PAKα, a Putative PAK Family Member, Is Required for Cytokinesis and the Regulation of the Cytoskeleton in Dictyostelium discoideum Cells during Chemotaxis

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Abstract. We have identified a Dictyostelium discoideum gene encoding a serine/threonine kinase, PAKα, a putative member of the Ste20/PAK family of p21-activated kinases, with a kinase domain and a long NH₂-terminal regulatory domain containing an acidic segment, a polyproline domain, and a CRIB domain. PAKα colocalizes with myosin II to the cleavage furrow of dividing cells and the posterior of polarized, chemotaxing cells via its NH₂-terminal domain. paka null cells are defective in completing cytokinesis in suspension. PAKα is also required for maintaining the direction of cell movement, suppressing lateral pseudopod extension, and proper retraction of the posterior of chemotaxing cells. paka null cells are defective in myosin II assembly, as the myosin II cap in the posterior of chemotaxing cells and myosin II assembly into cytoskeleton upon cAMP stimulation are absent in these cells, while constitutively active PAKα leads to an up-regulation of myosin II assembly. PAKα kinase activity against histone 2B is transiently stimulated and PAKα incorporates into the cytoskeleton with kinetics similar to those of myosin II assembly in response to chemoattractant signaling. However, PAKα does not phosphorylate myosin II. We suggest that PAKα is a major regulator of myosin II assembly, but does so by negatively regulating myosin II heavy chain kinase.

Key words: Dictyostelium discoideum • PAK • myosin II • chemotaxis • Rac/Cdc42

The actin cytoskeleton, composed of actin filaments and multiple actin-binding proteins, plays important roles in cell motility, cytokinesis, phagocytosis, and probably intracellular transport processes (Cooper, 1991; Mabuchi, 1994). Cell movement requires the protrusive and contractile forces generated via polymerization, depolymerization, and cross-linking of actin filaments and myosin motors. The Rho family of small G proteins are key regulators of changes in the actin cytoskeleton (Nobes et al., 1995; Tapon and Hall, 1997; Hall, 1998). In yeast, Cdc42 is required for cell polarization and bud formation (Ziman et al., 1993; Koon and Gow, 1995; Li et al., 1995), whereas a mutation in a putative Rac exchange factor leads to cell polarization defects during Drosophila melanogaster embryogenesis (Häcker and Perrimon, 1998). In mammalian cells, Cdc42 regulates the formation of filopodia, whereas Rac regulates lamellipodia formation and membrane ruffling, and RhoA regulates the formation of stress fibers. These pathways can be regulated through growth factor signaling and are important in controlling cellular movement and differentiation.

Conventional myosin (myosin II) is a key component in the regulation of cell motility, cytokinesis, and phagocytosis, functioning as an essential force-generating motor in these processes (Spudich et al., 1995). Myosin II localizes to the contractile ring (cleavage furrow) during cytokinesis of metazoan and Dictyostelium discoideum cells (Fukui, 1990; Otto and Schroeder, 1990; Mores et al., 1996). In Dictyostelium, myosin II null cells show a conditional growth phenotype: the cells cannot undergo cytokinesis in suspension and form large, multinucleated cells (De Lozanne and Spudich, 1987; Knecht and Loomis, 1988). However, when grown on a substratum, the cells cleave by traction-mediated cytokinesis (Fukui et al., 1990). Myosin II is also involved in the regulation of chemotaxis. In polarized, chemotaxing cells, myosin II is predominantly localized to the posterior of the cell and forms a C-shaped cap that contracts during the last stage of cell movement, causing the posterior of the cell to detach from the substratum and move forward (Mores et al., 1996; Clow and McNally, 1999). Wild-type cells predominantly form a single pseudopod at the leading edge; myoII null cells produce lateral pseudopodia, possibly due...
to reduced cortical tension (Pasternak et al., 1989) and have difficulty lifting the cell’s posterior (J ay et al., 1995). This leads to a slower and less efficient chemotaxis.

Other key regulators of the cytoskeleton are PAKs, p21-activated Ser/Thr protein kinases, which are thought to lie downstream of R ac and Cdc42. The first members of this kinase family to be identified were Ste20 and Cla4 from Saccharomyces cerevisiae, which are regulated by Cdc42 (H erskowitz, 1995). Members of the PAK family have an NH2-terminal regulatory domain that contains a CRIB (Cdc42/Rac binding) domain and a related COOH-terminal kinase domain. Mammalian p65PAK can be activated via autophosphorylation in vitro upon the binding of activated (GTP-bound) Rho family members, Cdc42 and Rac (M anser et al., 1994). The yeast PAK, Ste20, plays important roles in activating the MAP kinase cascade in the pheromone-response pathway (H erskowitz, 1995), although mammalian PAKs have been implicated in the activation of the R ac/Cdc42-regulated MAP kinase cascade leading to the stimulation of stress-induced MAP kinases p38 and J NK (B agrodia et al., 1995; Z hang et al., 1995).

There is increasing evidence that PAK family kinases regulate cytoskeletal changes mediated by Cdc42 and Rac. Ste20, along with its homologue Cla4, regulates polarized cell growth, presumably by controlling the actin cytoskeleton (Chant and Stowers, 1995; Cvrckova et al., 1995). Microinjection of activated PAK1 protein into quiescent Swiss 3T3 cells induces the rapid formation of lamellipodia, filopodia, and membrane ruffles (Sells et al., 1997), which is very similar to the effect produced by microinjection of activated Rac and Cdc42 (N obes and H all, 1995). Moreover, the Drosophila homologue of mammalian PAK1 is a key regulator of the cytoskeletal changes required for axonal guidance (Hi ng et al., 1999). Expression of various constitutively active forms of αPAK induces disassembly of stress fibers and focal adhesion complexes (M anser et al., 1997). A Dictyostelium myosin I heavy chain kinase (M1HCK)1 homologous to PAK and Ste20 has been cloned and proposed to provide a direct link between Cdc42/Rac signaling pathways and motile processes requiring myosin I molecules (L ee et al., 1996; B rzeska et al., 1997). PAKs also appear to regulate the function of myosin II, as the regulatory myosin light chains are phosphorylated by the Jurkat and placenta (M anser et al., 1997). These findings suggest that different PAKs may regulate different cytoskeletal components.

We describe the properties of a putative Dictyostelium PAK (PAKa) that contains a potential polyproline SH3 binding domain, a highly acidic domain in its NH2 terminus, a CRIB domain, and a related kinase domain. Paka null cells or wild-type cells expressing a kinase dead (putative dominant negative) PAKa produce many random, lateral pseudopodia and have a much higher frequency of making wrong turns than wild-type cells. Similarities in the distributions of PAKa and myosin II and in the phospho-1.Abbreviations used in this paper: GFP, green fluorescent protein; H 2B, histone 2B; M IH CK, myosin I heavy chain kinase; mP Ak a, a PAKa carrying the NH2-terminal myristoylation signal from Src; PAKaA, a putative constitutively active PAKa containing only the kinase domain; PAKaΔN, kinase dead, putative dominant, negative mutant of PAKa; PAKaAΔN, a PAKa mutant containing the CRIB and kinase domains.

types of paka and myoII null cells suggest that PAKa might be involved in the regulation of myosin II filament assembly in the posterior cortex. Furthermore, the increase of myosin II in the cytoskeleton in wild-type cells in response to cAMP was lacking in paka null cells, suggesting the assembly of myosin II into the cytoskeleton requires PAKa function. PAKa kinase is activated in response to stimulation with the chemoattractant cAMP, suggesting it controls chemotaxis by regulating myosin II function.

Materials and Methods

Materials

Sodium orthovanadate, β-glycerophosphate, aprotinin, and leupeptin were obtained from Sigma Chemical Co. Histone 2B was purchased from Boehringer Mannheim Corp. γ32PATP was from ICN Biomedicals. Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech Inc. Purified myosin II was a generous gift from Dr. T. Egelhoff (Case Western Reserve U niversity, Cleveland, OH).

Cloning of PAKa

To clone PAKa, a pair of degenerate primers was made based on the amino acid sequences conserved among the kinase domains of yeast Ste20 and human PAK6 (the two primers corresponded to the amino acid sequences VAIKK and DFGLAR). A full-length cDNA clone (3.6 kb) was obtained by screening a 12–16 h developmental cDNA library with a probe amplified by PCR. PAKa was disrupted in the thymidine auxotroph JH 10 by inserting the Thy gene cassette into codon 791, and transformants were selected. For overexpression experiments, PAKa and PAKa mutants were subcloned into the DIP-J expression vector (G askins et al., 1996). PAKa was tagged at the COOH terminus with the FLAG epitope by using primer 5′-TTACCTGTATGCTGCTTGGATTGCATGTCATCTAGTT-3′. The HA epitope tag was added to the NH2 terminus of PAKa by using primer 5′-AAAATGAGCTCCCCATCAGATGTGTTCTCGATAGAAGACAAATCCAC-3′. mPAK was obtained by PCR using primer 5′-ATGGGTGTCAATCAAAATCCAACAAATCAGAACCTCACTACGCCGTCATGT-3′, a sequence coding for the first 16 amino acids of chicken c-Src, composed of the myristoylation signal and the basic amino acid cluster sufficient for stable membrane association.

Selection of Clones

To select for strains expressing a high level of PAKa, transformants were selected at 40 μg/ml of G418, which in our hands produces clones that express elevated proteins at high levels. To select clones that express at a low level, we selected and maintained cells at 15 μg/ml of G418. Clones were screened to identify those exhibiting a low level of exogenous PAKa expression and exhibiting wild-type phenotypes in actin staining.

Indirect Immunofluorescence Staining

For immunofluorescence staining, cells were starved in 12 mM sodium phosphate buffer (pH 6.2) for >5 h and fixed with 4% formaldehyde for 5 min. Cells were permeabilized with 0.5% Triton X-100, washed, and incubated with 1.4 μg/ml anti-FLAG mAb or rabbit anti-HA antibody (1:200 dilution) in PBS containing 0.5% BSA and 0.05% Tween-20 for 1 h. Cells were washed in 0.5% BSA containing PBS and incubated with FITC-labeled anti-mouse or anti-rabbit antibodies for 1 h. After washing, cells were observed with a 60× oil immersion lens on a Nikon Microphot-FX microscope. Images were captured with a Photometrics SenSys camera and IPLAB Spectrum software. Anti-HA antibody was purchased from Santa Cruz Biotechnology. Monoclonal anti-FLAG antibody was obtained from Kodak. F-actin was stained with FITC-labeled phalloidin (Sigma Chemical Co.). Rabbit antitrypsin II antibody was a generous gift from Dr. J ames A. Spudich (Stanford U niversity, Stanford, California).

PAKa Kinase Activity Assay

Log-phase vegetative cells were washed and resuspended at a density of
2–3 × 10⁶ cells/ml in Na/K phosphate buffer and pulsed for 5 h with 30 mM cAMP every 10 min. The cells were collected by centrifugation and resuspended at a density of 2–3 × 10⁶ cells/ml. The cAMP receptor activity was downregulated by bubbling air through the cell suspension for 10 min (M a et al., 1997). The 200-µl samples were lysed with mixing with an equal volume of 2× lysis buffer (50 mM Tris, pH 7.6, at room temperature, 200 mM NaCl, 20 mM NaF, 2 mM vanadate, 50 mM β-glycerophosphate, 6 mM d-sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 4 µg/ml leupeptin, 4 µg/ml aprotinin, 2% NP-40, 20% glycerol, 2 mM DTT). 1 µl of antibody was added to 200 µl supernatant and incubated on ice for 1 h. The formed immune complexes were collected with 50 µl of a 1:1 slurry of protein A beads in lysis buffer by incubation under agitation for 1 h at 4°C. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM MOPS, pH 7.4, at room temperature, 200 mM magnesium chloride, 1 mM DTT, 30 mM KCl for myosin II assay). PAKa activity was measured in an immunocomplex kinase assay after immunoprecipitation with the anti-FLAG antibody. The beads were incubated with 75 µl kinase buffer containing 5 µCi γ³²P]ATP, 5 µg cold ATP, and 5 µg histone 2B or myosin II as a substrate. The reaction was stopped by the addition of 25 µl 4× sample buffer and boiling for 5 min. The samples were separated by SDS-PAGE (12%), blotted onto a PVDF membrane (Millipore Corp.), and exposed to film.

**Chemotaxis Assay**

Cells were pulsed with 30 mM cAMP at 6-min intervals for 5 h, and cells were washed and resuspended in Na/K phosphate buffer containing 200 µM CaCl₂ and MgCl₂. A small volume of cells was plated on glass-bottomed microwell plates (MatTek, Inc.) and allowed to adhere to the surface for ~20 min. A micropuffette filled with 150 µl cAMP was positioned to stimulate cells by using a micromanipulator (Eppeendorf-Netheler-Hinz GmbH), and the response and movement of cells was recorded by using a time-lapse video recorder and NIH Image software (1 image every 6 s). The movement of cells and changes of cell shape were analyzed with the DIAS program (Wessels and Soll, 1998).

**Isolation of Cytoskeletal Proteins**

Cytoskeletal proteins were isolated as proteins insoluble in detergent NP-40. Cells were harvested by centrifugation and resuspended in 100 vol/100 ml cells/ml. CA M P (100 µM) was added to cells and 500 µl of cells were taken at each time point and immediately added to 500 µl of 2× lysis buffer as described for the kinase assay. After vortexing a few times, the tubes were placed on ice for 10 min, then allowed to warm to room temperature for 10 min. The samples were spun for 4 min at 11,000 g and the supernatant was discarded. After washing with 1× lysis buffer, the protein pellet was discarded by boiling in 2× SDS-PAGE sample buffer and boiling for 5 min. The samples were separated by SDS-PAGE (12%), blotted onto a PVDF membrane (Millipore Corp.), and exposed to film.

**Two-Hybrid and Overlay Analysis**

Rac1B, RacE, RasG, and human Cdc42 were amplified by PCR and cloned into yeast two-hybrid fish vector pG4-5 as described previously (Lee et al., 1997, 1999). The clones were sequenced to confirm the reading frame and the absence of mutations. Point mutations corresponding to Q61L in Ras were made with the Transformer Site Directed Mutagenesis Kit from CLONTECH Laboratories, Inc. Construct containing site-directed mutations were sequenced to confirm the reading frame and the absence of mutations. Point mutations corresponding to Q61L in Ras were made with the Transformer Site Directed Mutagenesis Kit from CLONTECH Laboratories, Inc. Constructs containing site-directed mutations were sequenced to confirm the reading frame and the absence of mutations. Point mutations corresponding to Q61L in Ras were made with the Transformer Site Directed Mutagenesis Kit from CLONTECH Laboratories, Inc. 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**Results**

**Cloning of PAKa**

We identified PAKa in a PCR screen for PAKa/Ste20 family kinases using degenerate primers based on conserved sequences in the kinase domains of Ste20 and mammalian PAKa. Sequenced PCR products carrying part of a potential PAKa family member were used to screen a cDNA library and several family members were identified, one being PAKa. One ~3.5-kb PAKa cDNA clone contained the complete PAKa open reading frame (Fig. 1 A). The domain structure of PAKa is similar to that of mammalian PAKa (Fig. 1 B). PAKa has a predicted Cdc42/Rac binding domain (CRIB), a COOH-terminal kinase domain, and a long NH₂-terminal putative regulatory domain containing a highly acidic region and a potential SH3-interacting polypeptide stretch. In addition, the NH₂-terminal domain contains an A kinase anchor (PKA) consensus phosphorylation site. Comparisons of the kinase and CRIB domains of PAKa and other members of this family are shown in Fig. 2, A and B, respectively. The PAKa kinase domain has the highest sequence identity to the kinase domain of Dicyostelium M1HCK (59% identity) and human PAKa, mouse PAK3, and Saccharomyces pombe PAKa (~48% identity each).
The PAKα CRIB domain shares strong homologies with the CRIB domain of Dictyostelium MIHCK and PAKs from other species (Fig. 2B). To examine the specificity of the PAKα CRIB domain for small G proteins, we used the yeast two-hybrid system (Gyuris et al., 1993) to assay the interactions of the PAKα CRIB domain with activated forms of Dictyostelium Rac1B, human Cdc42, and RasG, the latter two of which are required for cytokinesis in Dictyostelium (Vithalani et al., 1996; Tuxworth et al., 1997). As we show in Fig. 2C, the PAKα CRIB domain preferentially interacts with DdRac1B and HsCdc42. We confirmed the interaction of DdRac1B and HsCdc42 with the PAKα CRIB using an overlay assay (Manser et al., 1994). We observed strong interaction of a GST-CRIB/PAK fusion protein with DdRac1B<sub>GTP</sub><sup>S</sup> and Cdc42<sub>GTP</sub><sup>S</sup> (data not shown). These results are consistent with, but do not prove, that PAKα is a member of the PAK family of Rac/Cdc42-regulated protein kinases.

Northern blot analysis indicates that PAKα encodes a single developmentally regulated transcript (3.7 kb) that is expressed in vegetative and early developing cells, and is upregulated during aggregation and multicellular stages (Fig. 1C). No hybridization signal was detected when the probe was used on a blot carrying RNA isolated from paka null cells (data not shown).

**paka Null Cells Have a Defect in Cytokinesis and Development**

PAKα was disrupted by homologous recombination (see Materials and Methods). Random transformants were selected and screened for disruption of PAKα by Southern and RNA blot analysis. All clones that showed disruption of PAKα did not exhibit an mRNA signal on RNA blots, and had the same developmental and growth phenotype. As depicted in Fig. 3A, paka null cells exhibit a defect in completing cytokinesis when grown in suspension. Under these conditions, the increase in cell number indicates that the lack of PAKα might be responsible for cytokinesis, but karyokinesis is normal. The cytokinesis defect could be complemented by overexpression of PAKα from the A ct15 (Act15) promoter (data not shown).

To examine the effect of disrupting PAKα on multicellular development, we plated paka null and wild-type cells on nonnutrient agar and followed development. Compared with wild-type cells, development of paka null cells is very delayed (Fig. 4). Tightly aggregated cells do not form until 16–17 h, and some mounds arrest at this stage. For those that proceed further, the first fingers are not formed until ~24 h. Mature fruiting bodies form at ~36 h, containing sori that are smaller than those of wild-type cells (data not shown). To examine the role of PAKα further, we created a series of PAKα mutant constructs: a kinase dead, putative dominant negative mutant of PAKα made by changing the Lys residue in the ATP binding site in the kinase domain to Ala (PAKα<sup>K394A</sup>, designated PAKα<sup>DN</sup>); a puta-
tive constitutively active PAKa (PAKαCA) containing only the kinase domain (the NH₂-terminal regulatory domain is thought to function as a negative regulatory module and its deletion in mammalian PAKs leads to a constitutively active kinase; Cairns et al., 1992); a PAKa mutant containing the CRIB and kinase domains (PAKαG₁K); and a PAKa carrying the NH₂-terminal myristoylation signal from Src (myrPAKα), which has been demonstrated to constitutively target mammalian and Dictyostelium proteins uniformly to the plasma membrane (Buser et al., 1994; Meili et al., 1999). The structures of these constructs are shown in Fig. 1B.

We overexpressed the mutant PAKs and wild-type PAKα from the constitutive Act15 promoter. For these experiments, the overexpression mutants were selected at high G418 concentrations to yield clones expressing a high level of the specific PAKa protein (see Materials and Methods). Cells expressing PAKαDN have a phenotype similar to, but more severe than, that of paka null cells (Fig. 4). Aggregation is very delayed and most mounds arrest and do not develop further. The more severe phenotype of PAKαA⁰DN-expressing cells compared with paka null cells may be due to the inhibition of another PAK-like kinase by PAKαA⁰DN. Cells overexpressing PAKαCA exhibit severe aggregation defects and never form mounds.

We targeted PAKα to the plasma membrane by expressing myrPAKα. Indirect immunofluorescence indicates that myrPAKα, also tagged at the COOH terminus with the FLAG epitope, localizes uniformly to the plasma membrane around cells (see Fig. 8, B, f). These cells exhibit very delayed aggregation and development, suggesting that proper subcellular localization of PAKα might be important for regulating the in vivo function of PAKα during aggregation (Fig. 4). Cells overexpressing wild-type PAKα or PAKαG₁K exhibit a wild-type pattern of aggregation and multicellular development, and form normal-looking fruiting bodies (data not shown).

Aberrant F-actin Organization in paka Null Cells and Cells Expressing PAKα Mutants

To examine whether overexpression of PAKα mutants has an impact on the actin cytoskeleton, we performed phalloidin staining of aggregation-competent cells. Wild-type cells are usually polarized and show localized F-actin assembly at lamellipodia of the leading edge and sometimes, to a lesser degree, at the posterior cortical region of the retracting cell body (Fig. 5). paka null cells also appear elongated, but are not as polarized in the distribution of the actin cytoskeleton as wild-type cells. paka null cells have multiple, randomly localized F-actin-rich pseudopodia-like protrusions (Fig. 5, arrows). Cells overexpressing wild-type PAKα (PAKαOE), although lacking a developmental phenotype, exhibit changes in F-actin organization. PAKαOE cells have multiple F-actin–enriched crowns along the periphery of cells (Fig. 5). This pattern is very similar to that of racgap1 null cells and cells overexpressing constitutively active DDrac1B (DDrac1B Q61L; Chung, C.Y., S. Lee, C. Briscoe, and R.A. Firtel, manuscript submitted for publication), suggesting that the activity of PAKα might be regulated via Rac1B. Despite this aberrant F-actin organization, PAKαOE cells are elongated.

Cells expressing PAKαA⁰DN lack prominent actin-rich lamellipodia and actin staining is very diffuse and scattered around the cell periphery (Fig. 5). Some of the cells show microspikes (see lower panel for PAKαA⁰DN, Fig. 5), but these microspikes do not appear to be enriched in F-actin. In contrast, cells expressing PAKαA⁰⁰ accumulate assembled F-actin in multiple membrane ruffles over the entire cell. Some of these cells are multinucleate, although not to

Figure 3. Multinucleated paka null cells due to defective cytokinesis. A, Defective cytokinesis in paka null cells. Phase-contrast and epifluorescent micrographs of multinucleated paka null cells grown in shaking culture for 5 d. Nuclei were visualized with DAPI. B, Increase of the number of nuclei in paka null cells. Wild-type KAx3 and paka null cells were transferred from plates into axenic medium in shaken culture at 0 h and the number of nuclei in a cell were counted at intervals thereafter. Cells were counted using a hematocytometer. When paka null cells are grown in suspension for 5 d, there are an average of eight nuclei per cell.

Figure 4. The number of nuclei/cell in paka null cells. Wild-type KAx3 and paka null cells were transferred from plates into axenic medium in shaken culture at 0 h and the number of nuclei in a cell were counted at intervals thereafter. Cells were counted using a hematocytometer. The number of nuclei per cell increased with time, with an average of eight nuclei per cell when paka null cells are grown in suspension for 5 d.
the extent of paka null cells, suggesting that a putative nonregulated form of PAKa, as well as a lack of PAKa, can result in cytoskeletal defects that prevent proper cytokinesis. Moreover, although these cells elongate, they fail to show a polarization of the actin cytoskeleton, consistent with their inability to aggregate properly. These results suggest that PAKa can mediate rearrangements in the actin cytoskeleton.

Cells expressing PAKaG1K do not exhibit a prominent change in F-actin distribution (data not shown) compared with wild-type cells and in contrast to cells expressing PAKaCA, suggesting that the CRIB domain might negatively regulate kinase function, consistent with work on human PAK1 (Sells et al., 1997). As wild-type PAKa has the CRIB domain, it is possible that the upstream, NH2-terminal sequences deleted in PAKaG1K are involved in regulating the function of the CRIB domain in controlling PAKa activation. Cells expressing myrPAKa exhibit domains of F-actin along the entire membrane cortex, consistent with a uniform distribution of myrPAKa along the cortex of the cells (see Fig. 8 f). These cells fail to elongate or polarize (Fig. 5). We expect that the upregulated assembly of F-actin results from the activity of the membrane-localized PAKa.

Abnormal Chemotactic Movement of paka Null Cells and Cells Expressing PAKa Mutants

To examine whether the changes in the cytoskeletal organization described above alter chemotactic-induced cell migration, we compared the chemotactic movement of paka null cells to that of wild-type cells by examining their movement toward the chemotactic signal using the DIA5 image analysis software (Wessels and Soll, 1998). Cells competent to chemotax toward cAMP (aggregation-competent cells) were obtained by pulsing cells in suspension for 5 h with 30 nM cAMP, conditions that mimic the cAMP signaling during the phase leading up to aggregation in vivo (Devreotes et al., 1987; Mann and Firtel, 1987; Saxe et al., 1991; Insall et al., 1994). This protocol maximally induces the expression of aggregation stage genes required for aggregation, including the cAMP receptor, cAR1, and the coupled G protein α subunit, Gα2. Wild-type cells are usually well-polarized and move quickly and...
linearly toward the cAMP source. As we show in Fig. 6 A, wild-type cells produce pseudopodia almost exclusively at the edge in the direction of the micropipette, and produce very few random lateral or rear pseudopodia (an average of 1.5 lateral pseudopodia per 10 min). Wild-type cells make few changes in the direction of movement, as shown in Fig. 6 A. In sharp contrast, paka null cells produce more random pseudopodia (an average of 6 lateral pseudopodia per 10 min, some of which are marked with open arrowheads in Fig. 6 B) in various directions. In addition, paka null cells exhibit random changes in the direction of movement, which might result from the protrusion of lateral pseudopodia, leading to an inefficient chemotaxis toward the cAMP source. The movement of the four paka null cells shown in Fig. 6 B illustrates these phenotypes. Cells protrude multiple pseudopodia and, in cell c, two pseudopodia in the same cell become dominant (rather than one), resulting in the formation of two independent leading edges and the independent movement of the two halves that remain connected.

In addition, paka null cells do not retract their rear cell body as efficiently as wild-type cells. The posterior of wild-type cells contracts and lifts from the substratum after the leading-edge pseudopod has been extended (Jay et al., 1995); paka null cells appear defective in this process (Fig. 6 B: asterisk in a and c marks the rear cell body of the paka null cells, which remain tightly associated with the substratum). As illustrated by Fig. 6 B, cells a and c, the anterior of the paka null cell continues to extend its cell body toward the cAMP source until the cell reaches a certain length and then the posterior retracts very rapidly. Probably due to the combination of random changes of the direction of movement and the difficulty in retracting the rear cell body, the speed of movement of paka null cells (4.95 μm/min) is about half that of wild-type cells (8.63 μm/min).

PAKaDN cells produce more random pseudopodia than wild-type cells and have an increased frequency of turning, similar to paka null cells (Fig. 6 C). PAKaDN cells exhibit difficulty in retracting the rear cell body as depicted in Fig. 6, C, c. As the leading edge of a cell moves forward, the rear cell body elongates, due to the lack of retraction, and then retracts very rapidly. PAKaCA cells, as expected from the upregulated F-actin assembly over the entire cell, are
Figure 6. Abnormal chemotactic movement of paka null cells and cells expressing PA Kα mutants. Wild-type and paka null cells were washed and pulsed for 4.5 h with 30 nM cAMP every 6 min (see Materials and Methods). Cells were plated on a plastic petri dish containing a hole, over which a glass microscope slide was glued. The tip of the micropipette containing 100 μM cAMP was placed near the cell as described in detail previously (Meili et al., 1999). The movement of cells toward the tip was recorded with NIH Image software at 6-s intervals and the movement of cells and shape changes were analyzed with the DIAS program, a newly developed image analysis system.

A. Wild-type cells are well-polarized and move toward the cAMP gradient without making lateral pseudopodia. Left panel shows superimposed images representing cell shape at 1-min intervals. Right panel shows the change of direction in which the leading edge of the migrating cell headed. Solid arrows point out the direction of the micropipette filled with 100 μM cAMP. Note that wild-type cells do not make lateral pseudopodia and do not change their direction of movement very often. Bar, 20 μm. The number of newly formed pseudopodia was counted in each image for the 10-min time period of the experiment.

B. paka null cells protrude many random lateral pseudopodia (average ~6 lateral pseudopodia in 10 min, some of which are marked with open arrows) and the posterior cell bodies (marked with asterisks; a and c) of null cells are elongated, due to the difficulty in retracting. Due to frequent changes of the direction of movement, the speed of chemotactic movement of paka null cells is slower than that of wild-type cells.

C. Cells expressing PA KαDN make many random lateral pseudopodia and show elongated posterior cell bodies due to the difficulty of retraction (c). D. Cells expressing PA KαCA are very flattened and stationary. They are not polarized and move very slowly in random directions.
flattened and appear adherent and stationary. The cell-substratum contact area of these cells is significantly larger than that of wild-type cells (Fig. 6 D). Upon stimulation by cAMP, PAKaCA cells do not polarize and produce few dominant pseudopodia. These cells are not capable of changing their shape; hence, their migration is very slow. Our results suggest that PAKa plays an important role in the regulation of cytoskeletal organization, which is presumably required for maintaining cellular polarity and preventing the formation of random, lateral pseudopodia. The inhibition of the formation of random pseudopodia might prevent cells from making random changes of direction when moving up a chemoattractant gradient.

**Subcellular Localization of PAKa**

To examine where PAKa is localized in polarized, aggregation-competent cells, PAKa was tagged with FLAG at the COOH terminus or HA at the NH2 terminus, and expressed in wild-type cells. The transformants carrying a construct of tagged PAKa were selected and maintained at a low concentration of G418 to select strains that expressed PAKa at a low level. Strains were selected for those that did not exhibit the altered actin cytoskeletal defects shown in the high overexpression strains used in Figs. 4 and 5. The localization of tagged PAKa was determined in aggregation-competent cells by indirect immunofluorescence. FLAG-tagged PAKa mostly localizes to the posterior cortical region of the cell body (Fig. 7 B) and is not found in the leading edge where F-actin is concentrated (Fig. 7 A). The staining pattern of FLAG-PAKa in the posterior cortex overlaps with the phalloidin staining of F-actin in that region. Similarly, HA-tagged PAKa localizes to the posterior cortex (Fig. 7, D and E). Knowing that paka null cells have a cytokinesis defect, we examined the localization of HA-tagged PAKa in wild-type cells undergoing cytokinesis. As depicted in Fig. 7, F and G, HA-PAKa localizes to the cortex of the cleavage furrows of dividing cells. These results are consistent with involvement of PAKa in cytokinesis.

The subcellular localization of PAKa during chemotaxis and cytokinesis is very similar to that of myosin II. This
raises the possibility that PAKα might regulate myosin II and, in turn, control the cytoskeletal organization in the posterior cell body. To test this possibility, we examined the distribution of a myosin II–green fluorescent protein (GFP) fusion protein (Moores et al., 1996) in wild-type cells and paka null aggregation stage cells. Myosin II–GFP mainly localizes at the cortex of the rear cell body in wild-type cells (Fig. 8 A) as has been described previously (Yumura et al., 1984; Yumura and Fukui, 1985; Moores et al., 1996). However, in paka null cells, we observe a much more diffuse staining pattern of myosin II–GFP throughout the cells and no posterior cortical cap. We examined the localization of myosin II by indirect immunofluorescence staining using the antimyosin II antibody (Egelhoff et al., 1991). Myosin II is concentrated in the posterior cell body in aggregation-competent wild-type cells (Fig. 8 B) as shown by the myosin II–GFP fusion, whereas the staining of myosin II in paka null cells does not exhibit a strong subcellular localization. In contrast, we found strong myosin II staining along the membrane cortex of cells expressing myrPAKα, suggesting that anchoring PAKα to the plasma membrane promotes assembly of myosin II filaments at the membrane cortex (Fig. 8 B). This increased myosin II assembly at the membrane cortex is not found in cells expressing myrPAKαDN (Fig. 8, B, d), reflecting that myosin II assembly at the membrane cortex is due to the PAKα activity. However, cells expressing myrPAKαDN do not have a posterior that is enriched in myosin II. We expect this is because myrPAKαDN functions (like non–myr-tagged PAKαDN) as a dominant negative protein and inhibits intrinsic PAKα activity. Like myrPAKα, myrPAKαDN predominantly localizes to the membrane cortex as determined by immunostaining (Fig. 8, B, f; data not shown for myrPAKαDN).

Cells expressing PAKαCA have regions highly enriched in myosin II throughout the cell. These highly enriched myosin II-containing regions probably contain highly assembled myosin II. This result strongly suggests that PAKα might be involved in the regulation of myosin II assembly into the cytoskeleton. To test this idea, we determined the amount of myosin II in cytoskeleton fraction in wild-type cells, paka null cells, and cells expressing PAKαCA in both vegetative growth and aggregation stages. In vegetatively growing cells, the level of myosin II assembled into the cytoskeleton in both wild-type and paka null cells is low, compared with cells at the aggregation stage (Fig. 9 A). However, cells expressing PAKαCA display a higher level (approximately four times higher than wild-type cells) of myosin II assembly in the cytoskeleton. This result is consistent with PAKα activity being involved in regulating myosin II assembly.

The level of myosin II in the cytoskeleton in aggregation-competent, wild-type cells is significantly higher (seven to eight times higher) than that in growth-stage,

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![Figure 8](image-url)

**A** GFP–myosin II

**B** Immunostaining of myosin II

- **a** Wild-type: anti-myosin II
- **b** paka null: anti-myosin II
- **c** myrPAKα: anti-myosin II
- **d** myrPAKαDN: anti-myosin II
- **e** PAKαCA: anti-myosin II
- **f** myrPAKα: anti-FLAG

of myrPAKα–FLAG by immunofluorescence staining (f). The intracellular staining is nuclear, which was sometimes observed in Dictyostelium cells using the FLAG antibody. Bar, 5 μm.
vegetative cells (Fig. 9 A). This difference is probably due to myosin II assembly in the rear cell body upon cell polarization in aggregation-competent cells. In paka null cells, the level of assembled myosin II is ~65% of that of wild-type cells, suggesting that PAKa is required to control the level of myosin II assembly. Interestingly, there is no striking difference of myosin II level between wild-type cells and cells expressing PAKa<sup>CA</sup> at the aggregation stage (probably because the assembly of myosin II is already activated in aggregation-competent cells).

We examined whether PAKa function is important in controlling changes in myosin II assembly into the cytoskeleton upon cAMP stimulation (Liu and Newell, 1988). As shown in Fig. 9 B, in response to cAMP stimulation, the level of myosin II heavy chain in the cytoskeletal fraction in wild-type cells rises to a peak at 20–30 s and returns to the basal level by 60–80 s, consistent with previous observations (Liu and Newell, 1988). However, this peak of myosin II assembly upon cAMP stimulation into the cytoskeleton is absent in paka null cells. These results suggest that PAKa might be involved in regulating myosin II assembly via either direct phosphorylation of myosin II or regulation of kinases controlling myosin II assembly.

**Regulation of PAKa in Kinase Activity and its Localization upon Chemoattractant Stimulation**

To determine whether Dictyostelium PAKa activity is stimulated in response to the chemoattractant, aggregation stage cells expressing FL<sub>AG</sub>-PAKa were stimulated by cAMP, lysed at various times after stimulation, and PAKa-FL<sub>AG</sub> was immunoprecipitated from the detergent-soluble fraction (see Materials and Methods). Fig. 10 A shows that the kinase activity of immunoprecipitated PAKa towards a nonspecific substrate, H<sub>2</sub>B, rapidly increases three- to fourfold in response to cAMP. Maximal H<sub>2</sub>B phosphorylation is obtained within 30 s, after which the activity decreases slowly with kinetics similar to those of the adaptation of cAMP receptor-stimulated ERK2 kinase activity (Mead and Firtel, 1997). The kinetics of PAKa activation are very similar to those of PAK activation in human neutrophils stimulated by fMet-Leu-Phe (Knaus et al., 1995).

In a process of verifying that there is an equal amount of PAKa-FL<sub>AG</sub> in each immunoprecipitate, we observed that the amount of PAKa-FL<sub>AG</sub> in the detergent-soluble fraction is rapidly decreased in response to cAMP. This decrease of PAKa-FL<sub>AG</sub> in the detergent-soluble fraction is due to the incorporation of PAKa-FL<sub>AG</sub> into a detergent-insoluble cytoskeletal fraction (Fig. 10 C). The kinetics of PAKa relocalization into the cytoskeleton are very similar to the kinetics of myosin II assembly into the cytoskeleton upon cAMP stimulation. This result suggests that PAKa is involved in the regulation of myosin II assembly. When PAKa kinase activity is normalized for the amount of PAKa protein, activity increases approximately ninefold.

We tested whether PAKa might directly regulate myosin II through its phosphorylation in the COOH-terminal tail fragment (Egelhoff et al., 1991; Y umura and Kitanishi-Y umura, 1992). As shown in Fig. 10 A, we do not observe myosin II phosphorylation by PAKa, suggesting that...
PAKα indirectly regulates the assembly of myosin II, probably via the regulation of MHCKs. As expected, PAKα immunoprecipitated from aggregation-competent cells has three to four times higher basal activity against H2B (Fig. 10 B), but the activity is not regulated in response to cAMP stimulation, probably because the activity is already maximal.

Translocation of PAKα upon Change of Cell Polarity

To access the localization of PAKα in live, migrating cells, we fused the NH2-terminal regulatory domain of PAKα (excluding the CRIB and kinase domains) to GFP. Cells expressing N-PAKα-GFP chemotax normally (data not shown). As we demonstrate in Fig. 11 A, N-PAKα-GFP fusion protein localizes to the cortex at the cell’s posterior, as we observed with FLAG- or HA-tagged PAKα, suggesting the NH2-terminal domain used in this fusion is sufficient for proper subcellular localization of PAKα. As cells migrate, the N-PAKα-GFP fusion protein remains at the rear cortical region (Fig. 11 A), suggesting that PAKα activity is localized in the posterior and thus, may be important to maintain the polarity of cells during chemotaxis and retract the posterior cell body. To determine whether the localization of PAKα can be altered by disrupting cell polarity, we examined the distribution of N-PAKα-GFP by overlaying the cells with a receptor-saturating cAMP solution (Fig. 11 B). As cells lose their polarity and round up, N-PAKα-GFP remains associated with the cortex, but diffuses away from its previous location to be distributed along the membrane cortex over the entire cell. Fig. 11 C shows the quantification of the stimulus-induced relocalization of N-PAKα-GFP at the plasma membrane. The differences in fluorescence intensities before and after the addition of cAMP over the cell were measured along a thin line through the central portion of cells. In the polarized cell (frame 1), the fluorescence intensity in the rear cell body is tenfold higher than the intensity in the leading edge. However, the fluorescence intensity in the rear cell body is remarkably reduced by 50 s after cAMP stimulation. The intensity in the leading edge is increased ~2.5 times. The kinetics of the translocation of N-PAKα-GFP from the rear cell body to leading edge are presented in Fig. 11 D as a measure of the fluorescence intensity along the thin line through the anterior part of the cell. The translocation of N-PAKα-GFP reached maximum ~30–40 s after stimulation, which is similar to the kinetics of PAKα kinase activity upon the cAMP stimulation.

To examine whether the localization of holop-PAKα shows similar changes upon cAMP stimulation, cells expressing PAKα-FLAG were pulsed and plated on coverslips. Cells were bathed with 100 μM cAMP and fixed at 0, 25, and 50 s after cAMP stimulation and the localization of PAKα-FLAG was determined by indirect immunofluorescence staining. PAKα was localized in the posterior and thus, may be important to the relocation of N-PAKα-GFP. In contrast to the distribution of N-PAKα-GFP, PAKα staining in cells fixed at 50 s was absent in the cortex where new pseudopodia were formed, suggesting PAKα might be excluded in newly formed pseudopodia. The difference in the behavior between PAKα and N-PAKα-GFP might result from the lack of a CRIB domain in N-PAKα-GFP.

Discussion

PAKα Exhibits a Dynamic Subcellular Localization

We have identified a gene encoding a serine/threonine ki-
nase, PAKa, that is a putative member of the PAK family of protein kinases. We find that PAKa has a dynamic subcellular localization. PAKa localizes to the cleavage furrow of cells undergoing cytokinesis and the posterior cortex of polarized cells and colocalizes in the same region of the cell with myosin II. Using a GFP fusion, we demonstrate that the NH2-terminal domain lacking the CRIB domain is sufficient for the proper subcellular localization of PAKa in live, chemotaxing cells. If cells are overlaid with a receptor-saturating concentration of cAMP, the N-PAKa-GFP fusion proteins remain associated with the cell cortex, but delocalize along the cortex of the cell as it loses its polarized shape. Our data suggest that the NH2-terminal domain associates with a dynamically localized component of the cell cortex that is found in the posterior of polarized cells. Moreover, we suggest that the establishment of polarity in the cortical region responsible for this localization and the subsequent rearrangements of the cortical region are controlled through the chemoattractant receptors. We suggest that the localization of PAKa to a specific subdomain of the cell is a mechanism for restricting its activity to a specific site in the cell. This might be particularly impor-
Figure 12. Redistribution of PAKa-FLAG upon cAMP stimulation. Cells expressing PAKa-FLAG were pulsed with 30 nM cAMP for 5 h and plated on a coverslip. Cells were bated with 100 μM cAMP and fixed at 0, 25, and 50 s after stimulation. The localization of PAKa-FLAG was examined by indirect immunofluorescence staining with anti-FLAG antibody. Arrows indicate newly formed pseudopodia. Bar, 5 μm.

PAKα is a Regulator of the Myosin Component of the Cytoskeleton: Regulation of Cytokinesis

PAKα null cells exhibit two major phenotypes associated with an inability to properly regulate the cytokinetic: the cells fail to complete cytokinesis when grown in suspension and the cells exhibit defects in chemotaxis. Our results provide the first identification of an essential role of a putative PAK in cytokinesis. Dictyostelium cells and mammalian cells undergo cytokinesis via the formation and constriction of a myosin II-rich contractile ring in the cleavage furrow. myoII null cells do not divide in suspension due to the inability to perform cytokinesis, but undergo cytokinesis on an adhesive surface by traction-mediated cytokinesis in which dividing daughter cells are pulled apart (Yumura et al., 1984; Yumura and Fukui, 1985; De Lozanne and Spudich, 1987; Knecht and Loomis, 1988; Manstein et al., 1989; Fukui et al., 1990; Moores et al., 1996). Several small G proteins and GAPs are required for assembly of myosin II (Varnum-Finney et al., 1987), is important for suppressing lateral pseudopod formation. Both myoII and paka null cells form more lateral pseudopodia than do wild-type cells (Wessels et al., 1988; Wessels and Soll, 1995; this manuscript). The inability to inhibit lateral pseudopod formation in myoII null cells may be associated with a reduction in cortical tension of these cells (Paternak et al., 1989). The higher frequency of protrusion of lateral pseudopodia in paka null cells probably results from the reduction in cortical tension due to the lack of myoII filament assembly at the posterior cortex. These cytoskeletal defects might also be a major cause of the morphological defects during multicellular development of paka null cells and myoII null cells (Springer et al., 1994; Chen et al., 1998).

Possible Model for PAKα Function in Controlling Myosin II Assembly

We demonstrate that PAKα kinase activity against H2B is stimulated in response to cAMP, however, our data indicate that myosin II is not phosphorylated by PAKα. This observation is consistent with a requirement of PAKα for the maintenance, but not the disassembly, of myosin II fi-
bers. The regulation of myosin II filament assembly occurs through receptor-mediated phosphorylation of myosin II heavy chain tail and myosin II light chain (Berlot et al., 1985; Gerisch et al., 1993). Phosphorylations at threonine residues in the myosin II tail by two kinases, MHC-KA and MHC-PKC, lead to the disassembly of myosin filaments (Berlot et al., 1985; Côté and McCrea, 1987; Vaillancourt et al., 1988; Nachmias et al., 1989; Ravid and Spudich, 1989; Egelhoff et al., 1991). MHC-KA is expressed during growth and development (Futey et al., 1995). Cells overexpressing MHC-KA display reduced myosin II assembly in the cytoskeleton and become large and multinucleated in suspension, which is very similar to phenotypes of myoII mutants and a protein containing an activated Rac and Cdc42. The interaction between the proline-rich domain of PAK1 and a protein containing an SH3 domain appears to be important for regulating PAK1 activity. Nck, an adapter protein containing SH2 and SH3 domains, binds PAK1 and induces PAK1 relocalization to the membrane, leading to its activation (Lu et al., 1997). Other studies demonstrate that localization of PAK to membranes stimulates the kinase activity of PAK (Bokoch et al., 1996; Daniels et al., 1998) and our data with myrPAK1 are consistent with this model. Although we did not examine whether the proline-rich domain of PAK interacts with proteins containing an SH3 domain such as Nck, PAK probably associates with an SH3 domain via the PPxP sequence, resulting in the localization of PAK to the cortex. We note that PAK1 leads to a hyperpolymerization of actin. We do not know if this is a direct effect of PAK on regulators of the actin cytoskeleton or is indirect through the control of myosin II. It is also possible that the PAK1 kinase domain, when expressed by itself, may not show the same level of substrate specificity as the whole protein. Our observation that the expression of myrPAK1 results in a hyperpolymerization of myosin and actin along the periphery of the cell, reinforces the model that the subcellular localization of PAK1 to the posterior of the cell may be essential for its proper function. We suggest that this overexpression along the entire membrane or the localization by the myristoylation leads to a constitutive activation of the kinase.

Although we have not demonstrated that PAK1 is directly regulated by Rac1 and/or Cdc42, the GTP-bound, but not GDP-bound, forms of Rac1B and HsCdc42 bind to the PAK1 CRIB domain. These data are consistent with, but do not prove, that PAK1 is a bone fide PAK. In contrast to the kinase domain alone, overexpression of the entire PAK1 protein or the kinase and CRIB domains (PAK1 mE-K) does not cause a major change in the actin or myosin cytoskeleton. This finding is consistent with observations of mammalian PAKs in which the CRIB domain and an adjacent domain negatively regulate PAK1 kinase activity (Sells et al., 1997). We expect that binding of activated Rac or Cdc42 to the CRIB domain of PAK1 regulates the activation of the kinase, whereas the more NH2-terminal domain regulates PAK1’s localization.

Cells overexpressing PAK1 exhibit an upregulated assembly of F-actin, resulting in multiple actin-enriched crowns, which is very similar to the F-actin organization of cells expressing Rac1BQL or of null cells of the Rac1 GAP, DdracGAP1 (Chung, C.Y., S. Lee, C. Briscoe, and R.A. Firtel, manuscript submitted for publication). These similarities of F-actin organization suggest PAK1 may be important in regulating F-actin organization (Maner et al., 1997; Sells et al., 1997). A Dictyostelium Ste20 family kinase that phosphorylates severin, a Ca2+-dependent F-actin fragmenting protein, was purified and cloned recently (Eichinger et al., 1998), indicating a direct signal transduction from the plasma membrane to the cytoskeleton by phosphorylating actin-binding proteins. PAK1 might control F-actin organization by phosphorylation of actin-binding or actin-bundling proteins. The identification of the downstream substrates of PAK1 and the determination of the role of phosphorylation by PAK1 in regulating the function of the substrate should resolve these issues.

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