Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways

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Summary

Cdc42, a Rho family GTPase, is a key regulator of cell polarity. In *Saccharomyces cerevisiae*, it is required for polarized bud formation and pheromone gradient sensing, while in higher eukaryotes, it participates in asymmetric cell division, directional sensing during migration, and morphogenesis. Using a scratch-induced fibroblast migration assay, we previously showed that Cdc42 controls the polarization of both membrane protrusions and the Golgi/centrosome. We now find that Golgi/centrosome polarity is mediated through activation of the Par6/aPKC complex, as previously described in astrocytes. However, this complex is not involved in Cdc42-dependent polarization of protrusions, which instead is mediated by Pak acting through the Rac guanine nucleotide exchange factor, βPIX. Pak kinase activity is essential for spatially restricting Rac-dependent actin polymerization to the leading edge of the migrating cells, though it is not required for actin polymerization per se. We conclude that in migrating cells, Cdc42 co-ordinately regulates the polarity of the microtubule and actin cytoskeletons through two distinct pathways.

Supplementary material available online at
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Key words: Cdc42, Cell migration, Microtubules, Actin

Introduction

Directed cell migration is crucially important during embryonic development, for example during the long-range migration of neural crest cells, as well as in the adult in innate immune responses or in tumour cell metastasis (Etienne-Manneville and Hall, 2002). The mechanisms through which cells can polarize their actin cytoskeleton with respect to an extra-cellular cue have been the subject of great interest and the chemotactic responses of *Dictyostelium discoideum* and mammalian leukocytes have been used extensively to examine this in vitro (Allen et al., 1998; Funamoto et al., 2002; Iijima and Devreotes, 2002; Srinivasan et al., 2003). In both cases, actin polymerization is restricted to the front, or leading edge, forming a protrusive structure called a pseudopod or lamellipodium. Two major players in regulating this response are phosphatidylinositide 3 kinase (PI 3-kinase) and the small GTPase Rac, which are both recruited to the front of the cell to promote spatially localized activation of the actin polymerization machinery, in particular Arp2/3 (reviewed by Merlot and Firtel, 2003). These molecules are also involved in random migration, where cells spontaneously polarize their actin cytoskeleton. Thus PI 3-kinase and Rac are probably 'downstream' of the directional sensing machinery involved in chemotaxis.

There is growing evidence that another small GTPase, Cdc42, is a key regulator of directional sensing. It was first implicated in the establishment of cell polarity in *Saccharomyces cerevisiae*, where it is required both for polarized bud formation and the formation of polarized mating projections in response to pheromone gradients (see Etienne-Manneville, 2004; Nelson, 2003). In leukocytes, Cdc42 is essential for directional migration, but not for random migration, suggesting that it is required to spatially localize actin polymerization in response to an external cue (Allen et al., 1998; Srinivasan et al., 2003). Using a monolayer of primary fibroblasts in a scratch-induced migration assay, Cdc42 is also required for spatially localizing membrane protrusions at their leading edge, as well as for promoting reorientation of the Golgi/centrosome to face the direction of migration (Nobes and Hall, 1999). Further analysis of centrosome reorientation using primary astrocytes in a similar assay has revealed that Cdc42 mediates its effects through a complex containing Par6, a scaffold protein, and PKCζ, an atypical protein kinase C (aPKC) (Etienne-Manneville and Hall, 2001). Par6 is a direct target of Cdc42 and interestingly the same complex is involved in numerous other polarity-establishing processes, such as asymmetric cell division, epithelial cell morphogenesis and axon formation in neurons (Etienne-Manneville and Hall, 2003b; Henrique and Schweisguth, 2003; Shi et al., 2003).

We now show that the Cdc42/Par6/aPKC pathway is also required for centrosome/Golgi reorientation in fibroblasts, but

that this pathway does not control the spatial localization of protrusive activity. The latter is instead controlled by a Cdc42-dependent activation of the Ser/Thr kinase Pak, which controls the localization of Rac activity through recruitment of its associated βPIX Rac-GEF to the front of the cell. We conclude that Cdc42 controls the polarity of the actin and microtubule cytoskeletons during cell migration through two distinct signal transduction pathways.

Materials and Methods

Reagents

Unless specified, all reagents were from Sigma-Aldrich. Rabbit polyclonal anti-MYC, JAC6 rat monoclonal anti-MYC, 16B12 mouse monoclonal anti-HA and 3F10 rat monoclonal anti-HA were from RDI, Serotec, Cancer Research UK and Roche respectively. Mouse monoclonal anti-p115, anti-βPIX and Cdc42/Rac1 antibodies were from Cancer Research UK, Transduction laboratories and Upstate Technologies, respectively. CPITC- or Alexa Fluor 350 phalloidin were from Sigma and Molecular Probes. Rabbit polyclonal antibodies against panPIX (directed against the αPIX SH3 domain and cross-reacting with rat α and βPIX) and phospho-Pak (directed against pS199/204 of hPak1, but also cross-reacting with pS192/197 of hPak2) were kind gifts from G. Bokoch (Stofega et al., 2004) and M. E. Greenberg (Shamah et al., 2001). Rabbit polyclonal anti-PKCζ was from Santa Cruz Biotechnologies. Rabbit polyclonal Pak1 and Pak2 were from Cell Signaling Technology. Secondary antibodies coupled to HRP, FITC, Cy5 and Texas Red were from Jackson Immunotech. Those coupled to Alexa Fluor 488 and Alexa Fluor 568 were from Molecular Probes. PKC inhibitors (from Calbiochem, 5 μM GF109203X or 10 μM Ro318220) or GSK-3 inhibitor (20 μM SB216763, Tocris, Bristol, UK) were used to block Golgi reorientation.

Cloning procedure for constructs

pRK5-MYC::Cdc42N17, RacN17 and WASp-CRIB have been described previously (Nobes and Hall, 1999). pMT21::PKCζ wt and KD have been described by Etienne-Manneville and Hall (Etienne-Manneville and Hall, 2001). pXJ40-HA::wt βPIX and DH* (L238R,L239S) have been described by Manser et al. (Manser et al., 1998). pEGFP-F (Clontech) encodes enhanced GFP fused to the 20-amino-acid farnesylation signal from c-Ha-Ras. pRFPC1 and pRFP-F were built by removing the *Nhe*I/*Bsp*EI GFP insert of pEGFPC1 and pEGFP-F and ligating a PCR fragment encoding monomeric RFP (from pRSET-mRFP, a gift from R. Tsien, UCSA, San Diego, CA). PAR6 wt and Nt (aa 2-130 of hPar6C) were cloned by PCR into *Bam*HI/*Eco*RI sites of pRK5-

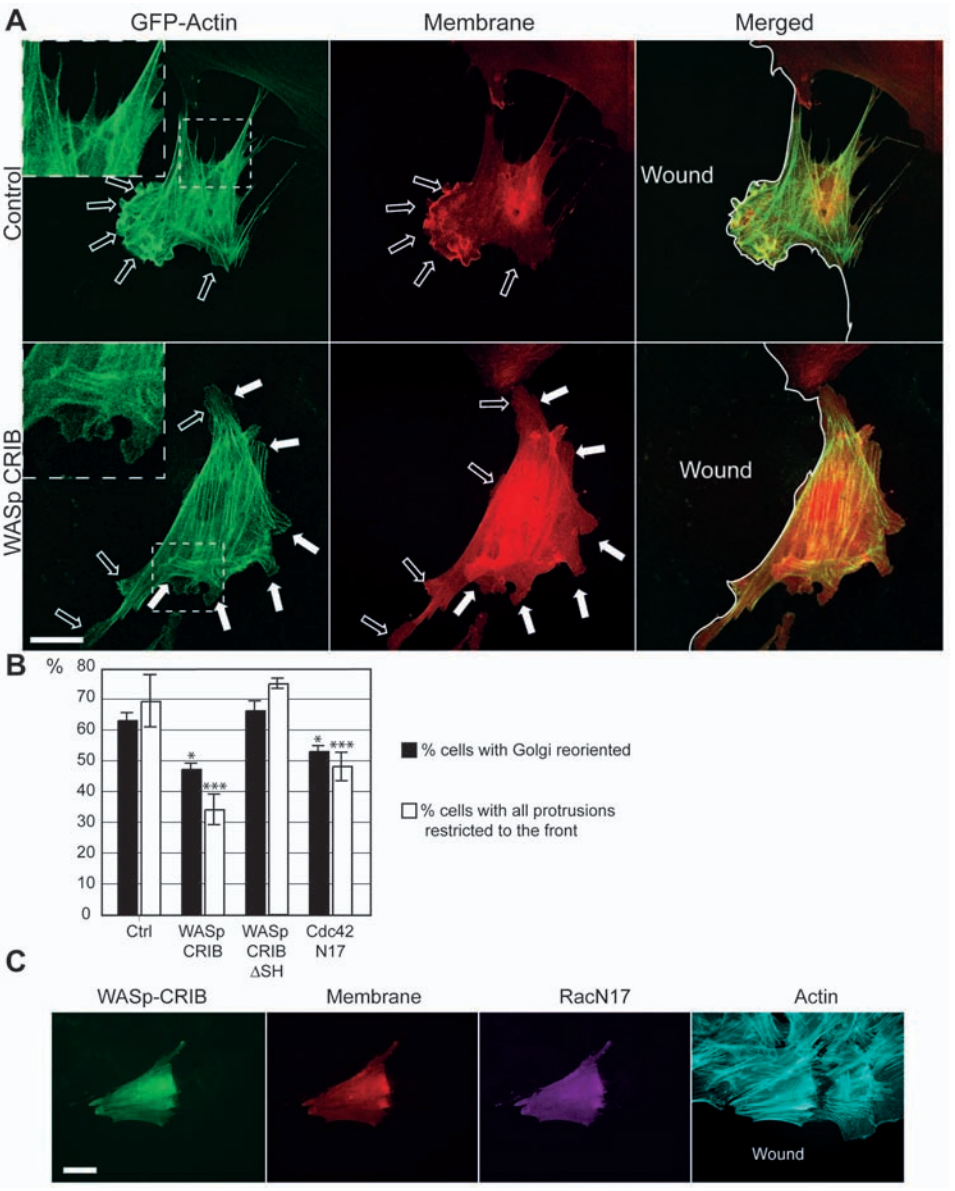

Fig. 1. Cdc42 controls Golgi reorientation and the polarized localization of Rac-dependent protrusions. (A) A representative cell injected with pcDNA3::GFP-β-actin (Actin) and pRFP-F (Membrane) along with either control pRK5-MYC or pRK5-MYC::WASp CRIB. Expression of MYC-WASp-CRIB was confirmed by immunofluorescence (not shown). The front edge of the scratched monolayer is visualized with phalloidin staining (not shown) and is indicated on the merged image as a white line. Protrusions, identified by their typical lamellipodium-like, smooth convex shape, are normally found at the front (open arrows), but upon inhibition of Cdc42 by WASp-CRIB expression, protrusions are depolarized and can now be seen at the back (filled arrows). Insets are magnified (2×) views of the boxed areas. (B) The effects of various constructs on Golgi reorientation (black bars) or protrusion polarization (white bars). Error bars represent standard deviation. Asterisks mark significant (*P<0.01) and highly significant (***P<0.0001) differences compared with control.
(C) Representative cell co-injected with pEGFP::WASp-CRIB, pRFP-F and pRK5-MYC::RacN17. The cell shows no typical protrusions. Bars, 20 μm.

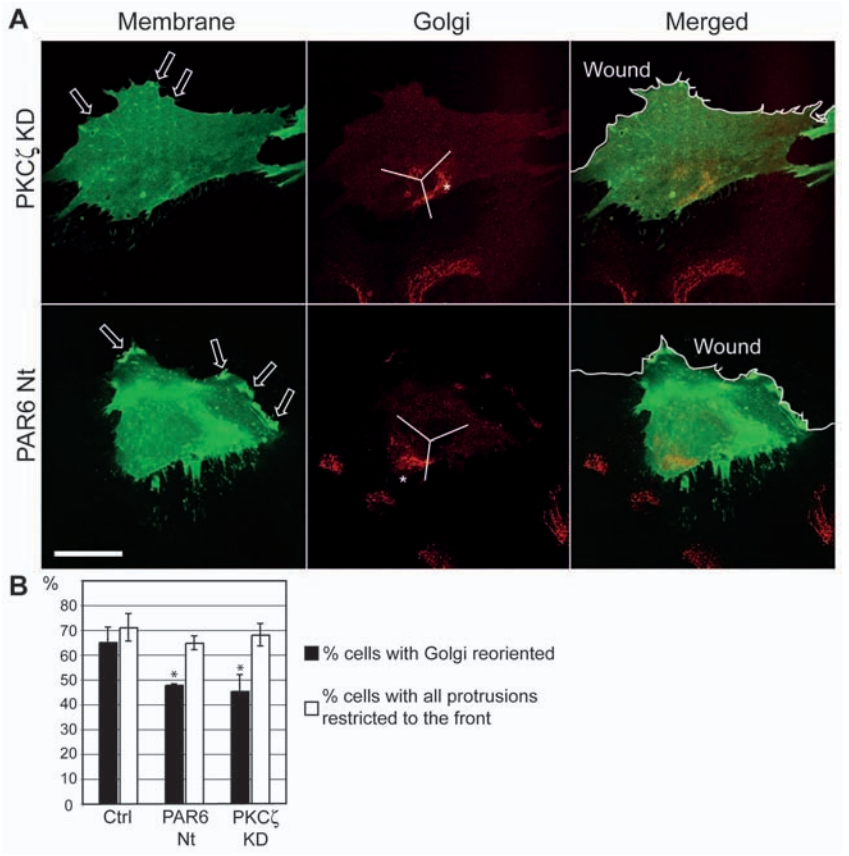

Fig. 2. The Par6/aPKC pathway controls Golgi reorientation but not localization of protrusions. (A) A representative cell injected with pEGFP-F (membrane) and either pMT21::PKCζ kinase dead (KD) or pRK5-MYC::Par6 Nt. Expression was checked by immunofluorescence (not shown). Protrusions are mainly found at the front (open arrows), while Golgi (asterisk) reorientation into the 120° sector facing the wound is inhibited. (B) Effects of various constructs on Golgi reorientation (black bars) or protrusion polarization (white bars). Error bars represent standard deviation. Asterisks mark significant (**P*<0.01) and highly significant (***P*<0.001) differences compared with control. Bars, 20 μm.

MYC. WASp-CRIB (aa 201-321 of hWASp), AID (aa 83-149 of hPak1), AID L107F Pak1, PID100 (aa 147-231 of hPak1), βPIX SH3 (aa 11-63 of rat βPIX) and βPIX SH3* (W43P/W44G) were either sub-cloned from existing constructs or cloned by standard PCR techniques into *Bgl*II/*Eco*RI sites of pEGFPC1 (Clontech) and pRFPC1. All constructs were carefully sequenced. Full constructs, maps and primer sequences are available upon request.

Cell culture and microinjection

Primary rat fibroblasts were prepared as described previously (Nobes and Hall, 1999) with some modifications: only passage two cells were used and cells were plated on n1.5 coverslips (Marienfield), 35 mm glass-bottom dishes (Mattek), or for biochemistry on 92 mm dishes, at a density of 4×10^4 cells/cm^2. Cells were then incubated for 72 hours and injected as previously described (Nobes and Hall, 1999). DNA concentration at injection was either 0.15 μg/μl for tracer constructs such as pEGFP-F, pRFP-F, pcDNA3::GFP-βactin, pEGFP-Cofilin or for single injection of pEGFP-AID±L107F. When double and triple co-injection experiments were carried out, DNA concentration of the plasmid of interest was 0.4 μg/μl to allow expression as early as 1 hour post-injection. Cells were injected exactly 30 minutes after wounding for no more than 30 minutes.

Immunofluorescence and microscopy

Cells were washed once in BRB80 (2 mM EGTA, 2 mM $MgCl_2$, 80 mM K-Pipes pH 6.8), and fixed in 4% paraformaldehyde (PFA) in BRB80 4.5 hours after scratching. 0.2% saponin was added to the wash and fixative to reduce background during staining for endogenous PIX with the panPIX antibody. Unreacted PFA was quenched, cells were permeabilized and non-specific binding blocked by incubating cells for 1 hour in 2% BSA 0.2% saponin in BRB80. This buffer was further used for all washings and incubations. Images were taken with a MRC1024 (Bio-Rad) confocal OptiphotII (Nikon) microscope using a 60× planapochromatic objective (NA 1.4) and a Kr/Ar laser (Fig. 1A, Fig. 2A and Fig. 3C). Otherwise, quadruple channel images were taken with a Axioplan microscope using a 63× planapochromatic oil immersion objective (NA 1.4) and Zeiss FS02 (Ex G365 Dichroic FT395 Em LP420), FS10 (Ex BP450-490 Dichroic FT510 Em BP515-565), FS43 (BP545/25 Dichroic FT570 Em BP605/70) and FS26 (Ex BP575-625 Dichroic FT645 Em BP660-710) filter sets. The same range of fluorochrome intensity was empirically set-up to minimize bleed-through of RFP (red fluorescent protein), when used, into the far-red channel due to unequal intensities. Acquisition was performed with an ORCA-ER (Hamamatsu) camera driven by Openlab software (Improvision). For video-microscopy, an average of five cells were imaged on an inverted fully motorized Axiovert 200M microscope fitted with a temperature-controlled Plexiglas box and an anti-vibration table (Improvision). Images were acquired using a 40× oil immersion planapochromatic objective (NA 1.35), Zeiss FS37 (Ex BP450/50 Dichroic FT480 Em BP510/50) and FS00 (Ex BP530-585 Dichroic FT600 Em LP615), Ludl transmission- and in-built fluorescence shutters, an ORCA-ER camera (Hamamatsu), and the Openlab acquisition software. Images were analysed and processed for presentation with Metamorph (UIC).

Quantification of Golgi and protrusion polarization

All fixations were performed 4.5 hours after scratching. The cell periphery was visualized with phalloidin staining and indicated on the merged images as a white line. For the analysis, all cells located behind the first row or without detectable expression of the construct of interest were automatically excluded. Protrusions, detected with GFP-β-actin or farnesylated, membrane-targeted GFP or RFP, have a typical smooth, convex shape very different from retraction fibres or retracting concave edges usually observed at the back of control cells. Cells with no protrusions (less than 10% at 3 days after plating) were excluded. Golgi reorientation was monitored using an antibody directed against the cis-Golgi protein p115. Cells where Golgi localization was difficult to interpret because the Golgi was dispersed, or because cells were bi-nucleate were not taken into account. Mean values represent a minimum of three independent experiments and a minimum of 180 cells. Error bars are standard deviations. Statistical significance was assessed with a two-tailed Fisher test. Unless specified by asterisks, differences with control were not significant (P>0.05).

RNAi experiments

All siRNAs used were from Ambion, obtained at standard purity.

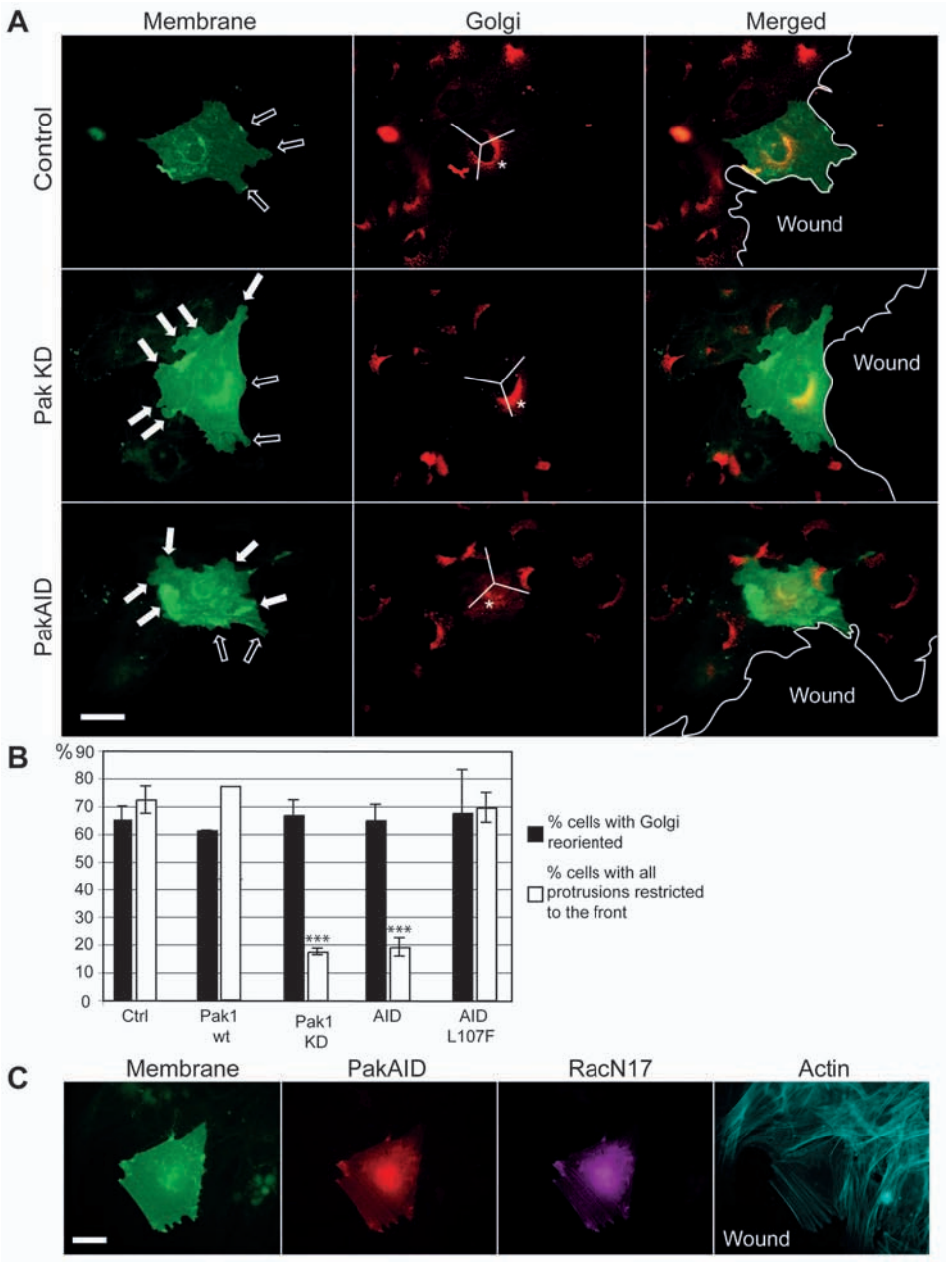

Fig. 3. Pak kinase is required for polarization of protrusions but not Golgi reorientation. (A) Pak kinase activity controls the spatial localization of protrusions. Representative cells injected with pEGFP-F (membrane) along with a control plasmid, pRFP::Pak1 K299A (Pak KD), or pRFP::Pak1 AID (PakAID). Expression of constructs was confirmed by immunofluorescence (not shown). Protrusions are found at the front (open arrows) in control cells, but are delocalised in cells in which Pak is inhibited (filled arrows). None of the constructs inhibit Golgi reorientation (asterisk). (B) The effects of various constructs on Golgi reorientation (black bars) or protrusion polarization (white bars). Error bars represent standard deviation. Asterisks mark highly significant (***P<0.0001) differences compared with control. (C) The delocalized protrusions, induced after Pak inhibition are dependent on Rac. Representative cell injected with pEGFP-F (Membrane), pRFP::Pak1 AID and pRK5-MYC::RacN17. Bars, 20 μm.

Cells were trypsinized, resuspended in 10% FCS/DMEM (Gibco) and centrifuged. Pellets were then washed with PBS at room temperature, and cells were centrifuged again to remove any traces of DMEM. 10^6 cells were resuspended in 100 μl of nucleofection solution V (Amaxa). 360 pmol siRNA against mock (GFP-22 siRNA, Qiagen) or the Rac guanine nucleotide exchange factor βPIX (GGGUUCG-AUACGACUGCCAtt) were added and cells electroporated with program G-09. Another siRNA for βPIX gave the same results (UCUAUCAGGAUGAUAAUCCtc). Cells were rapidly resuspended in 10% FCS/DMEM + 10 mM Hepes pH 7.2 (Gibco) and left in suspension for 1 hour at 37°C. Cells were then centrifuged, resuspended in 10% FCS/DMEM and seeded at 4-5×10^4 cells/cm^2 and left for 4 days, rather than the usual 3 days, to allow maximal depletion. Cells were then either lyzed in immunoprecipitation lysis buffer (IPLB) [10 mM Tris pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% IGEPA®, 1 mM sodium orthovanadate supplemented with 2 mM phenyl methyl sulfonyl fluoride (PMSF) and one Roche complete tablet/50 ml] or scratched and microinjected. For rescue experiments, cells were nucleofected with βPIX siRNA and injected 4 days later with pXJ40-HA::ratβPIX wt.

Immunoprecipitations and western blots

Cell lysates were prepared from confluent monolayers (in 92 cm^2 dishes) that were scratched 200 times with a multi-tip Pipetman fitted with P2 tips, (approx. 1 mm wide srcatches). Cells were washed with PBS and lyzed in 18 μl/cm^2 of cold IPLB. 100 μg protein were used for immunoprecipitation with protein G-Sepharose beads and 1 μl Pak1 antibody. Beads were further washed with cold IPLB (10 mM Tris pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% IGEPAL, 1 mM sodium orthovanadate supplemented with 2 mM PMSF and one Roche complete tablet/50 ml). 20-25 μg were used for western blotting of total extracts. Extracts were processed using standard SDS-PAGE and western blotting procedures.

Results

Cdc42 controls reorientation of the Golgi and localization of Rac-dependent protrusions

Directed migration was induced in primary rat embryo fibroblasts (REFs) by scratching a confluent monolayer. Immediately after scratching, the orientation of the Golgi with respect to the direction of migration is random with 33% of leading edge cells having their Golgi in a virtual sector of 120° drawn to face the wound. 4.5 hours later, the Golgi is polarized towards the direction of migration in 65% of the cells. Expression of either dominant-negative Cdc42 (N17Cdc42), or

the Cdc42-binding domain of WASp (WASp-CRIB) inhibits reorientation of the Golgi (Fig. 1B) and the centrosome (data not shown) (Nobes and Hall, 1999). The same migrating cells extend actin-rich protrusions specifically at their leading edge (Fig. 1A, top panels and B), which can be seen with phase-contrast optics, or more clearly after expression of either GFP-β-actin or membrane-targeted RFP. 4.5 hours after scratching, 70% of migrating leading edge cells show protrusions only at the front (Fig. 1A, open arrows and Fig. 1B) and the remaining 30% have additional minor protrusions at the back and sides of the cell. After microinjection of WASp-CRIB, only 34% of cells have protrusions restricted to the front and now the majority of cells have extensive, delocalized actin-rich protrusions all around the cell periphery (Fig. 1A, filled arrows, Fig. 1B). Dominant-negative Cdc42 also results in delocalization of leading edge protrusions (see Movies 1 and 2 in supplementary material). A WASp-CRIB domain lacking two key residues necessary for Cdc42 binding (WASp-CRIB ΔSH) has no effect (Fig. 1B). Rac controls actin polymerization and the formation of protrusions in many cell types. We find that expression of dominant negative Rac (RacN17) inhibits localized protrusive activity in control cells (data not shown) as well as delocalized protrusive activity in cells co-injected with WASp-CRIB (Fig. 1C). In addition, delocalized protrusions found in cells after inhibition of Cdc42 were as sensitive to cytochalasin D as protrusions in control cells (data not shown), showing they are dependent on actin polymerization. We conclude that the protrusive activity is mediated through Rac-dependent actin polymerization, while localization of the protrusive activity is mediated by Cdc42.

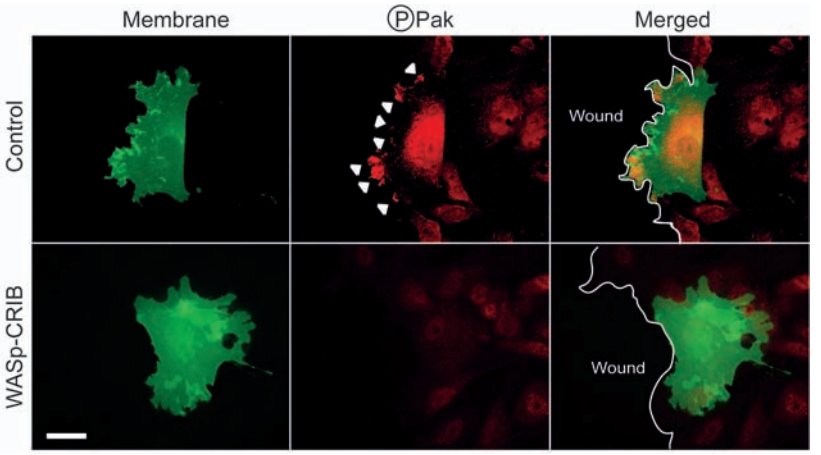

Fig. 4. Cdc42-activated Pak is concentrated at the leading edge. Active Pak is localized at the front of the cell upon wounding. Phospho-Pak (PPak) is specifically detected at the front of cells microinjected with a wild-type pRK5-MYC::Pak1 construct (not shown), in discrete foci that are distinct from thick ruffled areas of the cell. Upon inhibition of Cdc42 by co-injection of pRK5-HA::WASp CRIB (not shown), Pak is no longer activated (expression level of pRK5-MYC::Pak1 wt is comparable to control cells, not shown). Bars, 20 μm.

Par6 and aPKC mediate Golgi reorientation, but not the localization of protrusions

In migrating astrocytes, Cdc42 activates Par6/aPKC to induce inactivation of glycogen synthase kinase 3 (GSK-3) and promote reorientation of the centrosome/Golgi (Etienne-Manneville and Hall, 2003a). To determine whether the same pathway operates in primary fibroblasts to control actin and microtubule cytoskeleton polarization, we expressed either the domain of Par6 necessary for aPKC binding (Par6 Nt), or a kinase dead (KD) version of the aPKC PKCζ (Fig. 2A,B). Both constructs inhibit Golgi reorientation, but neither interferes with the polarized localization of protrusions. Small molecule inhibitors of PKCs (GF109203X, Ro318220), or of GSK-3 (SB216763) produced similar inhibition of Golgi reorientation, but had no effect on protrusions (data not shown). We conclude that the signal transduction pathway linking Cdc42 to Golgi reorientation is conserved between astrocytes and fibroblasts, but that the localization of protrusions is mediated by a distinct pathway.

Pak controls the localization of Rac-dependent protrusions, but has no effect on Golgi reorientation

p21-activated kinase (Pak), one of the first targets of Cdc42 and Rac to be identified, has been implicated in regulating the actin cytoskeleton. In particular, it influences persistence during the random migration of fibroblasts (Sells et al., 1999). Thus, to determine whether Pak plays a role in polarization during cell migration, we microinjected leading edge cells with Pak constructs. Kinase dead (Pak1 KD), but not wild type (Pak1wt) constructs, dramatically inhibit localized protrusions (Fig. 3A,B), with 83% of injected cells now showing delocalized protrusions all around the cell periphery. Neither construct had any effect on Golgi reorientation (Fig. 3A,B). Similar results were obtained with Pak2 constructs (data not shown). Pak1 KD is still able to interact with a large number of proteins, and, therefore, to determine specifically whether its kinase activity is required, we expressed an auto-inhibitory domain derived from Pak1 (PakAID), which has been shown to interact with and inhibit the kinase activity of Paks 1, 2 and 3 (Zenke et al., 1999; Zhao et al., 1998). PakAID also dramatically increased the number of cells with delocalized protrusions (Fig. 3A,B, and Movie 3 in supplementary material). A control construct containing a point mutation that blocks its activity had no effect (L107F, Fig. 3B). Moreover, dominant negative Rac blocked the formation of delocalized protrusions seen after expression of PakAID (Fig. 3C). We conclude that Pak controls the localization of Rac activity at the front of the cell, but that neither Pak kinase activity nor Cdc42 are required for Rac activation, during scratch-induced cell migration.

Pak is activated by Cdc42 at the front of the cell

To examine the localization of Pak during scratch-induced migration, we used antibodies to visualize active, auto-phosphorylated Pak. As previously reported with overexpressed constructs (Sells et al., 2000), phosphorylation of endogenous Pak1/2 on S199-S204/S192-S197 increases upon wounding (see Fig. S1 in supplementary material) and phosphorylated wild-type Pak1 is found concentrated in discrete puncta at the leading edge of migrating cells (Fig. 4).

The fluorescence signal disappears after expression of either PakAID (see Movie S4 in supplementary material), or WASp-CRIB (Fig. 4), showing that it is dependent on both Pak and Cdc42 activities. Expression of dominant negative Rac, blocked the formation of protrusions, but it did not affect Pak activation (see Fig. S2 in supplementary material), showing that Pak does not act downstream of Rac in this system. We conclude from this experiment that Pak is activated at the front of the cell by Cdc42.

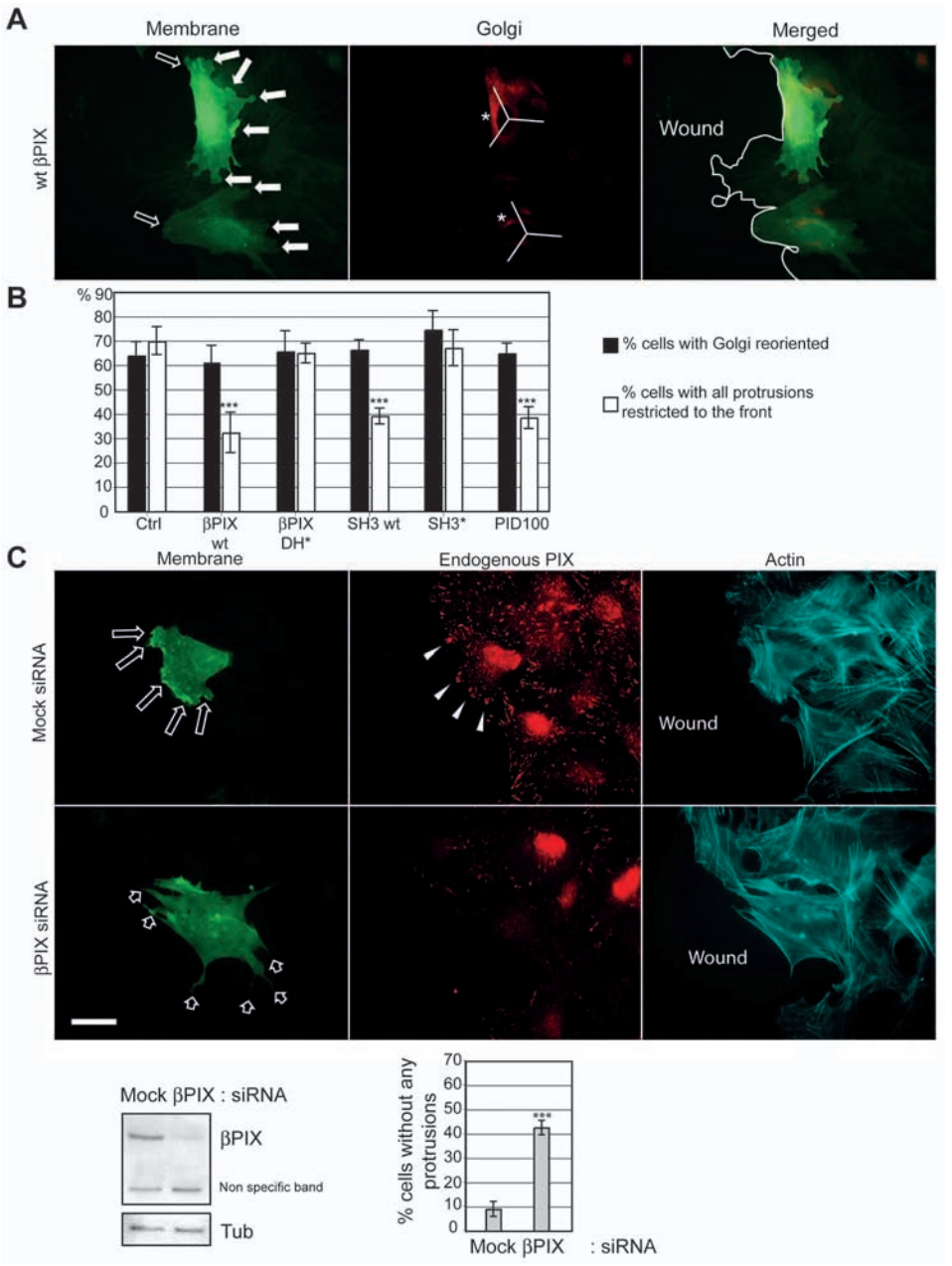

Fig. 5. βPIX accumulates at the front of the cell to confer localized Rac activation. (A) βPIX controls the formation of protrusions. Representative cells injected with pEGFP-F (Membrane) along with pXJ40-HA::wt βPIX. Expression of constructs was confirmed by immunofluorescence (not shown). Protrusions are found at the front (open arrows) and also at the back (filled arrows). Expression of wt βPIX has no effect on Golgi reorientation (asterisk). (B) Interfering with the endogenous Pak/βPIX interaction blocks polarization of protrusions. Effects of various GFP-fusion constructs on Golgi reorientation (black bars) or protrusion polarization (white bars). (C) Down-regulation of βPIX affects formation of the protrusions. 4 days after nucleofection with siRNA, monolayers were scratched and front row cells injected with pGFP-F to determine the localization of protrusions. siRNA-mediated down-regulation of βPIX was visualized by western blotting (bottom left). The representative control cell extends protrusions at the front (open arrows) and has βPIX localized in front focal-complex-like puncta (arrowheads). Down-regulation of βPIX increases the proportion of cells having no detectable protrusions (bottom panel). Instead, cells have elongated, thick filopodia-like extensions (small open arrows). In A and B, error bars represent standard deviation and asterisks mark a highly significant (***P<0.0001) difference compared with control. Bars, 20 μm.

Pak binding to βPIX is required to restrict protrusions to the front of migrating cells

To explore the mechanism by which Cdc42/Pak localizes protrusions to the front, we examined βPIX/p85^{COOL-1}, a guanine nucleotide exchange factor (GEF) for Rac that is known to interact directly with Pak (Bagrodia et al., 1998; Manser et al., 1998; Obermeier et al., 1998). Expression of wild-type (but not catalytically dead, DH*) βPIX in leading edge cells induces delocalized protrusions all around the cell periphery (Fig. 5A,B). To block the interaction of endogenous Pak1/2 and βPIX, we expressed either the βPIX-binding domain of Pak1 [PID100 (Obermeier et al., 1998)] or the Pak-binding SH3 domain of βPIX [SH3wt (Obermeier et al., 1998)] (Fig. 5B). Both constructs induced significant delocalization of protrusions, while a control SH3 mutant unable to bind Pak had no effect (Fig. 5B, SH3*) (Obermeier et al., 1998). We conclude that an interaction between βPIX and Pak is necessary for the correct localization of protrusions, but not for the formation of protrusions.

To analyse further the role of endogenous βPIX during scratch-induced cell migration, we used an RNAi approach. Western blot analysis (Fig. 5C, bottom-left panel) reveals an 80-90% reduction in βPIX protein after nucleofection. Immunofluorescence analysis after nucleofection reveals that the monolayer is heterogenous, with some cells lacking any detectable βPIX, while others still contain βPIX, albeit at reduced levels. When control monolayers were scratched 4 days after nucleofection with mock siRNA, only 10% of front row cells showed no detectable protrusions (Fig. 5C, bottom right panel). However, after

nucleofection with βPIX siRNA, 42% of front row cells showed no detectable protrusions, but instead had elongated, thick filopodia-like extensions (Fig. 5C top panel and small open arrows in bottom-right panel). To analyse protrusion formation specifically in cells lacking βPIX, we examined βPIX levels in individual cells by immunofluorescence. We found that over 95% of the cells that completely lack detectable βPIX have no detectable protrusions. Importantly, siRNA nucleofection had no effect on Golgi reorientation, irrespective of the ability of cells to form protrusions (data not shown). We conclude that βPIX, is the major Rac-GEF required for Rac-dependent formation of protrusions.

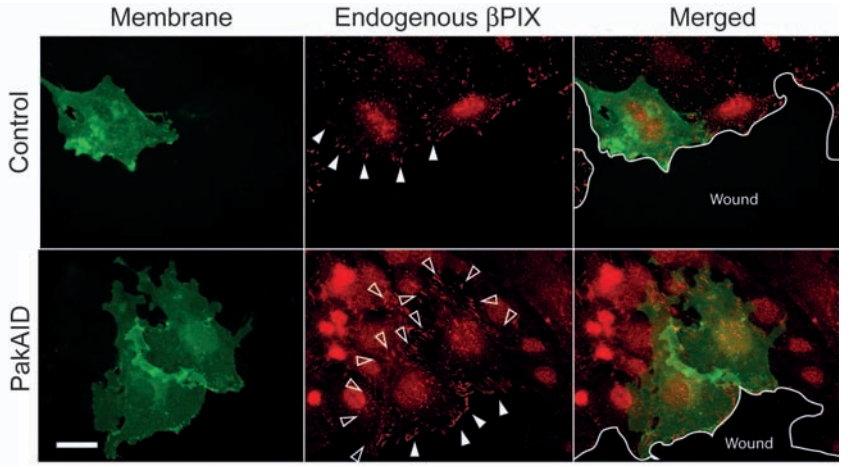

Fig. 6. Pak activity controls the specific localization of βPIX to the leading edge. Pak activity is needed to sequester βPIX at the front. Endogenous PIX is normally found in focal-complex-like puncta (filled arrowheads) located at the front in control-injected cells (top panel). Inhibition of Pak by injection of pEGFP::Pak1 AID (PakAID) delocalizes PIX throughout the cell [bottom panel; at the front (filled arrowheads) and also at the back (open arrowheads)]. Bars, 20 μm.

Pak activity is required to localize βPIX and Rac to the front of the cell

βPIX is found primarily in focal adhesion complexes (Manser et al., 1998). In control monolayers, βPIX can be seen in classical focal adhesions and peripheral focal complexes located throughout the cell (data not shown). After scratching, however, βPIX relocalizes and accumulates in focal complexes at the front of the cell (Fig. 5C, top panel, Fig. 6, top panel). After expression of PakAID in front row cells (but not inactive L107F AID, data not shown), βPIX localization is depolarized and accumulates in adhesion complexes all around the cell periphery (Fig. 6, bottom panel, arrowheads, the membrane localization of GFP-F outlines the limits of the two injected cells). We conclude from these experiments that Pak activity controls the specific accumulation of βPIX at the front of the migrating cells and thus restricts Rac activation towards the direction of migration.

Discussion

We show that upon scratching a fibroblast monolayer, Cdc42 activates Pak1/2 specifically at the front of leading edge cells to promote the recruitment of βPIX and the localized activation of Rac (Fig. 7). The activation of βPIX and Rac are not, however, dependent on Cdc42 or Pak kinase. Cdc42 activates another signal transduction pathway at the front of these cells leading to activation of the Par6/aPKC complex. This second pathway promotes the assembly of distinct molecular complexes at the leading edge that capture microtubule plus ends, leading to polarization of the microtubule cytoskeleton, the centrosome and the Golgi (Fig. 7) (Etienne-Manneville and Hall, 2003a). We have noticed that expression of dominant negative Cdc42 has a milder effect than expression of the CRIB domain of WASp, kinase dead aPKC or PakAID. This could be due to only partial inhibition of Cdc42, but an alternative interesting possibility is that another small GTPase can act similarly to Cdc42. In fact, the CRIB domain of WASp, Pak1 and Par6 also bind to TCL, TC10, Wrch and Chp to varying extents (Aspenstrom et al., 2004). This possibility is currently under investigation.

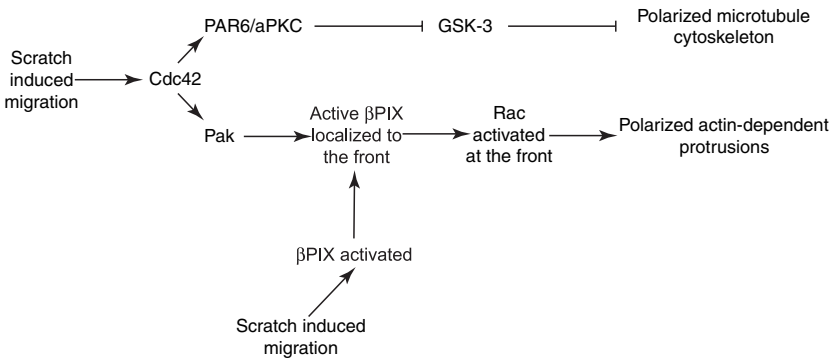

Fig. 7. Cdc42 controls two distinct polarity pathways in migrating cells. Microtubule cytoskeleton polarization occurs through activation of Par6/aPKC and subsequent inhibition of GSK-3, whereas Pak controls the localization, though not the activity, of the Rac-GEF βPIX, allowing the polarization of actin-rich, Rac-dependent protrusions.

βPIX is usually found in central focal adhesions and peripheral focal complexes (Manser et al., 1998). We think it is probable that immediately after scratching the monolayer, βPIX is activated and targeted to the cell periphery in an unpolarized fashion. Localized activation of Cdc42 and Pak then promotes the accumulation or retention of active βPIX at the front of the cell. The relationship between Pak and PIX with respect to localization is complex and contradictory. On one hand, PIX complexed with GIT (a member of the adhesions-bound Arf-GAP protein family) has been reported to target Pak to focal complexes (Manabe Ri et al., 2002; Manser et al., 1998; Stofega et al., 2004; Turner et al., 1999). On the other hand, it has been found that localization of Pak to focal complexes is mediated by Nck or paxillin, i.e. independently of PIX (Bokoch et al., 1996; Hashimoto et al., 2001; Zhao et al., 2000a), and that Pak then

localizes PIX/GIT complexes (Zegers et al., 2003; Zhao et al., 2000b). Such Pak-induced sequestration of βPIX has previously been documented in MDCK cells (Zegers et al., 2003). The identity of the Pak substrate involved in localization of βPIX in these scratched fibroblast monolayers is not clear. βPIX is itself a substrate, although the significance of this is unknown – phosphorylation on S525 and T526 for example is not thought to affect its Rac-GEF activity (Koh et al., 2001; Shin et al., 2002). Paxillin and GIT are also substrates of Pak (Bokoch, 2003) and it is possible that βPIX, GIT and/or paxillin phosphorylation by Pak modulates βPIX interactions with other molecules, affecting localization to the membrane and incorporation into leading edge focal complexes. These different possibilities are currently being examined.

Pak has been implicated in cell migration of many cell types in *Drosophila* and mammals (Adam et al., 2000; Harden et al., 1996; Kiosses et al., 1999; Kiosses et al., 2002; Sells et al., 1999; Vadlamudi et al., 2000) and there have been numerous reports linking Pak to the formation of protrusions, but in many cases the relationship has been unclear (Bokoch, 2003). For example, Pak affects the persistence of protrusions in cells randomly migrating (i.e. in the absence of any directional cue), but these protrusions were reported to be Rac-independent (Sells et al., 1999). Studies in *Dictyostelium* (Chung and Firtel, 1999; Labruyere et al., 2003) show that Paks are crucial for polarization of the actin cytoskeleton during directed migration, but it is not known how they themselves are polarized in the first place. Recently, Pak1 has been shown to be required for directional sensing in leukocytes (Li et al., 2003). In that case however, Pak1 acts as a scaffold protein required for the activation of Cdc42 by the GEF αPIX and by Gβ. The role of its kinase activity, if any, was not explored in this assay. αPIX is found mainly in haematopoietic cells and is not detectable in REFs (our unpublished results) and furthermore, RNAi knock-down of βPIX in REFs, or inhibition of Pak activity does not affect Golgi reorientation, suggesting that Cdc42 is still activated in cells lacking PIX or Pak kinase activity. We cannot rule out, however, the interesting possibility that Pak may play two roles in directional sensing; first as a scaffold in the activation of Cdc42 (kinase independent) and second as a mediator for polarization of Rac activity (kinase dependent). Further experiments will be required to address this issue.

In conclusion, Cdc42 orchestrates polarization of the actin and microtubule cytoskeletons through two different signal transduction pathways. A direct interaction with Par6 leads to activation of the associated atypical PKC and polarization of microtubules, while a direct interaction with Pak activates this kinase, leading to the localization of active βPIX/Rac and polarized actin polymerization.

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References

Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J. and Kumar, R. (2000). Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. *J. Biol. Chem.* **275**, 12041-12050.

Allen, W. E., Zicha, D., Ridley, A. J. and Jones, G. E. (1998). A role for Cdc42 in macrophage chemotaxis. *J. Cell Biol.* **141**, 1147-1157.

Aspenstrom, P., Fransson, A. and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem. J.* **377**, 327-337.

Bagrodia, S., Taylor, S. J., Jordon, K. A., van Aelst, L. and Cerione, R. A. (1998). A novel regulator of p21-activated kinases. *J. Biol. Chem.* **273**, 23633-23636.

Bokoch, G. M. (2003). Biology of the p21-activated kinases. *Annu. Rev. Biochem.* **72**, 743-781.

Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A. and Knaus, U. G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J. Biol. Chem.* **271**, 25746-25749.

Chung, C. Y. and Firtel, R. A. (1999). PAKa, a putative PAK family member, is required for cytokinesis and the regulation of the cytoskeleton in Dictyostelium discoideum cells during chemotaxis. *J. Cell Biol.* **147**, 559-576.

Del Pozo, M. A., Kiosses, W. B., Alderson, N. B., Meller, N., Hahn, K. M. and Schwartz, M. A. (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat. Cell. Biol.* **4**, 232-239.

Etienne-Manneville, S. (2004). Cdc42 – the centre of polarity. *J. Cell Sci.* **117**, 1291-1300.

Etienne-Manneville, S. and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489-498.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

Etienne-Manneville, S. and Hall, A. (2003a). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. *Nature* **421**, 753-756.

Etienne-Manneville, S. and Hall, A. (2003b). Cell polarity: Par6, aPKC and cytoskeletal crosstalk. *Curr. Opin. Cell Biol.* **15**, 67-72.

Funamoto, S., Meili, R., Lee, S., Parry, L. and Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* **109**, 611-623.

Harden, N., Lee, J., Loh, H. Y., Ong, Y. M., Tan, I., Leung, T., Manser, E. and Lim, L. (1996). A *Drosophila* homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol. Cell. Biol.* **16**, 1896-1908.

Hashimoto, S., Tsubouchi, A., Mazaki, Y. and Sabe, H. (2001). Interaction of paxillin with p21-activated Kinase (PAK). Association of paxillin alpha with the kinase-inactive and the Cdc42-activated forms of PAK3. *J. Biol. Chem.* **276**, 6037-6045.

Henrique, D. and Schweisguth, F. (2003). Cell polarity: the ups and downs of the Par6/aPKC complex. *Curr. Opin. Genet. Dev.* **13**, 341-350.

Iijima, M. and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* **109**, 599-610.

Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M. and Schwartz, M. A. (1999). A role for p21-activated kinase in endothelial cell migration. *J. Cell Biol.* **147**, 831-844.

Kiosses, W. B., Hood, J., Yang, S., Gerritsen, M. E., Cheresh, D. A., Alderson, N. and Schwartz, M. A. (2002). A dominant-negative p65 PAK peptide inhibits angiogenesis. *Circ. Res.* **90**, 697-702.

Koh, C. G., Manser, E., Zhao, Z. S., Ng, C. P. and Lim, L. (2001). Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. *J. Cell Sci.* **14**, 4239-4251.

Labruyere, E., Zimmer, C., Galy, V., Olivo-Marin, J. C. and Guillen, N. (2003). EhPAK, a member of the p21-activated kinase family, is involved in the control of Entamoeba histolytica migration and phagocytosis. *J. Cell Sci.* **116**, 61-71.

Li, Z., Hannigan, M., Mo, Z., Liu, B., Lu, W., Wu, Y., Smrcka, A. V., Wu, G., Li, L., Liu, M. et al. (2003). Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* **114**, 215-227.

Manabe Ri, R., Kovalenko, M., Webb, D. J. and Horwitz, A. R. (2002). GIT1 functions in a motile, multi-molecular signaling complex that regulates protrusive activity and cell migration. *J. Cell Sci.* **115**, 1497-1510.

Manser, E., Loo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan,

I., Leung, T. and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol. Cell* **1**, 183-192.

Merlot, S. and Firtel, R. A. (2003). Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J. Cell Sci.* **116**, 3471-3478.

Nelson, W. J. (2003). Mum, this bud's for you: where do you want it? Roles for Cdc42 in controlling bud site selection in Saccharomyces cerevisiae. *BioEssays* **25**, 833-836.

Nobes, C. D. and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* **144**, 1235-1244.

Obermeier, A., Ahmed, S., Manser, E., Yen, S. C., Hall, C. and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. *EMBO J.* **17**, 4328-4339.

Sells, M. A., Boyd, J. T. and Chernoff, J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145**, 837-849.

Sells, M. A., Pfaff, A. and Chernoff, J. (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. *J. Cell Biol.* **151**, 1449-1458.

Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A. et al. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell* **105**, 233-244.

Shi, S. H., Jan, L. Y. and Jan, Y. N. (2003). Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112**, 63-75.

Shin, E. Y., Shin, K. S., Lee, C. S., Woo, K. N., Quan, S. H., Soung, N. K., Kim, Y. G., Cha, C. I., Kim, S. R., Park, D. et al. (2002). Phosphorylation of p85 beta PIX, a Rac/Cdc42-specific guanine nucleotide exchange factor, via the Ras/ERK/PAK2 pathway is required for basic fibroblast growth factor-induced neurite outgrowth. *J. Biol. Chem.* **277**, 44417-44430.

Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D. and Bourne, H. R. (2003). Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. *J. Cell Biol.* **160**, 375-385.

Stofega, M. R., Sanders, L. C., Gardiner, E. M. and Bokoch, G. M. (2004). Constitutive p21-activated kinase (PAK) activation in breast cancer cells as a result of mislocalization of PAK to focal adhesions. *Mol. Biol. Cell* **15**, 2965-2977.

Turner, C. E., Brown, M. C., Perrotta, J. A., Riedy, M. C., Nikolopoulos, S. N., McDonald, A. R., Bagrodia, S., Thomas, S. and Leventhal, P. S. (1999). Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling. *J. Cell Biol.* **145**, 851-863.

Vadlamudi, R. K., Adam, L., Wang, R. A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M. C. and Kumar, R. (2000). Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. *J. Biol. Chem.* **275**, 36238-36244.

Zegers, M. M., Forget, M. A., Chernoff, J., Mostov, K. E., ter Beest, M. B. and Hansen, S. H. (2003). Pak1 and PIX regulate contact inhibition during epithelial wound healing. *EMBO J.* **22**, 4155-4165.

Zenke, F. T., King, C. C., Bohl, B. P. and Bokoch, G. M. (1999). Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *J. Biol. Chem.* **274**, 32565-32573.

Zhao, Z. S., Manser, E., Chen, X. Q., Chong, C., Leung, T. and Lim, L. (1998). A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol. Cell. Biol.* **18**, 2153-2163.

Zhao, Z. S., Manser, E. and Lim, L. (2000a). Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation. *Mol. Cell. Biol.* **20**, 3906-3917.

Zhao, Z. S., Manser, E., Loo, T. H. and Lim, L. (2000b). Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. *Mol. Cell. Biol.* **20**, 6354-6363.