A Mechanism of COOH–Terminal Binding Protein–Mediated Repression

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Abstract
The E2F4 and E2F5 proteins specifically associate with the Rb-related p130 protein in quiescent cells to repress transcription of various genes encoding proteins important for cell growth. A series of reports has provided evidence that Rb-mediated repression involves both histone deacetylase (HDAC)–dependent and HDAC-independent events. Our previous results suggest that one such mechanism for Rb-mediated repression, independent of recruitment of HDAC, involves the recruitment of the COOH-terminal binding protein (CtBP) corepressor, a protein now recognized to play a widespread role in transcriptional repression. We now find that CtBP can interact with the histone acetyltransferase, cyclic AMP–response element–binding protein (CREB) binding protein, and inhibit its ability to acetylate histone. This inhibition is dependent on a NH2-terminal region of CtBP that is also required for transcription repression. These results thus suggest two complementary mechanisms for E2F/p130-mediated repression that have in common the control of histone acetylation at target promoters. (Mol Cancer Res 2005;3(10):575–83)

Introduction
E2F transcription activity involves a family of DNA-binding activities that function in both transcriptional activation and transcriptional repression (1, 2). The E2F1-3a proteins seem to function as transcriptional activators, whereas E2F4 and E2F5, in conjunction with Rb family proteins, and E2F6 independently of Rb, seem to act as transcriptional repressors (3, 4). Initial studies of Weintraub et al. showed that E2F/Rb-mediated repression was a dominant event capable of shutting off an otherwise active promoter (5, 6). A series of reports has provided evidence that one mechanism for this Rb-mediated repression involves the recruitment of histone deacetylase (HDAC) to E2F site–containing promoters, presumably resulting in an alteration of chromatin conformation that hinders transcription (7-9).

Several observations suggest that additional events, independent of HDAC recruitment, may contribute to the repression. For example, many genes subject to E2F/Rb-mediated repression are not derepressed by treatment with the HDAC inhibitor trichostatin A (7). Moreover, Rb mutants that can no longer interact with HDAC are still capable of repressing transcription (10, 11). Based on these observations, it would seem that HDAC-independent mechanisms of transcriptional repression contribute to the Rb control of transcription. Indeed, several Rb-interacting proteins, such as the chromatin remodeling complex proteins BRG1 and human BRM, the methyltransferase protein SUV39H1, and RBP1, have been shown to mediate transcriptional repression in a HDAC-independent fashion (12-16).

Employing a yeast two-hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we identified a protein known as COOH-terminal binding protein (CtBP)–interacting protein (CtIP) that interacts with the p130 pocket domain (17). Similarly, others have shown that the CtIP/Rim protein interacts with Rb in vivo (18). CtIP functions as a transcriptional co-repressor in part by recruiting CtBP, a protein first identified as an adenovirus E1A-interacting protein (19, 20). Other work has shown that CtBP functions as a short-range corepressor in Drosophila (21-24), and numerous experiments have shown a role for CtBP as a transcriptional repressor in mammalian cells (17, 25-30). Although the exact mechanism of transcriptional repression remains unclear, several reports have shown that CtBP has both HDAC-dependent and HDAC-independent mechanisms of repression (27, 29, 31-34). We now describe experiments that show that CtBP interacts with the cyclic AMP–response element–binding protein (CREB) binding protein (CBP) transcriptional coactivator and inhibits its ability to acetylate histones dependent on CBP sequences that are critical for transcription repression activity. It thus seems that E2F/Rb-mediated repression may involve multiple complementary events that cumulatively lead to a loss of histone acetylation at target promoters.

Results
Control of Transcription by CtBP
Recent work has shown that the CtBP protein, from either mammals or Drosophila, plays a role as a transcription

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corepressor through physical interactions with a variety of promoter-specific transcription factors (17, 21, 23-30). Indeed, expression of a Gal4 binding domain (Gal4BD)-CtBP fusion protein resulted in a substantial repression of the major late promoter (MLP) when assayed in C33A cells. Interestingly however, no repression was observed when the same assay was carried out in 293 cells, possibly due to the previously observed ability of CtBP to also function as a transcriptional activator in certain cell lines (Fig. 1A; ref. 35). Because CtBP was originally identified as a protein that bound to the COOH-terminus of E1A (20) and 293 cells express high levels of E1A, it is possible that E1A might also disrupt the ability of CtBP to mediate repression. This is interesting in light of our previous findings that CtBP plays a role in mediating transcriptional repression through the Rb/p130 pathway that is well known to be disrupted by E1A (17, 36).

To directly test this possibility, we again did the Gal4BD-CtBP repression assay in C33A cells, this time adding E1A. Whereas the addition of E1A alone repressed the MLP 2-fold relative to cells expressing no E1A, expression of E1A together with Gal4BD-CtBP prevented any significant CtBP-mediated repression of the MLP (Fig. 1B). It would thus seem that E1A is able to disrupt the ability of CtBP to repress transcription in C33A cells.

**NH2-Terminal Sequences of CtBP Are Required for Repression**

Previous work has shown that the interaction of E1A with CtBP is dependent on a PLDLS sequence in the COOH-terminal region of E1A (20, 37). In light of the finding that E1A can disrupt CtBP-mediated repression, it seemed possible that the domain of CtBP through which it interacts with PLDLS motif-containing proteins might be important in mediating repression. Previous work has identified a centrally located dimerization domain within the 440-amino acid CtBP1 protein, and more recent data suggest that the dehydrogenase domain (28-353) is involved in the interaction with PLDLS proteins (29, 38). Given the possible role of the PLDLS-interacting region of CtBP in mediating repression, we generated a series of NH2-terminal deletions to map a region involved in the PLDLS interaction and to examine if these CtBP sequences are important for transcriptional repression. Examination of the amino acid conservation of CtBP found in humans, *Drosophila*, and *Xenopus* reveals that the highest degree of identity begins at amino acid 27. Five mutants were therefore created lacking the first 10, 30, 40, 70, or 120 amino acids of the CtBP protein (Fig. 2A). These mutants were tested in a yeast two-hybrid assay for their ability to interact with a PLDLS motif-containing protein, in this case the CtIP protein. Whereas wild-type CtBP and mutants lacking the first 10 or 30 amino acids retained the ability to interact with CtIP, mutants lacking the first 40, 70, or 120 amino acids all failed to interact with CtIP (Fig. 2B). As a control, we find that all of the mutants can dimerize with wild-type CtBP and are therefore functionally expressed in yeast.

We next assayed the ability of these CtBP mutants to repress transcription of the SV40 promoter in C33A cells. Wild-type CtBP and the CtBP mutants were created as Gal4 DNA-binding domain (DBD) fusion proteins and transfected into C33A cells along with the SV40 reporter-containing upstream Gal4 DBD sites. Whereas the SV40 promoter was dramatically repressed by the wild-type CtBP as well as the mutants with the first 10 or 30 amino acids deleted, mutants containing deletions of the first 40, 70, or 120 amino acids were significantly impaired in their ability to repress the SV40 promoter (Fig. 2C). As the SV40 promoter has been shown previously to be insensitive to repressors, such as MAD, which repress transcription via a HDAC-independent mechanism, CtBP likely represses, at least in part, through a HDAC-independent mechanism (7). Similar repression results were also obtained using a variety of other active promoters, including the MLP, the 14D promoter, the herpes virus thymidine kinase promoter, and the basal promoter pG5Luc (Fig. 2D). Western analysis reveals that all fusion proteins were expressed at relatively equal amounts (Fig. 2E). These results thus define a NH2-terminal region that is essential for CtBP-mediated transcription repression that also coincides with a region of CtBP that is essential for the interaction with PLDLS-domain proteins.
Interaction of CtBP with CBP

Due to the importance of the CtBP NH₂ terminus in mediating repression, we examined the possibility that CtBP might repress transcription by targeting a cellular factor that contains a PLDLS-like motif. By examining the group of proteins that are currently known to interact with CtBP through a PLDLS-like motif, it seems that most CtIPs contain P-X-[D/S/N]-L-[S/T/V] motif (28). We therefore searched the SwissProt + SPTreMBL, Protein Identification Resource, and ProClass protein databases for proteins that contain this motif (39-41). The search revealed a large number of proteins, from a variety of species, containing this motif. We limited the number of candidate proteins by examining only human proteins with established function in transcription regulation (Table 1). Among this list were many transcription factors known to act as negative regulators of transcription, most of which are already known to bind CtBP (25, 27, 28, 30, 42-45). Interestingly, we also identified several transcription corepressors, such as the proteins N-CoR, 5'-TG-3' interacting factor, and methyl CpG–binding protein, proteins found in complexes with HDAC. Indeed, the 5'-TG-3'-interacting factor protein has already been shown to interact with CtBP (46). In addition, the nuclear receptor–interacting repressors TIF1α and Rip140 also contain this motif (47). As CtBP has been described to interact with HDAC and HDAC complexes and to exhibit sensitivity to trichostatin A on certain promoters, it is

**FIGURE 2.** Identification of a domain within CtBP essential for PLDLS interaction and transcription repression. A. A schematic representation of CtBP along with the NH₂-terminal deletion mutants. B. HF7C yeast were transformed with plasmid encoding Gal4 DBD-CtBP or each of the mutants along with Gal4AD-CtIP, Gal4AD-CtBP, or Gal4AD. Yeast were then plated on medium lacking Trp, Leu, and His that is selective for protein/protein interactions. Yeast that grew on selective medium were then assayed for the presence of β-gal. An interaction was scored positive if yeast could both grow on selective medium and express β-gal. C. The Gal4-CtBP wild-type and mutants were cloned into mammalian expression vectors and transfected into C33A cells with β-gal and pSVECG (SV40) reporter plasmid. Cells were harvested 40 hours after transfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. D. Experiment was done as in (C) using MLP, pGLuc basal promoter, 14D promoter, and the herpes virus thymidine kinase promoter (TK). E. Western blot using Gal4 DBD antibody of C33A-transfected Gal4-CtBP and mutants. β-gal was used as a loading control for transfection efficiency.
likely that one mechanism of CtBP-mediated transcription repression is through a HDAC-dependent mechanism potentially through interactions with one or more of these proteins (25, 31, 48, 49). However, given our previous results that CtBP can repress HDAC-resistant promoters, along with similar results from other groups, it is likely that CtBP also represses transcription through HDAC-independent mechanisms (17, 27, 29).

Several coactivators were also found to contain P-X-[D/S/N]-L-[S/T/V] motifs, such as the 60-kDa subunit of the mammalian SWI/SNF complex, a chromatin remodeling ATPase involved in transcription activation, and the thyroid hormone receptor coactivator. An interesting set of coactivators, and transcription coactivators, and basal transcription machinery.

**Table 1. Identification of PXDLS-Containing Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>DNA-binding transcription factors Two-handed zinc finger protein ZEB</td>
<td>PLDLS</td>
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<tr>
<td>Transcription factor 8 (Elf1)</td>
<td>PLDLS</td>
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<tr>
<td>Negative regulator of interleukin-2</td>
<td>PLDLS</td>
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<tr>
<td>CREB (activating transcription factor 2)</td>
<td>PLDLS</td>
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<td>Zinc finger protein p40</td>
<td>PLDLS</td>
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<tr>
<td>DNA-binding protein Ikaros</td>
<td>PEDLS</td>
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<tr>
<td>Ras-responsive element–binding protein 1</td>
<td>PIDLS</td>
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<tr>
<td>E1K3</td>
<td>PLNL-S</td>
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<tr>
<td>Ev1-1</td>
<td>PLDS-PEPLDT</td>
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<tr>
<td>AREB6</td>
<td>PEDLT/PLNLS/PLDLS</td>
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<tr>
<td>NF-AT</td>
<td>PLSLT</td>
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<tr>
<td>Nuclear factor-κB</td>
<td>PLDLS</td>
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<tr>
<td>TCF-4</td>
<td>PLSLV</td>
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<tr>
<td>Rh protein-interacting zinc finger protein</td>
<td>PLDLS</td>
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<tr>
<td>Transcription corepressors</td>
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<tr>
<td>CBP</td>
<td>PLDLS</td>
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<tr>
<td>3'-TG^3'-interacting factor</td>
<td>PLDLS</td>
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<tr>
<td>Methyl CpG-binding protein 2</td>
<td>PQDLS</td>
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<tr>
<td>Receptor-interacting protein 140</td>
<td>PIDLS</td>
</tr>
<tr>
<td>Transcription intermediary factor 1a</td>
<td>PMDLS</td>
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<tr>
<td>N-CoR</td>
<td>PENLV</td>
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<td>Transcription coactivators</td>
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<td>E1A-associated protein p300</td>
<td>PMDLS</td>
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<tr>
<td>CBP</td>
<td>PMDLS</td>
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<tr>
<td>Thymidylate receptor coactivating protein</td>
<td>PMDLS</td>
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<tr>
<td>SWI/SNF complex 60-kDa subunit</td>
<td>PGDLS</td>
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<tr>
<td>GCN5</td>
<td>PENLT</td>
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<tr>
<td>Basal transcription machinery</td>
<td></td>
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<tr>
<td>TFIIF</td>
<td>PGbLS</td>
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<tr>
<td>TAFII-250</td>
<td>PQDLT</td>
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**NOTE:** The SwissProt + SPTENBL, Protein Identification Resource, and ProClass protein databases were searched for instances of the pattern P-X-[D/S/N]-L-[S/T/V] using the Institut de Biologie et Chimie des Protéines and Protein Identification Resource Web sites. This list was then limited to human proteins known to play a role in transcription. These proteins were then grouped in four general categories: DNA-binding transcription factors, transcription corepressors, transcription coactivators, and basal transcription machinery.

CtBP Inhibits CBP-Mediated Histone Acetylation

Given the observed interaction of CtBP with CBP, together with the role of the intrinsic histone acetylase activity in the function of CBP as a coactivator for many, if not all, promoters, we next measured the effect of CtBP on the histone acetylase activity of CBP in vitro. As shown in Fig. 4A, preincubation of baculovirus-produced CBP with baculovirus-produced CtBP, followed by the addition of [3H]acetyl CoA and free histone as a CBP substrate, resulted in a substantial inhibition of histone acetylation relative to a bovine serum albumin (BSA) protein control. This effect of CtBP on histone acetylation was retained across at 20-minute time course.

To further examine this inhibition of histone acetylation, we assessed the effect of increasing amounts of the CtBP protein on CBP. Figure 4B shows that a 20-minute preincubation of CBP with doses of CtBP ranging from 2.5 to 15 μg produced an increasingly dramatic inhibition of CBP’s histone acetylase activity relative to the BSA control (Sigma-Aldrich, St. Louis, MO and New England Biolabs, Beverly, MA). These data indicate that the CtBP corepressor can significantly inhibit the ability of CBP to acetylate histone in vitro.

To further examine the effects of CtBP on CBP-mediated histone acetylase activity and to determine if this inhibition correlates with CtBP-mediated transcription repression, we assayed the baculovirus-produced wild-type and mutant CBP proteins for their ability to affect CBP-mediated histone acetylation. We preincubated BSA, CtBP, and CtBP^1-120, or CtBP^1-10 with baculovirus-produced CBP for 20 minutes followed by the addition of histone and [3H]acetyl CoA. Whereas wild-type CBP and CtBP^1-120 dramatically inhibited the acetylation of histones by CBP relative to the BSA control, CtBP^1-10 was severely impaired in its ability to inactivate CBP (Fig. 4C). The same result was obtained in multiple

interacting motif was interesting in light of previous data showing that the E1A, E6, and Twist proteins can inactivate the acetyltransferase activity of p300/CBP leading to transcriptional repression (51-53).

Given the precedence of transcriptional repression via HAT inhibition, we first tested for an interaction between CtBP and CBP. The two proteins were coexpressed by baculovirus infection of SF9 cells, and cell extracts were immunoprecipitated using Gal4 DBD antibody recognizing the Gal4 DBD-tagged CtBP protein. The CBP Western analysis shown in Fig. 3B reveals that Flag-CBP could be immunoprecipitated with the Gal4 DBD-CtBP fusion protein, indicating that CtBP and CBP can indeed interact when coexpressed in SF9 cells. As a further test for interaction, ^35S-labeled CtBP and the PLDLS binding–deficient mutant, CtBP^1-120, were produced by in vitro transcription/translation and then assayed for interaction with a bacterially expressed glutathione S-transferase (GST)-CBP (amino acids 720-1,677) fusion protein. Protein bound to either GST-CBP fusion or GST alone was analyzed by SDS-gel electrophoresis. As shown in Fig. 3C, wild-type CtBP interacted specifically with GST-CBP, whereas the interaction of the CtBP^1-120 mutant was diminished compared with the wild-type CtBP protein.
experiments and with different preparations of wild-type and mutant CtBP proteins. As an additional control, the CBP histone acetylase inhibition experiment was carried out using samples of BSA, CtBP, and the CtBP mutants that were preboiled to denature and inactivate the proteins. Figure 4D shows that boiled CtBP is incapable of inactivating CBP histone acetylase activity, thereby implicating some function of the CtBP protein in the inactivation of histone acetylase activity. These results thus correlate the ability of CtBP to repress transcription with its ability to inhibit CBP-mediated histone acetylation, indicating that one mechanism of CtBP-mediated transcription repression is likely through the inactivation of the histone acetylase activity of CBP.

Discussion

The role of localized histone acetylation, as a mechanism for control of transcription activity, has been shown in numerous instances. This includes both the recruitment of histone acetylases as a mechanism for transcription activation and the recruitment of HDACs as a mechanism of transcriptional repression. Our finding that the CtBP corepressor can physically interact with and inhibit the histone acetylase activity of CBP provides yet another mechanism for transcription control through acetylation. Because CBP-like coactivators with intrinsic histone acetylase activity are likely to be a common component of many transcriptional activation events, the role of a histone acetylase inhibitor, such as CtBP, could be of widespread importance.

In some instances, CtBP is recruited to a promoter complex through a direct interaction with DNA-binding transcription factors. This includes the Drosophila proteins Snail, Knirps, and Hairy as well as the mammalian proteins BKL, dEF, ZEB, Net, AREF6, Evi-1, Ikaros, FOG, HPC2, etc. (22, 23, 25-29, 42, 44). In other instances, CtBP is recruited to a promoter through an interaction with the CtIP protein, as is the case for BRCA1 and E2F/Rb (17, 18, 54). In either case, CtBP is tethered to a target promoter where it acts to inhibit transcription. Several mechanisms for CtBP-mediated transcriptional repression have been identified, including an ability of CtBP to repress the activity of adjacent transcriptional activators (24, 33). As transcriptional activators often recruit HATs, perhaps CtBP functions to inactivate CBP or HATs recruited by these nearby transcription factors or by the basal transcription machinery. It is also of interest to note that several CtBP-binding proteins are...
themselves acetylated. This acetylation disrupts the ability of these binding proteins to bind CtBP (55). It is therefore possible that the inhibition of CBP-mediated acetylation of other proteins by CtBP may also restore the binding between CtBP and its binding partners, further promoting transcriptional repression.

It remains to be determined how CtBP might inactivate the histone acetylase activity of CBP. A recent report, published while this work was in preparation, described the interaction of CtBP with the CBP paralogue, p300 (56). This study supports a role for CtBP in blocking the accessibility of p300 to histones by binding to the bromodomain. Although it is likely that CtBP can act as an inhibitor of histone acetylation by similarly blocking the accessibility of CBP to histone, other mechanisms of action are also possible. It has been shown previously that the function of the bromodomain, the acetylated histone-binding domain, is necessary for the acetylation of nucleosomal histone but not necessary for acetylation of free histone (57). Furthermore, unacetylated histones are not believed to bind to the CBP bromodomain (58). As our in vitro experiments use free, unacetylated histones, we suggest that CtBP may also have the ability to directly inhibit the HAT activity of CBP as has been shown for E1A 12S, Twist, PU.1, and EBF (51, 52, 59-61). Interestingly, another Rb-interacting protein, E1D, also binds to p300 and inhibits its histone acetylase activity (62, 63). CtBP may act to directly inhibit histone acetylase activity by creating a binding-induced conformational change in CBP that affects the proximal HAT domain. Alternatively, as the CtBP protein is highly related to the D-hydroxy acid dehydrogenases, possesses slow enzymatic dehydrogenase activity, and binds NAD, it is also possible that the oxidation/reduction activities of CtBP may regulate CBP acetylase enzymatic activity (38, 64-66). Indeed, Kumar et al. finds that the dehydrogenase domain (amino acids 28-353) of CtBP is capable of repressing transcription. Moreover, the PXDLS motif of CtBP interacting proteins was found to interact directly with the dehydrogenase active site (38). Regardless of the mechanism, data presented here further emphasize a direct function for CtBP in regulating localized histone acetylation.

**FIGURE 4.** CtBP inhibits CBP-mediated histone acetylation. A. Baculovirus-expressed Flag-CBP was preincubated for 20 minutes with 15 μg baculovirus-expressed 6His-CtBP or an equal amount of BSA as a control. [3H]acetate CoA and 25 μg histone were then added to the reaction, which was allowed to proceed for the indicated time. Products were analyzed in a 15% acrylamide gel. B. Baculovirus-expressed Flag-CBP was preincubated for 20 minutes with 2.5, 5, 10, or 15 μg baculovirus-expressed 6His-CtBP or BSA. [3H]acetate CoA and histone were then added to the reaction, which was allowed to proceed for 20 minutes. C. Baculovirus-expressed Flag-CBP was preincubated for 20 minutes with baculovirus-expressed 6His-CtBP, CtBPD1-10, CtBPD1-120, or an equal amount of BSA as a control. [3H]acetate CoA and histone were then added to the reaction, which was allowed to proceed for 20 minutes. D. Assays were carried out as described in (C), except using BSA, CtBP, CtBPD1-10, and CtBPD1-120 that were first boiled before incubation with CBP.
In E2F/Rb-mediated repression, the inhibition of p300/CBP-mediated histone acetylation suggests a complementary mechanism for E2F/Rb-mediated repression. In one case, the recruitment of HDAC through Rb or p130 can lead to transcriptional repression through the localized alteration of chromatin structure (7-9). This action alone, however, might be expected to be less than complete given the expected continued histone acetylation mediated by promoter-bound acetylase. Our observation that E2F/Rb can also recruit an inhibitor of histone acetylation would then allow for a more complete inhibition of histone acetylation at a given promoter. Taken together, these data are consistent with the model diagrammed in Fig. 5. We propose that, because Rb has been shown to have the ability to bind simultaneously to HDAC and BRG1, both of which contain LXCXE motifs, perhaps HDAC and CtBP can coexist in one E2F/Rb or E2F/p130 complex (67). In this manner, the presence of both HDAC and a HAT-inactivating protein on the same promoter would insure the hypoacetylation of local chromatin at target promoters, thus resulting in transcriptional repression. Although we have only tested the effects of CtBP on CBP-mediated acetylation of histones, future work aims to examine the possibility that CtBP might also inhibit the acetylation of other CBP targets.

Materials and Methods

Cell Culture

C33A and 293 cells were grown in DMEM with 10% fetal bovine serum. SF9 cells were grown in suspension in HyQ SFX-Insect without serum.

Plasmids and Reagents

The SV40 promoter with upstream Gal4 sites (pSVECG) was a kind gift from D. Dean, Washington University School of Medicine, Saint Louis, MO (5). The MLP and 14D promoter with Gal4 sites were a kind gift from D. Dean and R. Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA. The thymidine kinase-Luc plasmid was prepared for the CAT assays to 590 gal values.

Reactions were incubated at 37°C for 3.5 hours. Raw CAT activity was measured by adding 10 μL of the extract prepared for the CAT assays to 590 μL of 0.1 mg/mL CPRG in Z buffer. Absorbance was measured at 570 nm. In all instances of comparison, Western analyses were done to determine that equal protein levels are expressed from transfected plasmids.

Mechanism of CtBP Repression

Mechanism of CtBP Repression

FIGURE 5. Complementary mechanisms for E2F/Rb-mediated transcription repression. HDAC is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. HDAC then modifies the histones proximal to the promoter causing transcriptional silencing. Rb and p130 also recruit CtBP to E2F complexes. CtBP bridges the interaction between CBP and the E2F/Rb complex. CtBP, acting as a dimer, then functions by inhibiting p300/CBP bound to the promoter through basal transcription machinery or other transcription factors.

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Yeast Two-Hybrid Assays

The yeast two-hybrid assay was done as recommended in the Clontech (Mountain View, CA) protocol.

Baculovirus-Expressed Proteins

Purified baculovirus-expressed proteins were made by infecting 50 mL of 2 x 10^6 Sf9 cells/mL with a given virus. Forty-eight hours after infection, cells were harvested and lysed in 50 mmol/L Tris-HCl, 1% NP40, 10 mmol/L BME, 1 mmol/L phenylmethylsulfonyl fluoride, 2 µg/mL pepstatin, and 2 µg/mL aprotinin. Extracts were then incubated for 30 minutes at 4°C with 1 mL of a 50% slurry of nickel beads. Beads were loaded onto a column and washed with lysis buffer. Protein was eluted in 20 mmol/L Tris-HCl, 100 mmol/L KCl, 100 mmol/L imidazole, 10% glycerol, and 10 mmol/L BME. Fractions (0.5 mL) were collected and peak fractions 2 to 4 were pooled.

In vitro Protein Interaction Assay

CtBP and CtBPΔ1-120 were synthesized by the T7 TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). BL21 cells expressing GST or GST-CtBP (amino acids 720-1,677) were lysed in 150 mmol/L NaCl, 80 mmol/L Tris (pH 7.5), 0.5% NP40, 2 mmol/L EDTA, and the protease inhibitor, pepstatin, leupeptin, AEBSF, and aprotinin. Bacterial cells were sonicated for 20 seconds and then centrifuged at 4°C. Equal amounts of GST or GST-CtBP protein were incubated with glutathione-Sepharose beads for 1 hour and the beads were washed thrice in lysis buffer. The in vitro translation mix of CtBP or CtBPΔ1-120 was split in half and diluted to 800 µL with lysis buffer. The diluted translation mix was incubated with GST or GST-CtBP for 4 hours at 4°C. Beads were washed four times in wash buffer [500 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% NP40] and then analyzed by SDS-gel electrophoresis. Gels were fixed, enhanced, dried, and then exposed to film overnight.

Coimmunoprecipitation Assays

Sf9 cells were infected with baculovirus expressing Flag-CtBP or Gal4 DBD-tagged CtBP. Cells were lysed in 500 µL lysis buffer (150 mmol/L NaCl, 1% NP40, 50 mmol/L Tris, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin). Extracts were precleared with protein A + G beads for 1 hour at 4°C. Extracts were then incubated for 1.5 hours at 4°C with protein A + G beads that were prebound to 1 µg anti-Gal4 DBD antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were then washed thrice in 1 mL lysis buffer, boiled in Laemmli sample buffer, and analyzed in an SDS-acrylamide gel. Western analysis was done using polyclonal CBP antibody (Santa Cruz).

Histone Acetylase Assays

Baculovirus-purified Flag-CtBP (~150 ng) was preincubated with 2.5 to 15 µg BSA (an 80- to 500-fold molar excess of CtBP or BSA to CtBP), baculovirus-purified CtBP, or CtBP mutants in a 15 µL volume with buffer containing 50 mmol/L Tris-HCl, 10% glycerol, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L EDTA, 75 mmol/L NaCl, and 10 mmol/L butyric acid for 20 minutes at 30°C. After preincubation, 25 µg histones and 125 µCi [3H]acetyl CoA were added and the volume was raised to 30 µL keeping the buffering conditions constant. The reaction was allowed to proceed at 30°C for 20 more minutes after which the reactions were immediately boiled and run on a 15% acrylamide gel. The gel was fixed, amplified, dried, and exposed to film. After exposure, the gel was rehydrated and stained with Coomassie blue.

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References


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