Phosphatidylinositol 4-phosphate 5-kinase β regulates growth cone morphology and Semaphorin 3A-triggered growth cone collapse in mouse dorsal root ganglion neurons

doi: 10.1016/j.neulet.2013.04.062

Phosphatidylinositol 4-phosphate 5-kinase β regulates growth cone morphology and Semaphorin 3A-triggered growth cone collapse in mouse dorsal root ganglion neurons

Masakazu Yamazaki[a,1,2], Yohei Yamauchi[b,1], Yoshio Goshima[c], Yasunori Kanaho[b,*]

[a]*Research Center for Biosignal, Akita University, 1-1-1 Hondo, Akita 010-8543, Japan*

[b]*Department of Physiological Chemistry, Faculty of Medicine & Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan*

[c]*Department of Molecular Pharmacology and Neurobiology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan*

*Correspondence to: Yasunori Kanaho

Department of Physiological Chemistry

Faculty of Medicine & Graduate School of Comprehensive Human Sciences

University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan

Tel: (Japan: +81) 29-853-3282

Fax: (Japan: +81) 29-853-3271

E-mail: ykanaho@md.tsukuba.ac.jp

[1] These authors equally contributed to this work.

[2] Present address: Department of Cell Biology and Morphology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

Abstract

Growth cone motility and morphology, which are critical for axon guidance, are controlled through intracellular events such as actin cytoskeletal reorganization and vesicular trafficking. The membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] has been implicated in regulation of these cellular processes in a diverse range of cell types. The main kinases involved in the production of PI(4,5)P_2 are the type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K) family, which consist of three isozymes, α, β and γ. Here, we demonstrate the involvement of PIP5Kβ in growth cone dynamics. Overexpression of a lipid kinase-deficient mutant of PIP5Kβ (PIP5Kβ-KD) in mouse dorsal root ganglion (DRG) neurons stimulated axon elongation and increased growth cone size, whereas wild-type PIP5Kβ tended to show opposite effects. Furthermore, PIP5Kβ-KD inhibited growth cone collapse of DRG neurons induced by semaphorin 3A (Sema3A). These results provide evidence that PIP5Kβ negatively regulates axon elongation and growth cone size and is involved in the cellular signaling pathway for Sema3A-triggered repulsion in DRG neurons.

Keywords: phosphatidylinositol 4-phosphate 5-kinase; semaphorin 3A; dorsal root ganglion neurons; growth cone dynamics

Abbreviations: PIP5K, phosphatidylinositol 4-phosphate 5-kinase; DRG, dorsal root ganglion; Sema3A, semaphorin 3A; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BHK cells, baby hamster kidney cells

1. Introduction

A highly ordered neuronal network is established by the precise regulation of axon guidance, which is controlled by extracellular guidance cues that attract or repel neurites during embryogenesis [23,34]. A specialized structure at the axon tip, the growth cone, detects and translates guidance information into changes in morphology to direct the motility necessary for navigation to an appropriate target [24,33]. Several lines of evidence indicate that growth cone morphology and directed motility involve cellular signaling pathways that regulate cytoskeletal dynamics and membrane trafficking [4-6,18,25,29,30]. Several intracellular molecules, including actin-binding proteins, Rho family small GTPases and protein kinases, regulate actin cytoskeleton reorganization and are implicated in the signaling pathways that regulate growth cone morphology and motility [17,29]. However, the molecular mechanisms through which extracellular cues regulate these growth cone dynamics remain unclear.

A candidate for a signaling mediator involved in the regulation of growth cone dynamics is type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which catalyzes phosphorylation of phosphatidylinositol 4-phosphate [PI(4)P] at the D-5 position of the inositol ring to generate the versatile signaling lipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] [2,15,19,20,26]. The mammalian PIP5K family consists of three gene products, α, β and γ, including three γ spliced variants, γ635, γ661 and γ687 [10,19,20,26]. The potential relevance of this candidate is supported by the following reports: (1) the PIP5K product, $PI(4,5)P_2$, reorganizes the actin cytoskeleton through direct interaction with and regulation of actin-associated proteins [15,35], and (2) we [39] and van Horck et al [37] have demonstrated that mouse PIP5Kα regulates lysophosphatidic acid (LPA)- and semaphorin 3A (Sema 3A)-induced neurite retraction of mouse neuroblastoma N1E-115 cells, respectively. In addition, PIP5Kγ687 and PIP5Kγ661 have been implicated in axon elongation and Sema3A-mediated repulsion of outgrowing axons, respectively (10,36). However, the role of PIP5Kβ in axon guidance is poorly understood. In this study, therefore, we investigated whether PIP5Kβ is involved in the regulation of growth cone morphology, axon elongation and

chemorepulsion of mouse dorsal root ganglion (DRG) neurons using wild-type PIP5Kβ and a lipid kinase-deficient mutant of PIP5Kβ (PIP5Kβ-WT and PIP5Kβ-KD, respectively). The results indicate that mouse PIP5Kβ negatively regulates axon elongation and growth cone size and is involved in the signal transduction pathway for semaphorin 3A (Sema3A)-induced growth cone collapse in DRG neurons.

2. Materials and methods

2.1. Generation of Sindbis pseudovirions

Mouse PIP5Kβ cDNA was amplified from mouse brain total RNA using RT-PCR, and the cDNA sequence, verified. cDNA for PIP5Kβ-KD was constructed using a PCR-based strategy. To coexpress EGFP and PIP5Ks, cDNAs for PIP5Kβ and its mutant were inserted into the *Xba*I/*Stu*I site in pSinRep5-IRES-EGFP [1], a generous gift from Dr Sehara. The plasmids were linearized and transcribed *in vitro* as previously reported [38]. These transcripts and the transcript of DH (26S) helper DNA (Invitrogen, Carlsbad, CA, USA) were cotransfected into BHK cells by electroporation. After 48 h, Sindbis pseudovirions were harvested and stored at -80°C until use.

2.2. Preparation and culture of DRG neurons, and PIP5K expression

Dorsal root ganglia were dissected from embryonic day 12.5 mice and plated on glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA) precoated sequentially with poly-D-lysine (PDL) and laminin (Biomedical Technologies, Stoughton, MA, USA). DRG explants were then cultured for 6 h in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 20 ng/ml of nerve growth factor (NGF) (Alomone Labs, Jerusalem, Israel) and infected with Sindbis pseudovirions for 2 h to express PIP5Ks. To analyze PIP5K localization, axon elongation and growth cone morphology, the infected DRG explants were further cultured in the growth medium for 10 h. For analysis of growth

cone collapse, the infected DRG explants were cultured in DMEM/F12 medium supplemented with 0.5% FCS and 20 ng/ml of NGF for 6 h and then incubated with recombinant chick Sema3A, which was purified as previously described [12].

2.3. Immunofluorescence microscopy

The DRG explants treated as described above were fixed, permeabilized and blocked according to the method reported previously by Fournier et al [9]. DRG neurons were then immunostained for EGFP using a polyclonal rabbit EGFP antibody (MBL, Nagoya, Japan; 1:500 dilution) and for PIP5Kβ using a polyclonal rabbit anti-PIP5Kβ antibody (Upstate Biotechnology, Lake Placid, NY, USA; 1:1000 and 1:100 dilutions for overexpressed and endogenous PIP5Kβ staining, respectively). EGFP and PIP5Kβ were visualized with Alexa488- and Cy3-conjugated secondary antibodies, respectively (Molecular Probes, Eugene, OR, USA and Jackson ImmunoResearch Laboratories, West Grove, PA, USA, respectively). Images of the immunofluorescently stained DRG neurons were obtained by means of an immunofluorescence microscope (Axiovert S100; Zeiss, Gottingen, Germany).

2.4. Measurement of axon length and growth cone area

Morphological analyses of DRG neurons expressing EGFP, PIP5Kβ-WT or PIP5Kβ-KD were performed using IPLab image analysis software (Scanalytics, Fairfax, VA, USA). The length of an axon was defined as the length of trace from the contour limit of the DRG explant to the tip of the process. To quantify axon lengths and growth cone areas, more than 100 neurons from 3-4 explants were analyzed in an individual experiment. Statistical significance was evaluated using the Tukey-Kramer multiple comparison test.

2.5. PIP5K activity assay

cDNAs for Myc-PIP5Kβ-WT and Myc-PIP5Kβ-KD were subcloned into a pcDNA3-myc vector and transfected into COS7 cells using Lipofectamine Plus (Invitrogen). Proteins were

overexpressed by incubation of the cells for 24 h. After cells were lysed in lysis buffer and centrifuged as previously described [16], expressed proteins were immunoprecipitated with the anti-Myc antibody affinity resin 9E10 (Santa Cruz Biotechnology). The immunoprecipitates, which contained PIP5Kβ-WT and PIP5Kβ-KD, were incubated at 37°C for 30 min in the presence of 50 μM of phosphatidic acid (PA), 50 μM of PI(4)P (Sigma, St Louis, MO, USA), and 50 μM of [γ-^{32}P]ATP (1 μCi/assay). [^{32}P]PI(4,5)P_2 produced by the incorporation of ^{32}Pi from [γ-^{32}P]ATP (Amersham Biosciences, Arlington Heights, IL, USA) into PI(4)P was detected by thin-layer chromatography as previously reported [16].

2.6. Immunoblot analysis

Immunoblot analysis was performed as previously reported [40]. In brief, proteins contained in the samples were separated by SDS-PAGE on 10% gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with anti-PIP5Kβ antibody (1:400 dilution) or anti-Myc antibody (Santa Cruz Biotechnologies; 1:500 dilution) and then with peroxidase-conjugated rabbit anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive proteins were detected with an ECL immunoblotting detection reagent (Amersham).

2.7. Assay for Sema3A-induced growth cone collapse

Growth cone collapse of DRG explants was assayed using purified recombinant chick Sema3A as previously described [11]. The individual growth cone area of each explant was scored as previously described [12].

3. Results

3.1. PIP5Kβ is enriched in the axon and growth cone

As shown in Figure 1A and B, endogenous PIP5Kβ was detected in DRG neurons by Western blot analysis and found to be localized in the punctate structures of the growth cone and in the axon shaft as analyzed by immunostaining. Overexpressed PIP5Kβ in DRG explants was also localized in the growth cone as well as in the axonal shaft and soma (Fig. 1C and D). These results raised the possibility that PIP5Kβ is implicated in the regulation of growth cone morphology and axon guidance of DRG neurons.

3.2. PIP5Kβ negatively regulates axon elongation and growth cone size

To investigate whether PIP5Kβ is implicated in the regulation of axon elongation and growth cone morphology, PIP5Kβ-WT and PIP5Kβ-KD, in the latter of which Ala was substituted for Asp^{307}, which is a critical amino acid residue for the putative ATP and PI(4)P binding site [31], were overexpressed in DRG neurons, and their effects, examined. This PIP5Kβ-KD mutant was shown to be almost completely deficient of kinase activity (Fig. 2A). As shown in Figure 2B and C, overexpression of PIP5Kβ-WT slightly inhibited axon elongation when compared with EGFP-expressing control neurons. In contrast, overexpression of PIP5Kβ-KD significantly stimulated axon elongation (Fig. 2B and C), leading us to conclude that this PIP5Kβ-KD functions as a dominant negative mutant of PIP5Kβ. Taken together, these results suggest that PIP5Kβ negatively regulates axon elongation of DRG neurons.

Several reports have indicated that molecules such as small GTPase RhoA and LIM-kinase, which possess the ability to reduce the elongation rate of neurites, cause growth cone collapse [1,3,8,28]. Since PIP5Kβ phenocopied these molecules in its effect on axon elongation, we examined the effects of PIP5Kβ-WT and PIP5Kβ-KD on growth cone morphology. When PIP5Kβ-WT was overexpressed in DRG neurons, growth cone size tended to be reduced in comparison with EGFP-expressing control cells (Fig. 2D and E), although the difference was not statistically significant (Fig. 2E). In contrast, overexpression of PIP5Kβ-KD markedly increased the growth cone area (Fig. 2D and E). Thus, PIP5Kβ also negatively regulates growth cone morphology.

3.3. Implication of PIP5Kβ in the signal transduction pathway of the Sema3A-induced repulsive response

To investigate whether PIP5Kβ is involved in the repulsive response induced by Sema3A, we employed an assay of growth cone collapse of DRG neurons, which is most useful and a characteristic response upon Sema3A stimulation (Fig. 3A and B). Overexpression of PIP5Kβ-WT and PIP5Kβ-KD tended to decrease growth cone collapse in the unstimulated control cells, but the difference was not statistically significant. Again, overexpression of PIP5Kβ-WT had little, if any, effect on Sema3A-induced growth cone collapse when compared with control EGFP overexpression. On the other hand, PIP5Kβ-KD significantly suppressed Sema3A-induced growth cone collapse, suggesting that PIP5Kβ participates in the signaling pathway for Sema3A-induced growth cone collapse in DRG neurons. Although we currently cannot clearly explain why overexpression of PIP5Kβ-WT had no effect on the number of collapsed growth cones in unstimulated and Sema3A-stimulated cells, it is conceivable that endogenous PIP5Kβ is sufficient to transduce the signaling for growth cone collapse.

4. Discussion

In the present study, we demonstrated that PIP5Kβ negatively regulates the axon elongation and growth cone size of mouse DRG neurons. Hernandez-Deviez et al [13] have recently reported that overexpressed PIP5Kα in hippocampal neurons localizes in the axon and suppresses axon elongation and branching induced by the dominant negative form of Arf6. We and van Horck et al have demonstrated that PIP5Kα regulates LPA- and Sema3A-induced neurite retraction of neuroblastoma N1E-115 cells [37,39]. These results support the notion that $PI(4,5)P_2$ produced by PIP5K functions as a negative regulator of axon elongation, although the PIP5K isozymes used for the experiments of these two studies were different. In conflict with these findings, the

neuron-specific splice variant of PIP5Kγ, PIP5Kγ687, has been reported to positively regulate axon elongation and branching [10], suggesting that $PI(4,5)P_2$ produced by PIP5K functions as a positive regulator of axon elongation. Although we currently cannot clearly explain why the effect of PIP5Kγ687 differs from that of PIP5Kα and β, it is plausible that the $PI(4,5)P_2$ pool produced by PIP5Kγ687 differs from those produced by PIP5Kα and β. It would be interesting to investigate the expression and localization of PIP5Kγ687 in DRG neurons in comparison with those of other PIP5K isozymes.

The chemorepellant Sema3A restricts axonal elongation and causes growth cone collapse through its specific receptor, the plexin-neuropilin complex, located on the surface of growth cones [17,29]. Sema3A-induced growth cone collapse is believed to be triggered by actin cytoskeletal reorganization [17,29]. Accumulating evidence suggests that growth cone morphology is regulated by actin cytoskeleton reorganization [5,6,24,33]. Since the PIP5K product, $PI(4,5)P_2$, regulates actin dynamics through direct binding to and regulation of actin-associated proteins such as alpha-actinin, gelsolin and cofilin [15,35], the effects of PIP5Kβ shown in this study may be mediated by reorganization of actin filaments. However, we could not detect any significant change in actin filaments in DRG neuron growth cones overexpressing PIP5Kβ-WT and PIP5Kβ-KD as assessed by phalloidin staining (data not shown). Although we cannot totally rule out the possibility that overexpression of PIP5Kβ causes subtle changes in actin filaments that cannot be detected by phalloidin staining, it is plausible that PIP5Kβ regulates growth cone morphology by regulating membrane dynamics such as endocytosis. This notion is supported by reports that $PI(4,5)P_2$ regulates both clathrin-dependent and clathrin-independent endocytosis [21,27] and that Sema3A-induced growth cone collapse is accompanied by macropinocytosis, which is categorized as clathrin-independent endocytosis [9]. Therefore, it could be speculated that PIP5Kβ regulates Sema3A-induced collapse response of growth cones through clathrin-independent membrane invagination regulated by $PI(4,5)P_2$. On the other hand, it has been reported that Sema3A induces a repulsive response through clathrin-dependent endocytosis [9] and that the GSK3β/axin-1/β-catenin complex plays an important role in Sema3A-induced axon repulsion via

an endocytic mechanism [14]. Taken together, the findings of these reports and of the present study suggest that PIP5Kβ and the GSK3β/axin-1/β-catenin complex coordinately regulate Sema3A-induced axon repulsion through clathrin-dependent and -independent endocytosis, respectively. In addition, it has been reported that several molecules such as CRMP, Rac1, LIM-kinase, Fyn and Cdk5 are involved in the signaling pathway for Sema3A-induced responses [1,7,12,22,32]. PIP5Kβ may also coordinate with these molecules in Sema3A-induced axon repulsion.

Recently, it has been reported that PIP5Kγ661, a crucial mediator of neuronal adhesion, is inactivated by sequestration from talin through interaction with FARP2 in response to Sema3A in DRG neurons [36]. The authors demonstrated that this decrease in PIP5Kγ661 activity leads to suppression of neuronal adhesion and is required for Sema3A-mediated repulsion of outgrowing axons [36]. Thus, PIP5Kγ661 negatively regulates Sema3A-mediated repulsion of outgrowing axons, whilst PIP5Kβ seems to function as a positive regulator, as was demonstrated by the present study. These observations led us to speculate that each PIP5K isozyme produces a distinct $PI(4,5)P_2$ pool in spatially and temporally distinct manners, thereby playing a role in different cellular events. Further studies on spatial and temporal analyses of $PI(4,5)P_2$ localization will provide insight into the molecular mechanisms by which $PI(4,5)P_2$ regulates the chemorepulsive response of axonal growth cones.

Acknowledgments

We are grateful to Drs S. Wakatsuki and A. Sehara for providing the pSinRep5-IRES-EGFP vector. This work was supported in part by a research grant (20247010) from the Ministry of Education, Science, Sports and Culture of Japan to Y.K.

References

[1] H. Aizawa, S. Wakatsuki, A. Ishii, K. Moriyama, Y. Sasaki, K. Ohashi, Y. Sekine-Aizawa, A. Sehara-Fujisawa, K. Mizuno, Y. Goshima, I. Yahara, Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse, Nat Neurosci 4 (2001) 367-373.

[2] R.A. Anderson, I.V. Boronenkov, S.D. Doughman, J. Kunz, J.C. Loijens, Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes, J Biol Chem 274 (1999) 9907-9910.

[3] H. Bito, T. Furuyashiki, H. Ishihara, Y. Shibasaki, K. Ohashi, K. Mizuno, M. Maekawa, T. Ishizaki, S. Narumiya, A critical role for a Rho-associated kinase, p160ROCK, in determining axon outgrowth in mammalian CNS neurons, Neuron 26 (2000) 431-441.

[4] M.E. Dailey, P.C. Bridgman, Vacuole dynamics in growth cones: correlated EM and video observations, J Neurosci 13 (1993) 3375-3393.

[5] E.W. Dent, F.B. Gertler, Cytoskeletal dynamics and transport in growth cone motility and axon guidance, Neuron 40 (2003) 209-227.

[6] B.J. Dickson, Molecular mechanisms of axon guidance, Science 298 (2002) 1959-1964.

[7] B.J. Eickholt, F.S. Walsh, P. Doherty, An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling, J Cell Biol 157 (2002) 211-217.

[8] M. Endo, K. Ohashi, Y. Sasaki, Y. Goshima, R. Niwa, T. Uemura, K. Mizuno, Control of growth cone motility and morphology by LIM kinase and Slingshot via phosphorylation and dephosphorylation of cofilin, J Neurosci 23 (2003) 2527-2537.

[9] A.E. Fournier, F. Nakamura, S. Kawamoto, Y. Goshima, R.G. Kalb, S.M. Strittmatter, Semaphorin3A enhances endocytosis at sites of receptor-F-actin colocalization during growth cone collapse, J Cell Biol 149 (2000) 411-422.

[10] M.L. Giudici, P.C. Emson, R.F. Irvine, A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isoform gamma, Biochem J 379 (2004) 489-496.

[11] Y. Goshima, T. Kawakami, H. Hori, Y. Sugiyama, S. Takasawa, Y. Hashimoto, M. Kagoshima-Maezono, T. Takenaka, Y. Misu, S.M. Strittmatter, A novel action of collapsin: collapsin-1 increases antero- and retrograde axoplasmic transport independently of growth cone collapse, J Neurobiol 33 (1997) 316-328.

[12] Y. Goshima, F. Nakamura, P. Strittmatter, S.M. Strittmatter, Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33, Nature 376 (1995) 509-514.

[13] D.J. Hernandez-Deviez, M.G. Roth, J.E. Casanova, J.M. Wilson, ARNO and ARF6 regulate axonal elongation and branching through downstream activation of phosphatidylinositol 4-phosphate 5-kinase alpha, Mol Biol Cell 15 (2004) 111-120.

[14] T. Hida, N. Yamashita, H. Usui, F. Nakamura, Y. Sasaki, A. Kikuchi, Y. Goshima, GSK3beta/axin-1/beta-catenin complex is involved in semaphorin3A signaling, J Neurosci 32 11905-11918.

[15] K.A. Hinchliffe, A. Ciruela, R.F. Irvine, PIPkins1, their substrates and their products: new functions for old enzymes, Biochim Biophys Acta 1436 (1998) 87-104.

[16] A. Honda, M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A.J. Morris, M.A. Frohman, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation, Cell 99 (1999) 521-532.

[17] A.B. Huber, A.L. Kolodkin, D.D. Ginty, J.F. Cloutier, Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance, Annu Rev Neurosci 26 (2003) 509-563.

[18] M. Igarashi, S. Kozaki, S. Terakawa, S. Kawano, C. Ide, Y. Komiya, Growth cone collapse and inhibition of neurite growth by Botulinum neurotoxin C1: a t-SNARE is involved in axonal growth, J Cell Biol 134 (1996) 205-215.

[19] H. Ishihara, Y. Shibasaki, N. Kizuki, H. Katagiri, Y. Yazaki, T. Asano, Y. Oka, Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase, J Biol Chem 271 (1996) 23611-23614.

[20] H. Ishihara, Y. Shibasaki, N. Kizuki, T. Wada, Y. Yazaki, T. Asano, Y. Oka, Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family, J Biol Chem 273 (1998) 8741-8748.

[21] T. Itoh, T. Takenawa, Regulation of endocytosis by phosphatidylinositol 4,5-bisphosphate and ENTH proteins, Curr Top Microbiol Immunol 282 (2004) 31-47.

[22] Z. Jin, S.M. Strittmatter, Rac1 mediates collapsin-1-induced growth cone collapse, J Neurosci 17 (1997) 6256-6263.

[23] R.J. Keynes, G.M. Cook, Repulsive and inhibitory signals, Curr Opin Neurobiol 5 (1995) 75-82.

[24] C.H. Lin, C.A. Thompson, P. Forscher, Cytoskeletal reorganization underlying growth cone motility, Curr Opin Neurobiol 4 (1994) 640-647.

[25] R.O. Lockerbie, V.E. Miller, K.H. Pfenninger, Regulated plasmalemmal expansion in nerve growth cones, J Cell Biol 112 (1991) 1215-1227.

[26] J.C. Loijens, R.A. Anderson, Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family, J Biol Chem 271 (1996) 32937-32943.

[27] T.F. Martin, PI(4,5)P(2) regulation of surface membrane traffic, Curr Opin Cell Biol 13 (2001) 493-499.

[28] P.J. Meberg, J.R. Bamburg, Increase in neurite outgrowth mediated by overexpression of actin depolymerizing factor, J Neurosci 20 (2000) 2459-2469.

[29] R.J. Pasterkamp, A.L. Kolodkin, Semaphorin junction: making tracks toward neural connectivity, Curr Opin Neurobiol 13 (2003) 79-89.

[30] S. Popov, A. Brown, M.M. Poo, Forward plasma membrane flow in growing nerve processes, Science 259 (1993) 244-246.

[31] V.D. Rao, S. Misra, I.V. Boronenkov, R.A. Anderson, J.H. Hurley, Structure of type IIbeta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation, Cell 94 (1998) 829-839.

[32] Y. Sasaki, C. Cheng, Y. Uchida, O. Nakajima, T. Ohshima, T. Yagi, M. Taniguchi, T. Nakayama, R. Kishida, Y. Kudo, S. Ohno, F. Nakamura, Y. Goshima, Fyn and Cdk5 mediate semaphorin-3A signaling, which is involved in regulation of dendrite orientation in cerebral cortex, Neuron 35 (2002) 907-920.

[33] E. Tanaka, J. Sabry, Making the connection: cytoskeletal rearrangements during growth cone guidance, Cell 83 (1995) 171-176.

[34] M. Tessier-Lavigne, C.S. Goodman, The molecular biology of axon guidance, Science 274 (1996) 1123-1133.

[35] A. Toker, The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate, Curr Opin Cell Biol 10 (1998) 254-261.

[36] T. Toyofuku, J. Yoshida, T. Sugimoto, H. Zhang, A. Kumanogoh, M. Hori, H. Kikutani, FARP2 triggers signals for Sema3A-mediated axonal repulsion, Nat Neurosci 8 (2005) 1712-1719.

[37] F.P. van Horck, E. Lavazais, B.J. Eickholt, W.H. Moolenaar, N. Divecha, Essential role of type I(alpha) phosphatidylinositol 4-phosphate 5-kinase in neurite remodeling, Curr Biol 12 (2002) 241-245.

[38] H. Watanabe, M. Yamazaki, H. Miyazaki, C. Arikawa, K. Itoh, T. Sasaki, T. Maehama, M.A. Frohman, Y. Kanaho, Phospholipase D2 functions as a downstream signaling molecule of MAP kinase pathway in L1-stimulated neurite outgrowth of cerebellar granule neurons, J Neurochem 89 (2004) 142-151.

[39] M. Yamazaki, H. Miyazaki, H. Watanabe, T. Sasaki, T. Maehama, M.A. Frohman, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling, J Biol Chem 277 (2002) 17226-17230.

[40] M. Yamazaki, Y. Zhang, H. Watanabe, T. Yokozeki, S. Ohno, K. Kaibuchi, H. Shibata, H. Mukai, Y. Ono, M.A. Frohman, Y. Kanaho, Interaction of the small G protein RhoA with the C terminus of human phospholipase D1, J Biol Chem 274 (1999) 6035-6038.

Figure legends

Fig. 1. Localization of endogenous and overexpressed PIP5Kβ in isolated DRG explants.

(A) Western blot analysis of endogenous PIP5Kβ expression in mouse DRG neurons. DRG neuron homogenates prepared from embryonic day 12.5 mice were immunoblotted with anti-PIP5Kβ antibody. (B) Immunofluorescent staining of endogenous PIP5Kβ in DRG neurons prepared from embryonic day 12.5 mice. Scale bar: 10 μm. (C and D) PIP5Kβ-WT was overexpressed in mouse DRG explants and then immunostained. (D) Magnified image of the growth cone shown in (C). Scale bars: 200 μm and 10 μm in C and D, respectively.

Fig. 2. Effects of overexpressed PIP5Kβ-WT and PIP5Kβ-KD on axon elongation and growth cone size of mouse DRG neurons.

PIP5Kβ-WT and PIP5Kβ-KD were overexpressed in mouse DRG neuron explants. EGFP was also expressed as a control. (A) Lipid kinase activities of recombinant PIP5Kβ-WT and PIP5Kβ-KD expressed in COS7 cells were analyzed (upper panel). The lower panel shows the expression levels of PIP5Kβ-WT and PIP5Kβ-KD as assessed by Western blot analysis. (B and C) DRG explants expressing EGFP, PIP5Kβ-WT or PIP5Kβ-KD were immunostained for EGFP and PIP5Kβ (B). Scale bar: 200 μm. (C) The axon lengths were determined, and the results presented as the means ± SEMs of 4 independent experiments. *$p<0.01$. (D and E) Growth cone morphology of DRG neurons expressing EGFP, PIP5Kβ-WT or PIP5Kβ-KD. Proteins overexpressed in DRG neurons were immunostained (D). Scale bar: 10 μm. (E) The growth cone areas of DRG neurons were determined, and the results presented as the means ± SEMs of 3 independent experiments. N.S., not significant. *$p<0.01$.

Fig. 3. Inhibition of Sema3A-induced growth cone collapse by PIP5Kβ-KD.

DRG explants overexpressing EGFP, PIP5Kβ-WT and PIP5Kβ-KD were incubated with or

withou 4 U/ml of Sema3A for 30 min. (A) DRG neurons were then immunostained for EGFP, PIP5Kβ-WT and PIP5Kβ-KD. Scale bar: 10 μm. (B) Collapsed growth cones were scored, and the results presented as the means ± SEMs of 3 independent experiments. N.S., not significant. *$p<0.05$.

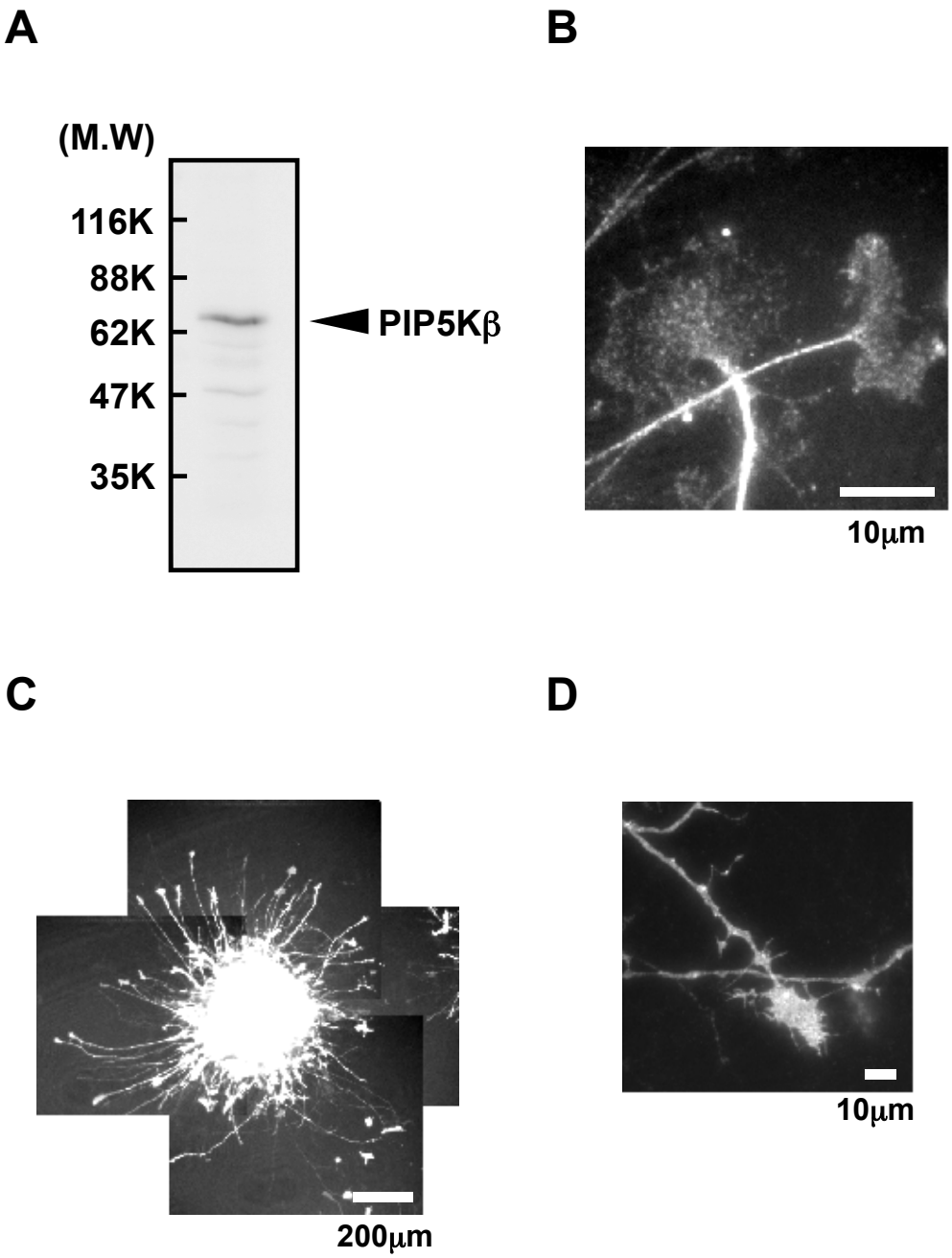


Fig.1. Yamazaki et al.

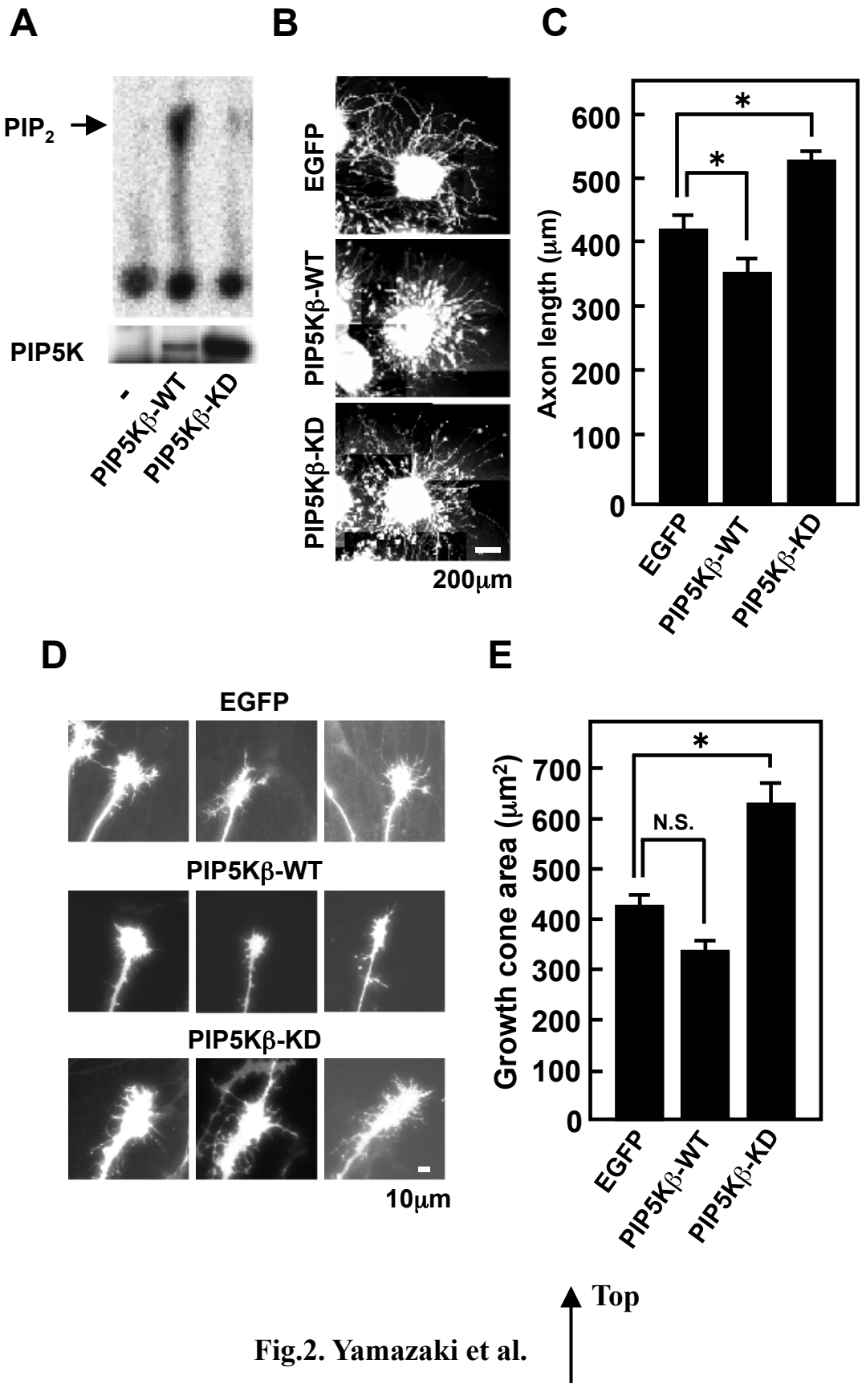
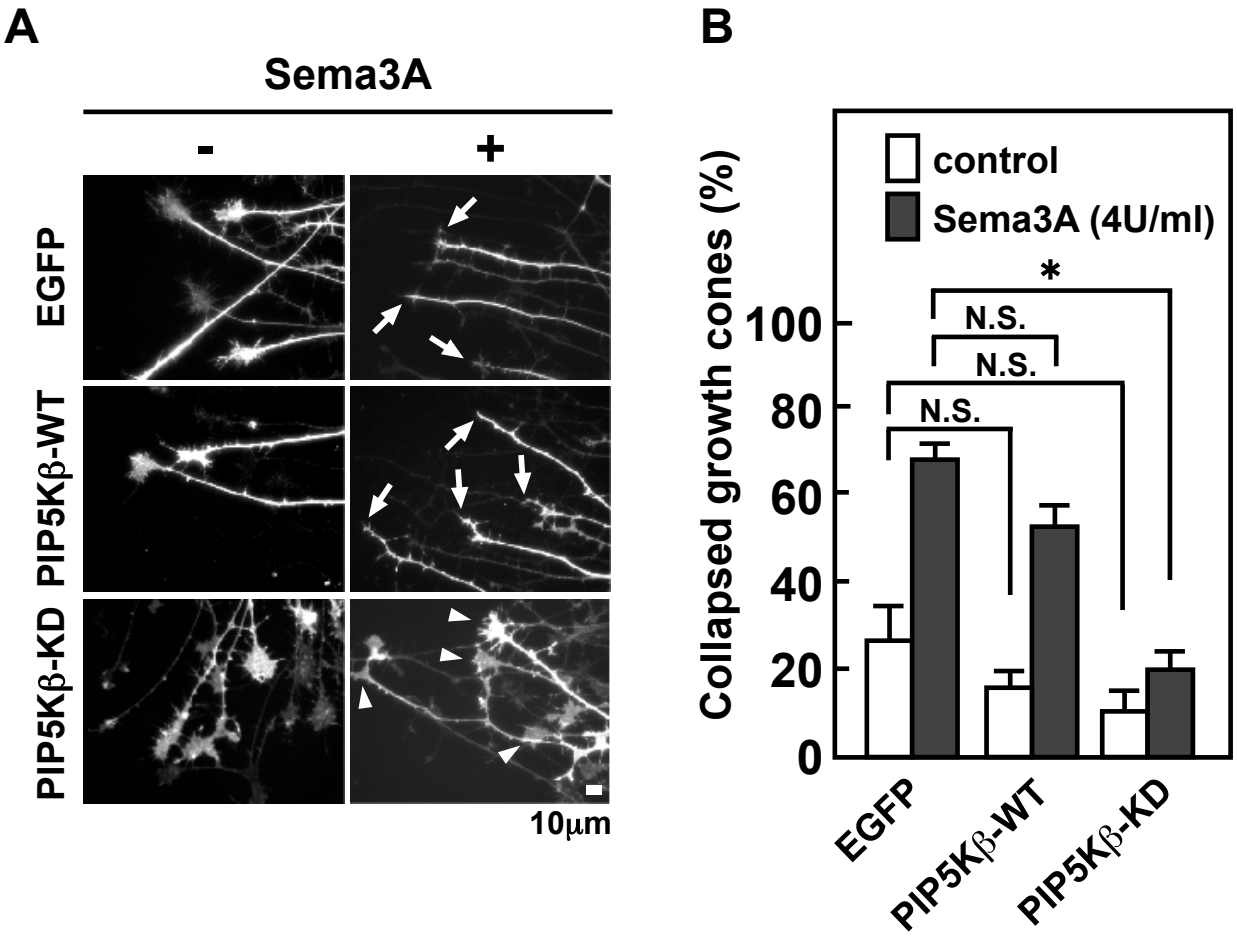


Fig.2. Yamazaki et al.



Top

Fig.3. Yamazaki et al.