The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex

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Abstract Binding of the C-terminal binding protein, CtBP, to the adenovirus E1A moiety of a Gal4-E1A fusion protein abolishes conserved region (CR) 1-dependent transcription activation. In contrast, a non-promoter targeted E1A peptide, capable of binding CtBP, can induce transcription from the proliferating cell nuclear antigen (PCNA) promoter. CtBP is shown here to bind the histone deacetylase HDAC1, suggesting that a promoter targeted CtBP-HDAC1 complex can silence transcription from the PCNA promoter through a deacetylation mechanism. Expression of the CtBP binding domain of E1A is sufficient to alleviate repression, possibly due to the displacement of the CtBP-HDAC1 complex from the promoter.

Key words: Adenovirus E1A; Transcription; C-terminal binding protein; Proliferating cell nuclear antigen; Histone deacetylase

1. Introduction

The major adenovirus E1A proteins (E1A-243R and E1A-289R) [1] can redirect cellular processes by interacting with key cellular regulatory proteins in order to achieve optimal conditions for virus growth. Three conserved regions (CR) have been identified and two of these, CR1 and CR2, are present in both E1A-243R and E1A-289R, whereas the third region, CR3, is unique to E1A-289R.

The E1A-289R protein is required for efficient expression of all adenovirus early genes and activates transcription through a CR3-mediated interaction with both basal and upstream binding transcription factors, and by inducing phosphorylation of specific transcription factors (reviewed in [2]).

Transcription regulation by E1A-243R protein has strong implications for the capacity of E1A to induce cellular transformation. The activities of E1A-243R engage the CR1, CR2 and the amino-terminus in physical interactions with proteins controlling cell-cycle progression (reviewed in [3]). Targeting of cellular regulatory complexes by E1A can result in their dissociation, as exemplified by the E1A induced release of the transcription factor E2F from inhibitory complexes with members of the family of retinoblastoma proteins (reviewed in [4]), leading to induction of S-phase and DNA synthesis. Alternatively, E1A binding may directly interfere with the activity of the targeted protein, as in the case of E1A binding and inhibition of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, thereby restoring the activity of the cyclin E-cdk2 kinase complex [5].

E1A-243R can also regulate transcription by directly interacting with specific transcription factors or the transcription co-activator proteins p300 and CREB binding protein (CBP). Binding of E1A has been shown to prevent the co-activator function of p300/CBP most likely by disrupting a complex between p300/CBP and the histone acetylase P/CAF [6], thereby interfering with a proposed chromatin remodelling activity of p300/CBP-P/CAF. Direct targeting of transcription factors and disruption of inhibitory complexes are also a common theme in transcription regulation by E1A-243R. Activation of the c-fos promoter by E1A involves the relief of YY1 mediated repression through a disruption of the ATF/CREB-YY1 complex [7] and E1A activation of the hsp70 promoter has been suggested to occur through the dissociation of the transcription inhibitor Dr1 from TBP [8].

Although transcription regulatory activities have also been demonstrated for the second exon of E1A, little is known about their mechanisms. Redundant sequences encoded by the second exon have been shown to activate viral gene expression independently of the first exon [9]. Specifically, the auxiliary regions (AR) 1 and 2 individually enhance CR3 dependent activation of the E4F transcription factor [10]. Furthermore, AR2 is also specifically required for the restricted metastatic properties of co-transformed cells, possibly through its ability to repress metalloprotease gene expression [11]. The second exon of E1A is dispensable for co-transformation with activated *ras* [12] and has in fact a general ability to inhibit in vitro transformation, tumorigenesis and metastasis. These events correlate with a specific interaction between the carboxy-terminus of E1A and the cellular C-terminal binding protein (CtBP) [13,14].

We have previously shown that a minimal region of E1A, encompassing mainly CR1, constitutes a potent transcription activator when expressed as a Gal4 fusion protein [15]. In contrast, a larger Gal4E1A fusion protein, expressing also the CtBP binding site, was inactive as a transactivator protein, suggesting that recruitment of CtBP to a promoter results in silencing of CR1 dependent transactivation [16]. Here we show that the second exon of E1A alone was able to activate transcription of the proliferating cell nuclear antigen (PCNA) promoter. Since we also demonstrate that CtBP can interact with a histone deacetylase (HDAC1), we propose that the second exon of E1A can activate transcription by disrupting a promoter bound complex containing CtBP and HDAC1, thereby possibly preventing negative chromatin remodeling.

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2. Materials and methods

2.1. Plasmids

PCNACAT [17] contains a region spanning from −87 to +60 from the PCNA promoter driving the CAT gene. PEREx3Luc contains a trimer of the E1A responsive PCNA promoter element (−59 to −45) upstream of the basal PCNA promoter (−45 to +60). PCNAΔPERELuc is a derivative of PEREx3Luc lacking the PERE repeats. Plasmids encoding the E1A-243R protein and its derivatives have been described (pML00512S, pML00512SΔCR1, pML00512SΔ225–238 and pML00512SΔCR1,Δ225–238) [16,18]. E1A-243R denotes the full-length protein, 243RΔCR1 lacks amino acids 38–65, 243RΔCB lacks amino acids 225–238 and 243RΔCR1,CB lacks amino acids 38–65 and 225–238. Exon2 denotes an E1A protein expressed from the second exon of E1A only (dl1119, [9]) and exon2ΔCB a variant lacking amino acids 225–238 (dl1135, [14]). Glutathione *S*-transferase (GST) fusion protein GSTctE1A [16] expresses amino acids 200–243 of E1A-243R and GSTCtBP [13] expresses the full-length CtBP protein. pcDNA$_3$-CtBP expresses the full-length CtBP protein [13].

HDAC1 encoding plasmids for in vitro translation (pING14A-HDAC-1) and haemagglutinin-tagged for in vivo expression (pCMV5′3THDAC-1) were kindly provided by Dr. T. Kouzarides.

2.2. Cell culture conditions and transfection and reporter gene analysis

The human osteosarcoma cell line U2OS was maintained in DMEM supplemented with 10% NCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Transfections were done by the calcium phosphate co-precipitation technique essentially as described in [19], or by using the Lipofectamine transfection system from Gibco as described by the manufacturers. The amounts of transfected plasmids are indicated in the figure legends. Cells were harvested at approximately 48 h post-transfection.

Chloramphenicol acetyltransferase assays were performed essentially as described previously [20]. The cell extracts were prepared by freeze-thawing three times in 0.25 M Tris-HCl, pH 7.5, and the results were quantitated using the ImageQuant computer program on a Phosphorimager (Molecular Dynamics). Luciferase assays were performed by using the Luciferase Assay System from Promega according to the manufacturers' instructions. The results were quantitated using a Luminometer (Labsystem).

2.3. In vitro binding analyses

The commercially available TNT T7 or SP6 coupled wheat germ or coupled reticulocyte system from Promega were used according to the manufacturers' instructions. DNA templates for E1A-243R and E1A-234RΔCB were synthesized by PCR using an N-terminal primer harboring a T7 promoter and a sequence complementary to the translation initiation region of E1A. The DNA templates were purified by using the PCR Purification Kit from Boehringer Mannheim Biochemicals according to the manufacturers' instructions. Full-length CtBP and HDAC1 were synthesized directly from plasmid pcDNA3-CtBP [13] and pING14AHDAC-1, respectively.

U2OS cells were labeled with 600 μCi [^{32}P]orthophosphate/60-mm Petri dish for 4 h, washed with PBS and disrupted in WCE buffer (25 mM HEPES pH 7.6, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin and 0.4 mM pefablock) rotating at 4°C for 30 min. Cell debris was removed by centrifugation. Cell lysate was diluted four times in WCEΔ buffer (25 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) supplemented with protease inhibitors as above, and precleared with glutathione agarose beads (Pharmacia).

GST fusion proteins were produced in *E. coli* and bound to glutathione Sepharose beads (Amersham Pharmacia Biotech) essentially as described in Current Protocols in Molecular Biology. Protein concentration was estimated on a Coomassie stained SDS-polyacrylamide gel. Approximately equal amounts of GST fusion proteins were mixed with [^{32}P]orthophosphate labeled crude protein extract or ^{35}S-labeled in vitro translated proteins in binding buffer (final concentration: 25 mM HEPES pH 7.6, 75 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.025% Triton X-100) and incubated rotating at 4°C for 3 h. Beads were washed four times in HEPES binding buffer (20 mM HEPES pH 7.6, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100 supplemented with a cocktail of protease inhibitor from Boehringer Mannheim Biochemicals) and bound proteins were separated on a 12% SDS-polyacrylamide gel and visualized by autoradiography.

2.4. Immunoprecipitations and Western blots

U2OS cells were labeled with 200 μCi [^{35}S]methionine/60-mm Petri dish for 4 h, washed with PBS and disrupted in lysis buffer (0.1% NP-40, 250 mM NaCl, 50 mM HEPES pH 7.0, supplemented with a cocktail of protease inhibitors from Boehringer Mannheim Biochemicals), on ice for 20 min. Cell debris was removed by centrifugation. The lysate was precleared with protein A Sepharose CL-4B conjugate (Amersham Pharmacia Biotech) followed by 1 h incubation with 1.0 μg of the anti-haemagglutinin (HA) antibody SCP-12CA5-1 (Berkeley Antibody Company) or with CtBP antiserum (kindly provided by Dr. Chinnadurai). Protein A Sepharose was added and incubation was continued rotating at 4°C for 1 h followed by centrifugation to collect immunoprecipitates. Precipitates were washed in lysis buffer four times and bound proteins were separated on a 12% SDS-polyacrylamide gel and visualized by autoradiography.

For Western blot the immunoprecipitated proteins were transferred from the 12% SDS-polyacrylamide to nitrocellulose filters. The filters were blocked in 5% non-fat dry milk in TBS-T (20 mM Tris pH 7.5, 140 mM NaCl, 0.25% Tween 20) for 1 h at room temperature and incubated overnight at 4°C with anti-haemagglutinin (HA) antibody, diluted 1:750. Hybridized proteins were detected using enhanced chemiluminescence as described by the manufacturers (Amersham).

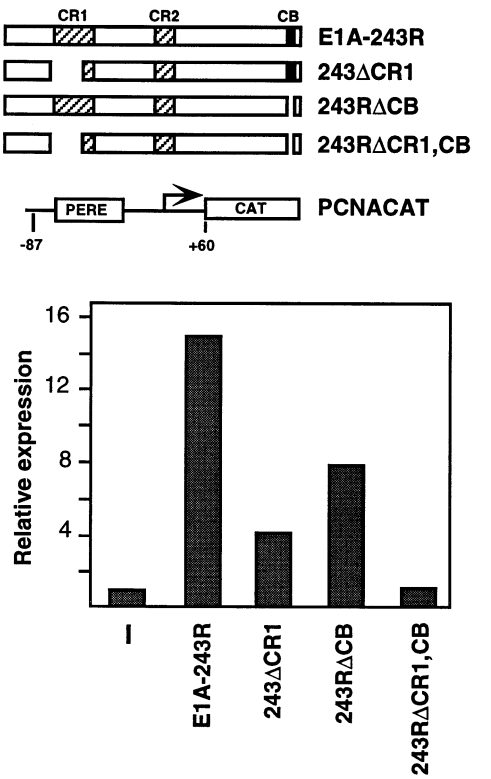

Fig. 1. Requirements for CR1 and the CtBP binding region in transactivation of the PCNA promoter. U2OS cells were cotransfected with 8 μg of PCNACAT and 8 μg the indicated E1A constructs. At 48 h post-transfection, cell extracts were prepared and analyzed for CAT activity. E1A-243R denotes the full-length E1A protein. 243RΔCB, 243RΔCR1 and 243RΔCR1,CB indicate derivatives of E1A-243R harboring a deletion of aa 225–238, 38–65 or both, respectively. Schematic representation of the E1A constructs and PCNACAT are shown at the top. CB; CtBP binding region. The relative expression from PCNACAT in the presence of vector alone (−) was set at 1. The result shown is a representative from at least three individual experiments.

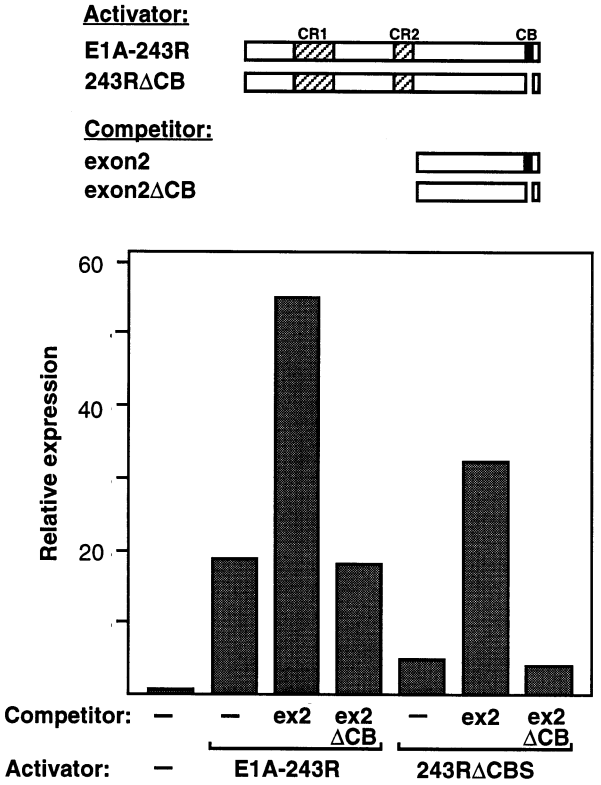

Fig. 2. Co-expression of a E1A second exon encoded protein with binding site for the CtBP restored maximal activation of the PCNA promoter. U2OS cells were co-transfected with 8 μg of plasmids encoding either E1A-243R or 243RΔCB (Activator) and 4 μg of plasmids encoding a protein from the second exon either with (exon2) or without the CtBP binding region (exon2ΔCB) (Competitor). Transcription activation was measured on the PCNACAT reporter (6.5 μg). The relative expression from PCNACAT in the presence of vector alone (−) was set at 1. The result shown is a representative from at least three individual experiments.

3. Results

3.1. E1A-243R transactivation of the PCNA promoter requires the CtBP binding region of E1A

We have previously shown that transactivation by a Gal4 fusion protein expressing the conserved region 1 (CR1) of the adenovirus type 2 E1A protein is efficiently repressed by the presence of the CtBP binding region (CB, amino acids 225–238 of E1A-243R) [16]. Since these experiments were performed using artificial DNA binding of the CR1 activator to a synthetic promoter, we continued to investigate the effect of the CtBP binding region on a previously characterized E1A-243R-responsive promoter. In this report we present data using the proliferating cell nuclear antigen (PCNA) promoter, but similar results were also obtained using the adenovirus major late promoter (Sundqvist et al., unpublished).

To study the effect of CtBP on E1A-243R mediated transactivation, a CAT reporter under the transcriptional control of the proximal PCNA promoter element (−87 to +60) (Fig. 1), was tested in a co-transfection assay in U2OS cells. As expected from previous reports [21], E1A-243R activation of PCNACAT was significantly reduced by deletion of CR1 (243RΔCR1). Interestingly, a mutant lacking the CtBP binding region (243RΔCB) also displayed reduced ability to induce transcription and the double mutant 243RΔCR1,CB, lacking both CR1 and the CtBP binding region, was severely impaired in its capacity to induce transcription from the PCNA promoter. From these results we conclude that efficient E1A-243R mediated transactivation of the PCNA promoter requires both CR1 and the CtBP binding region.

Western blot analyses confirmed that the observed effects were not due to poor expression of any of the E1A constructs (data not shown).

3.2. The defective transactivation capacity of the E1A-243R mutant lacking the CtBP binding domain is restored by co-expression of the second exon of E1A

The requirement of the CtBP binding region for E1A-243R-mediated activation of the PCNA promoter can be explained by either of two different models; CtBP (or possibly another protein binding to the same region of E1A) is an essential co-factor for E1A-243R mediated transactivation, or alternatively, CtBP might act as a repressor of transcription, which is sequestered and inactivated through its interaction with E1A.

To discriminate between these two models, experiments were performed in which E1A-243R derivatives expressing only the second exon of E1A were used as competitors for CtBP binding (Fig. 2). U2OS cells were transfected with E1A-243R or 243RΔCB in the presence of indicated competitor and the level of induction of the PCNA promoter was deter-

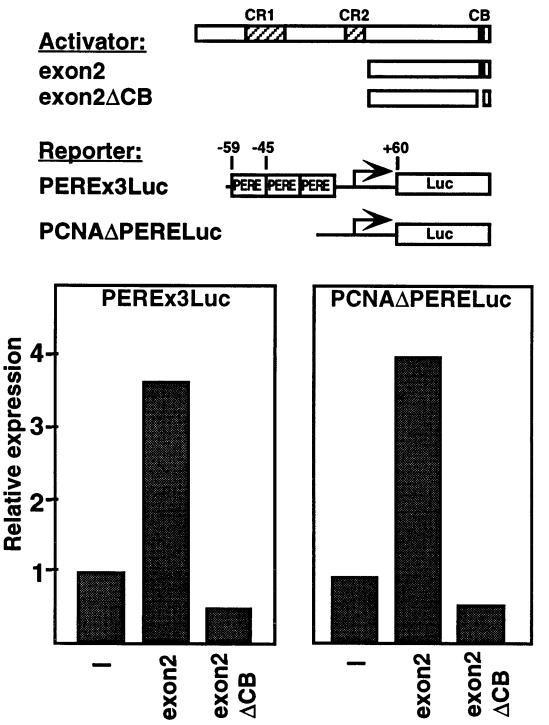

Fig. 3. The second exon encoded protein activates transcription independent of the first exon encoded region and the CR1 responsive PERE. Activation of PEREx3Luc and PCNAΔPERELuc were determined after cotransfection of U2OS cells with 2 μg reporter with 1 μg of plasmids encoding exon2 or exon2ΔCB. The relative expression from each reporter in the presence of vector alone (−) was set at 1. The result shown is a representative from at least three individual experiments.

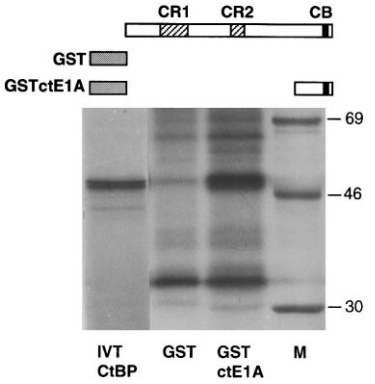

Fig. 4. Interaction between a 48 kDa cellular phosphoprotein and a GST fusion protein expressing the last 44 amino acids of E1A. [^{32}P]Orthophosphate-labeled whole cell extract from U2OS cells was bound to the indicated GST fusion proteins. Following electrophoretic separation bound proteins were visualized by autoradiography. A 48 kDa protein was specifically pulled down by GSTctE1A. Schematic representation of the GST fusion proteins are shown at the top. IVT CtBP; control lane showing the migration of in vitro translated [^{35}S]methionine-labeled CtBP. M, protein size marker.

mined. The competitor devoid of the CtBP binding region, exon2ΔCB, had no effect on PCNACAT expression, induced by either E1A-243R or 243RΔCB (Fig. 2). In contrast, the intact exon2 competitor, which encoded a functional CtBP binding site, efficiently restored the reduced transactivation capacity of 243RΔCB (Fig. 2). Moreover, CAT activity in cells expressing E1A-243R was also increased further in the presence of exon2.

These results supported the second model which suggests that CtBP acts as a repressor of PCNA promoter directed transcription. Sequestering of CtBP, by binding to an excess of the E1A second exon, alleviates the repression.

3.3. The second exon of E1A-243R is sufficient to induce transcription from the PCNA promoter

The second exon of E1A alone has previously been postulated to use redundant mechanisms to activate transcription of several viral genes [9]. The ability of the second exon alone, and particularly the CtBP binding region, to transactivate the cellular PCNA promoter was therefore tested. As seen in Fig. 3, co-transfection of the PEREx3Luc reporter with exon2 resulted in an approximately 3.5-fold increase in expression. In contrast, exon2ΔCB, lacking the CtBP binding site, failed to induce PEREx3Luc expression (Fig. 3). The level of protein expression from the second exon constructs were determined by Western blot analysis and found to be identical (data not shown).

Induction of the PCNA promoter by E1A-243R has previously been shown to require the PERE (PCNA-E1A Responsive Element) and to act via the CREB-CBP pathway [22]. To analyze whether second exon activation required the same target sequence, the PERE element was deleted from PEREx3Luc creating PCNAΔPERELuc, which contains only the PCNA basal promoter element (−47 to +60). As expected [21,23], PEREx3Luc, but not PCNAΔPERELuc, served as a target for CR1 dependent transactivation (data not shown). In contrast, co-expression of the E1A second exon constructs showed that both PEREx3Luc and PCNAΔPERELuc were activated by exon2, but not by exon2ΔCB (Fig. 3).

Taken together with the results presented in Fig. 2, the data suggests that the CtBP binding region constitutes a novel transactivating domain within E1A-243R and that this domain activates transcription of the PCNA promoter in the absence of first exon sequences and independent of the PERE. Based on the specific behavior of mutants lacking the CtBP binding region, we propose that the protein repressing the PCNA promoter directed transcription is indeed CtBP.

3.4. CtBP expressed in U2OS cells binds to the C-terminus of E1A-243R

Human CtBP is an abundant 48-kDa phosphoprotein [14]. To verify that CtBP was also present in U2OS cells an in vitro binding experiment was performed. In a GST pull-down experiment GSTctE1A, expressing the last 44 amino acids of E1A, was challenged with [^{32}P]orthophosphate labeled U2OS whole cell extract (Fig. 4). An interaction with a 48-kDa protein was seen with GSTctE1A but not with GST alone. The identification of the 48-kDa protein as CtBP was corroborated by the similar migration pattern of in vitro translated radiolabeled CtBP (Fig. 4). Furthermore, since CtBP previously has been shown to be the only phosphoprotein in the 48-kDa size range interacting with the C-terminus of E1A [14], this experiment indicated that CtBP capable of binding E1A was present in U2OS cells.

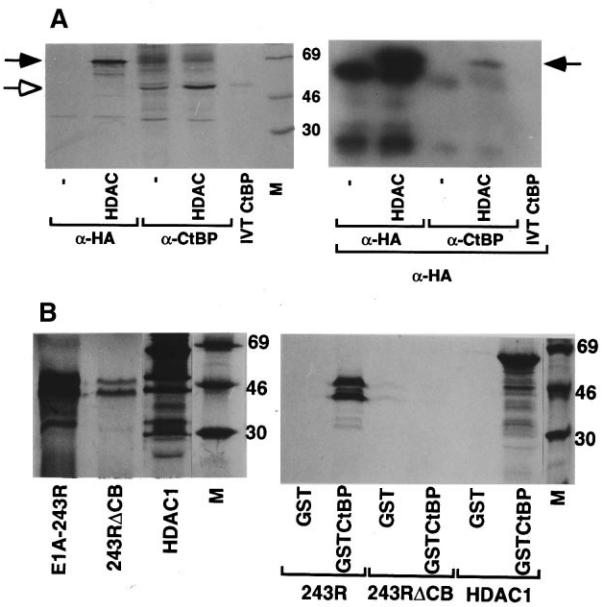

Fig. 5. Interactions between CtBP and HDAC1 or E1A. A: GST pull down experiment. Binding of in vitro translated E1A-243R, 243RΔCB, or HDAC1 to GST and GSTCtBP, respectively. The in vitro translated proteins are shown in the left panel. Equal amounts of the in vitro translated proteins were incubated with indicated GST proteins. B: Left panel: [^{35}S]Methionine labeled extracts from U2OS cells untransfected (−) or transfected with a plasmid encoding hemagglutinin tagged HDAC1 (HDAC1) were immunoprecipitated with the α-HA antibody (SCP-12CA5-1) or polyclonal antiserum raised against CtBP. Right panel: The same immunoprecipitates were subjected to Western blotting and hybridized to the α-HA antibody. For both panels: IVT CTBP; control lane showing the migration of in vitro translated [^{35}S]methionine-labeled CtBP. Filled arrows points to HA-HDAC1 and the open arrow to CtBP.

3.5. CtBP interacts with the histone deacetylase HDAC1 both in vitro and in vivo

In the Gal4-E1A experiments, the ability to recruit CtBP to a promoter obscures CR1 transactivation [16]. Since histone deacetylation appears to be an important mechanism controlling gene activity, we decided to investigate whether the repressive effect of CtBP involved recruitment of a histone deacetylase.

Using a pull-down experiment, we could demonstrate that GSTCtBP interacted with in vitro translated HDAC1 as well as full length E1A-243R (Fig. 5A). No binding was seen using in vitro translated E1A-243RΔCB (Fig. 5A). To investigate whether CtBP also interacted with HDAC1 in vivo, U2OS cells were transfected with a plasmid expressing haemagglutinin (HA) tagged HDAC1. Immunoprecipitation with α-HA mainly precipitated the HDAC1 protein (Fig. 5B). In contrast, immunoprecipitation with antiserum against CtBP co-precipitated proteins corresponding in size to both CtBP and HDAC1 (Fig. 5B). To verify the presence of the HA tagged HDAC1 in the α-CtBP precipitates, the protein gel was subjected to Western blot analysis using α-HA. As can be seen in the left panel of Fig. 5B, hybridization with α-HA detects HDAC1 both in α-HA- and α-CtBP-precipitates from HA-HDAC1 transfected cells. In summary, these results demonstrate that CtBP and HDAC1 can form complexes both in vitro and in vivo.

4. Discussion

The ability of E1A-243R to induce transcription has been ascribed mainly to regions encoded by the first exon of the E1A gene. Here we show that efficient induction of the PCNA promoter, in addition to first exon sequences, required the presence of the CtBP binding region of the E1A second exon. The CtBP binding region expressed as a separate second exon encoded protein was able to restore transactivation by the E1A-243RΔCB deletion mutant, itself unable to bind CtBP (Fig. 2), and furthermore able to activate transcription in the absence of additional E1A proteins (Fig. 3). These results suggests that E1A binding to CtBP alleviates a negative function of CtBP on PCNA promoter activity. We further postulate that CtBP in the absence of E1A acts as a promoter associated transcription repressor. In agreement with this, we have previously shown that the Gal4CR1 transactivator [15] is inactivated by the covalent fusion of the CtBP binding domain [16]. This repression was also relieved by co-expression of a CtBP binding competent derivative of E1A [16]. By competing for CtBP binding, excess of E1A could therefore disrupt potential repressor complexes involving CtBP and cellular transcription factors. In the case of the E1A-243R induction of the PCNA promoter, excess of the second exon encoded protein surprisingly further increased the level of transactivation. This may indicate that insufficient amounts of wild-type E1A-243R protein are expressed after transfection to sequester all CtBP.

The observation that CtBP associates with HDAC1 both in vitro and in vivo suggests a possible mechanism by which CtBP acts as a transcription repressor. Transcriptionally active genes correlate with hyperacetylated histones and several transcription factors recruit cofactors with histone acetyltransferase (HAT) activity [24]. Regulated gene expression involves the opposing activities of HATs and at least three different histone deacetylases (HDAC1–3) [25–27]. The HDACs are members of large co-repressor complexes [28] which can be recruited to different transcription factors [29,30]. In line with what is known about co-repressor complexes we propose that the CtBP-HDAC1 interaction constitutes a member of this family of complexes.

The recently identified *Drosophila* CtBP (dCtBP) was shown to interact with the *Drosophila* transcription repressors, Knirps and Snail [31]. Interestingly, the E1A motif P-DLS-K, which interacts with CtBP, is also present in Knirps and Snail and furthermore essential for the interaction with dCtBP [31]. These results support the model where E1A activates transcription by sequestering of the inhibitory CtBP-HDAC1 complex, thereby alleviating its repressive effect on transcription. Cloning of a cellular protein (CtIP) that binds to the mammalian CtBP through the same motif was recently described [32]. Since this interaction is disrupted by the second exon of E1A, the CtBP-CtIP complex is suggested to relate to the tumorigenic potential of transformed cells [32].

The E1A-243R responsive element in the PCNA promoter has been mapped to a sequence around −45 to −59 called the PCNA E1A-responsive element (PERE) [17]. PERE can bind heterodimers between ATF-1 and CREB and it has been suggested that E1A-243R induces transcription of the PCNA promoter through the CREB-CBP pathway [22]. Activation mediated by the second exon of E1A was shown here to be independent of PERE. Our results therefore suggest that dual mechanisms are involved in E1A-243R transactivation of the PCNA promoter. In addition to the E1A exon 1 transactivation through PERE, the CtBP binding domain in the second exon activates through the basal PCNA promoter. The basal PCNA promoter element conferring response to the second exon harbors an initiator site that binds YY1 [33]. YY1 has been suggested to negatively regulate transcription by tethering HDAC2 to the promoter [26]. It will therefore be of interest to determine whether recruitment of the CtBP-HDAC1 complex also involves YY1.

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