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Integrin-Mediated Activation of Cdc42 Controls Cell Polarity in Migrating Astrocytes through PKCζ

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Summary

We describe here a signal transduction pathway controlling the establishment of mammalian cell polarity. Scratching a confluent monolayer of primary rat astrocytes leads to polarization of cells at the leading edge. The microtubule organizing center, the microtubule cytoskeleton, and the Golgi reorganize to face the new free space, and directed cell protrusion and migration specifically occur perpendicularly to the scratch. We show here that the interaction of integrins with extracellular matrix at the newly formed cell front leads to the activation and polarized recruitment of Cdc42, which in turn recruits and activates a cytoplasmic mPar6/PKCζ complex. Localized PKCζ activity, acting through the microtubule motor protein dynein, is required for all aspects of induced polarity in these cells.

Introduction

The establishment and maintenance of cell polarity is an essential feature of all eukaryotic cells, both during development and in the adult. However, relatively little is known about the molecular mechanisms by which metazoan cells establish polarity, or whether the mechanisms are conserved between different organisms and cell types and in different biological contexts.

The genetic analysis of the budding yeast, *S. cerevisiae*, has revealed that the small GTPase Cdc42 is an essential regulator of cell polarity (Johnson and Pringle, 1990). Cdc42 has also been shown to regulate polarity in mammalian cells. For example, inhibition of Cdc42 prevents (1) the reorientation of the microtubule organizing center (MTOC) in T cells in response to an antigen-presenting cell (Stowers et al., 1995), (2) the directional movement of macrophages toward a chemotactic signal (Allen et al., 1998), (3) the directional movement and the reorientation of the Golgi of fibroblasts in an in vitro wound assay (Nobes and Hall, 1999), and (4) polarized basolateral secretion/endocytosis in the epithelial cell line, MDCK (Kroschewski et al., 1999).

Over 20 target proteins for Cdc42 have been identified in mammalian cells, including protein kinases such as p65PAK, MRCK, and MLK as well as structural proteins such as WASp and IQGAP (Bishop and Hall, 2000). Interestingly, one of the targets is mPar6, the mammalian homolog of the *C. elegans* PAR6 protein (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000). PAR6 belongs to the *par* (partitioning defective) family of genes (*par 1–6*), required to establish polarity in the single cell embryo of *C. elegans* (Guo and Kemphues, 1996). PAR6 contains a single PDZ domain and a CRIB-like motif (a Cdc42/Rac binding site), and it localizes to the anterior cortex of the embryo along with PKC3, an atypical protein kinase C (aPKC). Both proteins are required for establishing anteroposterior cell polarity in the zygote (Hung and Kemphues, 1999). The recent finding that *Drosophila* proteins related to PAR6 and PKC-3 regulate apical/basal polarity in both epithelial and nonepithelial cell types suggests that the mechanisms underlying cell polarization may be conserved in different organisms (Petronczki and Knoblich, 2001).

To explore the molecular mechanisms controlling polarity establishment in mammalian cells, we have used a relatively simple in vitro wound assay with monolayers of primary rat astrocytes. This assay has allowed us to examine both the biological and the biochemical parameters involved in setting up polarity in the migrating cell. A scratch through the monolayer initiates directional movement of the cells perpendicularly to the wound. Cell migration is initiated by a change in the morphology of the cells at the wound edge, which extend a long protrusion into the space, generating an elongated shape. Cell polarity is characterized by (1) the orientation of the protrusion and cell migration in a direction perpendicular to the wound and (2) the reorientation of the MTOC, the microtubule cytoskeleton, and the Golgi to face the direction of migration. We report here that the localized activation of integrins at the front of the cell initiates a signal transduction pathway, leading to the activation of Cdc42 and the recruitment of an mPar6/PKCζ complex that, along with the microtubule motor dynein, is essential for the establishment of cell polarity.

Results

Characteristics of Astrocyte Migration during Wound Healing

Astrocyte migration was stimulated in a monolayer using an in vitro scratch-wound assay. The wound width was approximately 300 to 400 μm and astrocytes closed the gap typically in 36 to 48 hr (Figure 1A). Cells moved unidirectionally, perpendicular to the direction of the wound, and the average velocity from time lapse recording was 0.104 ± 0.008 μm/min, which is around 5 times slower than that seen with fibroblasts or epithelial cells in a similar type of assay.

A polarized morphology developed 4 to 6 hr following wounding and became pronounced after 8 hr (Figure 1B). The wound edge cells, originally fibroblast-like in shape, adopted a highly elongated shape through the extension of a pseudopodium-like structure. We refer to this as a protrusion, and its length increased to around 100 μm 16 hr after wounding. Cell protrusion occurred in a direction perpendicular to the wound and was ac-

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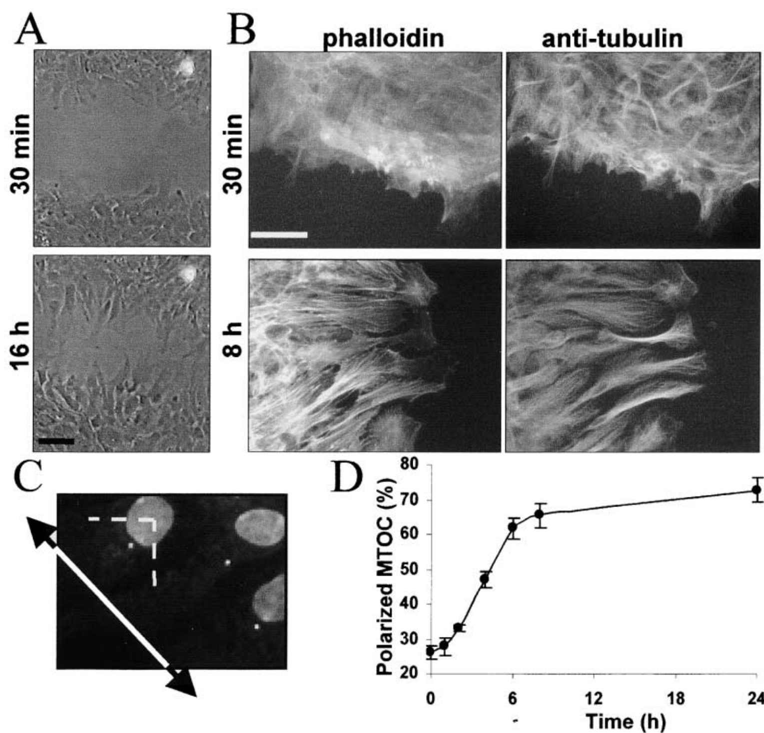

Figure 1. Characteristics of Polarized Astrocyte Migration

(A) Phase contrast images of astrocytes 30 min and 16 hr after wounding. Bar, 100 μm.

(B) Cells were fixed 30 min or 8 hr after wounding and stained with phalloidin or anti-tubulin antibodies. Bar, 50 μm.

(C) Astrocytes were fixed and stained with anti-pericentrin antibodies (to visualize MTOC) and Hoechst. The large arrow indicates the direction of the wound.

(D) The percentage of cells in the front row having their MTOC in the forward-facing quadrant was measured at the indicated time following wounding. Random orientation of the MTOC with respect to the wound edge corresponds to a value of 25% at 0 hr. For each time point and each experimental condition, a minimum of 100 cells were scored. Values represent means ± SEM of 3 independent experiments.

companied by a striking elongation of microtubules reaching the extreme tip of the cells (Figure 1B). In contrast, filamentous actin structures were seen mainly in the cell body, and only a fine line of cortical actin could be seen at the tip of the protrusion (Figure 1B). This polarization of astrocyte morphology occurred concomitantly with the reorientation of the MTOC and the Golgi in the direction of movement (Figure 1C and data not shown). The percentage of cells exhibiting a polarized MTOC increased during the first 8 hr following wounding and reached a plateau of around 70%.

To investigate the role of actin dynamics in astrocyte migration, the morphological changes and MTOC polarization were determined after treatment with cytochalasin D. Although cytochalasin D completely inhibited cell migration, it did not prevent the formation of protrusions or the polarization of the MTOC (data not shown). In contrast, treatment with even low concentrations (100 nM) of nocodazole or taxol, sufficient to block microtubule dynamics but not to destroy the microtubule cytoskeleton, prevented the formation of protrusions and polarization of the MTOC (data not shown). These observations show that astrocyte wounding induces an actin-independent, but microtubule-dependent, polarization of leading edge cells, characterized by the formation of a polarized, elongated morphology and by the reorientation of the MTOC and the Golgi in the direction of migration.

Role of the Rho GTPases in Astrocyte Polarization

To investigate the role of Rho GTPases in the microtubule-dependent establishment of polarity, DNA expression constructs for dominant negative Rho, Rac, or Cdc42 were microinjected into the first row of wound-edge cells immediately after wounding. Protein expression was detected as soon as 1 hr after microinjection. Polarization of the MTOC was dramatically inhibited by dominant negative Cdc42 (N17Cdc42), but not by dominant negative Rac (N17Rac) or Rho (N19Rho) (Figure 2A). The treatment of cells with toxin B10463 (from *Clostridium difficile*), which inhibits Rho, Rac, and Cdc42, also blocked MTOC polarization. Expression of wild-type Cdc42 had no effect, but constitutively activated Cdc42 (L61Cdc42) had a partial effect, indicating either that cycling between its GTP- and GDP-bound form or correct spatial activation, or both, are essential. To distinguish these two possibilities, FGD-1, a GEF specific for Cdc42, was used. It completely abolished MTOC polarization (Figure 2A), suggesting that the polarized spatial activation of Cdc42 is required for the establishment of MTOC polarity.

Rac activity is not required for MTOC polarization, but it is absolutely required for the formation of protrusions, since N17Rac expressed either immediately or even 4 hr after wounding totally abolished protrusions (Figure 2B). The effect of N17Cdc42 on protrusion formation was time dependent, with greatest inhibition seen when injected immediatly after the wounding (Figure 2B, N17Cdc t0). Microinjection 4 hr after wounding resulted in only a small (30%) inhibition of protrusions (Figure 2B, N17Cdc t4). This suggests that Cdc42 is required for initiating the formation of protrusions, but that Rac activity is essential for the development of the protrusions.

Role of mPar6 and PKCζ in Cell Polarization

Recent observations indicate that active Cdc42 can associate with mPar6, which in turn, through its N-terminal domain, interacts with atypical protein kinase Cs (aPKCs) (Joberty et al., 2000; Lin et al., 2000; Qiu et

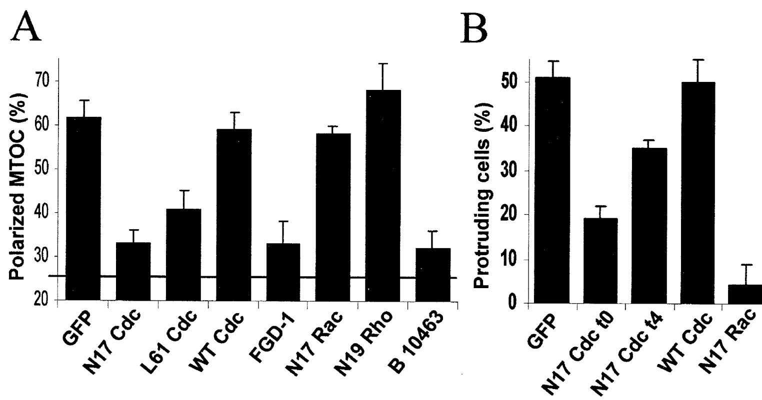

Figure 2. Role of Rho GTPases in Astrocyte Polarization

Astrocytes were either pretreated 2 hr with toxin B10463 before wounding or microinjected immediately, or at the times stated, following wounding with different mutants of the GTPases, or pEGFP (GFP) as a control.
(A) MTOC reorientation 8 hr after wounding.
(B) Protrusion formation. Cells were scored as protruding when their length was at least 4 times their width. For each experiment, approx. 100 cells were scored. Results shown are the mean ± SEM of 3–5 independent experiments.

al., 2000). mPar6 is the mammalian ortholog of the *C. elegans* polarity protein PAR6 (Doe, 2001), and in both worms and flies, PAR6 is required for asymmetric cell division (Doe, 2001). Figure 3A shows that overexpression of full-length mPar6C, one of the 4 known mammalian Par6 genes (Joberty et al., 2000), completely blocks MTOC polarization. In contrast, expression of the kinase inhibitory domain of p65PAK, another Cdc42 target,

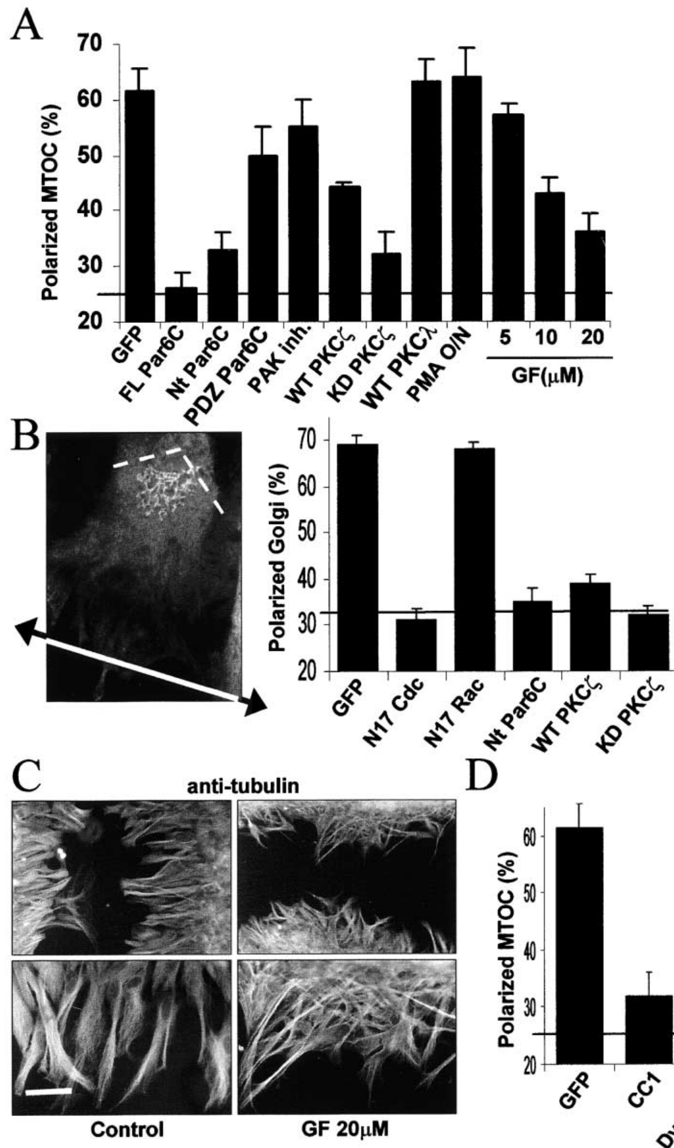

Figure 3. Role of mPar6, PKCζ and Dynein in Astrocyte Polarization

Astrocytes were either pretreated with phorbol ester (PMA, 160 nM) overnight, or with the PKC inhibitor GF109203X (GF) for 1 hr, or microinjected immediately following wounding with constructs. Abbreviations: FL, full length; Nt, N-terminal; WT, wild-type; KD, kinase-dead; CC1, CC1 fragment of p150^{Glued}.
(A and D) MTOC reorientation 8 hr after wounding (as in Figure 1 legend).
(B) Golgi reorientation. Left panel: Cells stained with anti-βCOP. Right panel: The percentage of cells with the Golgi in the forward-facing 120° sector was measured in front row cells.
(C) Cells were allowed to migrate for 16 hr, fixed, and stained with anti-tubulin antibodies. Bar, 50 μm. Pictures shown are representative of 5 independent experiments.

blocked protrusion (data not shown), but had no effect on polarity (Figure 3A). Since mPar6C could potentially block polarity simply by interacting with and interfering with Cdc42, we also expressed other regions of the protein. The PDZ domain of mPar6C (which interacts with another PAR gene product, mPar3) had only a slight effect on polarity, but the N-terminal domain of mPar6C (Nt Par6C) almost completely abolished MTOC reorientation (Figure 3A).

The N-terminal region of mPar6 interacts with aPKCs, and treatment of astrocytes with the inhibitor GF109203X to inhibit most known PKC isoforms, including aPKCs, prevented MTOC polarization (GF, Figure 3A). Overnight pretreatment with phorbol ester to deplete conventional PKCs, on the other hand, had no effect (PMA, Figure 3A). To identify the atypical PKC involved, we expressed a kinase-dead version of PKCζ in cells, and this blocked MTOC polarization (Figure 3A). Overexpression of wild-type PKCζ also resulted in a significant inhibition, suggesting that, like Cdc42, PKCζ activity must be properly localized. Overexpression of another aPKC, PKCλ, had no effect, suggesting that this closely related kinase does not have the same substrate specificity as PKCζ.

Reorientation of the Golgi was also abolished by microinjection of N17Cdc42, N-terminal mPar6C, wild-type PKCζ, or kinase-dead PKCζ, while N17Rac had no effect (Figure 3B).

Since Cdc42 is required to initiate the formation of protrusions, we next examined whether mPar6 or PKCζ might also play a role in this process, and found that neither protein was required (data not shown). Although PKCζ activity was not required for the formation of protrusions, inhibition of PKC (with GF 109203X) did lead to abnormal protrusions (Figure 3C). Instead of protrusions normally elongating in a direction perpendicular to the wound, their orientation in GF109203X-treated astrocytes was random, and a substantial proportion of cells displayed 2 or even 3 protrusions. We conclude that the Cdc42 target complex, mPar6/PKCζ, is not required for protrusion formation, but it is required for the correct orientation of the protrusion as well as the MTOC.

Role of Cytoplasmic Dynein in Cell Polarization

Since the peripheral plus ends of microtubules play a key role in the establishment of astrocyte polarity, we investigated whether the microtubule-based motor dynein was involved in mediating Cdc42/mPar6/PKCζ signaling to the MTOC. Dynein has been proposed to serve as a cortical anchor for cytoplasmic microtubules in *Dictyostelium*, thereby determining centrosome position (Koonce et al., 1999). Mammalian cytoplasmic dynein is associated with dynactin, and overexpressing one of the dynactin subunits, dynamitin, or a fragment of another, p150^{Glued}, has been shown to disrupt dynein function (Burkhardt et al., 1997; Quintyne et al., 1999). Overexpression of dynamitin or of CC1 (a fragment of p150^{Glued}) completely blocked astrocyte MTOC polarization (Figure 3D), but had no effect on protrusion formation, or on the overall integrity of the microtubule network (data not shown). We conclude that cytoplasmic dynein is involved in the signaling pathway leading to MTOC polarization.

Activation and Relocalization of Cdc42, mPar6C, and aPKC upon Wounding

To determine whether Cdc42 and PKCζ are activated upon wounding, around 50% of 10^7 confluent astrocytes were scraped away with a multichannel pipette. Cdc42 activity was assessed in the remaining attached cells by precipitating Cdc42-GTP with a GST-PAK-CRIB fusion protein. Cdc42 was rapidly activated upon wounding (Figure 4A) and showed a 4-fold increase 30 min after wounding, which gradually decreased to basal levels 24 hr after wounding. Wounding also induced a strong activation of aPKCs, which reached a maximum 1 hr after wounding and was sustained for at least 8 hr (Figure 4B).

Cdc42 localization in polarized migrating cells was addressed by microinjecting a GFP-tagged wild-type Cdc42 construct into cells. It has previously been shown that GFP-Cdc42 localizes to the same cellular location as endogenous Cdc42 (Michaelson et al., 2001). We observed that in a confluent monolayer of nonmigrating cells, GFP-Cdc42 is cytoplasmic and mainly associated with Golgi elements and perinuclear membranes (Figure 4C, panel a, white triangles). Moreover, time lapse recording showed that GFP-Cdc42 associates with motile, vesicle-like structures. In migrating astrocytes, GFP-Cdc42 was still visible in perinuclear membranes and Golgi, but in addition displayed two new and prominent characteristic features: (1) GFP-Cdc42 clearly associated with an increased number of vesicles, which were larger in size than in confluent cells and which were generally moving from the tip of the protrusion toward the nucleus (Figure 4C, panel c, white angles), and (2) GFP-Cdc42 fluorescence was also enhanced at the very tip of the protrusion, apparently associated with the plasma membrane at the leading edge (Figure 4C, panel c, white arrowheads).

Since the anti-mPar6C antibody was unsuitable for immunofluorescence, DNA encoding myc-tagged full-length mPar6C was injected into already polarized migrating astrocytes. Cells were incubated for only 2 hr after injection to allow relatively low levels of expression without perturbing cell polarity. mPar6C localized mainly in the nucleus, as previously described (Johansson et al., 2000), but could also be seen clearly at the leading edge of migrating cells (Figure 4C, panel f). When expressed together, GFP-Cdc42 and myc-mPar6C colocalized at the leading edge of migrating cells (Figure 4C, panels e and f). Finally, we also visualized endogenous aPKC, which was mainly diffusely distributed in the cytoplasm, but a fraction could be seen associated with the plasma membrane at the leading edge (Figure 4C, panel d). Membrane/cytosol fractionation confirmed the translocation of endogenous Cdc42, mPar6C, and PKCζ to the membrane following wounding (data not shown). These results show that upon wounding, Cdc42 and aPKCs are activated and become associated with the plasma membrane at the leading edge of migrating cells.

To determine whether dynein function was required for recruitment of Cdc42, mPar6C, and PKCζ to the leading edge, dynamitin was overexpressed in wounded astrocytes. Although MTOC polarity was perturbed, GFP-Cdc42 and PKCζ recruitment to the leading edge was still visible (data not shown), indicating that dynein is acting downstream of Cdc42 and PKCζ.

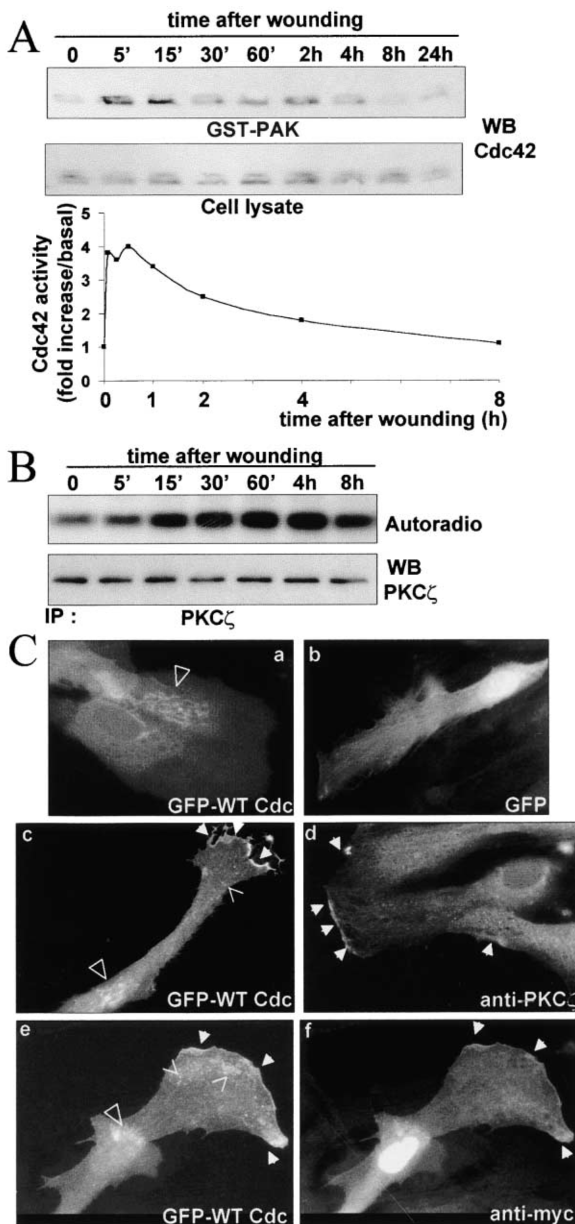

Figure 4. Activation and Relocalization of Cdc42 and aPKCs after Astrocyte Wounding

(A) Lysates from wounded astrocytes were incubated with agarose beads coupled to GST-PAK-CRIB (GST-PAK). Top panel: GTP-bound Cdc42 visualized on Western blots. Middle panel: Total Cdc42 in lysates. Bottom panel: GTP-Cdc42 to total Cdc42 ratio.

(B) aPKC activity in cell lysates was assayed by immunoprecipitation and in vitro kinase assays using MBP as a substrate. Upper panel: Radiolabeled MBP. Lower panel: Western blots showing total aPKC precipitated.

(C) Confluent (a) or 14 h-migrating (b, c) astrocytes were microinjected with pEGFP-WTCdc42 (a, c) or pEGFP (b), incubated for 2 hr and then fixed. Alternatively, 6 hr after wounding, cells were coinjected with pEGFP-WT-Cdc42 and myc-FL-mPar6C and incubated for 2 hr before fixation. GFP fluorescence (e) and myc-staining (f) of the same cell. (d) 8 hr after wounding, cells were fixed and stained with anti-nPKCζ antibody. All results are representative of 3 independent experiments.

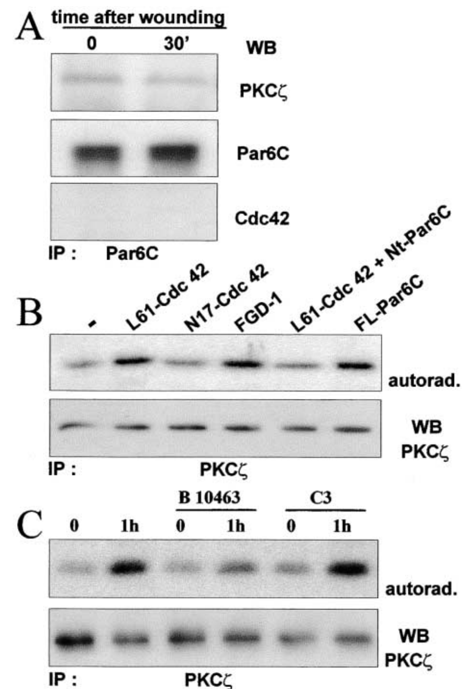

Figure 5. Formation and Regulation of a Cdc42/mPar6/aPKC Complex

(A) Astrocytes were wounded and incubated for the indicated period of time. Endogenous mPar6C was immunoprecipitated and associated proteins were analyzed by Western blotting as indicated.

(B) COS cells were transfected with pRK5-myc vectors encoding indicated GTPase mutants. 48 hr following transfection, cells were lysed and endogenous aPKCs immunoprecipitated and subjected to in vitro kinase assay using MBP as a substrate.

(C) Astrocytes were pretreated with toxin B 10463 (3 hr, 1 pg/ml) or with C3 toxin (overnight, 5 μg/ml) and wounded in the presence of the inhibitor and in serum-containing medium. Cells were immediately lysed or incubated for 1 hr. aPKC activity in each cell extract was recovered by immunoprecipitation of aPKCs followed by in vitro kinase assays using MBP as a substrate. The products of kinase reactions were analyzed by SDS-PAGE and transferred onto nitrocellulose. Radiolabeled MBP was detected by autoradiography (upper panels) and blots were probed with anti-nPKCζ (lower panels). Pictures shown are representative of at least 3 independent experiments.

Role of Cdc42 in the Regulation of aPKCs

To examine the relationship between Cdc42 and mPar6/aPKCs, endogenous mPar6C was immunoprecipitated from confluent and wounded astrocytes. mPar6C is constitutively associated with aPKCs before and after wounding (Figure 5A), but under these conditions, we were unable to detect any association of Cdc42 with this complex. Nevertheless, wound-induced aPKC activation was blocked by the Rho/Rac/Cdc42 inhibitor toxin B10463, but not by the Rho inhibitor C3 transferase (Figure 5C).

When overexpressed in COS cells, L61Cdc42 (but not N17Cdc42 or L63Rho) could be immunoprecipitated with an endogenous mPar6C/aPKC complex, as reported by others (data not shown) (Joberty et al., 2000; Lin et al., 2000). Furthermore, expression of L61Cdc42, FGD-1 (a Cdc42 exchange factor), or full-length mPar6C led to an increase in the activity of endogenous aPKCs

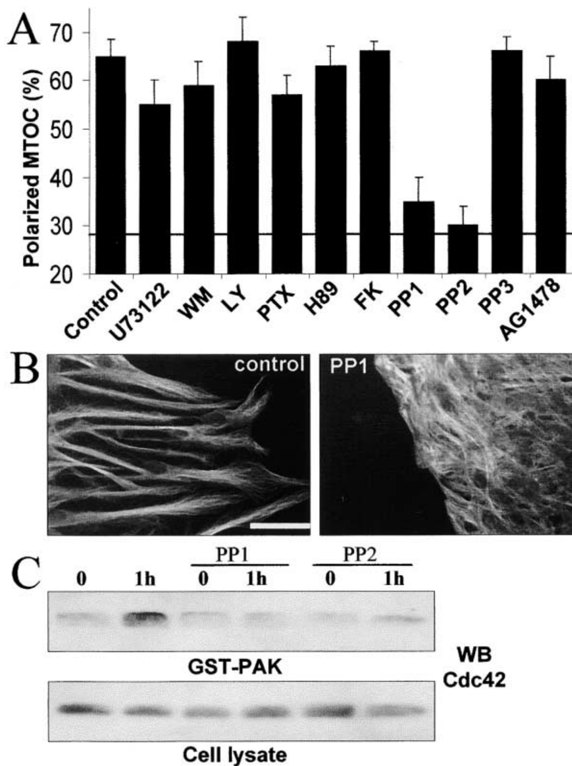

Figure 6. Src-like Tyrosine Kinase Activity Is Required for Astrocyte Polarization

Astrocytes were pretreated with the wortmanin (WM, 20 nM), LY294002 (LY, 20 μM), H89 (20 μM), forskolin (FK, 10^{-5} M), PP1 20 μM, PP2 20 μM, PP3 20 μM or AG1478 (AG, 0.1 μg/ml) for 1 hr, or Pertussis toxin (PTX, 0.1 μg/ml) overnight.

(A) MTOC reorientation 8 hr after wounding (see legend Figure 1).

(B) Cells were allowed to migrate for 16 hr, fixed, and stained with anti-tubulin antibodies. Bar, 50 μm. Pictures shown are representative of 3–5 independent experiments.

(C) GTP-Cdc42 levels in the presence or absence of PP1 or PP2 were determined as in Figure 4 legend.

in COS cells (Figure 5B). Finally, the Cdc42-induced aPKC activation was inhibited by overexpression of the N-terminal domain of mPar6C (Figure 5B). These results suggest that the interaction of Cdc42 with mPar6 leads to activation of an associated aPKC. We conclude that Cdc42 activation mediates wound-induced aPKC activation.

Integrin Activation Is Required for Cell Polarization

To identify the polarity signals that initiate Cdc42 activation, we tested a wide range of inhibitors for their ability to prevent MTOC polarization and protrusion formation. Of the inhibitors tested, only PP1 and PP2, two relatively specific inhibitors of Src-like tyrosine kinases, perturbed MTOC polarization (Figure 6A), protrusion formation (Figure 6B), as well as Golgi reorientation (data not shown). In contrast, neither PP3, an inactive analog of PP2, nor the EGF-receptor tyrosine kinase inhibitor AG 1478 had any effect. Pretreatment of astrocytes with PP1 and PP2 abolished both Cdc42 (Figure 6C) and aPKC activation (data not shown).

To determine which proteins are tyrosine-phosphorylated upon astrocyte wounding, cells were visualized by immunofluorescence using an anti-phosphotyrosine antibody. Increased staining at the leading edge of the cells could be seen, which first formed as a dotted pattern (Figure 7A, upright panel) and later matured into dashed structures reminiscent of focal adhesions (Figure 7A, 4 h). This increase in tyrosine phosphorylation was abolished by PP1 or PP2 treatment (Figure 7A). Western blots showed a strong increase in total tyrosine phosphorylated proteins during the first hour following wounding (data not shown), and immunoprecipitations revealed that the typical focal-adhesion associated proteins, FAK, paxillin, and $p130^{Cas}$ were strongly phosphorylated (Figure 7B).

Finally, given these results, the potential role of integrins in initiating astrocyte polarization was examined. Integrin function was blocked using a cyclic RGD peptide, added immediately before wounding. The peptide completely abolished Cdc42 and aPKC activation (Figure 7C) and as a consequence inhibited MTOC polarization (Figure 7D), Golgi reorientation (data not shown), and protrusion formation (data not shown).

Discussion

To study the molecular mechanisms underlying cell polarization in mammalian cells, we have used an in vitro assay of wound-induced astrocyte migration. Wounding induces astrocyte polarization, which we have visualized in two ways; (1) the development of a polarized morphology, which we refer to as protrusion, perpendicular to the wound edge, and (2) the reorientation of the MTOC and the Golgi to face the wound edge.

Surprisingly, treatment of cells with cytochalasin D (or latrunculin, data not shown) did not prevent protrusion formation, though it did block cell migration. In fact, most of the filamentous actin seen in migrating cells, mainly actin bundles, is localized at the rear, and very little is seen in protrusions. Only a very thin line of cortical actin was detectable at the very tip of the protrusion. By contrast, low doses of nocodazole (0.1 μM), which perturb microtubule dynamics while preserving an intact microtubule system, totally inhibited protrusion formation. These results suggest that microtubule dynamics, and not actin dynamics, provides the driving force for elongation at the leading edge. We previously reported that the microtubule cytoskeleton did not play a significant role in the migration of primary rat embryo fibroblasts in a similar wound-induced assay, whereas in neuronal cells, microtubules are known to be required for axon elongation (Nobes and Hall, 1999; Tanaka et al., 1995). Clearly, the microtubule cytoskeleton makes different contributions to cell migration in different cell types.

A role for the three Rho GTPases, Rho, Rac, and Cdc42 in controlling the organization of the actin cytoskeleton is well established in many different cell types (Hall, 1998). However, there is growing evidence that they can also influence the microtubule cytoskeleton. Rho, for example, leads to the stabilization of microtubules (as visualized with antibodies to detyrosinated tubulin) in the direction of migration (Cook et al., 1998; Ishizaki

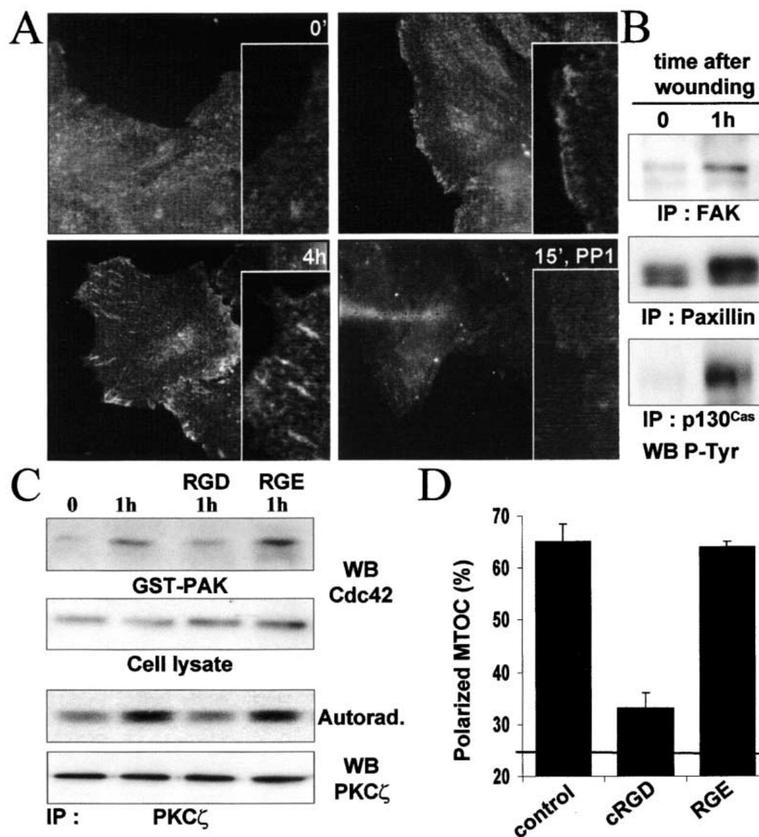

Figure 7. Integrin Engagement Is Required for Astrocyte Polarization

Astrocyte monolayers untreated or pretreated with PP1 (1 hr, 20 μM) were wounded and incubated with or without cyclic RGD peptide (cRGD, 200 μM), or control RGE peptide (RGE, 200 μM).

(A) Immunofluorescence using anti-phosphotyrosine antibodies (clone PT-66). Insets show enlargement of the leading edge of the cells.

(B) Western blots using anti-phosphotyrosine antibodies (clone 4G10) of immunoprecipitated FAK, paxillin or $p130^{Cas}$.

(C) GTP-Cdc42 levels and aPKC activity with or without cRGD or RGE peptides were determined as in Figure 4 legend.

(D) MTOC reorientation 8 hr after wounding with or without cRGD or RGE peptides (see legend Figure 1).

et al., 2001). We also find these nocodazole-resistant microtubules in protrusions after wounding (unpublished data), suggesting that Rho might affect the microtubule cytoskeleton during astrocyte migration. However, Rho is not required for MTOC polarization or for formation of protrusions. We show here that Rac is essential for the microtubule-dependent formation of protrusions in migrating astrocytes. It appears, therefore, that Rac mediates cell elongation either by directly affecting microtubule dynamics or by regulating an activity that is dependent on microtubule dynamics. We recently reported that Rac, via p65PAK, can potentially regulate microtubule dynamics through phosphorylation and inhibition of the microtubule-destabilizing protein stathmin (Daub et al., 2001). We are currently investigating whether stathmin phosphorylation is involved.

The results we present here now provide a mechanism through which Cdc42 regulates the microtubule-dependent establishment of cell polarity. We have shown that the Cdc42 target protein, mPar6, and its associated protein, PKCζ, are essential for defining cell polarity as determined by the reorientation of the MTOC and the Golgi to face the wound edge and by the direction of cell elongation. mPar6 is the mammalian ortholog of the *C. elegans* PAR6 protein. PAR proteins were identified as key regulators of cell polarity in early *C. elegans* development (Kemphues et al., 1988), and our results show that mPar6C and PKCζ are involved in a signaling pathway directly responsible for the establishment of polarity during migration of mammalian cells. Furthermore, we show that the kinase activity of PKCζ is essential for polarity. A number of other PAR gene products have been shown to be essential in establishing polarity in the *C. elegans* embryo, including a PDZ-containing protein, PAR3, that binds directly to the PDZ domain of PAR6. The mammalian homolog of PAR3, together with aPKCs, has recently been reported as essential for the establishment of epithelial junctional structures (Suzuki et al., 2001). However, overexpression of the mPar6C PDZ domain did not dramatically affect polarity of the migrating astrocytes; moreover, endogenous Par3 did not localize at the leading edge, but rather at cell-cell contacts (data not shown).

Our data also show that Cdc42 is essential for the formation of protrusions leading to the elongated morphology, though only at early times after wounding. Rac, on the other hand, is essential for both the development and the maintenance of protrusions during migration. Interestingly, Cdc42-mediated initiation of protrusions is independent of mPar6 and PKCζ. We think that the most likely explanation for this is that at early times, Cdc42 is required to activate Rac in a cascade as previously described (Nobes and Hall, 1995; Price et al., 1998). At later times, Rac recruitment, perhaps by integrin complexes, and activation at the leading edge becomes Cdc42-independent. Nevertheless, inhibition of aPKCs with the GF109203X inhibitor leads to a disorganized orientation of protrusions. It seems likely that suppression of MTOC reorientation leads to mislocalized targeting of proteins required for the formation of the protrusion. Our results indicate that Cdc42 coordinately controls two distinct pathways: one, involving PKCζ, is

essential for MTOC polarization, and the other, involving Rac, is essential for the formation of protrusions. The consequences of this are that inhibition of PKCζ blocks MTOC polarization but has no effect on protrusion formation, while inhibition of Rac blocks protrusion but has no effect on MTOC polarization.

Recent reports have shown that GTP-bound Cdc42 can bind to mPar6 in vitro as well as in vivo using overexpressing transfected cells (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000). Here, we show that Cdc42 activation leads to the recruitment of mPar6C and PKCζ to the plasma membrane, specifically at the leading edge, and to activation of PKCζ. The target of PKCζ phosphorylation is unknown, but one of the obvious candidates is the microtubule-based dynein motor complex. We have shown that dynein function is required for MTOC polarization, but not for protrusion formation or for localization of Cdc42 and PKCζ at the leading edge. These results suggest the possibility that dynein is downstream of a Cdc42/mPar6/PKCζ complex. The function and cellular localization of dynein/dynactin can be directly regulated via multiple sites of phosphorylation (Bloom, 2001; Karki and Holsbaur, 1999), and it is tempting to speculate that localized PKCζ regulates localized recruitment and/or activation of dynein. Dynein activity would then provide a link between the plasma membrane at the leading edge and the microtubule system and through its minus end directed motor activity would lead to the reorientation of the MTOC toward the front of the cell.

We also note that ectopic expression of L61Cdc42, FGD-1, wild-type mPar6C, or wild-type PKCζ leads to an increase in PKCζ activity; yet, this inhibits MTOC and Golgi polarization. Clearly, activation of Cdc42 or PKCζ per se is not sufficient, and the establishment of polarity depends on their localized activation, in this case at the front of the migrating cell. These results are analogous to those observed in *Saccharomyces cerevisiae*, where Cdc42 is required for establishing polarized bud growth and where both constitutively activated and dominant negative Cdc42 cause loss of polarity (Ziman et al., 1991).

In migrating astrocytes, the activation of Cdc42 appears to be the key to establishing directionality. We have observed by total internal reflection (TIRF) microscopy that Cdc42 associates with the plasma membrane at the extremity of the protrusion (data not shown). Since translocation of Cdc42 and other small GTPases to the membrane fraction correlates with their activation, it is tempting to speculate that Cdc42 activation occurs specifically at the leading edge of the migrating astrocyte. Indeed, its colocalization with mPar6C suggests that the active form of Cdc42 is essentially restricted to the leading edge. One simple hypothesis is that Cdc42 directly translocates from the cytosol to the plasma membrane, possibly via dissociation from a Rho GDI as suggested by others for Rac and Rho (Michaelson et al., 2001). However, in agreement with recent observations, we find Cdc42 localizes to endomembranes (mainly Golgi, but also vesicles) in live cells (Michaelson et al., 2001). Upon wounding, there is a significant increase in Cdc42 associated with vesicular structures and mostly concentrated at the extremity of the protrusion. Cdc42 activation might, therefore, first involve transport of vesicles to the leading edge followed by the interaction of the GTPase with a membrane-bound GEF. In agreement with this, we have found that complete disruption of the microtubule cytoskeleton with high concentration (10 μM) of nocodazole blocks activation of Cdc42 and its localization to the leading edge (data not shown).

Finally, we show that activation of Cdc42 is mediated by integrin signaling, perhaps involving a Src-like tyrosine kinase. The Scr family kinases Fyn and Src are closely associated with integrins and can be activated downstream of integrin activation (Cobb et al., 1994; Wary et al., 1998). A number of previous observations have shown that Rho GTPases can be activated upon integrin engagement (Cox et al., 2001; Ren et al., 1999). However, not all integrins lead to Cdc42 activation, and therefore not all would be expected to participate in polarity establishment (Caron and Hall, 1998; Lowin-Kropf et al., 1998). Cytochalasin D treatment had no effect on Cdc42 activation and astrocyte polarization, although it did prevent the formation of focal adhesions; this leads us to the conclusion that Cdc42 activation by integrin engagement does not require integrin clustering or actin bundle recruitment (Leng et al., 1998). We do not know which integrins are involved in initiating astrocyte polarization, but this is currently under investigation.

In conclusion, our results define a signal transduction pathway controlled by Cdc42 that links integrin engagement to the establishment of polarity in migrating cells. The recognition of space by integrins appears to be the first polarity signal, which rapidly leads to the activation and localized recruitment of Cdc42. Cdc42 then establishes and maintains the polarity of the migrating cell by promoting Rac-dependent protrusion and PKCζ/dynein-dependent reorientation of the MTOC, the Golgi, and microtubule network toward the leading edge.

Experimental Procedures

Materials

Antibodies used: α-tubulin and phosphotyrosine (clone PT-66) from Sigma (St. Louis, MO), anti-FAK (clone 2A7) from Upstate Biotechnology (Lake Placid, NY), anti-paxillin from Zymed Laboratories, Inc. (San Francisco, CA), anti-p130^{Cas} and anti-PKCλ from Transduction Lab. (Lexington, KY), anti-nPKCζ and anti-Cdc42 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-pericentrin from BabCO (Richmond, CA), anti-β-COP from Prof. C. Hopkins (London, UK), anti-Par6C (against amino acid residues 2–16) from P. Aspenström (Ludwig Institute, Uppsala, Sweden) (Johansson et al., 2000). Secondary antibodies were from Jackson ImmunoResearch Labs, Inc. Rhodamine-phalloidin, cytochalasin D, nocodazole, Taxol (paclitaxel), and PMA were from Sigma (St. Louis, MO), and GF109203X, PP1, PP2, PP3 were from Calbiochem (San Diego, CA). Toxin B 10463 was provided by Dr. C. von Eichel-Streiber (Gutenberg University, Mainz, Germany). The peptides GPenRGDSPCA and GRGESP were from Life Technologies, Ltd. (Paisley, UK).

Cell Culture and Wounding

Primary astrocytes were prepared as previously described (Etienne-Manneville et al., 1999). For wound-healing assays, cells were seeded on (poly)L-ornithine-precoated coverslips or 90 mm diameter dishes, grown in the presence of serum to confluence, and the medium was changed 16 hr before wounding. The wound was made by scraping either a microinjection needle across the coverslip or a 8-channel pipette (with 0.1–2 μl tips) several times across the 90 mm dish. COS-7 cells were grown and passaged in DMEM supplemented with 10% FCS and penicillin/streptomycin (100 IU/ml and 100 μg/ml), and incubated at 37°C and 10% CO_2.

DNA Constructs

GTPases contructs in the pRK5-myc vector were used (Lamarche et al., 1996). PKCζ was in pEF-myc vectors. Myc-PKCλ was from P. Parker (ICRF, London, UK). PCMV-CC1 and pcDNA3-myc-GFP-dynamitin and the anti-CC1 antibody were from Dr. T. Schroer (Johns Hopkins, Baltimore, MA). GFP-WT-Cdc42 was in the pEGFP vector (Clontech Laboratories, Inc., Palo Alto, CA, USA). pCAN-full length-mPar6C and pCAN-N terminal-mPar6C were from Dr. G. Martin (U.C. Berkeley, CA). PDZ-mPar6C was cloned into pRK5-myc using PCR primers (5′-GAGGATCCGAGACCCACCGACGG-3′; 5′- GGGAATTC CTAGGGCTTGACAGTGAC-3′).

Astrocyte Microinjection and COS Cell Transfection

Nuclear microinjections in the first row of wound edge astrocytes were performed immediately after wounding. Expression vectors were used at 100–200 μg/ml. COS cells were transfected by the DEAE-dextran method.

Immunofluorescence

Cells were stained as previously described (Nobes and Hall, 1999). Fluorescence images were recorded on a CCD camera and processed using Openlab software.

MTOC/Golgi Reorientation and Protrusion Formation

MTOC or Golgi were localized by immunolabeling using anti-pericentrin or anti-β-COP antibodies, respectively. Cells in which the MTOC was within the quadrant facing the wound were scored positive, and for each time point at least 100 cells were examined. Golgi localization was determined in a similar way, except that 120° sectors were used. To assess the formation of polarized morphology, astrocytes were microinjected with pGFP as a control or indicated DNA constructs and Biotin-dextran. Cells were fixed 16 hr after wounding. Cell morphology was observed by staining for the expressed protein and streptavidin-Cy5. Expressing cells were scored as polarized when their length was at least 4 times their width. Cell morphology was determined in all expressing cells of the front row.

Immunoprecipitation

Cells were washed with ice-cold PBS containing 1 mM orthovanadate and were lysed at 4°C in Nonidet P-40 buffer (10 mM Tris/HCl [pH 7.5], 140 mM NaCl, 1 mM orthovanadate and 1% Nonidet P-40, 2 mM PMSF, 5 mM EDTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Nuclei were discarded following centrifugation at 10,000 g for 10 min. Lysates were incubated for 2 hr at 4°C with specific antibodies and protein G-sepharose, and immunoprecipitates were collected by centrifugation and extensively washed in Nonidet P-40 buffer. Immunoprecipitated proteins were eluted with SDS-sample buffer and analyzed by 8% SDS-PAGE.

Cdc42 Activity

GTP-bound Cdc42 was affinity purified using GST-PAK-CRIB attached to gluthatione agarose beads (Kjoller and Hall, 2001). Proteins were eluted with SDS-sample buffer and analyzed by 15% SDS-PAGE and anti-Cdc42 Western blotting.

aPKC Activity

The kinase reaction was performed as previously described (Diaz-Meco et al., 1994). aPKCs were immunoprecipitated with the anti-nPKCζ rabbit polyclonal antibody, and the kinase reaction performed for 10 min at 37°C using myelin basic protein as substrate.

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