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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 ($1.2 \times 10^7$ 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 ($4 \times 10^6$ 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

12 kDa which corresponded to the molecular weight of V-1 [2] (Fig. 1A). Immunoprecipitation followed by Western blotting demonstrated that these anti-V-1 antibodies actually immunoprecipitated V-1 in the cell extract from V-1 transfectant (Fig. 1B). Accordingly the tested antibodies were employed for Western blotting, immunoprecipitation or immunohistochemistry in this study.

Next, to search for proteins that interact with V-1 in cells, metabolic labelling of V-1-overexpressing PC12D transfectants was followed by immunoprecipitation with anti-V-1 antibodies. The anti-V-1 antibodies co-immunoprecipitated labelled V-1 with two labelled proteins which had the molecular weights of 33 and 35 kDa, respectively (Fig. 2A). The molecular weights of the two co-immunoprecipitated proteins appeared to correspond to those of CapZ-α and β described in our earlier report, respectively [10]. On the other hand, neither V-1 nor the two proteins were immunoprecipitated with the preimmune serum (Fig. 2A). Further, to examine whether V-1 associated with both the proteins via a physical interaction or a chemically covalent bond, the metabolically labelled cell extract was denatured by boiling it in the presence of 1% SDS prior to immunoprecipitation with the anti-V-1 antibody. As shown in Fig. 2B, it was observed that when the denatured sample was immunoprecipitated with the anti-V-1 antibody, labelled V-1 alone was immunoprecipitated. In addition V-1 was more effectively immunoprecipitated with the anti-V-1 antibody after denaturation than before. These findings indicate that V-1 specifically associates with both the proteins via a physical interaction, as reported previously [10].

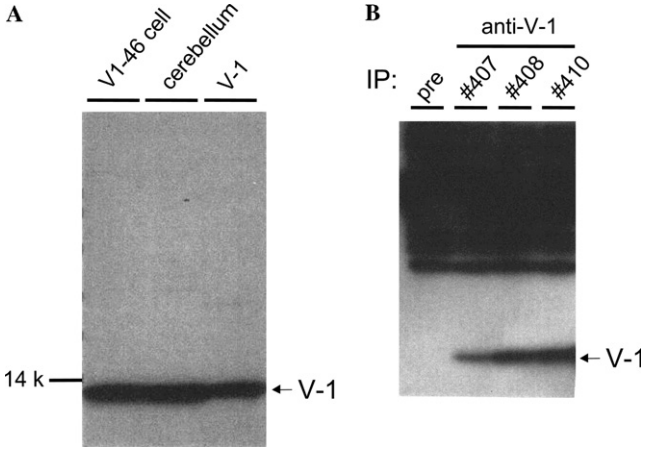


Fig. 1. Analyses of the specificity (A) and immunoprecipitation activity (B) for different anti-V-1 antibodies (#407, 408, and 410) by Western blotting and immunoprecipitation. Anti-V-1 antibody #407 was prepared as the antibody against the C-terminal amino acid sequence (CDGLTALEATDNQAIKALLQ) of V-1, while antibodies #408 and 410 recognize the N-terminal amino acid one (ALKNGDLDEVKDYVAKGED). V-1 and pre denote a purified recombinant V-1 protein bacterially expressed and a preimmune rabbit serum, respectively. Recombinant V-1 protein was used as a positive control. Similar results were obtained in at least two independent experiments.

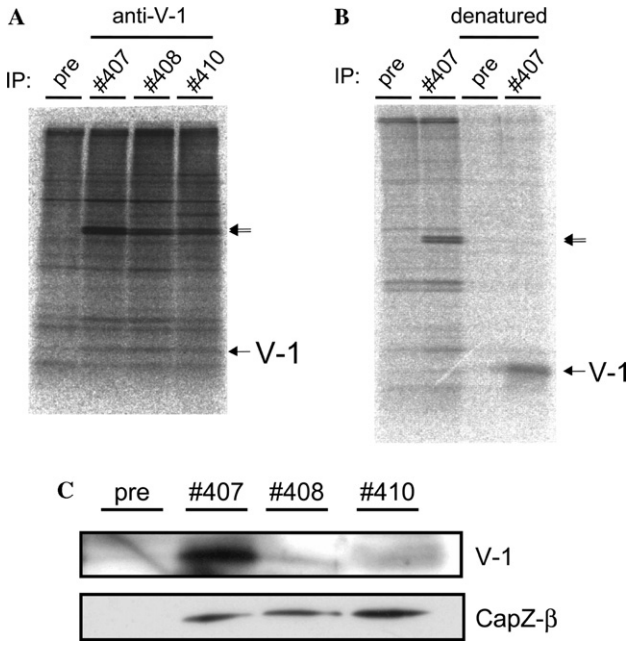


Fig. 2. Physical association of V-1 with CapZ in V-1 transfectants. (A) Specific immunoprecipitation of two metabolically labelled proteins with different anti-V-1 antibodies. Metabolic $^{35}$S-labelling of V-1 transfectant, immunoprecipitation, and the subsequent SDS–PAGE followed by detection labelled proteins as described under Materials and methods. (B) Prevention of co-immunoprecipitation of V-1 with the proteins with 33 and 35 kDa due to protein denaturation prior to the immunoprecipitation with anti-V-1 antibody. Arrows indicate the positions of 33 and 35 kDa proteins. (C) Specific co-immunoprecipitation of V-1 with CapZ-β from the cell extracts of V-1 transfectant. The cell extract of V-1 transfectant (V1-69) was incubated with an anti-V-1 antibodies (#407, 408, and 410) or preimmune rabbit serum. The resulting immunoprecipitates were collected on protein A–Sepharose CL-4B beads, subjected to SDS–PAGE, and analyzed by Western blotting with the anti-V-1 antibody (upper panel) or the anti-CapZ-β antibody (lower panel). Pre denotes a preimmune rabbit serum. Similar results were obtained in two or three independent experiments.

To test the question of whether the co-immunoprecipitated proteins are CapZ-α and -β proteins, cell extract from V-1 transfectant was immunoprecipitated with three kinds of anti-V-1 antibodies, and then the immunoprecipitate was analyzed by Western blotting using anti-V-1 and anti-CapZ-β antibodies. This biochemical analysis showed that V-1 was co-immunoprecipitated with CapZ-β from the cell extract from V-1 transfectant with the antibodies, with different immunoprecipitation efficiencies, whereas V-1 and CapZ-β were not immunoprecipitated with preimmune serum (Fig. 2C). CapZ-α was also observed to be co-immunoprecipitated with V-1 (data not shown). Based on the results described above, we concluded that V-1 physically associates with CapZ in the V-1 transfectants.

Furthermore, our immunohistochemical analysis showed that in cerebella of developing and mature rats,

V-1 co-localized with CapZ-β in the soma and primary dendrites of Purkinje cells (Fig. 3A). Interestingly in postnatal 14-day-old rats, co-localization of V-1 with CapZ-β was observed in glomerular synaptic junctions in the innergranule layer as well as in the cell bodies of Purkinje cells where active development of the dendrites is known to take place. Consistently in fact, CapZ was co-immunoprecipitated with V-1 in cerebellum extract from mature rats (Fig. 3B). These observations suggest that the association of V-1 with CapZ-β may participate in morphogenesis, including dendrite development and synapse formation via interaction with CapZ in the CNS neurons in vivo.

It is of great importance to identify the intracellular factors which control the association of V-1 with CapZ in the endocrine system and the CNS in order to elucidate the functional significance of V-1 in both the systems. We used the V-1 transfectants and cultured bovine adrenal medullary cells as model systems to study the effects of forskolin, a potent activator of adenylate cyclase which is the cAMP-producing enzyme, and dibutyryl cAMP (DB-cAMP), on the association of V-1 with CapZ. In the V-1 transfectants, forskolin at 3 μM decreased the amount of CapZ which associated with V-1 at 2 h after treatment, and thereafter increased the amount of CapZ associating physically with V-1 to reach its initial level at 12 h after treatment (Fig. 4A). On the other hand, expression of V-1 and CapZ showed no change in response to forskolin in V-1 transfectant (Figs. 4B and C). A similar time-dependent alteration in the physical association was also observed upon treatment with 0.5 mM DB-cAMP as well (Figs. 4D–F). Furthermore, a 24-h treatment of cultured bovine adrenal medullary cells with 1 mM DB-cAMP stimulated the association of V-1 with CapZ (Fig. 5). These findings for the first time demonstrate that this protein association is negatively and positively regulated by a cAMP-dependent signalling pathway in cultured mammalian cells.

Capping protein (CP) is one of the F-actin-binding proteins that cap the barbed end of actin filament to regulate actin assembly. CapZ distributes in a wide variety of tissues and organisms, and exhibits the biological roles as follows: (1) nucleation of actin assembly, (2) capture of preexisting F-actin, and (3) regulation of actin assembly at the barbed ends of F-actin [16]. In addition, like other capping proteins, CapZ is negatively regulated by the second messengers, such as PIP and $PIP_2$, by promoting removal of this protein from F-actin in vivo. This enables actin polymerization to proceed in the local region of cells [17]. Recently, Taoka et al. [10] have reported that V-1 binds to the heterodimer composed by CapZ-α and -β to inhibit both the nucleation of actin assembly and the actin assembly at the barbed ends of F-actin, indicating that V-1 acts as a novel negative regulator of CapZ in vitro. In the present study, we confirmed the co-localization of V-1 and CapZ in the Purkinje cell soma and the primary dendrites in rat cerebellum as well as in V-1 transfectants and cultured adrenal medullary cells. Neurite extension requires organized actin polymerization to push the cell membrane forward [18]. CapZ has been recently demonstrated to serve as a negative regulator of filopodia formation [19]. Therefore, these findings provide the notion that V-1 accelerates morphological differentiation of neurons by formation of filopodia through stimulation of dissociation of CapZ with actin filament. The most important finding of the present study is the fact that such a protein association between V-1 and CapZ is controlled by intracellular cAMP. It has been documented that in cerebellar Purkinje cells, a PKA-dependent signalling

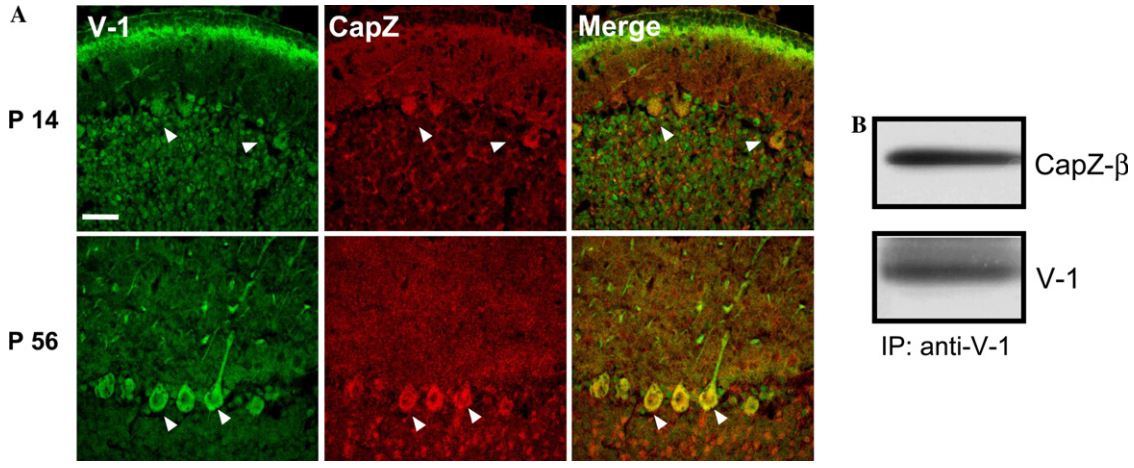


Fig. 3. Co-localization of V-1 and CapZ in the soma of Purkinje cells of rat cerebellum (A), and physical association of V-1 with CapZ in the rat cerebellum (B). (A) These microphotographs show sections of rat cerebellum double-stained with antibodies to V-1 (left) and CapZ-β (middle), and the merged image (right). Arrowheads indicate Purkinje cells which show a co-localization of V-1 with CapZ in the cell body. (B) Co-immunoprecipitation of V-1 with CapZ-β from the cerebellum extract. Immunoprecipitation of the cerebellum extracts with anti-V-1 antibody and the subsequent Western blot analysis with anti-CapZ-β antibody were performed as described in the legend of Fig. 2C. Scale bar, 40 μm.

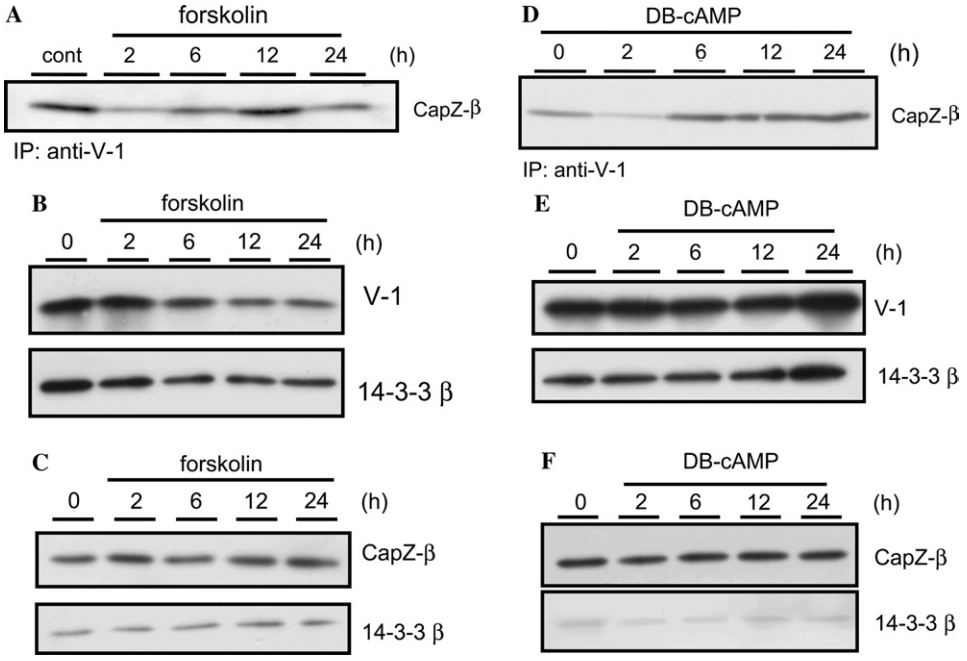


Fig. 4. Regulation of the association of V-1 with CapZ (A and D) and the expression of V-1 (B and E) and CapZ (C and F) by a cAMP-dependent signalling pathway in the V-1 transfectant. V-1 transfectant (V1-69) was cultured in the presence of a vehicle (0.1% DMSO) as control, or 3 μM forskolin (A–C) or 0.5 mM dibutyryl-cAMP (DB-cAMP; D–F) for indicated times. Immunoprecipitation of the cell extracts with anti-V-1 antibody and the subsequent Western blot analysis with anti-CapZ-β antibody were performed as described in the legend of Fig. 2C. Similar results were obtained in three independent experiments.

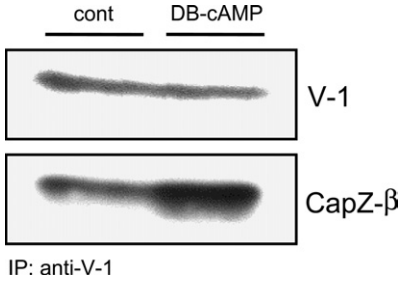


Fig. 5. Enhancement of the association of V-1 with CapZ by a 24-h treatment with DB-cAMP in bovine adrenal medullary cells in culture. Cells were cultured in the presence of a vehicle (0.1% DMSO) as control, or 1 mM DB-cAMP for 24 h. Cell extract preparation and immunoprecipitation with anti-V-1 antibody followed by Western blotting for V-1 and CapZ were conducted as described above. Similar results were obtained in three independent experiments.

pathway triggers cytoskeletal actin reorganization through an activation of cdc42 leading to filopodia formation and facilitation of the dendritic differentiation [20]. In this study, a negative and positive regulation of the association of V-1 with CapZ by intracellular cAMP also showed a cell type- and time-dependency. Accordingly it is fairly plausible to consider that this negative and positive control of association of V-1 with CapZ by intracellular cAMP induces filopodial or lamellipodial formation.

In summary, we found V-1 association with CapZ in clonal and primary endocrine cells in culture and in the CNS, and that the protein association was regulated in a cAMP-dependent manner. These results suggest that the association of V-1 with CapZ plays a critical role in actin dynamics under cAMP-dependent control. The elucidation of the regulatory mechanism of actin dynamics in the CNS neurons that is controlled by V-1 via protein–protein interaction with CapZ may give us a new insight into the molecular mechanism underlying morphogenesis of the CNS.

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