Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP

P. Balasubramanian, Ling-Jun Zhao, G. Chinnadurai*

Institute for Molecular Virology, Saint Louis University Health Sciences Center, 3681 Park Avenue, St. Louis, MO 63110, USA

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Abstract The C-terminal region of adenovirus E1A interacts with the transcriptional corepressor, CtBP. The mechanism of transcriptional regulation by CtBP is not known. CtBP shares a significant homology with NAD^+-dependent D2-hydroxy acid dehydrogenases. CtBP binds to NAD^+ and NADH. Both forms of the dinucleotide stimulate oligomerization of native CtBP and enhance complex formation with E1A. CtBP also has a slow dehydrogenase activity. Interaction of CtBP with E1A reduces the dehydrogenase activity. Our results raise the possibility that the oxidation/reduction reactions of CtBP may regulate transcription. Thus, CtBP is a unique transcriptional regulator with an enzymatic activity similar to metabolic dehydrogenases. The levels of intracellular nicotinamide adenine dinucleotide may modulate transcriptional activity of CtBP.

Key words: CtBP; Corepressor; E1A; NAD^+/NADH; Dehydrogenase

1. Introduction

The CtBP family proteins are highly conserved in vertebrates and invertebrates and they are linked to important cellular processes during development and oncogenesis [1]. The founding member of this family, hCtBP1, was identified as a cellular protein that binds to the C-terminal region of adenovirus E1A protein through a sequence motif PLDLS [2,3]. Interaction of CtBP with the C-terminus of E1A antagonizes a transcriptional activation function mediated by the N-terminal region of E1A [4]. Searches for cofactors of various transcriptional repressors such as *Knirps*, *Snail* and *Hairy* that function during *Drosophila* embryo development identified a CtBP homolog, dCtBP [5,6]. Numerous other invertebrate and vertebrate transcriptional repressors have now been recognized to mediate their activities through recruitment of CtBP [1,7]. Studies with mutant mice lacking CtBP reveal that the activities of a large number of transcription factors are compromised in the absence of CtBP and the mutant mice exhibit severe developmental defects [8]. In addition to transcriptional repression, CtBP has also been reported to activate transcription in a context-dependent manner [8,9]. The mechanisms by which CtBP modulates transcriptional repression and activation are not known. The animal CtBPs share a high degree of homology with NAD^+-dependent D2-hydroxy acid dehydrogenases (D2-HDHs) [3]. The homology between D2-HDH and CtBP extends over a conserved signature Rossman fold motif [10] required for nicotinamide adenine dinucleotide (NAD) binding and a catalytic triad (His/Glu/Arg) conserved in all D2-HDHs [3]. Consistent with the presence of the Rossman fold motif, hCtBP1 and a splice variant of bovine CtBP2 have been reported to bind with NAD^+ and NADH [11,12]. It has also been suggested that CtBP may regulate transcription in a redox-dependent manner [11]. Here, we report that both NAD^+ and NADH stimulate oligomerization of native CtBP and interaction with E1A. Further, CtBP1 also possess an intrinsic dehydrogenase activity suggesting that CtBP may modulate transcription by novel mechanisms.

2. Materials and methods

2.1. Proteins

The cDNA for hCtBP1 was cloned in the expression vector pET-11a (Novagen) and used for transformation of *E. coli* strain BL21 (DE3). Expression of the recombinant protein was induced by treatment of log phase bacterial cultures with 1 mM isopropyl thiogalactose for 5 h. The cells were collected and resuspended in 20 mM imidazole in phosphate-buffered saline (PBS) containing cocktail protease inhibitors (one tablet/10 ml; Roche), sonicated and centrifuged at $20\,000 \times g$ for 30 min at 4°C. The supernatant was mixed with 10 ml of Ni^{2+}-NTA agarose (Qiagen), rotated overnight and packed in a column. The column was washed with 50 ml of 20 mM imidazole in PBS and CtBP was eluted with 250 mM imidazole, dialyzed twice against PBS for 1 h at 25°C and once overnight at 4°C (using slide A lyser dialysis cassette, Pierce). The protein was concentrated using Centricon tubes (Millipore) and stored in 20% glycerol (containing protease inhibitors) at −70°C. The recombinant E1A 243R protein was expressed and purified as described by Madison et al. [13] and was a gift from James Lundblad, Oregon Health Sciences University. Glutathione-*S*-transferase (GST) and GST fusion proteins were expressed in *E. coli* BL21 (DE3) cells transformed with pGEX-5X3 or GST-Cter [2] or pGST-CtBP [14]. GST fusion proteins were purified by affinity chromatography on glutathione agarose beads as described in [2,3].

2.2. Oligomerization

Purified CtBP protein (11.5 μM) was incubated with or without NAD^+/NADH (46 μM) in 400 μl of PBS on ice for 2 h, mixed with 100 μl of molecular weight (MW) standards (Bio-Rad Biologic LP; Cat. No. 151-1901) and loaded on a 1×50 cm column (Pharmacia Biotech) packed with Sephadex G-150 (equilibrated with PBS containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol with or without NAD^+/NADH). The samples were subjected to liquid chromatography (Bio-Rad Biologic LP) at a flow rate of 0.1 ml/min. 1 ml fractions were collected and aliquots were analyzed by SDS–PAGE followed by Western blot analysis using a CtBP polyclonal antibody.

*Corresponding author. Fax: (1)-314-577 8406.
E-mail address: chinnag@slu.edu (G. Chinnadurai).

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2.3. Protein interactions

Interactions between E1A and CtBP were carried out using purified proteins that were expressed in bacteria. Full-length CtBP or E1A proteins (10 μg) were mixed with immobilized (on glutathione agarose beads) GST or GST–E1A Cter or GST–CtBP proteins (10 μg) in the presence of various concentrations of NAD^+ or NADH in 1 ml binding buffer (150 mM NaCl, 0.1% NP40, 50 mM Tris–HCl, pH 7.5) containing protease inhibitors aprotinin (20 μg/ml) and leupeptin (200 μg/ml) and incubated at 4°C for 3 h. The glutathione agarose beads were washed with the binding buffer three times, resuspended in 2× SDS sample buffer and boiled for 2 min. The beads were pelleted and the supernatants were analyzed by SDS–PAGE and Western blot analysis using CtBP or E1A antibodies.

2.4. Dehydrogenase activity

Assays were carried out in 0.2 M Tris–HCl, pH 7.3, containing 150 μM NADH and 375 μM pyruvate (Sigma Chemical) at 25°C with indicated amounts of bacterially expressed CtBP protein as described by Adams et al. [15] and outlined in http://www.worthington-biochem.com/manual/L/LDH/html. The change in absorbance (340 nm) was measured in VERSA max turnable microplate reader (Molecular Devices) for 30 min at intervals of 5 min.

3. Results and discussion

3.1. Oligomerization of CtBP

CtBP family proteins share extensive homology with NAD^+-dependent D2-HDHs [3]. Although our original attempts to demonstrate nucleotide binding and DH activity of hCtBP1 were unsuccessful [3], we reexamined these activities using native proteins expressed and purified by improved methods. We observed a significant nucleotide binding activity with hCtBP1 similar to the observations by other investigators [11,12] (data not shown). We then investigated the effect of NAD^+ and NADH on various CtBP activities. Since CtBP is highly homologous to various NAD^+-dependent DHs that function in oligomeric forms, we determined the effect of NAD^+ and NADH on oligomerization of full-length hCtBP1. For this purpose, CtBP1 was expressed as a His-tagged protein in bacteria and purified over the Ni^{2+} affinity matrix. CtBP was incubated in the presence or absence of NAD^+ or NADH and analyzed by gel filtration chromatography. The fractions were analyzed by Western blot analysis (Fig. 1). In the absence of NAD^+, CtBP1 migrated with an apparent MW of 65–70 kDa. Both NAD^+ and NADH had striking effects on oligomerization and doubled the apparent MW of CtBP1.

3.2. E1A–CtBP interaction

We then examined the effect of NAD^+ on binding of CtBP with E1A. For this purpose, purified CtBP1 was incubated with GST or a GST–E1A fusion protein (GST–E1A–Cter) that contains the C-terminal region (67 aa) of E1A. The protein complexes were collected over the glutathione agarose beads and analyzed by Western blot analysis using an antibody specific for CtBP1 (Fig. 2A). Both NAD^+ and NADH enhanced interaction of CtBP1 with E1A at dinucleotide concentrations of 10–100 μM and had an inhibitory effect at higher concentrations (1 mM). We also carried out a reverse experiment in which binding of purified full-length E1A (243R) to GST and GST–CtBP fusion proteins was analyzed (Fig. 2B). These results indicated that E1A 243R protein interacted at comparable levels in the absence or presence of nicotinamide adenine nucleotides. It is possible that dinucleotide binding may alter the conformation of native CtBP, thereby enhancing interaction with E1A. In contrast, GST–CtBP fusion protein efficiently binds with E1A in the absence of dinucleotide, suggesting that the GST–CtBP fusion protein may be in a different conformation conducive for constitutive interaction with E1A.

3.3. Dehydrogenase activity of CtBP

The CtBP family proteins from animals exhibit compelling amino acid homology to NAD^+-dependent D2-HDHs including a catalytic triad (His315/Glu295/Arg266) conserved in all D2-HDHs. We examined if CtBP1 has an intrinsic DH activity by measuring the conversion of NADH to NAD^+ in a prototypical DH reaction in which pyruvate is reduced to lactic acid. We observed increasing DH activity with increasing concentrations of CtBP (Fig. 3). In contrast, a CtBP mutant in the catalytic His residue (His315→Val), did not exhibit detectable DH activity, suggesting that the enzyme activity is intrinsic to CtBP. We also determined the effect of E1A on DH activity. Incubation with E1A reduced the DH activity modestly (Fig. 4).

We have shown that the binding of NAD^+ causes oligomerization of CtBP1. Since the CtBP proteins of invertebrates and vertebrates contain a conserved Rossman fold motif, it is likely that other animal CtBPs may also bind to NAD^+ and NADH. A splice variant of bovine CtBP2 has been shown to bind NAD^+ [12]. We have observed that both NAD^+ and NADH enhance interaction of CtBP with the prototypical target protein, Ad E1A. It has previously been reported that the effect of NADH is far more effective than NAD^+ in facilitating binding of CtBP1 with E1A [11]. This observation has led to the suggestion that CtBP may be a redox-sensing transcriptional regulator. Although we did not observe dramatic effects of NADH on protein interaction as previously reported, we observed that the effect of NADH was better than that of NAD^+. While we observed a significant effect of NAD^+ and NADH on interaction between native CtBP

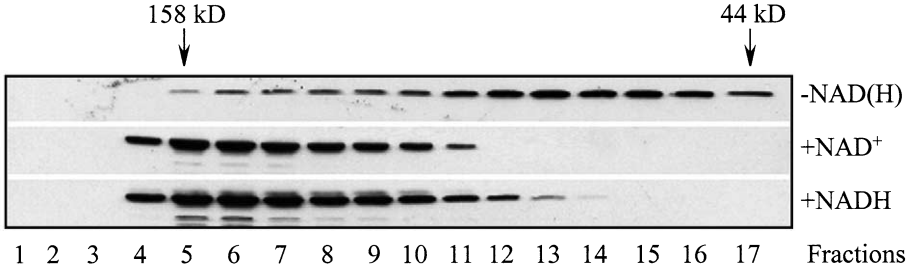

Fig. 1. Effect of NAD^+ and NADH on oligomerization of CtBP. CtBP1 was incubated in the presence or absence of NAD(H) and subjected to exclusion chromatography. Aliquots of various fractions were analyzed by Western blot analysis using a polyclonal CtBP antibody. Among the various MW markers used, the positions of bovine γ-globulin (158 kD) and chicken ovalbumin (44 kD) are indicated.

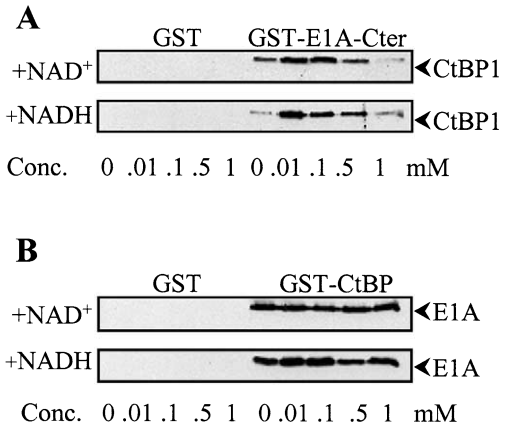

Fig. 2. Effect of NAD^+ and NADH on CtBP–E1A interactions. Purified CtBP or E1A proteins were incubated with GST or GST fusion proteins containing either CtBP or the C-terminal region of E1A in the presence or absence of indicated concentrations of NAD(H).

and GST–E1A there was no significant stimulatory effect on interaction between native E1A and GST–CtBP. It is possible that this differential effect may be due to conformational changes induced by the dinucleotide on native CtBP.

We have shown that CtBP has an intrinsic dehydrogenase activity. Demonstration of such an activity of CtBP, in light of its compelling overall aa sequence homology and the presence of a signature NAD^+-binding motif and catalytic aa residues, has long been anticipated. Although the observed enzymatic DH activity of CtBP is slow, it is specific, as the mutant lacking the catalytic His315 residues has no significant activity. The slow activity may be attributed to the fact that the cognate substrate for CtBP remains to be identified and the substrate (pyruvate) used here may be non-optimal. It should be noted that the activities of various dehydrogenases are highly substrate-specific. During the preparation of the manuscript a dehydrogenase activity of CtBP has been reported by other investigators [16].

The important issue is whether the transcriptional regulatory activity of CtBP is linked to the enzymatic activity. Previous transcriptional tethering studies have shown that mutating the His residue of the catalytic triad did not significantly affect the transcriptional repression activity of mCtBP2 [17] and dCtBP [9]. However, this mutation impaired a context-

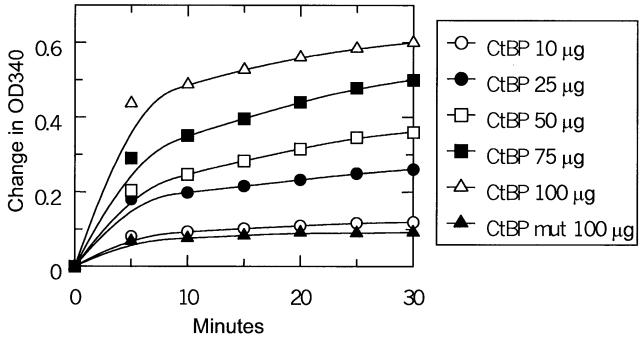

Fig. 3. Dehydrogenase activity of CtBP. The DH activity was determined on the basis of reduction of pyruvate to lactic acid and oxidation of NADH to NAD^+ by measuring change in absorbance at 340 nm.

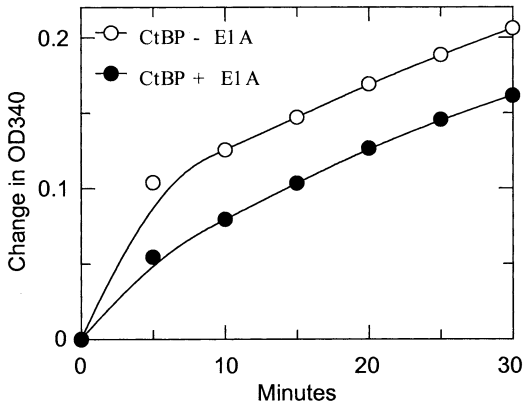

Fig. 4. Effect of E1A on dehydrogenase activity of CtBP. CtBP (25 µg) was incubated with E1A (38 µg) and the DH activity was measured as in Fig. 3.

dependent transcriptional activation function of dCtBP [9]. Parkhurst and colleagues [9] have also reported that co-expression of dCtBP with the corepressor mSin3 in mammalian cells attenuated the repressor activity of mSin3. They postulated that an intrinsic oxido-reduction activity of CtBP might alter the activity of Sin3. These observations suggest that the oxidation/reduction reactions mediated by CtBP may play a regulatory role in modulating the transcriptional activities of associated factors. However, detailed mutational analysis of full-length CtBP would be required to clearly delineate the role of the DH activity in transcriptional repression and activation functions of CtBP. In this context, it should be noted that the recently discovered plant CtBP homolog, angustifolia (AN), has neither the signature motif for NAD^+-binding nor the catalytic triad residues [18,19]. The *AN* gene has also been postulated to function as a transcriptional repressor [18]. Thus, the dinucleotide binding activity and the DH activity may be unique to animal CtBPs.

CtBP joins the Sir2 silencing protein as transcriptional corepressors with intrinsic NAD^+-dependent enzymatic activities [20,21]. Sir2 has a NAD^+-dependent deacetylase activity and regulates transcription depending on the energy status of the organism [22]. Since CtBP appears to play important roles in normal development and in oncogenesis, expression of the CtBP target genes may be influenced by the cellular redox status. The energy status of the cells may also play a role in adenovirus replication, which is critically dependent on the transcriptional regulatory activities of E1A proteins. The intracellular levels of NAD(H) during viral infection may influence the activities of E1A through recruitment of CtBP.

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