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Lysine 263 residue of NPM/B23 is essential for regulating ATP binding and B23 stability

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Abstract **Here, we show that Nucleophsomin/B23 provides lysine 263 as a critical binding site for ATP. Mutagenesis of lysine 263 to asparagine (K263N) disrupts B23 from ATP binding. While B23 WT exclusively localizes to the nucleolus, the B23-K263N is redistributed from the nucleolus to the nucleoplam. Notably, the K263N mutant is unstable, and displayed rapid degradation. Alteration of K263 induced B23 instability through increased ubiquitination and proteaosomal degradation. Moreover, mutation of K263 impedes the mitogenic effect of B23 in PC12 cells. Thus, K263 is a critical site for ATP binding and required for B23 stability, confining B23 in the nucleolus.**

Keywords: Nucleolus/Nucleoplasm; Protein stability; Ubiquitination; Proliferation; Apoptosis

1. Introduction

B23/Nucleophosmin, a phosphonucleolar protein, has been implicated in several cellular processes, including cell proliferation [1], anti-apoptotic activity [2], cytoplasm/nuclear shuttling [3] and nucleolar/nucleoplasm transportation [4]. Downregulation of B23 mRNA delays the entry of cells into mitosis [5] and overexpression of B23 induces cell cycle arrest in normal fibroblasts, whereas it accelerates the entry into S phase in cells lacking p53, suggesting that B23 may have oncogenic potential [6–8]. In contrast, knocking down B23 prevents pre-ribosomal processing and causes cell death. Previous studies have suggested that B23 transport is critical for its function in the cell. Indeed, not only does B23 shuttle nucleus/cytoplasm [3], but also translocates from nucleolus to nucleoplasm during S phase [4]. The depletion of the GTP pool in culture condition caused B23 translocation to nucleoplasm [9]. B23 translocation can also be induced by certain anti-cancer drugs that cause growth inhibition [10]. Moreover, ATP promotes B23 translocation from nucleolus to nucleoplasm in isolated permeabilized cells [11], and positively charged lysine residues in the C-terminus of B23 have been implicated in binding ATP [12]. However, the ATP binding residue is unknown, and the physiological meaning of this event is not clear as well.

In this study, we identify lysine 263 in B23 as a specific binding site of ATP. Mutation of this site into asparagines (K263N) disrupts ATP binding and impedes B23 nucleolar residency. Moreover, K263N mutation increases the instability of B23 protein via ubiquitination and proteaosomal degradation, affecting cell proliferation. This demonstrates that K263 of B23 is critical for efficient nucleolar localization and protein stability as well as ATP binding.

2. Materials and methods

2.1. Cell cultures and reagent

PC12 cells were maintained in medium A (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% horse serum, and 100 units of penicillin–streptomycin). Stably-transfected PC12 cells with stably inducible B23 DNA were cultured in medium B (medium A with 100 μg/ml G418, 100 μg/ml hygromycin B, and 2 μg/ml tetracycline) and induced in medium B without 2 μg/ml tetracycline for 24 h. Anti-Akt, anti-GST, anti-NPM, anti-HA, anti-PARP and anti-Myc antibodies were obtained from Cell Signaling (Danvers, MA). Anti-α-Tubulin, anti-fibrillarin were acquired from Santa Cruz (Santa Cruz, CA). Adenosine triphosphate-agarose conjugated beads (ATP-beads) were from Fluka (Milwakuee, WI). All the chemicals not included above were from Sigma (St. Louis, MO).

2.2. ATP binding assay

ATP binding assays were performed as previously described [13]. Briefly, ATP-beads were incubated with 10 μg of purified GST-B23 protein in 400 μl binding buffer (40 mM HEPES, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 5% glycerol) at 4 °C for 2 h. The beads were washed three times and associated protein was eluted in binding buffer containing 10 mM of soluble ATP.

2.3. Immunostaining and confocal analysis

PC12 cells grown on coverslips in 6-well plates were treated with antimycin A (5 μg/ml) for 1 h. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Immunolocalization was determined using anti-B23 and anti-fibrillarin antibodies with the Alexa Flour 488 goat anti-mouse and Alexa Flour 594 goat anti-rabbit fluorescent dye-conjugated antibodies. Cells were microscopically visualized fluorescent illumination. GFP-B23 WT and GFP-B23 K263N transfected PC12 cells were visualized using a Zeiss LSM confocal fluorescence microscope (Carl Zeiss, Jena, Germany).

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2.4. Pulse-chase determination of protein half-life

PC12 cells were transfected with myc-B23 WT and myc-B23 K263N and labeled with 50 μl/ml of [^{35}S]methionine for 2 h, and were chased by culturing in media for the indicated times. Total cell lysates were immunoprecipitated with anti-myc antibody and incubated with protein agarose A/G. The reaction mixtures were analyzed by autoradiography.

2.5. Measure of ATP concentration

PC12 cells were treated with or not antimycin A (5 μg/ml) or sodium azide(5 μM) for 12 h. Same amount of proteins were incubated with ATP bioluminescent assay mix (Sigma). Then reaction mixture was measured by luminotor.

2.6. Purification of nucleoli

Nucleoli were isolated from PC12 cells as previously described (18). In brief, purified nuclei were added to equal volumes of 0.88 M sucrose containing 0.05 mM $MgCl_2$. After centrifugation at $1200 \times g$ for 10 min at 4 °C, the nuclei were resuspended in 10 volumes of 0.34 M sucrose with 0.05 mM $MgCl_2$. Equal volumes of 0.88 M sucrose containing 0.05 mM $MgCl_2$ were centrifuged at $2000 \times g$ for 20 min at 4 °C. The supernatant containing the nucleoplasmic fraction was collected for further analysis. The nucleolar pellet was washed twice in 0.34 M sucrose containing 0.05 mM $MgCl_2$ and analyzed by Western blotting.

2.7. Ubiquitination assay

PC12 cells were co-transfected with myc-B23 and HA-ubiquitin. Transiently transfected PC12 cells obtained after 24 h were treated with or without 5 μg/ml antimycin A for 1 h and then harvested. Cells were lysed in TBS (20 mM Tris–Cl, pH 7.2, 137 mM NaCl) containing 2% sodium dodecyl sulfate (SDS) at 95 C, 10 min and then were added to four volumes of TBS containing 1% Triton X-100. These lysates were sonicated for 2 min and centrifuged at 4 C. Myc-B23 in the resulting lysate was immunoprecipitated with 2 μg anti-myc antibody and protein G/A agarose beads at 4 C for 4 h with rotation. The beads were washed three times with lysis buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride and associated proteins were eluted in double-strength SDS sample buffer at 95 C for 7 min). Eluted proteins were subjected to immunoblotting against HA-tag to detect ubiquitinated B23. Immunoblotting against α-tubulin and myc-tag were a loading control.

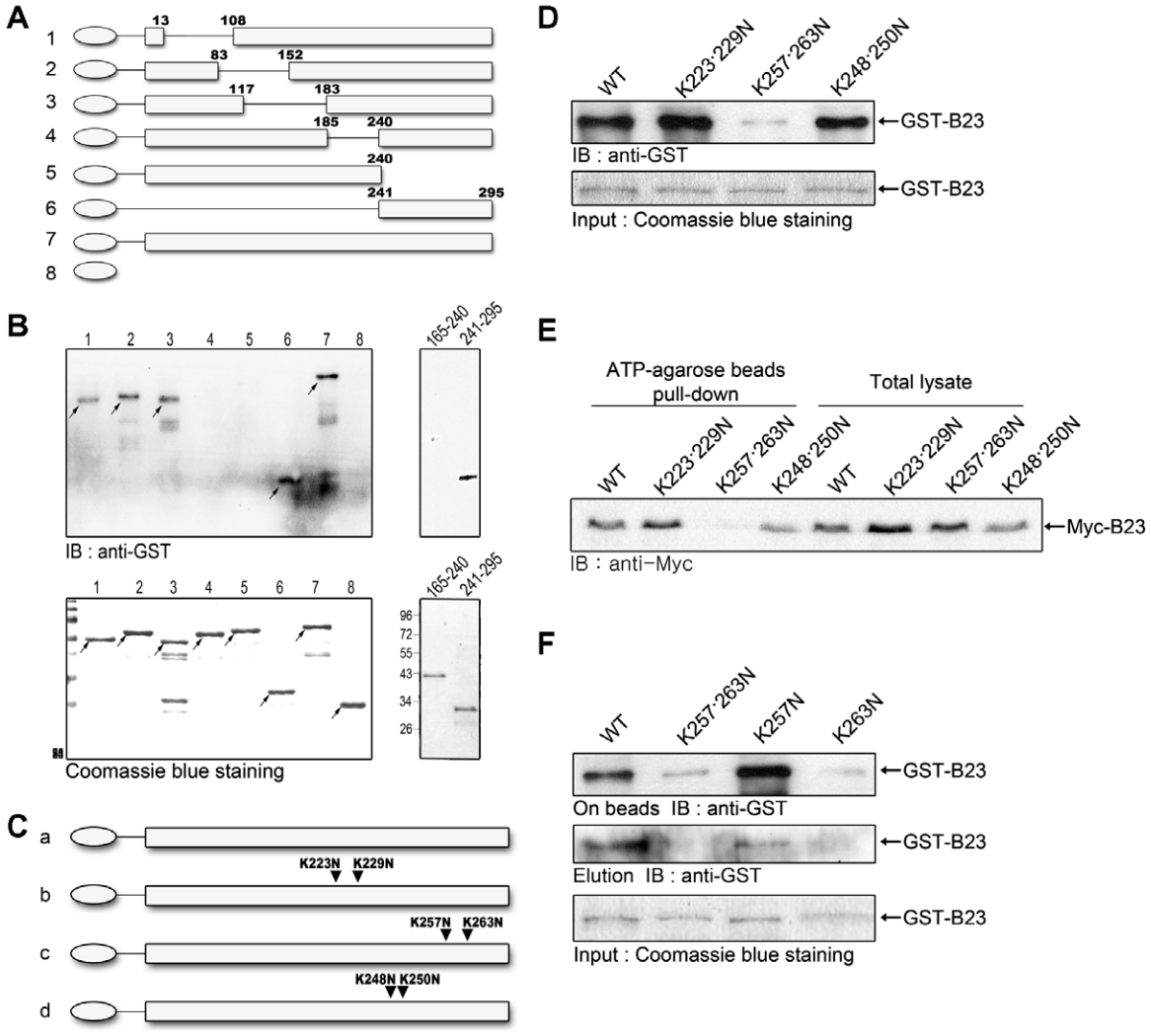

Fig. 1. Lysine 263 residue in B23 is critical docking site for ATP binding. (A) Diagram of GST-B23 deletion constructs. GST tag is depicted by the circles and B23 by squares. (B) GST-B23 deletion mutants were bound to ATP-beads (upper panel) and presented by Coomassie Brilliant Blue staining (bottom panel). B23 proteins are indicated by arrows. (C) Diagram of GST or Myc-B23 lysine mutant proteins. GST tag is depicted by the circles and Myc-tag by the squares. (D) In vitro binding assay. Purified GST-B23 mutant C fails to bind with ATP-beads. (E) ATP-pull-down assay using stably tranfected PC12 cells with inducible forms of Myc-B23 WT and mutants. (F) In vitro ATP binding assay using purified GST-B23 WT and mutants proteins. Associated GST-B23 proteins with ATP-beads were eluted in 10 mM soluble ATP.