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Coronin 1A inhibits neurite outgrowth in PC12 cells

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HIGHLIGHTS

- Remodeling of actin cytoskeleton plays an important role in axonal sprouting.
- Overexpression of Coronin 1A in PC12 cells effects neurite outgrowth.
- Coronin 1A has an inhibitory effect on neurite outgrowth *in vitro*.

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ABSTRACT

Regenerative response to central nervous system damage in mammals is limited because of inhibitor signals which consist of myelin associated inhibitor proteins and chondroitin sulfate proteoglycans. Inhibitor signals mainly affect cytoskeleton elements which are important for axonal sprouting and neurite outgrowth. Coronin 1A is an actin cytoskeleton associated protein. Coronin 1A shows its effect on actin cytoskeleton through binding to the Arp2/3 complex which is a key nucleator of actin polymerization and regulates its activation on actin cytoskeleton. Coronin 1A–Arp2/3 interaction is regulated by phosphorylation of Coronin 1A from the C and N terminal region. Thus, Coronin 1A–Arp2/3 complex is one of the targets of inhibitory signaling cascades. The aim of this study was to investigate the effect of Coronin 1A on neurite outgrowth in PC12 cells *in vitro*. The results showed that Coronin 1A is expressed in differentiated PC12 cells and localized along axonal sprouting region of the neurites. Other results showed that overexpression of Coronin 1A in PC12 cells effects neurite outgrowth. Neurite lengths of the Coronin 1A overexpressing PC12 cells were lower than the untransfected ($p < 0.001$) and control transfected ($p = 0.002$) PC12 cells. These results indicate that Coronin 1A has an inhibitory effect on neurite outgrowth *in vitro*.

1. Introduction

Cytoskeleton organization and remodeling are essential cellular events during sprouting and neurite outgrowth [1]. Correct spatial and temporal organization of actin cytoskeleton, which is regulated by the assembly and disassembly processes, is important for the establishment of neuronal connections [2–4]. A number of actin binding proteins have been shown to orchestrate actin cytoskeletal dynamics, and these include the coronin family of proteins [5,6]. Coronin was originally identified as an F-actin binding protein enriched at the leading edge and at phagocytic cup structures of the slime mold *Dictyostelium discoideum* [5]. It was shown that one of the coronin protein family members Coronin 1A directly binds to Arp2/3 protein complex [7], and inhibits F-actin nucleation by freezing the Arp2/3 complex in its inactive "open" conformation [8–11]. The interaction between Coronin 1A and Arp2/3 is regulated by phosphorylation of Coronin 1A from both the C and N terminal regions with protein kinase C [7,8,12,13]. However, so far the role of Coronin 1A is only investigated in the immune system, due to the specific expression pattern in the phagocytic cells [14]. On the other hand, it was reported that the expression of Coronin 1A was significantly upregulated in rats following the spinal cord injury [15]. In addition, while this paper was prepared, Jayachandran et al. [16] published their data about the effects of Coronin 1A in nervous system, and showed that Coronin 1A deficiency may cause severe neurobehavioral and cognitive defects in mouse. The above observations prompted us to postulate that Coronin 1A could play a role in regulating cytoskeleton organization during axon formation or neurite outgrowth in neuronal cells. Although the role of Coronin 1A in immune system cells has already been investigated in numerous studies, this is the first study where the effects of Coronin 1A on neurite outgrowth were investigated, and the inhibiting effects on neurite outgrowth were shown.

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2. Materials and methods

2.1. Cell culture

PC12 cells (ATCC CRL-1721) were cultured in RPMI 1640, 15% horse serum, 5% fetal calf serum supplemented (media and serums from Gibco/Invitrogen) with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37 °C in a humidified and 5% CO_2 incubator. The cells were grown on collagen type 4-coated (Sigma) (40 μg/μl) flasks and glass coverslips. To provide the differentiation of PC12 cells, only 1% horse serum was used in complete medium (differentiation medium).

2.2. Immunofluorescence staining

PC12 cells were grown on collagen type 4-coated glass coverslips. The cells were cultured for 24 h in differentiation medium and treated with and without NGF (50 ng/ml) (Millipore) for the times indicated (0, 24, 48, 72, 96, and 120 h). The cells were fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS. The localizations of Coronin 1A and cytoskeletal elements (neuron specific βIII-tubulin and actin) were documented by incubation with Coronin 1A mouse monoclonal antibody (1:100) (Novus Biologicals), rabbit polyclonal anti-actin antibody (1:100) (Abcam), and mouse monoclonal anti-βIII tubulin antibody (1:250) (Abcam), respectively. Alexa Fluor 488 and 568 anti-rabbit, and anti-mouse (Molecular Probes/Invitrogen) were used as secondary antibodies (1:500 for both of them). Nuclei staining were carried out by incubation with DAPI. The coverslips were mounted in ProLong Gold antifade reagent (Molecular Probes/Invitrogen) and immunofluorescence images were acquired with trinocular inverted microscope with fluorescent attachment (Leica DMIL, Leica Microsystems, Heidelberg, Germany).

2.3. Western blot analysis

PC12 cells were grown on collagen type 4-coated 25 cm^2 culture flasks. For the experimental procedure, the cells were cultured for 24 h in differentiation medium and treated with and without NGF (50 ng/ml) (Millipore) for the times indicated (0, 24, 48, 72, 96, and 120 h). Lysed in blending buffer (62.5 mM Tris–HCl (pH 6.8), 5 mM EDTA, 10% SDS (pH 7.2), and mini protease inhibitor tablet). Bicinchoninic acid (BCA) Protein Assay Kit (Pierce) was used to determine the protein concentrations of cell lysates. Proteins (40 μg per lane) were separated using SDS-polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membrane (Thermo Scientific). Blots were probed with anti-Coronin 1A antibody (1:500). Goat anti-rabbit IgG HRP-conjugated secondary antibody (1:3000) (Molecular Probes/Invitrogen) was used for protein band detection. The blots were developed by ECL Plus chemiluminescence kit (Amersham Biosciences). To monitor the amount of protein loaded, membranes were stripped and reprobed with mouse anti-β-actin antibody. For quantitative Western blotting, densitometric analyses of the blots were made by Scion Image software (www.scioncorp.com). Data were calculated as the ratio of arbitrary densitometric units of Coronin 1A immunoreactive bands normalized to values obtained for β-actin bands on the same immunoblots.

2.4. Transfection

PC12 cells were plated 24 h before the transfection with antibiotic free medium at a density of 72,000 cells/cm^2 and transfected using Fugene HD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations using the following constructs: pQE-TriSystem-6-Coronin 1A (Qiagen) and pEGFP C2 (Clontech, Palo Alto, CA). Transfection mix was prepared as follows: 2 (μg):10 (μl) DNA to Fugene HD ratios. The transfection complex medium was removed 48 h later and replaced with differentiation medium with or without addition of 50 ng/ml NGF. The efficiency was calculated by observing pEGFP expression 24 h after transfection.

2.5. Neurite outgrowth analysis

We performed neurite outgrowth analyses in four groups: The first group was untransfected (T(−)) PC12 cells, the second groups was Coronin 1A overexpressing (T(+)) PC12 cells, the third and the fourth groups were control groups where transfection agent (Fugene HD), and pEGFP vector were applied alone to cells to evaluate the effects of transfection procedure, and also expression vector on neurite outgrowth, respectively. Neurite outgrowth analysis was performed as described by Fournier et al. [17] with some modifications. Briefly, the induction of differentiation in PC12 cells was evaluated by counting the proportion of cells bearing neurites and measuring the length of the longest neurite in individual cells. Cells bearing neurites with length greater than twice the cell body diameter were considered positive and neurite lengths were measured by using Leica Application Suite program (V2.4.0 R1). Tissue culture plates were photographed with 20× magnification, and 120 ± 20 cells were analyzed per tissue culture plate. The experiments were performed in biological triplicates. Mean values (SD) were determined for each time point and the length of the Coronin 1A overexpressing neurite cones were compared to that of control cultures.

2.6. Statistics

Nonparametric Kruskal–Wallis test was used for analysis of difference between groups. Significant differences between groups were determined using Mann–Whitney *U* test. A p value less than 0.05 was considered as statistically significant. All statistical analyses and tests were performed with the SPSS statistical package (SPSS 11.0 for Windows, Chicago, IL, USA).

3. Results

3.1. Localization and expression of Coronin 1A in PC12 cells

Localization of Coronin 1A and its association with cytoskeleton (actin and microtubules) were documented by using immunofluorescence (Fig. 1). Immunofluorescence staining (Fig. 1A) showed that Coronin 1A has been localized at the cortical cytoskeleton, cytoplasm, neurites and the neurite outgrowth cone overlapping with the actin cytoskeleton.

To determine the expression of Coronin 1A in the course of PC12 cell differentiation, immunoblotting was performed (Fig. 2). We determined the Coronin 1A specific band at 57 kDa by immunoblotting. Coronin 1A expression level was evaluated with densitometric analysis by using Scion Image program during differentiation of PC12 cells.

3.2. Localization and expression of Coronin 1A in Coronin 1A/pEGFP co-transfected PC12 cells

Coronin 1A overexpressing (T(+)) PC12 cells were analyzed by immunofluorescence staining to document any changes of Coronin 1A localization. Results of immunofluorescence staining revealed that intracellular distribution of Coronin 1A was similar to T(−) PC12 cells and was localized at the cortical cytoskeleton, cytoplasm, neurites and the neurite outgrowth cone overlapping with the actin cytoskeleton (Fig. 3).