Changes in C-terminal Binding Protein 2 (CtBP2) Corepressor Complex Induced by E1A and Modulation of E1A Transcriptional Activity by CtBP2*

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The N-terminal region of adenovirus E1A interacts with histone acetyl transferases (HATs) such as p300, P/CAF, and GCN5. The C-terminal region interacts with the transcriptional corepressors CtBP1 and CtBP2. The functional significance of co-recruitment of HATs and CtBPs by E1A is not well understood. In this study, we have shown that E1A enhanced acetylation of CtBP2 by recruitment of p300 to the CtBP2 complex. Additionally, E1A also displaced the histone methyltransferase G9a and the E-box repressor ZEB from the CtBP2 complex through the C-terminal CtBP-binding domain. A transcriptional activation function encoded by the E1A N-terminal region was efficiently inhibited by CtBP2 but not by a mutant with an N-terminal deletion or by a mutant deficient in interaction with E1A. Two isoforms of CtBP1 (CtBP1-L and CtBP1-S) poorly inhibited transcriptional activity of the E1A N-terminal region. Thus, the N-terminal domain of CtBP2 may contribute a unique transcriptional regulatory activity of CtBP2. Our results provide new insights by which CtBP might modulate the biochemical activities of E1A.

E1A is the first viral regulatory protein expressed during adenovirus (Ad)2 life cycle, and is essential for transcriptional activation of other viral early regions (reviewed in Ref. 1). The E1A proteins are expressed as two major isoforms from two alternatively spliced mRNA of 12 S encoding 243 residues (243R) and 13 S encoding 289 residues (289R). Although both E1A proteins exhibit transforming activities, the 289R protein functions as a transcriptional activator of other viral early genes. The 243R and 289R proteins share three conserved regions, CR1, CR2, and CR4, whereas the 289R protein contains an extra 46-amino acid region (CR3). The CR3 region contributes to the trans-activation function of 289R (2) in association with a cellular mediator complex (3). The 243R protein is sufficient for transformation of primary cells in cooperation with oncogenes such as the activated Ras oncogene (4).

The transforming activities of E1A are critically dependent on the N-terminal 80-amino acid region encompassing CR1 and the region between amino acids 120 and 140 (CR2) (5). The N-terminal 80-amino acid region interacts with the global transcriptional coactivators p300 and its paralog CBP, which function as histone acetyl transferases (HATs) (6–8). The CR1 region interacts with a protein complex designated the TRRAP-p400 complex (9–13). The TRRAP-p400 complex contains components of the SWI/SNF family chromatin remodeling complex (9, 10) and associates with one of the two HATs, GCN5 or Tip60 (14). Among these two HATs, the E1A-associated TRRAP complex has been shown to contain GCN5 in Ad-infected cells (15). The potential presence of Tip60 in E1A-associated TRRAP-p400 has not been investigated thus far. In addition to p300/CBP and GCN5, the CR1 region also binds P/CAF directly (15, 16) as well as through p300/CBP (17). Thus, the N-terminal 80-amino acid region of E1A recruits multiple HATs either directly or through adapter proteins such as TRRAP and p400 to regulate cell proliferation and to contribute to the transforming activities of E1A.

Although it is generally believed that sequestration and suppression of the activities of various HATs such as p300 and CBP by E1A (7, 17, 18) might contribute to cell proliferation and the oncopgenic activity of E1A, the precise mechanism by which the interaction of E1A with various HATs contributes to cell proliferation remains unclear. A chromatin immunoprecipitation (ChIP) study has suggested that E1A-mediated targeting of HATs to promoters of cell cycle regulatory genes might contribute to cell proliferation and transformation. In contrast to the lack of clear understanding of the mechanism by which the N-terminal region of E1A contributes to cell proliferation and transformation, the mechanism by which the interaction of E1A with pRb family proteins (pRb, p130, and p107) contributes to these activities is better understood (20,
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The CR2 region interacts with pRb family members through the prototypical Rb-binding motif, LXCXE, with high affinity and displaces various E2F family members from the RB protein complex resulting in transcriptional activation of the S-phase genes. Although activation of S-phase genes by the E1A CR2 region is sufficient for driving cells into S-phase, it is not sufficient to drive cells through the entire cell cycle. The activity of the N-terminal 80-amino acid region (i.e. interaction with p300 and other HATs) is required for conferring the full cell proliferation promoting activity of E1A (22, 23).

The CR4 region interacts with two highly related proteins, collectively termed as C-terminal binding protein (CtBP) through a sequence motif, PLDLS, conserved among all primate Ad E1A proteins (24, 25). The CR4 region modulates the transforming activity of E1A in opposing manners. It is essential for immortalization and cooperative transformation with E1B (26–28). However, CR4 exerts a strong negative regulatory effect on cooperative transformation with the activated Ras oncogene in primary epithelial cells (24, 25, 29, 30). Cells transformed by Ras and E1A CR4 mutants are highly tumorigenic and invasive (24, 29, 31). Several CR4 mutants that cooperate with Ras more efficiently are deficient in interaction with CtBP (24, 25) suggesting a link between recruitment of CtBP and retardation of the oncogene cooperating activity of E1A.

The CtBP family members CtBP1 and CtBP2 are highly homologous, and a number of transcriptional repressors bind to both homologs through motifs that resemble the canonical CtBP-binding motif (PLDLS) of E1A (32, 33). A proteomic analysis of the nuclear protein complex of CtBP1-L has revealed the presence of CtBP2 as well as enzymatic constituents that mediate histone modifications, in addition to DNA-binding repressors such as ZEB (34, 35). These studies have suggested that DNA sequence-specific repressors recruit the CtBP corepressor complex to mediate coordinated histone modifications by deacetylation, demethylation, and methylation. Additionally, CtBP1 has been reported to interact with p300/CBP and inhibit their HAT activity (36–38), which may contribute to a global repression activity. The presence of both CtBP homologs in the CtBP1 complex is consistent with the observation that CtBP1 and CtBP2 play redundant transcriptional regulatory roles during mouse development (39). Genetic studies with mice have revealed that CtBP1 and CtBP2 also play distinct transcriptional regulatory roles. The determinants for the unique regulatory activities of the two CtBPs are not known. Despite extensive sequence similarities, the vertebrate CtBPs differ in the N-terminal 20-aa acid region. CtBP2 contains a unique 19-amino acid Lys/Arg-rich N-terminal domain, which is modified by acetylation by p300 and is required for nuclear localization of CtBP2 (40). The two isoforms of CtBP1 vary by the presence of a 13-amino acid N-terminal domain in CtBP1-L or by the absence of this domain in CtBP1-S (reviewed in Ref. 41). The availability of mouse cells that are null for both CtBP1 and CtBP2 has provided an experimental system to investigate the activities of CtBP1 and CtBP2 individually.

The mechanism by which the E1A C-terminal region downregulates Ras cooperative transformation and restrains the oncogenic and metastatic potentials of transformed cells is not fully understood. Some of the activities of the E1A C-terminal region may be linked to relief of CtBP-mediated transcriptional repression of various epithelial genes such as E-cadherin by cellular repressors (42, 43). Additionally, CtBP associated with the E1A C terminus might also modulate the activities of protein complexes associated with the E1A N-terminal region. Such a possibility was initially suggested from an observation that a transcriptional activation function encoded by the N-terminal region of E1A was efficiently inhibited by the C-terminal region of E1A (44). The hypothesis that the N- and C-terminal activities of E1A may oppose each other becomes attractive considering the opposing biochemical activities (e.g. HATs versus HDACs) of the cellular proteins associated with E1A. Because CtBP2 is specifically acetylated by p300 (40), we have tested a hypothesis that the recruitment of p300 and CtBP2 into a single protein complex by E1A would result in changes in the activities of these cellular proteins. Here, we report that E1A-mediated juxtaposition of p300 and CtBP2 results in increased acetylation of CtBP2 and the E1A C-terminal region antagonizes the transcriptional activity of the N-terminal region of E1A through specific recruitment of CtBP2. Additionally, E1A caused changes in the composition of the CtBP2 corepressor complex.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Proteins and Purification—The GFP open reading frame was PCR-amplified from pEGFP-N1 with primers N45 (ACTGCATCTGAGGTCGGCACCAGTGAGGAAGGCATGAGACATATTATCTGCTCGTCCATGCC) and N46 (CATCGAGATCTCTTGTAGACGAGCTGGGCTGTTACAGCTCGTCCATGCC) and cloned into the XhoI/BamHI sites of pHA-IRESngeo (Clontech) to generate pHA-GFP. E1A (Ad2 12 S) was PCR-amplified with primers N103 (ACTGACGGATCCGGCGACAGAGAAGGCATGAGACATATTATCTGCTCGTCCATGCC) and N20 (TTCGAGATCTCTTGTAGACGAGCTGGGCTGTTACAGCTCGTCCATGCC) and N46 (CATCGAGATCTCTTGTAGACGAGCTGGGCTGTTACAGCTCGTCCATGCC) into the EcoRI/Xbal fragment of the pIRESpuro3 vector (Clontech) to generate pGFP-E1A. All E1A mutants and the E1A 13 S (Ad5) were prepared by a similar approach using PCR. To express His$_{6}$-tagged CtBP proteins in Escherichia coli, the CtBP2 N-terminal coding sequence was PCR-amplified with primers N135 (AGACTGCCATCTGAGGTCGGCACCAGTGAGGAAGGCATGAGACATATTATCTGCTCGTCCATGCC) and N136 (GATGGGTGTGGTACATCATGGCGCC) and cloned into the XhoI/BamHI sites of pFH-CtBP2. All His$_{6}$-tagged proteins were expressed in E. coli strain BL21(DE3) and purified using Ni$_{2+}$-nitrilotriacetic acid affinity chromatography.
To express p300 in insect cells using the baculovirus vector, the full-length p300 open reading frame and the aa 1063–2414 region of the p300 open reading frame were PCR-amplified with primers N104 (ACATGGCCTCAGGCAATGCGGCAAAATGTGTGGTGAACCGG) and N107 (AGCTAATAGCCGGCCTAGTGTATTGTCTAGTGTACTGTTGAGAGG), and N123 (ACTAGCGTGCAGGCATGGACACTTACGTCAGGATC) and N107, respectively. The PCR-amplified DNAs were digested with XhoI/NotI and ligated to the Stul/NotI fragment of pBacPak8 (Clontech) and the EcoRV/Xhol fragment of pFH-CtBP2 (40). Recombinant baculoviruses were generated in Sf21 cells following recommended conditions (Clontech). Both the full-length p300 and the p300BD(+) constructs carry a FLAG-HA tag at the N terminus. For protein expression and purification, Sf21 cells grown in T75 flasks were infected with baculoviruses for 2 days. Cell lysates were used for partial purification of the FLAG-HA-tagged proteins using the FLAG antibody beads (Sigma).

**Cell Culture, Transfection, and Adenoviral Infection—**HeLa and the mouse embryo fibroblasts (MEF90) null for CtBP1 and CtBP2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Insect cells (Sf21) were cultured in SF-900 II SFM medium (Invitrogen) with 2% fetal bovine serum. HeLa cells were transfected for luciferase assays using the JetPEI reagent (Mid gate, #1014992 (Qiagen); GFP, #632460 (BD Biosciences); p300, SC-584 (Santa Cruz Biotechnology); GCN5; #SC-20698 (Santa Cruz Biotechnology); PCAF, #SC-13124 (Santa Cruz Biotechnology); G9a, #07–551 (Upstate Biotech); ZEB, #SC-25388 (Santa Cruz Biotechnology); CoREST, #612146 (BD Biosciences); HDAC1, #SC-8410 (Santa Cruz Biotechnology); and HDAC2, #SC-9959 (Santa Cruz Biotechnology). Signal intensities in Western blots were quantified on a QuantityOne gel scanner (Bio-Rad).

**In Vitro Acetylation of CtBP2 and p300 Binding—**For CtBP2 acetylation, ~1 μg of purified H6-CtBP2 was incubated with p300 (~20 ng) for 30 min at 30 °C in 40 μl of acetylation buffer containing 30 mM Tris–HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.1 mM acetyl-CoA, 1 mM sodium butyrate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. For p300/CtBP binding assays, p300 was first bound to the FLAG antibody beads, washed, and then incubated with various CtBP proteins (1 μg) in the presence or absence of GST-E1A (0.5 μg) in 100 μl of the acetylation buffer with a gentle rocking at 4 °C for 30 min. The supernatant of this assay was treated as the unbound fraction, and the bound proteins were washed and examined by Western blots. Radioactive acetylation of CtBP2 was performed with [14C]acetyl-CoA under recommended conditions (Upstate Biotechnology). Reaction products were precipitated with trichloroacetic acid and examined by electrophoresis on a 4–15% gel, followed by Coomassie Blue staining or phosphorimaging analysis (Storm 850, Amersham Biosciences).

**Chromatin Immunoprecipitation—**ChIP assay was performed under recommended conditions (EZ ChIP kit, Upstate) with the following modifications. HeLa cells or HeLa/CtBP2 cells in 100-mm dishes were fixed with 1% formaldehyde, collected, and resuspended in 0.6 ml of the IP buffer supplemented with 20 mM EDTA. The cell suspension was sonicated, and the chromatin preparation was precleared with protein G-agarose. Pre-cleared chromatin preparations were bound to FLAG antibody beads for 1 h at 4 °C. Antibody beads were washed twice with the IP/EDTA buffer, once with half-strength IP/EDTA buffer with 0.25 mM LiCl, and twice with TE. Bound protein-DNA complexes were eluted and cross-linking reversed, and the DNA was phenol-extracted and ethanol-precipitated. Precipitated DNA was PCR-amplified with primers N187 (CTCGACCTCAGCTGGTGAAAAGATGTG) and N179 (GGTGCGGTGGCTGCAGCGGTGGAGCC) to detect the human E-cad promoter region. PCR products were electrophoresed on a 1.5% agarose gel and photographed with the Quantity One system (Bio-Rad). Control ChIP analysis of the glyceraldehyde-3-phosphate dehydrogenase promoter region was performed as recommended (EZ ChIP kit, Upstate Biotechnology).
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**RESULTS**

**E1A Enhances Acetylation of CtBP2 in Vivo**—CtBP2 has three Lys residues at positions 6, 8, and 10 (Fig. 1A) that can be acetylated by p300 in vivo (40). Because Ad E1A interacts with p300 (6, 7) as well as CtBP (24, 25), we examined whether E1A affects acetylation of CtBP2. To facilitate immunoprecipitation analyses, CtBP2 was engineered to carry a FLAG-HA tag at the N terminus, and E1A was expressed as a GFP-E1A fusion protein. Throughout the text, E1A refers to the 12 S isoform, whereas the 13 S isofrom is designated as such. Cell lysates from transfected cells were immunoprecipitated with the FLAG antibody and examined by Western blot analysis using different antibodies. As shown in Fig. 1B, co-expression of CtBP2 with GFP-E1A resulted in a dramatic enhancement of CtBP2 acetylation (compare lanes 1 and 2, top panel). In contrast, co-expression of CtBP2 with the mutant GFP-E1A-C*, which carries a mutation (DL→AS) within the PLDLS motif, had a much smaller effect (lane 3). GFP-E1A also enhanced acetylation of the CtBP2 mutant (AS8E) deficient in binding to the PLDLS motif (lanes 4 and 5) but to a lower extent than the wt CtBP2. The CtBP2 mutant (3KR), which contains substitution of Lys→Arg at positions 6, 8, and 10 (Fig. 1A), also yielded a low level of acetylation in the presence of GFP-E1A (Fig. 1B, lanes 6 and 7). No acetylation of CtBP2 was detectible in the presence (lane 8) or absence of GFP-E1A (not shown). Probing the immunoprecipitates with the GFP antibody revealed that CtBP2, 3KR-CtBP2, and CtBP2 wt all interacted well with GFP-E1A, whereas AS8E-CtBP2 bound to GFP-E1A only weakly (Fig. 1B, third panel from top). As expected, wt CtBP2 also bound to the GFP-E1A-C* mutant poorly. The enhanced CtBP2 acetylation by GFP-E1A was correlated with the relative levels of interaction between CtBP2 and GFP-E1A.

Immunofluorescence analysis revealed that the 3KR-CtBP2 mutant was localized predominantly in the cytoplasm when expressed alone (Fig. 1C, top panel). The cytoplasmic localization of 3KR-CtBP2 is partly due to nuclear export, because inhibition of the CRM-1-mediated nuclear export by leptomycin B rendered 3KR-CtBP2 to be localized predominantly in the nucleaus (40). Interestingly, co-expression of 3KR-CtBP2 with GFP-E1A resulted in almost exclusive nuclear localization of 3KR-CtBP2 (Fig. 1C, bottom panel). Examination of GFP-E1A revealed that it was co-localized with 3KR-CtBP2 (data not shown). Co-localization of GFP-E1A with 3KR-CtBP2 is consistent with the observation that 3KR-CtBP2 interacts with GFP-E1A efficiently (Fig. 1B, third panel, lane 7).

To examine the effect of E1A produced during Ad infection on acetylation of CtBP2, HeLa cells stably expressing FLAG-HA-tagged CtBP2 were infected with Ad 12 S wt or with a E1A 12 S C-terminal mutant (ΔC, lacking aa 178–238 (29). CtBP2 was immunoprecipitated from the virus-infected cells and examined for its acetylation status. As shown in Fig. 1D, wt E1A
12 S (lane 3), but not the C-terminal mutant (ΔC) of E1A 12 S (lane 4), enhanced CtBP2 acetylation. Thus, expression of E1A in transfected cells as well as in Ad-infected cells enhanced acetylation of CtBP2.

**E1A and p300 Synergistically Enhance CtBP2 Acetylation in Vivo**—We examined if p300 and other HATs are recruited into the CtBP2 protein complex through E1A in Ad-infected cells. For this purpose, HeLa cells stably expressing FLAG-HA-tagged CtBP2 were infected with Ad 12 S wt or Ad 12 S-ΔC, the protein complex was immunoprecipitated with the FLAG antibody (as in Fig. 1D) and probed for various HATs, E1A, and CtBP2. As shown in Fig. 2A, p300 was preferentially recruited into the CtBP2 complex by E1A (lane 3), whereas GCN5 was only moderately recruited by E1A. P/CAF was not detectable. The presence of p300 in the CtBP2 complex prompted us to examine the effect of E1A and p300 co-expression on acetylation of CtBP2. First, we examined the effect of the E1A C-terminal mutant deficient in interaction with CtBP2 (Fig. 2C). Co-expression of a mutant (Δ2–74) with a deletion within the N-terminal region of E1A did not significantly enhance acetylation of CtBP2 in the presence (Fig. 2D, lane 8) or absence of exogenous p300 (Fig. 2D, lane 4) compared with cells without E1A (lanes 1 and 5). In contrast, GFP-E1A (lanes 2 and 6) or a mutant with a deletion in the middle region of E1A (Δ91–199) (lanes 3 and 7) induced enhancement of CtBP2 acetylation. It should be noted that the activity of the mutant Δ91–199 was higher than GFP-E1A despite the lower level of mutant protein expression (Fig. 2D, panels 3 and 4). The level of CtBP2 acetylation was much higher in the presence of exogenous expression of p300 (lanes 6 and 7). Thus, the results presented in Fig. 2 clearly demonstrate that co-expression of both E1A (N-terminal region) and p300 synergistically enhances acetylation of CtBP2 associated with the C-terminal region of E1A.

**p300 and E1A Synergize to Acetylate CtBP2 in Vitro**—We then examined if the synergistic enhancement of CtBP2 acetylation by E1A and p300 can also be observed in vitro using purified proteins. His₉-tagged CtBP2 wt, 3KR-CtBP2 (Fig. 3A) and GST-E1A (243R) were purified from E. coli. FLAG-HA-tagged versions of full-length p300 or an N-terminally truncated version containing the bromo and HAT domains (p300BD(+) (Fig. 3A) were purified from insect cells. All purified proteins were found to be the major species in the preparations (Fig. 3B). When CtBP2 was incubated with p300 or p300BD(+) in the presence of different concentrations of Ac-CoA, strong acetylation of CtBP2 was observed (Fig. 3C). Acetylation of CtBP2 was more efficient with p300BD(+) (lanes 2–5) than with full-length p300 (lanes 9–12) and was inversely correlated with the concentration of Ac-CoA. In contrast, acetylation of the 3KR-CtBP2 mutant by either p300 (lane 13) or p300BD(+) (lane 6) was low. Subsequently, increasing amounts of GST-E1A (Fig. 3D, lanes 2–4) or GST (lanes 5 and 6) were included in the acetylation reaction. CtBP2 acetylation was strongly enhanced by GST-E1A, whereas the GST control had only a marginal effect. These results are consistent with the
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E1A Promotes the Formation of p300-E1A-CtBP Ternary Complex—in vitro observation that CtBP2 acetylation is enhanced by p300 and E1A synergistically (Figs. 1 and 2).

Because acetylation of CtBP2 with normal acetyl-CoA appears to be sensitive to acetyl-CoA concentrations, we performed a CtBP2 acetylation assay with p300 and 14C-labeled acetyl-CoA. As shown in Fig. 3E, CtBP2 (lane 2) was acetylated by p300, whereas the 3KR-CtBP2 mutant (lane 5) was more weakly acetylated. Addition of GST-E1A in the reaction caused enhanced CtBP2 acetylation (lane 3) but not acetylation of 3KR-CtBP2 (lane 6). Thus, p300 acetylation of CtBP2 and the enhancement by GST-E1A are specific events that can be observed under a low acetyl-CoA concentration (radiolabeled) as well as under a high acetyl-CoA concentration (non-radiolabeled, Fig. 3D). It was noted, however, CtBP2 acetylation was enhanced by GST-E1A to a lower extent when radiolabeled acetyl-CoA was used (Fig. 3E), possibly due to the limiting acetyl-CoA concentration.

E1A Causes Changes in the Composition of the CtBP2 Protein Complex—A proteomic analysis of a CtBP2 protein complex revealed the presence of several enzymatic constituents that mediate coordinated histone modifications as well as certain DNA-binding repressors (34). These constituents included euchromatic histone methyl transferases, lysine-specific demethylase 1, CoREST, HDAC1/2, the E box repressor ZEB, and other factors with less well characterized functions. Although CtBP1 has also been reported to interact with p300/CBP in separate biochemical studies (36–38), p300 was not detected in the protein complex characterized by Shi and colleagues (34). Therefore, it was of interest to determine if a CtBP2 protein complex contains p300 and other constituents of the corepressor complex and if E1A enhances recruitment of p300 into such a complex.

HeLa cells stably expressing FLAG-HA-tagged CtBP2 were transfected with GFP-E1A or mutants with N-terminal (∆N, E1A aa 2–74) or C-terminal (∆C E1A aa 178–238) deletions within E1A. Protein complexes were prepared by immunoprecipitation with the FLAG antibody, and the major components were detected by Western blot analysis. The CtBP2 complex contained several proteins (G9a, ZEB, CoREST, and HDAC2) found in the CtBP1 complex (Fig. 5, lane 2). Similar to the observation with the Ad-expressed E1A (Fig. 2A), p300 was recruited more efficiently into the CtBP2 complex in cells expressing GFP-E1A (lane 3). Consistent with enhanced recruitment of p300 by GFP-E1A, there was substantial recruitment of p300 by GFP-E1A, there was substantial

then incubated with various bacterially expressed and purified CtBP proteins. As shown in Fig. 4A, both CtBP2 and CtBP1 and various mutant derivatives were capable of interacting with p300 in the absence of E1A (first panel), albeit CtBP1-L interacted at a lower level. When the assay was performed in the presence of GST-E1A (Fig. 4A, lower panels), interaction between p300 and CtBP was enhanced for all CtBP proteins except the A58E-CtBP2 mutant (lane 4). The ability of different CtBP proteins to bind E1A was examined in the absence of p300 (Fig. 4B) by using a GST-tagged E1A (GST-E1A). All CtBP proteins, except the A58E-CtBP2 mutant (lane 4), bound to GST-E1A efficiently. This was consistent with the failure of E1A to enhance binding of A58E-CtBP2 to p300 (Fig. 4A, lane 4). These results are consistent with the interpretation that E1A promotes the formation of a ternary complex containing p300, E1A, and CtBP2.
increase in acetylation of CtBP2 when GFP-E1A was expressed (lane 3, second panel from bottom). The expression of mutant proteins ΔN (E1A aa 2–74) and ΔC (E1A aa 178–238) did not significantly enhance p300 recruitment (lanes 4 and 5), because these mutants lack the binding sites for p300 or CtBP2, respectively. In addition, the presence of ZEB in the CtBP2 complex was slightly reduced in the presence of GFP-E1A (lane 3), almost abolished in the presence of ΔN (lane 4), and slightly increased in the presence of ΔC (lane 5). The presence of G9a in the CtBP2 complex was competed strongly by ΔN (lane 4) and enhanced to different degrees by GFP-E1A (lane 3) or ΔC (lane 5). In contrast, interaction of CoREST and HDAC2 with CtBP2 was affected to a lower extent by the expression of GFP-E1A or mutants ΔN and ΔC. Both GFP-E1A and ΔN bound well to CtBP2, and the mutant ΔC bound to CtBP2 to a much lower extent (third panel from bottom). Thus, the different CtBP2 cofactors may bind to CtBP2 with different affinities. E1A C-terminal domain appears to reduce recruitment of G9a and ZEB to the CtBP2 complex and enhance recruitment of p300 in collaboration with the N-terminal domain.

E1A is normally expressed from two alternatively spliced mRNAs of 12 S and 13 S. To examine if the 13 S product functions similarly with respect to its effects on CtBP2 acetylation and the composition of the CtBP2 complex, the 13 S form of E1A was also expressed as a GFP-13 S fusion protein by plasmid transfection into the HeLa/CtBP2 cell line. As shown in Fig. 5A, similar to GFP-E1A (12 S), GFP-13 S also strongly enhanced CtBP2 acetylation and facilitated p300 recruitment to the CtBP2 complex (lane 6). In addition, GFP-13S (lane 6) consistently enhanced CtBP2 binding to G9a much more efficiently than GFP-E1A (lane 3). Thus, the E1A 12 S and 13 S products appear to have a significant difference with respect to their effects on CtBP2 binding to G9a (see “Discussion”).

E1A/CtBP2 Interaction Affects CtBP2 Binding to the Cellular E-cad Promoter—CtBP serves as a transcriptional co-repressor by binding to repressors that bind specific promoters. Because E1A was capable of interfering with CtBP2 binding to ZEB, a well characterized cellular repressor involved in repression of the E-cad promoter, it is possible that E1A could also affect CtBP2 association with the E-cad promoter-containing chromatin. To examine this possibility, ChIP was performed with the HeLa/CtBP2 cell line transfected with different GFP-E1A constructs. Normal HeLa cells were used as a control. As shown in Fig. 5B, in the HeLa/CtBP2 cell line, CtBP2 was found associated with the E-cad chromatin (lane 2), whereas the normal HeLa cells yielded a low background (lane 1). Transfection of HeLa/CtBP2 cells with GFP-E1A 12 S (lane 3) and GFP-E1A 13 S (lane 6) caused a small reduction in CtBP2 binding to the E-cad promoter (lane 3), whereas transfection with GFP-E1AΔΔ2–74 (lane 4) resulted in a more dramatic inhibition of CtBP2 binding to the E-cad promoter. Transfection with GFP-E1AΔ178–238 also reduced CtBP2 binding to the E-cad promoter (lane 5) through unknown mechanisms. Thus, E1A appears to inhibit CtBP2 binding to the E-cad promoter through the C-terminal CtBP-binding domain. Control ChIP analysis for the glyceraldehyde-3-phosphate dehydrogenase promoter using an RNA polymerase II antibody suggested that E1A and its mutants did not have significant effects on RNA polymerase II binding to the glyceraldehyde-3-phosphate dehydrogenase promoter (Fig. 5B, third panel).

Because ZEB binding to CtBP2 was only moderately competed by the wt E1A proteins (Fig. 5, A and B), an RT-PCR analysis was performed for the cellular endogenous E-cad mRNA in HeLa/CtBP2 cells transfected with various GFP-E1A
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FIGURE 5. Recruitment of p300 into the CtBP2 complex and displacement of G9a and ZEB from CtBP2 by E1A. A, HeLa/CtBP2 permanent cell line was transfected with GFP (lane 2), GFP-E1A (lane 3), GFP-E1A Δ2–74 (lane 4), GFP-E1A Δ178–238 (lane 5), or GFP-13S (lane 6) and the CtBP2 complex was immunoprecipitated with the FLAG antibody. Bound proteins were examined by Western blots as indicated. Relative levels of binding for ZEB and G9a were normalized to lane 2 and indicated on the bottom of the panels. B, ChIP analysis for CtBP2 binding to the E-cad promoter. HeLa (lane 1) or HeLa/CtBP2 cell lines (lanes 2–6) was transfected with the indicated plasmids, fixed, and processed for ChIP analysis using the FLAG antibody. The Input controls represent 0.5% of the amount used for ChIP analysis. Relative levels of the E-cad promoter PCR signals were normalized to that of lane 2. Control ChIP analysis for the glyceraldehyde-3-phosphate dehydrogenase promoter was performed with the anti-RNA polymerase II C terminus antibody (Upstate Biotechnology). C, RT-PCR analysis for endogenous E-cad mRNA. HeLa/CtBP2 cells transfected with various GFP-E1A constructs as indicated were used for mRNA isolation and RT-PCR assay. Relative levels of the E-cad RT-PCR signals were normalized to that of lane 2. Lane 1: same as lane 2 except reverse transcriptase was omitted during RT reaction.

constructs (Fig. 5C). The E1A mutant Δ2–74 strongly activated E-cad mRNA synthesis (lane 4), whereas the wt E1A 12S (lane 3), the Δ178–238 mutant (lane 5), and the E1A 13S product (lane 6) all inhibited E-cad mRNA synthesis. Similar effects were also observed with E-cad promoter-driven luciferase reporter assays (data not shown). Thus, E1A appears to inhibit the cellular endogenous E-cad promoter through the N-terminal domain and activate it through the C-terminal CtBP-binding domain.

CtBP2 Antagonizes the Activity of N-terminal Region of E1A—We then sought to determine the functional consequence of recruitment of a coactivator (i.e., p300) and a corepressor (i.e., CtBP) simultaneously by E1A. For this purpose, we exploited a Gal4-based tethering transcriptional assay developed by Sollerbrant et al. (44). In this assay, the N-terminal region of E1A linked to the Gal4 DNA-binding domain strongly activated transcription from a basal promoter containing one

Gal4-binding site. In contrast, the presence of the E1A C-terminal region in the Gal4-E1A chimeric construct inhibited the transcriptional activity mediated by the N-terminal region. The transcriptional inhibition mediated by the C-terminal region was relieved by a deletion encompassing the PLDLS motif, implicating a potential role for CtBP in antagonizing the activity of the N-terminal region. The availability of MEF cells from CtBP1/2 double knock-out embryos (MEF90 cells) (39) has provided an opportunity to directly determine the role of CtBP1 and CtBP2 on the activity of the N-terminal region of E1A.

Various CtBP constructs were transfected into MEF90 cells together with the pG5-Luc promoter-reporter and pGal4-E1AΔ91–199 which expresses a truncated E1A containing both the HAT and CtBP-recruiting domains. Expression of CtBP2 strongly repressed the trans-activation activity of Gal4-E1AΔ91–199 (Fig. 6A, lane 3). Similar levels of repression were also observed in cells expressing the CtBP2-3KR mutant (lane 4), whereas deletion of the unique N-terminal domain or a mutation (A58E) within the PLDLS-binding cleft (46) abolished the repressive activity (lanes 5 and 6). Surprisingly, both isoforms of CtBP1 (L and S) did not significantly repress the trans-activation activity of Gal4-E1AΔ91–199 (lanes 7 and 8). Western blot analysis showed that the CtBP proteins were expressed at similar levels (Fig. 6, bottom panel). These results suggest that the unique N-terminal domain of CtBP2 is a critical determinant for antagonizing the transcriptional activity of the N-terminal domain of E1A that interacts with p300 and other HATs. Interestingly, the repressive activity of the N-terminal domain may not be significantly altered by acetylation of Lys 6, 8, and 10 as the activity of the 3KR mutant was similar to that of CtBP2 wt. The apparently normal repression activity of the 3KR mutant was similar to that of MEF cells from CtBP1/2 double knock-out embryos (MEF90 cells) (39) has provided an opportunity to directly determine the role of CtBP1 and CtBP2 on the activity of the N-terminal region of E1A.

DISCUSSION

In this study, Ad E1A 243R protein was found to recruit both p300 and CtBP2 simultaneously (Figs. 1, 2, 4, and 5) and mod-
ulate the composition of the CtBP2 protein complex (Fig. 5) and the transcriptional activity of the N-terminal HAT-recruiting region of E1A (Fig. 6). CtBP1 has previously been reported to directly interact with p300/CBP at least through two different binding interfaces (36–38). Our results also suggest interaction between CtBP1/2 and p300 (Fig. 4). However, E1A 243R enhanced interaction of p300 with CtBP2 and CtBP1 significantly (Figs. 2, 4, and 5). This enhancement by E1A appears to depend on simultaneous interaction of E1A with CtBP1/2 (through the C-terminal PLDLS motif) and p300, because E1A did not enhance interaction of p300 with the A58E-CtBP2 mutant that is deficient in binding to E1A (Fig. 4). Interestingly, unlike E1A interaction with CtBPs, interaction between p300 and CtBP2 did not appear to require the PLDLS-binding cleft in CtBP2, because the A58E mutation, which abolishes PLDLS binding (46), did not affect CtBP2 interaction with p300 (Fig. 4). Simultaneous recruitment of both p300 and CtBP2 by E1A resulted in substantial increase in acetylation of CtBP2 (Figs. 2, 3, and 5), suggesting that p300 recruited by E1A retains the acetyl transferase activity. Previous results in this regard have been conflicting (18, 47). Consistent with our observation that E1A-associated p300 acetylates CtBP2, it has been reported that pRB associated with E1A was also acetylated by p300 (48). The retention of the HAT activity by E1A-associated p300 might be important for E1A-mediated chromatin remodeling during the cell cycle progression (19) (see below).

We have observed that CtBP2 recruited to the E1A C-terminal region efficiently antagonized a transcriptional activation function encoded by the HAT-recruiting N-terminal region of E1A, whereas both isoforms of CtBP1 (L and S) were less effective (Fig. 6). It has been generally assumed that CtBP1 and CtBP2 are functionally similar with regard to transcriptional repression due to their high degree of sequence homology. Indeed, the E-cadherin promoter was suppressed by CtBP1 and CtBP2 to comparable levels (40). However, our reporter-based transcriptional assay in CtBP-null MEF cells has revealed a clear difference between these two highly related homologs. Although the reporter system used here is somewhat artificial, it has facilitated the detection of a functional difference between CtBP1 and CtBP2 for the first time. The differential activity CtBP2 (compared with CtBP1) appears to be determined by its unique N-terminal sequence, which allows CtBP2 to form higher order oligomers that can be promoted by E1A (data not shown). We have observed that E1A enhanced oligomerization of CtBP2 (data not shown). The possibility that CtBP2 oligomerization may affect the assembly of the CtBP2 super complex and its interaction with gene promoters remains to be investigated. Because CtBP proteins can form dimers, it is possible that the HAT–E1A–CtBP complex is formed on one CtBP subunit and recruited to promoters by the PLDLS-containing transcription factors/respressors through the second CtBP subunit. This model would be consistent with the notion that E1A and associated HATs are targeted to the promoter region of some cell cycle regulatory genes. The data based on ChIP analysis (19) and studies using the E1A/c-Myc chimeric gene constructs (11, 12) support this notion.

We have observed major differences in the subunit composition of the CtBP2 protein complex in the presence of E1A (Fig. 5). The E1A C-terminal domain was found to displace G9a and ZEB from the CtBP2 complex and affect CoREST and HDAC2 binding to CtBP2 to a lower extent (Fig. 5A). Because ZEB (49, 50) and E1A interact with CtBP through PLDLS-related motifs, it is possible that E1A might competitively displace ZEB. However, the mechanism by which G9a is displaced remains to be investigated. Recent results suggest that G9a binding to CtBP is mediated by a zinc-finger protein Wiz, which has PLDLS-like motifs (51). The activity of G9a is associated with transcriptional repression due to histone H3K9 methylation (52–54). Because E1A expression has been reported to induce loss of histone H3K9 methylation during G0 to G1 progression of the cell cycle (19), it is possible that E1A-induced changes in CtBP2/G9a interaction may modulate cellular histone methylation patterns.

The endogenous E-cad promoter appears to be regulated by the N-terminal and the C-terminal domains of E1A in opposite fashions. The Δ178–238 mutant of E1A does not compete with ZEB for CtBP binding (Fig. 5A); however, it potently inhibited the E-cad mRNA synthesis (Fig. 5C). In contrast, the Δ2–74 mutant of E1A strongly competed with ZEB for CtBP binding (Fig. 5A) and strongly activated E-cad mRNA synthesis (Fig. 5C). Therefore, the effects of the E1A 12 S and 13 S products on the E-cad mRNA synthesis (Fig. 5C) appear to be the sum of
these two competitive activities of the N-terminal and C-terminal domains of E1A.

Although the activity of the N-terminal HAT-recruiting region of E1A is essential for E1A-induced cell proliferation and for the transforming activities (5), the precise mechanism is not well understood. Similarly, the role of the CR4 region in cell proliferation, which is required for immortalization and cooperative transformation with E1B, has not been sufficiently addressed. It has been believed that interaction of E1A with p300 may relieve a growth suppressive function of p300 (55). However, ChIP studies are consistent with a model that E1A induces chromatin remodeling around critical E2F target genes, which are transcriptionally repressed by p130 in quiescent cells (19). These studies have revealed that E2F-responsive genes such as cdc6 and Cyclin A were repressed predominantly by the occupancy of p130 at promoter regions and methylation of histone H3K9. Previous studies have suggested at least part of the repressive activity of p130 might be attributed to the recruitment of CtBP by p130 via CtIP (56). The ChIP studies have revealed that E1A activated recruitment of CtBP by p130 via CtIP (56). The ChIP studies of histone H3K9. Previous studies have suggested at least part of these two competitive activities of the N-terminal and C-terminal domains of E1A.

In summary, our results have demonstrated that both adenovirus E1A 243R and 289R proteins enhance recruitment of p300 into the CtBP2 complex. The E1A-associated p300 retains the acetyl transferase activity and causes substantial augmentation of acetylation of CtBP2. The E1A 243R protein also caused changes in the subunit composition of the CtBP2 corepressor complex by displacing H3K9 methyl transferase G9a and E-box repressor ZEB through the C-terminal domain. Recruitment of the CtBP2 corepressor complex to E1A antagonized a transcriptional activation function encoded by the HAT-recruiting N-terminal region of E1A. This transcriptional antagonism may provide an explanation for the transformation restraining activity of E1A C-terminal region. Our results suggest new insights into how the N- and C-terminal regions of E1A might regulate cell proliferation.

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