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Crystal structures of human CtBP in complex with substrate MTOB reveal active site features useful for inhibitor design

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ABSTRACT

The oncogenic corepressors C-terminal Binding Protein (CtBP) 1 and 2 harbor regulatory D-isomer specific 2-hydroxyacid dehydrogenase (D2-HDH) domains. 4-Methylthio 2-oxobutyric acid (MTOB) exhibits substrate inhibition and can interfere with CtBP oncogenic activity in cell culture and mice. Crystal structures of human CtBP1 and CtBP2 in complex with MTOB and NAD^+ revealed two key features: a conserved tryptophan that likely contributes to substrate specificity and a hydrophilic cavity that links MTOB with an NAD^+ phosphate. Neither feature is present in other D2-HDH enzymes. These structures thus offer key opportunities for the development of highly selective anti-neoplastic CtBP inhibitors.

1. Introduction

The paralogous transcription coregulators C-terminal Binding Proteins (CtBP) 1 and 2 are critical modulators of numerous cellular processes; overexpression of both has been linked to multiple human cancers. CtBP1 was initially identified due to its interaction with the C-terminal region of the adenovirus E1A oncoprotein and modulation of E1A transforming activities [1,2]. CtBP functions as a transcriptional regulator by tethering chromatin remodeling proteins, such as histone deacetylases, histone methyl transferases, and histone demethylases, to DNA bound transcription factors [3,4]. Alternative splice forms of CtBP1 and 2 also have non-nuclear functions in the Golgi and at synapses [5]. CtBP1 and 2 are unique among transcription factors in the incorporation of a D-isomer specific 2-hydroxyacid dehydrogenase (D2-HDH) domain, which reduces or oxidizes substrates utilizing coenzyme $NAD(P)^+$/NAD(P)H [6]. The D2-HDH family (Fig. S1), which is not otherwise involved in transcriptional regulation, includes bacterial D-lactate dehydrogenase (D-LDH) and D-hydroxyisocaproate dehydrogenase (D-HicDH) and, importantly for the development of inhibitors specific for human CtBP, the human enzymes glyoxylate reductase/hydroxypyruvate reductase (GRHPR) and phosphoglycerate dehydrogenase (PHGDH) [7–10]. CtBP recruitment of coenzyme nicotinamide adenine dinucleotide (NAD^+) or NADH induces dimerization [11], an event necessary for transcriptional repressor activity, linking enzymatic function and transcriptional activity [12]. With a reported 100 fold higher affinity for NADH than NAD^+ [13], CtBP may be able to respond to the redox state of the cell, increasing repressor function when stimuli, such as hypoxia and high extracellular glucose levels, increase the NADH/NAD^+ ratio [14,15].

Mounting evidence implicates CtBP repressor function in human cancer. CtBP corepression activity targets pro-apoptotic factors (Bik, Noxa), cytoskeletal/cell adhesion molecules (keratin-8, E-cadherin) [16], and tumor suppressors ($p16^{INK4a}$, $p15^{INK4b}$) [4], facilitating the epithelial to mesenchymal transition (EMT), conferring resistance to apoptosis and promoting metastasis and oncogenesis [17]. CtBP is targeted for degradation by individual or combined effects of multiple tumor suppressors including adenomatous polyposis coli (APC) [18,19], homeodomain-interacting protein kinase 2 (HIPK2) [20], JNK1 [21], and alternative reading frame (ARF) [22]. Consistent with these cellular effects of CtBP, overexpression of CtBP is observed in the majority of human colon, prostate, ovarian, and breast cancers [23–27].

A substrate for CtBP catalysis, 4-Methylthio 2-oxobutyric acid (MTOB) antagonizes CtBP transcriptional regulation [23,27,28]. MTOB induced apoptosis through displacement of CtBP2 from

Abbreviations: CtBP, C-terminal Binding Protein; MTOB, 4-Methylthio 2-oxobutyric acid; D2-HDH, D-isomer specific 2-hydroxyacid dehydrogenases; GRHPR, glyoxylate reductase/hydroxypyruvate reductase; PHGDH, phosphoglycerate dehydrogenase; NAD, nicotinamide adenine dinucleotide; ARF, alternative reading frame; HIPK2, homeodomain-interacting protein kinase 2; APC, adenomatous polyposis coli

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the *Bik* promoter in HCT116 colon cancer cells [23]. Administering MTOB in mouse xenograft models resulted in decreased tumor burden and prolonged survival for MTOB treated mice compared with untreated mice [23]. Additionally, MTOB evicted CtBP from target promoters in breast cancer cell lines, shifting phenotypic indicators (E-cadherin/vimentin) from mesenchymal to a more epithelial phenotype [27]. Although high MTOB concentrations (mM) are required for (substrate) inhibition of CtBP, MTOB's clear inhibitory effect on cancer cells provides proof of principle that small molecules could be developed to effectively treat cancers specifically regulated by CtBP activity.

Crystal structures of both CtBP1 and 2 have been reported [29–31], but none are in complex with potential substrates. We report here crystal structures for both human CtBP1 (28–353) and CtBP2 (33–364) minimal dehydrogenase domains in ternary complexes with coenzyme NAD^+ and ligand MTOB. These structures reveal unique active site CtBP features, including a prominent tryptophan and a hydrophilic channel, which may be useful for the design of highly selective inhibitors.

2. Materials and methods

2.1. Protein expression and purification

As described more fully in the Supplementary material, both CtBP1 and 2 were expressed as His_6-tagged proteins in BL21-CodonPlus®(DE3)-RIL competent cells (Stratagene). Both proteins were purified using NiNTA beads (Qiagen) followed by size-exclusion on a Superdex 75 column. The His_6 tag was cleaved from CtBP2 with Thrombin (Novagen) prior to the size exclusion step, but was not cleaved from CtBP1.

2.2. Crystallization of ternary CtBP2/NAD^+/MTOB complex

Protein (20–25 mg/ml) incubated with a 50 M excess of MTOB overnight at 4 °C was mixed in a 2:1 ratio with buffer and crystallized by hanging drop vapor diffusion in 24 well VDX plates. The highest quality crystals were grown in buffer containing 200 mM, potassium nitrate, 15–20% PEG 3350, and 100 mM bis tris propane pH 7.0. Crystals typically grew as multiple joined plates; microseeding resulted in large single plates suitable for diffraction. Crystals were cryoprotected by submersion in mother liquor supplemented with 20% ethylene glycol for 5–10 s and then flash frozen in liquid nitrogen.

2.3. Crystallization of ternary CtBP1/NAD^+/MTOB complex

Protein (~10 mg/ml) was mixed with a 50 M excess of MTOB immediately before hanging drops were setup. Bipyramidal crystals grew overnight at room temperature in 200–300 mM magnesium chloride, 0–140 mM sodium formate, 100 mM HEPES pH 7.5, and 2.5 mM NAD^+. Crystals were cryoprotected by adding well buffer solution supplemented with increasing amounts of glycerol. Once the drop containing the crystal reached 20% v/v glycerol, the crystal was moved to 30% glycerol for 5 s and flash frozen in liquid nitrogen.

2.4. Data collection and structure solution

Diffraction data for the CtBP1/NAD^+/MTOB and CtBP2/NAD^+/MTOB complexes were collected on the BioCARS 14-BM-C beamline at the Advanced Photon Source of Argonne National Laboratory. The initial models were obtained by molecular replacement with PhaserMR [32]. For CtBP1, the binary complex (1MX3 [29]) was used as a search model, whereas for CtBP2, individual cofactor and substrate binding domains of the binary CtBP2 complex (2OME) were used as described in the Supplementary material. Water molecules were automatically generated by Arp/Warp [33], and the structure was refined with RefMac5 [34]. All other

Table 1
Data collection and refinement statistics.

Data collection	CtBP1–MTOB	CtBP2–MTOB
Wavelength (Å)	0.99	0.99
Resolution range (Å)[a]	29.92–2.38 (2.47–2.38)	32.30–2.86 (2.96–2.86)
Space group	$P6_422$	$P2_1$
Unit cell		
a, *b*, *c* (Å)	88.97, 88.97, 161.54	86.15, 140.61, 135.13
α, β, γ (°)	90, 90, 120	90, 97.87, 90
Total reflections	167249	298092
Unique reflections	15538	73354
Multiplicity*	10.7 (11.6)	4.1 (4.0)
Completeness (%)*	97.9 (99.9)	99.3 (96.9)
Mean *I*/sigma (*I*)*	22.7 (7.1)	9.6 (3.3)
Wilson B-factor	52.8	54.3
R-sym (%)*	6.5 (39.0)	9.3 (38.9)
Refinement		
R-factor (%)*	20.0 (27.7)	21.4 (30.1)
R-free (%)*	23.9 (38.9)	25.1 (33.3)
Number of atoms	2644	20058
Protein	2463	19429
Ligands	97	424
Water molecules	84	205
Protein residues	327	2646
RMS (bonds)	0.009	0.009
RMS (angles)	1.27	1.15
Ramachandran favored (%)	96	96
Ramachandran outliers (%)	0	0
Clashscore	3.79	11.14
Average B-factor	55.5	52.7
Protein average B-factor	55.9	52.9
Water average B-factor	53.5	44.3

[a] Highest resolution shell shown in parentheses.

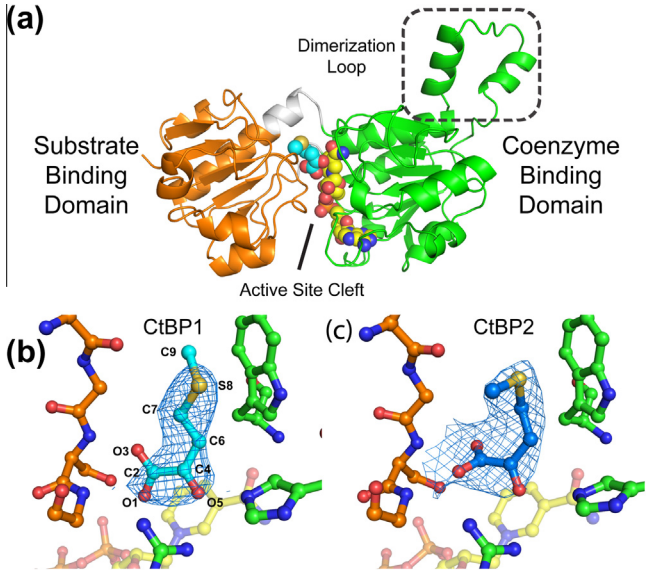

Fig. 1. Binding of MTOB to CtBP. (a) CtBP1 monomer domains; the substrate binding domain (orange) and coenzyme binding domain (green) are connected through a hinge (grey). MTOB (cyan) and NAD^+ bind in an active site cleft formed at the interface of the domains. The interface between subunits of the physiological dimer is primarily formed between the coenzyme binding domain, including extensive contacts formed by the dimerization loop. (b) and (c) The $2F_o-F_c$ maps for MTOB in CtBP1 (1σ; 0.20 e-/Å^3) and CtBP2 monomer A (1σ; 0.27 e-/Å^3), respectively. MTOB atom labels are shown in CtBP1 (b).