The Journal of Cell Biology

Transport of PIP₃ by GAKIN, a kinesin-3 family protein, regulates neuronal cell polarity

Kaori Horiguchi,¹,² Toshihiko Hanada,¹ Yasuhisa Fukui,² and Athar H. Chishti¹

¹Department of Pharmacology and Cancer Center, University of Illinois College of Medicine, Chicago, IL 60612
²Department of Applied Biological Chemistry, Graduate School of Agriculture and Life Science, University of Tokyo, Tokyo 113-0032, Japan

Phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃), a product of phosphatidylinositol 3-kinase, is an important second messenger implicated in signal transduction and membrane transport. In hippocampal neurons, the accumulation of PIP₃ at the tip of neurite initiates the axon specification and neuronal polarity formation. We show that guanylate kinase-associated kinesin (GAKIN), a kinesin-like motor protein, directly interacts with a PIP₃-interacting protein, PIP₃BP, and mediates the transport of PIP₃-containing vesicles. Recombinant GAKIN and PIP₃BP form a complex on synthetic liposomes containing PIP₃ and support the motility of the liposomes along microtubules in vitro. In PC12 cells and cultured hippocampal neurons, transport activity of GAKIN contributes to the accumulation of PIP₃ at the tip of neurites. In hippocampal neurons, altered accumulation of PIP₃ by overexpression of GAKIN constructs led to the loss of the axonally differentiated neurites. Together, these results suggest that, in neurons, the GAKIN–PIP₃BP complex transports PIP₃ to the neurite ends and regulates neuronal polarity formation.

Introduction

Phosphatidylinositol (PI) 3-kinase signaling plays an important role in neuronal development (Cantley, 2002; Higuchi et al., 2003; Menager et al., 2004; Aoki et al., 2005). It is essential for the NGF-induced neurite elongation of PC12 cells, as this process is inhibited by wortmannin, a relatively specific inhibitor of PI 3-kinase (Kimura et al., 1994). Also, overexpression or microinjection of active PI 3-kinase in PC12 cells is sufficient to induce neurite formation (Kobayashi et al., 1997; Kita et al., 1998). Recent technological developments made it possible to visualize the intracellular localization of PIP₃ by the use of pleckstrin homology (PH) domain of PI-(3,4,5)-trisphosphate (PIP₃) interacting proteins such as Akt (Varnai and Balla, 1998) and GRP1 (Sato et al., 2003) as PIP₃-specific probes. These studies revealed that PIP₃ is highly enriched at the tip of growing neurites in PC12 cells (Higuchi et al., 2003; Aoki et al., 2005). Similarly, in cultured hippocampal neurons, PIP₃ accumulates at the distal end of the longest neurite and induces the single longest neurite to develop into an axon (Shi et al., 2003; Menager et al., 2004). The downstream signaling mechanism coordinating the impact of concentrated PIP₃ at the tip of the neurite to form an axon has been extensively studied (Ming et al., 1999; Inagaki et al., 2001; Shi et al., 2003; Shi et al., 2004; Nishimura et al., 2005; Yoshimura et al., 2005).

However, how PIP₃ accumulates at the tip of neurite remains an intriguing, yet unresolved, question. One possible explanation is that the activity of PI 3-kinase and its upstream signaling mediators are concentrated at the tip of neurites, thereby producing PIP₃ locally (Shi et al., 2003). A possible positive-feedback loop in which PIP₃ activates upstream signaling of PI 3-kinase has been proposed (Aoki et al., 2005). Alternatively, PIP₃ might be transported from the cell body through neurites by the motor-dependent trafficking mechanism. Although historically PI 3-kinase activity and PIP₃ production have been considered to occur at the plasma membrane (Cantley, 2002), significant evidence has emerged supporting the idea that PIP₃ is also produced within intracellular membranes, such as the endocytosed vesicles containing activated receptors (Sato et al., 2003). Therefore, an attractive hypothesis predicts that such PIP₃-containing vesicles, laden with its upstream and downstream signaling components, are transported to specialized destinations where the local signaling activity is most needed.

*Correspondence to Athar H. Chishti: chishti@uic.edu*

Abbreviations used in this paper: DN, dominant-negative; FHA, forkhead-associated; GAKIN, guanylate kinase-associated kinesin; MAGUK, membrane-associated guanylate kinase; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol; PIP₂, PI-(4,5)-bisphosphate; PIP₃, PI-(3,4,5)-trisphosphate; PI₃P, PI-(3)-phosphate; PIP₃BP, PIP₃-binding protein; Rh-PE, rhodamine-phosphatidylethanolamine; Trx, thioredoxin.

The online version of this article contains supplemental material.

Supplemental Material can be found at: http://www.jcb.org/cgi/content/full/jcb.200604031/DC1
Kinesin-family proteins are microtubule-based motor proteins implicated in the transport of diverse cargos (Hirokawa and Takekura, 2004b). Some of these kinesins recognize phospholipids as their cargo molecules. A kinesin-3 family protein (Lawrence et al., 2004), Unc104/KIF1A has a PD domain located at the C-terminal tail, which directly interacts with PI-(4,5)-bisphosphate (PIP2) and transports PIP2-containing vesicles (Klopfenstein et al., 2002; Klopfenstein and Vale, 2004). Another kinesin-3 protein, KIF16B, transports PI-(3)-phosphate (PI3P) through direct binding, via its C-terminal Phox homology domain to PI3P (Hoepfner et al., 2005). We propose that guanylate kinase–associated kinesin (GAKIN; Hanada et al., 2000; Asaba et al., 2003), classified as a kinesin-3 protein, KIF13B (Lawrence et al., 2004), transports PIP2-containing vesicles through the interaction with an adaptor protein PIP3BP/centaurin-α (Hammonds-Odie et al., 1996; Tanaka et al., 1997). PIP3BP has two PH domains, which specifically interact with PIP3 and has been implicated in the regulation of PIP3 signaling function (Tanaka et al., 1997, 1999; Venkateswarlu et al., 1999, 2004). The PIP3BP was originally identified in other species and named as centaurin-α (Hammonds-Odie et al., 1996). Centaurin-α/PIP3BP was recently shown to exhibit Arf GAP (ADP-ribosylating factor GTPase activating protein) activity in vivo (Venkateswarlu et al., 2004). Our results provide the first evidence that PIP3 is transported by the motor-dependent mechanism.

Results

The forkhead-associated (FHA) domain of GAKIN mediates the biochemical interaction with PIP3BP

Using full-length bovine PIP3BP as bait, we performed a yeast two-hybrid screen in HeLa cell cDNA library and isolated a partial cDNA of human GAKIN, a kinesin-like protein. Recently, in a collaborative study, human centaurin-α1 was used as a bait to isolate rat homologue of GAKIN/KIF13B from rat brain cDNA library (Venkateswarlu et al., 2005). We confirmed the interaction between PIP3BP and GAKIN and mapped the binding site using the GST pull-down assay. Defined segments of GAKIN, covering the region originally isolated by the yeast two-hybrid screen, were expressed in 293T human kidney cells, and their biochemical interactions with GST-PIP3BP immobilized on glutathione–Sepharose beads were tested. The construct spanning the motor-FHA domains (1–486) and a short construct containing only the FHA domain (455–557) showed specific binding to PIP3BP. To further test whether the observed biochemical interaction is direct, we incubated GST-PIP3BP with thioredoxin (Trx) fusion protein of the GAKIN FHA domain immobilized on agarose beads. GST-PIP3BP specifically interacted with Trx-FHA but not with the control Trx (Fig. 1 D). Together, these results demonstrate that the FHA domain of GAKIN mediates its direct interaction with PIP3BP.

To demonstrate the in vivo association between PIP3BP and GAKIN, we performed coimmunoprecipitation experiments from COS-7 cells transiently expressing PIP3BP and full-length GAKIN. The COS-7 cells were transfected with myc-PIP3BP and FLAG-GAKIN, and an anti-myc antibody was used to immunoprecipitate the complex. Western blotting with anti-FLAG antibody detected PIP3BP and GAKIN. COS-7 cells were transfected with plasmids encoding full-length clones of FLAG-GAKIN or KIF13A and myc-PIP3BP. Immunoprecipitation was performed from the cell lysates using anti-myc monoclonal antibody. FLAG- and myc-tagged proteins were detected by Western blotting using anti-FLAG and anti-myc monoclonal antibodies.
in the coimmunoprecipitation experiments (Fig. 1 E, third lane). This result suggests that the interaction between PIP3BP and GAKIN is highly specific. It is noteworthy that the FHA domain is a widely conserved module in the kinesin-3 family members (Lawrence et al., 2004); however, its function is poorly understood in the context of motor proteins.

**GAKIN is a microtubule plus-end motor**

As PIP3BP specifically interacts with PIP3 via its PH domains (Tanaka et al., 1997), we hypothesized that PIP3BP mediates the physical linkage between GAKIN and PIP3-containing vesicles, thereby facilitating the microtubule-dependent transport of the cargo vesicles containing PIP3. To test this hypothesis, we first characterized the motor activity of GAKIN by the microtubule gliding assay. Recombinant GAKIN containing motor and FHA domains (1–557) was expressed in Sf9 cells using the baculovirus expression system, and purified protein was coated on the glass surface of the motility chamber. Microtubules were polarity labeled with Alexa 488 to highlight their minus ends, and their gliding movement was visualized using time-lapse fluorescence microscopy (Fig. 2 A). The smooth movement of microtubules toward their brightly labeled minus end was reproducibly observed in multiple trials, demonstrating that GAKIN is a plus end–directed microtubule motor. A histogram showing the velocity of labeled microtubules was generated from multiple tracings and is shown in Fig. 2 B. The mean velocity of moving microtubules was estimated to be 1.66 μm/s.

**PIP3BP and GAKIN form a complex on synthetic liposomes containing PIP3**

Next, we tested whether PIP3BP and GAKIN can form a macromolecular complex on the surface of PIP3-containing liposomes. Purified recombinant GST-PIP3BP and GAKIN (motor-FHA segment 1–557) were incubated with synthetic liposomes containing 10% PIP3 and 90% phosphatidylcholine (PC), and the liposome and soluble protein components were separated in the top and bottom fractions, respectively, by sucrose density gradient centrifugation. GAKIN was recovered from the top (liposome fraction) of the gradient when PIP3BP and PIP3 liposomes were present (Fig. 3, top, lanes 5 and 6). In contrast, GAKIN was recovered only from the bottom (soluble protein fraction) of the gradient when control GST was used instead of GST-PIP3BP (Fig. 3, top, lanes 3 and 4) or when 15% PIP2 liposomes were used instead of PIP3 (Fig. 3, top, lanes 7 and 8). The GST-PIP3BP was consistently recovered from the top fraction with PIP3 liposomes (Fig. 3, bottom, lanes 5 and 6) and only in the bottom fraction when 10% PIP2 liposomes were used (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1). When 15% PIP2 liposomes were used as a negative control, only
a trace amount (<0.5%) of GST-PIP3BP was recovered in the top fraction; most of the protein remained in the bottom fraction (Fig. 3, bottom, lanes 7 and 8). The lipid binding specificity of PIP3BP was documented previously (Hammonds-Odie et al., 1996; Tanaka et al., 1997; Venkateswarlu et al., 1999). Based on our Western blot results, the affinity of PIP3BP to 10% PIP3 liposomes appears to be >100 times better than 15% PIP2 liposomes. As a negative control, GST was recovered only from the bottom fraction (Fig. 3, bottom, lanes 3 and 4). Together, our data are consistent with the notion that PIP3BP is a specific binder of PIP3 and demonstrate that PIP3BP can bind both GAKIN and PIP3 simultaneously, thereby serving as a molecular linker connecting GAKIN to PIP3-containing lipid vesicles.

**GAKIN transports PIP3 liposomes via PIP3BP in vitro**

We then examined whether the complex of GAKIN and PIP3BP formed on PIP3 liposomes could support actual movement of the liposomes along microtubules. To demonstrate this possibility, we reconstituted the microtubule-dependent movement of liposomes using purified recombinant PIP3BP and GAKIN. A dense microtubule lawn was prepared on the glass surface of the motility chamber using Alexa 488–labeled microtubules. To visualize liposome movement by fluorescence microscopy, liposomes were labeled using rhodamine-phosphatidylethanolamine (Rh-PE). Processive movement of PIP3 liposomes along microtubules was observed in the presence of GAKIN, PIP3BP, and ATP (Fig. 4 A and Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1). Mean velocity of liposome movement was ~0.7 μm/s (Fig. 4 B). No processive movement was observed in the absence of GAKIN, PIP3BP, or ATP or when PIP2 was used instead of PIP3, demonstrating the specificity of the liposome movement (Fig. 4 C). This result suggests that GAKIN is capable of transporting PIP3-containing vesicles via direct interaction with PIP3BP.

**GAKIN contributes to the accumulation of PIP3 in the neurite ends of PC12 cells**

Next, we asked whether this PIP3 transport by GAKIN–PIP3BP complex plays a functional role in vivo. As endogenous GAKIN
and PIP3BP can be coimmunoprecipitated from rat brain lysate (Venkateswarlu et al., 2005), we surmised that the neuronal cell might be the site where such transport is important. Therefore, we selected PC12 cells, the widely used cell line with neuronal properties, for further studies. In PC12 cells, PIP3 is highly enriched at the tip of growing neurites (Higuchi et al., 2003; Aoki et al., 2005) and its signaling is important for neurite outgrowth (Kobayashi et al., 1997; Kita et al., 1998). First, we examined whether PIP3BP and GAKIN colocalize at specific sites in PC12 cells. When PC12 cells were cotransfected with myc-PIP3BP and GFP-GAKIN and treated with NGF, both proteins significantly colocalized and accumulated at the tip of neurites (Fig. 5 A). Higher magnification images show that both proteins are found at the growth cone protrusions (Fig. 5 A). To visualize the localization of PIP3, the PH domain construct of Akt (GFP-Akt-PH), which specifically interacts with PIP3 in the cells (Varnai and Balla, 1998), was used. As reported previously (Higuchi et al., 2003), accumulation of GFP-Akt-PH was observed at the tip of growing neurites (Fig. 5 B). Endogenous GAKIN was also found concentrated at the tip of the neurites, where it colocalized with GFP-Akt-PH (Fig. 5 B). To test whether the observed colocalization of GAKIN, PIP3BP, and PIP3 is the consequence of the transport of PIP3 by the GAKIN–PIP3BP complex, we decided to use the dominant-negative (DN) approach by blocking specific interactions. The DN construct of GAKIN is a motor-deleted mutant of GAKIN, which can still bind to PIP3BP via its FHA domain (unpublished data). Unlike full-length GAKIN, which accumulates only at the tip of neurites (Fig. 5 A and Fig. 6 B), the transfected DN-GAKIN in PC12 cells distributes along neurites and the cell body. The accumulation of DN-GAKIN is still evident at the tip (Fig. 6 B); however, it is not as intense and, consequently, the cell body and the entire length of neurites are visible. This distribution suggests the inability of DN-GAKIN to translocate its cargo to its final destination. Accumulation of PIP3, as monitored by the GFP-Akt-PH detector, at the tip of neurites is significantly inhibited by the overexpression of DN-GAKIN (Fig. 6, B and C). The relative intensity of GFP-Akt-PH and anti-FLAG staining was measured and is shown in the graphical format (Fig. 6 B and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1). Because the FHA domain of GAKIN is required for its interaction with PIP3BP, two additional constructs lacking the FHA domain of GAKIN were made and used as negative controls (Fig. 6). In the Δ-FHA construct of GAKIN, the FHA domain was deleted from the DN-GAKIN mutant. Thus, the Δ-FHA construct starts with the membrane-associated guanylate kinase (MAGUK) binding stalk domain and extends up to the Cap-Gly domain. A chimera construct (FHA-chimera) of GAKIN was also made where its FHA domain was replaced with the FHA domain of KIF13A, the closest homologue of GAKIN, which does not bind to PIP3BP. Thus the FHA-chimera contains the FHA domain of KIF13A and the MAGUK binding stalk to Cap-Gly domains of GAKIN. Unlike DN-GAKIN, neither of these constructs showed any effect on PIP3 localization (Fig. 6). These results suggest that PIP3 is transported to the distal end of neurites by a GAKIN-dependent mechanism.

![Figure 5. PIP3, PIP3BP, and GAKIN colocalize at the tip of growing neurites in PC12 cells.](http://www.jcb.org/cgi/content/full/jcb.200604031/DC1)
GAKIN-mediated transport of PIP3 regulates neuronal polarity

In cultured hippocampal neurons, the location of PIP3 was monitored by the GFP-Akt-PH detector and found highly enriched in the distal end of the longest neurite (Fig. 7, A and B) in contrast to the diffused fluorescence observed for GFP (Fig. 8 A and Fig. S2). This location of PIP3 is consistent with previously published studies (Shi et al., 2003; Menager et al., 2004). Endogenous PIP3 was found colocalized with GFP-Akt-PH at the end of the longest neurite (Fig. 7 A). As was the case in the PC12 cells, overexpression of full-length GAKIN was found highly enriched at the distal ends of neurites, often at their multiple termini (Fig. 7 B, middle). Significant colocalization of GFP-Akt-PH with overexpressed GAKIN was observed, and the accumulation of GFP-Akt-PH appeared to be enhanced under these conditions (Fig. 7 B, middle). On the other hand, overexpression of DN-GAKIN caused the loss of enriched accumulation of GFP-Akt-PH at the tip of the longest neurite (Fig. 7 B, right). Similar to the pattern observed in the case of GFP-Akt-PH expression alone in control neurons, some staining of GFP-Akt-PH could be visualized at the end of short neurites in DN-GAKIN transfected cells. As before, the relative intensity was measured and is shown in Fig. 7 B (Fig. S2). We quantified the GFP-Akt-PH accumulation by scoring the cells with the relative intensity ratio between neurite tip and cell body (Fig. 7 C), and the representative images are presented (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1). The data show that upon overexpression of DN-GAKIN,
a subpopulation of cells that show high accumulation at the tip (tip/cell body ratio $> 1$) disappeared. In contrast, cells with a high accumulation ratio are increased when full-length GAKIN is overexpressed. These results suggest that the accumulation of PIP$_3$ in hippocampal neurons is dependent on the activity of GAKIN.

Also, we noticed that the cells overexpressing GAKIN and DN-GAKIN often lost the typical morphology of the well-differentiated axon-dendrite structure, leading to the formation of multiple, highly branched neurites (Fig. 7 B). In the case of full-length GAKIN, this could be attributed to the hyperaccumulation of PIP$_3$ at the ends of multiple neurites. Generally, PIP$_3$ without the overexpression of GAKIN would accumulate in the longest neurite observed in normally developing neurons. In the case of DN-GAKIN, the reduction of PIP$_3$ accumulation in the longest neurite end might inhibit proper axon-dendrite specification.
Therefore, we quantified the number of cells with axon-dendrite polarity based on the morphological observation (Shi et al., 2003; Menager et al., 2004) upon overexpression of GAKIN and DN-GAKIN. It is noteworthy that a recent study examined the role of PIP3 in neuronal polarization and axon formation and established that the hippocampal neurons expressing low levels of the GFP-Akt-PH construct retained cell polarity (Menager et al., 2004). Following this experimental strategy, we used a highly purified preparation of GFP-Akt-PH plasmid and selected neurons expressing low levels of GFP-Akt-PH. Similar to the GFP transfected control neurons, the GFP-Akt-PH transfected cells maintained cell morphology as established by the observation of Tau-1–positive neurites in individual neurons (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1). However, to further eliminate the possibility of deleterious effects of the GFP-Akt-PH detector on the neuronal polarity (Shi et al., 2003), GAKIN and DN-GAKIN were cotransfected with GFP as a morphological marker. Representative images (Fig. 8 A) and the quantification data (Fig. 8 B) demonstrate that overexpression of GAKIN and DN-GAKIN caused the loss of neuronal polarity. To confirm that such an effect is mediated by the FHA–PIP3BP interaction, the effect of the motor-FHA (1–557) domain of GAKIN was tested. When expressed in primary neurons, the motor-FHA domain showed diffuse distribution throughout the cell and did not accumulate at the neurite ends (Fig. S5), suggesting that it is not capable of transporting cargo in vivo. The motor-FHA domain suppressed neuronal polarity, whereas the motor domain alone (1–486, without the FHA domain) did not (Fig. 8 B and Fig. S5). These results suggest the necessity of the FHA domain for the DN effect of GAKIN mutants on neuronal polarity. As a negative control, KIF13A had no effect on polarity.

To further confirm this effect on neuronal polarity, transfected neurons were stained for tau, a marker for axon differentiation (Shi et al., 2003; Menager et al., 2004). GFP-expressing control neuron exhibited a single long Tau-1–positive neurite, which is an axon (Fig. 8 C, left). However, neurons expressing full-length GAKIN or DN-GAKIN did not have Tau-1–positive neurites (Fig. 8 C). These results confirmed the loss of polarity observed by the morphological analysis.

Discussion

In this study, we demonstrate that a complex of PIP3BP and GAKIN provides a novel mechanism for motor-mediated transport of PIP3-containing vesicles along microtubules (Fig. 9). Previously, a model for the transport of PIP2-containing vesicles by Unc104/KIF1A, an evolutionally conserved kinesin-like motor protein, was proposed (Klopfenstein et al., 2002). In that model, a PH domain located at the C-terminal tail of Unc104

Figure 8. GAKIN-mediated transport of PIP3 regulates neuronal polarity of hippocampal neurons. (A) Overexpression of GAKIN constructs affects the neuronal polarity. GFP, as a morphological marker, was cotransfected with FLAG-GAKIN or FLAG-DN-GAKIN. Neuronal polarity was determined as described in Materials and methods. Overexpression of GAKIN, as well as DN-GAKIN, caused the loss of the axonally specified neurites. (B) Quantification of neuronal polarity formation as determined in A (percentage of neurons polarized: GFP, 62 ± 3%, n = 185; GFP-GAKIN, 26 ± 8%, n = 131, P < 0.002; GFP-DN-GAKIN, 19 ± 5%, n = 193, P < 0.0005; GFP-motor, 74 ± 8%, n = 55, P = 0.08; GFP-motor-FHA, 31 ± 13%, n = 43, P = 0.02; GFP-KIF13A, 74 ± 11%, n = 32, P = 0.16; FLAG-GAKIN, 19 ± 4%, n = 120, P < 0.0002; FLAG-DN-GAKIN, 6.4 ± 1%, n = 185, P < 0.00002; *, P < 0.05). (C) Loss of the Tau-1–positive axon structure by overexpression of GAKIN and DN-GAKIN. Bars, 20 μm.
interacts with PIP₂ specifically, thereby connecting the PIP₂ vesicles to microtubule-dependent transport (Fig. 9, left). Our results propose a new model in which PIP₂BP serves as a molecular linker connecting PIP₃ vesicles to a motor protein (Fig. 9, right).

The PIP₂BP binding site in GAKIN was localized to a small region encoded by the FHA domain (Fig. 1). The FHA domain was originally discovered in transcription factors and DNA repair proteins and is now well established as the phosphothreonine binding protein domain (Durocher and Jackson, 2002). All members of the kinesin-3 subfamily of proteins, including GAKIN, contain an FHA domain in close proximity to the N-terminal motor domain (Lawrence et al., 2004). However, it is not known whether the FHA domain of kinesins recognizes phosphothreonine residues, as do other conventional FHA domains. Our direct binding experiments used bacterially expressed GST-PIP₂BP and GAKIN FHA domain, suggesting that phosphorylation was not required for the ligand recognition of FHA, although we could not rule out the possibility that the recombinant GST-PIP₂BP was phosphorylated in the bacterial host. Nonetheless, our results provide the first evidence indicating that the FHA domain of kinesin-3 family protein is a cargo binding domain.

Using in vitro motility assays, previous studies have determined the velocity range of kinesins to be ~0.1–1.5 μm/s (for review see Hirokawa and Takemura, 2004a). We measured the velocity of the recombinant motor-FHA domain of GAKIN using a microtubule gliding assay in the presence of 1.0 mM ATP and ATP regeneration system, and it was determined to be ~1.66 μm/s. Using a liposome motility assay, the velocity was determined to be ~0.7 μm/s, which is slightly less than the velocity measured by the microtubule gliding assay. It may be speculated that in the microtubule gliding assay, multiple motor molecules contribute to the movement of each microtubule, whereas in the vesicle motility system, fewer motor molecules or possibly a single motor could be responsible for the movement of a single liposome. Overall, these values are consistent with the microtubule-dependent ATPase activity of the GAKIN motor domain that we reported previously (Asaba et al., 2003). These results indicate that the motor and FHA segment of GAKIN shows relatively fast anterograde activity; however, the motor activity of the full-length GAKIN remains to be established.

GAKIN was originally discovered as the binding partner for human discs large tumor suppressor protein (hDlg), a member of the MAGUK superfamily (Hanada et al., 2000). As a scaffolding protein with multiple protein–protein interaction motifs, hDlg is proposed to link specific cargo vesicles to GAKIN for their transport to specialized membrane regions (Asaba et al., 2003). Its Drosophila melanogaster homologue, Dlg, is an important polarity determination factor in epithelial cells and asymmetric cell division of neuroblasts (Knust and Bossinger, 2002). It is, therefore, possible that the transport of hDlg by GAKIN also has significant effects on cell polarity in general. However, in the current setting, we believe that the effect on the neuronal polarity is primarily regulated by GAKIN–PIP₂BP–mediated transport of PIP₃, mainly because (1) significant enhancement of PIP₃ accumulation that was observed upon overexpression of full-length GAKIN correlated with the loss of polarity and (2) the motor-FHA construct, which binds to PIP₂BP but not to hDlg, exhibited DN effects on neuronal polarity. The precise relationship of two potential cargos of GAKIN, mediated by PIP₂BP and hDlg, will be a subject of future studies.

Figure 9. A proposed model for the intracellular transport mechanism of PIP₃ and PIP₂ cargo vesicles by kinesins.
(Aoki et al., 2005). Therefore, it has been assumed that local production of PIP₃ at the tip of neurites and the enhancement of the signal by positive feedback, which activates further production of PIP₃, are sufficient to explain the sustained local accumulation of PIP₃ in neuronal cells (Menager et al., 2004; Aoki et al., 2005).

How then, does our proposed model of PIP₃ transport fit into the current paradigm? Our results demonstrate that full-length GAKIN overexpressed in hippocampal neurons accumulated at the distal ends of neurites and PIP₃ accumulation is enhanced at sites where GAKIN is accumulated. In contrast, overexpression of a related motor protein, KIF13A, did not significantly affect PIP₃ accumulation. These results suggest that GAKIN specifically transports PIP₃ itself or transports the factors that stimulate the production of PIP₃, to contribute to the local accumulation of PIP₃. From our present data, it is not possible to differentiate between these two possibilities at this stage. In any event, these two possibilities may not be mutually exclusive, as the transported PIP₃ can stimulate the local production of PIP₃ by the aforementioned positive-feedback mechanism. The transport of PIP₃-containing vesicles could move upstream and downstream components of the PIP₃ signaling complex residing on the same vesicles as PIP₃. Overall, our data suggests that GAKIN-dependent transport contributes to the local accumulation of PIP₃ at the tip of neurites, thus supporting the existence of a novel mechanism for the proposed transport of PIP₃ in vivo.

The conventional model of PIP₃, as a second messenger describes that it is produced on the plasma membrane by a signaling complex formed by the activated receptors and is metabolized quickly by the lipid phosphatase activity such as PTEN (phosphatase and tensin homologue deleted on chromosome 10), thus eliciting only the transient response on site (Cantley, 2002). Can PIP₃ be found on the intracellular vesicles that are transportable? Significant evidence has been reported that demonstrates the production of PIP₃ at the intracellular membranes (Sato et al., 2003). Also, in sympathetic neurons, locally administered NGF at the tip of axons leads to activation of PI 3-kinase at the cell body (Kuruvilla et al., 2000) by the retrograde transport of the internalized activated NGF receptor (Miller and Kaplan, 2001; Ye et al., 2003; Kuruvilla et al., 2004). Therefore, it is reasonable to assume that in developing neurons a significant amount of PIP₃ is continuously produced at intracellular membrane vesicles in the cell body.

In neurons, which often extend exceptionally long processes, transport of specific cargo molecules by motor proteins is essential for the proper targeting of cellular components (Hirokawa and Takeura, 2004b). For example, signaling components downstream of PIP₃ that regulate axonal specification, such as CRMP-2 (collapsin response mediated protein 2) and Par3/Par6/APKC, are transported by kinesin-1 and -2, respectively (Nishimura et al., 2004; Kimura et al., 2005). We propose that the GAKIN–PIP₃BP complex transports PIP₃-containing vesicles from the cell body to the distal end of the neurites. Directed transport of PIP₃ and its signaling components to the specialized membrane region contributes to the local enhancement of the signaling strength at the neurite ends, thus leading to a precipitous breakdown of the cellular symmetry.
Preparation of liposomes

Egg yolk PC, Rh-PE, synthesized unsaturated PIP2, and synthesized unsaturated PIP3 (all from Avanti Polar Lipids, Inc.) were stored in organic solvents. 1.0 μmol PC was mixed with 1.0 or 10% (g/g lipids) PIP2 or 10 or 15% PIP3 and 0.05% Rh-PE and dried in a glass vial under the stream of dry nitrogen. The lipid film was further desiccated under high vacuum overnight, and lipids were rehydrated by the addition of BRB80 containing 5% sucrose and incubated at 70°C for 30 min with occasional mixing by vortex. Three freeze/thaw cycles were performed in the dry ice box and a 37°C water bath. A mini-extruder device with 250-μl syringes (Avanti Polar Lipids, Inc.) was used to extrude liposomes through a 100-nm pore polycarbonate filter (Whatman) at 70°C. Liposomes were stored in the dark at 4°C and used within 2 wk.

Liposome flotation assay

Liposomes containing 10 or 15% PIP2, or 10% PIP3 (5.0 nmol) were incubated on ice for 30 min with 70 pmol GST-PP1-PB and 30 pmol His-motor-FHA. BRB80 containing 2.0 M sucrose was added to the incubation reaction to bring the final sucrose concentration to 1.6 M, and this mixture was overlaid with cushions containing 1.4, 0.4, and 0.25 M sucrose in the same buffer in a centrifuge tube (7 × 20 mm). After centrifugation at 40,200 rpm at 4°C for 30 min in a rotor (Type 42.2Ti; Beckman Coulter), the 0.25/0.4 M fraction (top fraction) and the loading fraction (1.6 M sucrose; bottom fraction) was collected and analyzed by SDS-PAGE followed by Western blotting with anti-GAKIN polyclonal antibody and anti-GST antibody. For anti-GST Western blot, the loading ratio of top/bottom fraction was 5:1. For anti-GAKIN Western blot, the ratio was 25:1. GST-PP1-PB loaded in the input lane was 15 ng. The His-motor-FHA amount was 20 ng.

Liposome motility assay

A lawn of Alexa 488-labeled microtubules was prepared on the motility chamber to visualize the movement of rhodamine-labeled liposomes by fluorescence microscopy. To ensure the attachment of microtubules on the glass surface, the flow cell was first coated with recombining His-GAKIN-CT (aa 1414-1826) that contains a microtubule binding CAP-Gly domain and binds avidly to microtubules in vitro. A similar approach was used before to immobilize microtubules on the glass surface coated with either anti-tubulin ofpoly-lysine (McGoldrick and Sheetz, 1998). After incubation of 0.125 mg/ml His-GAKIN-CT (in BRB80 containing 0.1 mg/ml BSA) in the flow cell for 10 min at room temperature, fluorescent-labeled microtubules (0.1–0.2 mg/ml in BRB80 supplemented with 10 mM taxol and 1.0 mM GTP) were introduced and incubated for 30 min at room temperature in a humidified chamber. The flow cell was washed twice with BRB80 supplemented with 5.0 mg/ml casein, 0.1 μM taxol, and 1.0 mM GTP and used immediately for the motility assay. Liposomes of various compositions were incubated with 0.15 nmol GST-PP1-PB on ice for 30 min, and stable liposome-PI3-PB complex was separated from unbound PIP2-PB by ultracentrifugation using sucrose gradient as described (see Liposome flotation assay). The top fraction of the gradient containing the liposome–PI3-PB complex was mixed with 0.5 mg/ml of His-motor-FHA in the motility buffer and introduced into the flow cell containing a lawn of microtubules. The final concentration of liposomes was ~50 μg/ml. Time-lapse imaging was captured as described for the microtubule gliding assay. Sequential photographs were taken every 1 s for 30 s. Imaging of the fluorescently labeled microtubules was captured for each frame and overlaid with the imaging of liposomes. The velocity of each moving liposome was measured using MetaMorph software by tracing the position of each liposome for every second. Videos were made using the MetaMorph software. Individual experiments were repeated more than three times for each sample (nine times for 1% PI3-containing liposomes and six times for 10% PI3-containing liposomes).

Immunofluorescence microscopy

PC12 cells were seeded onto coverslips (Fisher Scientific) coated with 0.01% poly-lysine (Sigma-Aldrich), stimulated with 5.0 ng/ml NGF for 36 h, and fixed with 4% PFA in PBS at room temperature for 15 min. Cells were then washed with PBS and permeabilized by PBS supplemented with 0.1% Triton X-100, 0.1% PI3-PB complex was separated from unbound PIP2-PB by ultracentrifugation using sucrose gradient as described (see Liposome flotation assay). The top fraction of the gradient containing the liposome–PI3-PB complex was mixed with 0.5 mg/ml of His-motor-FHA in the motility buffer and introduced into the flow cell containing a lawn of microtubules. The final concentration of liposomes was ~50 μg/ml. Time-lapse imaging was captured as described for the microtubule gliding assay. Sequential photographs were taken every 1 s for 30 s. Imaging of the fluorescently labeled microtubules was captured for each frame and overlaid with the imaging of liposomes. The velocity of each moving liposome was measured using MetaMorph software by tracing the position of each liposome for every second. Videos were made using the MetaMorph software. Individual experiments were repeated more than three times for each sample (nine times for 1% PI3-containing liposomes and six times for 10% PI3-containing liposomes).

Analysis of GFP-Akt-PH accumulation in PC12 cells

The intensity of GFP-Akt-PH was observed by fluorescence microscopy using a 40× magnification setting. The expression of several GAKIN constructs was detected by staining with an anti-FLAG antibody. The number of neurites with and without the accumulation of GFP-Akt-PH fluorescence was manually counted. At least three independent experiments were performed for each construct (＞70 cells and ＞300 neurites were observed for each experimental condition).

Hippocampal neuron culture

Methods for preparing the hippocampal cell cultures were essentially the same as those described previously (Banker and Goslin, 1998). Hippocampi were dissected from mice at embryonic day 16.5, and dissociated cells were plated in plating medium (DMEM supplemented with 10% FCS and 5% horse serum) onto glass coverslips coated with poly-lysine. After neurons attached to the substrate, the medium was exchanged to neuronal culture medium (MEM [Invitrogen] with B27 supplement [Invitrogen], 0.6% glucose, and 1 mM sodium pyruvate [Sigma-Aldrich]). Around 18 h after plating, neurons were transfected with various DNA constructs using Lipofectamine 2000.

Determination of neuronal polarity

Neuronal polarity was assessed by determining the percentage of neurons with a single long process that was at least twice as long as the other processes. For each construct, at least three independent experiments were performed. The t test was used for the statistical significance.

Online supplemental material

Fig. S1 shows that GAKIN and PI3-PB form a complex on PI3-PB liposomes. Fig. S2 shows that relative fluorescence intensity was measured to show the accumulation of PI3-PB at the tip of the neurite in PC12 cells and in neurons. Fig. S3 demonstrates that low-level expression of GFP-Akt-PH detector does not affect neuronal polarity. Fig. S4 shows the effect on PI3-PB accumulation by overexpression of full-length GAKIN and DN-GAKIN in hippocampal neurons. Fig. S5 depicts the distribution of several GAKIN constructs expressed in hippocampal neurons. Videos 1 and 2 show that GAKIN transports PI3-PB liposomes via PI3-PB in vitro. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200604031/DC1.

We are grateful to Drs. K. Venkateswarlu, Y. Gotoh, and T. Balla for reagents and advice. We thank Dr. H. Hashimoto for suggestions on liposome motility experiments.

This work was supported by the National Institutes of Health grants CA 94414 and HL 60755. T. Hanada is the recipient of a Campus Research Board Award from the University of Illinois at Chicago (2004–2005).

Submitted: 6 April 2006
Accepted: 25 June 2006

References


