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Protein complex formation and intranuclear dynamics of NAC1 in cancer cells

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ABSTRACT

Nucleus accumbens-associated protein 1 (NAC1) is a cancer-related transcription regulator protein that is also involved in the pluripotency and differentiation of embryonic stem cells. NAC1 is overexpressed in various carcinomas including ovarian, cervical, breast, and pancreatic carcinomas. NAC1 knock-down was previously shown to result in the apoptosis of ovarian cancer cell lines and to rescue their sensitivity to chemotherapy, suggesting that NAC1 may be a potential therapeutic target, but protein complex formation and the dynamics of intranuclear NAC1 in cancer cells remain poorly understood. In this study, analysis of HeLa cell lysates by fast protein liquid chromatography (FPLC) on a sizing column showed that the NAC1 peak corresponded to an apparent molecular mass of 300–500 kDa, which is larger than the estimated molecular mass (58 kDa) of the protein. Furthermore, live cell photobleaching analyses with green fluorescent protein (GFP)-fused NAC1 proteins revealed the intranuclear dynamics of NAC1. Collectively our results demonstrate that NAC1 forms a protein complex to function as a transcriptional regulator in cancer cells.

1. Introduction

Nucleus accumbens-associated protein 1 (NAC1), encoded by the *NACC1* gene, is a nuclear protein that harbors an *N*-terminal BTB/POZ (**b**road complex, **t**ramtrack, **b**ric-a-brac/**po**xvirus and **z**inc finger) (hereafter abbreviated BTB) and a *C*-terminal BEN (**B**ANP, **E**5R and **N**AC1) domain (Fig. 1A). The BTB domain is a ~100 amino acid highly conserved motif that mediates homodimerization and/or heterodimerization and interacts with other proteins [1,2]. NAC1 homodimerizes through its BTB domain [3] and heterodimerizes with Myc-interacting zinc-finger protein 1 (Miz1) through the respective BTB domain [4,5]. Most BTB proteins contain other *C*-terminal functional domains, such as DNA-binding zinc fingers. NAC1 lacks these characteristic DNA-binding domains but instead contains a *C*-terminal BEN domain, identified through computational analysis in 2008, which may mediate protein-DNA and protein-protein interactions [6].

NAC1 participates in various biological processes. NAC1 was originally identified and cloned as a cocaine-inducible transcript from the nucleus accumbens, a unique forebrain structure involved in reward motivation and addictive behavior [7]. NAC1 is known to be important for the pluripotency of embryonic stem cells [8,9]. Recently, NAC1 was shown to promote mesendodermal and repress neuroectodermal fate selection in embryonic stem cells, in cooperation with other pluripotency transcription factors, Oct4, Sox2, and Tcf3 [10]. *NACC1* knockout mouse embryos and newborns exhibit a lower survival rate for embryos or newborns, with surviving mice showing defective bony patterning in the vertebral axis [11].

NACC1 was also identified as a cancer-associated BTB gene by serial analysis of gene expression in ovarian cancer cells [12]. NAC1 is significantly overexpressed in several types of carcinomas including ovarian, colorectal, breast, renal cell, cervical, and pancreatic carcinomas is associated with tumor growth and survival, and increases the resistance of tumor cells to chemotherapy [12–22]. These reports suggested that NAC1 plays a driver role in cancer development and that it might be a potential therapeutic target.

Although NAC1 appears to play increasingly significant and

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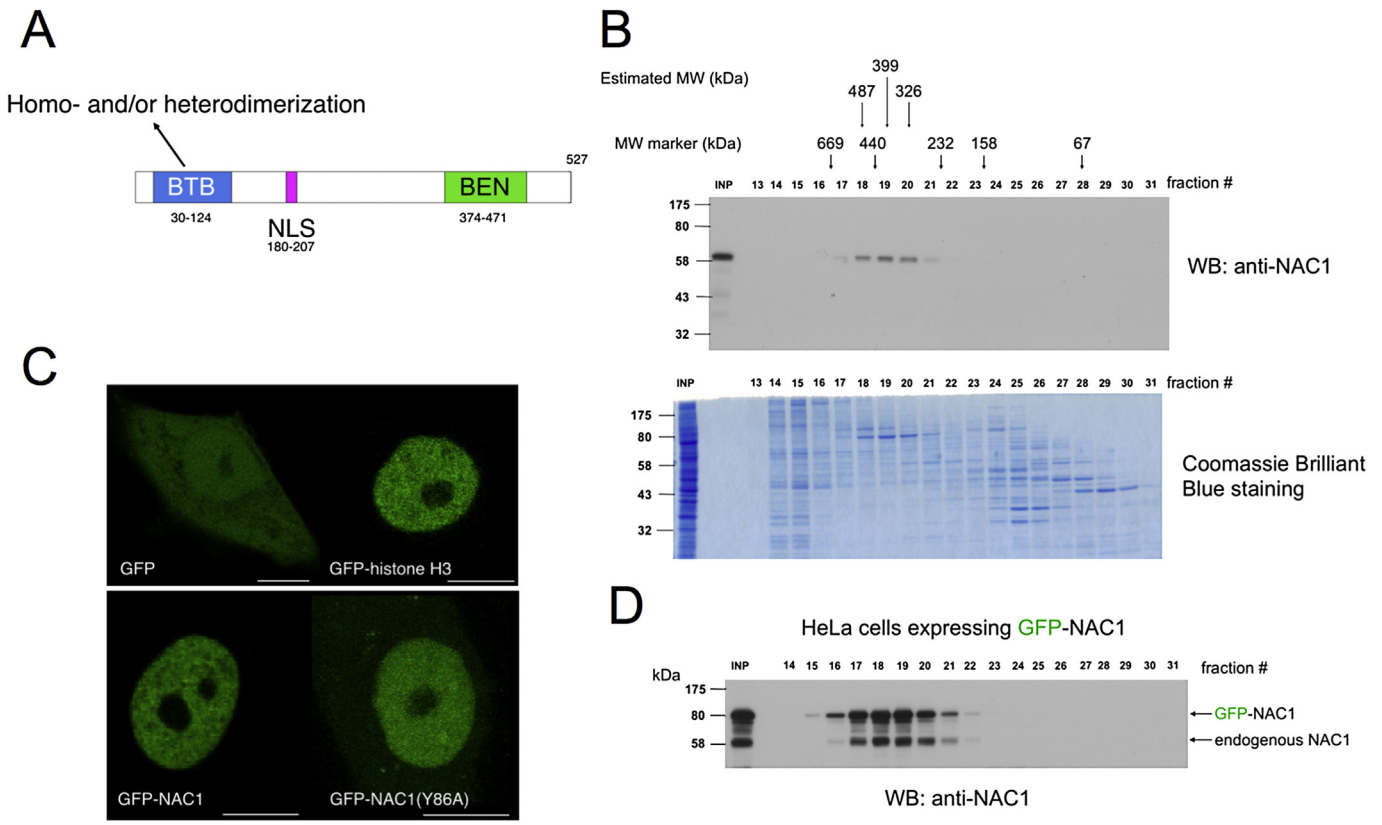

Fig. 1. NAC1 protein complex in cancer cells. (A) Schematic representation of human NAC1 protein showing the BTB domain, nuclear localization signal (NLS), and the BEN domain. (B) Protein extracts of HeLa cells were analyzed by size exclusion chromatography on a FPLC Superdex 200 column. Protein mass standards are indicated above the graph: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa). The estimated molecular weights of fractions 18 to 20 were calculated using the standard curve. The eluted fractions were analyzed by Coomassie staining and Western blot using anti-NAC1 antibody 9.27. (C) A representative HeLa cell stably expressing GFP, GFP-histone H3, GFP-NAC1, or GFP-NAC1(Y86A). Images were obtained under a 473 diode laser. Bars, 10 μm. (D) Protein extracts of HeLa cells stably expressing GFP-NAC1 were analyzed by size exclusion chromatography on a FPLC Superdex 200 column. Eluted fractions were analyzed by Western blot using anti-NAC1 antibody 9.27.

diverse functions in cancer and stem cell biology, protein complex formation and the dynamics of intranuclear NAC1 in cells are poorly understood. In this study, we fractionated NAC1-containing complexes according to mass by gel size-exclusion column chromatography and investigated the intranuclear dynamics of NAC1 by live cell photobleaching analysis.

2. Materials and methods

2.1. Plasmid construction

Human histone H3 full-length cDNA obtained by reverse transcribed PCR using the total RNA from HeLa cells was cloned into pMXs-FHG. All PCR-amplified cDNA products were fully sequenced using a 3130 genetic analyzer (ThermoFisher Scientific, Waltham, MA, USA) to confirm the sequences and to verify the absence of secondary point mutations.

2.2. Cell culture

The HeLa human cervical epithelioid carcinoma cell line was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (JCRB9004). To maintain authenticity of the cell line, multiple aliquots of frozen stocks were prepared from initial stocks, and every 3 months, a new frozen stock was used for the experiments. The cells were routinely inspected for identity by morphology and growth curve analysis and validated to be *mycoplasma* free. HeLa cells expressing GFP, GFP-NAC1, or GFP-NAC1(Y86A) have been described [23]. The GFP is Emerald GFP (ThermoFisher Scientific) (made monomeric by introducing the mutation A206K) (hereafter abbreviated GFP). HeLa cells expressing GFP-histone H3 were established by utilizing a retrovirus infection system [23]. Overexpression of NAC1 proteins gives rise to the formation of dense body-like structures in the nucleus [12,24]. Only GFP-positive cells expressing the lowest detectable amounts of fusion protein were isolated using a FACSAris II cell sorter and FACSDiva software (BD, Franklin Lakes, NJ, USA). All cells were grown in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Molecular mass determination

The molecular mass of endogenous NAC1 in HeLa cells was estimated by fast protein liquid chromatography (FPLC) gel filtration on a Superdex 200 Increase 10/300 GL column (GE Healthcare, Buckinghamshire, UK) by comparison with protein molecular weight markers. The markers were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa). One % (v/v) Triton X-100 lysate of HeLa cells and markers were separately loaded on the column and eluted with 0.50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. Fractions (0.5 ml) were collected and proteins were resolved by SDS-PAGE. NAC1 was detected on immunoblots with anti-NAC1 monoclonal antibody 9.27 [23]. The exponential equation of the standard curve was $y = 17816e^{-0.2x}$ ($R^2 = 0.9991$) (y = estimated