Biochemical and Biophysical Research Communications 496 (2018) 1109–1114

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The interaction between calcineurin and α-synuclein is regulated by calcium and calmodulin

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ARTICLE INFO

Article history:
Received 16 January 2018
Accepted 24 January 2018
Available online 7 February 2018

Keywords:
Alpha-synuclein
Calcineurin
Microscale thermophoresis
GST pull-down assays
Co-immunoprecipitation
Thapsigargin

ABSTRACT

Calcineurin (CN) is a protein phosphatase and widely distributed in eukaryotes, with an extremely high level of expression in mammalian brain. Alpha-synuclein (α-syn) is a small soluble protein expressed primarily at presynaptic terminals in the central nervous system. In our present study, we explored the interactions between CN and α-syn *in vitro*. Based on the data from microscale thermophoresis, GST pull-down assays, and co-immunoprecipitation, we found that CN binds α-syn. Furthermore, this interaction is mediated by calcium/calmodulin (Ca^{2+}/CaM) signaling. Additionally, thapsigargin (TG) triggered an increase in CN activity and α-syn aggregation in HEK293 cells stably transfected with α-syn. Our previous study *in vivo* suggest that overexpression of α-syn in transgenic mice significantly promoted CN activity and subsequent nuclear translocation of nuclear factor of activated T-cells (NFAT) in the midbrain dopaminergic (mDA) neurons. These *in vivo* and *in vitro* studies have been complementary with each other, representing the changes in the CN-dependent pathway affected by overexpression of α-syn.

1. Introduction

Calcineurin (CN) is a highly conserved calcium/calmodulin (Ca^{2+}/CaM)- dependent phosphatase. It is widely distributed in mammals, especially abundant in nerve cells and T lymphocytes [1]. CN is a heterodimeric protein composed of a catalytic subunit (CNA) and a regulatory subunit (CNB) at a ratio of 1:1. It plays an important role in a series of cell processes and Ca^{2+}-dependent signal transduction pathways involved in neurodegenerative diseases, cytoskeletal stabilization, long-term memory, neurite extension, and apoptosis [2,3].

Alpha-synuclein (α-syn) is a small soluble protein (14 kDa) expressed primarily in the presynaptic terminals of dopaminergic neurons, such as frontal cortex, striatum, and midbrain [4]. The normal function of α-syn remains unclear, although it plays an important role in neuroplasticity and in response to neuronal cell damage. It has also been shown to interact with and affect the activities of important enzymes in neurons [5]. For example, Peng et al. found that α-syn regulates dopamine synthesis by binding and inhibiting tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis. Additionally, this group described a protein phosphatase PP2A-dependent mechanism by which α-syn inhibits tyrosine hydroxylase phosphorylation and its activity in stably transfected dopaminergic neurons [6].

In our present study, we explored the interaction between CN and α-syn, which is upstream of a CN/NFAT-dependent pathway. Using microscale thermophoresis (MST), GST pull-down assays, and co-immunoprecipitation (Co-IP), we found that α-syn can bind CN. Furthermore, this interaction is mediated by Ca^{2+}/CaM signaling. Previously, using the midbrain dopaminergic (mDA) neurons derived from α-syn transgenic mice, we reported that CN and nuclear factor of activated T-cells- (NFAT-) mediated signaling pathway are actively involved in α-*syn*-mediated degeneration of mDA neurons in PD [7–9]. Our results showed that overexpression of the α-syn protein containing the A53T missense mutation

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https://doi.org/10.1016/j.bbrc.2018.01.148
0006-291X/

promoted CN activity and subsequent nuclear translocation of NFAT in mDA neurons. These *in vivo* and *in vitro* studies have been complementary with each other, representing the changes in the CN-dependent pathway affected by overexpression of α-syn.

2. Materials and methods

2.1. Materials

RIPA lysis buffer was purchased from Beyotime Biotechnology (Shanghai, China). Thrombin was purchased from Merck Serono Co., Ltd. (Germany). Thapsigargin (TG) was purchased from J&K Scientific Ltd. (Beijing, China). Peptides were synthesized by Scilight-Peptide Co. (Beijing, China). All other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

2.2. Expression and purification of proteins

The primers used to amplify *α-syn* to generate constructs for the GST fusion proteins were as follows: sense, 5′-TCCCCCGGGTATG-GATGTATTCA-3′; antisense, 5′-CCGCTCGAGTTAGGCTTCAGGTT-3′. The recombinant plasmid encoding α-syn fused to GST was obtained by cloning a gene fragment of *α-syn* into a *Sma I/Xho I*-digested pGEX-4T-1 plasmid. The GST fusion protein of α-syn was expressed in *Escherichia coli* BL21 cells and purified with glutathione-agarose beads. The GST tag was cleaved from the fusion proteins by digestion with thrombin.

The cDNAs for the CNA and CNB were isolated from rat brain cDNA libraries. CNA and CNB were expressed in *E. coli.* and purified in our lab. The purification scheme of CNB involved sequential hydrophobic chromatography, DEAE chromatography, and gel filtration. CaM from the bovine brain was purified by DEAE-cellulose 52 and Phenyl-Sepharose column in our lab [10]. CaM-Sepharose was prepared by coupling to CNBr-activated Sepharose. The CNA subunit was purified by CaM-Sepharose 4B affinity column. The enzyme activities of the reconstituted CN complex were found to be comparable to that of the bovine brain enzyme [11].

2.3. Microscale thermophoresis (MST)

The MST method has been previously described in detail [12,13]. We used a Monolith NT.115 from NanoTemper Technologies to measure the dissociation constant (K_d) of binding of Dylight488-labeled CNA to α-synuclein [14]. All proteins were dissolved in phosphate-buffered saline (PBS) buffer. CNA was labelled with the dye DyLight 488 NHS Ester (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Next, unconjugated dye was removed using the Zeba Spin Desalting Column (Thermo Scientific) and CNA was resuspended in PBS. The labelled CNA (10 μl) was kept at a constant concentration of about 500 nM and added to a dilution series of α-syn buffer (10 μl, from 40 μM to 1 nM). The samples were completely mixed and incubated at 25 °C for 5 min. They were then loaded into silica capillaries (Polymicro Technologies). Measurements were taken at 20 °C using 20% LED power and 20% IR laser power. Data were analyzed using NanoTemper Analysis software, v.1.2.101.

2.4. Preparation of mouse brain lysates

Male Kunming mice (weight 16 ± 2 g, 4 weeks of age) were supplied by the Vital River Corp (a joint venture with Charles River Laboratories). The animals were housed in groups under the following laboratory conditions: temperature 20 ± 1 °C, humidity 40–60%, and 12 h light/dark cycle with food and water *ad libitum*. The mice were killed under sodium pentobarbital anesthesia to minimize suffering. The brains were removed and homogenized at 4 °C by passing through a syringe into a solution of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM dithiothreitol, 0.2% NP-40, 1.0 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 μg/ml pepstatin. After sonication and centrifugation for 60 min at 16,000 × *g* and 4 °C, the supernatant was used as a source of CN for subsequent GST pull-down assays. Ca^{2+} and CaM were added to the lysis buffer in advance.

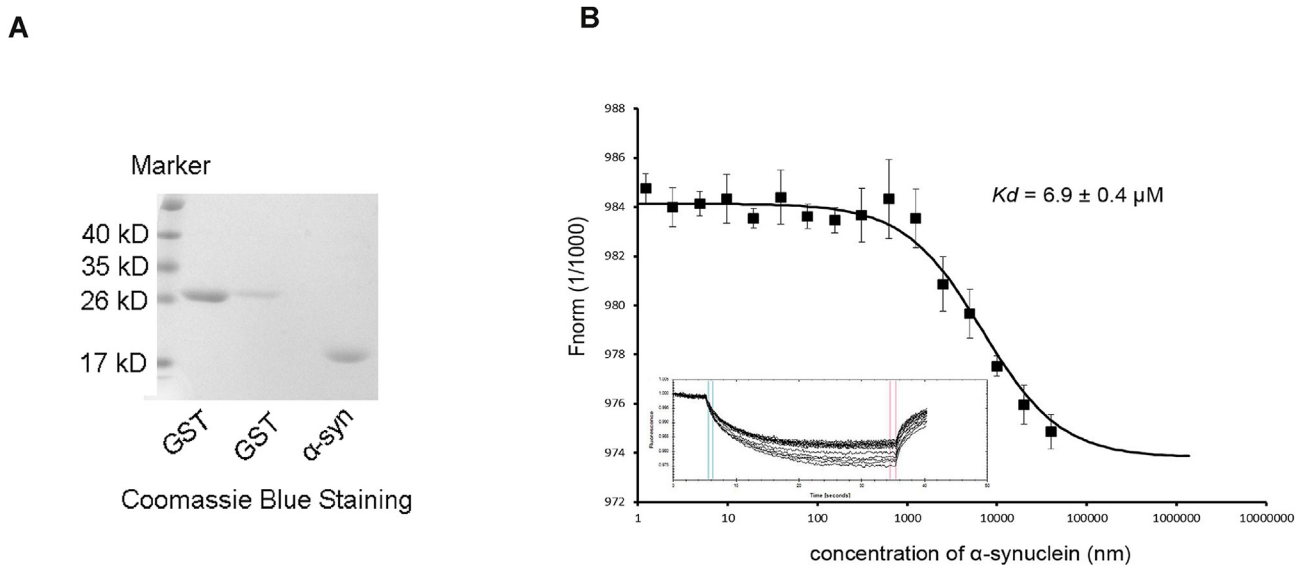

Fig. 1. α-syn binds purified recombinant CNA directly.
(A) SDS-PAGE of the purified proteins. The proteins were run on a 14% acrylamide gel and stained with Coomassie Blue. (B) The affinity of the Dylight 488-labeled CNA to α-syn was quantified. The α-syn protein was titrated using a fixed concentration of CN (500 nM). The top panel shows the isotherm derived from the raw data and fitted to a sigmoidal dose-response curve. The bottom panel shows the raw data for thermophoresis recorded at 20 °C using 20% LED and 20% MST power. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)