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Mechanistic studies for the role of cellular nucleic-acid-binding protein (CNBP) in regulation of *c-myc* transcription

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ABSTRACT

Background: Guanine-rich sequence of *c-myc* nuclease hypersensitive element (NHE) III_1 is known to fold in G-quadruplex and subsequently serves as a transcriptional silencer. Cellular nucleic-acid-binding protein (CNBP), a highly conserved zinc-finger protein with multiple biological functions, could bind to *c-myc* NHE III_1 region, specifically to the single strand G-rich sequence.
Methods: In the present study, a variety of methods, including cloning, expression and purification of protein, EMSA, CD, FRET, Ch-IP, RNA interference, luciferase reporter assay, SPR, co-immunoprecipitation, and co-transfection, were applied to investigate the mechanism for the role of CNBP in regulating *c-myc* transcription.
Results: We found that human CNBP specifically bound to the G-rich sequence of *c-myc* NHE III_1 region both *in vitro* and *in cellulo*, and subsequently promoted the formation of G-quadruplex. CNBP could induce a transient decrease followed by an increase in *c-myc* transcription *in vivo*. The interaction of CNBP with NM23-H2 was responsible for the increase of *c-myc* transcription.
Conclusions: Based on above experimental results, a new mechanism, involving G-quadruplex related CNBP/NM23-H2 interaction, for the regulation of *c-myc* transcription was proposed.
General significance: These findings indicated that the regulation of *c-myc* transcription through NHE III_1 region might be governed by mechanisms involving complex protein–protein interactions, and suggested a new possibility of CNBP as a potential anti-cancer target based on CNBP's biological function in *c-myc* transcription.

1. Introduction

Aberrant *c-myc* transcription is a common feature in many human malignancies [1]. It has been shown that dysregulation of *c-myc* proto-oncogene is a result through various mechanisms, such as gene amplification [2], increased transcription [3] and most often alterations in cell signaling which lead to an increase in *c-myc* transcription [4]. *c-myc* transcriptional regulation is governed by complex mechanisms. Notably, nuclease hypersensitive elements (NHEs) have been documented to play important roles in *c-myc* transcriptional regulation [5]. Among them, NHE III_1, upstream of P1 and P2 promoters, controls 75% to 95% of the total *c-myc* transcription [6]. The NHE III_1 region harbors a 31-base-pair element consisting of five repeats of the sequence (C/T)C(C/T)TCCCCA. Under negative supercoiled conditions, the purine-rich strand in single stranded DNA of the NHE III_1 region has been shown to adopt a G-quadruplex (G4) structure by Hoogsteen hydrogen bonds [7]. An overwhelming number of studies, focused on both small molecular ligands [7–14] and transcriptional factors [15,16], demonstrated that G-quadruplex, especially *c-myc* G-quadruplex, serves as a transcription silencer. The recent *in vivo* visualization of G-quadruplex [17] further persuasively demonstrated its existence, biological significance and druggability [18].

CNBP, also known as zinc-finger protein 9 (ZNF9), is a highly conserved zinc-finger protein with seven tandem repeats of CCHC zinc finger knuckles and one RGG box [19]. CNBP has been found to be involved in myotonic dystrophy type 2 (DM2) [20] and formation of embryonic craniofacial structures [21,22]. It has also been reported to both negatively and positively regulate DNA transcription [23–25]. On top of that, CNBP functions as nucleic acid chaperone [26] with both single strand DNA [24] and RNA [27] binding activity. CNBP has been found to activate *c-myc* transcription both *in vivo* and *in vitro* [19,22,24]. A

Abbreviations: CD, circular dichroism; ChIP, chromatin immunoprecipitation; CNBP, cellular nucleic-acid-binding protein; Co-IP, co-immunoprecipitation; EMSA, electrophoretic mobility shift assay; FAM, 6-carboxyfluorescein; FRET, fluorescence resonance energy transfer; NHE, nuclease hypersensitive element; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SPR, surface plasmon resonance; TAMRA, tetramethylrhodamine; TMPyP4, meso-tetra(*N*-methyl-4-pyridyl)porphine

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recent study has indicated that *Chaunus arenarum* CNBP promotes the formation of parallel G-quadruplex to the detriment of anti-parallel G-quadruplex *in vitro* [28]. Within this research, a model is proposed, in which CNBP activates *c-myc* transcription by promoting the formation of parallel *c-myc* G-quadruplex, which is eventually released by CNBP, to create a relatively open DNA structure that facilitates the reinitiation of transcription [28]. However, this hypothesis is discrepant to the idea of G-quadruplex being a transcriptional silencer. Understanding of *c-myc* transcriptional regulation mechanism in relation with the formation and dissociation of G-quadruplex is indispensible for understanding cancer biology and discovery of G-quadruplex based anti-tumor drugs [29]. However, so far, the mechanism of CNBP's regulation of *c-myc* transcription through G-quadruplex is still elusive.

Thus, it is important to know whether human CNBP possesses the same binding pattern and G-quadruplex catalytic capability, and if so, by how does it influence *c-myc* transcription. In the present study, a variety of methods, including EMSA, CD, FRET, Ch-IP, siRNA, luciferase reporter assay, SPR, Co-IP, protein depletion and overexpression, were applied to investigate the mechanism for the role of CNBP in regulating *c-myc* transcription. Our results showed that CNBP bound to single strand G-rich sequence of *c-myc* NHE III_1 region and facilitated the formation of G-quadruplex. Overexpression of CNBP induced a transient decrease followed by an increase in *c-myc* mRNA. Our study further indicated an interesting interplay between CNBP and NM23-H2, which could be one of major factors for regulating *c-myc* transcription. We also testified the hypothesis of CNBP as a potential anti-cancer drug target.

2. Materials and methods

2.1. Cells, antibodies, oligomers, siRNAs and reagents

Human cervical cancer cell line HeLa was obtained from the American Type Culture Collection (ATCC) and preserved in our lab. The cell culture was maintained in complete DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under humidified atmosphere with 5% CO_2. Cell line was tested negative for mycoplasma contamination prior to and after experimentation.

All antibodies were commercially available: β-actin rabbit mAb (#4970, Cell Signaling), c-Myc rabbit mAb (#1472-1, Epitomics), CNBP goat polyclonal antibody (sc-51052X, Santa Cruz), NM23-H2 rabbit mAb (5969-1, Epitomics), Anti-rabbit IgG-HRP (#7074, Cell Signaling), donkey anti-goat IgG-HRP (sc-2020, Santa Cruz) and anti-mouse IgG-HRP (#7076, Cell Signaling).

All oligomers were purchased from Invitrogen. Sequences of oligomers used in plasmid construction, EMSA, CD and FRET are listed in Table 1. Sequences of PCR primers are listed in Table S1. For EMSA or FRET, oligomers were 5′-FAM labeled or 5′-FAM and 3′-TAMRA dual-labeled respectively. For experiments where G-quadruplexes were needed, oligomers were heated at 95 °C for 10 min and slowly cooled to room temperature in the presence of 100 mM KCl.

Table 1
Sequence of all oligomer used in this study.

Name	Sequence (5′ → 3′)
His-CNBP for.	CAGGTCGCTAGCAGCAATGAGTGCTTCAAGTG
His-CNBP rev.	GTCCAGCTCGAGTTAGGCTGTAGCCTCAATTGTGC
EGFP-CNBP for.	CAGGTCGCTAGCAGCAATGAGTGCTTCAAGTG
EGFP-CNBP rev.	GTCCAGGGATCCGGCTGTAGCCTCAATTGTGCATTC
Pu27	TGGGGAGGGTGGGGAGGGTGGGGAAGG
Py27	CCTTCCCCACCCTCCCCACCCTCCCCA
Random oligo	AGTTAGAATCCTTCAACATCTGCATAC
Pu27(Δ8A17A)	TGGGGAG**A**GTGGGGAG**A**GTGGGGAAGG

CNBP Stealth siRNAs (HSS111485, HSS111486, HSS187750, Cat. # 1299001) and Stealth RNAi™ siRNA Negative Control Med GC (Cat. # 12935-300) were purchased from Invitrogen. NM23-H2 siRNAs (5′-GCACUACAUUGACCUGAAAT-3′; 5′-UUUCAGGUCAAUGUAGUGCT T-3′) were purchased from RiboBio (Guangzhou).

All enzymes used for reverse transcription and PCR were purchased from TaKaRa. All enzymes used for plasmid construction were purchased from Fermentas.

2.2. Plasmid construction

Total mRNAs were isolated from HeLa cells and subjected to reverse transcription. First strand cDNAs were subsequently applied to PCR for Human *cnbp* gene cloning using primers His-CNBP for and His-CNBP rev. Xho I and Nhe I digested PCR products were inserted into pET-28a(+) vector (Novagen) for pET-28a-CNBP plasmid construction.

Cnbp sequence was sub-cloned using primers for EGFP-CNBP and EGFP-CNBP rev, digested by Nhe I and BamH I, and inserted into pEGFP-N_3 vector for pEGFP-CNBP plasmid construction. pEGFP-NM23-H2 plasmids were constructed as described previously [9].

c-myc promoter sequences, both wild-type and mutant, were sub-cloned from pBV-Luc-*c-myc* (wt) and pBV-Luc-*c-myc* (mut) plasmids (gifts of professor Laurence H. Hurley, University of Arizona), and EcoR I and BamH I were digested and inserted into pMetLuc vector (Clontech) for pMetLuc-*c-myc* (wt) and pMetLuc-*c-myc*(mut) luciferase reporter plasmid construction respectively. All reconstructed plasmids were sequenced and proved to be correct by BLAST.

2.3. Protein expression, purification, identification and quantification

The pET-28a-CNBP fusion plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen) for protein expression. Protein expression was induced in the presence of 0.1 mM IPTG for 14 h at 16 °C, at 160 rpm. The cells were lysed using a Scientz-II D sonicator (Scientz) with the addition of EDTA-free Protease Inhibitor Cocktail Tablets (Roche). Recombinant protein was purified using HisTrap HP columns (GE Healthcare) to apparent homogeneity, following the manufacturer's protocol. The purified protein was dialyzed against dialysis buffers (20 mM Tris, 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM DTT, 5% glycerol, pH 6.9) overnight for EMSA, CD and FRET or dialyzed against SPR running buffer overnight (20 mM Na_3PO_4, 150 mM NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100, pH 6.9) for SPR. Protein was analyzed by using SDS-PAGE and identified with Bruker Ultraflex III MALDI-TOF/TOF MS spectrometer. MALDI-TOF/TOF MS data was analyzed in Mascot software (Matrix Science). The concentration of purified protein was determined using Thermo Scientific *NanoDrop 3300* UV–vis Spectrophotometer. His-tagged NM23-H2 protein was expressed, purified and identified in a similar way. The purified NM23-H2 was dialyzed overnight against dialysis buffer (20 mM Tris, pH 7.4).

2.4. Electrophoretic mobility shift assay (EMSA)

Briefly, 2 μM 5′FAM-labeled oligonucleotides were mixed with diverse amounts of recombinant CNBP in EMSA binding buffer (20 mM HEPES, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, pH 6.9) for 1 h at 37 °C in a final binding reaction volume of 20 μL. For binding reactions requiring G-quadruplex DNA or double-strand DNA, pre-annealed oligonucleotides were incubated with CNBP for 1 h at 37 °C in binding buffer with or without 100 mM KCl respectively. The reaction products were electrophoresed on 8%–10% native polyacrylamide gels at 60 V for 3 h in the presence of ice-cooled 0.5× TBE buffer.

2.5. CD spectroscopy

The oligomers at a final concentration of 2 μM were resuspended in CD binding buffer (20 mM Tris/HCl, 2 mM $MgCl_2$, 5% glycerol,