Introduction

Neurite outgrowth is essential for the proper formation of neuronal networks in the developing nervous system and is ultimately dependent on cytoskeletal dynamics, which often requires the activity of the low molecular weight GTPases of the Rho family. Rho GTPases are molecular switches, which, upon binding GTP, undergo conformational changes that enable the regulation of downstream target proteins (Jaffe and Hall, 2005). Hydrolysis of GTP to GDP turns off and limits the extent of downstream signalling. Members of the Rho family of GTPases, which include RhoA, Rac1 and Cdc42 have been extensively studied in the context of neurite remodelling (Govek et al., 2005).

Neurite dynamics, controlled by Rho GTPase, have been widely studied in model systems such as the neuronal cell line N1E-115 (Gebbink et al., 1997; Hirose et al., 1998; Jalink et al., 1993; Jalink et al., 1994; Kozma et al., 1997; Krabbe et al., 1999; Postma et al., 1996). These cells maintain a spherical morphology in the presence of serum; however, withdrawal from serum results in rapid cell flattening, differentiation and the formation of neurites. Stimulation of serum-starved cells with different repulsive agonists, such as lysophosphatidic acid (LPA), trigger a rapid collapse of neurites that is dependent upon the activation of RhoA. Using N1E-115 cells, we and others reported that activity of phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks). PIP5Ks synthesise the phosphoinositide lipid second messenger phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2], and overexpression of active PIP5K is sufficient to induce neurite retraction in both N1E-115 cells and cerebellar granule neurons. However, how PIP5Ks are regulated or how they induce neurite retraction is not well defined. Here, we show that neurite retraction induced by PIP5Kβ is dependent on its interaction with the low molecular weight G protein Rac. We identified the interaction site between PIP5Kβ and Rac1 and generated a point mutant of PIP5Kβ that no longer interacts with endogenous Rac. Using this mutant, we show that Rac controls the plasma membrane localisation of PIP5Kβ and thereby the localised synthesis of PtdIns(4,5)P_2 required to induce neurite retraction. Mutation of this residue in other PIP5K isoforms also attenuates their ability to induce neurite retraction and to localise at the membrane. To clarify how increased levels of PtdIns(4,5)P_2 induce neurite retraction, we show that mutants of vinculin that are unable to interact with PtdIns(4,5)P_2, attenuate PIP5K- and LPA-induced neurite retraction. Our findings support a role for PtdIns(4,5)P_2 synthesis in the regulation of vinculin localisation at focal complexes and ultimately in the regulation of neurite dynamics.

Key words: Lipid kinase, PIP5K, Rac, G-proteins, Neurite retraction, PtdIns(4,5)P_2, PIP2

Summary

In N1E-115 cells, neurite retraction induced by neurite remodelling factors such as lysophosphatidic acid, sphingosine 1-phosphate and semaphorin 3A require the activity of phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks). PIP5Ks synthesise the phosphoinositide lipid second messenger phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2], and overexpression of active PIP5K is sufficient to induce neurite retraction in both N1E-115 cells and cerebellar granule neurons. However, how PIP5Ks are regulated or how they induce neurite retraction is not well defined. Here, we show that neurite retraction induced by PIP5Kβ is dependent on its interaction with the low molecular weight G protein Rac. We identified the interaction site between PIP5Kβ and Rac1 and generated a point mutant of PIP5Kβ that no longer interacts with endogenous Rac. Using this mutant, we show that Rac controls the plasma membrane localisation of PIP5Kβ and thereby the localised synthesis of PtdIns(4,5)P_2 required to induce neurite retraction. Mutation of this residue in other PIP5K isoforms also attenuates their ability to induce neurite retraction and to localise at the membrane. To clarify how increased levels of PtdIns(4,5)P_2 induce neurite retraction, we show that mutants of vinculin that are unable to interact with PtdIns(4,5)P_2, attenuate PIP5K- and LPA-induced neurite retraction. Our findings support a role for PtdIns(4,5)P_2 synthesis in the regulation of vinculin localisation at focal complexes and ultimately in the regulation of neurite dynamics.
**Results**

**PIP5K activity drives neurite retraction**

We previously demonstrated that PIP5K activity was essential for neurite retraction in response to LPA and other agents in N1E-115 cells. To demonstrate that overexpression of PIP5K attenuates neurite outgrowth in a more physiological setting, we examined the effects of PIP5K\(\beta\) overexpression in primary murine cerebellar granule neurons (CGNs). Similarly to N1E-115 cells, transient expression of PIP5K\(\beta\) potently inhibited neurite formation in CGNs (Fig. 1A). These data are in agreement with a previous study that

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**Fig. 1.** PIP5K\(\beta\) blocks neurite outgrowth in both primary neurons and neuronal cell lines and is dependent upon Rac. (A) Cerebellar granule neurons (CGNs), isolated from postnatal day 2 mice, were seeded onto laminin- and poly-L-lysine-coated dishes. CGNs were transfected with the constructs shown on the left and subsequently fixed and stained for the neuronal marker GAP-43 and imaged by confocal microscopy. Neurite length was quantified and is shown on right. Scale bar: 50 \(\mu\)m. A total of 20 transfected cells were included for each condition. Error bars represent ± s.e.m. (B) N1E-115 cells were treated with the siRNA oligonucleotides as shown and after 36 hours cells were lysed and the RNA was extracted and used for qRT-PCR analysis to determine the levels of PIP5K\(\alpha\), PIP5K\(\beta\) and PIP5K\(\gamma\) (left). After treatment with siRNA, N1E-115 cells were grown in serum and assessed for the number of cells with neurites. The first two bars show controls in which cells have been maintained in serum (control) or have been serum starved to induce neurites (–FCS). (C) Short hairpins targeting the murine PIP5K\(\alpha\) (herein defined using human nomenclature PIP5K\(\beta\)) were cloned into pSuper and transiently transfected into HEK293 in the presence of each of the indicated isoforms of Myc-tagged PIP5K. After 72 hours, the cells were imaged by light microscopy. Shown are representative images of control cells (left) and cells expressing RNAi for murine PIP5K\(\beta\). Scale bar: 20 \(\mu\)m.
demonstrated that PIP5Kα overexpression attenuated axonal growth induced by inhibition of Arf in hippocampal neurons (Hernandez-Devieze et al., 2004). To assess whether PIP5Ks are required for controlling neurite growth, we suppressed PIP5K expression in N1E-115 and N2A cells using RNAi and assessed neurite formation in the presence of serum. In N1E-115 cells, siRNA oligonucleotides abrogated the expression of their corresponding PIP5Ks (Fig. 1B) although suppression of PIP5Kγ induced an increase in the mRNA encoding PIP5Kβ. Suppression of PIP5Kα potently induced neurite formation whereas suppression of PIP5Kγ or PIP5Kβ had little effect (Fig. 1B). The lack of neurite outgrowth on suppression of PIP5Kγ might be a result of upregulation of expression of PIP5Kβ.

The in vitro activity of PIP5Kβ is already extremely low in N1E-115 cells and therefore, we tested whether the suppression of PIP5Kβ in N2A cells, which express higher levels, could induce neurite outgrowth. The RNAi construct against PIP5Kβ suppressed PIP5Kβ expression, but did not suppress the expression of PIP5Kα or PIP5Kγ (Fig. 1C). In N2A cells, siRNA-mediated knockdown of PIP5Kβ induced neurite outgrowth in the presence of serum, whereas transfection with the control plasmid (pSuper) failed to induce neurites (Fig. 1C). siRNA-mediated suppression of PIP5Kα in N2A cells also induced neurite outgrowth (data not shown).

These data support the concept that modulation of PIP5K activity can regulate neurite outgrowth or retraction in model systems such as N1E-115 and N2A cells. The data, however, do not support a role for a specific PIP5K isoform in the regulation of neurite outgrowth or retraction but rather for a role of PtdIns(4,5)P_2 synthesis in regulating this process. In support of this conclusion, we also show that overexpression of various PIP5K isoforms induced retraction in N1E-115 cells (see below).

The decrease in the number and length of neurites in N1E-115 cells induced by overexpression of PIP5K could be a result of enhanced retraction or attenuation of neurite outgrowth. To investigate this, we developed a drug-activatable PIP5K. Fusion of an estrogen-binding domain to kinases and transcription factors often generates an inactive protein (Barthel et al., 2002; Kohn et al., 1998; Pritchard et al., 1995), which can be activated by the addition of the estrogen antagonist 4-hydroxytamoxifen (4-OHT).

The in vitro activity of PIP5Kβ, when fused to an estrogen-binding domain (ER-PIP5Kβ) was not significantly altered by 4-OHT treatment (supplementary material Fig. S1A,B). However, ER-PIP5Kβ, unlike wild-type PIP5Kβ, was predominantly localised in the cytosol and only translocated to the plasma membrane upon treatment with 4-OHT (supplementary material Fig. S1C). Overexpression of wild-type PIP5K in HeLa cells leads to dramatic vacuolisation in the cell; however, this did not occur with ER-PIP5Kβ until after treatment with 4-OHT (data not shown). These data demonstrate that fusion of PIP5Kβ to the ER domain prevents PIP5Kβ from localising to the membrane and attenuates its activity in vivo until 4-OHT is added.

In contrast to the transfection of N1E-115 cells with wild-type PIP5Kβ, cells expressing ER-PIP5Kβ developed neurite-like structures that were comparable with those in serum-starved wild-type cells. As expected, ER-PIP5Kβ localised in the cytosol and translocated to the plasma membrane only after treatment with 4-OHT (Fig. 2). 4-OHT treatment also induced retraction of preformed neurites, which eventually led to rounding of the cells (Fig. 2). Cells transfected with the ER-vector alone and treated with 4-OHT did not show neurite retraction (Fig. 2, right column). These data suggest that increased synthesis of PtdIns(4,5)P_2 can lead to the retraction of preformed neurites in N1E-115 cells.

Neurite retraction induced by PIP5Kβ and LPA is dependent on the low molecular weight G protein Rac

Although PIP5K activity is essential for LPA- and Rho-driven neurite retraction in N1E-115 cells, it does not lead to the activation of Rho (supplementary material Fig. S3). Furthermore, it is unclear how these retraction agonists regulate PIP5K. Rac1, Cdc42 and Arf6 regulate PIP5K activity and localisation and therefore we investigated whether their activities are important in regulating PIP5Kβ-mediated neurite retraction. Neither dominant-negative (DN)-Arf6 nor DN-cdc42 blocked the PIP5Kβ-induced neurite retraction in N1E-115 cells (supplementary material Fig. S2). By contrast, expression of DN-Rac1 (RFP-DN-Rac1) blocked PIP5Kβ-induced neurite retraction (Fig. 3A). PIP5Kβ interacts with the C-terminus of Rac1 (Rac1CT; Fig. 3A inset) and cell-permeable Rac1CT peptides can perturb Rac-mediated actin polymerisation and migration (van Hennik et al., 2003). Overexpression of RFP-Rac1CT also blocked PIP5Kβ-mediated neurite retraction (Fig. 3A; for quantification see Fig. 3B). Expression of Rac1CT and DN-Rac1 did not alter the expression level of PIP5Kβ in N1E-115 cells (Fig. 3C). Both Rac1CT and DN-RacN17 inhibited LPA-mediated retraction (Fig. 3D), demonstrating that active Rac is also required in LPA-mediated neurite retraction in N1E-115 cells. However, these experiments do not indicate whether Rac acts upstream or downstream of PIP5K. To explore this further, we investigated how PIP5Ks interact with Rac.

Mutation of Glu61 to leucine in PIP5Kβ is sufficient to reduce its association with Rac in vivo

We first demonstrated that endogenous Rac interacts with endogenous PIP5K in neuronal cells. Rac was immunoprecipitated with an isotype-specific antibody against PIP5K. Scale bar: 25 μm. The number and length of the neurites was assessed and is shown on the right. In the far right column, cells were transfected with ER alone and stimulated with 4-OHT for 4 hours as a control. The data are expressed as the mean ± s.d. and are representative of at least two different experiments.

Fig. 2. Induction of PIP5Kβ activity induces neurite retraction. N1E-115 cells were transiently transfected with ER-PIP5Kβ and maintained as controls or stimulated with 4-OHT for the times indicated, after which the cells were fixed and immunostained using an isotype-specific antibody against PIP5K. Scale bar: 25 μm. The number and length of the neurites was assessed and is shown on the right. In the far right column, cells were transfected with ER alone and stimulated with 4-OHT for 4 hours as a control. The data are expressed as the mean ± s.d. and are representative of at least two different experiments.
from N1E-115 cells and the amount of co-precipitating PIP5K activity was assessed. The Rac-specific antibody immunoprecipitated 3.5-times more endogenous PIP5K activity than was immunoprecipitated with a nonspecific antibody (Fig. 3E). Comparable data was obtained after immunoprecipitation of Rac from N2A cells (data not shown).

We identified a peptide region located at the N-terminus of PIP5Kβ, designated peptide 1 (Fig. 4A), which interacted with both recombinant and endogenous Rac (Fig. 4B). The interaction of this peptide with Rac was specific because peptide 1 did not associate with RhoA, another GTPase regulator of PIP5Kβ (Fig. 4C). Furthermore, a peptide corresponding to a C-terminal region of PIP5Kβ (peptide 2), did not associate with either Rac or RhoA (Fig. 4C). We identified a point mutation in full-length PIP5Kβ, PIP5KβE61L that, when compared with wild-type PIP5Kβ, displayed reduced association with both the RacCT peptide (Fig. 4D) and full-length Rac1 (Fig. 4E).

Fig. 3. Active Rac is required for PIP5Kβ and LPA induced neurite retraction. (A,B) DN-Rac1 attenuates the inhibition of neurite outgrowth by PIP5Kβ. N1E-115 cells expressing GFP-PIP5Kβ and RFP (top) or GFP-PIP5Kβ and mRFP-DN-Rac1 (middle) or GFP-PIP5Kβ and RFP-Rac1CT (bottom) were serum starved overnight and imaged using confocal microscopy. Representative images are shown, from which neurite length and number were quantified (B). Scale bar: 10 μm. The inset demonstrates that Rac1CT is able to interact with PIP5Kβ. HeLa cells expressing Myc-tagged PIP5Kβ were lysed before addition of either biotinylated control peptide or biotinylated Rac1CT peptide. Peptide–protein complexes were purified using streptavidin-coated beads. Associated PIP5Kβ was visualised by western blot (top) and associated PIP5K activity was determined (bottom). (C) Lysates from N1E-115 cells transiently transfected with the indicated constructs were lysed and western blotted using the antibodies shown. (D) DN-Rac1 attenuates LPA-mediated neurite retraction. N1E-115 cells, expressing RFP (control) or mRFP-DN-Rac1 (RacN17) or RFP-Rac1CT (Rac1CT), were serum starved overnight, treated with LPA (0.5 μM) fixed and the number of round cells was quantified. The data are from two experiments where at least 30 cells were assessed for each treatment. The data are shown as the mean ± the range from the two experiments. (E) Cells lysates from N1E-115 cells were immunoprecipitated using either a non-immune (NI) or an anti-Rac antibody (Rac). Immunoprecipitates were then split and a portion was used to assess the presence of co-precipitating PIP5K activity (middle and quantified in the top panel) or used for western blotting to demonstrate the specific immunoprecipitation of Rac (bottom).
data are consistent with a role for Rac in the regulation of plasma membrane localisation of PIP5Kβ. The E61L mutation could potentially affect PIP5K folding or inhibit its interaction with other unidentified interacting proteins. PIP5KβE61L had the same in vitro PIP5K activity as the wild-type enzyme after immunoprecipitation [GFP-PIP5K synthesised 3250 U PtdIns(4,5)P₂, whereas GFP-PIP5KβE61L synthesised 3600 U], suggesting that it was unlikely that the E61L mutation affected protein folding. To demonstrate that the reduced affinity for endogenous Rac was primarily responsible for the delocalisation of PIP5KβE61L, we overexpressed Rac, expecting that this might drive the interaction between Rac and PIP5KβE61L in vivo. Although PIP5KβE61L was cytosolic when overexpressed alone (Fig. 4G, left panel), co-expression with active Rac led to the localisation of PIP5KβE61L on the plasma membrane (Fig. 4G, right panels) and colocalisation with Rac.
Fig. 5. See next page for legend.
The interaction between PIP5Kβ and Rac is required to induce neurite retraction in N1E-115 cells

Unlike the overexpression of wild-type PIP5K, PIP5KβE61L did not induce neurite retraction in N1E-115 cells after serum starvation (Fig. 5A). PIP5KβE61L localised in the cytosol in N1E-115 cells (Fig. 5B, top panel), but translocated to the plasma membrane when co-expressed with wild-type Rac (Fig. 5B, bottom panels). Furthermore, PIP5KβE61L induced neurite retraction when co-expressed with wild-type Rac (Fig. 5B, bottom panels and quantification in Fig. 5C). These data show that Rac controls the plasma membrane localisation of PIP5K and that the interaction between Rac and PIP5K is required for neurite retraction. In HeLa cells, expression of PIP5K induces rounding and vacuolisation. Expression of PIP5KβE61L was cytosolic and did not induce these phenotypes. However, when co-expressed with wild-type Rac, PIP5KβE61L was present on the membrane and induced phenotypes that were similar to wild-type PIP5Kβ. PIP5KβE61L did not relocalise to the membrane nor did it induce rounding or vacuolisation when it was co-expressed with a mutant of Rac that does not interact with PIP5K (supplementary material Fig. S4). These data show that PIP5KβE61L localises in the cytosol because of reduced association with endogenous Rac. Peptide 1 and Glu61, which mediate the interaction between PIP5Kβ and Rac are both highly conserved in all PIP5K isoforms. The corresponding glutamate in the PIP5K isoforms (PIP5Kα, Glu117; PIP5Kβ, Glu61; PIP5Kγ, Glu111) was mutated to leucine and we analysed their ability to induce neurite retraction in N1E-115 cells. As expected, PIP5Kα, PIP5Kβ and PIP5Kγ all induced neurite retraction when overexpressed, whereas the corresponding glutamate mutants did not (Fig. 5D). The glutamate mutants in all PIP5K isoforms no longer localised at the plasma membrane whereas their wild-type counterparts did (data not shown).

To demonstrate the requirement for endogenous Rac in the localisation and activity of PIP5Kβ, we suppressed the expression of endogenous Rac and assessed PIP5K localisation and ability to induce neurite retraction. When GFP-PIP5K was coexpressed with an RNAi construct that targeted the expression of both Rac1 and Rac3 (Hajdo-Milasinovic et al., 2007), GFP-PIP5K plasma membrane staining was strongly reduced (Fig. 5E). The suppression of Rac also attenuated PIP5K-induced neurite retraction (Fig. 5E, bottom panels). Previous studies in N1E-115 cells showed that Rac3 was required for LPA-induced rounding and we found that specific suppression of Rac3 levels (Hajdo-Milasinovic et al., 2007), also attenuated neurite retraction induced by PIP5K (Fig. 5F). These data demonstrate that Rac is required to localise PIP5K at the membrane and that Rac is required for PIP5K-induced neurite retraction.

As PIP5KβE61L did not itself induce neurite retraction, we assessed its role in LPA-induced neurite retraction. In control cells, LPA stimulation triggered neurite retraction and cell rounding, which as previously demonstrated, was blocked by the overexpression of a kinase-inactive PIP5K (PIP5KKD) (Fig. 5G). Surprisingly, overexpression of the PIP5KKD (Fig. 5G) also attenuated LPA-induced neurite retraction. In vitro, PIP5KβE61L is active, and it is therefore surprising that it acts as a dominant negative. To further explore this, we assessed the levels of PtdIns(4,5)P2 in cells overexpressing either wild-type PIP5K or PIP5KβE61L. Surprisingly, we found that PIP5KβE61L elevated PtdIns(4,5)P2 levels to the same extent as wild-type PIP5Kβ in HeLa, HEK293 and N1E-115 cells (Fig. 5H). We hypothesised that uncoupling PIP5Kβ from Rac (PIP5KβE61L mutant) might lead to aberrantly localised synthesis of PtdIns(4,5)P2, which inhibits LPA-driven retraction. To test this concept, we introduced a point mutation into the PIP5KβE61L that inactivated its PIP5K activity (PIP5KβKDE61L). In accordance with our hypothesis, PIP5KβKDE61L no longer attenuated LPA-induced neurite retraction in N1E-115 cells (Fig. 5G, bottom panel). Together, these data suggest that the interaction between Rac and PIP5K is required to drive the localised synthesis of PtdIns(4,5)P2, which is required for neurite retraction.

**PIP5Kβ induced neurite retraction is dependent on the interaction between PtdIns(4,5)P2 and vinculin**

Neurite extension is facilitated by interactions with the extracellular matrix through the formation of focal contacts (Suter and Forscher, 1998; Suter and Forscher, 2000), which can be stabilised by recruitment of vinculin (Xu et al., 1998). Vinculin is maintained in an inactive conformation by the interaction of its globular head domain with its tail domain (Ziegler et al., 2006) and can be

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**Fig. 5. Characterisation of the effects of PIP5Kβ and PIP5KβE61L upon neurite remodelling.** (A) Unlike mRFP-PIP5Kβ, mRFP-PIP5KβE61L fails to induce neurite retraction in N1E-115 cells after serum starvation. After transfection with the constructs indicated, cells were serum starved for 24 hours and the number of cells with neurites were quantified. (B) N1E-115 cells transfected with either Myc-PIP5KβE61L alone (top) or with wild-type Rac (bottom). Cells expressing Rac were identified by the presence of GFP synthesised from an internal ribosomal entry site. The cells were fixed, stained with Myc antibody and analysed by confocal microscopy. Scale bar: 10 μm. The length of the neurites was quantified. (C) The mean neurite length of cells expressing PIP5KβE61L is significantly higher (P<0.005) than those coexpressing PIP5KβE61L and Rac. (D) N1E-115 cells transfected with GFP-labelled wild-type or glutamate mutants of PIP5K isoforms (α, β or γ). Cells were serum starved and the numbers of round cells were assessed and presented graphically. All wild-type isoforms of PIP5K induce neurite retraction and rounding, which is severely attenuated in the corresponding glutamate mutants. (E) N1E-115 cells co-transfected with a control siRNA (left) or an siRNA targeting the expression of Rac (right), with GFP-PIP5Kβ. Cells were fixed and GFP expression was analysed by confocal microscopy (top). A line was drawn across the cells as (indicated) and the mean pixel intensity was plotted (middle). The membrane to cytosol ratio was determined (bottom left) and demonstrated a loss of membrane staining upon suppression of endogenous Rac. Neurite length (bottom middle) or neurite numbers (bottom right) were also determined and demonstrated that suppression of endogenous Rac expression attenuated PIP5K-induced neurite retraction. (F) N1E-115 cells were transfected as shown and then serum starved overnight. The cells were fixed and the number of cells with neurites was assessed. The data show that GFP-PIP5K-induced neurite retraction requires the expression of endogenous Rac3. (G) PIP5KβE61L and PIP5KβE61L block LPA-induced neurite retraction in N1E-115 neuroblastoma cells. After transfection with the constructs shown, cells were serum starved for 3 hours to induce neurite outgrowth before stimulation with 0.5 μM LPA. Representative images are shown of fluorescence images (left) before LPA stimulation and phase-contrast images, before LPA stimulation and 15 minutes after LPA stimulation (middle and right, respectively). Scale bar: 20 μm. The data were quantified and shown in the graph below. In this experiment, an additional point in which a mutation has been introduced into GFP-PIP5KβE61L (H) HeLa (left), HEK293 (middle) and N1E-115 (right) cells were transfected with the constructs shown and were labelled with [32P]orthophosphate for 2 hours after which phosphoinositides were extracted and the levels of PtdIns(4,5)P2 were analysed and quantified. A duplicate transfection was used to determine the expression levels of GFP-PIP5K (bottom). Error bars represent s.e.m.
activated by numerous mechanisms, including its interaction with actin-binding proteins and acidic phospholipids such as PtdIns(4,5)P₂. Activation stimulates vinculin recruitment to focal adhesions (Chen et al., 2005; Chen et al., 2006). Interestingly, the role of PtdIns(4,5)P₂ in the activation of vinculin has recently been challenged, and PtdIns(4,5)P₂ also appears to stimulate the dissolution of vinculin from the focal adhesion complex (Chandrasekar et al., 2005; Saunders et al., 2006). This led us to investigate whether vinculin has a role in PIP5K-induced neurite retraction.

Expression of PIP5Kβ in HeLa cells led to a decrease in the average number of focal adhesions in a cell staining positive for vinculin. (Fig. 6A,B). In contrast to PIP5Kβ, expression of either PIP5KβKD or PIP5KβE61L did not disrupt vinculin staining in HeLa cells, nor did they change the average number of vinculin-positive structures per cell (Fig. 6A, quantified in Fig. 6B). It should be noted that endogenous vinculin levels were unaffected by expression of the wild type or mutant PIP5Kβ constructs. Western blot showing vinculin levels (top) and actin levels (bottom) in duplicate for each condition. (C) Endogenous vinculin levels are not altered by expression of the wild type or mutant PIP5Kβ constructs.

Expression of mRFP-PIP5KβKD or mRFP-PIP5KβE61L did not change the distribution of vinculin-positive structures compared with expression of mRFP. (D) N1E-115 cells transfected with the constructs indicated on the left and placed in serum-free conditions overnight to induce neurite outgrowth. Cells were left untreated (top) or incubated with 0.5 μM LPA for 15 minutes where indicated (+). Cells were fixed and stained for endogenous vinculin. Scale bar: 5 μm. (E) Neurites were imaged using confocal microscopy and the number of vinculin-positive focal-adhesion-like structures at neurite tips was determined. For each set of transfected cells, a total of 30 neurites were imaged and analysed. Error bars represent s.e.m.

Fig. 6. PIP5Kβ expression leads to the removal of vinculin from focal adhesions, whereas PIP5KβKD and PIP5KβE61L inhibit vinculin loss from focal adhesions during neurite retraction by LPA. (A) HeLa cells transfected overnight with the constructs indicated. Cells were fixed and stained using an anti-vinculin antibody and imaged by confocal microscopy. Red in the merge indicates the cells that are expressing the mRFP construct. Scale bar: 10 μm. (B) The number of vinculin-positive structures on the basal membrane of these cells was counted in a minimum of 35 cells and the average number + the s.e.m. is depicted, *P<0.000863 for distribution of the number of vinculin-positive structures in mRFP and mRFP-PIP5KWT. Western blot showing vinculin levels (top) and actin levels (bottom) in duplicate for each condition. (D) N1E-115 cells transfected with the constructs indicated on the left and placed in serum-free conditions overnight to induce neurite outgrowth. Cells were left untreated (top) or incubated with 0.5 μM LPA for 15 minutes where indicated (+). Cells were fixed and stained for endogenous vinculin. Scale bar: 5 μm. (E) Neurites were imaged using confocal microscopy and the number of vinculin-positive focal-adhesion-like structures at neurite tips was determined. For each set of transfected cells, a total of 30 neurites were imaged and analysed. Error bars represent s.e.m.
between PtdIns(4,5)P2 and vinculin, expression of VincLD significantly delayed neurite retraction induced by LPA treatment (Fig. 7B). These data suggest that the synthesis of PtdIns(4,5)P2, either in response to LPA stimulation or PIP5K overexpression, regulates vinculin interaction at focal contacts, which in turn affects contact stability.

**Discussion**

In N1E-115 cells, PIP5Kβ- or LPA-induced neurite retraction, requires active Rac to regulate the synthesis of PtdIns(4,5)P2. Increased PtdIns(4,5)P2 synthesis is sensed by the phosphoinositide-binding domain of vinculin, leading to less vinculin in focal contacts and ultimately to focal adhesion disassembly and neurite retraction. Our data are consistent with PtdIns(4,5)P2 synthesis driving neurite retraction in N1E-115 cells and in primary neurons. However, they are not consistent with any specific PIP5K isoform controlling neurite retraction. Rather, we suggest that PIP5K isoforms are regulated by specific inputs to control PIP5K-mediated neurite retraction. It should be noted that unlike previous data showing that overexpression of the talin-binding splice variant of PIP5Kγ, PIP5Kγ661, or its kinase-inactive counterpart can destabilise focal adhesions (Ling et al., 2002), PIP5Kβ-mediated loss of focal adhesions in N1E-115 cells is dependent on PtdIns(4,5)P2 synthesis.

PIP5K interacts with many low molecular weight G proteins, including Arf (Honda et al., 1999), Rho (Chong et al., 1994; Ren et al., 1996) and Rac (Tolias et al., 1998; Tolias et al., 2000). PIP5K-induced neurite retraction is not dependent on either Arf or Rho, but is dependent on active Rac. Rac interacts with PIP5K in a GTP-independent manner and the differential cellular localisation of activated Rac, compared with its inactive counterpart, might regulate the localisation and activity of PIP5K at the plasma membrane. In our study, dominant-negative Rac (RacN17) attenuated PIP5Kβ-induced neurite retraction, although surprisingly, it did not prevent PIP5K localisation at the membrane. However, immunostaining revealed that RacN17 was localised at the plasma membrane. RacN17 also interacts with PIP5K and this is probably...
responsible for maintaining PIP5K at the membrane. The fact that RacN17 attenuates PIP5K-induced neurite retraction suggests either that active Rac is required for PIP5K mediated PtdIns(4,5)P2 synthesis as well as regulating PIP5K localisation, or that active Rac is also required for neurite retraction downstream of PIP5K.

We have identified a point mutant of PIP5Kβ (PIP5KβE61) that has decreased affinity for Rac. PIP5KβE61 is catalytically active, predominantly cytosolic and unlike its wild-type counterpart, does not induce neurite retraction upon overexpression. The use of PIP5KβE61 to study the role of Rac in regulating PIP5Ks gives clearer and more interpretable results than either suppression of Rac expression or the dominant-negative versions of Rac, which both can inhibit or activate many other upstream and downstream Rac targets. Importantly, we show that co-overexpression of PIP5KβE61 with wild-type Rac targeted PIP5KβE61 back to the membrane and induced neurite retraction. These data are consistent with a decrease in the affinity for Rac being the primary defect in PIP5KβE61 localisation. All PIP5Ks tested interact with Rac in a GTP-independent manner and all have the conserved glutamate residue. Expression of all three isoforms of PIP5Ks induced neurite retraction in N1E-115 cells and mutation of the equivalent Glu61 residue to leucine blocked PIP5K-induced neurite retraction (Fig. 5D) and led to their localisation in the cytosol (data not shown). These data suggest that the regulation of PIP5K localisation by Rac is conserved in PIP5K isoforms. It also suggests that mutation of Glu61, or its equivalent residue in PIP5Ks, will be a useful tool in determining the role of Rac in PIP5K-regulated processes. PIP5Ks phosphorylate PtdIns4P at the plasma membrane, and in many lipid kinases, the ability to interact with the membrane is a key regulatory step in controlling their activity. Interaction with PtdIns4P mediates PIP5K membrane interaction (Kunz et al., 2000), as does an electrostatic interaction with the negative charge of the inner surface of the plasma membrane (Fain et al., 2009). Our data show that interaction with Rac also controls membrane localisation of PIP5K. How Rac regulates membrane localisation of PIP5K is not completely clear. It might purely be a consequence of Rac tethering PIP5K at the membrane, or the interaction with Rac might lead to post-translational modifications of PIP5K, which ultimately control localisation. How these various mechanisms control localisation at the membrane are co-ordinated to regulate PtdIns(4,5)P2 synthesis is not clear.

Suppression of Rac expression also leads to cytosolic localisation of GFP-PIP5K and reduced neurite retraction. Of the three isoforms of Rac, Rac1 is ubiquitously expressed, whereas Rac2 is limited to haematopoietic cells and Rac3 is expressed in the brain. Although Rac1 and Rac3, share 92% overall homology and have identical switch loop regions, they do have antagonistic functions when overexpressed or silenced in N1E-115 cells (Hajdo-Milasinovic et al., 2007). Rac1 overexpression induces neurite outgrowth, whereas Rac3 expression induces cell rounding. Interestingly, Rac3 and PIP5K-mediated neurite retraction show similar characteristics: they are independent of Rho signalling; they do not require myosin light chain kinase activity and they are dependent on the loss of adhesion complexes (Hajdo-Milasinovic et al., 2007; Hajdo-Milasinovic et al., 2009). Furthermore, both PIP5K and Rac3 modulate LPA-induced retraction. These data suggest that Rac3 and PIP5K work in the same pathway. Indeed, overexpression of a kinase-inactive PIP5K attenuates Rac3-induced neurite retraction (our unpublished data). The induction of neurite retraction by Rac3 is determined by the polybasic region (PBR) in the C-terminus of the protein, which is different to the PBR in Rac1. As the PBR mediates the interaction with PIP5K, we assessed the interaction of Rac3 with PIP5K and demonstrated that Rac3 interacts better than Rac1 with PIP5Kβ (supplementary material Fig. S5).

Do PIP5Ks and PtdIns(4,5)P2 synthesis have a physiological role in the determination of neurite dynamics in vivo? PIP5Kα (Sasaki et al., 2005) or PIP5Kβ (Wang et al., 2008) knockout mice do not show an overt neuronal phenotype, whereas PIP5Kγ knockout mice are embryonic lethal. However, our data in N1E-115 cells suggest that the level of PtdIns(4,5)P2 is important in controlling neurite outgrowth. In HeLa cells, knockdown of any one single isoform does not lead to a significant decrease in PtdIns(4,5)P2 levels, and knockdown of PIP5Kγ, which is highly expressed isoform in the brain, decreases PIP5K activity in an embryonic brain lysate by only 50% (Wang et al., 2008). These data suggest that double or triple knockouts of the PIP5K isoforms might be required to reveal a neuronal phenotype. In this study, to demonstrate physiological importance, we show that overexpression of PIP5K in primary cerebellar granular neurones also induces neurite retraction, which is similar to a previous study showing that axonal extension of hippocampal neurones is attenuated by PIP5K overexpression (Hernandez-Deviez et al., 2004). Furthermore, we show that siRNA-mediated suppression of PIP5K, or the overexpression of a kinase-inactive PIP5K, leads to neurite induction in N1E-115 cells, implicating the importance of local levels of PtdIns(4,5)P2. Previous studies have also shown that neuronal outgrowth, in response to guidance cues such as brain-derived growth factor and Netrin-1, is dependent on phospholipase-C-mediated PtdIns(4,5)P2 hydrolysis (Xie et al., 2005). How decreases in PtdIns(4,5)P2 lead to neurite extension is not clear. A reduction in PtdIns(4,5)P2 could lead to a decrease in membrane tension (Niebuhr et al., 2002), which might enable cytoskeletal invasion and the initiation of neurite outgrowth.

PIP5K-mediated retraction can be attenuated by overexpression of a mutant vinculin that is unable to interact with PtdIns(4,5)P2. Furthermore, LPA-mediated loss of focal contacts and retraction are attenuated by overexpression of a kinase-inactive PIP5K. In contrast to previous results (Gilmore and Burridge, 1996), our data suggest that enhanced PtdIns(4,5)P2 synthesis leads to vinculin dissolution from focal adhesions and stimulates focal adhesion turnover. How might increased PtdIns(4,5)P2 stimulate the loss of focal adhesions? The interaction site between PtdIns(4,5)P2 and vinculin overlaps with the interaction site between actin and vinculin, and PtdIns(4,5)P2 can inhibit the actin–vinculin interaction (Steimle et al., 1999). The activation of vinculin and its localisation at focal adhesions depends on its interaction with both actin and talin (Chen et al., 2006). Increased PtdIns(4,5)P2 synthesis at focal adhesions might decrease the interaction of vinculin with the actin cytoskeleton and thereby favour vinculin inactivation, leading to its dissolution from focal adhesions. The role of vinculin in stabilising focal adhesions is well established. Focal adhesions in vinculin-knockout mouse embryonic fibroblasts are less stable and are generally smaller (Xu et al., 1998). Overexpression of activating mutants of vinculin stimulates vinculin interaction with focal adhesions and enhances the number and size of focal adhesions (Humphries et al., 2007). Furthermore, and relevant to this study, embryos derived from vinculin-knockout mice show poorly developed neuronal circuitry, and suppression of vinculin expression in PC12 cells leads to fewer extended neurites, filopodia and lamellipodia (Varnum-Finney and Reichardt, 1994).

At the rear of a migrating cell, the disassembly of focal adhesions is required for the release of the cell from the extracellular matrix.
to enable directional migration. We suggest that activation of PIP5K and increased local synthesis of PtdIns(4,5)P_2 at the trailing edge of a migrating cell regulates vinculin interactions, to decrease focal adhesion stability. Interestingly, a recent study has demonstrated that PIP5K is localised to the uropod and that PtdIns(4,5)P_2 synthesis is required for uropod retraction during neutrophil retraction (Lokuta et al., 2007).

Materials and Methods

**Chemicals**

GAP-43, NeuN, and GFAP antibodies were purchased from Chemicon and DMEM medium and supplements were obtained from Gibco (Karlsruhe, Germany).

**Cell lines and cultures, protein preparation and immunoblotting**

N1E-115 cells were cultured in DMEM (Gibco) medium containing 10% FCS (Biochrom, Berlin, Germany) and antibiotics (Gibco) in a humidified atmosphere at 37°C and 5% CO2. Cells were transfected using FuGene6 (Roche) according to the manufacturer’s protocol. For total protein extraction, cell pellets were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride with the inclusion of a protease inhibitor cocktail (Roche). After centrifugation at 15,000 g for 10 minutes at 4°C, 20 μg of the supernatant was subjected to SDS-polyacrylamide gel electrophoresis, electroblotted onto a nitrocellulose membrane and immunostained as indicated in the results. Equal loading amounts of the probes were estimated using immunostaining with anti-mouse β-tubulin or actin monoclonal antibodies (Sigma).

**Dissociated cerebellar granule neuron cultures**

The whole cerebellum was isolated from postnatal FVB mice (P2–P4) and meninges were carefully removed. Tissues were transferred to culture medium [Neurobasal medium supplemented with 30 nM sodium selenite (Sigma), B27 (Gibco), penicillin and streptomycin] and gently triturated with a fire-polished Pasteur pipette and trypsinised for 5 minutes at 37°C. Cells were seeded on glass coverslips pre-coated with laminin and poly-L-lysine (20 μg/ml and 0.2 mg/ml, respectively) with a density of 200,000 cm² and cultured at 37°C under 5% CO2. Cytochrome arabinofuranoside (10 μM, Sigma) was added after 16 hours to inhibit replication of non-neuronal cells. FuGene6 was used for transfection. Cultured cells were fixed with 4% parafomaldehyde, stained for GAP-43 (Chemicon) and processed for confocal microscopy.

**Statistical significance**

Data from all experiments were obtained from at least three independent experiments. Data from immunoblots shown in the figures are selected representatives from performed experiments. Data analysis was performed using the Mann–Whitney test or, as described (StatviewII, Abacus). The level of significance was set at *P<0.05, **P<0.01, ***P<0.001. Error bars represent s.d.

**Metabolic labelling with [32P]orthophosphate**

Transfected or non-transfected cells were orthophosphate labelled as previously described (Halstead et al., 2001). After extraction, phospholipids were treated with monoethylamine and the deacylated products were analysed using PEI–cellulose thin-layer chromatography (Humphries et al., 2007). Vinculin controls focal adhesion formation by direct interactions with talin and α-actinin. We would also like to thank all the members of the Science Program Organization (HFSPO).

**Rac regulates synthesis of PtdIns(4,5)P_2**

Lysates were incubated with appropriate antibodies overnight and were captured using Protein-G Sepharose (1 hour 4°C). Immunoprecipitates were washed three times in the above buffer and then resuspended in SDS loading buffer, heated to 73°C for 10 minutes and separated by SDS–PAGE (Invitrogen pre-cast gels). The separated proteins were transferred to nitrocellulose and then immunoblotted using the appropriate antibodies. Detection was carried out with either ECL or SuperSignal (Pierce).

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