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Intracellular cAMP controls a physical association of V-1 with CapZ in cultured mammalian endocrine cells

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Abstract

V-1, an ankyrin repeat protein with the activity to control tyrosine hydroxylase (TH) gene expression and transmitter release in PC12D cells, associates with CapZ, an actin capping protein, and thereby regulates actin polymerization in vitro. In this study, immunoprecipitation and Western blot analysis showed that V-1 was physically associated with CapZ-β in PC12D transfectants overexpressing V-1. These proteins were co-localized in the soma of Purkinje cells of rat cerebellum as assayed by immunohistochemistry. Furthermore, in the V-1 transfectants, the amount of CapZ which physically associated with V-1 was steeply reduced at 2 h after treatment with forskolin, but was thereafter increased to reach its initial level at 12 h after forskolin-treatment. These results suggest that the association of V-1 with CapZ is controlled by a cAMP-dependent signalling pathway probably to play a functional role in the regulatory mechanism of actin dynamics in the endocrine system and the central nervous system.

Keywords: V-1 protein; CapZ; Physical association; cAMP

V-1 is an ankyrin repeat protein, of which the expression is transiently upregulated during postnatal murine cerebellar development [1,2], and localized in the multiple CNS and PNS neurons, and the endocrine system as well [3–6], suggesting the possible involvement of V-1 not only in the development and maintenance of the neuronal circuit but also in the function of the endocrine system. V-1 is a soluble protein consisting of 117 amino acids that contains 2.5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat [1,2]. This motif has been demonstrated to be crucial for protein–protein interactions between various ankyrin repeat proteins with multiple physiological functions and the specific partner proteins [7–9], raising the possibility of the presence of the specific partner proteins for V-1. Thus, we have tried to search for the proteins with which V-1 interacts. Taoka et al. [10] have recently

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demonstrated that V-1 binds to two proteins in human embryonic kidney 293T cells ectopically overexpressing V-1 and rat cerebellum, and have identified these proteins as the α and β subunits of the actin capping protein called as CapZ [11] or β-actinin [12]. Furthermore, V-1 has been found to inhibit CapZ-regulated actin polymerization in a dose-dependent manner in vitro [10]. Here we provide evidence that V-1 physically associates with CapZ in PC12D transfectants overexpressing V-1 and cultured adrenal medullary cells, but also in the soma of rat cerebellar Purkinje cells in vivo, and that the association of V-1 with CapZ is regulated by intracellular cAMP in the cultured cells.

Materials and methods

Cell culture. V-1 transfectants (V1-69 and V1-46), the PC12D cells which stably overexpress V-1, and vector control transfectants (C-9) were established and cultured as previously reported [5]. Primary culture of bovine adrenal medullary cells was carried out as reported previously [13].

Anti-V-1 antibody preparation and recombinant V-1 production. Preparation and purification of rabbit polyclonal anti-V-1 antibodies and purification of rat recombinant V-1 bacterially expressed were performed as described previously [3,4].

Analyses of the specificity and immunoprecipitation activity for anti-V-1 antibodies. Cell extract preparation was performed as described previously [6]. For examining the specificity of each anti-V-1 antibody, cell extract (50 μg of protein/lane), tissue extract from adult rat cerebellum (50 μg of protein/lane), and a purified recombinant rat V-1 protein bacterially expressed (2 ng of protein/lane) were separated by SDS–PAGE and subjected to Western blotting using the tested anti-V-1 antibodies, as described previously [3,5]. For checking immunoprecipitation activity for different anti-V-1 antibodies, 4 μg of each anti-V-1 antibody was used for immunoprecipitation according to the following procedure: cell extracts including 1 mg proteins were incubated with 4 μg anti-V-1 antibodies or a preimmune serum comparable to 4 μg IgG for 12–18 h at 4 °C. Forty microliters of protein A–Sepharose CL-4B beads (50%(v/v) suspension, Amersham Biosciences) was then added to each tube and incubated for 1 h at 4 °C. The immunocomplex-bound beads were washed with ice-cold RIPA buffer. The beads were then mixed with 40 μl of twofold concentration of SDS–PAGE sample buffer and boiled to elute the bound immunocomplex as described previously [3,5]. Protein concentration was determined using Bio-Rad Protein Assay Reagent (Bio-Rad).

Metabolic labelling and immunoprecipitation. V-1 transfectants (1.2×10^7 cells) were plated on a 100-mm dish and cultured for 24 h. For metabolic labelling, cells were washed with DMEM minus cysteine (Gibco) and cultured for 12 h in 4 ml DMEM containing 5% dialyzed fetal calf serum, 10% dialyzed horse serum, and L-[^{35}S]cysteine (ICN Pharmaceuticals), and L-[^{35}S]cysteine then further added to the culture medium. Three hours later cells were washed with ice-cold 150 mM NaCl and 10 mM Hepes–KOH (pH 7.5). Five hundred microliters of RIPA buffer including protease inhibitors was then directly added to the dish to lyse cells with scrapers and by sonication. The cell lysates were centrifuged to obtain the supernatants as cell extracts. One hundred microliters of the cell extract was transferred to four 1.5-ml microcentrifugation tubes, respectively. Three different anti-V-1 antibodies and a preimmune rabbit serum comparable to 4 μg IgG were added to these tubes, respectively. For preparation of denatured cell extract, the cell extract was boiled in the presence of 1% SDS for 2 min and then a final concentration of 0.1% SDS was diluted by addition of RIPA buffer including 1% NP-40 prior to immunoprecipitation with anti-V-1 antibody or a preimmune rabbit serum. Immunoprecipitation was performed as described previously [14] except that Dynabeads M-280 sheep anti-rabbit IgG (Dynal) was used instead of Dynabeads M-280 sheep anti-mouse IgG. Immunoprecipitates were subjected to SDS–PAGE followed by visualization using an image analyzer BAS2000 (Fuji).

Immunohistochemistry. Postnatal 14- and 56-day-old rats were transcardially perfused with ice-cold Zamboni's fixative [15] following perfusion with 0.1 M PBS containing 50 U/ml heparin sodium (Novo Nordisk) warmed at 37 °C. Cerebella were dissected out and post-fixed in the same fixative overnight at 4 °C. Cerebellar tissues were immersed in 0.1 M phosphate buffer (PB, pH 7.2) containing 20% sucrose overnight at 4 °C for cryoprotection after rinsing them three times in 70 % ethanol and then in 0.1 M PB. They were then embedded in OCT compound (Sakura Finetechnical) and frozen in a mixture of dry ice and acetone. Cryostat sections were cut at a thickness of 8 μm and incubated with 5% normal donkey serum (Jackson ImmunoResearch) in a dilution buffer (0.1 M PBS with 0.2% Triton X-100) for 30 min at room temperature for blocking. Sections were then incubated with 1:400 diluted mouse monoclonal anti-chicken CapZ-β2 subunit antibody overnight at 4 °C. After a three-time rinse in 0.1 M PBS, sections were incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 1:100 in the dilution buffer) for 1 h at room temperature and rinsed in 0.1 M PBS. Thereafter sections were incubated with rabbit anti-V-1 antibody (1:1000) overnight at 4 °C, rinsed in 0.1 M PBS, incubated with FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, 1:100) for 1 h at room temperature, and rinsed in 0.1 M PBS. Sections were coverslipped with glycerol and observed by a confocal laser scanning microscopy (Bio-Rad). As a negative control, sections were treated with the same protocol described above except omitting the primary antibodies. We also confirmed no cross-reactivity of FITC-conjugated donkey anti-rabbit IgG with mouse anti-chicken CapZ-β2 subunit antibody.

Assay for association of V-1 with CapZ in V-1 transfectant. For this experiment, V-1 transfectants (4×10^6 cells) were cultured on a 60-mm culture dish for 48 h and then treated with or without forskolin or dibutyryl cAMP for indicated times. Cell and tissue extract preparation and immunoprecipitation were performed as described by Taoka et al. [10]. Immunoprecipitation was performed as described above, except that 5 μg IgG was added to each tube and that the immunocomplex-bound beads were washed with ice-cold washing buffer [10] instead of RIPA buffer. Cell extracts and immunoprecipitates were separated by SDS–PAGE and transferred to polyvinylidene difluoride (Bio-Rad) or nitrocellulose membrane (Schleicher and Schuell). Blotted membrane was blocked in PBST buffer (PBS including 0.05% Tween 20). The membrane was thereafter incubated successively with mouse monoclonal anti-CapZ-α1 and anti-CapZ-β2 antibodies (The Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences) in 5% skim milk/PBST and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (CHEMICON International). Western blot analysis of immunoprecipitates with anti-V-1 antibody was performed as described previously [6]. Immunoreactivities were visualized with enhanced chemiluminescence detection reagents (Pierce).

Results and discussion

First, to ascertain the specificity of anti-V-1 antibodies utilized for the present study, the immunoreactivity of each anti-V-1 antibody to cell extract from the V-1-overexpressing transfectants and rat cerebellum extract was tested by Western blot analysis. Western blot analysis evidently showed that anti-V-1 antibodies exhibited positive immunoreactivities to a single band with