Mechanism of polarized protrusion formation on neuronal precursors migrating in the developing chicken cerebellum

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Summary

Directed cell migration results from the polarization of the cellular motile apparatus by integration of extracellular signals, which are presented in a three-dimensional, spatio-temporal manner in living organisms. To investigate the mechanism underlying the highly polarized and directional nature of migration in vivo, we have developed an imaging system for observing rhombic lip cell migration in the developing chicken cerebellum. First, we show that Cdc42 is the central regulator of the overall polarity, morphology and protrusion formation in these cells. However, perturbation of canonical polarity effectors of Cdc42, e.g. the Par6-Par3-aPKC complex, does not disrupt the cell asymmetry, whereas it affects orientation of the tip of the leading process. In contrast to Cdc42, Rac is required for the generation of protrusions but not the overall polarity. Function interference of class IA phosphoinositide 3-kinase abrogates both directional extension and maintenance of the long leading process, whereas PTEN modulates the size of the protrusion. Actomyosin contractility is important for coordinated spreading of the tip of the leading process in situ. Finally, ErbB4 functions in the generation of protrusions on the rhombic lip cells. These results suggest that polarized protrusion formation on neuronal precursors may occur by a more divergent and complex mechanism than that seen in studies of other cell types growing on planar substrates.

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Key words: Cell migration, Polarity, Rho GTPases, Phosphoinositide-3 kinase, PAK, Actomyosin

Introduction

Directed cell migration, an essential process in the development of multicellular organisms, relies on the integration of extracellular signals from the three-dimensional environment in which the cells reside (Gilbert, 2003; Ridley et al., 2003). Presentation of these migration cues, which comprise soluble factors, cell-cell and cell-matrix interactions, changes spatially and temporally during development. Although little is known about the cellular mechanisms that drive and regulate directed migration in living tissues, studies using cells in culture have revealed that it involves the integration of several steps including: cell polarization, formation of protrusions and adhesions at the cell front, and disassembly of adhesions and retraction at the rear (Ridley et al., 2003; Lauffenburger and Horwitz, 1996).

Cell polarity is intrinsic to all of these steps. From cell culture studies, it appears that a polarized cell shape results from local cell-surface-activated signals that produce a polarized positioning of microtubules through a Cdc42-mediated pathway. In addition, cells can generate a protrusion in the direction of a chemotactic gradient through the local generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3) and activation of Rac at the cell front (Devreotes and Janetopoulos, 2003; Fukata et al., 2003; Merlot and Firtel, 2003; Etienne-Manneville, 2004). Although the mechanisms by which Cdc42 generates cell polarity are only beginning to emerge, a number of observations point to the importance of a complex that includes Par6, Par3 and aPKC as well as IQGAP1. These intermediates are thought to regulate microtubule organization via the microtubule binding proteins, APC, EB1/3 and CLIP-170. One model envisages creation of a directionally polarized cell shape through the orientation of the MTOC by microtubule plus-end capturing at the cell front (Fukata et al., 2002; Etienne-Manneville and Hall, 2003; Fukata et al., 2003; Etienne-Manneville, 2004; Watanabe et al., 2004; Galjart, 2005).

Recent studies of *Dictyostelium* and neutrophil chemotaxis have revealed that phosphoinositide 3-kinase (PI3K) is an early, crucial intermediate in gradient sensing which catalyzes the localized production of PIP_3 at the leading edge in response to extracellular signals (Funamoto et al., 2001; Wang et al., 2002; Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003). The resulting PIP_3 gradient can be enhanced by the polarized distribution of the PIP_3 phosphatase, PTEN, along the sides of the cell (Iijima and Devreotes, 2002). The high concentration of PIP_3 recruits downstream effectors with PH domains to produce localized protrusive activity through activation of Rac and PAK (Hawkins et al., 1995; Sells at al., 2000; Papakonstanti and Stournaras, 2002; Srinivasan et al., 2003; Welch et al., 2003).

Neuronal migrations that mediate the development of the vertebrate nervous system are particularly attractive for

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studying polarity and directed cell migration in vivo (Hatten, 2002). The migration of neuronal precursors tends to be robust, and the cells often show a single, very long, dominant protrusion in the direction of migration. During the development of chicken embryonic cerebellum, for example, the marginal region called the rhombic lip produces a subset of neuronal precursors in a temporally regulated manner (Wingate, 2001). These cells display a highly polarized leading protrusion and execute robust directional migration to their destination (Wingate and Hatten, 1999; Gilthorpe et al., 2002). These characteristics make them easy to image and thus analyze their polarized migration in living tissues.

To study the polarity of these neuronal precursors, we have developed a novel imaging system for observing their polarity and directed migration in the developing chicken cerebellum. The combination of electroporation-based gene transfer with an organotypic explant culture enabled us not only to image the dynamics of GFP-labeled cells, but also to ectopically express gene products in these cells. With this system, we elucidated the signaling responses that lead to the highly polarized and directional rhombic lip cell migration. We show a pivotal role for Cdc42 in formation of the polarized leading process in vivo, whereas perturbation of individual polarity effectors, e.g. the Par6-Par3-aPKC complex, affects only the orientation of the protrusion at the tip of the leading process (TLP). We also show that Rac is required for generation of protrusion at the TLP. Class IA PI3K controls both directional extension and maintenance of the long leading process, and PTEN modulates the magnitude of protrusion. Actomyosin contractility and its regulators, e.g. PAK, Rho, and Rho-kinase, are involved in spreading of the TLP. Finally, we show that the receptor tyrosine kinase (RTK) ErbB4 plays an essential role in generation of protrusions.

Results

Imaging the migration of rhombic-lip-derived neuronal precursors in situ

To investigate the molecular mechanisms underlying directed migration in situ, we developed a method for imaging the migration of cerebellar rhombic-lip-derived neuronal precursors in chicken embryos. This system has the following advantages: (1) the cells migrate highly directionally; (2) the major migration path penetrates in the sub-pial space, thus facilitating imaging in the embryonic tissue; (3) the embryos are easy to manipulate. Our system uses electroporation-based gene transfer for ectopic gene expression in the rhombic lip cells of organotypic cerebellar explants prepared from stage 28 chicken embryos (Fig. 1A,B). We used EGFP to visualize the electroporated cells. By using the neuron-specific α-tubulin promoter, ectopic gene expression was restricted to the neuronal lineage and the background from other cell types reduced (Gloster et al., 1999; Köster and Fraser, 2001). Fluorescent images of the EGFP-visualized migrating neuronal precursors were acquired by optical penetration into the organotypic explant tissue, culture insert, and dish (Fig. 1C). After incubation for 24 hours, most EGFP-positive cells had a long leading process that was elongated toward the ventral midline (Fig. 1D,E). Translocation of the cell soma followed

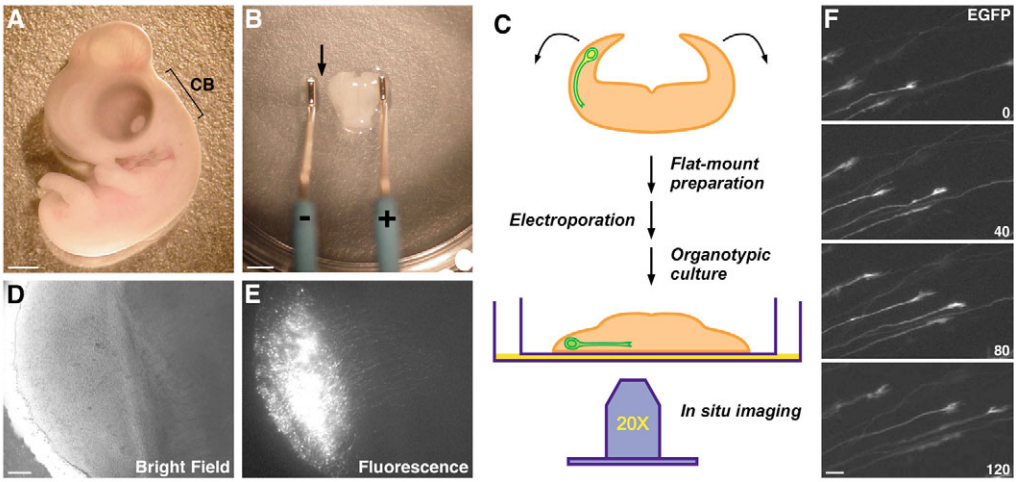

Fig. 1. In situ imaging of chicken embryonic neuronal migration. (A) Image of stage 28 chicken embryo. Organotypic explants were prepared from embryonic cerebellum (CB). (B) Experimental design for electroporation. A cerebellar explant is placed on the culture insert membrane with its pial surface facing the membrane insert. In this photograph, rostral is on top. The cathode (–) was placed near the left side of the cerebellar rhombic lip. The anode (+) was touched to the right side of the rhombic lip. 2 μl of plasmid solution were applied between the cathode and rhombic lip (arrow), followed by electroporation five times using 30 V pulses for 50 milliseconds at 100 millisecond intervals. (C) Schematic view of in situ imaging of neuronal precursors migrating within the organotypic cerebellar explant. An open-book style flat-mount explant was prepared from stage 28 chicken embryonic cerebellum; cross section images of the cerebellar tissue and explant are shown (top and bottom). The explant was electroporated as shown in Fig. 1B. Fluorescent images of GFP-expressing cells were acquired from the pial-side of the explant through the culture insert and dish by using a 20× long-working-distance objective lens (bottom). (D-E) In situ imaging of cultured cerebellar explants. Bright field (D) and EGFP fluorescence (E) images of 24-hour cerebellar explant cultures are shown. In these micrographs, the left margin of the explant is the rhombic lip, the ventral midline is along the right end of the micrographs, and rostral is to the top. Neuron-specific α-tubulin promoter-driven EGFP fluorescence is detected around the electroporated rhombic lip. Rhombic-lip-derived EGFP-positive cells have an elongated, single long leading process directed toward the ventral midline. (F) Fluorescent time-lapse imaging of leading protrusions elongating from rhombic-lip-derived neuronal precursors expressing EGFP. Fluorescent images were acquired every 2 minutes for 2 hours (see supplementary material Movie 1). Images at 0, 40, 80, 120 minutes are shown. In these micrographs, the rhombic lip is to the left, the ventral midline is to the right, and rostral is at the top. The same orientation is used in all the following micrographs and movies. The leading protrusions from EGFP-expressing cells show highly directed extension toward the ventral midline. Bar, 2 mm (A,B); 200 μm (D,E); 10 μm (F).

Table 1. Characteristics of leading processes

Treatment	Extension speed (μm/hour)†	Morphological change
EGFP	29.8±10.8 (*n*=30)	
N17-Cdc42	NA	Defective formation of polarized process
Par6-NT	18.9±10.1** (*n*=26)	TLP branching
S9A-GSK	16.4±12.6*** (*n*=26)	TLP branching
APCΔCT	21.2±13.8* (*n*=30)	TLP branching
Nocodazole	NA	Fragmentation of leading process
EB3-GFP	NA	Random extension of leading process
N17-Rac	1.64±7.86*** (*n*=28)	Suppressed protrusive activity
Rac	9.99±8.26*** (*n*=16)	Multiply branched protrusive structure on TLP
Δp85α PI3K	NA	Random migration and loss of long leading process
KD-p110γ PI3K	28.4±14.0 (*n*=28)	Reduced size of TLP
PTEN	26.6±11.6 (*n*=30)	Reduced size of TLP
C124A-PTEN	18.3±12.5** (*n*=26)	Increased size of TLP
KD-PAK	29.8±9.82 (*n*=26)	Branching and destabilization of TLP
PAK	12.0±9.05*** (*n*=26)	Enhanced spreading of TLP
Blebbistatin	10.6±12.2*** (*n*=30)	Reduced spreading of TLP
C3 toxin	11.4±15.3*** (*n*=30)	Reduced spreading of TLP
DN-Rho-kinase	17.8±11.6** (*n*=30)	Reduced spreading of TLP
AG1478	7.74±12.0*** (*n*=29)	Suppressed protrusive activity
ErbB4ΔIC	7.17±17.4*** (*n*=28)	Suppressed protrusive activity

†Mean values obtained from three to four independent experiments are shown ± s.d. Asterisks indicate the value with a statistically significance change over control EGFP cells determined by Student's *t*-test: *P<0.05, **P<0.01, ***P<0.001. NA, not applicable because of their loss of directed extension.

extension of the long leading process. These features are consistent with previous observations on DiI-labeled cerebellar rhombic lip cells (Wingate and Hatten, 1999; Gilthorpe et al., 2002).

To observe the dynamics of the TLP on these cells, we performed fluorescent in situ time-lapse imaging of the EGFP-expressing cells. At the terminus of extending processes, active generation and retraction of thin microspikes and lamella were observed (Fig. 1F; supplementary material Movie 1). These protrusive structures formed primarily near the TLP and little protrusive activity was observed at the lateral side of the processes. The average extension rate for leading process was ~30 μm/hour (Table 1). Translocation of the soma followed extension of the long leading process (supplementary material Movie 2). The highly polarized and directional nature of the migration provided an in situ assay for determining the roles of signaling components that are thought to function in cell polarization and directed protrusion formation (Fig. 2).

The role of Cdc42 signaling and microtubules in polarization of the leading process on migrating neuronal precursor in vivo

Cdc42 regulates cell polarization in some types via the Par6-Par3-aPKC complex, GSK3β, APC, EB1/3, IQGAP1 and CLIP-170 (Fukata et al., 2003; Etienne-Manneville, 2004). To determine the role of this network on the polarity of neuronal precursors in situ, we first studied Cdc42. We co-expressed a dominant-negative mutant, N17-Cdc42, which has been shown to interfere with the MTOC reorientation and directed migration of astrocytes (Etienne-Manneville and Hall, 2001), with EGFP at a 5:1 ratio to visualize the N17-Cdc42-expressing cells. We observed a bipolar, fusiform morphology for most of the cells, whereas some others were round. The morphology arose from a dramatic retraction of the major process on the N17-Cdc42-expressing cells (Fig. 3A; supplementary material Movie 3) and inhibition of protrusion formation; the round cells did not move.

Par6 is a component of the Par6-Par3-aPKC complex downstream of Cdc42. It interacts with aPKC, GTP-bound Cdc42 and Par3 through its N-terminus, CRIB and PDZ domains, respectively (Lin et al., 2000). The N-terminal portion of Par6 (Par6-NT) functions as a dominant-negative mutant by forming a defective complex with aPKC; its expression in migrating astrocytes perturbs the orientation of the protrusion and MTOC while showing polarized protrusion (Etienne-Manneville and Hall, 2001). In striking contrast with the suppressive effect of N17-Cdc42 on maintenance of monopolar process (Fig. 3A), Par6-NT expression in migrating neuronal precursors did not perturb the formation of the leading process. Instead, it produced an aberrant branching morphology of TLP (Fig. 3B; supplementary material Movie 4) and inhibited the extension of the leading process (Table 1). This phenotype is consistent with the in vitro observation that Par6-NT affected the orientation of protrusion but not the polarized shape of migrating astrocytes.

GSK3β is a target of the Par6-Par3-aPKC complex in astrocytes via its inactivation by phosphorylation on Ser9 (Etienne-Manneville and Hall, 2003). We assayed the role of GSK3β on polarity using a constitutively active mutant of GSK3β (S9A-GSK). Like Par6-NT, this mutant also induced branching of TLP and inhibited efficient extension of the leading process, although its effect on branching was weaker than that of Par6-NT (Fig. 3C; Table 1). Localized inactivation of GSK3β is thought to alter the microtubule-binding property of APC, which is essential for controlling the orientation of the protrusion and MTOC in astrocytes (Etienne-Manneville and Hall, 2003; Zumbrunn et al., 2000). Therefore, we expressed a microtubule-binding-deficient mutant of APC (APCΔCT). Cells expressing this mutant showed a branched TLP and inhibited process extension (Fig. 3D; Table 1).

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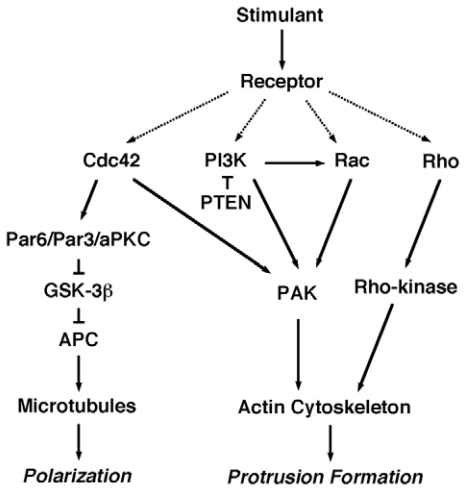

Fig. 2. Simplified network of putative signaling intermediates that regulate cell polarization and protrusion formation in directionally migrating cells. Regardless of the receptor type, localized activation of receptor by stimulant is thought to elicit subsets of signaling events similar to those depicted here and regulate cell polarization and directed protrusion formation.

To further evaluate the role of microtubules in directed migration of neuronal precursors, we first treated the explant with the microtubule-depolymerizing drug nocodazole (5 μM). Disruption of microtubules by nocodazole caused fragmentation of leading processes (Fig. 3E; supplementary material Movie 5), showing that microtubule integrity is essential for the integrity of the thin, long leading process. To assess the function of the microtubule plus-end in directional extension of the leading process, we overexpressed EB3-GFP, an APC-interacting microtubule plus-end binding protein. Although the overexpression lead to a large background of non-localized EB3-GFP-derived fluorescence that was not obviously associated with plus-ends, these cells displayed non-directional, random extension of the leading process (Fig. 2F; supplementary material Movie 6). By contrast, expression of EGFP-tubulin did not affect the direction of extension of the leading process (data not shown). Taken together, these observations support a notion that the directional extension of leading process involves the function of microtubules and their plus end.

Rac generates localized protrusions at the tip of the leading process

We next investigated the mechanism by which localized protrusions form on the migrating neuronal precursors. In vitro studies on fibroblasts and other cell types implicate localized Rac activation in the formation of lamellar protrusions (Ridley et al., 1992; Itoh et al., 2002). However, less is known about the requirement for Rac in the generation of protrusions in vivo and on these highly polarized cells. Therefore, we expressed a function-interfering mutant of Rac, N17-Rac, which inhibits Rac-mediated protrusion in vitro (Ridley et al., 1992). The function-interfering mutant suppressed protrusive activity and impaired extension of the leading process of the rhombic lip neuronal precursors (Fig. 4A; supplementary material Movie 7; Table 1). In clear contrast to the dramatic effect of N17-Cdc42 on cell polarization in these cells (Fig. 3A), N17-Rac did not alter their overall polarity. This suggests that Rac is required for the generation of protrusions at the TLP but not for the generation or maintenance of the monopolar morphology. Overexpression of wild-type Rac produced a large, multiply branched protrusion composed of unstable, thin spikes at the TLP and inhibited the extension of the leading process (Fig. 4B; supplementary material Movie 8; Table 1); however, the overall monopolar morphology was not altered.

PI3K regulation of directed protrusion formation

The formation of a localized PIP_3 gradient by PI3K is a key mechanism in generating protrusions at the front of some cell types in vitro (Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003). Therefore, we assayed the effect of PI3K mutants on the directed migration of rhombic lip neuronal precursors in situ. Two classes of PI3K, class IA and IB PI3Ks, function downstream of activated receptor tyrosine kinases (RTKs) or G protein coupled receptors (GPCRs), respectively. To interfere with the signaling through class IA PI3Ks, we used a deletion mutant of the adaptor subunit, Δp85α, which does not associate with any of the three types of catalytic subunits but interacts with activated RTKs and other signaling molecules via its SH2 and SH3 domains. Expression of Δp85α promoted frequent changes in the direction of migration and caused a drastic shortening of the leading process on rhombic-lip-derived neuronal precursors (Fig. 5A; supplementary material Movie 9). Importantly, the non-directional migration induced by Δp85α was different from non-directional extension of the leading process on EB3-GFP-overexpressing cells, which still had a long leading process. We next assayed the effect of a kinase-dead mutant of class IB PI3K, K799R p110γ (KD-p110γ) on these cells. In contrast to Δp85α, expression of KD-p110γ did not affect the directional extension of the leading process although it reduced the size of the TLP (Fig. 5B; supplementary material Movie 10). Likewise, expression of pertussis toxin in these cells did not alter the direction of the leading process (data not shown). These results suggest that the signaling cascade through RTK-class IA PI3K but not GPCR-class IB PI3K participates in directional extension and formation of the long leading process in these cells.

PTEN, a PIP_3 phosphatase, can act reciprocally with PI3K in regulating directional migration. In *Dictyostelium*, its exclusion from the cell front enhances the PIP_3 gradient produced by local activation of PI3K (Iijima and Devreotes, 2002). However, in chemotactic leukocytes, PTEN is distributed homogenously (Lacalle et al., 2004). These observations suggest that the role of PTEN in directed migration is dependent on the cell type or context. To investigate its role in neuronal precursors, we overexpressed wild-type PTEN and its phosphatase-deficient mutant, C124A-PTEN (Tamura et al., 1998). PTEN overexpression primarily reduced the size of TLPs with little effect on their directional extension (Fig. 5C). By contrast, the phosphatase-dead C124A-PTEN enlarged the TLP and significantly inhibited extension of the leading process (Fig. 5D; supplementary material Movie 11; Table 1). These results indicate a function of PTEN in modulating the protrusive activity in the TLP of migrating neuronal precursors. Intriguingly, it has been reported that *PTEN* conditional mutant mice under control of *engrailed 2 cre* exhibit a dramatic migration defect in many cell types in

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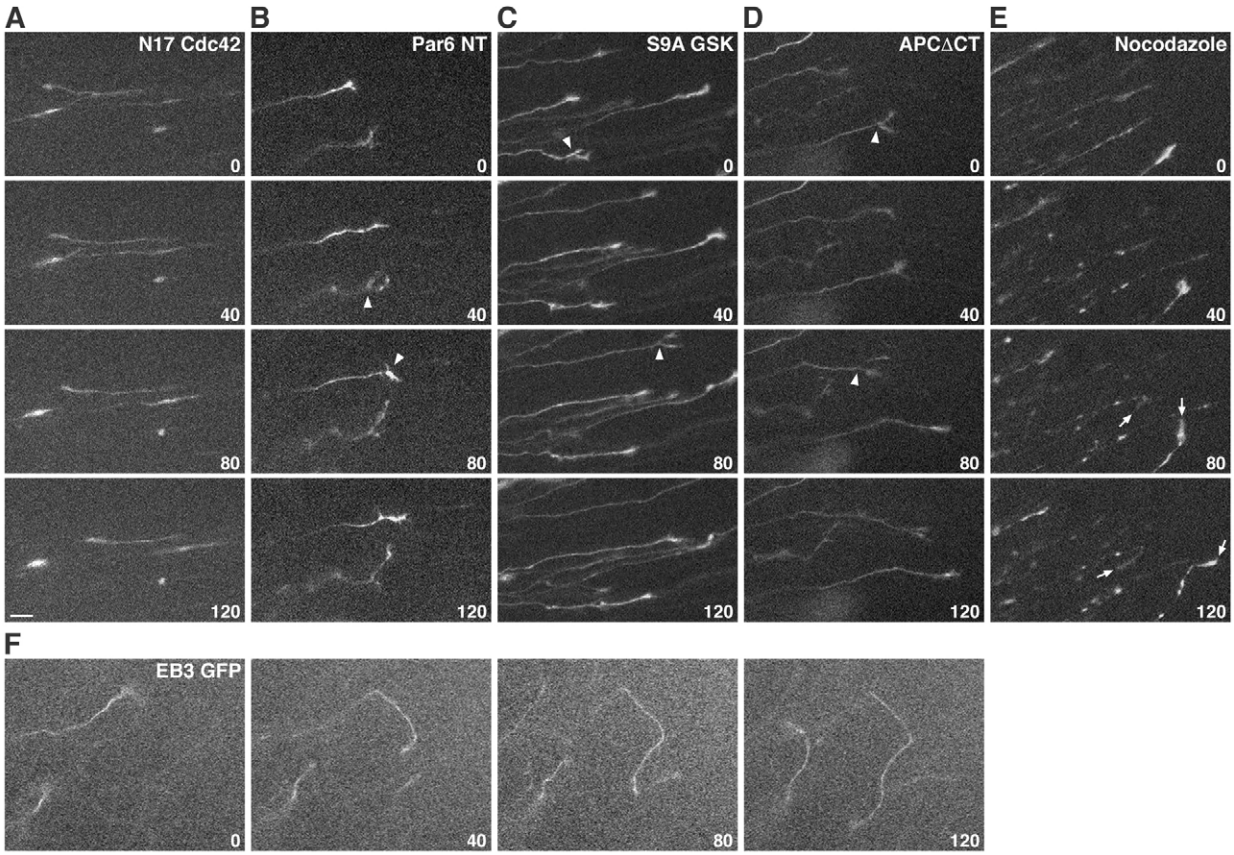

Fig. 3. Functional analysis of Cdc42 signaling and microtubules in polarized protrusion formation of directionally migrating neuronal precursors. (A) Dominant-negative mutant of Cdc42 (N17-Cdc42) was co-expressed with EGFP in rhombic-lip-derived neuronal precursors. EGFP fluorescent images were captured every 2 minutes for 2 hours (see supplementary material Movie 3), and images from 0, 40, 80, 120 minutes time points are displayed. N17-Cdc42-expressing cells showed a bipolar morphology and some were rounded up. (B) Function-interfering mutant of Par6 (Par6-NT) altered orientation of TLPs and frequently induced branching (arrowheads) (see supplementary material Movie 4). (C) Neuronal precursors expressing a constitutively active mutant of GSK3β (S9A-GSK) showed a branching phenotype that was somewhat weaker than that seen in Par6-NT-expressing cells (arrowheads). (D) Microtubule binding-deficient mutant of APC (APCΔCT) produced branched TLPs (arrowheads). (E) Cerebellar explants electroporated with EGFP were pre-cultured for 24 hours before treatment with 5 μM nocodazole (microtubule-depolymerization drug), for 15 minutes, followed by in situ fluorescent imaging. The same drug-containing medium was used during the time-lapse imaging. Disruption of microtubule structure by nocodazole induced fragmentation of leading processes (see supplementary material Movie 5). In some cases, altered orientation of TLPs were observed (arrows). (F) Fluorescent images of leading processes visualized by microtubule plus-end binding protein EB3-GFP. EB3-GFP showed a broad localization that increased toward the TLPs. These processes displayed non-directional extension of the polarized process (see supplementary material Movie 6). Bar, 10 μm.

the developing cerebellum (Marino et al., 2002). Taken together, PTEN modulation of protrusive activity on migrating cells might be important for proper execution of developmental cell migration in vivo.

Actomyosin contractility regulates the spreading of protrusions at the tip of the leading process

PAK is an effector of Rac and Cdc42 as well as downstream of PI3K (Manser et al., 1994; Sells at al., 2000; Papakonstanti and Stournaras, 2002; Bokoch, 2003). Interestingly, it can either promote or suppress actomyosin contractility via direct phosphorylation of myosin light chain or the inactivation of myosin light chain kinase by its phosphorylation (Ramos et al., 1997; Chew et al., 1998; Sanders et al., 1999). LIM-kinase-mediated modulation of the actin severing and depolymerizing function of ADF/cofilin is also downstream of PAK (Edwards et al., 1999; Blanchoin et al., 2000). Thus, it has multiple, potential effects on cell migration (Sells et al., 1999). When a kinase-dead mutant of PAK (KD-PAK) was expressed in the neuronal precursors in situ, coordinated spreading of TLP was disturbed, and frequent branching of TLP was observed (Fig. 6A; supplementary material Movie 12). However there was no detectable effect on the rate of extension of the leading process (Table 1). These results suggest that the kinase activity of PAK is important for stabilization of protrusion direction but not for the extension of the process itself. By contrast, overexpression of wild-type PAK produced a large, relatively stable, TLP and inhibited extension of the process (Fig. 6B; supplementary material Movie 13; Table 1).

Blebbistatin is an inhibitor of the myosin II ATPase, which is a target of PAK (Straight et al., 2003). Neuronal precursors treated with 100 μM blebbistatin for 15 minutes showed a less spread shape of the TLP (Fig. 6C; supplementary material Movie 14). When compared with the reduced size of

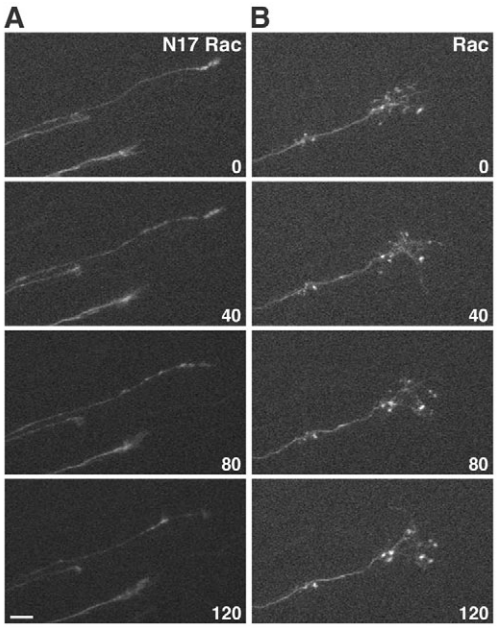

Fig. 4. Rac is required for generation of the protrusion on the tip of the leading process. (A) Dominant-negative mutant of Rac (N17-Rac) was co-expressed with EGFP in cerebellar rhombic-lip-derived neuronal precursors. EGFP fluorescent images were captured every 2 minutes for 2 hours (see supplementary material Movie 7), and images at 0, 40, 80, 120 minutes are shown. N17-Rac suppressed the formation of the protrusion on the TLPs and inhibited their extension. (B) Overexpression of Rac induced the formation of multiple protrusive structures at the TLP, whose extension was inhibited (see supplementary material Movie 8). Bar, 10 μm.

protrusions observed with KD-p100γ PI3K- or PTEN-overexpressing cells (Fig. 5C,D), the TLP on the blebbistatin-treated cells displayed a more angular shape and did not extend smoothly (Table 1). These results indicate a role for actomyosin contractility in the morphology of the protrusion.

To evaluate further the contribution of actomyosin system for the morphology of protrusions, we assayed the function of Rho and Rho kinase, which regulate myosin function (Amano et al., 1996). To inhibit the Rho activity, we expressed C3 toxin (Paterson et al., 1990; Yoshizaki et al., 2004). The C3-toxin-expressing cells showed a less spread TLP, suppressed extension of the leading process, and irregular extension of non-directed protrusion (Fig. 6D; supplementary material Movie 15; Table 1). We next inhibited Rho-kinase activity by expression of RB/PH (TT), which is a dominant-negative mutant of Rho-kinase (Amano et al., 1999). Expression of RB/PH (TT) also reduced spreading of TLPs and inhibited extension of leading processes, although the effect was somewhat milder than that of C3 toxin (Fig. 6E; Table 1). Similar phenotypes were observed when explants were treated with the Rho-kinase inhibitor, 50 μM Y-27632 (data not shown). Taken together, our data suggest that regulation of actomyosin contractility is involved in coordinated spreading and extension of TLP.

ErbB4 is involved in extension of leading process

Although the RTK-mediated activation of Rac promotes protrusion formation in fibroblasts and other cells in vitro (Ridley et al., 1992), the role of RTKs in the generation of protrusions in vivo is not known. Interestingly, the RTK ErbB4 is expressed in the developing chicken cerebellum together with neuregulin-1, an ErbB4 ligand (Dixon and Lumsden, 1999; Jones et al., 2003). Treatment of explants with 100 μM

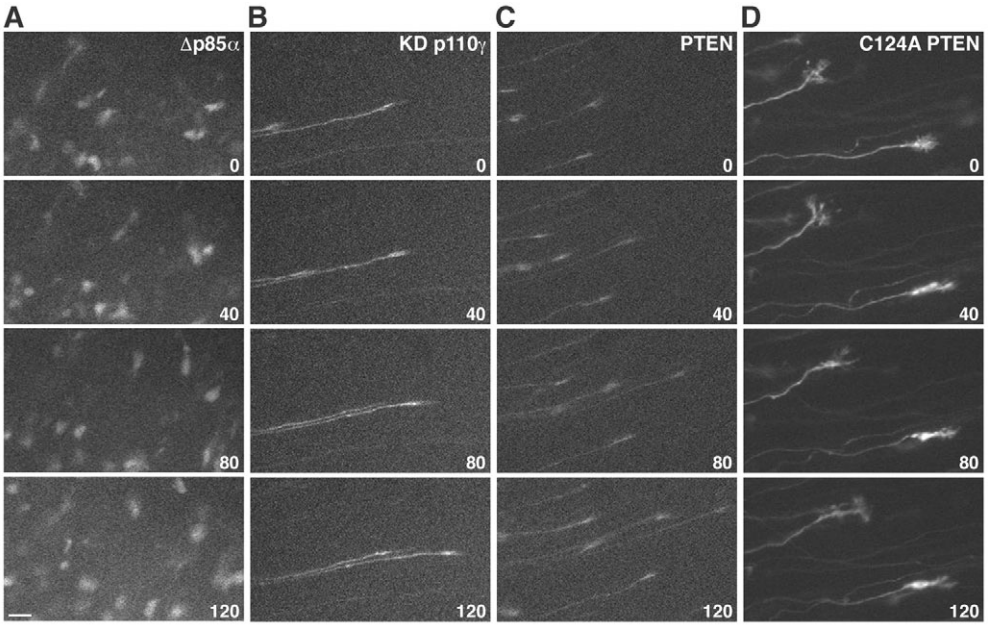

Fig. 5. PIP_3 signaling controls direction and magnitude of protrusion formation. (A) Class IA PI3K mutant, Δp85α, was co-expressed with EGFP in cerebellar rhombic-lip-derived neuronal precursors. EGFP fluorescent images were captured every 2 minutes for 2 hours (see supplementary material Movie 9), and images at 0, 40, 80, 120 minutes are shown. Δp85α-expressing neuronal precursors do not form long leading process and show random migration. Since these cells no longer extend their leading process directionally toward the ventral midline, images of cell soma-rich region near the rhombic lip are shown. (B) Dynamics of Class IB PI3K mutant, KD-p110γ-expressing neuronal precursors visualized with co-expressed EGFP. These cells do not show any apparent defect in directed extension of leading processes (see supplementary material Movie 10). (C) Overexpression of PTEN reduced the protrusive activity on the tip of the leading processes. However, it had no apparent effect on rate and direction of process extension. (D) Phosphatase-dead PTEN mutant, C124A-PTEN, upregulated the protrusive activity at the process tips. This significantly perturbed the extension of leading process (see supplementary material Movie 11). Bar, 10 μm.

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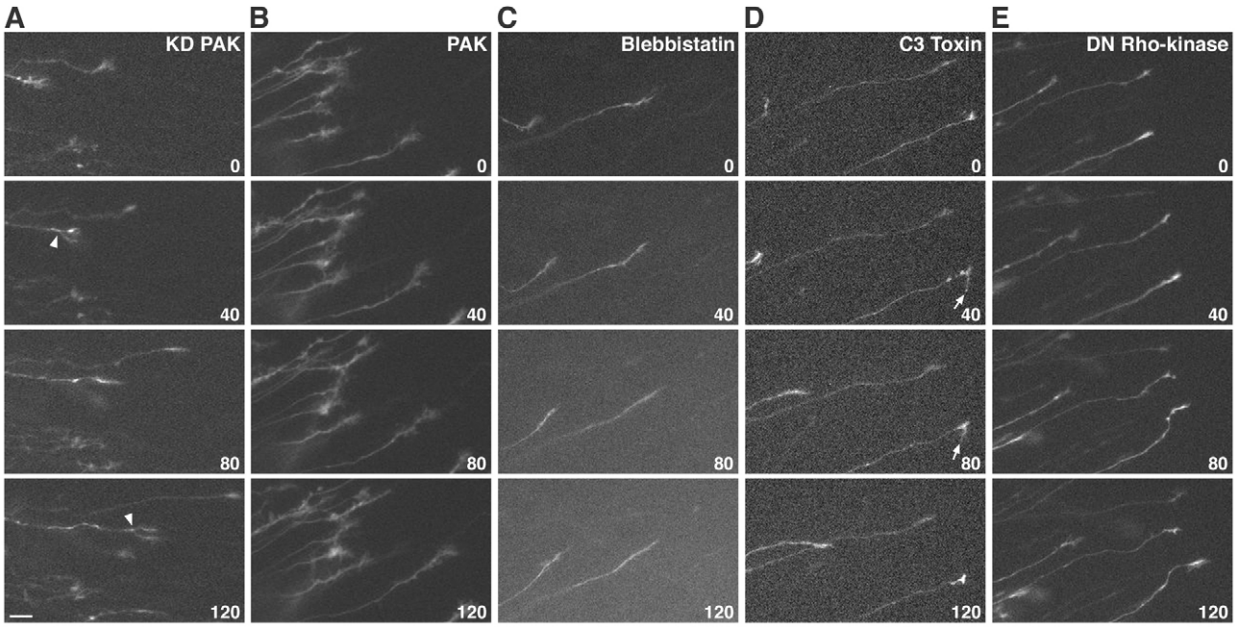

Fig. 6. PAK, Rho and actomyosin system regulate spreading of protrusions. (A) A kinase-dead mutant of PAK (KD-PAK) was co-expressed with EGFP in cerebellar rhombic-lip-derived neuronal precursors. EGFP fluorescent images were captured every 2 minutes for 2 hours (see supplementary material Movie 12), and images at 0, 40, 80, 120 minutes are shown. KD-PAK created unstable branches (arrowheads) and caused frequent changes in the growing direction of the leading process. (B) Overexpression of PAK induced a large, spread growth-cone-like protrusion at the TLPs and inhibited their extension. These large protrusions were relatively stable (see supplementary material Movie 13). (C) Cerebellar explants electroporated with EGFP were pre-cultured for 24 hours, before treatment with 100 μM blebbistatin, a myosin II ATPase inhibitor, for 15 minutes. Inhibition of myosin II reduced formation of spread lamella on the TLPs and decreased the rate of their extension (see supplementary material Movie 14). (D) C3-toxin-expressing neuronal precursors showed reduced spreading of leading protrusions. Termini of their leading process often displayed an angular shape and irregularly extended protrusion in non-directional manner (arrows; see supplementary material Movie 15). (E) Expression of DN-Rho-kinase reduced spreading of leading protrusions and perturbed their coordinated extension. Bar, 10 μm.

AG1478, an inhibitor of epidermal growth factor family RTKs, decreased protrusive activity at the TLP and inhibited extension of the leading process (Fig. 7A; Table 1). Expression of a dominant-negative mutant of ErbB4 (ErbB4ΔIC), which lacks the intracellular kinase domain, also inhibited extension of the leading process (Fig. 7B; supplementary material Movie 16; Table 1). These results indicate that the activation of ErbB4 is a crucial for extension of the leading process on the rhombic-lip-derived neuronal precursors.

Discussion

Directed cell migration results from the interpretation of extracellular signals by polarizing the motile apparatus with respect to the direction of migration. Based on studies of a few cell types in culture, the mechanisms underlying this process are only beginning to appear. It is likely, however, that the robustness of mechanisms will become clearer when studied in other cell types or cells migrating in vivo, where the surroundings are far more complex. A common feature, for example, of neuronal precursors and other cells growing in vivo is a highly polarized morphology with a leading process directed toward the direction of migration; this morphology is seldom observed in mesenchymal-like cells growing in planar tissue cultures.

In this study, we have addressed the origins of the highly polarized morphology seen in vivo by developing an imaging system for visualizing the dynamics of rhombic-lip-derived neuronal precursors migrating in the developing chick cerebellum in situ. With this system, we have assayed the contribution of putative polarity generating molecules on cell polarization, protrusion formation, and directional migration in situ (Fig. 8). The major observations are: (1) Cdc42 is required to produce the directed protrusion and the characteristic monopolar morphology of these cells, whereas the Par6-Par3-aPKC complex regulates the orientation of the protrusion. (2) Rac promotes the localized formation of protrusions on the TLP. (3) Class IA PI3K functions in directional extension as well as the formation of the long leading process, whereas PTEN modulates the size of the TLP. (4) Actomyosin contractility and Rho signaling regulate the coordinated spreading and extension of TLP. (5) ErbB4 is required for extension of the leading process.

From our study, Cdc42 has emerged as the major regulator of polarization in rhombic lip neuronal precursors. Cdc42 activity is crucial for both generating the monopolar morphology and the formation of the protrusion. Many in vitro studies, using other cell types, have demonstrated that the Par6-Par3-aPKC complex and microtubule-binding proteins such as APC mediate polarity-related Cdc42 signaling (Fukata et al., 2003; Etienne-Manneville, 2004). In our system, they regulate the orientation of the TLP but not the formation of the leading process. This is consistent with previous in vitro observations on directionally migrating astrocytes where Cdc42 is required for the generation of a polarized protrusion whereas the Par6-Par3-aPKC complex

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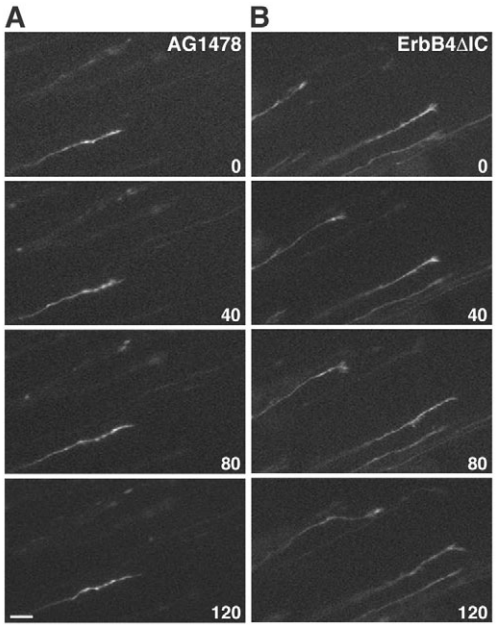

Fig. 7. ErbB4 signaling is essential for the extension of leading process on migrating cerebellar rhombic lip cells. (A) Cerebellar explants electroporated with EGFP were pre-cultured for 24 hours, before treatment with 100 μM AG1478, an RTK inhibitor, for 2 hours, followed by fluorescent in situ imaging. Fluorescent images were captured every 2 minutes for 2 hours, and images from 0, 40, 80, 120 minute time points are shown. Inhibition of RTK suppressed the protrusion formation at the TLP. Bar, 10 μm. (B) Expression of dominant-negative mutant ErbB4ΔIC inhibited extension of TLPs (see supplementary material Movie 16).

regulates its orientation (Etienne-Manneville and Hall, 2001). Taken together, these results suggest that similar mechanisms downstream of Cdc42 are shared in the formation and orientation of polarized protrusions in both cultured astrocytes in vitro and migrating neuronal precursors in situ.

The local activation of Rac appears to be largely responsible for the formation of localized protrusions at the TLP (Itoh et al., 2002; Ridley et al., 2003). Inhibiting Rac activity by expressing a dominant-negative mutant inhibits formation of the protrusion at the process tip without affecting the polarity of the leading process, itself. Likewise, expression of wild-type Rac or its effector, PAK, only affects the dynamics of the TLP. This indicates that Rac regulates the formation of the protrusion but not the overall polarity. This also points to a local engagement of activation signals for this pathway at the TLP. Recently, it was reported that moderate levels of Rac activation sustain the directionality of migration while suppressing the formation of extra, non-directed protrusions (Pankov et al., 2005). In our system, elevation of Rac activity by overexpression induced random extension of numerous protrusions, although the site of protrusion formation was restricted to the TLP. Thus, it is also likely that optimal level of Rac activation coordinates the directional extension of the leading process in situ.

PAK is an effector for both Rac and Cdc42 (Manser et al., 1994; Bokoch, 2003). Although KD-PAK did not recapitulate the effect of either N17-Rac or N17-Cdc42, its phenotype is analogous to a previous in vitro observation on migrating fibroblasts; overexpression of KD-PAK upregulated non-directional migration (Sells et al., 1999). Interestingly, KD-PAK is also reported to promote non-polarized, random formation of protrusions and thus inhibit directionally persistent migration of fibroblasts (Sells et al., 1999; Cau and Hall, 2005). Taken together, these results suggest that the kinase activity of PAK is important for the formation of a dominant polarized protrusion.

Myosin II is a downstream effector of PAK as well as Rho kinase (Ramos et al., 1997; Chew et al., 1998; Amano et al., 1996). Our observations support a positive role for actomyosin contractility in the spreading of protrusions on migrating neuronal precursors in situ. Furthermore, our results suggest that the Rho signaling positively regulates spreading of protrusions in these cells. Although the negative effect of the Rho and actomyosin system on cell spreading and neurite extension has been reported in many in vitro studies (Paterson et al., 1990; Aepfelbacher et al., 1996; Amano et al., 1998; Hirose et al., 1998), Rho can also positively regulate cell spreading in some cell types (Suidan et al., 1997). Accordingly, the effect of Rho on cell spreading is likely to be cell type-specific and/or context dependent. Thus, the present study expands our understanding of the function of actomyosin contractility and Rho signaling in the context of controlling protrusions on migrating neuronal precursors in living tissue.

Finally, we have provided new insight into the function of ErbB4 in the migration of neuronal precursors. The ErbB4ΔIC mutant, which lacks the intracellular kinase domain, dramatically impaired the formation of the protrusion on the TLP of rhombic-lip-derived neuronal precursors. By contrast, the same mutant did not alter protrusive activity but only altered the direction of migration of cortical interneurons in the developing mouse telencephalon (Flames et al., 2004). Interestingly, the directed migration of border cells during *Drosophila* oogenesis involves a spatially restricted activation of RTK signals at the cell front (Jekely et al., 2005). Thus, it is tempting to speculate that a similar focal activation of ErbB4 occurs at the TLP and leads to the targeted activation of PI3K, Rac and PAK in directionally migrating rhombic lip cells in vivo.

In conclusion, our studies of the rhombic-lip-derived neuronal precursors in situ have revealed features that are not readily seen in other cell types. The polarization of these cells is complex and exhibits features unique to both this cell type and its tissue environment. Our studies should stimulate investigation on the mechanisms by which complex cell morphologies are generated in situ.

Materials and Methods

Reagents

All the expression constructs were subcloned into a neuronal expression vector from F. Miller (Gloster et al., 1999), which uses the neuron-specific α-tubulin promoter. Wild-type and mutant forms of human Cdc42 and Rac1 (Rac) and of C3 toxin expression cDNA were obtained from M. Matsuda (Itoh et al., 2002; Yoshizaki et al., 2004). A dominant-negative mutant of human Par6C (Par6-NT; comprising amino acid residues 2 to 130) was obtained from A. Hall (Etienne-Manneville and Hall, 2001). A constitutively active mutant of human GSK3β (S9A-GSK) was obtained from M. Birnbaum (Summers et al., 1999). Microtubule binding-deficient mutant of APC (APCΔCT) was made by deletion the C-terminal 616 amino acid residues in the human APC, which was obtained from B. Gumbiner (University of Virginia, Charlottesville, VA); this mutant disrupts both the microtubule- and EB1/3-binding domains (Zumbrunn et al., 2000). EB3-GFP was obtained from N. Galjart (Stepanova et al., 2003). EGFP-tubulin was purchased from Clontech (Mountain View, CA). A dominant-negative mutant of class IA PI3K, Δp85α, was

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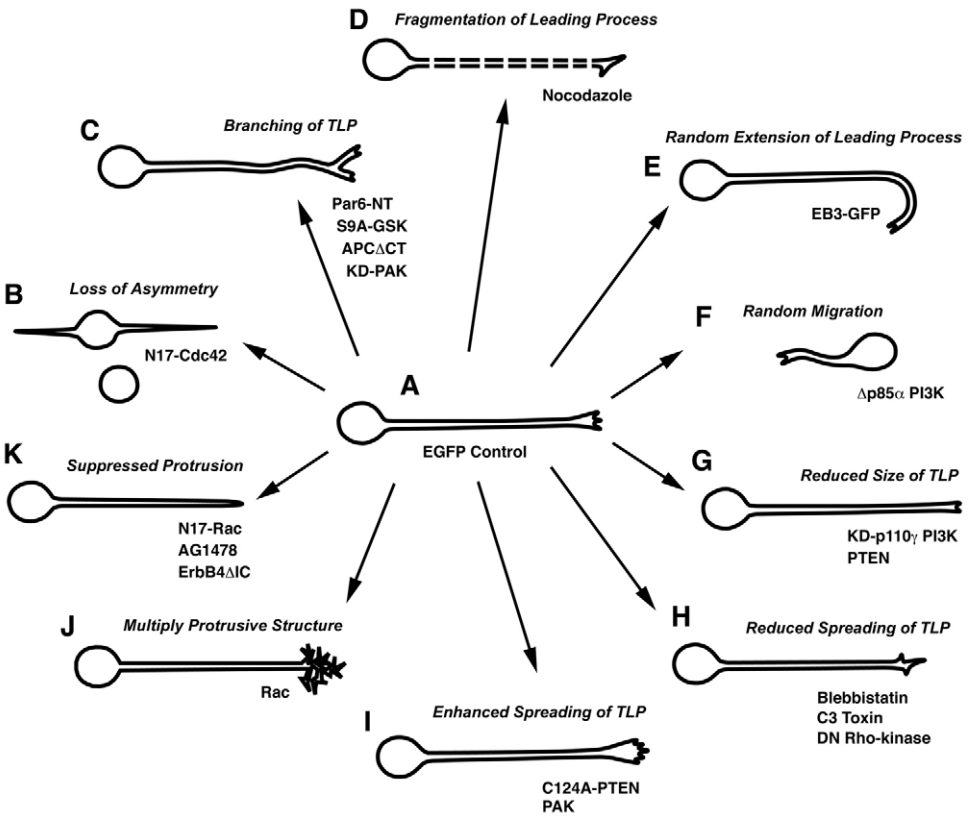

Fig. 8. Schematic summary of morphological changes on polarized processes of neuronal precursors observed in situ. (A) Control EGFP-expressing cells elongated a long leading process. The tip of the process formed protrusions in a spatially restricted manner. (B) Expression of N17-Cdc42 induced retraction of the major existing process and abrogated the monopolar morphology. (C) Perturbation of polarity effectors of Cdc42 (Par6-NT, S9A-GSK, APCΔCT) generated branched protrusions on the terminus of the leading process. Expression of KD-PAK also induced branching of the terminal protrusion. The duration of these branches were short; but the resulting processes often displayed a curved shape at the points the branches formed. (D) Depolymerization of microtubules by nocodazole caused fragmentation of the leading process. Altered orientation of TLP was often observed during the fragmentation. (E) Overexpression of microtubule plus-end binding protein EB3-GFP induced non-directional extension of the leading process. (F) Dominant-negative mutant of class IA PI3K (Δp85α PI3K) promoted random migration of rhombic-lip-derived cells. This mutant disturbed formation of long leading process as well as perturbed directional migration. (G) Kinase-dead mutant of class IB PI3K (KD-p110γ PI3K) affected directional extension but somewhat reduced the size of TLP. Overexpression of PTEN also reduced the size of TLP. The resulting TLPs extended normally compared with control EGFP cells. (H) Inhibition of actomyosin contraction by blebbistatin reduced the degree of spreading of TLP. Perturbation of the Rho-Rho-kinase pathway by expression of C3 toxin or DN-Rho-kinase also suppressed spreading of TLP. (I) C124A-PTEN increased the terminal spreading of leading process. Overexpression of PAK also enhanced the terminal protrusion and resulted in a well-developed growth cone-like structure. (J) Overexpression of Rac produced an aberrant multiply protrusive structure on the TLP. This structure displayed rapid extension and retraction of microspikes rather than lamella. (K) Perturbing the function of Rac (N17-Rac) as well as RTK (AG1478, ErbB4ΔIC) suppressed generation of protrusions at the TLP.

obtained from W. Ogawa (Hara et al., 1994). A kinase-dead mutant of class IB PI3K, K799R p110γ (KD-p110γ), was obtained from T. Noguchi (Takeda et al., 1999). Human PTEN and its phosphatase-dead mutant, C124A, were obtained from K. Yamada (Tamura et al., 1998). Wild-type human PAK1 (PAK) and its kinase-dead mutant K299R (KD-PAK) were obtained from J. Chernoff (Sells et al., 1999). A dominant-negative mutant of Rho-kinase (DN-Rho-kinase), RB/PH (TT), was obtained from K. Kaibuchi (Amano et al., 1999). ErbB4ΔIC, a dominant-negative, intracellular kinase domain-deleted mutant of human ErbB4, was obtained from D. Stern (Jones et al., 1999). The microtubule-depolymerizing drug nocodazole, myosin II ATPase inhibitor blebbistatin, and RTK inhibitor AG1478 were purchased from Calbiochem (San Diego, CA).

Electroporation and explant culture of chicken embryonic cerebellum

The cerebellar region from stage 28 embryonic chicken brain was dissected into Tyrode's saline and then cut open at the dorsal midline. The resulting organotypic explant was placed onto a membrane insert (Millicell-CM: pore size, 0.4 μm; diameter, 30 mm: Millipore, Bedford, MA) in a 35 mm dish filled with 1 ml ice-cold CCM1 medium (Hyclone, Logan, UT) supplemented with 10 mM HEPES (pH 7.4) and 5% horse serum. The explant was placed between the cathode and anode of BTX Genetrode Model 516 electrodes (Harvard Apparatus, Holliston, MA) allowing contact with a portion of the explant with the anode (Fig. 1B). A drop (2 μl) of plasmid solution was placed between the cathode and the cerebellar rhombic lip. The explant was pulsed five times with 30 V, 50 milliseconds at 100 milliseconds intervals by BTX ECM830 electroporator (Harvard Apparatus). The electroporated explants were allowed to express the ectopic genes by incubating for 24 to 30 hours at 37°C in 5% CO_2, followed by imaging with epifluorescence. In general, we co-electroporated the gene of interest with EGFP at a 5:1 ratio (plasmid/EGFP plasmid); the final concentration of the plasmid mixture used for electroporation was approximately 1 μg/μl. Several independent experiments have shown that virtually all EGFP positive cells also co-express the gene of interest construct. For the chemical inhibitor studies, the electroporated explants were precultured to allow the EGFP expression, followed by incubation with media containing the inhibitor compound for the indicated number of hours prior to the imaging.

In situ time-lapse imaging

The explant was cultured at 37°C on the filter insert in a 35 mm dish covered with Saran wrap and sealed with Parafilm. The sealed culture insert was placed into a thermo-controller unit (DH35 dish heater and TC-344B controller: Warner Instruments, Hamden, CT) on the stage of Nikon TE300 inverted microscope (Nikon, Melville, NY) equipped with a 20× long-working-distance objective lens (Plan Fluor ELWD 20×/0.45: Nikon); a 4× objective lens (Plan 4×/0.10: Nikon) was used to acquire low-magnification images. To observe GFP fluorescence, a Chroma Endow GFP filter cube (exciter HQ470/40, dichroic mirror Q495LP, emitter HQ525/50: Chroma, Rockingham, VT) was used. Fluorescent images were collected using a Hamamatsu Orca II cooled charge-coupled device camera (Hamamatsu, Bridgewater, NJ) and Unibritz model T132 shutter (Vincent Associates, Lochester, NY) controlled by MetaMorph software (Universal Imaging, West Chester, PA). The extension speed of the leading processes was calculated from translocation values acquired by tracking the tip position of leading process from EGFP-visualized cells using the track point application in MetaMorph.

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