A, we exploited the structural information based on the solution structure of the complex of the mSin3A PAH2 and the Mad1 Sin3 interaction domain (38). In the Sin3 interaction domain-PAH2 complex, the structure of PAH2 consists of a left-handed four-helix bundle, whereas the Sin3 interaction domain motif forms an amphipathic α helix that is wedged within a deep hydrophobic pocket defined mainly by...
two helices (α1 and α2) in PAH2; another two α helices also contribute side chains to the pocket (38). Because the amino acid sequences of PAH1 and PAH2 are highly conserved (38), it is generally assumed that they might adopt similar structures. We therefore introduced point mutations that changed Leu130 or Leu148 to Pro (Pro is known to disrupt α helix), and both mutations are expected to disrupt two α helices corresponding to α1 and α2 of PAH2. As shown in Fig. 3C, both mutations abolished the Ad12 E1B-mSin3A interaction, whereas mutations at Val168 and Leu178 to Pro within the putative α3 and α4 did not affect this interaction.

Next, we tested whether mutations in the corresponding region of PAH2 affect Ad12 E1B-mSin3A interaction. Val311 and Leu329 in PAH2 are at the equivalent positions to Leu130 and Leu148 of PAH1. Asp substitution at either Val311 or Leu329 markedly reduced mSin3A-Mad1 interaction with the former (i.e. V311D) being essentially inert in this interaction (38, 39). As shown in Fig. 3D, V311D or L329D did not affect Ad12 E1B-mSin3A interaction. Therefore, PAH2 is not required for the interaction between mSin3A and Ad12 E1B.

A Conserved Sequence Motif of E1B (LxLLA) Binds to mSin3A—We then wished to determine the sequence of Ad12 E1B required for interacting with mSin3A. Results from the yeast two-hybrid assays shown in Fig. 4A permit us to map the mSin3A-binding site to the NH2-terminal domain of the Ad12 E1B protein. It is likely that the sequence between amino acids 14 and 50 is required for binding to mSin3A, as the Gal4 DBD fusion construct containing Ad12 E1B aa 1–50 still binds to mSin3A (Fig. 4A, sector 14), but that with aa 1–13 failed to do so (Fig. 4A, sector 15). Sequence alignment revealed that the NH2 termini are conserved among the E1B proteins from different human and simian adenovirus serotypes (Fig. 4C). We thus mutated several conserved residues in this region to see if any point mutation could disrupt the interaction. Computer-based structure prediction indicated that this region of Ad12 E1B may form an α helix (Fig. 4C, lower part). We therefore mutated selected residues to Pro that may result in the disruption of the putative α helix. Mutating Leu11, Leu15 (outside of the putative α helix), and Val21 to Pro did not impact the mSin3A-Ad12 E1B interaction.
HDAC-independent Transcriptional Repression by E1B 55-kDa

A

B

C

D

α-Helix

Ad12 1c NAAVEGMAEEE---GLHLLLAGAAFPDH 40
Ad2/5 1c HAVESGGETQESPATVVFRPFGNTT 43

β-Strand

Consensus Motif

Φ = any bulky hydrophobic residue  X = any non-proline residue
interaction (see Fig. 4A, sectors 16–18). However, substitution of Leu30 or Leu33 with Pro abolished the interaction (see Fig. 4A, sectors 19 and 20).

**Ad12 E1B Protein Does Not Bind to mSin3A**—A previous study revealed direct interaction of Ad2 E1B and HDAC1 in vitro and coimmunoprecipitation of HDAC1 and mSin3A with this viral protein in total cell extracts (37). This prompted us to examine whether Ad2 E1B also interacts with mSin3A. As shown in Fig. 4B, full-length Ad12 E1B or a chimeric construct containing the Ad12 E1B sequence from the NH2 terminus to aa 340 and the COOH-terminal fragment of the Ad2 protein (aa 355–495) interacted with mSin3A (Fig. 4B, sectors 1 and 2). In contrast, another chimeric construct containing the Ad2 E1B NH2-terminal portion (1–149) and the Ad12 protein sequence from 136 to the COOH terminus did not bind to mSin3A (Fig. 4B, sector 3). Similarly, neither Ad2 E1B construct with a small COOH-terminal truncation (aa 1–437) nor another construct with NH2-terminal truncation (aa 155–495) interacted with mSin3A (Fig. 4B, sectors 4 and 5). We thus concluded that Ad2 E1B does not bind to mSin3A directly.

Sequence or structural differences might explain why Ad12 E1B, but not the Ad2 counterpart, binds to mSin3A. Fig. 4C shows an alignment of the NH2-terminal sequences of E1B from Ad12 along with several other human Ad serotypes, one from each of the six major Ad subgroups. The sequences around Leu30 and Leu33 are conserved among the E1B proteins of Ad12 (group A), Ad7 (group B), Ad9 (group D), Ad25 (group E), and Ad40 (group F). But the corresponding Ad2 sequence (group C) is quite divergent (Fig. 4C). Secondary structural predictions based on both Chou-Fasman and Robson-Garnier methods indicated that the Ad12 sequence encompassing the LHLLA motif might form an α helix, whereas the corresponding Ad2 sequence might be mostly non-structured with a short β-strand structure (Fig. 4C). Several recent studies identified proteins that specifically interact with PAH1 of mSin3A or B (40–42). Fig. 4D shows an alignment of the mSin3A-binding sequence of Ad12 E1B against that of several known PAH1-binding proteins. The E1B motif strongly resembles the conserved motif of this group of PAH1-binding proteins. Thus, the sequence and/or structural differences might underlie our observations that (i) Ad12 E1B but not the Ad2 protein binds to mSin3A and (ii) Ad12 E1B binds to PAH1 but not PAH2 of mSin3A.

**Ad12 E1B Binds to mSin3A in Cells**—To assess whether the Ad12 E1B binds to mSin3A in cultured cells, we infected 293 cells with a recombinant Ad carrying GFP-Ad12 E1B coding sequence. Cells were harvested 24 h after infection and fractionated into nuclear and cytoplasmic fractions. The nuclear extracts were subjected to IP with rabbit polyclonal antibody to mSin3A or control (against bacterial NusG) and the immunoprecipitates were assayed in Western blotting with anti-GFP antibody (lanes 1–3). The extracts were also immunoprecipitated with mouse monoclonal antibody to GFP or control antibody (against β-galactosidase). The immunoprecipitates were assayed in Western blotting (WB) with rabbit polyclonal antibody to mSin3A (lanes 4–6).

**mSin3A Is Not Required for Transcriptional Repression by E1B**—The mSin3A corepressor complex is involved in gene repression mediated by multiple transcriptional repressors (39, 43). To assess whether mSin3A is required for E1B to repress p53 target genes, we expressed WT E1B and the L30P mutant in LN-229 cells via lentiviral vectors. The E1B L30P mutant was unable to bind to mSin3A in yeast two-hybrid assays (see Fig. 4A), as well as failed to colocalize with mSin3A (see supplemental Fig. S3). As shown in Fig. 6, the L30P mutant was as potent as the WT E1B in repressing p21 expression without or with 5-FU treatment, whereas GFP had no effect on p21 expression (cf. lanes 3 and 4 with 1 and 2, or lanes 7 and 8 with 5 and 6, Fig. 6). Therefore, E1B-mSin3A interaction is not required for E1B-mediated transcriptional repression. To further substantiate this conclusion, we conducted luciferase reporter gene assays using the p21-Luc, in which the luciferase expression was under the control of the p21 promoter. As shown in Fig. 7A, in transfected LN-229 cells, WT E1B and the L30P mutant were equally effective in suppressing the reporter activities. We also treated a subset of the transfected cells with TSA. In agreement with

**FIGURE 4.** The NH2-terminal domain of Ad12 E1B is necessary and sufficient for interaction with mSin3A. A, the conserved NH2-terminal domain of Ad12 E1B binds to mSin3A. Depicted Gal4 DBD-Ad12 E1B hybrids that span different regions of Ad12 E1B or that with specific point mutations were tested for interaction with the Gal4 TAD-mSin3A (aa 1–849) hybrid in yeast two-hybrid assays as described in the legend to Fig. 3. The interaction data are summarized and the mSin3A-binding domain in Ad12 E1B is depicted with a gray box. The positions of the starting and ending residues or specific point mutations in each Ad12 E1B construct are also indicated. B, Ad2 E1B does not bind to mSin3A. Fusions of Gal4 DBD with the indicated Ad12, Ad2 E1B, or chimeric constructs between the two E1B proteins were individually tested for interaction with Gal4 TAD-mSin3A. C, sequence comparison of the NH2-terminal domain of Ad12 E1B (group A) with the corresponding sequences from Ad2, Ad7 (group B), Ad9 (group D), Ad25, and Ad40. Conserved residues are shown in shaded boxes. Gray arrows point to Ad12 E1B residues whose mutations did not affect interaction with mSin3A, whereas black arrows point to residues whose mutations eliminated the interaction. Predicted secondary structures of the NH2-terminal domain of Ad12 and Ad2 E1B based on Chou-Fasman and Robson-Garnier methods implemented in the MacVector software are shown below. D, sequence alignment of the PAH1-binding regions of selected proteins. Conserved residues are depicted in the gray boxes. Note that the mouse Sin-associated protein 25 sequence is shown in reverse orientation to maximize sequence identity.

**FIGURE 5.** Coimmunoprecipitation of Ad12 E1B and mSin3A in cells. The 293 cells were infected with a recombinant Ad carrying GFP-Ad12 E1B coding sequence. Cells were harvested 24 h after infection and fractionated into nuclear and cytoplasmic fractions. The nuclear extracts were subjected to IP with rabbit polyclonal antibody to mSin3A or control (against bacterial NusG) and the immunoprecipitates were assayed in Western blotting with anti-GFP antibody (lanes 1–3). The extracts were also immunoprecipitated with mouse monoclonal antibody to GFP or control antibody (against β-galactosidase). The immunoprecipitates were assayed in Western blotting (WB) with rabbit polyclonal antibody to mSin3A (lanes 4–6).
data shown in Fig. 1, TSA had no effect on the repression of the 
*p21* promoter mediated by either WT E1B or the L30P mutant 
(Fig. 7B). Similar results were obtained in HCT116 cells using 
the PG13-Luc, in which the luciferase reporter is under the 
control of a synthetic promoter containing 13 copies of the 
consensus p53 binding site (see supplemental Fig. S5). Because 
HDAC activity is essential for mSin3A-dependent gene repression, 
these data collectively reinforce the notion that mSin3A is 
not a co-repressor involved in the repression of p53-mediated 
transcription by E1B.

**Effects of Specific E1B Mutations on Its Transcriptional 
Repression Function**—Previous studies revealed that two 
conserved serine residues at positions 476 and 477 are important 
for E1B to repress p53-mediated transcription (7, 10). Indeed, 
the S476A/S477A double mutations completely abolished 
repression of the *p21* promoter by E1B (Fig. 7A). Interestingly, 
mutation of either serine residue had no effects on repression, 
suggesting that these two residues have redundant roles for 
E1B-mediated gene repression. One notable feature of the E1B 
proteins from different Ad serotypes is that there are numerous 
highly conserved phenylalanine, tryptophan, and histidine res-
idues throughout the conserved region. We therefore mutated 
several of these residues to assess whether these residues are 
important for gene repression. As shown in Fig. 7A, most of 
these point mutations had no obvious effects on the repression 
function of E1B. Nonetheless, the H245A mutation moderately 
relieved repression, whereas the H446A mutant was completely 
defective in eliciting gene repression. Residue His446 is close to 
the equivalent position of the R443 linker insertion in Ad2 E1B, 
which did not affect E1B-p53 interaction, but abolished the 
repression function of E1B (13), suggesting that the amino acids 
surrounding His446 are critical for gene repression. The find-
ings that both H446A and S476A/S477A mutants could not at 
all repress the *p21* promoter further substantiate the critical 
importance of the extreme COOH-terminal domain of E1B in 
gene repression. We also tested several truncation mutants of 
E1B in the reporter assays. Data represented in Fig. 7A show that 
all of them were completely devoid of repression activity. Therefore, both 
the NH2- and COOH-terminal sequences of E1B are essential for 
gene repression. The extreme NH2-terminal sequences of Ad E1B pro-
teins (~first 30 residues) are con-
served and this sequence motif is 
unique to the E1B protein family and 
is designed as Adeno_E1B_55K_N 
(pfam04623.6) in the NCBI conserved 
domain data base (see Fig. 4C). Thus, 
despite the fact that mSin3A is not 
required for E1B-mediated gene repression, this conserved NH2-
terminal motif appears to have an 
important role in the transcriptional 
repression mechanism, because dele-
tion of this motif (Fig. 7A, construct 
83–482) rendered E1B nonfunctional 
in gene repression.

**E1B Associates with the Chroma-
tin of the p21 Promoter**—It was 
shown that E1B must bind to p53 to
E1B did not appear to associate with the precipitated DNA in the proximal promoter region. In contrast, native p53-binding sites (p53BS1 and -2), and interestingly, also enriched chromatin DNA in the regions harboring the two cognate GFP-E1B. As shown in Fig. 8, anti-E1B antibody significantly repressed transcription in vitro (11). Accordingly, it is expected that E1B should associate with chromatin-bound p53 in vivo. To test this notion, we have done ChIP experiments using anti-E1B antibody and the cross-linked, fragmented chromatin from LN-229 or LN-229-E1B cells that constitutively express E1B antibody and the cross-linked, fragmented chromatins subjected to ChIP with rabbit polyclonal anti-Ad12 E1B antibody. The precipitated DNA was PCR amplified with primers specific to three regions of the p21 promoter as indicated in the schematic diagram of the p21 promoter. PCR of the Bax promoter region as well as the chromosome 4 centromere serves as a negative control.

Because E1B expression results in marked repression of p21 expression in LN-229 cells (Figs. 1 and 2), it is possible that E1B can still alter histone modifications associated with gene activation. Furthermore, p53 is required for E1B to associate with the p21 promoter, as E1B did not bind to the p21 promoter in the absence of p53 in HCT116 cells (see supplemental Fig. S6). These results suggest that E1B represses p53 by associating with it on the chromatin of its target promoters.

p21 promoter

FIGURE 8. E1B associates with the p21 promoter. Parental glioblastoma LN-229 cells or the derivatives stably transduced with LV-GFP-E1B were subjected to ChIP with rabbit polyclonal anti-Ad12 E1B antibody. The precipitated DNA was PCR amplified with primers specific to three regions of the p21 promoter as indicated in the schematic diagram of the p21 promoter. PCR of the Bax promoter region as well as the chromosome 4 centromere serves as a negative control.

DISCUSSION

This study provides some important insights into the mechanism by which E1B represses transcription. First, E1B inhibits the expression of several known p53 target genes such as p21 and Puma-α at their endogenous loci. Second, this repression does not require corepressor mSin3A and the HDAC activities that can be inhibited by TSA. Third, E1B associates with the chromatin of p53 target genes. Fourth, p53-activating chemotherapeutic agents could not alleviate E1B-mediated repression. Collectively, our study is consistent with a model in which E1B associates with p53 when it is bound to its cognate DNA-binding sites in the promoters of its target genes. In this way, E1B blocks p53-activated transcription. It can be envisioned that E1B could do so by preventing the recruitment of the components of PIC to p53 target promoters and/or inhibiting histone modifications associated with gene activation.

Transcription corepressors can preclude the recruitment of PIC to specific promoters. For instance, the human papillomavirus E2 protein associates with the bromodomain protein Brd4 and prevents the formation of PIC on the E2 target promoter within the human papillomavirus genome (44). In the case of E1B, Martin and Berk (11) demonstrated that it acts during PIC assembly in a study based on in vitro biochemical assays using purified components, as E1B cannot repress p53-activated transcription when PIC is fully assembled (11). Nonetheless, it remains unknown whether E1B precludes the recruitment of PIC to the promoter or prevents the assembled PIC to engage in transcription elongation. Recently, it has been shown that basal levels of p53 under normal cell growth conditions are required to assemble a poised Pol II initiation complex on the p21 promoter. This poised complex is quickly converted into an elongation form after treatment of the cells with doxorubicin (45). Because E1B expression results in marked repression of p21 expression in LN-229 cells (Figs. 1 and 2), it is possible that E1B could block the assembly of the poised Pol II complex, or prevent the conversion of poised PIC to an elongation complex.

Alternatively, or in conjunction with potential interference with PIC recruitment/transcriptional elongation, E1B can also impact histone modifications. Transcription from the p21 promoter is highly regulated by different histone modifying enzymes including acetylases and HDACs (33, 34, 46) and histone lysine methyltransferases (47, 48). Occupation of the p21 promoter by E1B likely affects histone modifications of the p21 promoter. Although our data demonstrated that mSin3A is not involved in E1B-mediated gene repression, E1B can still alter histone modifications including acetylation. For example, HDACs that cannot be inhibited by TSA such as the yeast Sir2 homologs in the humans (Sirt1–7) could in principal be associated with E1B. Further studies are required to determine whether E1B can influence histone modifications of the p53 target genes.

It came as a surprise that mSin3A is not involved in E1B-mediated repression, despite the fact that E1B binds to mSin3A in a highly specific manner. It should be noted that mSin3A is a multifunctional protein and its involvement in activities other than transcriptional repression has started to emerge. For example, the yeast Sin3-Rpd3 complex was shown to have a role in transcriptional activation under osmotic stress (49). Additionally, the yeast and Caenorhabditis elegans Sin3 proteins appear to participate in DNA repair, probably through modulating chromatin structure (50, 51). We have detected colocalization of mSin3A and E1B in both the nucleus and the cytoplasm (see supplemental Fig. S3), suggesting that E1B could employ mSin3A for unknown activities in both compartments.
HDAC-independent Transcriptional Repression by E1B 55-kDa

The precise role of mSin3A in E1B-mediated processes and adenovirology will be an interesting subject of future study.

Acknowledgments—We thank D. Ayer for mSin3A cDNA and L. Zhou for database search.

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