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Calcineurin B subunit interacts with proteasome subunit alpha type 7 and represses hypoxia-inducible factor-1α activity via the proteasome pathway

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ABSTRACT

The calcineurin (CN) B subunit (CNB) is the regulatory subunit of CN, which is the only serine/threonine-specific protein phosphatase regulated by Ca^{2+}/CaM. It has been shown to have potential as an anticancer agent, and has a positive effect on the phagocytic index and coefficient. We report here that CNB binds to proteasome subunit alpha type 7 (PSMA7) and inhibits the transactivation activity of hypoxia-inducible factor-1α (HIF-1α) via the proteasome pathway. In addition, we show that CNB represses the expression of vascular endothelial growth factor (VEGF), which is regulated by HIF-1α. These results indicate that CNB modulates cellular proteasome activity via a specific interaction with PSMA7. This may provide a molecular basis for its anticancer and antiviral activities.

1. Introduction

Calcineurin (CN), the only serine/threorine-specific protein phosphatase regulated by Ca^{2+}/CaM, is normally composed of a catalytic subunit calcineurin A (CNA) and a regulatory subunit calcineurin B (CNB) [1,2].

CNB is widespread among eukaryotes [3,4]. Recently CNB has been shown to play many important roles. It is required for vegetative hyphal growth of *Neurospora crassa* and determines its morphology and developmental state [5], and in *Naegleria gruberi* it appears to be involved in processes ranging from differentiation to changes in cell motility and shape, and perhaps also flagellar function [6]. In addition, CNB influences calcium signaling via a calcineurin-like pathway mediating the beneficial effects of calcium on plant salt tolerance [7]. CNB also plays an important role in the formation of calcineurin–immunophilin complexes, and in rat brain homogenates injected with the immunophilin, cyclosporin A (CsA), CNB and CNA have significantly different effects [8,9].

In some tumor tissues, the level of CNB is much higher than that of CNA [10]. In previous work we showed that recombinant human calcineurin B subunit (rhCNB) has anticancer potential, and has a positive effect on the phagocytic index and coefficient [11,12]. However, the basis of the anticancer action of rhCNB is not well understood.

Proteasome subunit alpha type 7 (PSMA7) is an alpha-type subunit of the 20S proteasome core complex, and participates in degrading proteins through the ubiquitin–proteasome pathway (UPP). It can interact with important proteins such as HBX, HIF-1α, Parkin, HIV, Rab7, and EMAP2 [13–18], which are involved in transcription factor regulation, cell proliferation and cell cycle control, virus replication, immune and stress responses, cell differentiation, apoptosis and even tumor initiation and progression. PSMA7 is differentially expressed in colorectal cancer; it is overexpressed and associated with liver metastases in these cancers, and so may play an important role in the progression of colorectal cancer [19]. These findings suggest that PSMA7 could be useful in clinical diagnosis and as a potential target for therapeutic drugs [20].

PSMA7 interacts specifically with two subdomains of the hypoxia-inducible factor-1α subunit (HIF-1α) [14]. HIF-1 is a key factor in the expression of mammalian genes that are involved in angiogenesis, vasculogenesis, glucose metabolism, and apoptosis due to low oxygen tension. It is a dimeric transcription factor composed of HIF-1α and HIF-1β subunits [21]. In normoxia, HIF-1α is rapidly degraded, while HIF-1β is stably expressed regardless of the oxygen tension. Under hypoxic conditions it up-regulates messenger RNAs encoding vascular endothelial growth factor (VEGF), erythropoietin, and glycolytic enzymes [22,23]. PSMA7 inhibits the transactivation function of HIF-1α and its action is associated with the proteasome pathway.

The aim of the present work was to gain further understanding of the anticancer activity of CNB by identifying the proteins with which it interacts, and the consequences of such interactions for cell function.

2. Materials and methods

2.1. Materials

Matchmaker Library Construction and Screening Kits were purchased from BD (USA). Anti-HA (mouse IgG) was purchased from

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Santa Cruz Biotechnology (USA). Anti-CnB (mouse IgG) was prepared in our laboratory. The Proteasome-Glo Assay System and Dual Luciferase Assay System were purchased from Promega (USA). All other reagents were of standard laboratory grade.

2.2. Methods

2.2.1. Cell culture

293T and RAW264.7 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM). U937 cells were routinely maintained in RPMI Medium 1640 medium, with 0.375% sodium bicarbonate, and 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 37 °C.

2.2.2. Yeast-two-hybrid screen

A cDNA encoding full-length (FL) CNB was subcloned into the two-hybrid vector pGBKT7 and used as bait to screen a U937 cell cDNA library. Screening was performed according to the User Manual of the BD Matchmaker Library Construction & Screening Kits.

2.2.3. Plasmid construction

The pGBKT7 vector and pGADT7-Rec were used in the yeast-two-hybrid assay. A glutathione-S-transferase (GST) fusion with PSMA7 (pGEX-4t-1/psma7) (for expression in *Escherichia coli*) was constructed by the polymerase chain reaction, followed by cloning into pGEX-4t-1 plasmid. An N-terminal hemagglutinin (HA)-tagged PSMA7 plasmid (for expression in mammalian cells) was constructed in pEGFP-N2 using a primer with the HA coding sequence. The CNB plasmid for expression in mammalian cells was constructed in the pcDNA3 vector. The luciferase reporter plasmid (pFR-Luc) containing a promoter with five tandem repeats of the GAL4 binding sites was kindly provided by Professor Jie Zhou.

2.2.4. In vitro binding assays

Pull-down assays were carried out essentially as described previously [24]. GST fusion proteins were expressed in *E. coli*. The GST fusion proteins (GST-PSMA7) were immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia), and incubated with purified CNB for 6 h at 4 °C in Triton x-100 lysis buffer. After being washed 3 times with the same buffer the bound proteins were eluted with SDS–PAGE sample buffer and separated by SDS–PAGE, followed by Western blotting.

2.2.5. Binding assays and immunoblotting in vivo

Co-IP assays were carried out according to a published protocol [25]. Transfected HEK293T cells were lysed with cell lysis buffer for Westerns and IP (Beyotime, China), followed by centrifugation. The supernatant was incubated with mouse anti-HA monoclonal antibody overnight at 4 °C. It was then incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 2 h and the beads were washed three times in the lysis buffer. The bound proteins were eluted with SDS–PAGE sample buffer, separated by SDS–PAGE and analyzed by Western blotting.

2.2.6. Luciferase assay

RAW264.7 cells were grown to 50–80% confluence in 6-well plates, and transfected with 1 μM of pFR-Luc using FuGENE HD Transfaction Reagent (Roche, USA). The cells were incubated for an additional 24 h and in the presence or absence of 2 μM CNB for 6 h using 100 μM $CoCl_2$ as a positive control. Luciferase activity was determined using a commercial assay system (Promega). All treatments were carried out in duplicate and repeated at least six times.

2.2.7. Assay of proteasomal activity

U937 cells were incubated with 0 to 9 μM CNB for 2 h, or were incubated with 2 μM CNB for 0 to 10 h. The cells were harvested, resuspended in 10 mM HEPES buffer (pH 7.6) and lysed on ice. After centrifugation at 4 °C, the supernatant was diluted with lysis buffer to a concentration of 20 μg/200 μl. The substrates were added to obtain a final concentration of 20 μM. [26] The total volume of 100 μl was in white-walled 96-well plates. After gentle mixing, the luminescent signal was recorded with a plate-reading luminometer during 20 min. The experiments were repeated three times.

2.2.8. Fluorescence microscopy

HEK293 cells stably expressing GFP-CNB were cultured on chambered coverglasses, transfected with pDsRed-N1 or pDsRed-N1/HA-PSMA7 and grown for 24 h. They were treated with Hoechst for 10 min, then with polyformaldehyde, and washed twice with phosphate-buffered saline (PBS, pH 7.4). Images were analyzed with Confocal Assistant software (Fluoview 4.0, Olympus).

2.2.9. RNA extraction and real-time RT-PCR analysis

Total RNA was extracted with purified RNA extracted reagent (Bio Teke Corporation, Beijing, China). Real-time reverse transcriptase-polymerase chain reactions (RT-PCR) were performed to detect transcripts of VEGF using GAPDH as an endogenous control gene [27].

2.2.10. Statistical analysis

All experiments were performed in at least triplicate and the results were expressed as means ± SD. Statistical significance was determined using SPSS 11.0 for Windows software (SPSS, Chicago, IL, USA). Student's *t*-test was used for paired samples and differences were deemed significant at $P < 0.05$.

3. Results

3.1. Interaction of CNB with proteasome complex subunit PSMA7

To identify novel proteins interacting with CNB, we performed pGADT7-rec screens in a U937 cell cDNA library using human CNB as bait. This led to the isolation of a cDNA clone encoding the 20S proteasome subunit alpha type 7 (NCBI: NM_002792). The interaction between CNB and PSMA7 was specific as co-transformation of CNB and PSMA7 activated the HIS3, URA3 and lacZ reporter genes in strain AH109 (Fig. 1A).

To confirm the interaction revealed by the yeast two-hybrid screen, we also performed binding assays both in vitro and in vivo. We first performed a GST-PULLDOWN assay. CNB bound specifically to a GST PSMA7 fusion (GST-PSMA7) construct but not to GST (Fig. 1B).

We also compared the localization of the two proteins by immunocytochemistry. HEK293 cells stably expressing GFP-CNB were transfected with expression plasmids for RFP and an RFP PSMA7 fusion. Both PSMA7 and CNB were localized throughout the cytoplasm (Fig. 1C).

We also tested for *in vivo* interaction by co-immunoprecipitation experiments in which HA-PSMA7 and CNB were overexpressed, and we detected the overexpressed CNB in HA immunoprecipitates (Fig 1D). We also detected the interaction in CNB immunoprecipitates when HA-PSMA7 was precipitated from HEK293 cells co-expressing CNB (Fig. 1E).

3.2. CNB stimulates proteasomal activity via interaction with PSMA7

PSMA7 is a subunit of the 20S proteasome complex and has a gating function in proteasomal degradation of poly-ubiquitinated proteins [28]. In order to determine the biological function of the interaction between CNB and PSMA7, we incubated U937 cells