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# Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold

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Initiation of cell migration requires morphological polarization with formation of a dominant leading pseudopodium and rear compartment. A molecular understanding of this process has been limited, due to the inability to biochemically separate the leading pseudopodium from the rear of the cell. Here we examine the spatio-temporal localization and activation of cytoskeletal-associated signals in purified pseudopodia directed to undergo growth or retraction. Pseudopodia growth requires assembly of a p130Crk-associated substrate (CAS)/c-CrkII (Crk) scaffold, which facilitates translocation and activation of Rac1. Interestingly, Rac1 activation then serves as a positive-feedback loop to maintain CAS/Crk coupling and pseudopodia extension. Conversely, disassembly of this molecular scaffold is critical for export and down regulation of Rac1 activity and induction of pseudopodia retraction. Surprisingly, the uncoupling of Crk from CAS during pseudopodium retraction is independent of changes in focal adhesion kinase activity and CAS tyrosine phosphorylation. These findings establish CAS/Crk as an essential scaffold for Rac1-mediated pseudopodia growth and retraction, and illustrate spatio-temporal segregation of cytoskeletal signals during cell polarization.



## Introduction

Directed cell movement or chemotaxis is exhibited during wound healing, angiogenesis, embryonic development, and immune function (Lauffenburger and Horwitz, 1996). This process is highly conserved, as prokaryotes and eukaryotes from *Dictyostelium discoideum* to human leukocytes exhibit the ability to sense and move in the direction of a chemoattractant (Parent and Devreotes, 1999; Jin et al., 2000; Servant et al., 2000). Recent evidence indicates that when eukaryotic cells encounter a chemoattractant gradient they respond by local activation and amplification of signals on the side facing the gradient (Parent et al., 1998; Meili et al., 1999; Jin et al., 2000; Servant et al., 2000). These signals facilitate localized actin polymerization leading to membrane protrusion in the direction of the gradient. The protrusion of a dominant leading pseudopodium (or lamellipodium) marks the first sign of morphological polarity with establishment of an anterior and posterior compartment, and occurs independently of cell body translocation or chemotaxis (Lauffenburger and Horwitz, 1996; Parent and Devreotes, 1999). Once a dominant pseudopodium is formed, cell movement commences in the direction of the gradient as the cell undergoes a cycle of membrane extension at the front and contraction at the rear.

Recent work has shown that integrin-cytoskeletal linkages, chemokine receptors, and actin regulatory proteins are spatially regulated in migratory cells (Ridley et al., 1992; Schmidt et al., 1993; Manes et al., 1999; Nobes and Hall, 1999; Eddy et al., 2000). Adhesive signals by integrins may also spatially localize to extending pseudopodia where they fine tune and maintain directional growth while suppressing retraction and detachment mechanisms (Smilenov et al., 1999; Kiosses et al., 2001; Laukaitis et al., 2001). Significant progress has also been made in determining the intracellular organization in migratory cells of pleckstrin homology (PH)*-domain proteins, integrins, and the small GTPase Rac1 using GFP technology (Meili et al., 1999; Jin et al., 2000; Servant et al., 2000; Kraynov et al., 2001; Laukaitis et al., 2001). However, this work is typically done in individual cells, preventing molecular and biochemical evaluation of

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*Abbreviations used in this paper ECM, extracellular matrix; ERK, extracellular-regulated kinase; FAK, focal adhesion kinase; LPA, lysophosphatidic acid; PH, pleckstrin homology; SD, substrate domain; SH, src-homology.

chemotactic signals and their spatio-temporal association with regulatory proteins and scaffolds. This is due to the lack of cellular material available for analysis, as well as the inability to specifically isolate the pseudopodium and cell body for biochemical comparison using previous techniques. The biochemical purification of the pseudopodium and cell body is necessary to understand the molecular detail of entire signaling networks and spatio-temporal mechanisms of regulation including protein translocation, activation/phosphorylation, and formation of complex multiprotein scaffolds. In this work we provide evidence for purification of pseudopodia induced to undergo growth or retraction, and demonstrate the spatial, dynamic assembly of the CAS/Crk/Rac signaling complex that controls this process.

## Results

### LPA induces cell chemotaxis, but not chemokinesis

Directional cell migration or chemotaxis requires cells to sense the direction and proximity of a chemoattractant. This requires activation of localized signals and actin polymerization on the cell membrane facing the gradient. On the other hand, random migration or chemokinesis on the other hand is persistent cell movement in the absence or presence of a uniform concentration of chemokine (Lauffenburger and Horwitz, 1996). To determine whether lysophosphatidic acid (LPA) (Fukushima et al., 2001) and insulin induced directed or random cell migration, NIH 3T3 and COS-7 cells were examined for cell migration through 8.0-μm porous membranes in the presence of either a gradient or uniform concentration of these chemokines. Only cells exposed to an LPA gradient were induced to migrate, indicating that LPA is a true chemoattractant and does not facilitate random cell migration (Fig. 1). LPA-induced chemotaxis was dose dependent and reached a maximum with 100–200 ng/ml. In contrast, insulin promoted random migration, but was a poor chemoattractant for both cell types (Fig. 1).

### Cells extend pseudopodia through 3.0-μm pores in response to a chemoattractant gradient, independent of cell body translocation

When cells respond to a chemoattractant gradient they extend pseudopodia in the direction of the chemokine before cell translocation. The formation of a dominant leading pseudopodium establishes cell polarity and the future direction of chemotaxis. These observations prompted us to determine whether cells would polarize by extending pseudopodia through 3.0-μm porous membranes toward a chemoattractant gradient, independent of cell body translocation. Indeed, cells exposed to a gradient, but not a uniform concentration of LPA, extend pseudopodia through small pores specifically in the direction of the gradient (Fig. 2, a–c). As expected, cells exposed to a gradient of insulin failed to polarize and extend pseudopodia through the pores (unpublished data). Using a confocal microscope sequentially focused at the upper and lower membrane surface, we determined that >90% of the cells polarize by extending pseudopodia in response to an LPA gradient (unpublished



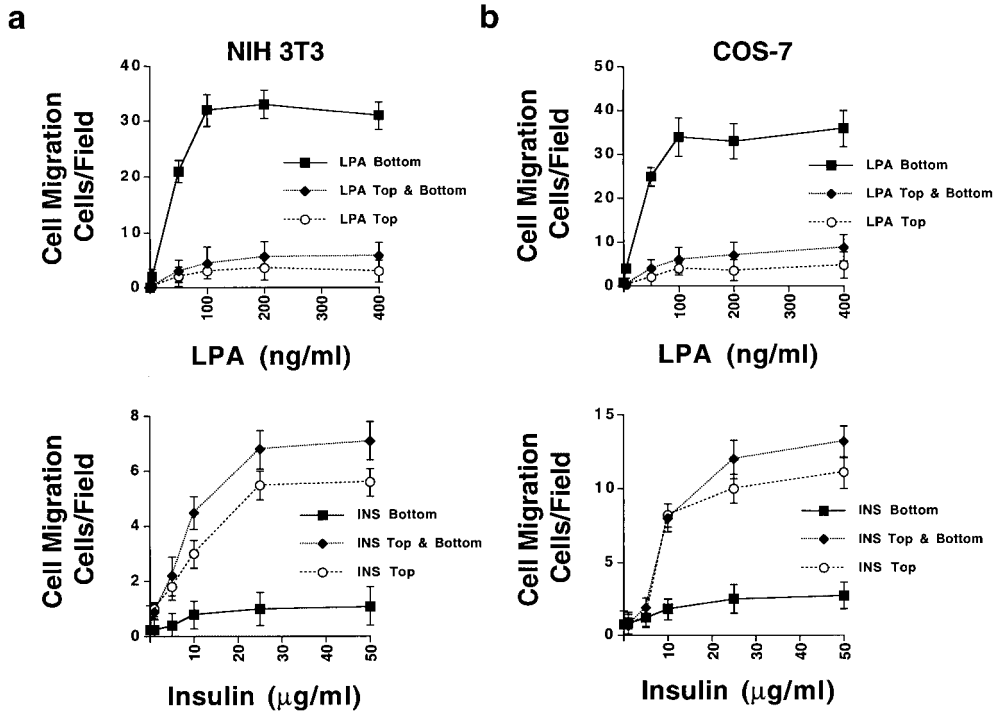


Figure 1. **Lysophosphatidic acid is a potent chemoattractant for NIH 3T3 and COS-7 cells.** (a) NIH 3T3 cells were examined for cell migration for 3 h in 8.0-μm porous Boyden chambers containing different concentrations of LPA or insulin placed in the bottom, top, or top and bottom compartments. The number of migratory cells per microscopic field (200×) on the underside of the membrane was counted as described in Materials and methods. (b) COS-7 cells were examined for cell migration as described in panel a. Each point represents the mean ± SEM of three triplicate migration chambers of three independent experiments.

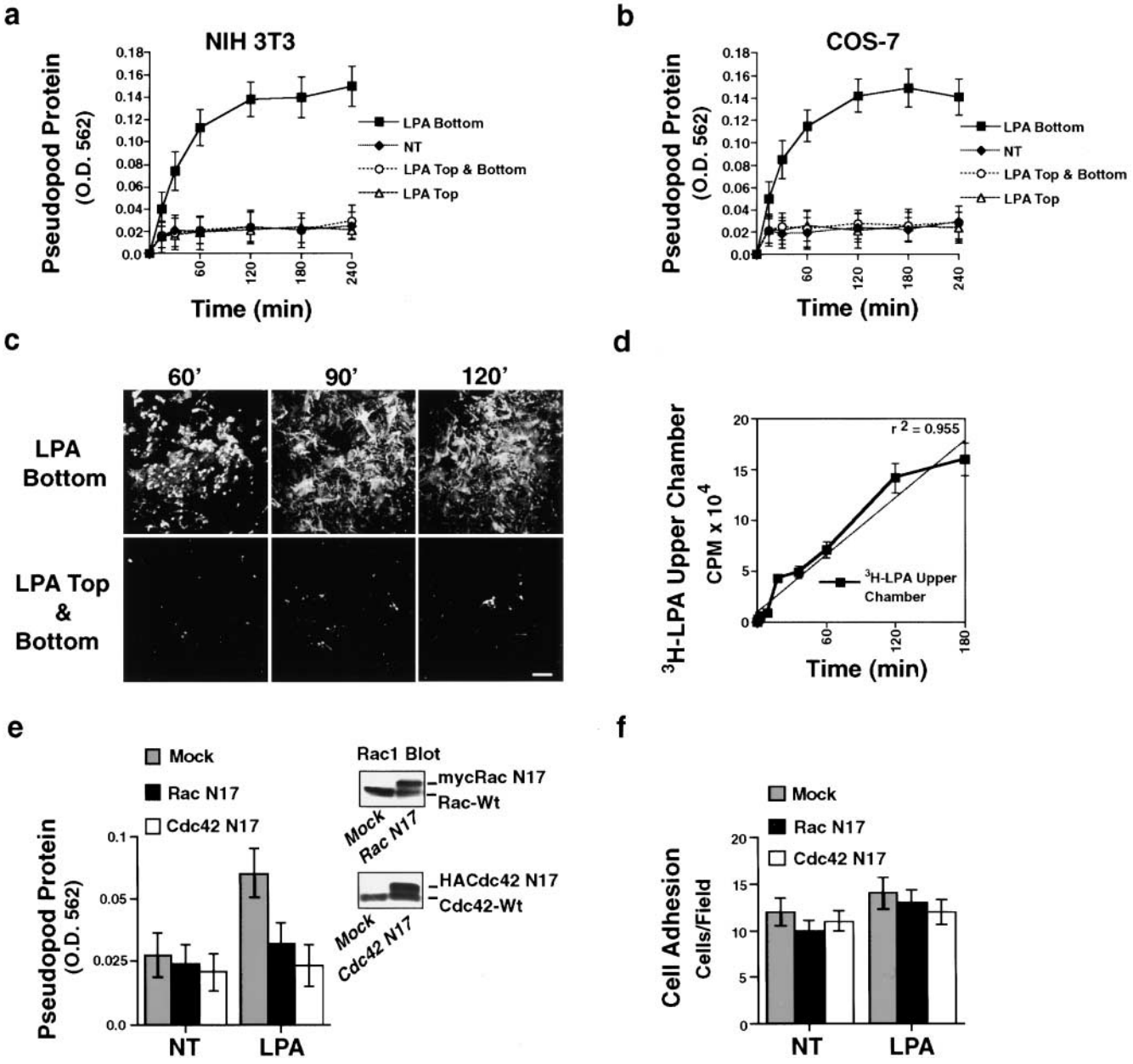


Figure 2. **Cells extend pseudopodia through 3.0-μm pores toward an LPA gradient, but not a uniform concentration of LPA.** (a) NIH 3T3 cells were allowed to attach to fibronectin coated 3.0- μm porous membranes for 2 h. Pseudopodia extension was then examined for various times in the absence (NT) or presence of LPA (100 ng/ml) in the bottom, top, or top and bottom compartments. Pseudopodia protein on the underside of the membrane was determined as described in Materials and methods. Each point represents the mean ± SEM of three triplicate membranes of three independent experiments. (b) COS-7 cells were examined for pseudopodia protrusion as described in panel a. (c) Confocal images of NIH 3T3 pseudopodia protrusion on the undersurface of a 3.0-μm porous membrane in response to LPA (100 ng/ml) as a gradient (LPA bottom) or uniform concentration (LPA top and bottom). Cells were labeled with cell tracker green and then fixed at the indicated times to visualize protruding pseudopodia on the undersurface of the membrane. Bar, 15 μm. (d) Diffusion of $^3$H-LPA (100 ng/ml) from the lower chamber to the upper chamber through a 3.0-μm porous membrane was measured at the times indicated. Each point represents the mean counts per minute (CPM) ± SEM of three triplicate chambers of three independent experiments. (e) COS-7 cells transfected with dominant negative Rac1 (Rac N17), Cdc42 (Cdc42N17), or the empty vector (mock) were examined for pseudopodia formation toward an LPA gradient for 60 min as described in (a). Each bar represents the mean ± SEM of three triplicate membranes of three independent experiments. An aliquot of cells used for the pseudopodia assay was also lysed and Western blotted for exogenous Rac and Cdc42 expression. RacN17 and Cdc42N17 are myc- and HA-tagged, respectively, and thus show reduced mobility in SDS-PAGE relative to endogenous proteins (Wt). (f) An aliquot of cells transfected as in panel e was examined for cell adhesion to collagen-coated dishes for 30 min in the presence or absence (NT) of LPA (100 ng/ml) as described in Materials and methods. Results are reported as cells attached per microscopic field (200×). Each bar represents the mean ± SEM of five fields of three triplicate wells of three independent experiments.

data). Extension of pseudopodia through 3.0-μm pores was first detected 10–15 min after exposure to the LPA gradient and proceeded linearly for 60–90 min (Fig. 2, a and b). Formation of the LPA gradient under these conditions was steep and linear for at least 3 h (Fig. 2 d). In addition, nuclei were not detected (DAPI staining) on the lower surface of

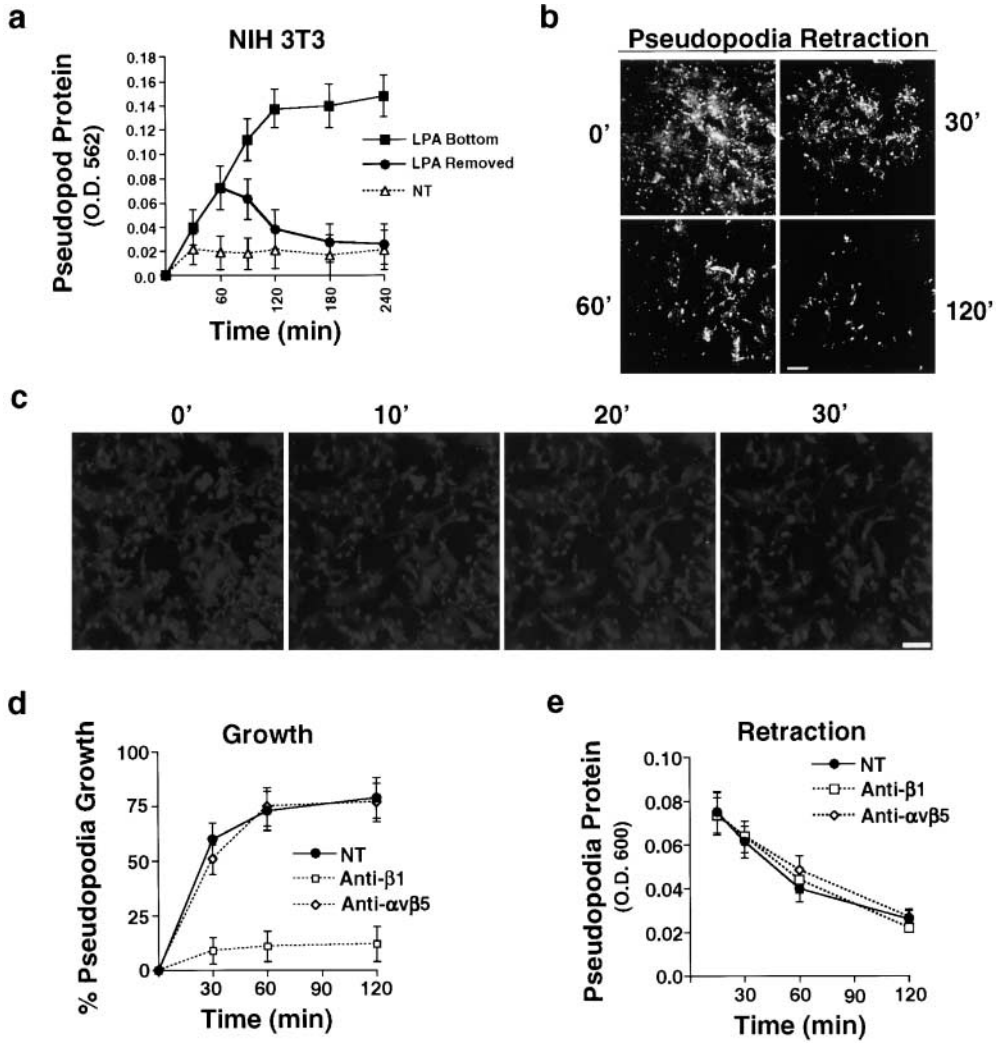


Figure 3. **Pseudopodia retract upon removal of an LPA gradient.** (a) Pseudopodia extension of NIH 3T3 cells through 3.0-μm pores toward an LPA gradient was examined for the indicated times, or the LPA gradient was removed after 60 min and pseudopodia were allowed to retract for the indicated times (LPA Removed). Total pseudopodia protein on the underside of the membrane was determined as described in Materials and methods. Each point represents the mean ± SEM of three triplicate membranes of three independent experiments. (b) Pseudopodia were allowed to extend for 60 min (time 0) toward an LPA gradient as described in panel a. The gradient was then removed and pseudopodia were fixed at the indicated times. Cells were labeled with cell tracker green (CTG) to visualize retracting pseudopodia on the undersurface of the membrane by confocal microscopy. Bar, 15 μm. (c) NIH 3T3 cells labeled with CTG were allowed to extend pseudopodia through 3.0-μm pores toward an LPA gradient for 60 min. The LPA gradient was then removed (time 0) and the pseudopodia allowed to retract for the indicated times. Time-lapse images of retracting pseudopodia were taken with a confocal microscope focused at the pore–membrane interface on the undersurface of the polycarbonate membrane. See Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200111032/DC1) for a movie illustrating pseudopodia retraction. Bar, 15 μm. (d) COS-7 cell pseudopodia extension toward an LPA gradient was determined in the presence or absence (NT) of function-blocking antibodies to αvβ5 and β1 integrins (25 μg/ml) for the indicated times. Percent pseudopodia growth is the amount of pseudopodia protein on the undersurface of the membrane induced by cells exposed to an LPA gradient relative to cells in the absence of LPA. (e) Retraction of COS-7 cell pseudopodia were determined as described in panel a in the presence or absence (NT) of antibodies to αvβ5 and β1 integrins (25 μg/ml) for the indicated times. Each point represents the mean ± SEM of three triplicate membranes of three independent experiments.

the membrane, but were restricted solely to the cell body on the upper surface, indicating that only pseudopodia protrude through the pores (unpublished data). Most importantly, pseudopodia extension was prevented in cells expressing dominant negative mutants of the small GTPase Rac1 (RacN17) or Cdc42 (Cdc42N17) (Ridley et al., 1992) (Fig. 2, e and f). Rac and Cdc42 are well established to control cell polarity through regulation of actin protrusive processes at the leading front of migratory cells (Allen et al., 1998; Nobes and Hall, 1999; Etienne-Manneville and Hall, 2001). Together, these findings indicate that cells establish polarity by extending leading pseudopodia through 3.0-μm pores toward a gradient of LPA in a Rac and Cdc42 dependent manner.

## Pseudopodia retract upon removal of a chemoattractant gradient

Given that the cells polarized toward a chemoattractant gradient, removing the gradient should induce pseudopodia retraction and loss of cell polarity. To investigate this possibil-

ity, cells were induced to extend pseudopodia toward an LPA gradient for 60 min. The gradient was then removed from the lower chamber and pseudopodia retraction was examined for various times. Loss of the LPA gradient was sufficient to reverse the polarized phenotype and induce pseudopodia retraction (Fig. 3, a and b). Confocal imaging and time-lapse determination revealed that pseudopodia retraction began within 5 min of removing the gradient and proceeded for 2 h (Fig. 3 c). No new pseudopodia extensions were observed during this time period, and >80% of the cells displayed pseuodopodia retraction under these conditions (unpublished data). As expected, restoring the LPA gradient to these cells inhibited the retraction process and induced pseudopodia growth (unpublished data). Therefore, cells retract their pseudopodia protrusions and lose cell polarity upon removal of the chemoattractant gradient.

Recent evidence indicates that integrin adhesion receptors play a critical role in facilitating and maintaining directional growth of pseudopodia on extracellular matrix (ECM) proteins (Bailly et al., 1998; Kiosses et al., 2001; Laukaitis et al., 2001). To investigate whether integrins were necessary for pseudopodia extension on a collagen substrate, COS-7 cells were allowed to attach to collagen-coated membranes for 2 h and then exposed to an LPA gradient in the presence of function-blocking antibodies to β1 integrins, which facilitates attachment of these cells to collagen (Cho and Klemke, 2000). The anti-β1 antibody prevented pseudopodia growth on collagen, whereas control antibodies to the vitronectin receptor αvβ5 present on these cells did not (Fig. 3 d). Importantly, the β1 blocking antibodies specifically prevented pseudopodia extension and did not cause detachment of the cell body from the substratum (unpublished data). The anti-β1 antibodies did not alter pseudopodia retraction, indicating that formation of new adhesion contacts were not necessary for the retraction process per se (Fig. 3 e). These findings demonstrate that integrins are necessary for pseudopodia growth on the ECM, and provide additional evidence that new pseudopodia do not extend through the pores after the gradient is removed, as this would depend on new integrin contacts.

## Purification of growing and retracting pseudopodia

The above findings indicate that pseudopodia growth and retraction is a dynamic process involving changes in focal adhesions and the actin cytoskeleton. To directly examine cytoskeletal components as well as complex signaling pathways that control cell polarity, we sought to biochemically separate the cell into its leading pseudopodium and cell body for protein analysis. Our findings that cells polarize by extending pseudopodia through 3.0-μm pores, only in the direction of a chemoattractant gradient, presented us with a unique opportunity to separate and purify these structures. Cells were allowed to extend pseudopodia for 60 min toward a chemoattractant gradient or the gradient was removed and cells were allowed to retract for 30 min. At these times, the chemoattractant gradient as well as pseudopodia growth and retraction are linear (Fig. 2). The cell body on the upper surface of the membrane was manually removed and the pseudopodia on the undersurface extracted with detergent. The cell body was purified in a similar manner except that pseudopodia on the lower surface were manually removed and the cell body on the upper surface extracted with detergent. The total profile of proteins isolated from the cell body and pseudopodium was normalized and then analyzed by one-dimensional SDS-PAGE. As expected, abundant proteins were similar in the cell body and pseudopodium, indicating that the samples were normalized appropriately (Fig. 4 a). However, nuclear histones were clearly absent from the pseudopodium, providing additional evidence for the selective purification of this structure (14–18- and 30-kD proteins) (Fig. 4 a).

## Analysis of cytoskeletal-associated proteins and tyrosine phosphorylation in growing and retracting pseudopodia

Next, we examined the expression of several cytoskeletal proteins previously associated with pseudopodia structures in cells. Caldesmon, dynamin II, paxillin, and filamin were all substantially increased in the pseudopodium (Fig. 4 b) (Ridley et al., 1992; Helfman et al., 1999; Ohta et al., 1999; McNiven et al., 2000; Laukaitis et al., 2001). In contrast, the actin-severing protein gelsolin was restricted to the cell body proper and was not present in the pseudopodium, whereas extracellular-regulated kinase (ERK)2 did not show a spatial change in polarized cells (Azuma et al., 1998). Importantly, Rho, Rac, and Cdc42 showed significantly increased activity in the extending pseudopodium compared with the cell body (Fig. 4 c). Associated with the increased GTPase activity was increased Rho and Rac, but not Cdc42 protein levels, in the pseudopodium. On the other hand, retracting pseudopodia showed decreased Rho, Rac, and Cdc42 activity, as well as decreased Rho and Rac protein levels. However, whereas Rho activity was clearly decreased during retraction, a notable amount of Rho activity remained in the pseudopodium under these conditions (Fig. 4 c). There was also a small increase in Rho activity in the body of retracting cells. Rho may play an important role in both growth and retraction processes through its ability to regulate Rac and Cdc42 activity as well as the actin/myosin contractile machinery (Schmitz et al., 2000). Pseudopodia isolated after 15 and 90 min of growth or 15 and 90 min of retraction showed identical results (unpublished data). It is important to note that during LPA-induced pseudopodia growth there is no change in Rho, Rac, or Cdc42 activity in the cell body relative to the nontreated whole cell group. Thus, it is clear that the cell body on the upper surface does not simultaneously extend pseudopodia, as this would also lead to increased GTPase activity in the upper compartment. Together, these findings demonstrate the biochemical purification of pseudopodia and the spatial segregation of cytoskeletal regulatory proteins in polarized cells.

Establishment of a leading pseudopodium requires spatial regulation of actin polymerization and formation of focal adhesions, which is associated with tyrosine phosphorylation of cellular proteins (Lauffenburger and Horwitz, 1996; Aplin et al., 1998). Therefore, we examined the phosphotyrosine protein profile in the cell body and pseudopodium of cells polarized towards a chemoattractant gradient. Phosphoproteins of 220, 125–130, 100, and 70 kD were present in purified pseudopodia and either absent or dephosphory-

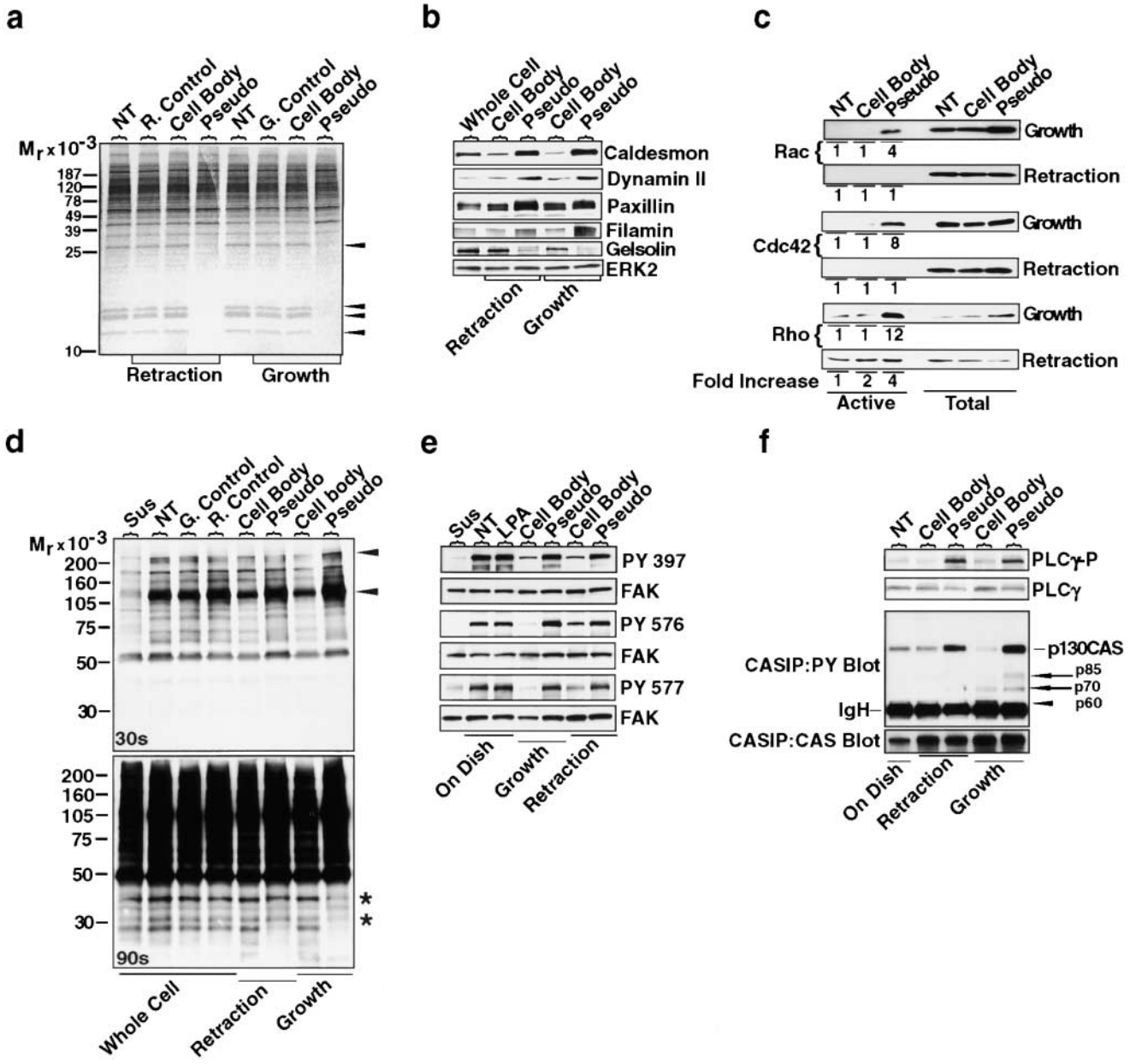


Figure 4. **Biochemical characterization of cytoskeletal-regulatory proteins in growing and retracting pseudopodia.** (a) NIH 3T3 cells were allowed to extend pseudopodia toward an LPA gradient (100 ng/ml) for 60 min (growth), or the LPA gradient was removed and pseudopodia allowed to retract for 30 min (retraction). Proteins (10 μg) isolated from the cell body on the upper membrane surface or pseudopodia (Pseudo) on the lower membrane surface were resolved by one-dimensional SDS-PAGE and GelCode Staining (Pierce Chemical Co.) as described in Materials and methods. Total cellular proteins were also isolated from cells attached to culture dishes either not treated (NT) or treated with a uniform concentration of LPA for 60 min (growth control). Cells were also treated with a uniform concentration of LPA for 60 min and then washed and proteins isolated after 30 min of retraction (retraction control). Arrowheads indicate nuclear histone proteins which are absent in purified pseudopodia. (b) Proteins isolated as described in panel a were analyzed by Western blotting using antibodies to the indicated proteins. Whole cell represents total cellular protein isolated from untreated cells attached to fibronectin coated dishes for 90 min. (c) Proteins prepared from NIH 3T3 cells as described in panel a were examined for GTP-bound activated Rac and Cdc42 using the p21-binding domain of PAK, which selectively binds Rac- and Cdc42-GTP. Rho-GTP was detected using the Rhotekin Rho binding domain. GTP bound (active) or total protein (total) in the corresponding whole-cell lysates was detected by Western blotting as described in Materials and methods. Densitometry was used to determine the ratio of GTP bound Rac, Cdc42, and Rho to the total GTPase protein present in an aliquot of the same protein lysates used for the activity assay. Fold increase represents the change in GTPase activity in the cell body and pseudopodial fractions relative to basal GTPase activity present in untreated whole cells (NT). (d) NIH 3T3 cells were either held in suspension for 30 min (sus) or allowed to attach for 2 h to either fibronectin coated culture dishes, or the upper surface of a 3.0-μm porous membrane coated with fibronectin. Whole cells on culture dishes or pseudopodia in the growth and retraction phase were isolated as described in panel a and analyzed for tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibodies. Blots treated with ECL reagent were exposed to film for 30 and 90 s. Arrowheads indicate proteins with increased phosphotyrosine in purified pseudopodia. Asterisk shows proteins with increased phosphotyrosine in retracting pseudopodia. (e) Total proteins isolated as described in (d) were analyzed by Western blotting with phosphorylation site–specific antibodies to FAK at tyrosine's 397 (autophosphorylation, c-src and PI3K binding sites), 576, and 577

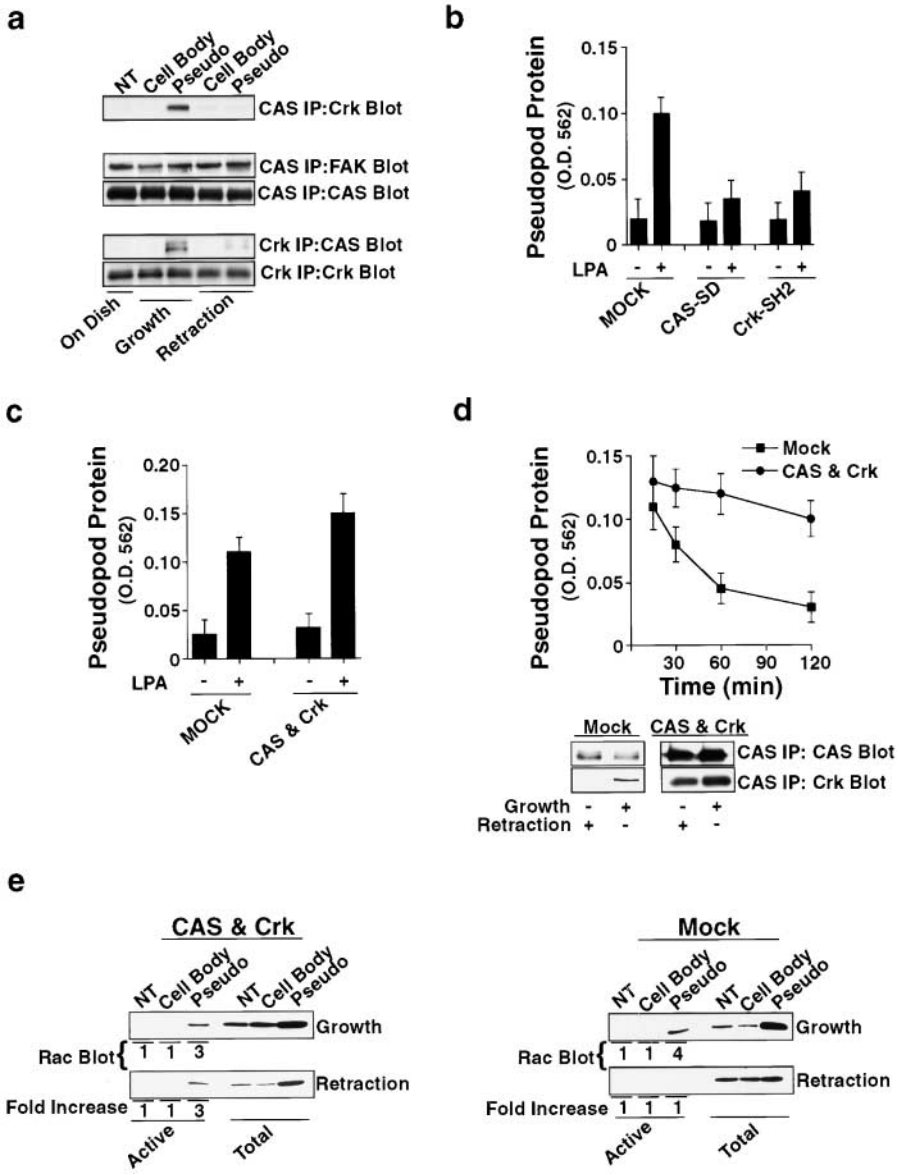


Figure 5. **CAS/Crk coupling and Rac activity regulate pseudopodia growth and retraction.** (a) NIH 3T3 cells attach to fibronectin coated 3.0-μm porous membranes were allowed to extend pseudopodia toward a LPA gradient (100 ng/ml) for 60 min (growth), or the LPA gradient was removed and the pseudopodia allowed to retract for 30 min (retraction). Proteins isolated from the cell body or pseudopodia under growth or retraction conditions were prepared as described in Fig. 4 a. Total cellular protein (NT) was also isolated from cells attached to culture dishes in the absence of LPA. CAS or Crk was then immunoprecipitated and Western blotted for associated Crk or CAS, respectively. Also, FAK association with CAS was examined in the CAS immunoprecipitates by Western blotting with FAK specific antibodies. (b) COS-7 cells were transfected with the empty vector (Mock) or the vector encoding either CAS with its substrate domain truncated (CAS-SD) or Crk with a mutated SH2 domain (Crk-SH2). Cells were then examined for pseudopodia extension in response to a LPA gradient (100 ng/ml) or left untreated for 60 min. Pseudopodia protein was determined as described in Materials and methods. (c) COS-7 cells were transfected with the empty vector (Mock) or vectors encoding CAS and Crk. Cells were then examined for pseudopodia extension in response to a LPA gradient (100 ng/ml) or not treated for 60 min. Pseudopodia protein on the underside of the membrane was determined as described in Materials and methods. (d) COS-7 cells transfected as in panel c were allowed to extend pseudopodia toward an LPA gradient (100 ng/ml) for 60 min (growth), or the LPA gradient was removed and the pseudopodia allowed to retract for 30 min (retraction). Pseudopodia protein was determined as described in Materials and methods. The bottom panel shows CAS/Crk coupling in purified pseudopodia undergoing growth (60 min) or retraction (60 min) from cells transfected as described in panel c. (e) Cell body and pseudopodia proteins prepared from cells treated as described in panel c were examined for activated Rac or total Rac protein in whole cell lysates as described in Fig. 4 c.

lated in the cell body (Fig. 4 d). Importantly, these proteins were dephosphorylated in suspension cells, indicating that their phosphorylation is dependent on cell adhesion to the ECM. In contrast, phosphoproteins of 74 and 28 kD were present in the cell body and either absent or dephosphorylated in the pseudopodium. Phosphotyrosine proteins appeared similar under growth and retraction conditions except for a prominent 40-kD protein that was phosphorylated only in pseudopodia undergoing retraction, but not growth, suggesting a possible role for this protein in the retraction process (Fig. 4 d).

Analysis of the focal adhesion–associated proteins FAK, CAS, and PLCγ-1 revealed that these p120–130-kD phosphoproteins were strongly activated/phosphorylated during pseudopodia growth as well as retraction, although there may be a small decrease in FAK 576 and 577 phosphoryla-

(catalytic activation sites). Blots were stripped and reprobed with antibodies to FAK protein to evaluate the level of FAK protein present in the lysates. (f) Proteins isolated as described in (a) were either analyzed by Western blotting with phosphorylation-site specific antibodies to tyrosine 783 of human PLCγ-1 or immunoprecipitated with antibodies to CAS and then immunoblotted with antiphosphotyrosine antibodies. NT is total cellular protein isolated from untreated cells attached to fibronectin coated dishes for 120 min. Arrows indicate tyrosine phosphorylated proteins of 85 (p85), and 70 kD (p70) that coimmunprecipitate with CAS specifically in growing, but not retracting pseudopodia. Arrowhead shows a tyrosine-phosphorylated protein of 60 kD that coimmunoprecipitates with CAS in the cell body, but not pseudopodia of polarized cells. IgH is the immunoglobulin heavy chain.

tion during retraction (Fig. 4, e and f). Importantly, the relative levels of FAK, CAS, and PLCγ-1 were similar in the pseudopodium and cell body of polarized cells during growth and retraction. Thus, the protein level and tyrosine phosphorylation of these signals do not change significantly (Fig. 4, e and f), even though pseudopodium growth/retraction involves dynamic focal contact remodelling and actin cytoskeletal changes. These findings suggest that general dephosphorylation or loss of focal adhesion–associated signals is not the primary mechanism responsible for pseudopodium retraction and detachment from the substratum. Thus, the process of pseudopodia retraction is not simply the reverse of the signaling processes that mediate growth. This is significantly different than cell detachment from the ECM, which is accompanied by complete dephosphorylation and inactivation of FAK, CAS, and PLCγ-1 (Aplin et al., 1998).

### Assembly and disassembly of a CAS/Crk complex controls Rac localization and activity, which is necessary for pseudopodia growth and retraction, respectively

What role then does tyrosine phosphorylation of these and other cytoskeletal regulatory proteins play during pseudopodia growth and retraction? One possibility is that these proteins assemble specific signaling scaffolds consisting of unique kinases, substrates, and effector proteins that regulate this process. To investigate this possibility, we examined the formation of a FAK/CAS/Crk protein complex, as the spatio-temporal assembly of this scaffold and its role in mediating cell migration are poorly understood (Klemke et al., 1998). Interestingly, Crk strongly associated with CAS in growing but was significantly reduced in retracting pseudopodia (Fig. 5 a). The interaction was specific to the pseudopodium, as CAS isolated from the cell body of polarized cells did not show Crk binding. In contrast, FAK, which binds to the src-homology (SH)3 domain of CAS, showed no difference in binding to CAS under these conditions (Fig. 5 a). In addition, we observed several tyrosine-phosphorylated proteins that coimmunoprecipitated with CAS in growing, but not retracting pseudopodia (Fig. 4 f). In contrast, a 60-kD phosphoprotein associated with CAS only in the cell body, but not the pseudopodium. Denaturation of cellular proteins before CAS immunoprecipitation prevented the association of these proteins with CAS, indicating that the interaction is specific and not related to antibody cross-reactivity (unpublished data). Thus, CAS/Crk complexes assemble and disassemble in a highly spatial manner during pseudopodia extension and retraction.

Cell adhesion to the ECM promotes Crk binding via its SH2 domain to phosphotyrosine residues present in the substrate domain of CAS, whereas cell detachment causes complete dephosphorylation of CAS and disassembly of CAS/Crk complexes (Vuori et al., 1996; Klemke et al., 1998). Therefore, it is surprising that the overall level of protein and tyrosine phosphorylation of CAS does not change during pseudopodium retraction and detachment from the ECM, but Crk binding does. This suggests that assembly and disassembly of CAS/Crk in the pseudopodium is tightly regulated through phosphorylation/dephosphorylation of a specific subset of tyrosine residues present in CAS. Alternatively, phosphorylation of the regulatory tyrosine 221 of Crk, which prevents CAS/Crk coupling in cells, may regulate this process (Kain and Klemke, 2001). However, we did not detect significant changes in tyrosine phosphorylation of Crk during pseudopodia growth and retraction (unpublished data). Therefore, tyrosine phosphorylation of the substrate domain of CAS may be a critical event involved in CAS/Crk coupling and pseudopodium formation. To directly test this possibility, we examined pseudopodia formation in cells expressing CAS with its substrate domain deleted (CAS-SD). CAS-SD or Crk with a mutated SH2 domain (Crk-SH2) serve as dominant negative proteins preventing CAS/Crk coupling and Rac activation in motile cells (Klemke et al., 1998). Expression of CAS-SD or Crk-SH2 prevented pseudopodia extension in response to an LPA gradient (Fig. 5 b). These findings demonstrate that CAS/Crk coupling is necessary for cell polarization and extension of a leading pseudopodium.

To investigate whether CAS/Crk coupling could enhance pseudopodia extension, we exogenously expressed full-length CAS and Crk in cells. However, these cells did not show a significant change in pseudopodia extension in response to an LPA gradient, suggesting that these molecular signals are not limiting to the process of gradient sensing and chemotaxis and that an additional component(s) is required

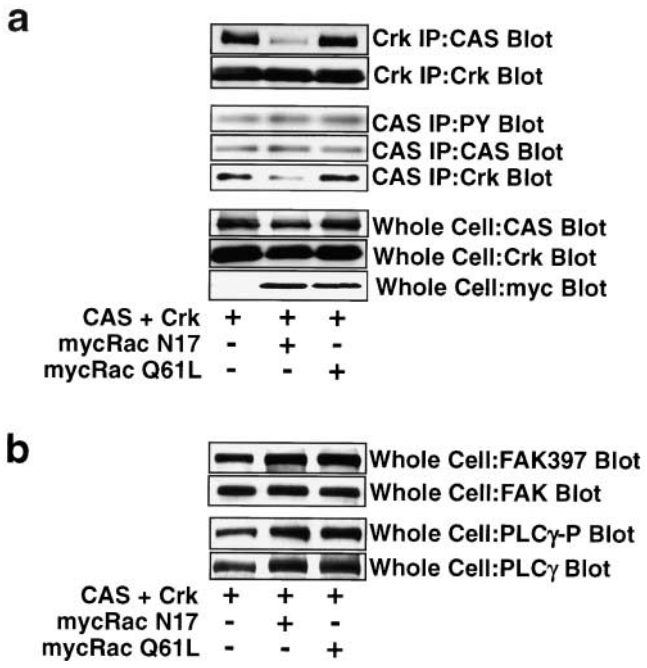


Figure 6. **Rac activity regulates assembly of CAS/Crk complexes in cells.** COS-7 cells were transfected with CAS and Crk along with either myc-tagged dominant negative (RacN17) or dominant positive (RacQ61L) Rac1. Cells were then lysed in detergent (whole cell lysate) and examined for either (a) assembly of CAS/Crk complexes as described in Fig. 5 a; or (b) changes in FAK and PLCγ-1 activity as described in Fig. 4, e and f. Blots were stripped and reprobed with antibodies to FAK and PLCγ-1 to confirm equal protein loading.

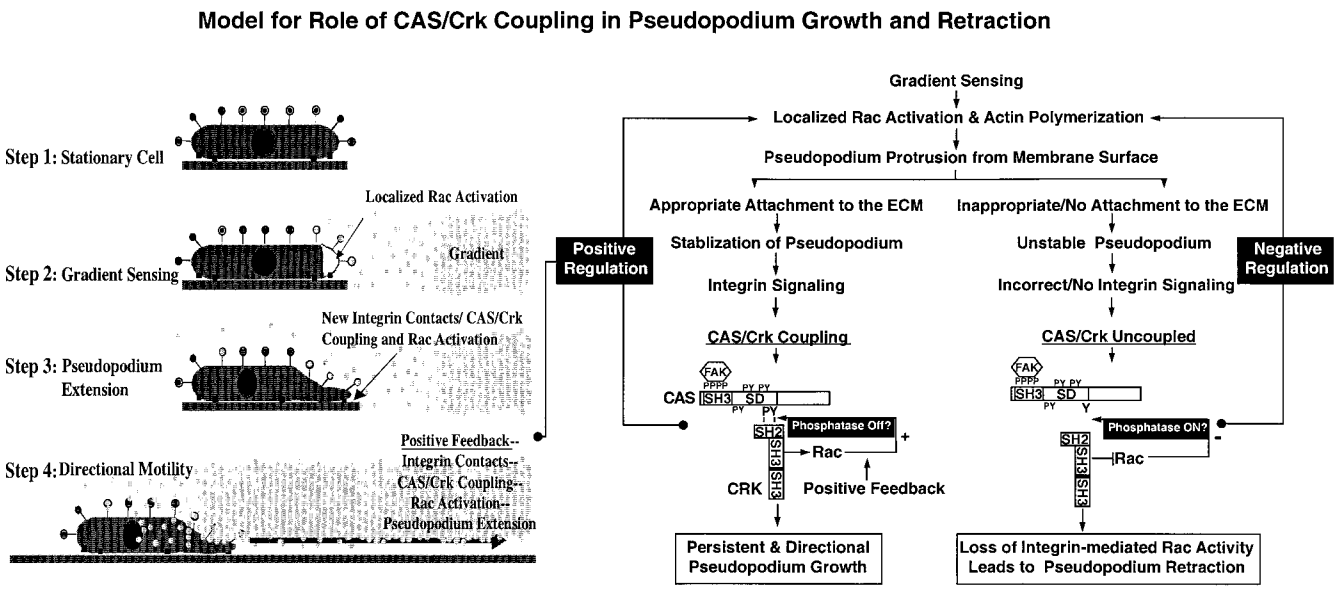


Figure 7. **Proposed model for the role of CAS/Crk coupling in regulation of Rac-mediated pseudopodial dynamics.** (Left) Model illustrating the basic steps of cell polarization and chemotaxis on the ECM. (Step 1) Stationary cells are attached to the underlying ECM through integrin receptors (squares). Cells are then exposed to a soluble gradient of growth/factor or chemokine (Step 2). This activates cell surface chemattractant receptors leading to activation and amplification of Rac signaling events on the side facing the gradient. (Step 3). Rho, Cdc42, and Rac then regulate localized actin dynamics as well as force requirements leading to membrane protrusion in the direction of the gradient. This process is independent of actual cell body translocation or chemotaxis and marks the first sign of morphological polarity with establishment of a dominant leading pseudopodium and posterior compartment. Importantly, evidence indicates that the initial protrusion of a pseudopodium at the cell surface is independent of integrins and the ECM contacts. However, integrins do play a critical role in pseudopodial dynamics by tethering the extending membrane to the substratum, which initiates the molecular coupling of CAS and Crk. CAS/Crk coupling in turn mediates Rac activity leading to sustained and directional pseudopodium growth. A pseudopodium that does not attach to the ECM rapidly retracts back to the cell body. (Step 4) Once a dominant pseudopodium is formed, cell movement commences in the direction of the gradient as the cell undergoes repeated cycles of membrane protrusion, adhesion to the ECM, and CAS/Crk/Rac activation. Importantly, our findings suggest that sustained Rac activity then provides a positive feedback signal to maintain CAS/Crk coupling and membrane extension at the leading front as the cell moves on the ECM (Fig. 7, right panel; Discussion). On the other hand, loss of the chemoattractant gradient or inappropriate contact with the ECM terminates pseudopodium extension, turning off CAS/Crk/Rac activity and chemotaxis. Therefore, in this model integrin ligation events at the leading front of the extending membrane cooperate with chemoattractant receptors to fine tune and maintain directional growth, while suppressing retraction mechanisms through regulation of CAS/Crk and Rac.

to mediate this process (Fig. 5 c). This is different from haptotaxis migration, as exogenous CAS/Crk coupling in cells is sufficient to facilitate strong migration as well as pseudopodia extension (unpublished data) toward an adhesive gradient of ECM protein in the absence of a soluble chemoattractant (Klemke et al., 1998). Surprisingly, whereas increased CAS/Crk coupling in these cells did not impact pseudopodia extension, it did prevent membrane retraction, when the gradient was removed (Fig. 5 d). In this case, CAS/Crk complexes were not disassembled, and the activation and translocation of Rac into pseudopodia were persistent (Fig. 5, d and e). These findings suggest that sustained Rac activity sends a positive feedback signal to upstream kinases/phosphatases that regulate CAS/Crk coupling and pseudopodium extension. To investigate this possibility, we examined CAS/Crk coupling in cells expressing dominant negative RacN17. Inhibition of Rac activity in cells significantly decreased CAS/Crk coupling, which was independent of changes in FAK and PLCγ-1 activity (Fig. 6, a and b). Moreover, the inhibition of CAS/Crk coupling was independent of changes in overall CAS tyrosine phosphorylation. Expression of activated RacQ61L in cells did not alter CAS/Crk coupling, indicating that this complex was maximally activated in these cells (Fig. 6 a). Therefore, Rac can operate upstream to specifically regulate assembly of CAS/Crk complexes in cells. This supports the idea that Rac activation serves as a positive feedback signal to modulate CAS/Crk coupling in migratory cells. Together, our findings demonstrate that assembly of a CAS/Crk complex is important for pseudopodia extension, whereas disassembly facilitates retraction through deactivation of Rac and its export from this cellular structure. A model depicting the role of CAS/Crk coupling in regulating Rac-mediated pseudopodium dynamics in chemotactic cells is shown in Fig. 7 and discussed below.

## Discussion

We provide several lines of evidence for the biochemical purification of pseudopodia. First, >90% of cells polarize by extending pseudopodia through 3.0-μm pores in the direction of a chemoattractant gradient, whereas cells exposed to a uniform concentration of chemokine do not. Importantly, pseudopodium extension was directional and proceeded in a linear manner, independent of cell body translocation, which allowed us to differentially isolate this structure from the cell body. Second, cytoskeletal regulatory proteins previously associated with pseudopodia-like structures were

present in protein extracts using this fractionation method (Fig. 4). In contrast, nuclear histones associated with the cell body region of polarized cells were not present in the pseudopodial extract. Finally, and most importantly, Rac and Cdc42 activity were increased in extending, but not retracting pseudopodia. Moreover, Rac and Cdc42 activity were necessary for this response as expression of a dominant negative forms of these proteins in cells prevented pseudopodia extension. Rac and Cdc42 are well documented to facilitate actin-based protrusive mechanisms leading to membrane extension and polarity in migratory cells (Ridley et al., 1992; Nobes and Hall, 1999). Together, these findings demonstrate that cells polarize by extending pseudopodia through 3.0-μm pores in the direction of a chemoattractant, and that it is possible to purify these structures for biochemical analysis.

An important aspect of this model is the ability to differentially control pseudopodia growth and retraction. Whereas nonpolarized cells in culture have been observed to extend and retract exploratory pseudopodia, the addition of a uniform concentration of chemoattractant only increases these random events, with little or no cell polarization (i.e., extension of one dominant pseudopodium). In our system, we were able to induce in a synchronous manner a leading pseudopodium in >90% of the cells. We found that removing the chemoattractant gradient also caused a synchronous loss of cell polarity and retraction of pseudopodia in the majority of cells. This allowed us to differentially monitor biochemical changes during pseudopodium growth as well as retraction. This aspect of the model is particularly attractive for studying components that regulate actin polymerization/depolymerization, myosin contractility, and adhesive signals that facilitate membrane attachment/detachment from the ECM. In addition, we show that it is possible with this model to quantify as well as visualize pseudopodia in living cells using confocal microscopy and time-lapse imaging. Therefore, analysis of signaling dynamics of growing and retracting pseudopodia in live cells is also possible with this system.

## Spatio-temporal assembly/disassembly of a CAS/Crk complex

We demonstrate that CAS and Crk are specifically assembled during pseudopodia growth and then disassembled during retraction. Surprisingly, this occurred without apparent change in the overall level of CAS tyrosine phosphorylation or FAK activation, which is an upstream activator of CAS (Vuori et al., 1996; Tachibana et al., 1997). One explanation is that Crk couples only to a specific subset of phosphotyrosine residues present in the substrate domain of CAS, which changes during pseudopodia growth and retraction. CAS/Crk association is mediated through the binding of the SH2 domain of Crk to phosphotyrosine residues present in the substrate domain of CAS (Matsuda et al., 1993). In fact, there are 15 tyrosine residues in this region of CAS that correspond to potential SH2 binding motifs, 9 of which conform to the Crk SH2 recognition sequence (YD(V/T)P (Klemke et al., 1998). Alternatively, regulation may occur through serine phosphorylation of CAS (Ma et al., 2001) or phosphorylation of the regulatory tyrosine 221 of Crk, which prevents CAS/Crk coupling in cells (Kain and Klemke, 2001). However, the latter is unlikely as we did not detect a significant change in Crk tyrosine phosphorylation. The upstream and downstream components that modulate the assembly/disassembly of this molecular scaffold in the pseudopodium are not yet clear, but likely candidates include c-src, PTP-PEST, and PTP-1B (Garton et al., 1996; Liu et al., 1996; Vuori et al., 1996).

## Translocation and activation of Rac in growing and retracting pseudopodia

The active translocation of Rac to the pseudopodium also implies that there is an import/export mechanism that controls Rac transport to the different poles of migratory cells. It is unlikely that CAS and Crk are directly involved in this process, as these proteins do not appear to translocate in and out of growing and retracting pseudopodia. Rather, it is more likely that assembly of CAS/Crk is important for maintaining localized Rac activity within the pseudopodium. For example, it may be that when cells encounter a chemoattractant gradient, Rac (and associated regulatory proteins) translocates to the side of the membrane facing the gradient as previously described for PH domain proteins like Akt (Servant et al., 2000) (Fig. 7). The localized Rac activity then induces actin polymerization leading to protrusion of a pseudopodium from the cell surface and recruitment of high-affinity integrins to this region (Kiosses et al., 2001). Interestingly, protrusion of the pseudopodium from the membrane surface is independent of integrin ligation and attachment to the ECM (Bailly et al., 1998). This clearly places Rac activation and actin protrusive mechanisms as an early response upstream of integrin ligation and CAS/Crk assembly. However, pseudopodia attachment to the ECM is critical to stabilize the protrusive structure, as protruding membranes that do not contact the ECM readily retract back to the cell body (Bailly et al., 1998). Therefore, it is likely that during pseudopodia attachment to the substratum, integrin activation and focal complex formation play a central role in driving assembly of CAS/Crk, leading to sustained Rac activation and persistent membrane protrusion. Our findings suggest that the persistent Rac activity then serves as a positive input to maintain a high level of CAS/Crk coupling at the leading front (Fig. 7). In contrast, withdrawal of the chemotactic signal terminates membrane extension and focal adhesion formation resulting in uncoupling of the CAS/Crk/Rac signaling module leading to pseudopodium retraction. Interestingly, the disassembly of this complex and membrane retraction occur independently of changes in FAK and PLCγ-1 activity, and CAS tyrosine phosphorylation. This suggests that the Rac feedback pathway specifically regulates CAS/Crk coupling and does not generally block upstream integrin signaling processes. The Rac feedback loop may target a phosphatase that dephosphorylates a specific tyrosine residue(s) in the substrate domain of CAS, which facilitates Crk binding. Apparently, integrin and cytoskeletal signals continue to play a prominent role during pseudopodium retraction by assembling and regulating new protein scaffolds that mediate this process.

However, it is clear that pseudopodium retraction is not the simple reversal of the adhesive-signaling processes that facilitate growth.

In summary, we purified pseudopodia in the process of growth or retraction and biochemically examined the spatial localization and activation of adhesion and cytoskeletal-associated signals that control this process. We demonstrate that pseudopodia extension and retraction require Rac activation/deactivation, respectively, through spatial assembly/disassembly of a CAS/Crk protein scaffold. Our findings provide a biochemical understanding of how cytoskeletal and adhesive signals control morphological changes necessary for cell migration, and illustrate the dynamic spatio-temporal complexity of signal regulation during cell polarization.

## Materials and methods

### Cell lines, reagents, and antibodies

The expression plasmid pUCCAGGS containing full-length as well as mutant forms of the c-Crk cDNA were constructed as described previously (Matsuda et al., 1993; Tanaka et al., 1993). The pEBG expression plasmid containing wild-type CAS or CAS with an in-frame deletion of its substrate domain (CAS-SD) has been described previously (Mayer et al., 1995). Myc-tagged dominant negative Rac1 (N17), dominant positive Rac1 (Q61L), and HA-tagged dominant negative Cdc42 (Cdc42N17) in pcDNA3 have been described previously (Klemke et al., 1998). Antibodies to Crk, CAS, and paxillin, were from Transduction Laboratories. Antibodies to myc (9E10), ERK2, and FAK were from Santa Cruz Biotechnology. Antibodies to phosphotyrosine (monoclonal antibody 4G10), Rho, Rac1, Cdc42, and the GTPase activity kits for these proteins were from Upstate Biotechnology. Goat anti–rabbit and anti–mouse antibodies were from Bio-Rad Laboratories. Antibodies to gelsolin, filamin, the β1 integrin subunit, integrin αvβ5, caldesmon, and QCM migration kit were from Chemicon International. Phosphospecific antibodies to FAK tyrosine 397, 576, 577, and PLCγ-1 tyrosine 783 were from Biosource International. Anti-dynamin II antibodies were provided by Dr. Sandra Schmid (The Scripps Research Institute, La Jolla, CA). LPA and anti-vinculin antibodies were from Sigma-Aldrich. $^{3}$H-LPA (50 Ci/mmol) was from NEN Life Science Products, Inc. Cell tracker green was from Molecular Probes, Inc. COS-7 cells were from American Type Culture Collection. Mouse NIH3T3 cells were provided by Dr. Tony Hunter (The Salk Institute, La Jolla, CA).

### Quantitative pseudopodia assay

Pseudopodia extension was monitored using a pseudopodia assay kit (ECM 650; Chemicon International) or Costar chambers. Serum-starved cells (75,000) were placed into the upper compartment of a chamber (6.5 μm) equipped with a 3.0-μm porous polycarbonate membrane coated on both sides with an optimal amount of ECM protein (5 μg/ml fibronectin or collagen type I). Cells were allowed to attach and spread on the upper surface of the membrane for 2 h, and then stimulated with LPA (Sigma-Aldrich), insulin, or buffer only, which was placed in the lower chamber to establish a gradient or placed in the upper and lower chamber to form a uniform concentration. Cells were allowed to extend pseudopodia through the pores toward the direction of the gradient for various times. To initiate pseudopodia retraction, the chemoattractant was removed or an equivalent amount of chemoattractant placed in the upper chamber to create a uniform concentration. The cell body on the upper surface was manually removed with a cotton swab and the total pseudopodia protein only on the undersurface determined using BCA and a microprotein assay system (Pierce Chemical Co.). It is also possible to stain pseudopodia with 1% crystal violet in 2% ethanol and then elute the dye with 10% acetic acid, which can be measured in an ELISA plate reader (OD 600) for comparison to a standard curve as previously described (Klemke et al., 1998).

### Purification of pseudopodia, western blotting, and GTPase activity assays

To specifically isolate proteins from growing pseudopodia, 1–1.5 × $10^6$ cells were induced to form pseudopodia for various times as described above or not treated using a pseudopodia isolation kit (ECM 660; Chemicon International) or chambers from Costar (24 mm 3.0-μm pores). Cells were rinsed in excess cold PBS and either rapidly fixed in 100% ice-cold methanol (Western blotting of whole-cell lysates) or not fixed (for GTPase activity and immunoprecipitation assays). We found that the protein profile of pseudopodia proteins from fixed and unfixed cells is identical under these conditions as determined by silver staining and SDS-PAGE (unpublished data). Cell bodies on the upper membrane surface were manually removed with cotton swab and pseudopodia on the undersurface scrapped into lysis buffer (100 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, protease inhibitors [cocktail tablet; Boehringer Mannheim Corp.]) containing the appropriate detergent for Western blotting of whole-cell lysates (1% SDS), CAS/Crk coupling (1% Triton X-100), or GTPase activity assays (Triton X-100 according to the manufacturer's recommendation; UBI). Cell bodies were purified in a similar manner except that pseudopodia on the undersurface were removed and the cell body on the upper surface scraped into lysis buffer and detergent. Retracting pseudopodia were induced for various times by removing or placing chemoatttractant in the upper chamber to create a uniform concentration as described above. We typically obtain 25–30 μg of pseudopodia protein from each 24-mm well stimulated with chemokine for 30–60 min. This represents ~4–5% of the total cellular protein. Cell culture, transient transfections and efficiency determinations, Western blotting, chemotaxis, and adhesion assays were performed at least three times, and a representative image is shown as previously described (Klemke et al., 1998). We routinely obtain transfection efficiencies in COS-7 and NIH 3T3 cells of 80 and 70%, respectively.

### Time-lapse imaging of retracting pseudopodia

Cells were labeled with cell tracker green according to the manufacturer's recommendation (Molecular Probes). Pseudopodia retraction was initiated by removing chemoattractant from the lower chamber as described above. The upper compartment (6.5 μm) was then fitted to an Attofluor cell chamber containing a glass coverslip (Molecular Probes) and placed into a stage heater (20/20 Technologies) on a Zeiss Axiovert 100TV microscope. A Bio-Rad 1024 confocal microscope was used to capture time-lapse images of pseudopodia on the undersurface of the membrane every 60 s with a Zeiss 633 Achostigmat lens and LaserSharp software (Bio-Rad Laboratories). QuickTime and Adobe photoshop software were used to prepare time-lapse movies and representative montage images for each time frame assembled for comparison. Immunofluorescent staining and confocal imaging of cells were performed as described (Cheresh et al., 1999).

### Online supplemental material

Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200111032/DC1) shows time-lapse images of pseudopodia retraction through 3.0-μm pores as depicted in Fig. 3 C.

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