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Expression and function of neuronal growth-associated proteins (nGAPs) in PC12 cells

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ABSTRACT

The growth cone plays crucial roles in neural wiring, synapse formation, and axonal regeneration. Continuous rearrangement of cytoskeletal elements and targeting of transported vesicles to the plasma membrane are essential to growth cone motility; however, the proteins directly involved in these processes and their specific functions are not well established. We recently identified 17 proteins as functional marker proteins of the mammalian growth cone and as neuronal growth-associated proteins in rat cortical neurons (nGAPs; Nozumi et al., 2009). To determine whether these 17 proteins are growth cone markers in other neuronal cell types, we examined their expression and function in PC12D cells. We found that all 17 nGAPs were highly concentrated in the growth cones of PC12D cells, and that knockdown of all of them by RNAi reduced or inhibited neurite outgrowth, indicating that all of the 17 nGAPs may be general growth cone markers. Among them, eight proteins were shown to regulate the amount of F-actin in PC12D growth cones. Two of these nGAP that are cytoskeletal proteins, Cap1 and Sept2, increased the mean growth cone area and the mean neurite length by regulating the amount of F-actin; Sept2 also induced filopodial growth. Taken together, our data suggested that some of the nGAPs were generalized markers of the growth cone in multiple neuronal cell types and some of them, such as Cap1 and Sept2, regulated growth cone morphology through rearrangement of F-actin and thereby controlled neurite outgrowth.

1. Introduction

The growth cone is a specialized structure formed at the tip of an extending axon in a developing neuron that is crucial to axonal growth, axon pathfinding, synaptogenesis, and ultimately neural network formation (Chilton, 2006; Gomez and Zheng, 2006). The molecular components and mechanisms functioning in the growth cone were mainly studied using genetic mutant analysis in model organisms (e.g., *Caenorhabditis elegans* and *Drosophila*) and pharmacological studies of the peripheral nervous system (PNS) neurons or neuronal cell lines. However, the molecular basis of growth cone behavior in mammalian central nervous system (CNS) neurons is less well studied.

In our recent proteomic studies, we revealed approximately 1000 species of proteins and many features of mammalian CNS growth cone proteins by comparing growth cones and mature synapses in the rat cortex (Nozumi et al., 2009). In addition, using immunolocalization and RNAi studies in combination with our proteomic analysis, we provided evidence that 17 of the proteins we identified are highly concentrated in growth cones and regulate axonal growth, and we concluded that these 17 proteins are useful as unique functional molecular markers of the growth cone. We named them neuronal growth-associated proteins (nGAPs; Nozumi et al., 2009).

To determine whether these proteins can function as growth cone markers in neuronal cell types other than cortical neurons, we examined their expression and functions in PC12D cells. The PC12D cell line, a derivative of the PC12 cell line, differs from the parent PC12 cells because PC12D cells extend neurites very quickly (within 24 h) in response to nerve growth factor (NGF).

We designed experiments to determine whether the nGAPs are concentrated in the growth cone of PC12D cells and whether any of the nGAPs regulate growth cone morphology and/or neurite outgrowth. We found that all of the 17 nGAPs, which were identified in rat cortical growth cones, were concentrated in PC12D growth cones. In addition, knockdown of all of 17 by RNAi reduced or inhibited neurite outgrowth, indicating that all of the 17 nGAPs may be general growth cone markers. Further tests with two of these

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Table 1
List of the nGAPs and their antibodies.

Official symbol	Protein name	Protein category	Antibody provider/references	Type of antibody	Fixation method
Cap1	Adenylate cyclase-associated protein 1	Cytoskeletal	Abnova	mAb	GA
Capzb	Capping protein (actin filament) muscle Z-line, beta	Cytoskeletal	Nozumi et al. (2009)	pAb	GA
Clptm1	Cleft lip and palate associated transmembrane protein 1	Receptor	Nozumi et al. (2009)	pAb	GA
Cotl1	Coactosin-like 1	Cytoskeleton	Abnova	mAb	GA
Crmp1	Collapsin response mediator protein 1	Signaling	Rosslenbroich et al. (2003)	pAb	PFA
Cyfip1	Cytoplasmic FMR1 interacting protein 1	GTP-binding	Nozumi et al. (2009)	pAb	GA
Fabp7	Fatty acid binding protein 7, brain	Signaling	Tachikawa et al. (2004)	pAb	GA
Farp2	FERM, RhoGEF and pleckstrin domain protein 2	GTP-binding	Koga, H., Nagase, T. (Kazusa DNA Inst, Japan; unpublished)	pAb	GA
Gap43	Growth associated protein 43	Signaling	Sigma–Aldrich	mAb	GA
Gnao1	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	GTP-binding	Asano et al. (1988)	pAb	GA
Gnai2	Guanine nucleotide binding protein (G protein), alpha inhibiting 2	GTP-binding	Asano et al. (1989)	pAb	GA
Pacs1	Phosphofurin acidic cluster sorting protein 1	Signaling	Xiang et al. (2000)	pAb	GA
Rtn1	Reticulon 1	Signaling	Mubio	mAb	PFA
Sept2	Septin 2	Cytoskeleton	Kinoshita et al. (1997)	pAb	GA
Snap25	Synaptosomal-associated protein 25	Membrane	WAKO	mAb	GA
Strap	Serine/threonine kinase receptor associated protein	Signaling	Nozumi et al. (2009)	pAb	GA
Stx7	Syntaxin 7	Membrane	Synaptic systems	pAb	PFA
Tmod2	Tropomodulin 2	Cytoskeleton	Nozumi et al. (2009)	pAb	GA

For nGAPs (Nozumi et al., 2009), the protein information and antibodies used are listed. Official symbol and protein name: they are followed in NCBI. Antibody provider/references: we purchased the antibodies from the companies shown in this list, or were provided by the researchers whose papers are shown here. Types of antibody: mAb, monoclonal; pAb, polyclonal. Fixation method: for immunostaining using these antibodies, PC12D cells were fixed with either glutaraldehyde (GA) or paraformaldehyde (PFA).

nGAPs, Cap1 and Sept2, demonstrated that these two cytoskeletal components regulated neurite extension by influencing the motility of growth cones. Our results indicated that expression levels of nGAPs may be precisely regulated to ensure normal F-actin organization.

2. Materials and methods

2.1. Cell culture

PC12D cells were seeded on poly-L-lysine-coated chamber slides with 4-wells made of Permanox Lab Tek Chamber Slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a density of 2×10^4 cells/cm^2. Cultured cells were grown in Dulbecco's modified Eagle's medium (DMEM; WAKO, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 50 ng/ml NGF (mouse nerve growth factor 2.5S Grade I; Invitrogen, Carlsbad, CA, USA) and kept in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C.

2.2. Antibodies and reagents

The primary antibodies used were listed in Table 1: CAP1 (monoclonal; Abnova, Taipei, Taiwan); COTL1 (monoclonal; Abnova); GAP-43 (monoclonal; Sigma–Aldrich, St. Louis, MO, USA); Reticulon-1C (monoclonal; Mubio, Maastricht, The Netherlands); mouse anti-SNAP-25 (WAKO, Japan); Syntaxin7 antibody (polyclonal; Synaptic Systems, Goettingen, Germany); rabbit anti-CapZβ, rabbit anti-CLPTM1, rabbit anti-CYFP1, rabbit anti-STRAP and rabbit anti-Tropomodulin 2 (newly produced for this research; see Table S7 in Nozumi et al., 2009); monoclonal anti-GFP and monoclonal anti-myc tag 9E10 (MBL, Japan). Other specific antibodies were introduced in the following references and generously provided by the respective authors: rabbit anti-B-FABP (Fabp7; Tachikawa et al., 2004); anti-mKIAA0793 (Farp2; Koga H., Kazusa DNA Inst, Japan); rabbit anti-Septin2 (Kinoshita et al., 1997); rabbit anti-$Gi_2\alpha$ (Asano et al., 1989); rabbit anti-$G_O\alpha$; (Asano et al., 1988); rabbit anti-CRMP1 (Rosslenbroich et al., 2003). Alexa 546-conjugated phalloidin (used to label F-actin) and Alexa 488- and 546-conjugated goat secondary antibodies (used to recognized mouse or rabbit immunoglobulin G (IgG)) were from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

2.3. Immunostaining and digital imaging analysis

The cultured PC12D cells were fixed in 2.5% glutaraldehyde (GA) or 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100. For glutaraldehyde fixation, we reduced background staining by treating with 1% sodium tetrahydroborate (Nozumi et al., 2009). The blocking solution was 1% bovine serum albumin (Sigma–Aldrich). Cells were then sequentially incubated with primary antibodies and secondary antibodies.

For immunostaining, primary antibodies against a target protein or a peptide tag were used together with a primary antibody recognizing α-tubulin (Abcam and Sigma–Aldrich), which labeled the growth cones. In each double-labeling experiment, one primary antibody was detected using an Alexa 488-labeled secondary antibody, and the other was detected with an Alexa 568-labeled secondary antibody. Rhodamine-phalloidin (Sigma–Aldrich) was included in the incubation with secondary antibodies to label F-actin.

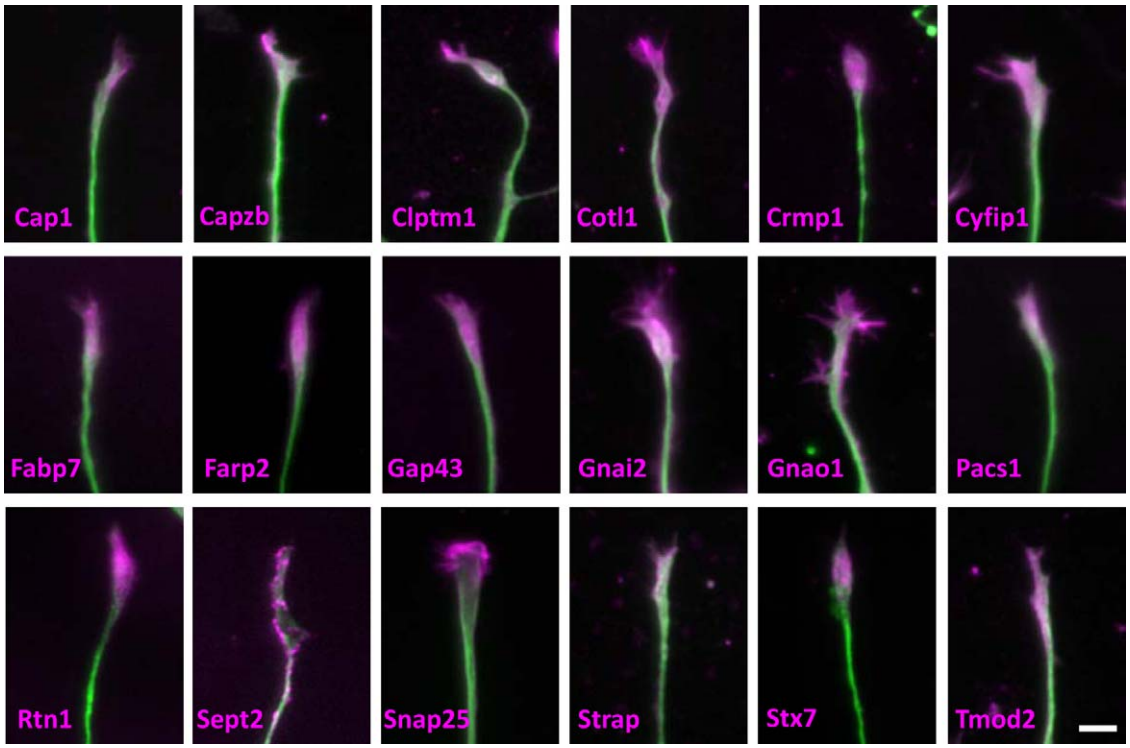

Fig. 1. Immunostaining of nGAPs and GAP-43 (Gap43) in the growth cone of PC12D cells. The micrographs shown are merged immunofluorescent images of anti-nGAPs (*magenta*) and anti-α-tubulin antibodies (*green*). See also Table 1 for the information of each protein. Scale bar: 5 μm. The order of micrographs is alphabetical.

To analyze digital images, micrographs of fluorescent immunostained specimens were taken using a digital CCD camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan) attached with an Axiovert 200 (Carl Zeiss). Regions (150 × 150 pixels) of the 1344 × 1024 pixel micrograph images that contained growth cones were excised. For each 150 × 150 pixel region, we determined the average fluorescence intensity per pixel, the fluorescence wavelength(s), and the pixel number in the growth cone area using ImageJ software. We measured growth cone areas using the method described by Takei et al. (2000).

2.4. RNAi experiments

For knockdown in expression of specific nGAPs, PC12D cells were transfected with siRNA targeting individual nGAPs. The siRNA sequences were described previously (Lu et al., 2008; Nozumi et al., 2009). PC12D cells grown on coverslips pre-coated with poly-L-lysine were transfected with siRNA using Lipofectamine 2000 reagent according to the manufacturer's protocol. The cells were treated with NGF 24 h after transfection and further cultured in the presence of NGF for 24 h. After this 48-h post-transfection culture, cells were fixed with 4% PFA, stained with Alexa 546-conjugated phalloidin, and observed by microscopy. Neurite length, growth cone area, and/or mean filopodia length was measured using ImageJ software (NIH). Control and mock transfection solution groups were treated without siRNAs and with only transfection solution, respectively.

2.5. Overexpression experiments for Cap1 and Sept2

PC12D cells (1×10^6) were transiently transfected with 1 μg/μl of vector-only DNA or the pEGFP-C1/Septin2 or myc-pCAGGS Cap1 expression construct by electroporation using CUY21Pro-Vitro (NEPA GENE, Co., Ltd., Ichikawa, Japan), according to the manufacturer's protocol. Transfected cells were grown on poly-L-lysine-coated coverslips for 24 h, then treated with NGF, and cultured for another 24 h in the presence of NGF. At 48 h after electroporation, the cells were fixed with 4% PFA and then observed with a microscope.

2.6. Statistics

All data are represented as means ± S.E.M. Student's *t*-test was used for statistical analysis as appropriate and a P value < 0.05 was considered statistically significant.

3. Results and discussion

Immunostaining results showed that all 17 nGAP proteins enriched in growth cones of rat cortical neurons were also more enriched in PC12D growth cones than in PC12D neuritis, as well as GAP-43, a classical growth cone marker (Fig. 1; Nozumi et al., 2009; also see Table 1). We used an RNAi-based approach to determine the function of these proteins in neurite growth. We found that reduced expression of all of the 17 proteins inhibited neurite outgrowth (Fig. 2A). These data are in agreement with our previous results from studies of cortical neurons (Nozumi et al., 2009). Thus, all of them may be generally useful as markers of the neuronal growth cone that are independent of neuronal cell type.

Next, we selected the 10 of the 17 nGAPs that are cytoskeletal, vescicular trafficking, or signaling proteins and measured the area of the occupied by the growth cone in RNAi-treated and control PC12D cells using phalloidin staining of F-actin (Fig. 3). RNAi-based knockdown of any one of 8 (Gnai2, Stx7, Snap 25, Gnao1, Rtn1, Cotl1, Cap1, Capzb, or Sept2) of the 10 proteins resulted in a reduction in growth cone size (Fig. 2B). Thus, several of the nGAPs affect growth cone morphology.

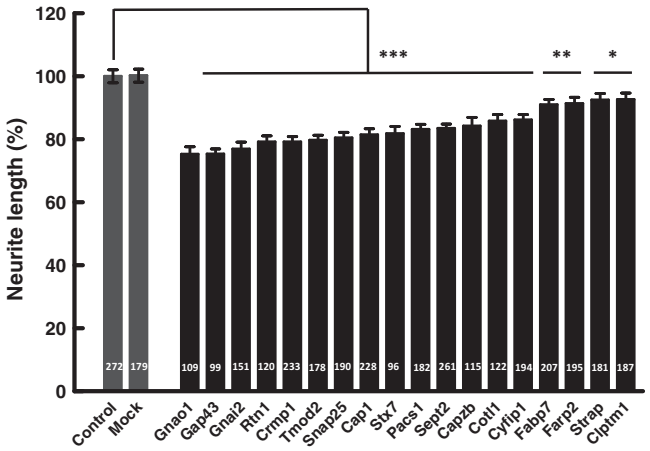

Fig. 2. Neurite length was decreased by siRNA-mediated knockdown of nGAPs and GAP-43 (Gap43). The values shown are means ± S.E.M. Control and mock transfection solution groups were treated without siRNAs and with only transfection solution, respectively. The mean neurite length in the control group was designated as 100%. Differences were compared by *t*-test (***$P < 0.001$; **$P < 0.01$; and *$P < 0.05$). The number at the bottom of each bar indicates the number of cells examined.

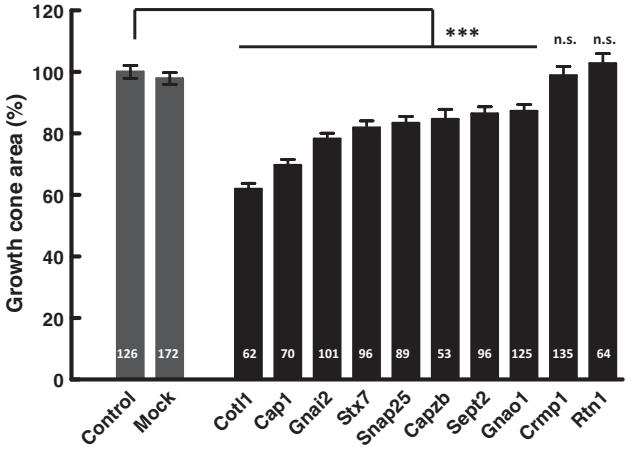

Fig. 3. Growth cone area was decreased by siRNA-mediated knockdown of 10 nGAPs. The values shown are means ± S.E.M. Control and mock transfection solution groups were treated without siRNAs and with only transfection solution, respectively. The mean growth cone area in control group was designated as 100%. Differences were compared by *t*-test (***$P < 0.001$; *n.s.*, $P > 0.05$). The number at the bottom of each bar indicates the number of cells examined.

RNAi-based knockdown of each cytoskeletal nGAP resulted in reduced growth cones areas; therefore, we selected the two cytoskeletal nGAPs, Cap1 (adenylate cyclase-associated protein 1) and Sept2 (Septin 2; Nedd5), with the largest RNAi-mediated effects on neurite outgrowth (Figs. 2 and 3) and performed overexpression experiments. Endogenous Cap1 localized predominantly to the central region of the growth cone in PC12D cells (Fig. 4A). RNAi of Cap1 decreased the amount of Cap1 and F-actin intensity (Fig. 4A and B). Overexpression of myc-Cap1 increased the length of the neurite (Fig. 4C), the area of F-actin staining (Fig. 4D), and the total amount of F-actin in the growth cone (Fig. 4A and E).

In undifferentiated PC12D cells, endogenous Sept2 localized to the plasma membrane and the cell cortex (data not shown), but endogenous Sept2 localized to the peripheral region of the growth cone when PC12D cells differentiated (Fig. 1). RNAi of Sept2 decreased the amount of Sept2 and F-actin intensity (Fig. 5A and B). Like myc-Cap1 overexpression, EGFP-Sept2 overexpression increased neurite length (Fig. 5C), the area of F-actin stain-

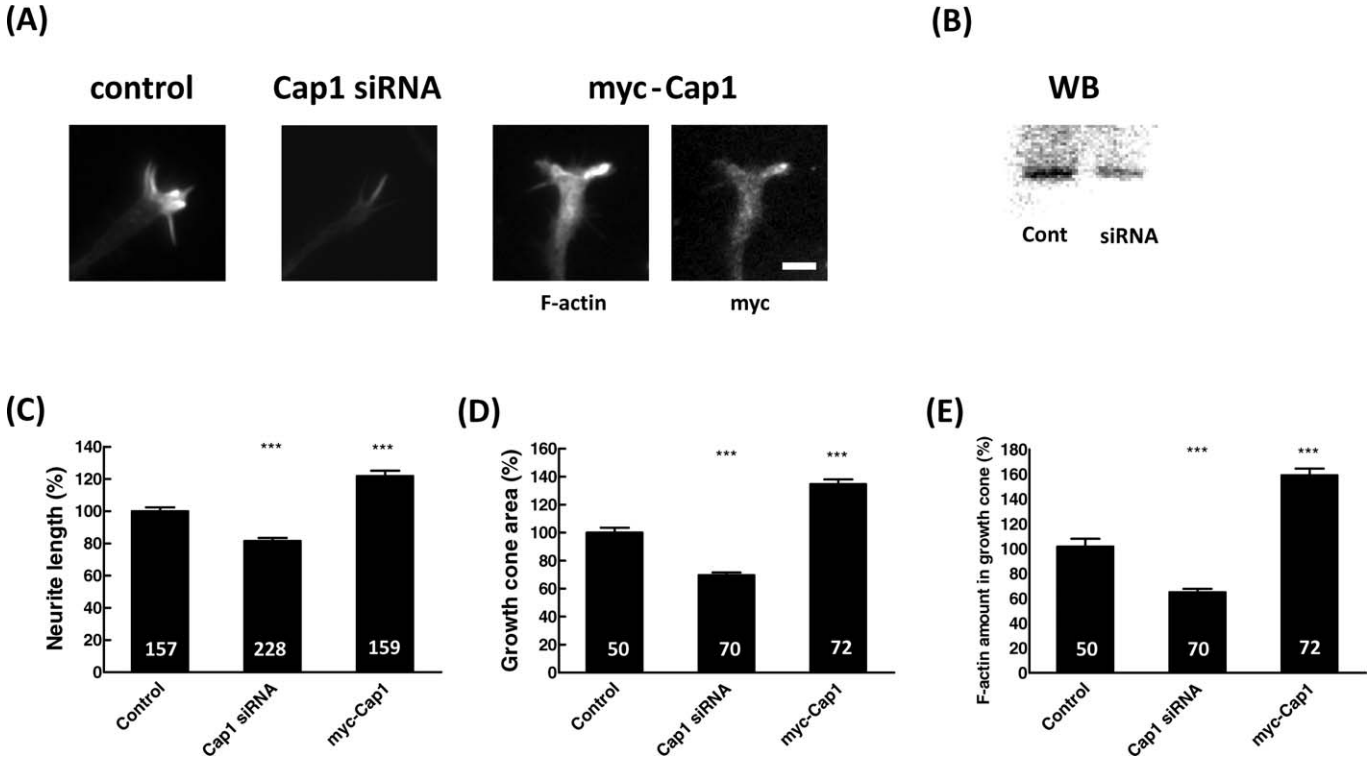

Fig. 4. Cap1 expression and function in PC12D growth cones. (A) Micrographs of phalloidin-staining in the growth cone of control (*control*), siRNA-knockdown (*Cap1 siRNA*), and Cap1-overexpressing PC12 cells stained with phalloidin (*F-actin*) or anti-c-myc tag antibody (*c-myc*). (B) Western blot of the Cap1 proteins after administration of siRNA (*siRNA*) compared with control (*control*). (C–E) Manipulation of Cap1 expression regulated neurite extension (C), influenced growth cone area (D), and altered the amount of F-actin in the growth cone (E). The mean neurite length (C), growth cone area (D), and F-actin in the growth cone area (E) of the control group were each designated as 100%. The number at the bottom of each bar (C–E) represents the observed number of growth cones. Differences were compared by *t*-test in (C)–(E) (***$P < 0.001$). Scale bar: 5 μm.

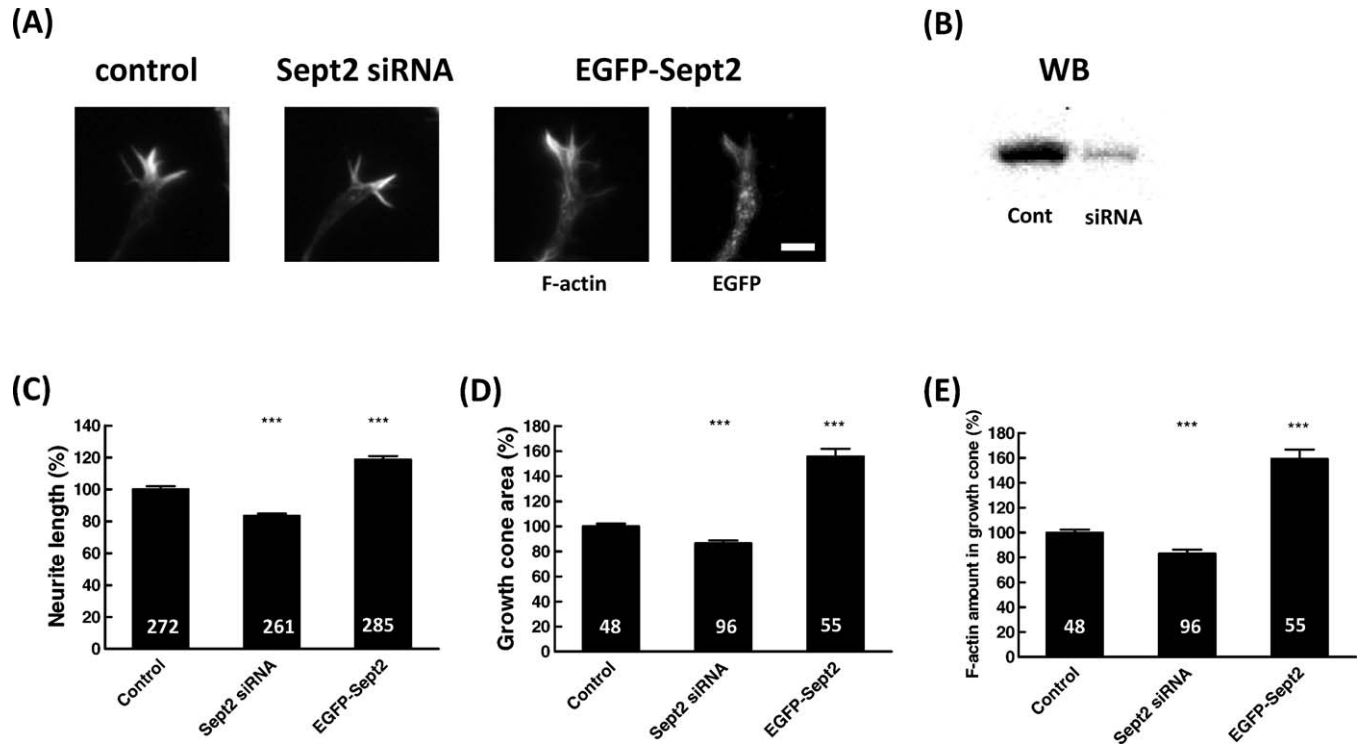

Fig. 5. Sept2 expression and function in PC12D growth cones. (A) Micrographs of phalloidin-staining in the growth cone of control (*control*), siRNA-knockdown (*Sept2 siRNA*), and Sept2-overexpressing PC12 cells stained with phalloidin (*F-actin*) or anti-EGFP tag antibody (*c-myc*). (B) Western blot of the Sept2 proteins after administration of siRNA (*EGFP*) compared with control (*control*). (C–E) Manipulation of Sept2 expression regulated neurite extension (C), influenced growth cone area (D), and altered the amount of F-actin in the growth cone (E). The mean neurite length (C), growth cone area (D), and F-actin in the growth cone area (E) of the control group were each designated as 100%. The number at the bottom of each bar (C–E) represents the observed number of the growth cones. Differences were compared by *t*-test in (C)–(E) (***$P < 0.001$). Scale bar: 5 μm.

ing (Fig. 5D), and the total amount of F-actin in growth cones (Fig. 5E).

Cap1 and Sept2 both affected the total intensity of F-actin staining in the growth cones (Figs. 4E and 5E); therefore, we counted the number of filopodia and mean filopodia length on the PC12D cell surfaces. Neither RNAi-mediated knockdown nor overexpression of either protein (Cap1 or Sept2) resulted in significant changes in filopodia number (Fig. 6A); however, overexpression of Sept2 (but not Cap1) increased the mean filopodial length (Fig. 6B).

Our present results clearly show that all of the nGAPs were also the growth cone markers in PC12D cells (Figs. 1 and 2). PC12D cells are pheochromocytoma cells related to the PNS neurons and are only distantly related to cortical neurons on the basis the cell lineage. Thus, our study indicated that these nGAPs may serve as general markers of the growth cone.

The growth cone is enriched in F-actin and the actin cytoskeleton undergoes extensive remodeling during growth cone motility (Geraldo and Gordon-Weeks, 2009). Moreover, neurite elongation is dependent upon the insertion of newly synthesized membrane at the growth cone (Igarashi et al., 1996, 1997; Pfenninger, 2009). Thus, continuous rearrangement of cytoskeletons and recruitment of transport vesicles to the plasma membrane are both essential to growth cone function; however, the identity of proteins directly involved in these processes has not been established. We suspect that the nGAPs identified in our previous study and those characterized in this study must be involved in these processes at least to some degree (Nozumi et al., 2009). Because of the importance of cytoskeletal rearrangements and of membrane remodeling to growth cone function, we focused our studies on the roles of the cytoskeletal proteins found in our collection of nGAPs. Cap1 has an adenylyl cyclase-binding domain and an actin-binding domain, suggesting a function in integrating signal transduction and actin cytoskeletal reorganization (Hubberstey and Mottillo, 2002). Mammalian Cap1 is a G-actin-binding protein and has a capping activity. Our present experiments show that Cap1 functioned in neurite growth by regulating the amount of F-actin in the growth cone (Fig. 4). In mammalian nonneuronal cells, Cap1 regulates F-actin through another actin-binding protein, cofilin (Moriyama and Yahara, 2002; Bertling et al., 2004), an important actin regulator in the growth cone (Pak et al., 2008), suggesting that Cap1 contributes to growth cone behavior in a similar mode.

Sept2, another cytoskeletal nGAP, belongs to the septin family, a conserved family of GTP-binding proteins present in organisms ranging from yeasts to mammals (Weirich et al., 2008). The septins have several roles in cytoskeletal organization and membrane-remodeling events (Beites et al., 1999; Vega and Hsu, 2003; Tada et al., 2007). We revealed that Sept2 was also involved in neurite outgrowth, probably by increasing the amount of F-actin staining in growth cone (Fig. 5C–E), and that it affected filopodial growth (Fig. 6B); however, we do not understand the molecular mechanisms by which Sept2 mediated these processes. Sept2 is a component of septin filaments, and these filaments interact with F-actin (Tada et al., 2007). Thus, it is very likely that like Cap1, Sept2 regulated the growth cone activity by regulating F-actin organization. Sept2 was more concentrated in the peripheral regions of the growth cone and tightly colocalized with F-actin staining (Fig. 1), and it is likely that overexpression of Sept2 induced filopodial growth. Because RNAi of either Sept2 or Cap1 did not alter the mean filopodial length (Fig. 6), we concluded that other actin-binding proteins, including other nGAPs, carried out activities similar to those of Sept2 and Cap1.

Our present results provided evidence that most nGAPs may be considered functional growth cone markers in multiple types of neurons. Moreover, we investigated the roles of two of these

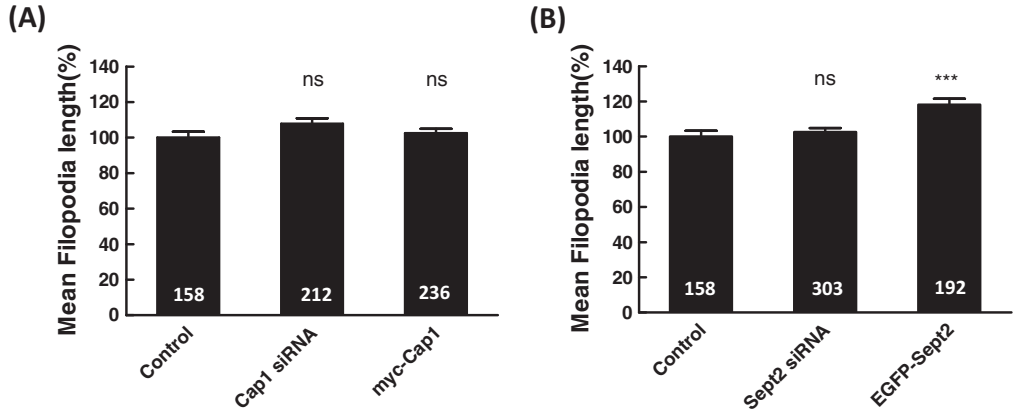

Fig. 6. Length of filopodial length extending from growth cones was increased by overexpression of Sept2, but of Cap1. The values of filopodial length in Cap1-overexpressing (A) and Sept2-overexpressing (B) cells shown are means ± S.E.M., and differences were compared by a *t*-test (***$P < 0.001$; ns, $P > 0.05$). The control group was not treated with siRNA or DNA, and the mean filopodia length of the control group was designated as 100%.

molecules, Cap1 and Sept2, in axonal growth to understand the mechanism of growth cone motility, providing a template for future studies of the other nGAPs.

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