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# Control of Cell Polarity and Chemotaxis by Akt/PKB and PI3 Kinase through the Regulation of PAKa

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## Summary

**We demonstrate that PI3 kinase and protein kinase B (PKB or Akt) control cell polarity and chemotaxis, in part, through the regulation of PAKa, which is required for myosin II assembly. We demonstrate that PI3K and PKB mediate PAKa's subcellular localization, PAKa's activation in response to chemoattractant stimulation, and chemoattractant-mediated myosin II assembly. Mutation of the PKB phosphorylation site in PAKa to Ala blocks PAKa's activation and inhibits PAKa redistribution in response to chemoattractant stimulation, whereas an Asp substitution leads to an activated protein. Addition of the PI3K inhibitor LY294002 results in a rapid loss of cell polarity and the axial distribution of actin, myosin, and PAKa. These results provide a mechanism by which PI3K regulates chemotaxis.**

## Introduction

Phosphatidyl inositols (PtdIns) are key mediators of intracellular signaling, required for controlling cell proliferation, actin cytoskeleton organization, and vesicle trafficking between intracellular organelles (Odorizzi et al., 2000; Toker and Cantley, 1997; Vanhaesebroeck and Waterfield, 1999). Ablation of phosphatidylinositol-3-kinase $\gamma$ (PI3K$\gamma$) function has demonstrated that PI3K$\gamma$ is also required for thymocyte development, chemotaxis of macrophage and neutrophils, and chemoattractant-mediated respiratory burst and activation of the serine/threonine kinase PKB (or Akt) in macrophage and neutrophils (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000; Vanhaesebroeck et al., 1999). Parallel studies in *Dictyostelium* have shown that the p110$\alpha$-related phosphatidylinositol-3-kinases PI3K1 and PI3K2 are required for proper development, pinocytosis, chemotaxis, and chemoattractant-mediated activation of PKB (Buczynski et al., 1997; Zhou et al., 1995, 1998; Meili et al., 1999; Funamoto et al., 2001).

Insights into the mechanism by which PI3K regulates chemotaxis derive from studies on PKB in mammalian leukocytes and *Dictyostelium* cells. PKB activation requires its translocation to the plasma membrane by binding of its PH domain to PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ produced upon activation of PI3K (Andjelkovic et al., 1997), leading to PKB activation (Leevers et al., 1999). In leukocytes and *Dictyostelium* cells, chemoattractants mediate PKB activation through a G-protein-coupled pathway that requires the activity of the respective PI3Ks (Meili et al., 1999; Stephens et al., 1996; Tilton et al., 1997). Chemoattractant stimulation of neutrophils and *Dictyostelium* cells results in a transient localization of a GFP fusion of the PH domains from the *Dictyostelium* and mammalian PKBs to the plasma membrane (Meili et al., 1999; Servant et al., 2000). When these cells are placed in a chemoattractant gradient, membrane localization of the PKB-PH-GFP fusion is restricted to the leading edge, as is the case for other PH-domain-containing proteins in *Dictyostelium* (Meili et al., 1999; Parent et al., 1998; Servant et al., 2000; Funamoto et al., 2001). In *Dictyostelium*, translocation of the PKB-PH domain GFP fusion is PI3K-dependent (Meili et al., 1999, 2000). Studies in *Dictyostelium* demonstrate that PKB is essential for proper cell polarization and movement in a chemoattractant gradient. Although these studies did not define the mechanisms by which PKB regulates cell polarity and chemotaxis, they provided the first direct link between PKB, PI3K, and chemotaxis.

Chemotaxing cells have a strongly biased axial polarity that is regulated through the differential control of the cytoskeleton in the front (leading edge) and posterior of the cell (Parent and Devreotes, 1999; Firtel and Chung, 2000; Sanchez-Madrid and del Pozo, 1999; Weiner et al., 1999). F-actin is localized to the leading edge and to a lesser extent to the posterior. Protrusion of pseudopodia results from the localized assembly of F-actin at the leading edge (Borisy and Svitkina, 2000; Weiner et al., 1999). In contrast, in a number of cell types (including leukocytes, lymphocytes, and *Dictyostelium*), myosin II assembly is localized to the rear cell body and along the lateral sides of the cell, where it is very important in defining axial polarity, retracting the posterior of the cell during chemotaxis, and biasing the direction of movement by repressing extensions of lateral pseudopodia through cortical tension (Clow and McNally, 1999; Sanchez-Madrid and del Pozo, 1999; Stites et al., 1998). Myosin II assembly and disassembly in response to extracellular signals is controlled by phosphorylation of Thr residues on the myosin II tail in *Dictyostelium* and some mammalian cell types (Egelhoff et al., 1993; van Leeuwen et al., 1999). Phosphorylation of these residues leads to myosin II fiber disassembly, whereas dephosphorylation leads to the formation of myosin II fibers.

We previously demonstrated that PAKa, a structural homologue of mammalian PAKs (p21-activated kinase), is essential for proper cell polarity, chemotaxis, and cytokinesis in *Dictyostelium* (Chung and Firtel, 1999). PAKa controls these processes, in part, by regulating myosin II assembly: *paka* null cells do not exhibit chemoattractant-mediated myosin II assembly, and expression of constitutively active PAKa results in hyper-assembly of myosin II and F-actin. *paka* and myosin II (*myoII*) null strains exhibit similar chemotaxis and cytokinesis defects (Chung and Firtel, 1999). PAKa does not directly phosphorylate myosin II and probably promotes myosin II assembly by negatively regulating myosin heavy-chain kinases (Chung and Firtel, 1999; Egelhoff et al., 1993). PAKa colocalizes with assembled myosin II in the posterior of chemotaxing cells and cleavage furrow of dividing cells. In chemotaxing cells, chemoattractants control PAKa's kinase activity and subcellular localization. Chemoattractant stimulation results in a rapid, transient increase in PAKa kinase activity and its association with

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the cytoskeleton. The localization of PAKa at the posterior of the cell and its association with the cytoskeleton, therefore, spatially restricts PAKa's site of action, leading to a localized myosin II assembly to this site. This localized myosin II assembly is key in maintaining cell polarity and cortical tension and retracting the posterior cell body (Clow and McNally, 1999; Egelhoff et al., 1996; Sanchez-Madrid and del Pozo, 1999; Stites et al., 1998).

In this study, we demonstrate that PAKa is an important downstream effector of the PI3K and PKB signaling pathway. We find that PAKa is phosphorylated by PKB in vitro, and this PKB phosphorylation site is essential for the in vivo regulation of PAKa kinase activity and its subcellular localization. In vivo, PAKa is not activated and exhibits an abnormal subcellular localization in either *pkbA* or *pi3k1/pi3k2* null cells. We suggest that direct phosphorylation of PAKa by PKB regulates PAKa and, in turn, controls cellular polarity and cell movement during chemotaxis. This model provides a mechanism by which PI3K and PKB regulate cell structure and chemotaxis. Consistent with this model, addition of the PI3K inhibitor LY294002 to chemotaxing cells results in a rapid loss of cell polarity, the actin/myosin cytoskeleton, and the posterior localization of PAKa.

## Results

### PKB and PI3K Are Required for Chemoattractant-Mediated Regulation of PAKa Subcellular Localization

PAKa has an N-terminal regulatory/localization domain, a $Rac1^{GTP}$ binding or CRIB domain, and a C-terminal kinase domain (Chung and Firtel, 1999). We previously demonstrated that the N-terminal domain of PAKa (N-PAKa, which lacks the CRIB and kinase domains; Figure 1A) is necessary and sufficient for PAKa's localization to the posterior cortex of polarized, chemotaxing cells. This enables us to use a GFP fusion of N-PAKa (N-PAKa-GFP) as a reporter to examine the dynamics of PAKa localization in response to stimulation with the chemoattractant cAMP (Figures 1B and 1C; Chung and Firtel, 1999). When this polarity is disrupted by globally stimulating cells (bathing the cells in cAMP to simultaneously activate all of the cell-surface cAMP receptors), PAKa and N-PAKa-GFP become uniformly distributed around the cells' periphery, suggesting a possible link between PAKa localization and cell polarity (Chung and Firtel, 1999).

Cells in which the genes encoding the two redundant PI3Ks, PI3K1/PI3K2, or PKB have been disrupted (*pi3k1/2* [strain MGP1, see Experimental Procedures] and *pkbA* null cells, respectively, do not exhibit a prominent axial polarity, even when placed in a chemoattractant gradient (Figures 1B and 1C) (Meili et al., 1999; Funamoto et al., 2001). Therefore, we investigated whether PAKa's subcellular localization is disrupted in these strains. In contrast to wild-type cells (strain KAx-3), N-PAKa-GFP is almost uniformly distributed along the cortex in both *pkbA* and *pi3k1/2* null cells, except for the positions of pseudopodia formation, where the presence of PAKa at the cortex is disrupted (Figure 1B). A similar distribution is observed with full-length PAKa in fixed cells (Figure 1C). These data suggest that PAKa localization might be regulated by PKB via a PI3K-dependent pathway. The absence of PAKa from the newly formed pseudopodia suggests that a disruption of the cortex and exclusion of PAKa activity from this site might be required for the reduction of cortical tension in this region, allowing dynamic ruffling and pseudopod extrusion.

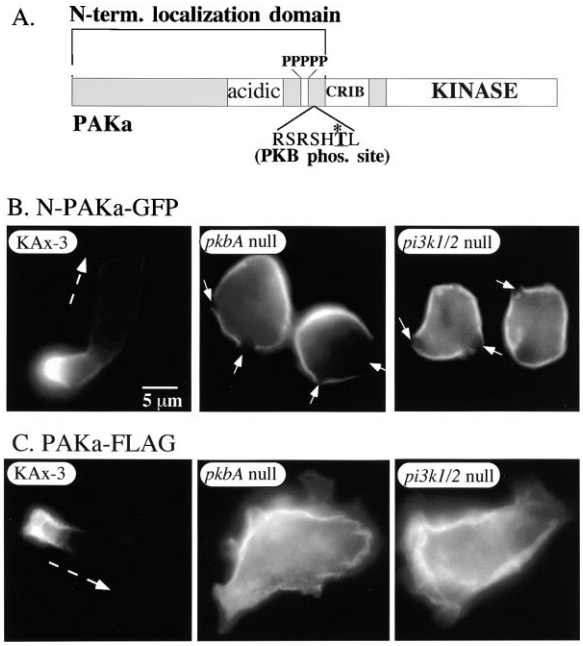



Figure 1. PKB and PI3K Regulation of the Subcellular Localization of PAKa

(A) Schematic diagram of PAKa domain structure.

(B) Localization of N-PAKa-GFP fusion protein in live wild-type, *pkbA* null, and *pi3k1/2* null cells (strain MGP1, which was used throughout the paper; see Experimental Procedures). The arrows show the position of forming pseudopodia in *pkbA* and *pi3k1/2* null cells. The dashed arrow indicates the direction of cell movement. Representative cells are depicted.

(C) Subcellular localization of PAKa-FLAG by indirect immunofluorescence.

### PAKa Is Phosphorylated by PKB in the N-Terminal Localization Domain

The minimum sequence motif for efficient phosphorylation by PKB is RXRXXS/T followed by a bulky hydrophobic residue (Andjelkovic et al., 1997). We identified a well-conserved PKB phosphorylation consensus sequence (RSRSH$T^{579}$L) within the N-PAKa targeting domain between the polyproline and CRIB domains (Figure 1A). To determine if this site can be phosphorylated by PKB in the context of the surrounding domains in PAKa, we made a GST-fusion protein, GST-N-PAKa*, containing the acidic, polyproline, and CRIB domains from PAKa. We tested the ability of activated PKB to phosphorylate this fusion protein in an in vitro phosphorylation assay. Figure 2Aa shows that PKB immunoprecipitated from cAMP-stimulated, wild-type cells phosphorylates GST-N-PAKa* but not GST. Control immunoprecipitates from stimulated *pkbA* null cells do not phosphorylate GST-N-PAKa*, suggesting that PKB is the kinase phosphorylating GST-N-PAKa* (Figure 2Ab). To determine if the putative PKB phosphorylate site is the potential site of this phosphorylation, we mutated Thr579 to Ala and tested whether it is a substrate for PKB in the in vitro kinase assay. As shown in Figure 2Ac, GST-N-$PAK^{*T579A}$ is not phosphorylated. The results

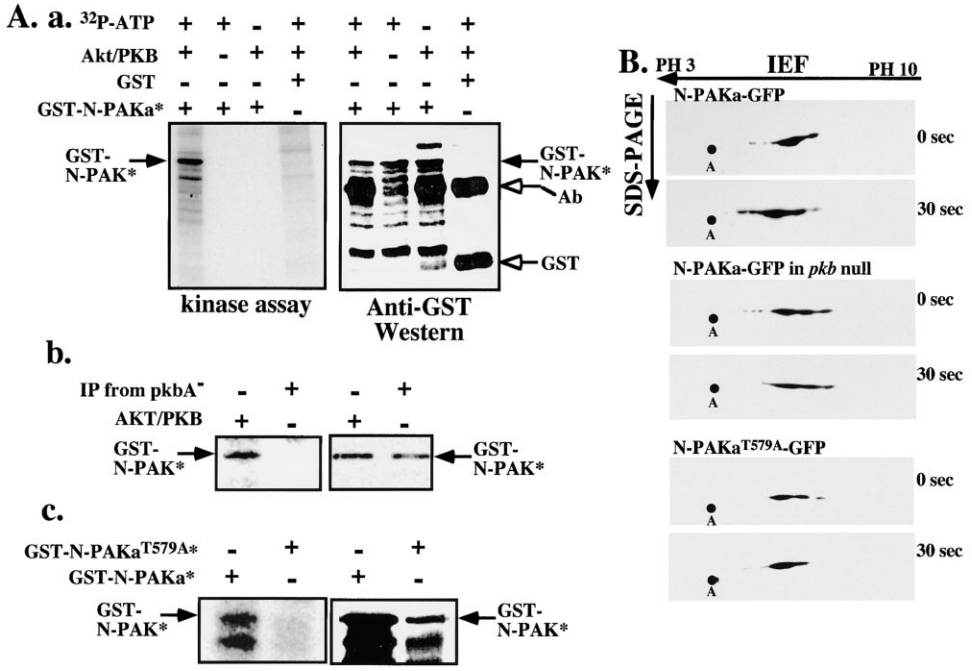



Figure 2. Phosphorylation of PAKa

(A) Direct phosphorylation of PAKa by PKB in vitro. (a) GST-N-PAK fusion protein is phosphorylated by PKB immunoprecipitated from the lysate of cAMP-stimulated cells (10 s after stimulation). (b) PKB immunoprecipitated from wild-type but not *pkbA* null cells phosphorylates GST-N-PAK. (c) Mutation of Thr579 to Ala in GST-N-PAK abolishes the phosphorylation by PKB.

(B) In vivo phosphorylation of Thr579 examined by two-dimensional (2D) electrophoresis. N-PAKa-GFP and N-PAKa$^{T579A}$-GFP were expressed in the respective cell lines. Cells were stimulated with cAMP and processed for 2D gels as described in Experimental Procedures. The GFP fusions were identified on Western blots with monoclonal anti-GFP antibody. The positions of N-PAKa-GFP in the gels were aligned using the positions of protein spots on the Coomassie blue-stained blots. The position of one such protein, labeled A, is shown.

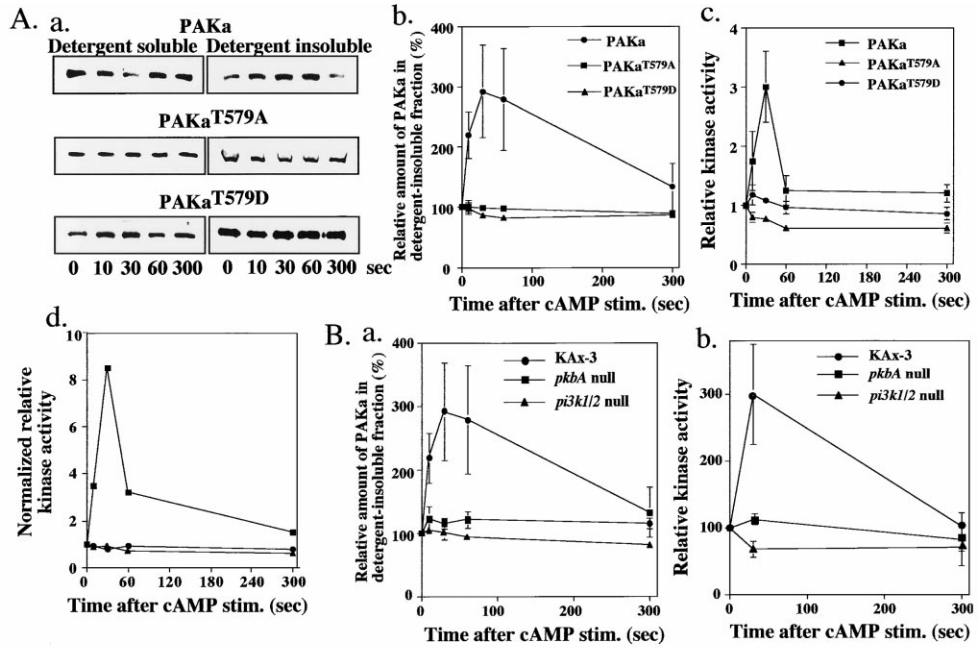
indicate that PAKa is an in vitro substrate for PKB and it is phosphorylated on Thr579.

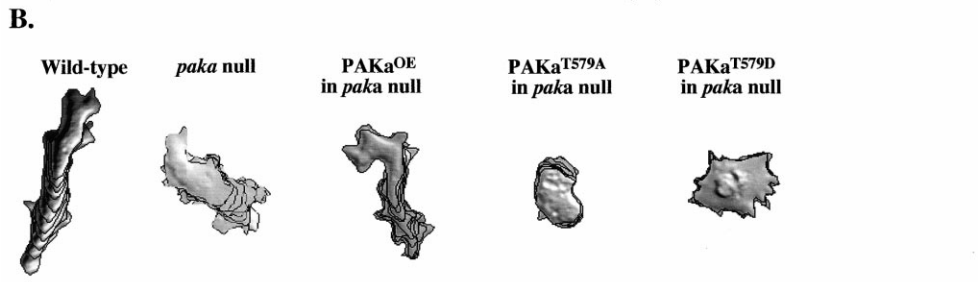
To determine if Thr579 is phosphorylated in vivo in response to cAMP stimulation, we used two-dimensional electrophoresis to examine changes in the isoelectric point (IP) of N-PAKa-GFP expressed in wild-type and *pkbA* null cells in response to cAMP stimulation (Figure 2B). We used N-PAKa-GFP in these studies, as it exhibits the same subcellular distribution in unstimulated cells and in response to cAMP stimulation as full-length PAKa and does not exhibit autophosphorylation. In unstimulated cells, N-PAKa-GFP runs as several spots in the isoelectric focusing gel (Figure 2B), which we expect is due to some N-PAKa-GFP phosphorylation, possibly resulting from the prior cAMP treatment. We treated cells with cAMP and took samples at 30 s, when PAKa activity and its translocation to the Triton-insoluble fraction are maximal (Chung and Firtel, 1999). Upon cAMP stimulation, we observed a shift of N-PAKa-GFP to a more acidic IP, consistent with chemoattractant-mediated phosphorylation of N-PAKa-GFP.

To examine the role of PKB and Thr579 in this process, we used *pkbA* null cells expressing N-PAKa-GFP. Only a very small fraction of N-PAKa-GFP shifts in IP in response to cAMP stimulation. We suggest that the low level of phosphorylation observed for N-PAKa-GFP in *pkbA* null cells results from PKBR-1, a related and partially redundant PKB protein that exhibits the same substrate specificity as PKB (Meili et al., 2000). We investigated whether the shift was dependent on Thr579. N-PAKa$^{T579A}$-GFP migrates predominantly as a doublet before stimulation and after stimulation; there is no change in its IP (Figure 2B), suggesting that Thr579 is the site of chemoattractant-mediated phosphorylation. As N-PAKa$^{T579A}$-GFP appears stable and exhibits some normal behaviors within cells (it localizes to the posterior of polarized cells), we think that N-PAKa$^{T579A}$-GFP is properly folded and the absence of phosphorylation is not the result of artifacts. The differences in the phosphorylation state of N-PAKa-GFP compared to N-PAKa$^{T579A}$-GFP suggest that the multiple phosphorylations observed on N-PAKa-GFP in wild-type cells require an initial phosphorylation on Thr579, to prime the other phosphorylations and/or to localize it in a subcellular domain where it can be phosphorylated by other kinases.

### Does Phosphorylation of Thr579 Regulate PAKa's Association with the Cytoskeletal Fraction?

Upon cAMP stimulation, PAKa translocates from the cytosolic, detergent-soluble fraction to the cytoskeletal, detergent-insoluble fraction (Figures 3Aa and 3Ab) (Chung and Firtel, 1999). The phosphorylation of Thr579 by PKB suggests that phosphorylation of Thr579 might be an important regulatory step for PAKa's subcellular localization and differential association with cellular components. To test this, we created two PAKa mutants, PAKa$^{T579A}$ and PAKa$^{T579D}$. The Thr→Ala substitution prevents phosphorylation,whereas the Thr→Asp substitution might mimic the phosphorylated state of Thr579. Mutation of Thr579 to Ala abrogates the ability of PAKa$^{T579A}$-FLAG to translocate to the cytoskeletal frac-




| | Wild-type | *paka* null | PAKa OE in *paka* null | PAKa$^{T579A}$ in *paka* null | PAKa$^{T579D}$ in *paka* null |
|---|---|---|---|---|---|
| Speed (μm/min) | 7.9 ± 2.6 | 4.4 ± 1.4 | 4.9 ± 0.59 | 2.2 ± 0.29 | 2.8 ± 0.36 |
| Roundness (%) | 42 ± 3.3 | 46 ± 10 | 38 ± 3.0 | 75 ± 6.7 | 58 ± 10 |
| Directionality | 0.85 ± 0.04 | 0.25 ± 0.05 | 0.33 ± 0.06 | 0.06 ± 0.02 | 0.05 ± 0.04 |
| Persistence | 3.54 ± 1.8 | 0.83 ± 0.28 | 0.98 ± 0.18 | 0.3 ± 0.06 | 0.28 ± 0.02 |

Figure 3. Regulation of PAKa by PKB and PI3K

(A) (a) Incorporation of PAKa-FLAG and mutant PAKa proteins into the cytoskeleton upon the stimulation of cAMP determined by immunoblotting with the anti-FLAG antibody. (b) The changes in the amount in the insoluble fraction are shown. The level of wild-type PAKa-FLAG in the detergent-insoluble fraction was rapidly increased upon the cAMP stimulation and was reciprocally decreased in the detergent-soluble cytoskeleton fraction ([Aa], graph not shown). Mutation on Thr579 abolishes this transition between detergent-soluble and -insoluble fractions. (c) Kinase activities of PAKa and its mutants after cAMP stimulation. We compared the kinase activity from cells stimulated with cAMP to that of the resting cells. The differences in the intrinsic (basal) kinase activities of the different PAKa proteins are not shown in this plot; the graph depicts kinase activities compared with activity in unstimulated cells plotted as 1.0. (d) The PAKa kinase activity was normalized to the amount of PAKa in the detergent-soluble fraction and plotted.

(B) Regulation of PAKa by PKB and PI3K. (a) The increased incorporation of PAKa-FLAG into the detergent-insoluble fraction is absent in *pi3k1/2* and *pkbA* null cells. (b) PAKa kinase activity upon cAMP stimulation in wild-type, *pi3k1/2* null, and *pkbA* null cells. (C). We analyzed chemotactic cell movement of wild-type cells, *paka* null cells, and *paka* null cells expressing PAKa$^{T579A}$ and PAKa$^{T579D}$ to a micropipette containing cAMP by using DIAS software (Soll and Voss, 1998; Wessels and Soll, 1998). Superimposed images representing cell shape at 45 s intervals are shown. Roundness is a measure (in percent) of how efficiently a given amount of perimeter encloses an area. More-polarized cells have a lower roundness. Directionality is the net path length divided by the total path length; a completely straight path has a value of 1.0. Directionality: the average angle of change in the direction of movement in each frame. Persistence is related to speed divided by the direction change. If an object is not turning, its persistence is the same as the speed. Wild-type cells are very polarized, their migration is rapid and directed toward the tip of micropipette, and the vast majority of pseudopodia are extended only in the direction of micropipette. *paka* null cells protrude many random lateral pseudopodia as described previously (Chung and Firtel, 1999). Cell migration is severely impaired in *paka* null cells expressing either PAKa$^{T579A}$ or PAKa$^{T579D}$.

tion (Figures 3Aa and 3Ab). Conversely, as we might expect if Thr→Asp mimics phospho-Thr579, PAKa$^{T579D}$ has a higher basal level of association with the cytoskeleton (Figure 3A). This suggests that the ability of PAKa to be phosphorylated at Thr579 is essential for the dynamic partitioning of PAKa between the detergent-soluble

and -insoluble fractions and that PAKa$^{T579A}$ associates with another cellular component to help partition it between the two fractions. Furthermore, PAKa-FLAG did not significant translocate to detergent-insoluble cytoskeletal fraction in either *pkbA* or *pi3k1/2* null cells (Figure 3Ba), indicating that both PKB and PI3K are required for PAKa translocation.

To examine the biological consequences of these mutants, we expressed them in *paka* null and wild-type cells. Overexpression of PAKa in *paka* null cells results in chemotaxis defects indistinguishable from PAKa overexpression in wild-type cells (Chung and Firtel, 1999). Compared to wild-type cells, *paka* null cells overexpressing PAKa produce an increased number of pseudopodia, although significantly less than exhibited by *paka* null cells, and a reduced directionality of movement (Figure 3C). These cells are significantly more polarized than *paka* null cells. Expression of PAKa$^{T579D}$ or PAKa$^{T579A}$ in *paka* null cells results in phenotypes that are more severe than those of *paka* null cells. Very few pseudopodia are produced and the speed (movement of the cell's centroid, not linear distance moved) is even more reduced. Moreover, these cells display almost no movement toward the chemoattractant source. These phenotypes suggest that both proteins function as dominant negative proteins, even in *paka* null cells. One possibility is that these proteins bind to and inhibit the function of other cellular components required for proper chemotaxis, producing a phenotype that is more severe than that of *paka* null cells. In contrast to PAKa$^{T579A}$, PAKa$^{T579D}$ expressing *paka* null cells produce multiple, randomly localized ruffles along the cell's plasma membrane. When these constructs are expressed in wild-type cells, they produce dominant negative phenotypes, although these phenotypes are less severe than when the constructs are expressed in *paka* null cells, presumably because of competition from the endogenous wild-type PAKa.

### PKB and PI3K Regulate cAMP-Mediated PAKa Activation

If PKB is required for PAKa translocation, it may also be required for PAKa activation and myosin II assembly. Indeed, Figure 3B indicates that cAMP does not stimulate PAKa activation in either *pi3k1/2* or *pkbA* null cells. We tested whether phosphorylation of Thr579 is required for PAKa activation in vivo. Consistent with our model, PAKa$^{T579A}$ shows a slightly lower basal activity and is not activated in response to cAMP stimulation, whereas PAKa$^{T579D}$ has a high basal kinase activity (Figure 4A). This higher basal activity is comparable to the activity we previously demonstrated for a "constitutively active" PAKa (PAKa containing only the kinase domain) in unstimulated cells (Chung and Firtel, 1999). This is consistent with PKB regulating PAKa activation directly through phosphorylation of Thr579 and being dependent on PI3K activity. We observe that, in response to cAMP stimulation, PAKa$^{T579D}$ shows no further increase in kinase activity (Figure 3Bb). We suggest that the absence of further activation of PAKa$^{T579D}$ is because PAKa$^{T579D}$ is already maximally activated in unstimulated cells. We note that the level of PAKa$^{T579D}$ kinase activity is lower than the activity of wild-type PAKa in response to cAMP (compare Figures 3Bb and 4A). There are two possible reasons for this difference: (1) an Asp at position 579 might not be as effective as the phosphorylated Thr in mediating a conformational or other type of change necessary for PAKa activation; or (2) a secondary activation response is required for maximal PAKa activation and this secondary response does not occur in PAKa$^{T579D}$.

We have demonstrated that PAKa is required for cAMP-mediated myosin II assembly (Chung and Firtel, 1999). If PAKa is not activated in *pkbA* null cells, we expect that myosin II is not assembled and does not exhibit the normal polarized localization in the rear of cells. Figure 4Bb shows that *pkbA* and *pi3k1/2* null cells do not display the robust, cAMP-mediated myosin II assembly into the cytoskeleton seen in wild-type cells. Moreover, myosin II assembly is blocked by a preincubation with LY294002 (data not shown). Consistent with this observation, GFP-myosin II, which localizes to the posterior of wild-type cells (Figure 4Ba) (Chung and Firtel, 1999), exhibits a very diffuse staining pattern in *pkbA* null cells. This lack of myosin II localization is similar to our observations in *paka* null cells (Chung and Firtel, 1999) and indicates that proper control of myosin II assembly requires PKB activity.

### The Dynamic Changes in the Subcellular Localization of PAKa Are Altered in PAKa$^{T579A}$ and PAKa$^{T579D}$

To examine dynamic changes in the subcellular localization of PAKa within the cell, we used GFP fusions of the PAKa N-terminal localization domain of PAKa, which localizes to the posterior cortical cap of chemotaxing cells (Figure 5A) (Chung and Firtel, 1999). When these cells are globally (uniformly) stimulated with cAMP, there is a rapid redistribution of N-PAKa along the cortex, which is concomitant with cell rounding and a loss of polarity (Figure 5Aa) (Chung and Firtel, 1999). N-PAKa$^{T579D}$-GFP behaves almost like wild-type N-PAKa-GFP, except the kinetics of relocalization are slightly faster. A sharp contrast is observed when the nonphosphorylatable form, N-PAKa$^{T579A}$-GFP, is examined. Whereas N-PAKa$^{T579A}$-GFP localizes to the posterior cortical cap of wild-type chemotaxing cells, which are polarized due to the endogenous PAKa activity, it does not redistribute even though the anterior of the cell reorganizes and rounds up normally (Figure 5Ac). Most cells expressing N-PAKa$^{T579A}$-GFP are not very highly polarized (Figure 5Ac, upper panels); however, some cells are (Figure 5Ac, lower panels). When a cell expressing N-PAKa$^{T579A}$-GFP is globally stimulated with cAMP, the anterior rounds up, similar to our observations of wild-type cells (Figure 5Aa). However, the posterior of the cell containing N-PAKa$^{T579A}$-GFP remains highly elongated and does not exhibit any shape change. Thus, N-PAKa$^{T579A}$ produces a highly localized, dominant-negative effect on chemoattractant-mediated changes in the cytoskeleton and cell polarity, suggesting that the phosphorylation of Thr579 by PKB is essential for the disruption and re-establishment of cellular polarity. The absence of cell polarization in *pkbA* and *pi3k1/2* null cells is consistent with the role of PKB in mediating this response and the requirement of PI3K for PKB activation.

We also examined the consequences of the Ala and Asp substitutions of Thr579 on the distribution of full-length, epitope-tagged PAKa in cells before and after global cAMP stimulation. As depicted in Figure 5B, PAKa$^{T579A}$-FLAG exhibits a defect in the ability to relocalize upon cAMP stimulation similar to that observed in N-PAKa$^{T579A}$-GFP. In unstimulated cells, PAKa$^{T579A}$-FLAG

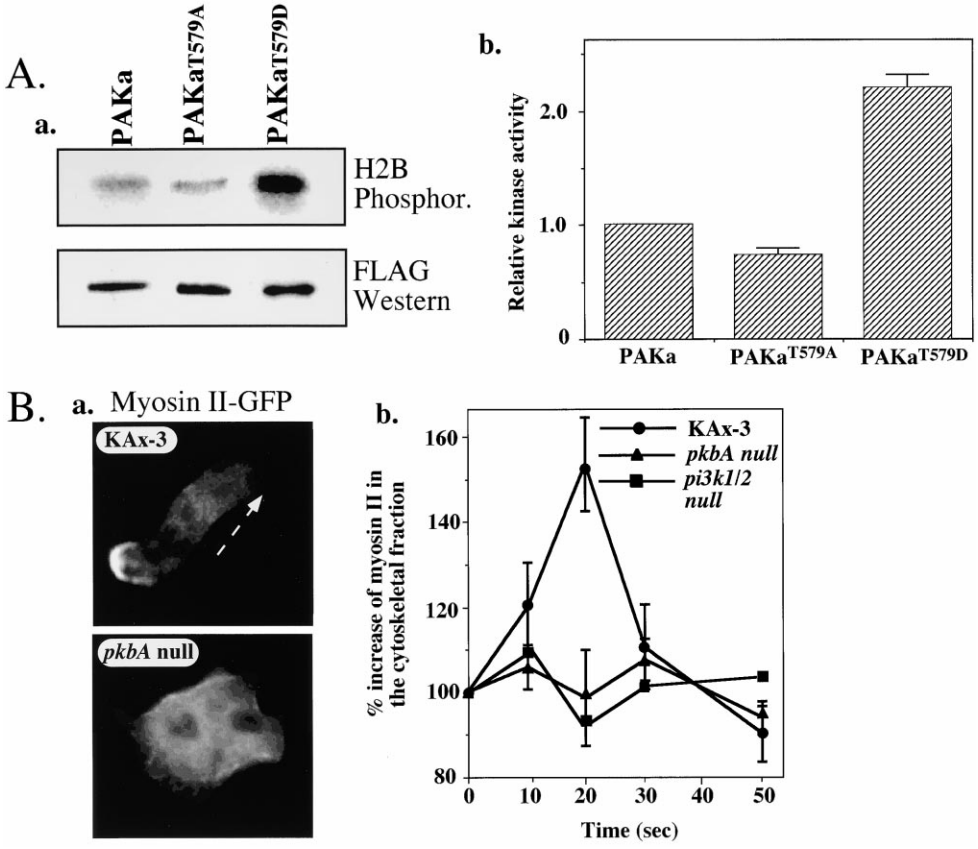



Figure 4. Regulation of PAKa Kinase Activity and Myosin Assembly

(A) Kinase activities of PAKa-FLAG, PAKa$^{T579A}$-FLAG, and PAKa$^{T579D}$-FLAG expressed in wild-type cells using Histone 2B as a substrate. Note: PAKa$^{T579D}$ exhibits a higher basal activity than wild-type PAKa.

(B) Localization of GFP-myosin II in aggregation-stage wild-type or *pkbA* null cells. (a) Myosin II is localized at the posterior cortex of wild-type cells but not in *pkbA* null cells. (b) We determined changes in myosin II content in the cytoskeleton upon cAMP stimulation as described previously (Chung and Firtel, 1999).

is less posteriorly restricted than wild-type PAKa-FLAG, being found in the posterior cortex and a diffuse gradient along the cortex that extends anteriorly (Figure 5B). This distribution of PAKaT579A-FLAG does not change upon cAMP stimulation, consistent with our conclusion based on real-time studies with N-PAKa$^{T579A}$-GFP. PAKa$^{T579A}$-FLAG exhibits a similar distribution before and after cAMP stimulation in *paka* null cells (data not shown), indicating the posterior distribution of PAKa$^{T579A}$-FLAG does not require wild-type PAKa. Unlike N-PAKa$^{T579D}$-GFP, PAKa$^{T579D}$-FLAG does not exhibit a restricted localization in unstimulated wild-type or *paka* null cells and is distributed along the entire cortex of cells (data for *paka* null cells not shown). These findings parallel the constitutive localization of PAKa$^{T579D}$-FLAG in the cytoskeletal fraction in unstimulated cells and may be related to the high, constitutive kinase activity of PAKa$^{T579D}$ (Figure 4A). As expected, PAKa$^{T579D}$ exhibits little observable change upon cAMP stimulation.

### PI3K Activity Is Required for Maintenance of Cell Polarity

Our results indicate that PI3K is required for the establishment of cell polarity and for the activation of pathways such as PKB that are essential for chemotaxis. To determine if PI3K is also required for the maintenance of cell polarity, we used the PI3K inhibitor LY294002, which blocks cAMP-mediated PKB activation (Meili et al., 2000). Addition of LY294002 to chemotaxing, polarized cells results in a rapid loss of cell polarity as determined by a redistribution of N-PAKa-GFP from the posterior to the entire cell cortex, a rounding of the cell, and a parallel loss of posterior myosin II localization (Figure 6A; data for myosin II-GFP not shown). When cells are pretreated with LY294002 and then stimulated with cAMP, there is no redistribution of myosin II-GFP to the cortex (data not shown). The redistribution of N-PAKa- and myosin II-GFP strongly suggests that basal (unstimulated) PI3K activity is essential for maintaining a cell's polarity.

The actin cytoskeleton controls the overall structure of cells and is highly polarized in chemotaxing cells, with F-actin localized predominantly in the anterior leading edge and to a lesser degree in the cell's posterior (Gerisch et al., 1995; Parent and Devreotes, 1999; Firtel and Chung, 2000) (Figure 6B). Similarly, addition of LY294002 to polarized cells causes a loss of the polarized actin cytoskeleton and an overall reduction in F-actin as determined by the level of FITC-phalloidin staining (Figure 6B). We performed time-lapse video experiments in live cells expressing GFP-coronin, which binds to F-actin

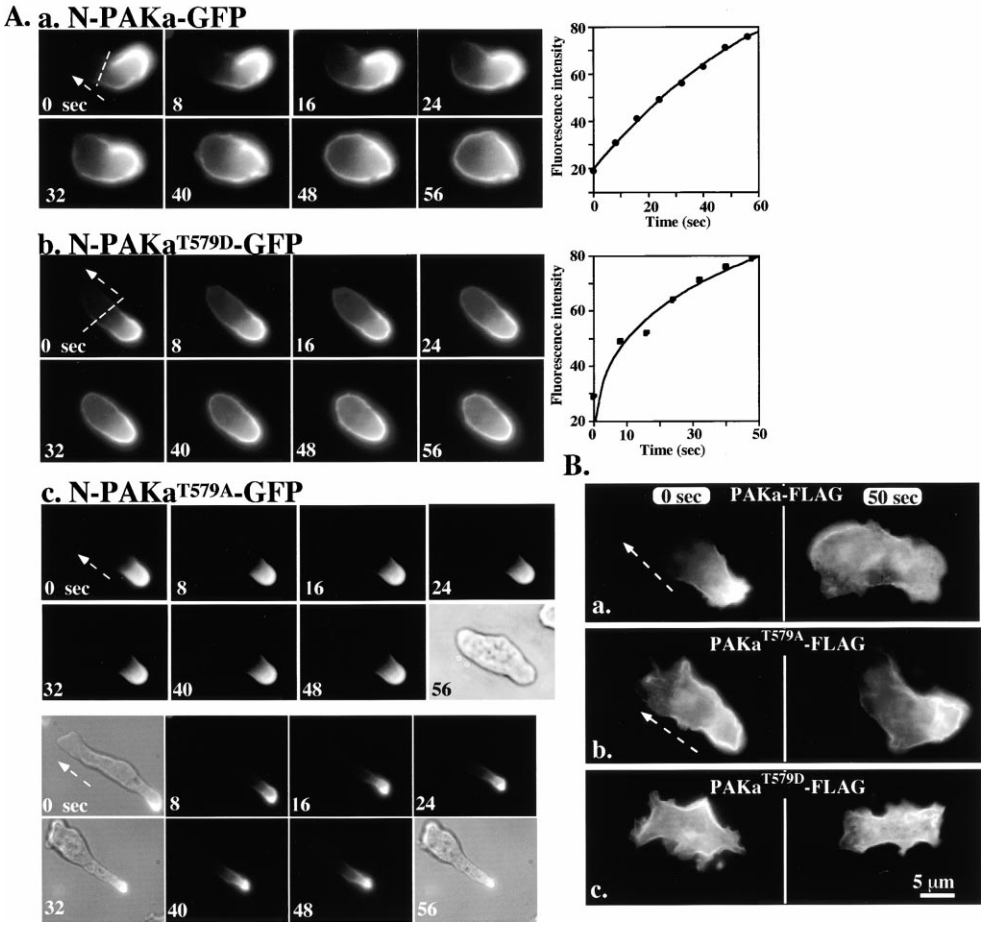



Figure 5. Changes in the Subcellular Localization of PAKa in Response to cAMP Stimulation

(A) Localization of N-PAKa-GFP and mutant proteins in live, migrating *Dictyostelium* cells. (a) N-PAKa-GFP, (b) N-PAKa$^{T579D}$-GFP fusion proteins were expressed in a wild-type cell. The arrow indicates the direction of the chemoattractant source, and (c) N-PAKa$^{T579A}$-GFP. We examined the localization of the fusion protein after overlaying the cell with a 150 μM cAMP solution. We viewed cells at 8 s intervals. Representative cells are shown.

(B) Redistribution of PAKa-FLAG upon cAMP stimulation. Cells expressing PAKa-FLAG were pulsed with 30 nM cAMP for 5 hr and plated on a coverslip. We fixed cells either immediately before or 50 s after bathing them with 100 μM cAMP. We examined the localization of PAKa-FLAG by indirect immunofluorescence staining with anti-FLAG antibody. PAKa-FLAG localizes in the rear cell body and redistributes along the membrane cortex after cAMP stimulation. However, PAKa$^{T579A}$-FLAG stays in the rear cell body after the stimulation. PAKa$^{T579D}$-FLAG distributes mainly along the membrane cortex even before the stimulation. Bar = 5 μm.

and functions as a reporter for F-actin distribution in live cells (Gerisch et al., 1995). Prior to LY294002 addition, GFP-coronin, and therefore F-actin, is highly localized to the leading edge (Figure 6C). Upon addition of LY294002, there is a rapid loss of the localization of GFP-coronin. These kinetics are more rapid than the redisruption of the posterior localization of PAKa or myosin II. Our results support those from the experiments examining F-actin in fixed cells and indicate that PI3K is required for the maintenance of polarized F-actin.

## Discussion

### PI3K and PKB Regulate Cell Polarity and Chemotaxis through Myosin II Assembly

Establishment of cell polarity is essential for directional cell movement. Chemotaxing mammalian and *Dictyostelium* cells have a strongly biased axial polarity, with F-actin assembled primarily in the leading edge and myosin II in the rear cell body. Genetic studies in *Dictyostelium* revealed that myosin contraction and thus myosin II assembly are required for retraction of the posterior part of the cell and the cortical tension that is necessary to suppress lateral pseudopod formation and maintain cell structure (Clow and McNally, 1999; Egelhoff et al., 1993, 1996; Wessels et al., 1988). This control of cell shape and cortical tension via regulated myosin II assembly is probably also a general property of mammalian cells. In PC12 cells, disruption of myosin II fibers leads to loss of contractility and cell spreading, whereas myosin assembly causes cell rounding and an increase in contractility (van Leeuwen et al., 1999). In *Dictyostelium*, this general property of motile cells is controlled through PAKa, which is also required for myosin II assembly in the contractile ring during cytokinesis (Chung and Firtel, 1999). We demonstrate that PKB, which is activated in response to chemoattractants in *Dictyostelium* cells and leukocytes, is required for chemoattrac-

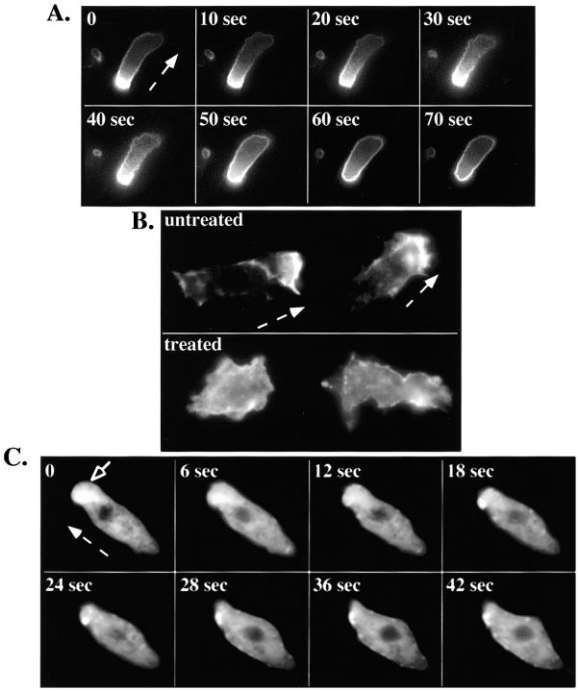



Figure 6. Disruption of Cellular Polarity of Chemotaxing Cells by the PI3K Inhibitor LY294002

(A) We treated *Dictyostelium* chemotaxing cells expressing N-PAKa-GFP with 20 μM LY294002 and followed the change in the subcellular localization of N-PAKa-GFP by time-lapse digital video microscopy.

(B) We treated chemotaxing cells with 20 μM LY294002 and determined the distribution of F-actin before and after treatment using FITC-phalloidin. The top cells are representative cells before treatment. The arrow indicates the direction of polarity and movement. The lower panel depicts cells after treatment.

(C) Chemotaxing cells expressing GFP-coronin were treated the same way as cells in (A). We followed changes in the localization of GFP-coronin by time-lapse video microscopy with digital images taken at 1 s intervals.

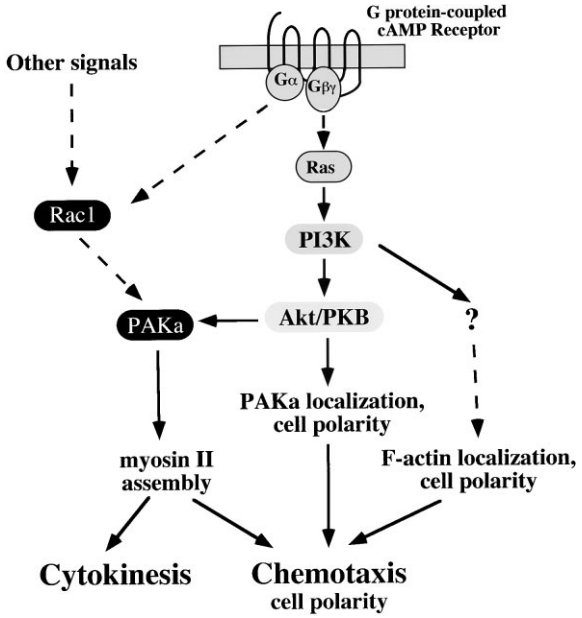



Figure 7. Model of the Role of PI3K and PAKa in Controlling Cellular Polarity and Chemotaxis

Proposed model of the pathway leading from the cAMP chemoattractant receptor to chemotaxis and cell polarity is shown. Aspects of the pathway are discussed in the text. The linkage between the cAMP receptor, heterotrimeric G protein, and other components of the pathway to PKB activation have been discussed previously (Firtel and Chung, 2000; Meili et al., 1999, 2000).

tant-mediated myosin II assembly in *Dictyostelium*, thus providing a direct link from PI3K and PKB to PAKa, myosin II assembly, and chemotaxis (Figure 7). At present, the functions of PKB in the chemotaxis of leukocytes are not known.

PI3K probably plays a broader role in regulating subcellular structure and cell polarity. *pi3k1/2* null cells show a significantly greater loss of axial polarity than do myosin II or *paka* null cells. Addition of LY294002 results in a loss of an already-established cell polarity, a reorganization of the cell's cortex resulting in a uniform distribution of N-PAKa-GFP and myosin II-GFP, and a loss of the localization of the F-actin cytoskeleton. These results suggest that continued PI3K activity is required for maintenance of cell polarity and controls events at the leading edge and the posterior of the cell. Recent studies of mice lacking PI3Kγ revealed that PI3Kγ is involved in the regulation of chemotaxis. Neutrophils purified from *pi3kγ* null mice retain relatively normal motility, but move in random directions, resulting in reduced chemotaxis (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). In addition, macrophages from PI3Kγ-deficient mice are much less chemotactic. These cells do not produce a detectable level of $PI(3,4,5)P_3$ in response to chemoattractant, suggesting that $PI(3,4,5)P_3$ production might be a key step in establishing cellular polarity. In fibroblasts migrating toward a shallow gradient of growth factor, a strong spatial correlation between polarized 3′ phosphoinositide production and rapid membrane spreading has been demonstrated (Haugh et al., 2000), implicating 3′ phosphoinositide lipids as direct mediators of polarized migration. Studies in leukocytes and *Dictyostelium* cells suggest that PI3K is one of the components that lie at the top of a signaling cascade and regulate cell polarity and chemotaxis (Parent et al., 1998; Meili et al., 1999; Servant et al., 2000). Our results provide a mechanistic pathway by which PI3K controls cell polarity and chemotaxis.

Is PI3K a general regulator of PAKa and myosin II assembly for other cellular processes? We believe it is not. PI3K regulation of myosin II may be restricted to pathways that control cell movement or other pathways regulated by upstream signals. Although we found that PAKa is required for myosin II assembly during cytokinesis, neither *pi3k1/2* nor *pkbA* null cells exhibit cytokinesis defects, indicating PI3K and PKB are not required for PAKa function in this process. As PAKa has a CRIB domain that binds tightly to $Rac1^{GTP}$ (Chung and Firtel, 1999), PAKa may be regulated by a Rac protein during cytokinesis. PAKa is probably not the only downstream effector of PI3K and PKB during chemotaxis. Analyses of the cellular phenotypes of *pi3k1/2*, *pkbA*, and *pakA* null cells indicate that *pi3k1/2* null cells have the strongest phenotype, followed by *pkbA* null cells, followed by *pakA* null cells, suggesting that there are multiple branch points in the pathways, PI3K has effectors other than PKB, and PAKa is not the only PKB effector (Figure 7).

### PI3K and PKB Regulate PAKa through Phosphorylation of Thr579

Our findings indicate that PAKa is an in vitro substrate of PKB and that phosphorylation on Thr579 is required for cAMP-mediated activation of PAKa and PAKa subcellular localization. Substitution of Thr579 for an Asp residue results in constitutive activation of PAKa, whereas a Thr→Ala substitution results in PAKa not being activated in response to cAMP. Other PAKs are regulated by the binding of a Rac family member to the CRIB domain (Knaus and Bokoch, 1998; Manser and Lim, 1999). PAKa has a homologous CRIB domain that tightly binds *Dictyostelium* $Rac1^{GTP}$ and $HsCdc42^{GTP}$, suggesting that PAKa is also regulated by Rac proteins, although this has not been demonstrated. Binding of $Rac1^{GTP}$ to the CRIB domain of PAKa is thought to release an inhibitory effect of the CRIB domain leading to PAKa activation (Zhao et al., 1998; Lei et al., 2000). Phosphorylation of Thr579, which lies near the CRIB domain, might result in a similar conformational change that leads to PAKa activation. In Rat-1 cells, activated PKB stimulates the activity of PAK1, whereas a dominant negative PKB inhibits the activation of PAK1 by Ras (Tang et al., 2000). Unlike PKB activation of PAKa, PAK1 activation by PKB is likely to be indirect because PAK1 lacks a conserved PKB phosphorylation site. However, mammalian PAK3 has a putative PKB phosphorylation motif (RQRKKSK), so it might be directly regulated by PKB.

Our data indicate that PKB phosphorylation of PAKa is required for PAKa's dynamic redistribution within the cell in response to a global cAMP stimulus. $PAKa^{T579A}$ and $N\text{-}PAKa^{T579A}$ do not relocalize in response to cAMP and do not translocate to the cytoskeletal fraction, suggesting that phosphorylation at Thr579 by PKB is required for these changes. Moreover, the N-terminal domain of PAKa carrying a Thr→Ala substitution functions as a dominant-interfering protein. We interpret these observations to suggest that PAKa interacts with different subsets of proteins in the phosphorylated and unphosphorylated states, and that an unphosphorylatable form blocks this transition. The observation that $N\text{-}PAKa^{T579A}$ inhibits the reorganization of the cell structure suggests that PKB plays a key role in this process. The SH3 domains of adaptor proteins can interact with PAKs via the polyproline motif (Bokoch et al., 1996; Hing et al., 1999; Yang et al., 1999) or a specific interaction motif (Manser et al., 1998) and, in the case of Nck, can mediate the relocalization and subsequent activation of PAK1 (Lu et al., 1997). The presence of a polyproline motif in very close proximity to Thr579 in PAKa raises the possibility that phosphorylation of Thr579 might alter the interaction(s) of PAKa with SH3 domains of similar adaptor proteins, leading to PAKa's dynamic relocalization to the detergent-insoluble fraction in response to cAMP. In the case of human PAK1, autophosphorylation of the Ser adjacent to the SH3 domain binding site (PXXPXRXXS) negatively regulates the binding of Nck to the PXXP motif and causes the release of PAK from the focal contacts to the cytosol fraction (Zhao et al., 2000). This regulation is very similar to the regulation of PAKa's subcellular localization, suggesting that regulation of the subcellular localization of PAKs via phosphorylation is widely utilized.

### Regulation of Localized Responses

We and others have suggested that the localized activation of PI3K at the leading edge is required for the localization and activation of signaling responses required for chemotaxis (Parent and Devreotes, 1999; Firtel and Chung, 2000; Rickert et al., 2000). This recruitment of PH-domain-containing proteins in leukocytes and *Dictyostelium* cells, including PKB (Meili et al., 1999; Parent et al., 1998; Servant et al., 2000; Funamoto et al., 2001), would result in the formation of an "activation domain" that dictates the position of the leading edge and leads to the formation of a dominant pseudopod and axial polarity. Several questions remain to be answered concerning the regulation of PAKa by PKB and PI3K. Previous studies in neutrophils and *Dictyostelium* support a model in which PKB is activated at the leading edge of chemotaxing cells: PKB activation requires its localization to the plasma membrane, and PKB-PH-domain-GFP fusions are found at the leading edge in chemotaxing cells (Meili et al., 1999, 2000; Servant et al., 2000). PAKa is localized at the cell's posterior, which restricts its activity and function to this subcellular compartment. PKB is required for a change in PAKa's subcellular localization and, we believe, in its initial localization. Once restricted to the posterior, PAKa's activation leads to myosin II assembly in the posterior.

How does PKB activate PAKa in the posterior of the cell? We suggest that activated PKB may translocate from the leading edge to the posterior of the cell. We note there is an ~20 s time differential between the time of peak PKB and PAKa activation. We cannot exclude the possibility that there is a lower level of membrane translocation and activation of PKB at the cell's posterior than is detectable with our assays. Another possibility is that phosphorylation of PAKa by PKB at the leading edge allows PAKa to interact with one or more proteins, leading to PAKa's localization in the posterior thus promoting myosin II assembly and the establishment of axial polarity in the cell. When a micropipette containing cAMP is placed near the posterior of a chemotaxing cell, the cortical domain containing PAKa disperses before relocalizing at the opposing side of the cell (C.Y.C. and R.A.F., unpublished observation). This disruption of the posterior cortex and the relocalization of PAKa require Thr579, suggesting that PKB is involved in excluding PAKa from the newly formed leading edge. Our results are consistent with a complex regulatory network in which PI3K, acting through PKB and PAKa, controls the formation of a stable subcellular domain in the posterior of the cell. As PI3K is required for chemotaxis in both mammalian and *Dictyostelium* cells, PKB may be the common link by which PI3K regulates cell polarity.

## Experimental Procedures

### Materials

We obtained sodium orthovanadate, β-glycerophosphate, aprotinin, and leupeptin from Sigma Chemical Co. (St. Louis, MO). [$\gamma^{32}$P]-ATP was from ICN Biomedicals (Costa Mesa, CA). We purchased glutathione-Sepharose and Protein A-Sepharose CL-4B from Pharmacia Biotech Inc. (Piscataway, NJ). The myosin II-GFP construct was a generous gift from Dr. James A. Spudich (Stanford University, Palo Alto, California). We obtained monoclonal anti-FLAG antibody from Sigma and rabbit anti-GST antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

### Cell Culture, Kinase Assays, and Cell and Molecular Biology

We remade the *pi3k1/2* null strain used in this study using KAx-3 as the parent and designated it MGP1. We obtained cells competent to chemotax toward cAMP (aggregation-competent cells) by pulsing cells in suspension for 5 hr with 30 nM cAMP. The chemotaxis

assays were done and analyzed as previously described (Chung and Firtel, 1999) and analyzed using DIAS software (Solltech, Oakdale, IA; Wessels and Soll, 1998). We performed site-directed mutagenesis for creating PAKa$^{T579A}$ and PAKa$^{T579D}$ using the Transformer Site Directed Mutagenesis Kit from Clontech Laboratories Inc (Palo Alto, CA); we sequenced the resulting constructs to confirm the nucleotide substitutions. To make GST-N-PAKa fusion protein, we amplified the N-terminal targeting domain by PCR and subcloned it into the pGEX-KG vector. We performed indirect immunofluorescence and phalloidin staining as previously described (Chung and Firtel, 1999). PAKa and PKB kinase activities were measured as described previously (Chung and Firtel, 1999; Meili et al., 1999). We used 1–3 μg of GST-N-PAKa or GST-PAKa$^{T579A}$ for the substrate instead of histone 2B.

### Isolation of Detergent-Insoluble Cytoskeletal Proteins

We isolated cytoskeletal proteins as proteins insoluble in detergent NP-40. Cells were pulsed and harvested by centrifugation and resuspended at $10^8$ cells/ml. We added cAMP (100 μM) to cells and lysed 500 μl aliquots of cells as described for the kinase assay. After vortexing a few times, we spun the samples for 4 min at 11,000 g. Proteins in the pellet were separated by SDS-PAGE on an 8% gel, blotted onto a PVDF membrane, and probed with anti-FLAG monoclonal antibody. For measurement of myosin II assembly, we ran cytoskeletal proteins on 6% SDS-PAGE gels and stained the gels with Coomassie blue. We scanned gels and blots and determined the intensities of myosin II and PAKa bands using IPLAB software.

### Two-Dimensional Electrophoresis

Cells were pulsed as described above and resuspended at $5 \times 10^8$ cells/ml. After stimulation with cAMP, we sequentially extracted cells with 4 vol of Extraction Reagent 2 and 1 vol of Reagent 3 from Bio-Rad (Hercules, CA) and combined the extracts. We loaded 20–40 μl of extract onto IEF tube gels (mini-PROTEAN Tube Gels) made with 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% of Bio-Lyte 5/7, and 0.4% of 3/10 ampholytes. We performed the IEF at 20°C for 8 hr at 700 V. Second-dimensional electrophoresis was performed on 0.7 mm SDS-PAGE gel (6%). We used prestained SDS-PAGE molecular weight markers. We detected N-PAKa-GFP and N-PAKa$^{T579A}$-GFP by immunoblot using anti-GFP monoclonal antibody from BABCO (Richmond, CA). We aligned the positions of the spots by staining the blots with Coomassie blue and determining the position of spots that did not change mobility under the different conditions.

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