Phosphatidylinositol 4-Phosphate 5-Kinase Is Essential for ROCK-mediated Neurite Remodeling

Phosphatidylinositol 4-phosphate 5-kinase (PIP-5kin) regulates actin cytoskeletal reorganization through its product phosphatidylinositol 4,5-bisphosphate. In the present study we demonstrate that PIP-5kin is essential for neurite remodeling, which is regulated by actin cytoskeletal reorganization in neuroblastoma N1E-115 cells. Overexpression of wild-type mouse PIP-5kin-α inhibits the neurite formation that is normally stimulated by serum depletion, whereas a lipid kinase-defective mutant of PIP-5kin-α, D266A, triggers neurite extension even in the presence of serum and blocks lysophosphatidic acid-induced neurite retraction. These results phenocopy those previously reported for the small GTPase RhoA and its effector p160 Rho-associated coiled coil-forming protein kinase (ROCK). However, the ROCK-specific inhibitor Y-27632 failed to block the inhibition by PIP-5kin-α of neurite extension, whereas D266A did block the neurite retraction induced by overexpression of ROCK. These results, taken together, suggest that PIP-5kin-α functions as a downstream effector for RhoA/ROCK to couple lysophosphatidic acid signaling to neurite retraction presumably through its product phosphatidylinositol 4,5-bisphosphate.

Axon guidance is a critical event in the establishment of neuronal networks during embryogenesis and is regulated by extracellular cues such as chemoattractants and chemorepellents, which are recognized by growth cones at the tips of axons (1, 2). Axons extend toward chemoattractants and away from chemorepellents through mechanisms involving actin cytoskeletal reorganization in the growth cone (3, 4).

Recently accumulated evidence suggests that the Rho family GTPases (Rho, Rac, and Cdc42) play crucial roles in reorganizing the actin cytoskeleton in neurons, thereby regulating the morphology of neurites and growth cones (5–8). In particular, Rho appears to be implicated in the repulsive signaling pathway. In mouse neuroblastoma N1E-115 cells, overexpression of a constitutively active mutant of Rho prevents neurite formation (9, 10), and inactivation of endogenous Rho by Clostridium botulinum C3 exoenzyme inhibits the growth cone collapse and neurite retraction induced by lysophosphatidic acid (LPA)1 (9, 10). Inhibition of growth cone collapse by inactivation of Rho has also been shown for myelin-stimulated central nervous system neurons (11) and Ephrin-A5-stimulated retinal neurons (12).

Rho-associated kinases termed ROCK, Rho-kinase, and ROK mediate Rho-induced actin cytoskeletal reorganization (13–15) and subsequent repulsive responses of neurites including growth cone collapse and neurite retraction. Evidence for this includes the findings that overexpression of ROCK as well as the active mutant of RhoA, RhoAV14, inhibits neurite formation in N1E-115 cells (16, 17). In addition, pharmacological inactivation of endogenous ROCK, similar to inhibition of Rho activity, interferes with LPA-induced neurite retraction in N1E-115 cells (16, 17) and Ephrin-A5-induced growth cone collapse in retinal neurons (12).

Although the downstream pathways that link ROCK signaling to actin cytoskeletal reorganization remain to be clarified, myosin II and LIM kinase (LIMK) appear to be involved (18–22). ROCK leads to an increase in the phosphorylation level of myosin light chain by direct and indirect mechanisms (18, 19). The indirect mechanism includes inactivation of myosin phosphatase by phosphorylation of its myosin binding subunit. Furthermore, the ROCK-dependent phosphorylation of myosin light chain has been reported to be involved in LPA-induced neurite retraction in N1E-115 cells (16, 17). ROCK also directly phosphorylates LIMK and thereby activates it, which in turn phosphorylates the actin-associated protein cofillin (20, 23). Recently it has been reported that semaphorin 3A increases and then subsequently decreases the phosphorylation level of cofillin at collapsing growth cones (24). Although myosin II and LIMK are required participants in the ROCK-mediated repulsion, activated myosin II or LIMK alone do not completely mimic ROCK-mediated effects in this system (16, 25), suggesting that an additional signaling pathway(s) is also required.

An additional ROCK effector candidate would be phosphatidylinositol 4-phosphate 5-kinase (PIP-5kin), which phosphorylates 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)P2.

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1 The abbreviations used are: LPA, lysophosphatidic acid; PIP-5kin, phosphatidylinositol 4-phosphate 5-kinase; PI(4)P, phosphatidylinositol 4-phosphate; ROCK, p160 Rho-associated coiled-coil forming protein kinase; FCS, fetal calf serum; HA, hemagglutinin; GFP, green fluorescent protein; LIMK, LIM kinase.

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(26–30). The potential relevance of this candidate is suggested by the following reports: 1) the PIP-5kin product, PI(4,5)P₂, reorganizes the actin cytoskeleton through direct interaction with actin-associated proteins (29, 31); 2) overexpression of PIP-5kin in COS-7 and CV1 cells promotes the formation of short actin fibers and robust actin stress fibers, respectively (32, 33); 3) PIP-5kin induces actin polymerization from membrane-bound vesicles to form motile actin comets (34); 4) although Jones et al. (36) and this group have reported that the small GTPase ARF directly activates PIP-5kin in a GTP-dependent manner (35), Rho and its effector ROCK also directly participate in neurite remodeling in this study we overexpressed wild type and a lipid kinase-defective mutant of PIP-5kin-α in N1E-115 cells and analyzed the effects on cell morphology and LPA-induced neurite retraction. The results obtained indicate that PIP-5kin-α at least in part functions as a downstream effector for RhoA/ROCK to couple it to neurite retraction.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pDNA3-Myc-mouse PIP-5kin-α was constructed as previously described (35). A cDNA for the lipid kinase-deficient mutant D266A (details in the text) was constructed using a PCR-based strategy and subcloned into pcDNA3-Myc and pcDNA3-HA vectors (a generous gift of Dr. Nakayama). pEF-BOS-HA-RhoAV14 and pCAG-Myc-ROCK were generous gifts of Drs. K. Kaibuchi and S. Narumiya, respectively.

Cell Culture, Transfection, and Cell Treatment—Mouse neuroblastoma N1E-115 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). To analyze by immunofluorescence microscopy cells were plated on glass coverslips at a density of 4 × 10⁴ cells per 2.5-cm dish, cultured overnight, and then transfected with plasmids using LipofectAMINE Plus (Invitrogen). For analysis of the effects of PIP-5kin-α and its mutant D266A on cell morphology, transfected cells were further cultured in the absence or presence of FCS for 18 or 48 h. To analyze the effects of D266A on LPA-induced neurite retraction and those of Y-27632 on the actions of ROCK1, ROCK2, and PIP-5kin-α, transfected cells cultured in the absence of FCS for 18 h were treated with 1 μM LPA (Sigma) for 10 min and 10 μM Y-27632 for 48 h, respectively.

Immunofluorescence Microscopy—Cells treated as described above were fixed, permeabilized, and blocked as previously described (35). The cells were then immunostained for Myc-tagged and HA-tagged proteins using monoclonal mouse anti-Myc (9E10, Santa Cruz) and monoclonal rat anti-HA (3F10, Roche Molecular Biochemicals) antibodies, respectively, followed by incubation with fluorescein isothiocyanate-conjugated secondary anti-mouse and anti-rat IgG antibodies (Jackson ImmunoResearch Laboratory), respectively. Tubulin and F-actin were visualized using a Cy3-conjugated monoclonal mouse anti-β-tubulin antibody (TUB2.1, Sigma) and rhodamine phalloidin (Molecular Probes), respectively. The immunofluorescently stained cells were imaged using an immunofluorescence microscope (Axiovert S100, Zeiss). The cell morphology of 100–150 cells was scored as follows: round, rounded cells without neurites; flat, flattened cells without neurites; neurite, cells with at least one process greater than the cell diameter. The data presented are the means ± S.E. of at least three independent experiments.

Assay of PIP-5kin Activity—Myc-PIP-5kin-α and Myc-D266A were transfected in N1E-115 cells and analyzed the effects of recombinant wild type PIP-5kin-α and D266A after expression in N1E-115 cells assayed as described under “Experimental Procedures.” C, the morphology of the transfected cells was scored as described under “Experimental Procedures,” and the results of three independent experiments are shown.

RESULTS AND DISCUSSION

PIP-5kin-α Inhibits Neurite Formation—Neuroblastoma N1E-115 cells are spherically shaped in the presence of serum, but when deprived of serum they become flat and then form neurites. When wild type PIP-5kin-α was overexpressed in these cells, neurite formation induced by serum depletion was inhibited, and the cells retained their spherical shapes, whereas GFP-expressing and non-transfected control cells flattened and formed neurites (Fig. 1, A and C). To examine whether PIP5-kin-α lipid kinase activity was essential for this effect we generated D266A, a PIP-5kin-α mutant in which Ala was substituted for a presumably critical Asp in the putative lipid kinase ATP-binding site (40). In vitro lipid kinase analysis of recombinant D266A revealed that it was inactive as anticipated (Fig. 1B). When overexpressed, D266A failed to interfere with the cell flattening and neurite extension induced by serum depletion (Fig. 1, A and C), demonstrating that the lipid kinase activity of PIP-5kin-α is essential for this effect. In fact, neurite
form by the D266A-overexpressing cells was modestly but significantly enhanced over that of the GFP-expressing control cells (Fig. 1C), possibly caused by the inhibition of endogenous PIP-5kin activity and suggesting possibly that there is a basal level of PIP-5kin activity that opposes neurite formation at all times. These results, taken together, provide evidence that elevating PIP-5kin-α activity signals N1E-115 cells to remain spherical and that this overrides opposing neurite formation signaling pathways.

The requirement for endogenous Rho and ROCK in preventing neurite formation when serum is present has been well documented (for example, Refs. 17 and 41). The observation above of neurite formation being enhanced by the expression of an inactive and potentially dominant negative PIP-5kin suggested that this might also be the case for PIP-5kin as well. To test this we examined whether overexpression of the lipid kinase-defective mutant D266A in N1E-115 cells would result in neurite formation in the absence of serum. Cells overexpressing D266A formed extended bipolar neurites even in the presence of serum (Fig. 2, A and B), demonstrating that D266A functions as a dominant negative mutant and that endogenous PIP-5kin activated by serum stimulation acts normally to block neurite formation. Although the extended bipolar neurites formed in D266A-overexpressing cells appeared somewhat different in morphology than the neurites found in control cells in the absence of serum (Fig. 1A), the control cell neurites became longer and bipolar if the cells were cultured for longer periods of time (Fig. 2C), suggesting that D266A may also be promoting more rapid maturation of the neurites formed. In D266A-overexpressing cells, about 80% of total cells formed neurites when cultured in the absence of FCS (Fig. 1C), whereas only 50% of cells did so in the presence of FCS (Fig. 2B). This difference may reflect the fact that the variable level of expression achieved for the D266A mutant did not suffice to override the endogenous inhibition of neurite formation in all transfected cells when serum signaling pathways were activated.

**Involvement of PIP-5kin-α in LPA-induced Neurite Retraction**—After formation by N1E-115 cells neurites can be triggered to retract in response to the bioactive phospholipid LPA, which is present in abundance in serum (42–44). Recent reports have shown that LPA stimulation activates PIP-5kin-α in endothelial cells and in NIH-3T3 cells (45, 46). These observations, taken together with the results described above, raised the possibility that PIP-5kin-α is also involved in LPA-induced neurite retraction. To explore this, we tested the effects of D266A expression on LPA-induced neurite retraction. Whereas the neurites formed by GFP-transfected and non-transfected control cells cultured in the absence of serum retracted in response to LPA stimulation, overexpression of D266A blocked the response (Fig. 3), suggesting that PIP-5kin-α activity is essential for LPA-induced neurite retraction.

**PIP-5kin-α Functions as a Downstream Effector for ROCK in Order to Couple to Neurite Retraction/Inhibition of Neurite Formation**—In N1E-115 cells the small GTPase RhoA and its downstream effector ROCK play crucial roles in the retention of spherical cell shapes in the presence of serum and in the retraction of extended neurites as triggered by LPA (9, 10, 16, 17, 41). The effects of PIP-5kin-α described above phenocopy those of RhoA and ROCK, raising three possibilities for mech-
PIPK-5kin Regulates Neurite Remodeling

PIPK-5kin-α couples the signal from ROCK to neurite remodeling. A, N1E-115 cells transfected with plasmids for GFP, HA-RhoAV14, Myc-ROCK, or Myc-PIPK-5kin-α were cultured in the absence of FCS for 18 h, and then incubated with or without 10 μM Y-27632 for 48 h. The cells were stained for the expressed proteins (green) and β-tubulin (red) as described under “Experimental Procedures,” and a representative of four experiments performed is shown. Scale bar, 20 μm. B, N1E-115 cells transfected with Myc-ROCK alone (red) and co-transfected with Myc-ROCK (red) and HA-D266A (green) were cultured in the absence of FCS for 18 h and stained for the expressed proteins as described under “Experimental Procedures,” Scale bar, 20 μm. C, the morphology of the transfected cells was scored as described under “Experimental Procedures,” and the cells exhibiting neurites greater than one cell diameter were counted.

PIPK-5kin activity in cell lysates (38); however, it remains imprecisely defined whether the regulation of PIP-5kin-α activity by ROCK is direct or indirect.

It has been reported that although the activation of myosin II and LIMK is involved in the repulsive responses of neurites, it does not suffice to trigger the repulsive events on its own (16, 25). Overexpression of PIP-5kin-α alone in neurite-bearing N1E-115 cells with the adenovirus expression system (similar to the activation of myosin II and LIMK) failed to trigger neurite retraction (data not shown) unlike RhoAV14 and ROCK (9, 48). These results, taken together with the findings described above, suggest that PIP-5kin-α is essential but not sufficient for neurite retraction and that cooperation of PIP-5kin-α with the other ROCK effectors (myosin II and LIMK) is required for the Rho/ROCK-mediated repulsive reactions. Nonetheless, the findings obtained in this study provide evidence for a novel function of PIP-5kin-α in the regulation of neurite remodeling.

Molecular Mechanism for the Regulation of Neurite Remodeling by PIP-5kin-α—Rho/ROCK regulation of the repulsive response by cultured neuronal cell lines and primary neurons is attributable to the regulation of actin cytoskeletal reorganization (5–8). The molecular mechanism by which these signaling molecules regulate actin cytoskeletal reorganization, however, remains unclear. Because in the present study we demonstrated that PIP-5kin-α at least in part functions as a novel downstream effector for ROCK to couple the LPA signal to neurite retraction in N1E-115 cells, actin cytoskeletal reorganization by Rho/ROCK might be mediated by PIP-5kin-α. Furthermore, it is most likely that the regulation by PIP-5kin-α of actin cytoskeletal reorganization is mediated by its product PI(4,5)P₂, which has been implicated in the reorganization of actin filament structures through the actin-associated proteins such as profilin, gelsolin, and α-actinin (29, 49–51). The PIP-5kin-α effect through PI(4,5)P₂ on the repulsive response of neurites seems to be attributable to its ability to polymerize actin and stabilize the formed F-actin, because in PIP-5kin-α-overexpressing cells intense staining of F-actin was observed at the plasma membrane of the rounded cell bodies (data not shown). This assumption is consistent with the finding by Rauch et al. with laser-based optical tweezers that PI(4,5)P₂ regulates actin cytoskeleton-plasma membrane adhesion to modulate membrane tension, which is critical for membrane dynamics and cell morphology (52). From these findings we propose that stabilization of cortical actin filaments by PI(4,5)P₂ produced by the action of PIP-5kin-α at restricted sites in the neuronal plasma membrane increases the membrane tension and thereby triggers neurite retraction.

Although we have proposed a simple explanation for the effects of PIP-5kin-α on the repulsive response of neurites through actin polymerization and stabilization of formed actin filaments, the underlying molecular mechanisms may be extremely complicated. It has been reported that depolymerization of actin filaments by cytochalasin D stimulates neurite outgrowth in cultured neuronal cell lines (including Neuro 2a
and PC12 cells) and primary neurons (53–56). Furthermore, overexpression of actin-depolymerizing factor in rat cortical and spinal cord neurons has been reported to stimulate neurite outgrowth (57). These findings are consistent with the idea that depolymerization of actin filaments initiates neurite formation and extension and conversely that actin polymerization triggers neurite retraction. On the other hand, however, it has been reported that chemorepellent semaphorin 3A, which induces growth cone collapse and subsequent axonal growth arrest, induces actin depolymerization (58, 59). Thus, at present it is controversial as to which type of actin cytoskeletal reorganization (depolymerization or polymerization) regulates which type of neurite remodeling (neurite formation or retraction). A possible explanation for this apparent contradiction is proposed by Bradke and Dotti, explaining that the neurite formation and retraction signals include actin polymerization and depolymerization in different places within the growth cone (60). This notion is supported by the report that F-actin concentration dramatically decreases in the leading edge of growth cone in response to semaphorin 3A, although at the center of growth cone it is relatively unchanged (58, 59). Alternatively, temporal reorganization of actin filaments may be critical for the type of neurite remodeling triggered. Further studies on spatial and temporal analysis of actin polymerization and depolymerization will provide insight into the molecular mechanisms through which neurite formation and retraction are regulated.

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