Nicotinamide Adenine Dinucleotide-induced Multimerization of the Co-repressor CtBP1 Relies on a Switching Tryptophan*

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Background: C-terminal binding protein 1 (CtBP1) assembles into a tetrameric transcriptional co-repressor but how it directs gene expression is not clear.

Results: CtBP1 requires NAD(H) for transition into multimers. Its biochemical activities are separable from transcriptional repression.

Conclusion: Tryptophan 318 permits CtBP1 to first dimerize and then tetramerize after the binding of NAD(H).

Significance: Clarification of how CtBP1 tetramerizes will permit development of CtBP inhibitors to target oncogenesis.

The transcriptional co-repressor C-terminal binding protein (CtBP) interacts with a number of repressor proteins and chromatin modifying enzymes. How the biochemical properties including binding of dinucleotide, oligomerization, and dehydrogenase domains of CtBP1 direct the assembly of a functional co-repressor to influence gene expression is not well understood. In the current study we demonstrate that CtBP1 assembles into a tetramer in a NAD(H)-dependent manner, proceeding through a dimeric intermediate. We find that NAD-dependent oligomerization correlates with NAD\(^+\) binding affinity and that the carboxyl terminus is required for assembly of a dimer of dimers. Mutant CtBP1 proteins that abrogate dinucleotide-binding retain wild type affinity for the PXDLS motif, but do not self-associate either in vitro or in vivo. CtBP1 proteins with mutations in the dehydrogenase domain still retain the ability to self-associate and bind target proteins. Both co-immunoprecipitation and mammalian two-hybrid experiments demonstrate that CtBP1 self-association occurs within the nucleus, and depends on dinucleotide binding. Repression of transcription does not depend on dinucleotide binding or an intact dehydrogenase domain, but rather depends on the amino-terminal domain that recruits PXDLS containing targets. We show that tryptophan 318 (Trp\(^{318}\)) is a critical residue for dimer assembly and likely functions as a switch for effective dimerization following NAD\(^+\) binding. These results suggest that dinucleotide binding permits CtBP1 to form an intranuclear homodimer through a Trp\(^{318}\) switch, creating a nucleation site for multimerization through the C-terminal domain for tetramerization to form an effective repression complex.

C-terminal binding protein (CtBP)\(^2\) is unique among transcriptional regulators in that it binds NAD(H), closely resembles the 2-hydroxyacid dehydrogenase family of enzymes, and was first identified as a binding target of the adenoviral oncoprotein E1A (1). CtBP1 and its paralogues bind their target proteins through a conserved PXDLS motif found in a number of transcriptional repressor proteins important in cellular growth, differentiation, and oncogenesis (2–4). Early organism studies in Drosophila null for CtBP show segmentation and patterning defects (5), whereas mouse knock-outs for CtBP1 and CtBP2 alleles exhibit dosage-dependent developmental defects and the dual knock-out is embryonic lethal (2). Due to its association with various cancer control genes, CtBP has gained attention as a possible therapeutic target; however, the mechanism used by CtBP to regulate gene expression in vivo is not well understood, underscoring the need for translating biochemical mechanisms to cellular single gene analyses.

Numerous studies have described the biochemical properties of CtBP:NAD(H) binding, dimerization, oligomerization, dehydrogenase homology, PXDLS target protein binding, and recruitment of DNA modifying enzymes (6). Determining the order in which CtBP uses each of these activities to assemble, maintain, and then dismiss a functional co-repressor during normal, regulated gene expression is critical to understanding the importance of CtBP in oncogene pathways. Most studies examine CtBP1, but CtBP2 (78% homology) is highly conserved across its functional domains and also binds dinucleotide to homodimerize (6). CtBP1 may be regulated by intranuclear NAD/NADH ratios or hypoxia (7), SUMOylation (8), interaction with tumor suppressors (9–11), and the recruitment of DNA modifying enzymes (12, 13). The variety of genes and mechanisms proposed for how CtBP1 functions shows the importance of understanding the specific mechanisms for CtBP1 assembly of a DNA-bound protein complex to direct gene repression, and subsequently how CtBP1 participates in de-repression.

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‡ The abbreviations used are: CtBP, C-terminal binding protein; NAD, nicotinamide adenine dinucleotide; GST, glutathione-S-transferase; HSV-tk, herpes simplex virus-thymidine kinase; IP, immunoprecipitation.
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How dinucleotide influences CtBP activity has been an active question. NAD(H) binding initiates dimerization and may influence dehydrogenase activity (14–16) although the substrate(s) and reaction are unknown. Recent Drosophila studies suggest that residues with high homology to dehydrogenase catalytic subunits (17) and the oligomeric state (18) are critical for CtBP function during development, although these studies also do not clarify the substrate. Prior reports suggest that NAD(H) binding is necessary for PXDLS target binding (7), although others observe relatively high affinity of CtBP for the PXDLS target protein E1A in vitro in the absence of dinucleotide binding (16, 19). In some CtBP-interacting proteins, mutation of the PXDLS motif both decreases binding of CtBP and correlates with either abolished or reduced repressor activity, whereas in others, mutation of this motif has little or no effect on repression (9). Another study suggested CtBP is likely able to recruit DNA modifying enzymes in the absence of target protein binding (20), but needs to retain PXDLS protein interactions for promoter localization. Collectively, these studies suggest that NAD(H) is likely the initiator for a molecular cascade that assembles a functional CtBP co-repressor, but the order of assembly and how other CtBP domains/residues participate is not clear.

Here we describe a mechanism for a switch following NAD(H) binding that permits CtBP1 to assemble a functional co-repressor complex. Similar to some previous studies, we show that the intrinsic co-repressor activity of CtBP1 does not require dinucleotides binding, the dehydrogenase domain, or interaction with PXDLS targets. We find that residues within the amino-terminal domain, but not NAD(H) binding are necessary for target protein recruitment and the carboxyl terminus is required for tetramerization. By equilibrium dialysis we report that CtBP1 binds both NAD(H) and NADH equivalently and similarly to others we confirm that rather than enhancing the binding affinity of CtBP1 for target proteins, dinucleotide induces CtBP1 self-association. Finally, we show that a tryptophan residue (Trp318) is critical for permitting full dimerization to occur following NAD(H) binding, a step necessary for progression to tetramerization. We propose a model suggesting Trp318 is a switching tryptophan required for effective dimerization following NAD(H) binding and is necessary to permit carboxyl-terminal-mediated tetramerization.

EXPERIMENTAL PROCEDURES

Plasmids—Human CtBP1 (CtBP) was a gift of Dr. G. Chinnadurai (St. Louis University). CtBP2 constructs were PCR cloned from a human CtBP2 construct (ATCC) (21). GST-CtBP was previously described (22). Histidine-tagged CtBP was constructed by PCR cloning into pET23d (Novagen). GST fusions of either full-length wild-type adenovirus serotype 2 12S E1A and the D235A/L236S mutant were constructed by PCR cloning into pGEX-KG. Epitope-tagged CtBP plasmids were constructed by fusing either 2 copies of the FLAG epitope (2×FLAG) or a single Myc epitope to the amino terminus of CtBP1 using fused oligonucleotides in pcDNA3 (Invitrogen). Fusions of the GAL4 DNA-binding (residues 1–147) and the VP16 transactivation domains to the amino terminus of CtBP1 were constructed by PCR in pcDNA3. For some experiments, a 2×FLAG-CtBP1 cassette was inserted into the carboxyl-terminal GAL4 and VP16 fusion vectors to permit quantitation in mammalian two-hybrid and reporter assays. Site-directed mutagenesis of CtBP1 was performed using oligonucleotide replacement and cloning and sequences were confirmed. The reporter plasmids HSV-tk-Renilla luciferase, G5-tk-luciferase, and G5-TATA-luciferase were obtained from Dr. R. Maurer, Oregon Health and Science University (23).

Purified Proteins—Wild type 12S E1A protein was expressed in Escherichia coli BL21(DE3) pT-‘Trx as previously described (22). GST fusions of full-length CtBP1 (GST-CtBP1), full-length wild type 12S E1A (GST-E1A), and the carboxyl-terminal end (amino acids 165–243) 12S E1A were expressed and purified by standard techniques. Full-length wild type and mutant histidine-tagged CtBP proteins were expressed in E. coli BL21(DE3). Cells were grown to log phase, induced with 0.4 mM isopropyl β-D-thiogalactopyranoside, and harvested after 3 h of shaking at 37 °C. Bacteria were washed once in cold PBS (pH 7.4) and resuspended in Buffer A (25 mM Tris, 150 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, 400 mM PMSF) including 50 mM imidazole, and lysed by two passages through a French pressure cell. Cell lysates were cleared of insoluble debris by centrifugation at 30,000 × g for 40 min. CtBP proteins partially purified by nickel-nitrosoacetic acid resin (Qiagen) were further purified by Q-Sepharose anion exchange column chromatography. Fractions were pooled, concentrated, and dialyzed into 25 mM Tris–Cl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT and stored at −80 °C.

Cell Culture, Transfection, and Reporter Assays—Monolayer cultures of U2OS or CtBP1/CtBP2 null cells (gift of Dr. J. Hildebrand, University of Pittsburgh) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. For transient expression assays, transfections were carried out using Trans-IT (PanVera, Madison, WI). For luciferase reporter assays, cells were plated in 12-well plates at 3 × 10⁴ cells/well and transfected at 24 h. For each experiment, the total molar concentration of the CMV enhancer-promoter was held constant by addition of pcDNA3 and all assays utilized a constant amount of a HSV-tk promoter-driven Renilla luciferase plasmid as an internal transfection control (10 ng). Cells were harvested 24 h post-transfection, and firefly and Renilla luciferase reporter assays were performed with the Dual Luciferase Assy kit (Promega). For mammalian two-hybrid assays, GAL4 fusion vectors were transfected at a subsaturating concentration of plasmid that achieved a half-maximal plateau effect. This concentration was then held constant for the experiments with varying effectors for VP-16 containing plasmids. For activation assays, a luciferase construct with low basal activity containing a minimal TATA promoter and GAL4 DNA-binding domain upstream activating sequences was used as a reporter (750 ng). Constant concentrations of GAL4 DNA-binding domain containing plasmids (20 ng) and increasing concentrations of VP-16 containing the plasmids (1–200 ng) indicated were transfected using optimized conditions in triplicate and harvested after 36 h. For repression assays, a luciferase construct containing the highly active thymidine kinase (TK) promoter and the 5 GAL4 DNA-binding domain upstream activating sequence was used as a reporter.
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GAL4 containing plasmids were transfected in increasing concentrations and the results analyzed as above. Results were plotted as a ratio of the three firefly means divided by the three Renilla means (ratio relative light units) for each condition, with a S.E. for each. Each experiment was performed at least three times and the results shown are from single experiments. Western analysis for the expressed proteins was performed as a pooled sample of each triplicate condition, representing 10% of the input material analyzed in each assay.

**Antibodies**—Monoclonal α-FLAG (M2), 9E10 (α-Myc), α-FLAG-HRP (horseradish peroxidase), and α-Myc HRP (direct conjugates) were from Sigma. Anti-His6 (Covance) was used at 1:6000. Anti-CtBP monoclonals were from Santa Cruz Biotechnology. Secondary goat anti-mouse IgG-HRP (Bio-Rad) or direct conjugated fluorescent antibodies (Invitrogen) were used.

**Immunoprecipitation Assays**—U2OS cells were plated as noted above and transfected with 1 μg each of a CtBP-Myc and CtBP-FLAG tagged construct. After 36 h cells were harvested by washing twice in cold PBS (pH 7.4) and then scraped into cold immunoprecipitation (IP) buffer, 20 mM HEPES (pH 7.4), 200 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM dithiothreitol, 50 μg/ml of nuclease-free bovine serum albumin (New England Biolabs). The cells were passively lysed in equal volumes of IP buffer at 4 °C for 15 min (plus or minus the addition of 5 μM NAD−) and the chromosomal precipitate was removed. CtBP complexes were immunoprecipitated with either α-FLAG (M2) or monoclonal antibody 9E10 (α-Myc) at 4 °C for 2 h followed by Protein A-Sepharose (Sigma) at 4 °C for 2 h, washed extensively in IP buffer (≥ 5 μM NAD−) and equal amounts of isolates, and input samples were analyzed by a Western blot probed with either α-FLAG-HRP (M2; 1:10,000) or α-Myc-HRP (9E10; 1:2000) detected by enhanced chemiluminescence (ECL; PerkinElmer Life Sciences).

**Cross-linking**—Full-length, bacterially produced, purified wtCtBP (100 ng) was incubated in vitro in the presence or absence of NAD+ (500 nM) and increasing concentrations of glutaraldehyde for 30 min, the products were resolved by SDS-PAGE and detected by Western blot.

**Fluorescence Resonance Energy Transfer (FRET)**—Constructs encoding full-length wild type or mutant CtBP fusions with ECFP and EYFP (enhanced cyan and yellow fluorescent proteins, respectively; Clontech) were generated by PCR cloning. These constructs or expression vectors for fused EYFP or ECFP were transiently transfected pairwise in Cos-1b cells. After 48 h, whole cells were resuspended in Hanks’ balanced salt solution, placed in a quartz cuvette, and kept in suspension with a stir bar for measurement of fluorescent spectra. Emission spectra (450–550 nm) were recorded following excitation at 430 nm using a PTI steady-state spectrofluorimeter.

**Binding Assays and Gel Filtration**—GST-CtBP and GST-E1A binding assays were performed in 20 mM HEPES (pH 7.4), 200 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM dithiothreitol, 25 μg/ml of nuclease free bovine serum albumin. Each 500 μl of binding reaction contained 25 nM GST-E1A or GST-CtBP, increasing concentrations of either CtBP proteins (wild type and mutants) or wild type 12S E1A and NAD+ where indicated. Binding reactions were performed at 4 °C for 2 h and captured with glutathione-Sepharose beads, washed extensively with binding buffer, and analyzed by Western blot with α-His6 (Covance). Under the conditions of these assays, GST fusion proteins are limiting with respect to glutathione-agarose. For gel filtration experiments 500 μl of 2 μM purified CtBP proteins were separated on a Sephacryl S-200 16/60 column (Amersham Biosciences) in binding buffer (25 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) in the presence or absence of 5 μM NAD or NADH. Fractions (0.5 ml) were collected, TCA was precipitated and analyzed by Western blot (anti-His6) and fluorescent secondary antibodies and the blots were quantified (LiCOR imager). The data are plotted as a relative scale for signal intensity to correct for differences in protein recovery and Western variability. Each separation was performed at least three times. These data are representative from a single experiment.

**Equilibrium Dialysis**—Equilibrium dialysis was performed using purified recombinant CtBP proteins (10 μM) in binding buffer (25 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) on one side of a 10-kDa molecular mass cutoff membrane with either [adenine-2,3-3H]NAD+ or [4-3H]NADH in varying concentrations from 12 nM to 50 μM at 4 °C for 48 h. Twenty percent of the volume from each side was collected and diluted in scintillation fluid and counts were measured and the bound and free fractions were determined using standard equations and fit to a nonlinear regression $Y = B_{max} \times X/(K_d + X)$ using Prism (GraphPad).

**RESULTS**

**NAD(H) Binds to CtBP with High Affinity**—The primary structure of CtBP resembles that of the D2-hydroxyacid dehydrogenases and includes a dinucleotide binding motif (GXXGXXG) and a homologous dehydrogenase domain (a His-Glu pair His315-Glu295 and Arg266). We used three-dimensional modeling (Fig. 1A) to examine the various structural motifs based on the related protein 3-phosphoglycerate dehydrogenase (24) and crystallographic models (14, 16) to design mutants predicted to disrupt critical functions in NAD(H) binding, target protein binding, and the catalytic residues. The CtBP dinucleotide-binding pocket is shown with a space-filling model of NAD+ to illustrate the amino acid residues (sticks) predicted to be important in the dinucleotide interaction. Previous crystallization studies (14) suggested that residues within the glycine-rich dinucleotide binding motif (Gly181-Gly186, gold) stabilize the pyrophosphate moiety of NAD, whereas the main chain (Arg184, Val185, Cys237, and Thr318) and side chain (Arg184, Asp204, Asn240, and Asp290) residues stabilize the different NAD moieties via hydrogen bonds. The Cys237 residue is not part of the conserved dinucleotide binding sequence, but is thought to lie within the binding pocket and therefore the bulky substitution of methionine for cysteine (C237M) was predicted to disrupt NAD+ binding.

To determine the role of dinucleotide in the biochemical functions of CtBP, we first examined the NAD+ binding properties of CtBP1 and mutant proteins by equilibrium dialysis. A representative set of binding isotherms are shown (Fig. 1B) and summarized based on their functional classification in CtBP1 (Fig. 1C). Wild type CtBP1 binds NAD+ and NADH with similar affinity with a calculated $K_d = 500 \text{ nM}$ ($K_d^{NAD^+} = 393 \pm 30$...
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FIGURE 2. NAD$^+$ enhances the binding of CtBP1 to E1A, but not E1A to CtBP1. A, a purified wild type CtBP1 in increasing concentrations (25 nM, 100 nM) exhibited increased binding to 25 nM GST-E1A with the addition of 5 μM NAD$^+$, whereas the addition of either 25 or 100 nM CtBP1(C237M), an NAD$^+$-binding mutant, does not. B, NAD$^+$ does not enhance the binding of wild type E1A (25, 50, 75, and 100 nM) to 25 nM GST-CtBP1 in the reciprocal experiment. Input was 20%. Under the conditions of these assays, GST fusion proteins are limiting with respect to glutathione-agarose.

FIGURE 1. NAD(H) binding to CtBP1 and effects of mutagenesis. A, NAD binding pocket of CtBP1 as derived from crystallization of full-length human CtBP1 with NAD (modified from Protein Data Bank entry 1MX3 (Refs. 14 and 37)). Backbone oxygens are omitted for clarity. The side and peptide chains of CtBP1 are shown with NAD (modified from Protein Data Bank entry 1MX3 (Refs. 14 and 37)). The predicted glycine-rich loop (G181GLGRGV185) interacts with the pyrophosphate moiety of NAD$^+$ (Fig. 1A) and mutation of these (G183A, R184D, V185D, and G186D or C237M) exhibited no significant binding above background up to 20 μM NAD$^+$ (not significant binding (nsb); Fig. 1C and not shown). The D-specific isomer 2-hydroxyacid dehydrogenases characteristically employ a histidine carboxylic acid pair as a proton shuttle necessary for catalytic activity (25) and mutants in this domain demonstrated reduced NAD$^+$ affinity (H315A $K_{d}^{\text{NAD}^+} = 7.5$ μM, and E295A $K_{d}^{\text{NAD}^+} = 2.6$ μM) relative to the wild type CtBP. A residue (Arg$^{266}$) that stabilizes the carboxylic acid of NAD$^+$ also exhibited a reduction in binding (R266A $K_{d}^{\text{NAD}^+} = 1.6$ μM), comparable with the catalytic mutants. These results demonstrate that structurally predicted disruptions correspond to relative and predicted changes in the NAD$^+$ affinity for CtBP allowing each class of mutant to be studied systematically based on these characteristics.

NAD$^+$ Enhances the Binding of CtBP1 to the Target Protein E1A, but Does Not Enhance the Binding of E1A to CtBP1—Previous studies have suggested that NAD(H) either changes affinity for target proteins (7), or induces dimer/oligomerization (14, 15, 26). We asked if CtBP1 self-associated in a dinucleotide-dependent manner to increase the stoichiometry of binding to PXDLS-containing targets or change the affinity for a target protein. Increasing amounts of both CtBP1wt or CtBP1(C237M) interacted with GST-E1A in the absence of NAD$^+$; however, only wild type CtBP1 demonstrated NAD$^+$-dependent enhanced binding (Fig. 2A). Under the conditions of these assays, GST fusion proteins are limiting with respect to glutathione-agarose and the added proteins. In the converse experiment, E1A binding to GST-CtBP1 increased in a concentration-dependent manner, but was not enhanced by NAD$^+$ (Fig. 2B). These data indicate that dinucleotide enhances CtBP1 stoichiometric association with a target protein and not the overall affinity.

Dinucleotide Binding Enhances the Association of CtBP1 to PXDLS-containing Targets by Promoting CtBP1 Self-association—To determine whether the interactions of CtBP1 with NAD$^+$ and target proteins are coupled, we performed GST-binding experiments with mutant CtBP proteins. By GST pulldown experi-
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**FIGURE 3.** An intact NAD$^+$-binding domain is required for self-association, but not for binding to the PXDLS containing target protein E1A. A, GST-pulldown assay using 25 nM GST-E1A-Cterm (125 E1A amino acids 165–243) reacted with the indicated increasing concentrations of wild type CtBP1, the NAD$^+$ binding mutants (C237M, V185D, G183A), or the catalytic mutants (E295A) were able to bind to E1A in the absence or presence of NAD$^+$. B, CtBP1 self-associates in direct proportion to its ability to bind NAD$^+$ as shown by significantly enhanced binding of wild type CtBP1 (wtCtBP) to 25 nM GST-CtBP1 (wild type) in the presence of 5 μM NAD$^+$ (left panels), minimal association with the catalytic mutants and no self-association of the NAD$^+$ binding mutants. Excess NAD$^+$ (30 μM, right panels) in parallel reactions enhances the self-association directly proportional to the $K_d$ for NAD$^+$. Under the conditions of these assays, GST fusion proteins are limiting with respect to glutathione-agarose. Recovery of proteins was consistent between experiments. Reactions were performed in parallel and a representative data set of at least 3 trials for each protein is shown.

We then asked if NAD$^+$ induced self-association based on the predicted $K_d$ for NAD$^+$. We then asked if NAD$^+$ induced self-association based on the predicted $K_d$ for NAD$^+$. For each protein because the catalytic mutants that retained NAD$^+$ binding activity (E295A, H315A) exhibited some self-association with higher NAD$^+$ concentrations (Fig. 3B, right panel, 30 μM). NAD$^+$ dose-response curves for CtBP1 binding to GST-CtBP1 reveal a half-maximal effect at ~500 nM, consistent with the affinity for NAD$^+$ and NADH we determined by equilibrium dialysis (Fig. 1C). These data demonstrate that CtBP1 will at least dimerize (self-associate) in response to NAD$^+$ in direct proportion to its $K_d$ and that in vitro, this activity is independent of its ability to bind to a target protein containing PXDLS.

**CtBP1 Forms a Tetramer in the Presence of NAD$^+$**—Previous studies have also suggested that CtBP might oligomerize in vitro (15) and its homologue 3-phosphoglycerate dehydrogenase forms a tetramer. A crystal structure of the central portion of CtBP1 (amino acids 28–353) bound to NAD$^+$ formed only a dimer (16); therefore, we asked whether NAD$^+$ could induce multimerization of CtBP1. Using gel-filtration, wild type CtBP1 was eluted within its predicted monomeric molecular mass of 48 kDa (Fig. 4A, Control). In the presence of NAD$^+$ or NADH CtBP1 eluted at a $M_r$ consistent with formation of both dimers and a homotetramer. Interestingly, wild type CtBP2 undergoes more NAD$^+$ independent homodimerization, and then tetramerizes in the presence of NAD(H) (Fig. 4B), confirming previous work that CtBPs can self-associate without dinucleotide (26) but also showing that CtBP2 will also form a homotetramer despite some sequence divergence from CtBP1 at the C terminus. These data confirm that both CtBP1 and CtBP2 will assemble into a homotetramer through a dimeric intermediate.

Structural modeling suggested that the C terminus might be important for dimer-dimer interactions and would explain why crystal structures of truncated CtBP1 revealed homodimers (16). We used a purified CtBP1 lacking the carboxyl-terminal domain and subjected it to gel filtration. CtBP1 (1–351) monomers eluted at their predicted molecular mass (39 kDa) but could only dimerize in the presence of NAD$^+$ (Fig. 4A, Control). In contrast, CtBP2 (1–351) monomers eluted at their predicted molecular mass but did not form a dimer (Fig. 4B, Control). Therefore, we used a mammalian two-hybrid system to establish if CtBP1 self-association is dependent on NAD$^+$ binding activity (E295A, H315A) exhibited some self-association with higher NAD$^+$ concentrations (Fig. 3B, right panel, 30 μM). NAD$^+$ dose-response curves for CtBP1 binding to GST-CtBP1 reveal a half-maximal effect at ~500 nM, consistent with the affinity for NAD$^+$ and NADH we determined by equilibrium dialysis (Fig. 1C). These data demonstrate that CtBP1 will at least dimerize (self-associate) in response to NAD$^+$ in direct proportion to its $K_d$ and that in vitro, this activity is independent of its ability to bind to a target protein containing PXDLS.

**Intrinuclear CtBP1 Self-association Requires Dinucleotide Binding But Not for Interaction with PXDLS Targets**—To determine whether the intranuclear requirements for CtBP self-association were consistent with our in vitro data we used a mammalian two-hybrid system to establish if CtBP1 self-association occurs in vivo. In these experiments, the association of a DNA bound, Gal4 DNA-binding domain fusion protein to a protein...
fused with the herpesvirus VP-16 transcription activation domain reconstitutes a strong transcriptional activator.

Wild type CtBP1 strongly activates in the mammalian two-hybrid assay indicating direct binding to E1A and is dependent upon the presence of an intact PXDLS because the mutant E1A(D235A/L236S) (∆233PLDLS → PLasS) protein is unable to interact with wild type CtBP1 (Fig. 6A). Both NAD⁺ binding mutants (C237M, R184D, V185D) and catalytic mutants (E295A, H315A, E295A/H315A) activate similar to CtBP1wt, demonstrating intact direct interaction with E1A even in the absence of NAD⁺ binding (Fig. 6B and data not shown). In replicate experiments, there were not specific differences in the two-hybrid experiments for the amount of expressed protein required for effects seen using wild type or mutant CtBPs. Variability in the amount of expressed CtBP proteins demonstrated similar results with plateau effects within the midrange of the transfection range. Using structural predictions from the CtBP/BARS protein (16), the CtBP1 N-terminal domain is likely crit-
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FIGURE 5. Purified CtBP1 can tetramerize in vitro by cross-linking. Wild type CtBP1 (wtCtBP) or the NAD-binding mutant CtBP1(C237M) were incubated in vitro with or without 5 μM NAD+(−/+) NAD in the presence of glutaraldehyde, separated by electrophoresis, and a Western blot for CtBP1 performed. Only wild type CtBP1 forms first a dimer then a tetramer at increasing glutaraldehyde concentrations in the presence of NAD+. The relative mobility (M₉) for the expected positions of CtBP monomers, dimers, and tetramers are shown at the right.

ical for target protein interaction. Modeling suggested that mutations in CtBP1 Ala²⁵² and Val⁶⁶ would disrupt contact with the X and Leu residues of the PXDLS motif. Purified CtBP1 A52D and V66R exhibited $K_\text{d}^{\text{NAD}^+}$ values similar to wild type CtBP1 (Fig. 1) and were unable to interact with E1Act in either GST pulldown or mammalian two-hybrid experiments, but retained self-association in response to NAD$^+$ (Fig. 6, C and D). These data demonstrate that CtBP1 interaction with PXDLS containing targets is not dependent upon binding to dinucleotide and requires both an intact PXDLS and key CtBP1 amino-terminal residues. Additionally, these data show that although the mammalian two-hybrid experiment is not a direct in situ binding assay, the interactions between the two proteins are direct.

Next we asked if intranuclear CtBP1 self-association depends on NAD$^+$ binding. By mammalian two-hybrid assay, CtBP self-association occurred in wild type CtBP1 and in direct proportion to the $K_\text{d}^{\text{NAD}^+}$ of the various mutant proteins with no binding seen in NAD$^+$ binding mutants (C237M shown; R184D, V185D), whereas the catalytic mutants (E295A/H315A and E295A) produced an interaction, whereas H315A, which has an almost 3-fold higher $K_\text{d}^{\text{NAD}^+}$ than E295A, did not produce a significant signal (Fig. 6E). These results are in agreement with our GST binding experiment (Fig. 3) and indicate that CtBP1 requires sufficiently intact NAD$^+$ binding for intranuclear self-association.

These experiments do not exclude the possibility that transected CtBP1 may indirectly interact with endogenous CtBP proteins in a multimeric complex. To address this possibility, we first performed a cross co-immunoprecipitation experiment by transfecting both single epitope-tagged FLAG-CtBP1wt or Myc-CtBP1wt into U2OS cells and immunoprecipitating with either anti-FLAG or anti-Myc, detecting the material by Western blot (Fig. 7A). 2×FLAG-CtBP1wt was recovered in the IP-Myc condition, demonstrating direct association of the two separate proteins. Recovery was enhanced by the inclusion of NAD$^+$ and the reciprocal experiment with anti-Myc yielded similar results (data not shown). Self-association was not seen with co-transfection with the NAD-binding mutant CtBP1(C237M) (Fig. 7A, lower panel). Furthermore, we utilized fluorescence resonance energy transfer (FRET) to determine if CtBP1 was in close proximity to other CtBP molecules in living cells and transfected U2OS cells with ECFP- or EYFP-fused CtBP1 constructs and controls. ECFP shows emission maxima at 475 and 501 nm, whereas close proximity of the ECFP-CtBP1 fusion with CtBP1-EYFP results in FRET and the appearance of a peak at 527 nm due to sensitized emission of YFP (blue curve, CtBP-CFP1 + CtBP1-YFP) (Fig. 7B). In the absence of CtBP1 fusion, no significant interaction with EYFP was seen (orange curve, CtBP1-CFP + EYFP), or with ECFP (violet curve, ECFP + CtBP-YFP) as determined by the ratio of fluorescence intensity, I₄₇₅ nm/I₅₂₇ nm. Taken together, these data demonstrate direct intranuclear CtBP self-association that is dependent upon an intact NAD$^+$-binding domain, although we cannot distinguish dimerization from tetramerization with these methods.

Recruitment of a Repression Complex by CtBP1 Does Not Depend Upon NAD$^+$ Binding—Our results demonstrate that although NAD(H) binding is not required for interaction with PXDLS containing repressor proteins, NAD(H) occupancy is required for tetramerization in vitro and intranuclear self-association. To determine the role of dinucleotide occupancy on the intrinsic activity of CtBP1 as a co-repressor, we examined CtBP1 and mutant proteins as GAL4 DNA-binding domain fusions in the repression of a GAL4 upstream activating sequence-regulated constitutively active HSV-thymidine kinase promoter-reporter (pG5-tk) with luciferase assays.

Both wild type CtBP1 and CtBP2 exhibited similar repression profiles (Fig. 8A). Wild type CtBP1 and its NAD$^+$ binding (C237M, R184D, V185D) and catalytic mutants (E295A/H315A, E295A/H315A) were equivalent (Fig. 8B and data not shown). Mutants (A52D, V66R) that fail to interact with a PXDLS target protein (Fig. 6, C and D) also act as repressors, similar to wtCtBP1 in this assay (Fig. 8C). To exclude the recruitment of endogenous wild type CtBP from the U2OS cells to the mutant protein GAL4 fusions we performed the same experiment in CtBP1/2 homozygous null cells (3) and found no difference in the ability of wild type CtBP1 (Fig. 8D) or CtBP2 mutants to repress transcription (data not shown). These data suggest that in a tethered assay, the recruitment of DNA/histone modifying enzymes to CtBP does not absolutely require NAD$^+$ binding, a functional catalytic domain or binding to a PXDLS containing target.

A Switching Tryptophan Mediates NAD$^+$-dependent CtBP1 Self-association—Our experiments demonstrate that NAD$^+$ induces a switch of full-length CtBP1 from monomer to tetramer through dimerization of dimers. The available crystal structures of CtBP lack resolution of the C-terminal tetramerization domain; however, by modeling the dimer structure we postulated a mechanism for a NAD-dependent dimerization switch (Fig. 9A). We hypothesized that a strand exchange mechanism contributes to the dimerization interface and residues 159–166 of α-helix C (αC) of the nucleotide-binding domain of one subunit that come in close contact with the “hinge” region between the N-terminal and nucleotide-binding domains of the opposite subunit (Fig. 9A). In particular, hydrophobic residues located on αC (Val¹⁵⁶ and Ile¹⁶²) contact Trp³¹⁸ within the NAD binding pocket where Trp³¹⁸ forms a
hydrogen bond to the nicotinamide moiety of NAD\(^+\) to communicate NAD\(^+\) binding to dimerization. CtBP1(W318F) binds NAD\(^+\) with reduced affinity (\(K_d\) \(NAD^+ = 2.4 \mu M\)), interacts with E1Act similar to wild type CtBP1, and self-associates with CtBP1 with reduced affinity comparable with the catalytic mutants (data not shown). By gel-filtration CtBP1(W318F) lacks tetramerization although it minimally dimerizes (Fig. 9B) in contrast to the catalytic mutants (E295A, H315A) that at least partially dimerize and tetramerize. These data suggest that NAD\(^+\) binding alone is not enough to complete dimerization and initiate tetramerization and that Trp 318 constitutes a switch following NAD(H) binding to initiate dimerization by indirectly recruiting and incorporating a helix from its dimeric partner into the structure of the nucleotide-binding domain.

**DISCUSSION**

The relationship of the biochemical activities of CtBP to its co-repressor function remains an open question with often conflicting data from within and between in vitro and in vivo studies. The mechanism and ordering for NAD\(^+\) binding, dehydrogenase activity, dimerization, oligomerization, binding of target proteins, recruitment of histone/DNA modifying enzymes, and the regulated dismissal of these individual components to affect individual gene expression are not clear. The role of CtBP in numerous oncogenesis pathways have made it a

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**FIGURE 6.** CtBP requires NAD for intra-nuclear self-association, but not binding to PXDL5 target proteins. A, mammalian two-hybrid experiment using a Gal4 DNA-binding domain-E1A fusion protein Gal4-E1Awt (wild type) or one mutated at the PXDL5 CtBP binding site (D235A/L236S). Transfected 2×FLAG-CtBP(wt)-VP16 constructs (μg of DNA), the G5-TATA reporter alone (C) or +Gal4-E1A (D) as controls are shown as triplicate luciferase assays from a single experiment. An intact PXDL5 is required for intracellular binding of wtCtBP1 to the target protein. Error bars = S.E. are indicated. Western blots with anti-FLAG indicate the transfected CtBP1 protein (below). B, mammalian two-hybrid using a Gal4 DNA-binding domain-E1A fusion protein and CtBP1-VP16 proteins and performed as in A. Gal5-TATA (G5-TATA) and Gal5-TATA+Gal4(DNA-binding domain)-E1A controls (Gal4-E1A) are indicated. Western blots as in A (below). C, GST-binding assays for target binding mutants CtBP1(A52D) and -(V66R). Top, 25 nM GST-wtCtBP1 reacted with increasing concentrations (above) of the (His\(_6\))-proteins indicated (right) or with GST alone (GST) in the presence or absence of 5 μM NAD. Western blots for anti-His\(_6\) were 20% input. Bottom, the same experiment in parallel using 25 nM GST-wtE1Acterm (G5-TATA) and Gal4(DNA-binding domain)-E1A controls (Gal4-E1A) are indicated. Western blot as described in A (below). D, mammalian two-hybrid analysis performed as in A using 2×FLAG-CtBP1-VP16 fusion constructs of A52D and V66R and GAL4-E1A show no interaction. Western blot as described in A (below). E, mammalian two-hybrid analysis as in A using the Gal4 DNA-binding domain CtBP1 fusion proteins indicated above the bars co-transfected with their corresponding CtBP1-VP16 fusions (below) and Western blot as described in A.
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A tempting target for modifying gene expression in certain cancer models; however, inhibiting NAD\(^+\) binding may not be adequate to disrupt the co-repressor function of CtBP because we and others suggest that in some contexts co-repression does not exclusively rely on NAD(H) binding (Fig. 8) (27, 28), suggesting both that monomeric CtBP1 (18) or directed assembly of CtBP1 are used to regulate intra-nuclear CtBP1 on and off chromatin and how monomeric, dimeric, or homotetrameric CtBP1 are not exclusively downstream of NAD\(^+\) (18, 30). Taken together, these results suggest that the activities of CtBP1 are not exclusively downstream of NAD\(^+\) binding and that CtBP1 can interact with its targets without dimerizing, but dispensable for target binding and repression activity. We localized target binding to residues at the N terminus that are predicted to contact the PDXLs motifs, similar to the structural predictions made for CtBP/BARS (16). These mutations (A52D, V66R) abrogate interaction with E1A, but do not interfere with NAD\(^+\)-induced dimerization (Fig. 6, C and D) nor the ability to change repression activity (Fig. 8B), suggesting that both the interaction with target proteins and dimerization are not required to recruit DNA/histone modifying enzymes. We previously observed that a soluble CtBP1(A52D) may act as a dominant negative mutant in a cellular model of p21 expression (21), further suggesting that CtBP1 can interact with its binding partners when not bound to chromatin. Interestingly, the possibility of a functional monomeric CtBP pool has been proposed (18, 30). Taken together, these results suggest that the activities of CtBP1 are not exclusively downstream of NAD\(^+\) binding and that CtBP1 can interact with its targets without dimerizing, potentially having a regulatory role as a monomer or dimer. These data further stress the need to understand the cycling of intra-nuclear CtBP1 on and off chromatin and how monomeric, dimeric, or homotetrameric CtBP1 are used to regulate both CtBP1 activity and gene expression.

Based on structural homology to other dehydrogenases, CtBP almost certainly harbors dehydrogenase activity, however, the substrate(s) and role in transcriptional repression remains unclear. Previous studies have demonstrated the oxidation of NADH to NAD\(^+\) only in the presence of pyruvate and very high concentrations of protein (14, 15, 32). In our experiments using highly purified recombinant CtBP1, we are unable to demonstrate either oxidation of NADH (with conversion of pyruvate to lactate) or the reduction of NAD\(^+\) to NADH in the presence of lactate (D\(_{\text{Absorbance}}\) at 340 nm; data not shown), suggesting that the previously reported dehydrogenase activity of CtBP1 may be due to co-purification of trace amounts of contaminating lactate dehydrogenase. Mutations in the catalytic triad impair developmental functions in Drosophila (20) and these residues are necessary for CtBP mutant rescue (17); however, other analyses have not found an intact dehydrogenase domain necessary for co-repressor activity (27, 28, 33). We find that either the human or Drosophila catalytic mutants self-associate/dimerize in direct relationship to their \(K_d\) for NAD\(^+\) and are unable to assemble into a tetramer (Figs. 3 and 6E and data not shown), yet retain the ability to repress transcription in a

for dehydrogenase activity (Figs. 3, 6, and 8). Similar to previous reports (14, 15), we find that CtBP1 binds NAD\(^+\) and NADH with near equal affinity (\(K_d \approx 500 \text{ nM}\)) at a level well below most estimates of intracellular NAD(H) concentrations of \(\approx 300 \mu\text{M}\) (38), suggesting that preferential binding of one does not play a regulatory role in CtBP activity as previously suggested (32). Mutations in the NAD\(^+\)-binding domain (G183A, R184D, V185D) or within the binding pocket (C237M) abrogate binding, whereas mutations in the dehydrogenase domain (E295A, H315A, E295A/H315A, R266A) reduce the \(K_d\) for NAD\(^+\) >5–10-fold. Mutation within either of these domains does not impair the ability to either interact with PDXLs containing target proteins (Figs. 2, 3, 6, and 7) or modify the ability to repress transcription from a heterologous promoter (Fig. 8). These results suggest that dinucleotide is important for CtBP dimerization, which is predicted to contact the P\(^R\) and E1A dimerization (Fig. 6, C and D) as previously observed that a soluble CtBP1(A52D) may act as a dominant negative mutant in a cellular model of p21 expression (21), further suggesting that CtBP1 can interact with its binding partners when not bound to chromatin. Interestingly, the possibility of a functional monomeric CtBP pool has been proposed (18, 30). Taken together, these results suggest that the activities of CtBP1 are not exclusively downstream of NAD\(^+\) binding and that CtBP1 can interact with its targets without dimerizing, potentially having a regulatory role as a monomer or dimer. These data further stress the need to understand the cycling of intra-nuclear CtBP1 on and off chromatin and how monomeric, dimeric, or homotetrameric CtBP1 are used to regulate both CtBP1 activity and gene expression.

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FIGURE 8. CtBP1 does not require NAD binding, catalytic domain, or target binding activities for repression. 2×FLAG-CtBP-Gal4 DNA-binding domain constructs (Gal4-CtBP) were co-transfected into U2OS cells with a Gal4 (5X) binding site-TK-luciferase reporter (TK-luc) and luciferase assays were performed on parallel, triplicate samples. A, wild type CtBP1 and wild type CtBP2 showed no differences in repression. B, similar results are seen with wild type CtBP1, NAD-binding mutant (C237M) dehydrogenase domain mutants (E295A, H315A), or C, PXDSLS-binding domain mutants (AS2D, V66R). D, repression by CtBP1wt in CtBP1 + 2 null cells is similar to that obtained in CtBP containing cells. Error bars ± S.E. are indicated. Western blot (anti-FLAG) indicating the transfected CtBP1 protein is shown below.

plasmid-based assay (Fig. 8). Similar results were seen for additional mutants involved in NAD+ binding or the dehydroge-
nase domain (G183A, R266A, R266Q) (Fig. 8 and data not shown). Part of the catalytic mutants effect is likely reliant upon their \(K_{d}^{NAD+}\) and potentially the ability of the protein to stabilize the interaction (16). Some studies (7) have used CtBP1(G183) to determine that dinucleotide binding was not required for co-repressor activity in plasmid-based assays, but we and others (18) have found this protein to be unstable in transfected cells. Differences in the expression system for purified CtBP (bacterial versus insect cells) and in vitro versus cell-based assays are likely to account for other experimental discrepancies. For example, it is likely that in tethered assays, which artificially bring CtBP to the context of DNA, that only the domains responsible for the recruitment of DNA modifying enzymes are required and therefore the elimination of target binding, NAD(H) binding, or the catalytic residues will not alter the ability of CtBP1 to recruit chromatin modifying enzymes. CtBP1 self-association by FRET, co-immunoprecipitation (Fig. 7), and two-hybrid experiments (Fig. 6) clearly show intra-nuclear self-association, but how CtBP1 maintains a pool of monomers or if CtBP1 is immediately directed to associate by NAD(H) or responds to varying intra-nuclear dinucleotide concentrations (7, 14) has not been definitively described using a cellular model of gene expression. CtBP1 monomers may have important biologic activities as recently shown in Drosophila for Wingless targets (18) and the ability of CtBP1 to recruit chromatin-modifying enzymes as a monomer may have important regulatory functions.

To date, CtBP crystal structures (CtBP1, CtBP/BARS) have been determined without the last 80–90 amino acids (14, 16, 31) and revealed dimers, whereas in vitro experimental data suggested the CtBP could multimerize (12, 15). Our data show that the discrepancy likely results from the carboxyl terminus being critical for assembling a tetramer because a CtBP1 truncated at amino acid 351 forms dimers, but not tetramers (Fig. 4). By examining the crystal structure of full-length CtBP1 bound to NAD+, modeling the binding pocket (Fig. 1A) and comparing with the previous structural data (16) and a similar mechanism in D-3-phosphoglycerate dehydrogenase (34), we hypothesized that Trp318 forms a critical switch between the binding of NAD+ and dimerization through a strand exchange mechanism with the opposite binding partner (Fig. 9), which subsequently allows tetramer formation. In this model, NAD+ occupancy of one CtBP1 subunit would stabilize the strand exchange mechanism for dimerization through interaction of hydrophobic residues on \(\alpha\)-helix C of one CtBP1 monomer with Trp318 of its dimeric partner. This positions the carboxyl termini of each subunit outward and able to contact a second CtBP1 dimer pair because effective dimerization would approximate each carboxyl terminus away from the center of the dimer pair, but available to interact and direct oligomerization. The twin PXDLS domains are available for docking on both the top and bottom of the homotetramer, positioned at 70–75 Å apart, which is about 2.5 DNA helical turns. The top PXDLS-binding domains are free to interact with CtIP or possibly further CtBP oligomers. The recruitment of DNA modifying enzymes to the exposed regions of CtBP1 would occur close to the heterochromatin surface. Mutating Trp318 diminishes NAD+ binding similar to that of the catalytic mutants (Fig. 1), but in contrast to those proteins a CtBP(W318F) protein minimally dimerizes but cannot tetramerize (Fig. 9). Trp318
may therefore be a critical residue for directing effective dimerization after NAD$^+$ binding. This step is required to position the carboxyl-terminal domains for tetramerization. The identification of this switch in CtBP1 is crucial for studying the dissociation of NAD$^+$ binding from dimerization and to determine the role of tetramer formation in CtBP1 transcriptional repression. This strand transfer mechanism will allow further investigation into CtBP1 structural disruptors that can modify gene expression and possibly have relevance for gene-specific targeting in cancer (35, 36).

Collectively, these results suggest a model for transcriptional repression by CtBP1 where NAD(H)-dependent dimerization followed by dimer positioning and homotetramerization permits recruitment of a repression complex through the bridging of additional CtBP1 subunits, depending on the total concentration of intra-nuclear NAD(H) and not its oxidation state. In
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this model (Fig. 10), CtBP1 binds to a DNA-bound repressor through the amino terminus at its PXDLS site. NAD(H)-dependent dimerization occurs with Trp318 required to effectively induce the strand switch, bringing the dimer pairs into a spatial context permissive for recruitment of addition CtBP1 subunits (homotetramerization) and finally forming a scaffold for the recruitment of a multiprotein histone modification complex, through the N terminus of each subunit. Potentially, intranuclear NAD(H) changes could lead to dissociation of CtBP1 oligomers and relief of repression of CtBP1-dependent promoters. Alternatively, the dehydrogenase activity of CtBP1 may serve a regulatory function with its associated histone modification complex or in regulated dismissal of the co-repressor complex. Further work in mammalian cells is needed to better understand the differentiation of CtBP1 function as monomers and multimers and relate the regulated transition directed by each of the biochemical properties of CtBP1 to its effects on gene expression.

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