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Rab5 Induces Rac-independent Lamellipodia Formation and Cell Migration

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Rab5 is a regulatory GTPase of vesicle docking and fusion that is involved in receptor-mediated endocytosis and pinocytosis. Introduction of active Rab5 in cells stimulates the rate of endocytosis and vesicle fusion, resulting in the formation of large endocytic vesicles, whereas dominant negative Rab5 inhibits vesicle fusion. Here we show that introduction of active Rab5 in fibroblasts also induced reorganization of the actin cytoskeleton but not of microtubule filaments, resulting in prominent lamellipodia formation. The Rab5-induced lamellipodia formation did not require activation of PI3-K or the GTPases Ras, Rac, Cdc42, or Rho, which are all strongly implicated in cytoskeletal reorganization. Furthermore, lamellipodia formation by insulin, Ras, or Rac was not affected by expression of dominant negative Rab5. In addition, cells expressing active Rab5 displayed a dramatic stimulation of cell migration, with the lamellipodia serving as the leading edge. Both lamellipodia formation and cell migration were dependent on actin polymerization but not on microtubules. These results demonstrate that Rab5 induces lamellipodia formation and cell migration and that the Rab5-induced lamellipodia formation occurs by a novel mechanism independent of, and distinct from, PI3-K, Ras, or Rho-family GTPases. Thus, Rab5 can control not only endocytosis but also actin cytoskeleton reorganization and cell migration, which provides strong support for an intricate relationship between these processes.

INTRODUCTION

Members of the superfamily of Ras-like GTPases have been implicated in a wide variety of biological processes: the Ras-family members such as Ras, R-ras, and Rap mainly in the regulation of proliferation, differentiation, and apoptosis (Bos, 1997); members of the Rho family such as Rho, Rac, and Cdc42 in cytoskeletal reorganization, gene transcription, and cell growth control (Zigmond, 1996; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998); and members of both the Rab family, such as Rab3 and Rab5, and the Arf family, such as Arf1 and Arf6, in vesicle fusion and transport involved in secretion and endocytosis (Lazar *et al.*, 1997; Novick and Zerial, 1997).

Rab5 has been implicated in receptor-mediated endocytosis and pinocytosis (Bucci *et al.*, 1992; Stenmark *et al.*, 1994). Introduction of active Rab5 in cells stimulates the rate of endocytosis and vesicle fusion, resulting in the formation of large endocytic vesicles, whereas dominant negative Rab5 inhibits vesicle fusion (Stenmark *et al.*, 1994, 1995). Rab5 appears to serve as a timer for docking between endocytic vesicles and early endosomes, with GTP–GDP exchange being required for membrane fusion, whereas GTP hydrolysis is required to stop the fusion process (Rybin *et al.*, 1996). Because it was found that a t-SNARE protein is activated by transient interaction with a Rab-like GTPase in yeast (Lupashin and Waters, 1997), Rab5 may regulate endosome docking and fusion by regulating the rate of SNARE complex assembly. Several Rab5 regulatory proteins have been identified. RabGDI, a Rab GDP-dissociation inhibitor, binds to Rab5 in the GDP-bound state and keeps Rab5 cytosolic by masking the geranyl–geranyl group of Rab5 (Ullrich *et al.*, 1994). Upon release of RabGDI, which may be induced by a GDI displacement factor (Dirac-Svejstrup *et al.*, 1997), Rab5 becomes membrane-associated. Rabex5 has been identified as a Rab5–guanine nucleotide dissociation stimulator, which can activate Rab5 by exchanging the bound GDP for GTP (Horiuchi *et al.*, 1997). Interestingly, the TSC2 product tuberin has been identified as a putative Rab5-GTPase–activating protein (Xiao *et al.*, 1997). Furthermore, Rabaptin5 has been identified as a Rab5 effector involved in endosome fusion (Stenmark *et al.*, 1995).

The actin and microtubule cytoskeleton plays an important role in vesicle transport (Cole and Lippincott-Schwartz, 1995; Lamaze *et al.*, 1996, 1997; Murphy *et al.*, 1996). With

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respect to endocytosis, recent studies have revealed that active mutants of the actin cytoskeleton–regulatory GTPases Rac1 and RhoA, which are involved in lamellipodia and stress-fiber formation, respectively, as well as agents that interfere with actin polymerization, inhibit receptor-mediated endocytosis (Lamaze *et al.*, 1996, 1997). Furthermore, an active mutant of RhoD, which induces plasma membrane rearrangements and a decrease in stress fibers, inhibits endosome motility (Murphy *et al.*, 1996). Given this important role for the cytoskeletal organization in endocytosis, and because vesicle transport has been proposed to be involved in membrane ruffling (Bretscher and Aguado-Velasco, 1998a) and cell migration (Bretscher, 1996a,b), we investigated whether the endocytosis–regulatory GTPase Rab5 itself may have the ability to control cytoskeletal reorganization and/or cell migration.

Here we show that Rab5 induces strong reorganization of the actin cytoskeleton resulting in lamellipodia formation. The lamellipodia formation by Rab5 does not require activation of Ras, PI3-K, or members of the Rho family of GTPases involved in cytoskeletal reorganization. In addition, insulin-, Ras-, or Rac-induced actin cytoskeletal reorganization does not require activation of Rab5. Furthermore, we observed a dramatic effect of Rab5 upon cell migration. These findings are discussed in the light of an apparent intricate relationship between the processes of endocytosis, cytoskeletal reorganization, and cell migration.

MATERIALS AND METHODS

Expression Plasmids

pToto2JC1 containing human L79-Rab5 or N34-Rab5 (Li and Stahl, 1993; Li *et al.*, 1995, 1997) was used to create the pMT2 expression plasmids encoding N-terminally HA-tagged L79- or N34-Rab5. pGBT8-V12-Rac1 and pGBT8-N17-Rac1 (Spaargaren and Bischoff, 1994) were used to create pcDNA3 encoding N-terminally myc-tagged V12-Rac1 and N17-Rac1. pRK5 encoding myc-tagged V12- or N17-cdc42 and pEXV encoding myc-tagged V14- or N19-RhoA were kindly provided by C. D. Nobes and A. Hall, and pCMV6 M-PAK-RBD encoding the myc-tagged domain of PAK comprising amino acids 67–150 was a generous gift by J. Chernoff. pSV-V12-Ras and pRSV-N17-Ras are as described by Medema *et al.* (1991).

Cell Culture and Transfection

NIH 3T3-A14 fibroblasts (Burgering *et al.*, 1991), grown on glass coverslips in DMEM with 10% FBS for 40 h, were transiently transfected by the calcium phosphate precipitation technique with 1 μg plasmid DNA (0.5 μg of each plasmid in case of cotransfection) for 8 h. Cells were grown for another 24 h in fresh medium, in case of insulin stimulation in the absence of serum. In general, 10–30% transfection efficiency was obtained. For immunofluorescence microscopy experiments, cells were stimulated with 5 μg/ml insulin for 5 min and/or treated with 100 nM Wortmannin (Sigma, St. Louis, MO) for 20 min, 50 μM LY294002 (Sigma) for 30 min, 0.1–2 μM cytochalasin D (Sigma) for 20 min, 33 μM nocodazole (Sigma) for 30 min, or 1 mM GRGDS peptide for 30 min.

Immunofluorescence Microscopy

After indicated treatments, cells were fixed in 3.9% paraformaldehyde, 0.02% TX-100–permeabilized, and stained with either anti-myc tag 9E10, anti-HA tag 12CA5, anti-ras (Transduction Laboratories), PY20 (Transduction Laboratories, Lexington, KY), anti-vinculin (Sigma), or anti-tubulin (Oncogene Science, Manhasset, NY) mAbs, followed by Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) with phalloidin-FITC (Sigma) and for anti-vinculin followed by a tertiary donkey anti-goat antibody (Jackson ImmunoResearch). Unless otherwise indicated in the legends, in case of cotransfection the primary antibody used for staining the cells shown in the figures was always directed against the tag of the dominant negative GTPase mutant or PAK-RBD, i.e., with 9E10 against the myc-tag of N17-Rac, N17-cdc42, N19-Rho, and PAK-RBD, with 12CA5 against the HA-tag of N34-Rab and with anti-Ras against N17-Ras; however, proper expression of the other cotransfected construct was also always inspected and confirmed. Samples were visualized with a Nikon immunofluorescence microscope. Every presented image is representative for at least six independent experiments; in each experiment (coverslip) at least 100 transfected cells were inspected.

MAP-Kinase Assay

A14 cells grown to subconfluency in a 5-cm dish were transiently transfected with 1 μg of either pMT2-HA encoding HA-tagged L79-Rab5 or N34-Rab5, pSV-V12-Ras, or empty pMT2-HA vector (for control and insulin stimulation), 3 μg carrier DNA, and 1 μg pcDNA3 encoding myc-tagged MAP-kinase. Forty hours after transfection, after stimulation with insulin (5 μg/ml) for 5 min as desired, cells were lysed in 500 μl lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1% TX-100, 50 mM NaF, 5 mM EDTA, 40 mM β-glycero-phosphate, 200 μM Na_3VO_4, and protease inhibitors). Myc-tagged MAP-kinase was immunoprecipitated, after preclearance with nonimmune serum, by antibody 9E10. Subsequently, MAP-kinase activity was assayed in vitro for 20 min at RT in 25 μl kinase buffer (30 mM Tris, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM [γ-^{32}P]-ATP [3 μCi]) containing 7.5 μg myelin basic protein as a substrate. Samples were analyzed by 15% SDS-PAGE.

Time-Lapse Video Microscopy

Cells grown and transfected on glass coverslips as above were placed in HEPES-buffered DMEM with 10% FBS and analyzed by video microscopy using low-light exposure at 37°C on a Leica (Nussloch, Germany) DMIRB inverted microscope with a Kappa CF 8/1 CCD camera connected to a Sony SVT-5000P time-lapse VCR. Recording was performed at either 2.08 (24 × reduced speed) or 1.25 (40 × reduced speed) fields per second. The video-recorded images were processed using Adobe photoshop. Transfected cells were identified by means of their unique characteristic morphology (lamellipodia) as compared with untransfected cells, as observed and confirmed by combined immunofluorescence and phase-contrast microscopy (described above). Cells were treated with 1 mM GRGDS, 10 μg/ml nocodazole, or 0.1–2 μM cytochalasin D (Sigma) as indicated. The presented images are representative for at least six independent experiments.

Analysis of Cell Adhesion and Migration for Substrate Dependency

For adhesion and substrate-dependency experiments, cells were released by 5 mM EDTA in PBS, washed, and replated in fresh medium on glass coverslips that were either uncoated or coated for 3 h at room temperature with 20 μg/ml poly-L-lysine (PLL) or 40 μg/ml fibronectin (Sigma). Replated cells were either fixed after 30 min for immunofluorescence microscopy to determine adhesive properties (which was not affected by Rab5) or analyzed after 16 h for migration by time-lapse video microscopy.

RESULTS

Activation of Rab5 Induces Lamellipodia Formation

To investigate possible effects on cytoskeletal organization, NIH 3T3-A14 fibroblasts were transfected with either a con-

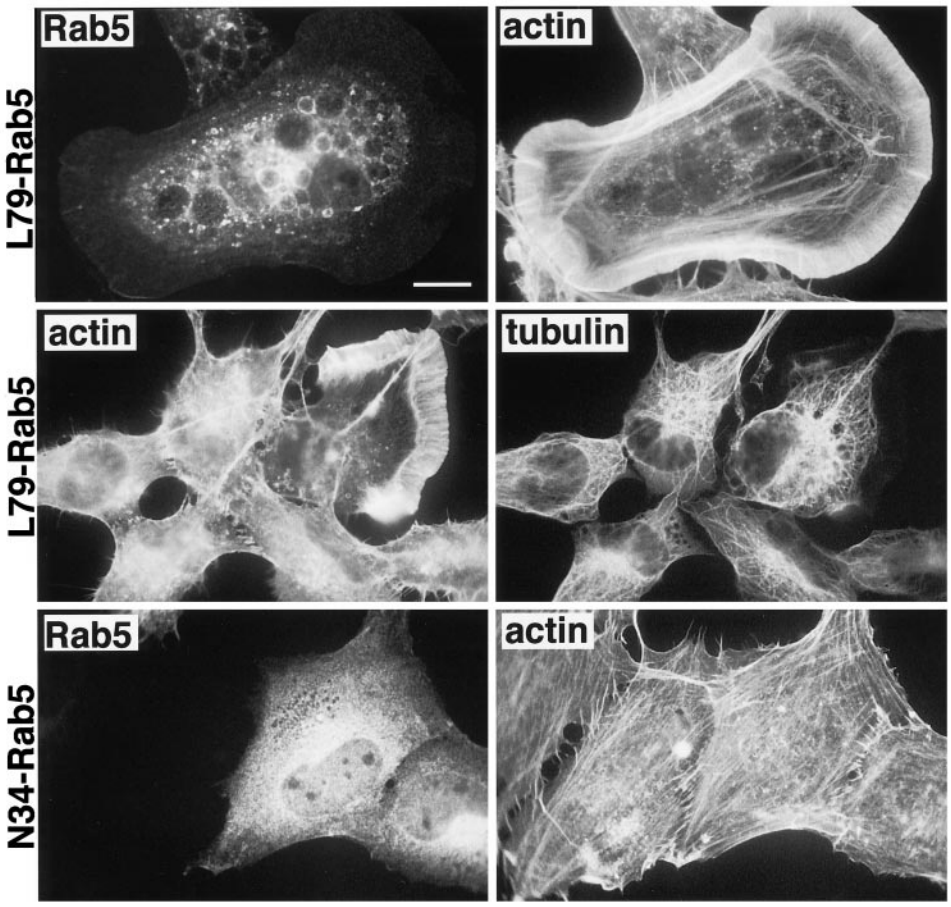

Figure 1. L79-Rab5, but not N34-Rab5, induces the formation of large endosomes and lamellipodia. Shown is immunofluorescence microscopy of A14 fibroblasts grown on coverslips and expressing either L79-Rab5 or N34-Rab5 as indicated. Rab5 expression and localization were visualized by anti-HA × GαM-Cy3 staining, the actin cytoskeleton was visualized by phalloidin-FITC, and microtubules were visualized by anti-tubulin × GαM-Cy3 (as indicated). The same cells are shown in the left and right panels. Bar, 30 μm.

stitutively active GTPase-defective Rab5 mutant L79-Rab5 or a dominant negative GTP binding-defective mutant N34-Rab5 (Stenmark *et al.*, 1994, 1995), both containing an HA epitope tag at their N terminus. Subsequently, the cells were analyzed for Rab5 expression by staining with a mouse anti-HA antibody followed by an anti-mouse-Cy3, and possible cytoskeletal effects were visualized using phalloidin-FITC by means of immunofluorescence microscopy. In agreement with previous studies in BHK cells (Stenmark *et al.*, 1994, 1995), L79-Rab5 displayed characteristic localization at irregular-sized, enlarged early endosomes that are formed as a consequence of enhanced endocytosis and endosome fusion by active Rab5, whereas N34-Rab5 showed typical diffuse, somewhat punctuate, cytoplasmic staining (Figure 1). Interestingly, L79-Rab5, but not N34-Rab5, induced strong reorganization of the actin cytoskeleton resulting in prominent lamellipodia formation (Figure 1). Typically, at the moment of fixation, 20 ± 8% (±SD, n = 10) of the L79-Rab5–expressing cells, as determined by immunofluorescence microscopy, displayed prominent lamellipodia, whereas in nonexpressing cells on the same coverslip or in cells that were not transfected at all, lamellipodia formation was never observed. This number, however, does not represent the percentage of L79-Rab5–expressing cells that have the potential to form lamellipodia, which will be much higher, because by means of video microscopy we noticed that the lamellipodia formation is a highly dynamic process, i.e., cells that do not display lamellipodia at a certain moment may exhibit prominent lamellipodia within 1 min (see below). The lamellipodia often displayed a very regular shape, contained radially directed, small, rib-like F-actin bundles, were positioned symmetrically around the cell's entire circumference, or showed a polar distribution in a fan shape to one side of the cell, and predominantly appeared to adhere to the substratum (Figure 1; see also Figures 2, 3, and 5). No change in the microtubule cytoskeleton organization was observed, but we noticed that microtubules were usually absent from the lamellipodia, except for an occasionally penetrating microtubule filament plus end (Figure 1). Similar effects on the actin cytoskeleton were observed in COS7 cells, whereas no effect was observed upon expression of an active mutant of Rab4, involved in early endosome recycling, Rab7, or Rab11 (our unpublished results).

Rab5-induced Lamellipodia Formation Resembles Insulin- but Not Ras- or Rac-induced Cytoskeletal Reorganization

Several small GTPases, such as Ras, Rac, Rho, and Cdc42, have been implicated in the organization of the cytoskeleton (Zigmond, 1996; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). A well established signal

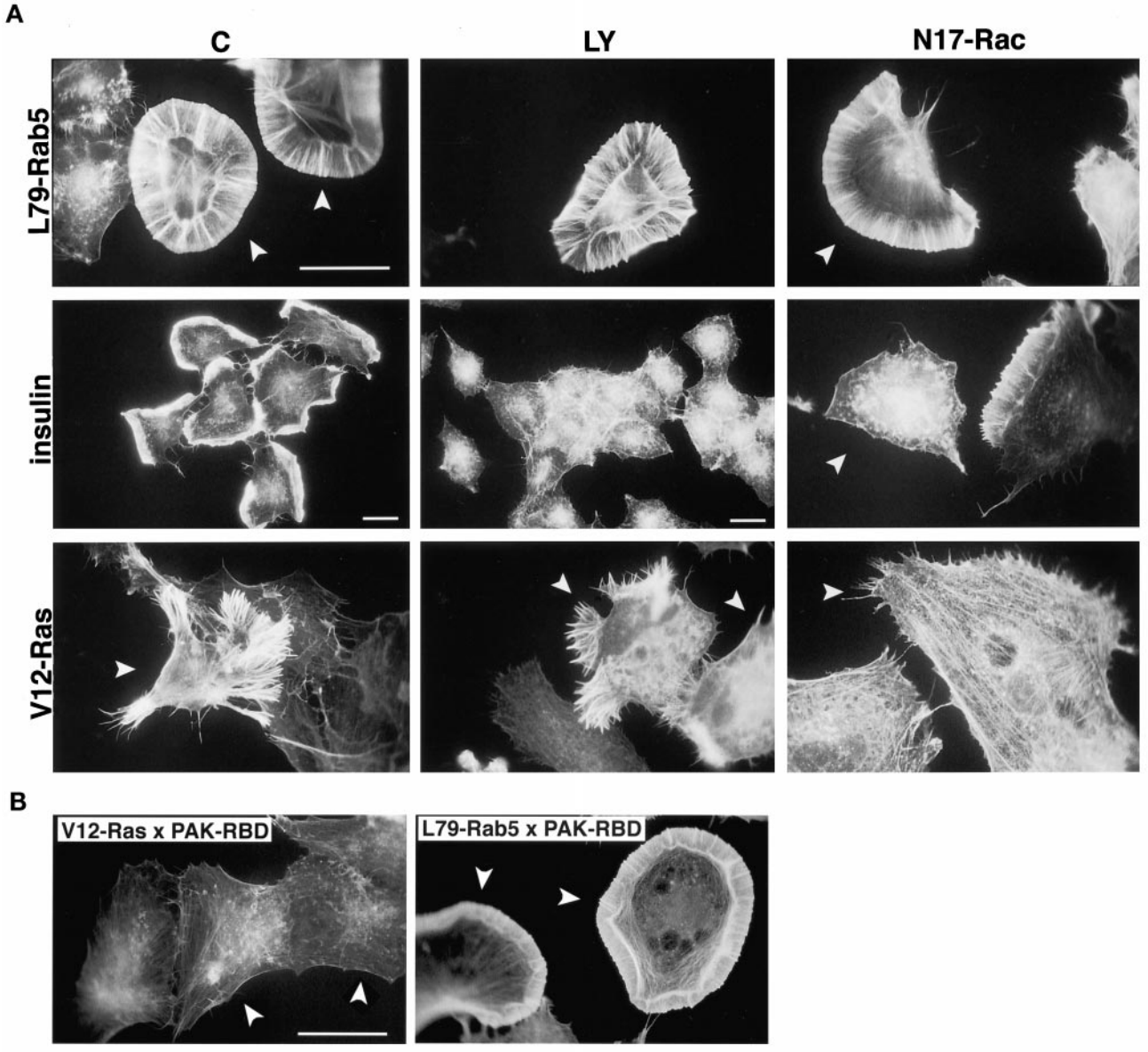

Figure 2. Rab5-induced lamellipodia formation is PI3-K– and Rac-independent. Shown is immunofluorescence microscopy of the actin cytoskeleton of A14 cells (A) transfected with either L79-Rab5 or V12-Ras or stimulated with insulin (as indicated per row) and (co)transfected with control plasmid or N17-Rac or treated with LY 294002 (as indicated per column), or (B) cotransfected with V12-Ras and PAK-RBD or L79-Rab5 and PAK-RBD, as indicated. Arrowheads indicate the Cy3-positive cells stained for the expression of L79-Rab5, V12-Ras, or in case of their cotransfection with L79-Rab5, N17-Rac, or PAK-RBD. Only the actin staining as detected by phalloidin-FITC is shown. Bar, 30 μm. The scale bar in the top left picture applies to all others, unless a different size scale bar is shown.

transduction pathway involved in cytoskeletal reorganization is the receptor tyrosine kinase/(Ras)/PI-3 kinase/Rac/Rho cascade (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kotani *et al.*, 1994; Zigmond, 1996; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). To study the nature of the cytoskeletal reorganization that occurred upon L79-Rab5 expression, we compared the cellular and cytoskeletal morphology of L79-Rab5–expressing cells with cells that were either insulin-stimulated or transfected with the constitutively active mutants V12-Ras, V12-Rac, V14-Rho, and V12-Cdc42, by phase-contrast and immunofluorescence microscopy. In comparison, we observed that although Rab5 induced regularly shaped lamellipodia, A14 cells expressing either active V12-Ras (Figure 2A) or V12-Rac (Figure 4) displayed typical membrane ruffling and sometimes small irregularly shaped lamellipodia, essentially as previously reported for other cell types (Bar-Sagi and Feramisco, 1986; Ridley *et al.*, 1992). V12-Cdc42–expressing cells showed the typical filopodia formation (Figure 4), as previously observed in Swiss 3T3 fibroblasts (Kozma *et al.*, 1995;

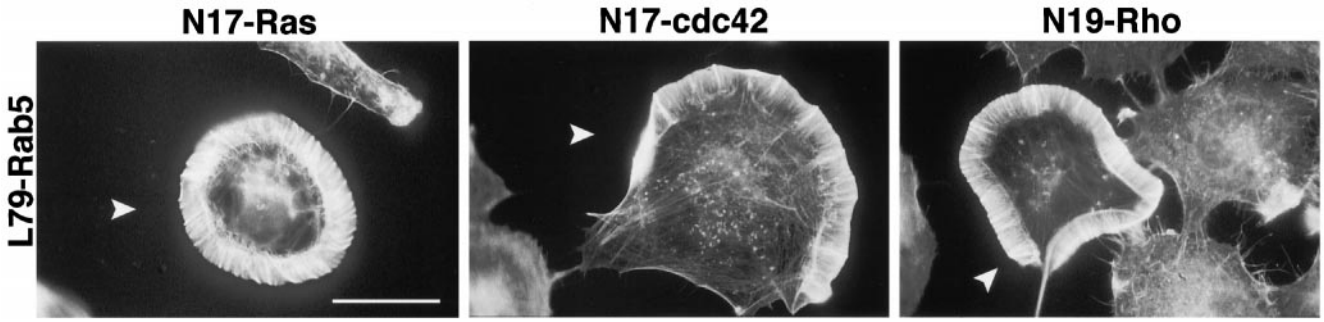

Figure 3. Rab5-induced lamellipodia formation is Ras-, Cdc42-, and Rho-independent. Immunofluorescence microscopy of the actin cytoskeleton of A14 cells cotransfected with L79-Rab5 and N17-Ras, N17-Cdc42, or N19-Rho is shown. Arrowheads indicate the Cy3-positive cells stained for expression of the dominant negative GTPases. Only the actin staining as detected by phalloidin-FITC is shown. Bar, 30 μm.

Nobes and Hall, 1995). V14-Rho–transfected cells were small, having a rather condensed appearance, and showed typical stress-fiber formation (Figure 4), as previously observed in Swiss 3T3 fibroblasts (Ridley and Hall, 1992). A14 cells stimulated with insulin for 5 min, like Rab5, induced regularly shaped lamellipodia (Figure 2A). Cells stimulated with insulin showed lamellipodia formation within 2 min, with an optimum between 5 and 10 min, then gradually the number of lamellipodia-containing cells declined, and lamellipodia were hardly observed after 1 h (our unpublished results). Most striking for both Rab5 and insulin-induced lamellipodia as compared with the Ras- and Rac-induced

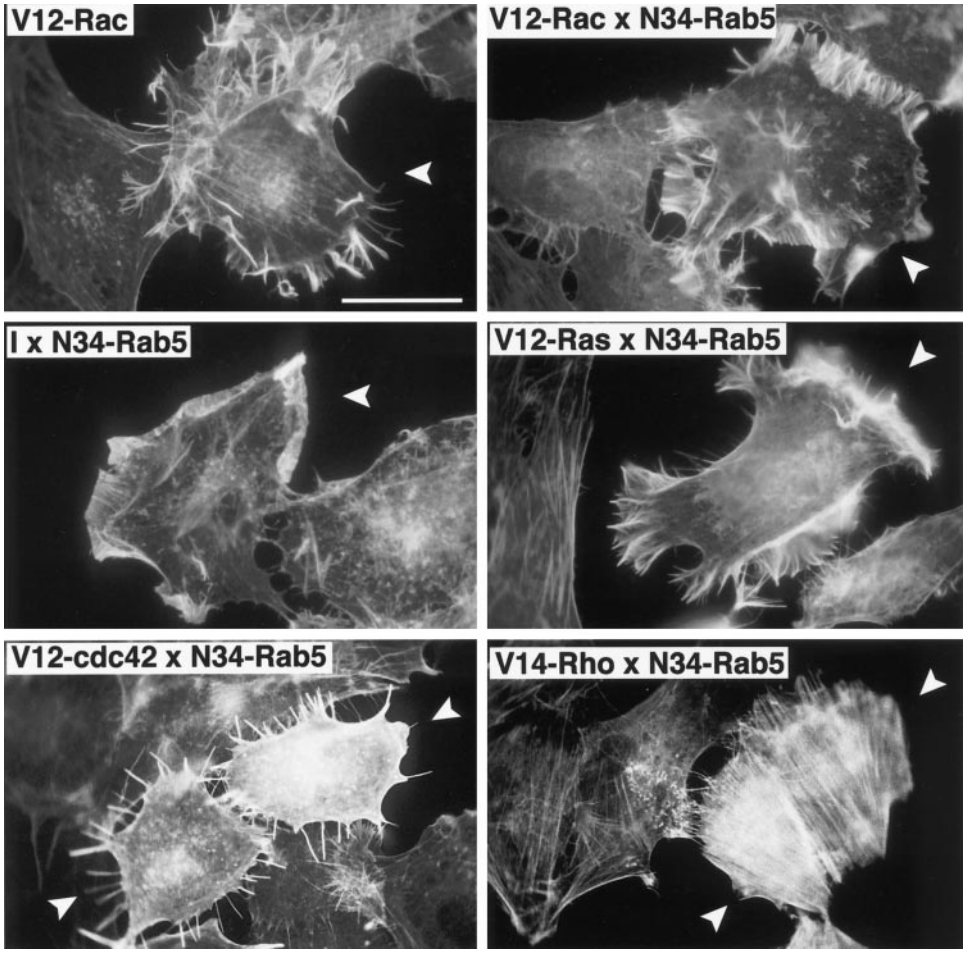

Figure 4. Insulin-, Ras-, and Rac-induced lamellipodia formation is Rab5-independent. Shown is immunofluorescence microscopy of the actin cytoskeleton of A14 cells transfected with V12-Rac alone, or cotransfected with V12-Rac, V12-Ras, V12-Cdc42, or V14-Rho and N34-Rab5, or transfected with N34-Rab5 alone and stimulated for 5 min with insulin (as indicated). Arrowheads indicate the Cy3-positive cells stained for expression of either V12-Rac or, in the case of cotransfection, N34-Rab5. Only the actin staining as detected by phalloidin-FITC is shown. Bar, 30 μm.

membrane ruffling are the regular morphology, the F-actin-rich radially directed rib-like microspikes in the lamellipodium, and the apparent predominantly adhering nature of the lamellipodia.

Given the close morphological resemblance between the Rab5 and insulin-induced lamellipodia, and because processes that affect receptor-mediated endocytosis may influence signal transduction by receptor tyrosine kinases (Vieira *et al.*, 1996), we investigated whether the effect of Rab5 may be due to activation of the insulin receptor, or any other receptor tyrosine kinase or protein upstream from Ras, by measuring the activity of MAP-kinase in the Rab5-expressing cells in an in vitro kinase assay; however, although insulin and V12-Ras induce a clear MAP-kinase activation, L79-Rab5 does not (our unpublished results). In addition, the formation of the lamellipodia is absolutely specific for the L79-Rab5–transfected cells and never occurs in nontransfected cells, indicating that a paracrine effect is not involved. Thus, the effect of Rab5 on cytoskeletal reorganization is unlikely to be due to the activation of the insulin receptor or any other signal upstream from Ras, and occurs in a Ras-independent manner, unless Rab5 is able to activate Ras but prevents it from activating the Ras/Raf/MEK/MAP-kinase route.

Rab5-induced Lamellipodia Formation Is Ras-, PI3-K-, and Rac-independent

To further characterize the Rab5-induced cytoskeletal reorganization, and in particular to investigate the possible involvement of the established Ras/PI3-K/Rac cytoskeleton regulatory pathway, we used Wortmannin and LY 294002, two unrelated inhibitors of PI3-K, and dominant negative mutants of the Ras, Rac, Cdc42, and Rho GTPases (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kotani *et al.*, 1994; Zigmond, 1996; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Interestingly, although insulin-induced lamellipodia formation is completely abolished by both PI3-K inhibitors, Rab5-induced lamellipodia formation is not affected (Figure 2A). V12-Ras–induced ruffling was not abolished by the PI3-K inhibitors either (Figure 2A), nor was V12-Rac–induced ruffling (our unpublished results) (Nobes *et al.*, 1995; Rodriguez-Viciana *et al.*, 1997). The observation that Wortmannin completely abolished insulin-induced lamellipodia formation, but not Ras- or Rac-induced membrane ruffling, is in agreement with previous observations in other cells (Kotani *et al.*, 1994; Nobes *et al.*, 1995). It should be mentioned, however, that in PAE endothelial cells V12-Ras–induced membrane ruffling was shown to be entirely dependent on PI3-K activity, as could be shown by means of dominant negative constructs of the p85 regulatory subunit of PI3-K (Rodriguez-Viciana *et al.*, 1997).

To investigate whether Rab5-induced lamellipodia formation is mediated by Rac, L79-Rab5 was cotransfected with dominant negative N17-Rac. Although N17-Rac was clearly expressed, as determined by immunofluorescence microscopy, it did not abolish Rab5-induced lamellipodia formation (Figure 2A). This is in striking contrast to the complete inhibition by N17-Rac of lamellipodia formation and membrane ruffling induced by either insulin or V12-Ras, respectively (Figure 2A). The observations that dominant negative Rac abolishes lamellipodia formation and membrane ruffling induced by insulin and Ras is in agreement with previous data obtained in Swiss 3T3 fibroblasts (Ridley *et al.*, 1992).

In an attempt to provide additional supportive evidence for the Rac independence of the Rab5-induced lamellipodia formation, as demonstrated by means of the dominant negative N17-Rac mutant, we investigated the possibility of using overexpression of the minimal Rac-binding domain of the Rac effector PAK (PAK-RBD) to suppress Rac signaling. Expression of this PAK domain has been shown to inhibit the Rac-dependent neurite outgrowth from NGF-stimulated PC12 cells (Daniels *et al.*, 1998), presumably by titrating out Rac activity. As shown in Figure 2B, overexpression of PAK-RBD, which by itself had no effect on cytoskeletal organization (our unpublished results), indeed was able to suppress the Rac-dependent V12-Ras–induced membrane ruffling. In contrast, PAK-RBD did not affect Rab5-induced lamellipodia formation (Figure 2B), which is in agreement with the inability of N17-Rac to abolish Rab5-induced lamellipodia formation. Thus, these data provide additional support for the Rac independency of the Rab5-induced lamellipodia formation. In conclusion, Rab5-induced lamellipodia formation is not mediated by PI3-K or Rac.

Finally, Rab5-induced lamellipodia formation is not abolished by coexpression of dominant negative N17-Ras, N19-Rho-, or N17-Cdc42 (Figure 3), or active V12-Ras or V12-Rac (our unpublished results). Dominant negative Ras does not affect insulin-induced lamellipodia formation either (our unpublished results), which is in agreement with previous observations in KB cells (Nishiyama *et al.*, 1994). Thus, although expression of constitutively active Ras is sufficient for membrane ruffling/lamellipodia formation (Figure 2A), activation of endogenous Ras is not required for insulin- or Rab5-induced lamellipodia formation. In a recent study, however, it was shown that although dominant negative Ras was not able to block TPA-induced Raf activation, basal levels of Ras-GTP were required for the activation of Raf (Marais *et al.*, 1998). This suggests that Ras activation is not, but Ras activity is, required for PKC-mediated Raf activation, and that a lack of effect by dominant negative N17-Ras does not necessarily completely exclude its involvement in a particular signaling pathway.

In conclusion, our data demonstrate that activation of Ras or Rac is sufficient for lamellipodia formation and membrane ruffling, that insulin-induced lamellipodia formation requires PI3-K and Rac activation, and that Ras-induced lamellipodia formation requires Rac activation; however, Rab5-induced lamellipodia formation does not require activation of Ras, PI3-K, or Rac.

Insulin-, Ras-, and Rac-induced Lamellipodia Formation Is Rab5-independent

Our results demonstrate that Ras, PI3-K, and Rac are not downstream components of the Rab5-induced signaling pathway resulting in lamellipodia formation. Therefore we next investigated whether Rab5 may be a downstream component of the insulin, Ras, or Rac signal transduction pathway resulting in lamellipodia formation and membrane ruffling. For this purpose cells were transfected with a dominant negative mutant of Rab5, N34-Rab5, and stimulated with insulin, or cotransfected with N34-Rab5 and either V12-Ras or V12-Rac; however, expression of dominant negative N34-Rab5 did not prevent insulin-, V12-Ras–, or

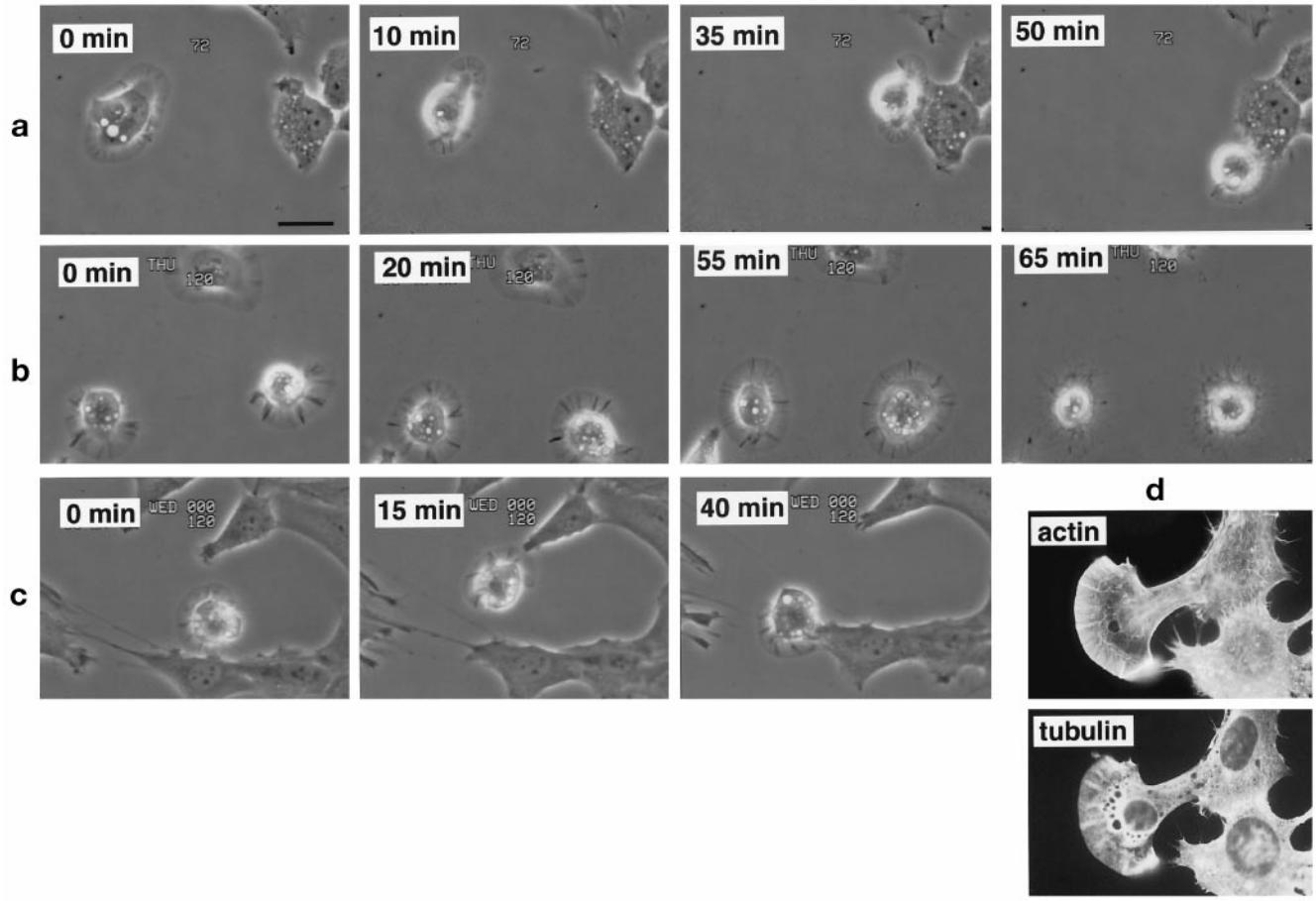

Figure 5. Rab5 induces cell migration that is actin polymerization-dependent but microtubule-independent. Shown is time-lapse video microscopy of the migration of A14 cells that were (A) transfected with L79-Rab5 (please note that the cell only starts to migrate after polarizing its lamellipodium), (B) L79-Rab5–transfected and treated at t = 58 min with either 1 μM cytochalasin D (please note that only the cell with the polarized lamellipodium in the bottom right corner shows migration between 0 and 20 min, until its lamellipodium depolarizes), and (C) L79-Rab5–transfected and treated at t = 0 min with 10 μg/ml nocodazole. The inset (D) shows the actin cytoskeleton and confirms depolymerization of microtubules (compare with Figure 1) of cells, including a cell displaying a lamellipodium as a consequence of L79-Rab5 transfection, after treatment with 10 μg/ml nocodazole for 30 min. Bar, 30 μm.

V12-Rac–induced lamellipodia formation and membrane ruffling (Figure 4), whereas the formation of endocytic vesicles was clearly diminished (Figure 1 and our unpublished results). In addition, V12-Cdc42–induced filopodia formation or V14-Rho–induced stress-fiber formation were not abolished either (Figure 4). Thus our data demonstrate that Rab5-induced lamellipodia formation is Ras-, PI3-K-, and Rac-independent, whereas activation of endogenous Rab5 is not required for insulin-, Ras-, or Rac-induced lamellipodia formation and membrane ruffling. Taken together, we show that Rab5 functions on a signaling pathway distinct from the (insulin) receptor tyrosine kinase/(Ras)/PI3-K/Rac pathway to regulate reorganization of the actin cytoskeleton.

Rab5 Activation Induces Cell Migration

Lamellipodia have been observed at the leading edge of motile cells, involved in the process of cell migration. Moreover, several recent studies have suggested a relationship between endocytosis or cytoskeletal reorganization and cell migration (Bretscher, 1996a, 1996b; Bretscher and Aguado-Velasco, 1998b). Furthermore, we noticed an apparent adhering nature of the lamellipodia formed upon L79-Rab5 expression. Therefore, we next investigated a possible effect of Rab5 on cell motility by time-lapse video microscopy. L79-Rab5–transfected cells could easily be identified on the basis of their unique morphological appearance, i.e., their prominent lamellipodia, because these are never observed in nontransfected cells. Indeed, we observed a striking effect of L79-Rab5 on cell motility because the lamellipodia-containing L79-Rab5–transfected cells displayed rapid cell migration, with some cells migrating a distance equal to their diameter within 10 min. The average speed of migrating cells was determined to be 3.7 $\pm$ 1.3 μm/min ($\pm$SD, n = 10), as measured over at least a 30 min period, with a maximum speed of 5.8 μm/min (Figure 5A). The lamellipodium is always at the leading edge of the cells, which migrate in a

regular continuous manner without detachment of the lamellipodia from the substrate. It is noteworthy that in the lamellipodia a continuous rearward or centripetal flow from the lamellipodium outline toward the cell body could be observed, which may reflect the retrograde flow of actin, membrane, and/or proteins (Cramer, 1997).

Rab5-transfected cells that displayed lamellipodia in a nonpolar symmetric manner along their entire circumference did not migrate until the lamellipodium became polarized asymmetrically to one side of the cell, which then becomes the leading edge of the cell. Apparently, the lamellipodia determine the direction of cell migration (Figure 5B). Furthermore, we noticed that the cell migration occurs in most, if not all, of the Rab5-expressing cells that have a polarized lamellipodium, but not in all of these cells at the same time in the same period of time. A cell without lamellipodia, and thus a nonmigrating cell, can form lamellipodia and start to migrate after a certain period of time, whereas cells that are migrating, and thus contain polarized lamellipodia, can lose their lamellipodia and stall for a certain period of time. Sometimes a polarized lamellipodia-containing cell can be seen that appears to try to migrate but is held in place by a neighboring cell; however, it is only a matter of time until the cell manages to migrate away. On the other hand, solitary cells with polarized lamellipodia are always migrating. Thus, the percentage of migrating cells largely depends on the period of time that the cells are monitored, but close to 100% of the polarized lamellipodia-containing L79-Rab5–expressing cells will migrate sooner or later.

Furthermore, time-lapse video microscopical analysis revealed that insulin treatment resulted in the occurrence of regularly shaped lamellipodia within 1 min over a period of 1 h. Expression of V12-Ras resulted in the induction of dynamic membrane ruffling and sometimes highly motile fan- or umbrella-shaped, small, lamellipodia-like structures that showed fast protrusion, substrate attachment, detachment, and retraction, followed by an eventual folding back onto the cell. The V12-Rac–transfected cells displayed Ras-like ruffling/lamellipodia (and sometimes filopodia-like extensions) that were, however, less motile in appearance. Finally, V12-Cdc42–transfected cells displayed strong filopodia formation, with the filopodia showing some motility as they detach and reattach to the substratum; however, insulin treatment or expression of activated Ras, Rac, or Cdc42 did not result in cell migration (our unpublished results). Thus, these data provide further support for the notion that the Rab5 effect on the cytoskeletal organization, as exhibited by the adhering lamellipodia formation (and cell migration), is not only morphologically but also functionally distinct from the insulin/(Ras)/PI3-K/Rac signaling pathway involved in cytoskeletal reorganization.

Rab5-induced Cell Migration Is Dependent on Actin Polymerization but Not on Microtubules

We investigated the L79-Rab5–transfected cells for the requirement for integrin-dependent adhesion in both lamellipodia formation and cell migration. Lamellipodia formation and cell migration by Rab5 was observed on the integrin-dependent adhesive substrate fibronectin as well as on the integrin-independent nonspecific adhesive substrate PLL (our unpublished results). In addition, we also observed Ras- and Rac-induced membrane ruffling on PLL (our unpublished results), as has recently been reported with respect to the actin reorganization induced by Rac or Rho in Swiss 3T3 cells (Machesky and Hall, 1997). These data suggest that integrin-mediated substrate adhesion may not be required for the Rab5-induced formation of lamellipodia or the induction of cell migration.

To investigate the involvement of the cytoskeleton on Rab5-induced lamellipodia formation and cell migration, cells were treated with either cytochalasin D, which prevents polymerization of actin, or nocodazole, which causes depolymerization of microtubules, and analyzed by both immunofluorescence microscopy and time-lapse video microscopy. Treatment of L79-Rab5–transfected migrating cells with 1 μM cytochalasin D, which causes depolymerization of actin (our unpublished results), resulted in the immediate loss of lamellipodia and concomitantly the cell migration stopped (cells without lamellipodia were morphologically unaffected) (Figure 5B). This demonstrates that the maintenance of lamellipodia and cell migration apparently requires continuous cycling of actin polymerization/depolymerization. Furthermore, at 0.25 μM cytochalasin D, which initially leaves the lamellipodia morphologically intact for ~30 min, both the rearward flow movement in the adhering lamellipodia as well as migration immediately slowed down (our unpublished results). Depolymerization of microtubules by nocodazole (compare Figure 5D with Figure 1), however, had no effect on Rab5-induced lamellipodia formation and cell migration (Figure 5C), which implies that microtubules are not required for those responses. It is noteworthy that the formation of enlarged endocytic vesicles by L79-Rab5 is not affected by the nocodazole treatment (our unpublished results), suggesting that microtubules are not involved in Rab5-induced endocytosis. Thus, the polymerization of actin, but not the microtubular cytoskeleton, is required for Rab5-induced lamellipodia formation and is either required or the driving force for the retrograde flow of membrane proteins and/or actin and for the cell migration induced by Rab5.

DISCUSSION

Rab5 is a member of the Rab family of GTPases that has been shown to be involved in the regulation of receptor-mediated endocytosis by regulating the fusion endocytic vesicles with early endosomes. Expression of the constitutively active mutant L79-Rab5 results in an increase in the rate of receptor-mediated endocytosis and pinocytosis (Li and Stahl, 1993; Stenmark *et al.*, 1994). Here we show that L79-Rab5 induces the formation of lamellipodia. Although insulin-induced lamellipodia formation was shown to be PI3-K– and Rac-dependent and Ras-induced membrane ruffling Rac-dependent, Rab5-induced lamellipodia formation was Ras-, PI3-K–, and Rac-independent. Furthermore, insulin-, Ras-, and Rac-induced lamellipodia formation were shown to be Rab5-independent. These results show that the signaling pathway involved in the Rab5-induced lamellipodia formation is distinct from the well known receptor tyrosine kinase/(Ras)/PI3-K/Rac pathway for lamellipodia formation. In addition, we show that the L79-Rab5–transfected cells show a dramatic stimulation of cell migration, in contrast to insulin-stimulated or V12-Ras– or V12-Rac–transfected cells. The lamellipodia formation and cell migration in the L79-Rab5–expressing cells is dependent on continuous actin polymerization but not on microtubules. In conclusion, our data for the first time show that a member of

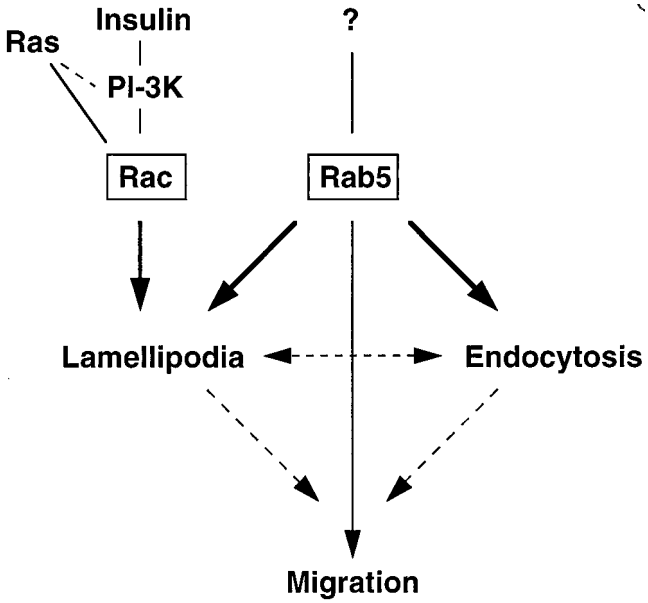

Figure 6. Distinct mechanisms for Rac- and Rab5-induced biological responses. The reorganization of the actin cytoskeleton is induced independently by Rac and Rab5. In addition, the Rab5-induced lamellipodia formation and endocytosis may be regulated by distinct mechanisms as well; however, there is a clear relationship between these two events, suggesting that Rab5 may control cytoskeletal reorganization to provide support and direction for the endocytic events. Furthermore, several models have been proposed in which the driving force for cell migration is either a polarized actin polymerization cycle or a polarized endocytic/exocytic cycle. We propose that the dramatic effect of Rab5 on cell migration, which was not observed with Ras or Rac, may be the consequence of the combined action of Rab5-induced actin cytoskeleton reorganization and endocytosis. See DISCUSSION and CONCLUSION for further details.

the Rab GTPase family, implicated in the regulation of vesicle fusion and trafficking, is able to induce lamellipodia formation and cell migration, the lamellipodia formation being mediated by a novel mechanism independent of the Rho GTPase family (Figure 6). Furthermore, our results provide support for a connection between endocytosis, cytoskeletal reorganization, and cell migration. Here we will discuss some of the additional evidence in favor of an intricate relationship between these processes.

Relationship between Endocytosis and Cytoskeletal Reorganization

Several lines of evidence have recently been obtained suggesting an intricate relationship between vesicle fusion/transport and cytoskeletal organization, as well as cross-talk between the regulatory GTPases involved in these processes. Although the GTPases Ras, Rac1, and RhoA are involved in the (growth factor-induced) formation of lamellipodia and stress fibers (Bar-Sagi and Feramisco, 1986; Ridley and Hall, 1992; Ridley *et al.*, 1992; Zigmond, 1996; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998), expression of their constitutively active mutants was also shown to result in enhanced secretion (Price *et al.*, 1995; Norman *et al.*, 1996) or endocytosis (Bar-Sagi and Feramisco, 1986; Ridley *et al.*, 1992; Schmalzing *et al.*, 1995). In a recent study using BHK fibroblasts, however, active Rac did not affect pinocytosis, whereas Ras-induced pinocytosis was shown to be mediated by Rab5 (Li *et al.*, 1997). The cytoskeleton regulatory GTPases have been implicated in the regulation of receptor-mediated endocytosis as well, because active mutants of Rac and Rho were shown to inhibit transferrin-receptor– and EGF-receptor–mediated endocytosis (Lamaze *et al.*, 1996). In addition, expression of an active mutant of RhoD, which results in membrane extensions and loss of stress fibers, resulted in a decrease of endosome motility and consequently of endocytosis (Murphy *et al.*, 1996). Moreover, actin polymerization-inhibitory reagents were recently reported to suppress receptor-mediated endocytosis (Lamaze *et al.*, 1997). Finally, PI3-K has been implicated both in regulation of cytoskeleton organization (Kotani *et al.*, 1994; Zigmond, 1996; Rodriguez-Viciana *et al.*, 1997; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998) and in receptor-mediated endocytosis (Li *et al.*, 1995). On the other hand, Rab8, a Rab-like GTPase involved in polarized membrane transport, induces the formation of membrane processes by reorganization of actin filaments (Peranen *et al.*, 1996). Furthermore, the GTPase Arf6, which like Rab5 has been implicated in the regulation of receptor-mediated endocytosis as expression of the active mutant results in decreased transferrin receptor endocytosis (D'Souza-Schorey *et al.*, 1995), induces subtle surface-localized actin polymerization (D'Souza-Schorey *et al.*, 1997). The results obtained by D'Souza-Schorey *et al.* (1997) even suggest that Rac and Arf6 share a common effector molecule, POR1, which is involved in membrane ruffling (Van Aelst *et al.*, 1996). It is noteworthy that Arfaptin1 (Kanoh *et al.*, 1997), a POR1 homologous protein, was recently identified by us as a putative Rab5 effector, interacting with Rab5 in a GTP-dependent manner (our unpublished observations). These data suggest that Rab5 may directly activate an effector molecule involved in lamellipodia formation. In a recent study by Bretscher and Aguado-Velasco (1998a), however, it was shown that membrane ruffles induced by either EGF or Rac arise by exocytosis of recycling membrane from the endocytic cycle. This implies that Rab5-stimulated endocytosis may be the driving force for the membrane ruffling/lamellipodia formation observed in the L79-Rab5–transfected cells. Thus, it will be interesting to establish whether Rab5-induced lamellipodia formation occurs in a direct manner independent of endocytosis, or whether it is the consequence of the stimulation of endocytosis.

Relationship between Cytoskeletal Reorganization and Cell Migration

Several studies suggest an intricate relationship between cell migration and cytoskeleton organization. On the basis of these studies, cell migration has been proposed to be the consequence of polymerization of monomeric G-actin at the leading edge of the cell, the subsequent retrograde flow of the F-actin (and F-actin–attached proteins such as integrins), and depolymerization at the cell's rear end, followed by recycling of the monomeric G-actin to the cell's leading edge to polymerize again. Thus, in this model the driving force for cell migration is a polarized actin polymerization cycle resulting in a retrograde flow of actin and actin-bound ad-

hesion proteins that pushes the cell forward (Cramer *et al.*, 1994; Bretscher, 1996b; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Welch *et al.*, 1997).

Members of the Rho family of Ras-like GTPases as well as Ras itself, which are involved in the regulation of cytoskeletal architecture, have been implicated in cell migration. Evidence has been presented showing the involvement of Ras, Rac, and Rho in hepatocyte growth factor/SF-induced motility/scattering of keratinocytes and epithelial cells (Takaishi *et al.*, 1994; Ridley *et al.*, 1995). Additional recent studies revealed that Rac and Rho are involved in E-cadherin–mediated cell–cell adhesions in Madin-Darby canine kidney cells and keratinocytes (Braga *et al.*, 1997; Hordijk *et al.*, 1997; Keely *et al.*, 1997; Takaishi *et al.*, 1997). Thus, possible effects of Ras, Rac, and Rho on (hepatocyte growth factor-induced) cellular motility, especially obtained in epithelial cells, may very well reflect an effect on intercellular cadherin-mediated adhesion rather than cell motility. In other cell systems, however, an effect of Ras-like GTPases on cell motility has been reported as well. Overexpression of RhoGDI or inhibition of Rho was shown to inhibit Swiss 3T3 fibroblast motility (Takaishi *et al.*, 1993), and Tiam1 and V12-Rac, but not V14-Rho, induce the invasiveness of T-lymphoma cells (Michiels *et al.*, 1995).

Relationship between Endocytosis and Cell Migration

Another model has been proposed in which cell migration is the consequence of a polarized endocytic and exocytic cycle that delivers membrane and membrane protein (e.g., adhesion proteins) for extension and substrate attachment to the leading edge of the migrating cell (Bretscher, 1996a,b; Bretscher and Aguado-Velasco, 1998b). Indeed, several studies have provided evidence for a relationship between cell migration and endocytosis. Several integrin subtypes involved in substrate adhesion and migration were found to participate in the endocytotic cycle (Bretscher, 1989, 1992b). In addition, B-cells are able to use their transferrin receptor for adhesion and locomotion on a surface coated with anti-transferrin receptor antibodies (Bretscher 1992a). If these cells were applied to a surface coated with anti-integrin $\alpha L\beta 2$ antibody, the cells did attach but did not migrate. Because the transferrin receptor is an efficiently circulating receptor, whereas in these cells integrin $\alpha L\beta 2$ is not (Bretscher, 1992b), this suggests that the endocytotic cycle can be the driving force for cell locomotion (Bretscher, 1992a). Moreover, in migrating fibroblasts the endocytosed transferrin receptors are exocytosed and emerge distributed over the surface of the leading lamellipodia at relatively much higher density as compared with surface elsewhere on the cell (Hopkins *et al.*, 1994). In addition, integrin $\alpha v\beta 3$ integrins were found to recycle upon endocytosis specifically to the front of migrating neutrophils as well, with higher concentrations of the integrin being found at the cell's leading edge than at the rear (Lawson and Maxfield, 1995). Although these studies suggest the cycling of integrins involved in cell adhesion and thus migration via the endocytotic machinery, it should be mentioned that the replenishment of integrins at the cell's leading edge can also occur via the cell surface (Schmidt *et al.*, 1993).

Mechanism of Rab5-induced Cell Migration

Thus, the question remains whether the driving force for cell migration is either a polarized actin polymerization/depolymerization cycle, which pushes the cell front forward (Cramer *et al.*, 1994; Bretscher, 1996b; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Welch *et al.*, 1997), or a polarized endocytosis/exocytosis cycle, which reinserts membrane and membrane proteins at the leading edge of the cell, thereby extending the front of the cell forward (Bretscher, 1996a,b; Bretscher and Aguado-Velasco, 1998b). For Rab5-induced cell migration we favor a combination of the two because it is easy to envision that for a polarized endocytotic cycle a polarized actin polymerization cycle may be very helpful in directing the vesicles and thus recycling adhesion proteins such as integrins to the leading edge of the cell. Obviously, both events occur in the L79-Rab5–transfected cells; Rab5 stimulates the endocytotic cycle and induces the formation of F-actin-rich lamellipodia; however, whether both processes are independently elicited by Rab5 activation remains to be established.

As discussed above, an apparent increase in cell migration may be due to either enhanced cell motility or decreased cell–cell adhesion. Several observations argue against the latter option to explain the cell migration as observed in the L79-Rab5–transfected cells. Very often, these cells seem to try to migrate away from their neighboring contacting cells but appear to be held in place by those cells. On the other hand, L79-Rab5–transfected migrating cells often get in touch with other cells during their migration, but do not show a tendency to stall and settle upon contacting those cells, suggesting that there is also no apparent increase in cell–cell adhesion formation. Finally, we do not see this dramatic cell migration in solitary untransfected cells. Thus, our data suggest that Rab5 does not induce a decrease or increase in the formation of intercellular adhesions, and the migrating behavior appears to be due to enhanced motility rather than decreased cell–cell adhesion.

In the case of Rab5-induced cell migration, the driving force for the cell migration may be the forced endocytosis and recycling of membrane and adhesive membrane proteins to the leading edge of the cell (i.e., the lamellipodia), the migration being the consequence of the polarized flow of membrane proteins (not necessarily integrins) floating along with the membrane, and/or of actin-bound transmembrane proteins moving along with the retrograde actin flux. The results obtained with cytochalasin D show that the polarized actin polymerization/depolymerization cycle is either required or the driving force for the Rab5-induced cell migration; however, although it has been reported that 2 μM cytochalasin D did not have an effect on the morphology of L79-Rab5–induced endosomal structures (Murphy *et al.*, 1996; our unpublished results) or on transferrin-receptor endocytosis measured in a permeabilized cell system (Lamaze *et al.*, 1996), it has been clearly demonstrated that other actin–polymerization inhibitory agents do affect receptor-mediated endocytosis (Lamaze *et al.*, 1997). Thus, we cannot exclude the possibility that cytochalasin D treatment influences the polarized endocytosis/exocytosis cycle as well. Therefore, it remains to be established whether the observed stimulation of cell migration upon Rab5 activation is a consequence of the stimulation of either the polarized endocytic cycle or polarized actin polymerization alone, or of the combination of these processes (Figure 6).

CONCLUSION

In conclusion, our data for the first time show that a member of the Rab GTPase family, implicated in the regulation of vesicle fusion and trafficking, is able to induce lamellipodia formation and cell migration by a novel mechanism independent of the Rho GTPase family (Figure 6). Our results strongly suggest an intricate relationship between endocytosis, cytoskeletal reorganization, and cell migration. Several models have been proposed in which the driving force for cell migration is either a polarized actin polymerization cycle resulting in a retrograde flow of actin and actin-bound adhesion proteins, which pushes the cell forwards, or a polarized endocytic/exocytic cycle, which delivers membrane and membrane protein (e.g., adhesion proteins) for extension and substrate attachment to the leading edge of the migrating cell. We propose that Rab5 may orchestrate the cytoskeletal architecture to support and direct endocytosis and that the combined action of a polarized actin polymerization and endocytic cycle, via the forced retrograde flow of adhesive membrane proteins, causes Rab5-induced cell migration (Figure 6).

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