The small GTP-binding protein Rab4 has been involved in the recycling of αvβ3 integrins in response to platelet-derived growth factor (PDGF) stimulation suggesting a role for Rab4 in cell adhesion and migration. In this study, we explored the role of Rabip4 and Rabip4v, two Rab4 effector proteins, in migration of NIH 3T3 fibroblasts. In these cells, Rabip4 and Rabip4v, collectively named Rabip4s, were partially co-localized with the early endosomal marker EEA1. PDGF treatment re-distributed endogenous Rabip4s toward the cell periphery where they co-localized with F-actin. In cells expressing green fluorescent protein (GFP)-Rabip4 or GFP-Rabip4v, constitutive appearance of GFP-Rabip4s at the cell periphery was accompanied by local increase in cortical F-actin in membrane ruffles at the leading edge. The expression of GFP-Rabip4 induced an increased migration compared with control cells expressing GFP alone, even in the absence of PDGF stimulation. On the contrary, in cells expressing a mutated form of Rabip4s unable to interact with Rab4, lack of typical leading edge was observed. Furthermore, PDGF treatment did not stimulate the migration of these cells. Furthermore, down-regulation of the expression of Rabip4s inhibited PDGF-stimulated cell migration. Endogenous Rabip4s were localized with αv integrins at the leading edge following PDGF treatment, whereas in cells expressing GFP-Rabip4s, αv integrins, together with GFP-Rabip4s, were constitutively localized at the leading edge. In contrast, reduction in Rabip4s expression levels using small interfering RNA was associated with impaired PDGF-induced translocation of αv integrins toward the leading edge. Taken together, our data provide evidence that Rabip4s, possibly via their interaction with Rab4, regulate integrin trafficking and are involved in the migration of NIH 3T3 fibroblasts.

Cell migration is a fundamental process that is required during embryonic morphogenesis, tissue repair, and regeneration, as well as, in progression of certain diseases including cancer, mental retardation, atherosclerosis, and arthritis (1, 2). Cell migration involves the formation of extended protrusions in the direction of migration, a process that is driven by actin polymerization (3), and subsequent stabilization of the leading edge protrusions via integrins adhering to the extracellular matrix (2, 4). Integrins are heterodimeric receptor molecules that participate in an endo-exocytic cycle in which they are continually internalized from the plasma membrane, delivered to the endosomes, and recycled to the plasma membrane for reutilization (5–7). Recent studies have shown that, in fibroblasts, platelet-derived growth factor (PDGF) stimulates recycling of αvβ3 integrins from the early endosomes is dependent on Rab4a, suggesting a role for Rab4a in the mechanisms regulating integrin trafficking during cell adhesion (7, 8).

Rab4a is a small GTPase implicated in endocytosis in multiple cell types (9, 10). We identified Rabip4, a ubiquitous 69-kDa protein, as an effector of Rab4a on the basis of its ability to bind specifically to the GTP-bound form of Rab4a in a yeast two-hybrid system (11). The human counterpart of Rabip4, RUFY1, has also been identified (12). Like Rab4a, expressed Rabip4 is predominantly associated with the early endosomes, and co-expression of Rabip4 with active Rab4a causes an enlargement of the early endosomes (11, 13, 14). In Chinese hamster ovary cells and 3T3 L1 adipocytes, expressed Rabip4 is partially co-localized with an effector of Rab5, the early endosome marker early endosome antigen 1 (EEA1) (15, 16) but is absent from the recycling endosomes and late endosomes as evidenced by the lack of its co-localization with Rab11 or Rab7, markers of recycling and late endosomes, respectively (11, 14). In addition to a FYVE finger domain that has been found present in several proteins including EEA1 (17), Fab1p (18), Vps27p (19), and Vac1p (20), Rabip4 contains a RUN domain and two coiled-coil domains (11, 13, 21). The C-terminal cysteine-rich FYVE finger of Rabip4 can bind PI(3)P in vitro and trigger membrane association of Rabip4, although it is unclear whether this interaction targets Rabip4 to the early endosomes (13, 21). The two isoforms of Rabip4, Rabip4 and Rabip4v, which originate from the same gene, have been described (21, 22). In the mouse, Rabip4...
and Rabip4’ differ by 112 amino acid residues that are present only at the N terminus of Rabip4’ (Fig. 1A). We hypothesized that Rabip4 and Rabip4’, Rab4a effectors, might be playing a role in some aspect of intracellular trafficking during cell adhesion and migration.

In the present study, we characterized the intracellular distribution of endogenous Rabip4 isoforms, collectively named Rabip4s, in NIH 3T3 fibroblasts in the presence or absence of PDGF stimulation, and we studied the effects of Rabip4s using cells expressing GFP-Rabip4s or the mutated Rabip4s form that is unable to bind Rab4a. In addition, we examined the effects of inhibition of Rabip4s expression levels using small interfering RNA (siRNA). We provide evidence that Rabip4 is involved in migration of NIH 3T3 fibroblasts and propose that Rabip4 may be regulating the trafficking of integrins to the leading edge of migrating fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies against EEA1, rat anti-mouse CD51 (integrin αv chain), were from BD Biosciences. Monoclonal antibodies against transferrin receptor were from Zymed Laboratories (San Francisco, CA). Monoclonal antibody against paullinx was from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies against Myc (9E10) and polyclonal antibodies against extracellular regulated kinases (ERK) were from Santa Cruz Biotechnology (Santa Cruz, CA). PDGF-BB was obtained from Pepro Tech. Inc. (Rocky Hill, NJ). Polyclonal antibody against Rabip4s was produced as previously described (11). Fluorescein isothiocyanate (FITC), Texas Red-conjugated, and horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Texas Red-conjugated phallloidin was obtained from Molecular Probes (Eugene, OR). Monoclonal antibody against tubulin (clone DM1A) was obtained from Sigma. Other chemicals were from Sigma, and molecular biology reagents were from New England Biolabs, Inc. (Beverly, MA), Invitrogen (Carlsbad, CA), or Qiagen (Hilden, Germany).

cDNA Constructs—The construction of pEGFP-Rabip4 and pEGFP-Rabip4Δ-(507–517) was previously described (11, 13). These construction vectors allow for the expression of Rabip4 or a Rabip4 form that is unable to interact with Rab4, as a C-terminal fusion with GFP. For the cloning of Rabip4’, a mouse heart cDNA TRIPLEX phage library (Clontech) was screened using as a probe a cDNA corresponding to the amino acid sequence 401–507 of Rabip4. Positive clones were selected after 3 screening rounds. The corresponding phages were used to infect Escherichia coli strain BM25.8 to transform phages into plasmids, before sequencing the inserted cDNA. The cDNA sequence of Rabip4 with a 5′ extension was subcloned in-frame with the 3′ region of Rabip4 into pcDNA3 and pEGFP vectors. This cDNA encodes for Rabip4’.

Cells and Transfections—NIH 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing glutamine and 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 7% CO2. For cDNA transfections, NIH 3T3 cells were transiently transfected once they achieved 70–80% confluence with FuGENE 6 (Roche Diagnostics) or jetPEI™ (Polyplus Transfection, Illkirch, France), as indicated by the manufacturer. One day following transfection, cells were passaged (50,000 cells per well) onto glass coverslips of a 12-well plate that was pre-coated with 20 μg/ml fibronectin overnight at 4 °C, blocked with 1% heat-inactivated bovine serum albumin for 30 min at 37 °C, and rinsed twice with PBS. Transfected cells were used 48 h following transfection and the transfection procedures led to 10–30% efficiency. Prior to each experiment, cells were serum deprived for 30 min with DMEM containing 1% FCS and were then treated with 20 ng/ml PDGF for 60 min. For Rabip4s siRNA experiments, cells were transfected by electroporation using the Nucleofector Kit R Solution (Amaxa Biosystems), as indicated by the manufacturer. Cells were transfected using 1.5 μg of non-targeting (control) or siRNA against Rabip4s and incubated for 72 h at 37 °C prior to further analysis. The siRNA sequences against Rabip4 and Rabip4’ were as following: sense 5′-CAAGCAGCTCCTAAATGAGTT-3′ and antisense 5′-CTCACCTAGGAGCCTGTAGT-3′.

Cell Fractionation—NIH 3T3 cells were harvested and homogenized in 250 μl of cold 10 mM Tris-HCl, pH 7.4, buffer containing 5 mM sucrose and a protease inhibitor mixture (Complete™, Roche Diagnostics). Cells were broken by 20 passages through a 22-gauge needle and centrifuged for 10 min at 600 × g at 4 °C to generate a postnuclear supernatant. A high-speed supernatant (cytosol) and a membrane pellet were obtained following centrifugation of the postnuclear supernatant for 1 h at 100,000 × g at 4 °C (Optima X100 ultracentrifuge, Beckman, Palo Alto, CA). Proteins from equal volumes of cytosol and membrane-associated fractions were resolved by SDS-PAGE and analyzed by immunoblotting using anti-Rabip4 antibodies.

Immunofluorescence Confocal Analysis—Cells grown on fibronectin-coated glass coverslips were fixed for 15 min with 4% paraformaldehyde in PBS. Following neutralization with 10 mM NH4Cl in PBS for 15 min, cells were washed three times with PBS, and permeabilized with PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin. To detect GFP fusion proteins, cells were directly mounted in Mowiol 4-88 (Calbiochem, Darmstadt, Germany). To detect endogenous Rabip4s as well as integrins, EEA1, paullinx, or transferrin receptor coverslips were incubated for 1 h at room temperature with the appropriate primary antibodies diluted in permeabilization buffer. Following three washes with PBS, coverslips were incubated with FITC-conjugated anti-rabbit antibodies and Texas Red-conjugated anti-mouse antibodies for 45 min at room temperature. Coverslips were finally mounted in Mowiol 4-88 on glass slides. Cells were then examined by sequential excitation (MultiTrack option) at 488 (GFP and FITC) and 568 nm (Texas Red) using a scanning confocal fluorescence microscope with a PL APO 63 × 1.40 oil objective (LSM 510, Zeiss, Götttingen, Germany). The images were combined and merged using Photoshop software (Adobe Systems, Mountain View, CA). Intensity profiles were determined by using LSM Image Browser.

Time Lapse Imaging—Cells plated on fibronectin-coated 35-mm dishes and incubated in DMEM containing 1% FCS were filmed for 4 h before and after the addition of 20 ng/ml PDGF under 5% CO2, 37 °C. Cells were observed by phase-contrast optics using a fully motorized Axiovert 200 micro-
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scope (Zeiss, Göttingen, Germany) and a cooled digital CCD camera (CoolSnap HQ, Roper Scientific, Evry, France) using ×20 lens objective. Images were recorded at 1 frame/30 s and processed using MetaMorph 2.0 image analysis software (Universal Imaging, Downingtown, PA) and QuickTime software (Apple Computer, Cupertino, CA). Migration of individual cells and their length were determined using the plugging “manual tracking” of the ImageJ software. The nucleus position of a particular cell in a constant field among all the conditions filmed is defined every 10 min.

Cell Migration Assay—Cells transfected with siRNA were washed twice with PBS, detached with trypsin, and resuspended (2.0 × 10^5 cells/ml) in DMEM containing 1% FCS. A suspension (0.5 ml) of cells was pipetted onto a fibronectin-coated Transwell insert (8-μm pores) and incubated (24 h) at 37 °C. After incubation, non-migratory fibroblasts were removed carefully from the upper face of the Transwell chambers with a cotton swab. Transwell membranes were fixed in 0.2% (v/v) glutaraldehyde and stained with crystal violet. Cells (blue) that had migrated and adhered to the Transwell membrane were microscopically counted. Multiple fields (up to 10) were counted per membrane using a ×20 objective.

RESULTS

Endogenous Rabip4s Translocate to the Cell Periphery of NIH 3T3 Fibroblasts following PDGF Treatment—NIH 3T3 fibroblasts were subjected to fractionation and immunoblotting using antibodies developed previously against the C-terminal part of Rabip4 (11). The antibodies detected both Rabip4 isoforms in NIH 3T3 fibroblasts, Rabip4b and Rabip4c, which differ by the presence of N-terminal 112 amino acid residues within Rabip4c, as observed earlier in other cell types (22) (Fig. 1A). The endogenous Rabip4s were detected both in cytosolic and membrane-associated fractions (Fig. 1A). We next analyzed the subcellular localization of endogenous Rabip4s. Under basal conditions, endogenous Rabip4s were mainly localized in punctuate spots enriched in the perinuclear region (Fig. 1B). Rabip4s partially co-localized with a marker of early endosomes, EEA1, in this region, indicating that, in fibroblasts, Rabip4s are likely localized to early endosomes, as previously reported for the expressed Rabip4 in other cell types (11, 13, 21). Co-localization between Rabip4s and transferrin receptors, the marker of recycling endosomes, was observed only around certain transferrin receptor-positive vesicles. Importantly, following PDGF treatment, in addition to perinuclear localization, Rabip4s were also detected at the cell periphery (Fig. 1C, right panel) in the majority of cells (Fig. 1D).

Because previous work reported Rab4a dependence in cell adhesion (8), we next decided to characterize intracellular localization of endogenous Rabip4s during cell adhesion. To do this, we checked co-localization of endogenous Rabip4s with focal adhesion marker, paxillin, and F-actin, after allowing cells to adhere onto fibronectin-coated coverslips in the presence of PDGF for 60 min. Rabip4s were not significantly co-localized with paxillin at the cell periphery although they appeared in close proximity (Fig. 2A, top). However, Rabip4s were significantly co-localized with F-actin at the cell periphery during cell adhesion (Fig. 2A, bottom). The presence of Rabip4s at the cell periphery was observed whether cells were allowed to adhere in the presence (Fig. 2A) or absence of PDGF (data not shown) indicating that Rabip4s-containing vesicles are likely targeted toward the sites at the cell periphery where initial events of cell adhesion occur. Similarly, in adhering cells that were subsequently stimulated with PDGF for 60 min, Rabip4s detected at the periphery of the cell were also co-localized with F-actin, indicating that Rabip4s are present at the PDGF-induced leading edge of migrating fibroblasts (Fig. 2B).

Overexpression of Rabip4 Stimulates Migration of NIH 3T3 Fibroblasts in the Absence of PDGF Treatment—The presence of endogenous Rabip4s at the PDGF-induced leading edge of
NIH 3T3 fibroblasts and their co-localization with F-actin prompted us to further examine whether Rabip4 might be playing a role in cell migration by looking at the effects of Rabip4 overexpression. In cells expressing GFP-Rabip4 or GFP-Rabip4’, Rabip4s could be observed at the cell periphery both under basal conditions (Fig. 3A, top) and following PDGF treatment (Fig. 3A, bottom). The percentage of cells expressing GFP-Rabip4 or GFP-Rabip4’ that presented Rabip4s at the cell periphery under basal conditions was almost equivalent to that observed following PDGF treatment (Fig. 3B). Under basal conditions, in cells expressing GFP-Rabip4, but not in cells expressing GFP alone, cortical F-actin was present in membrane ruffles at the leading edge, and it was significantly co-localized with GFP-Rabip4 (Fig. 3C) or GFP-Rabip4’ (data not shown). Importantly, GFP was not present in the membrane ruffles at the leading edge (Fig. 3C). Furthermore, under basal conditions, the majority of cells expressing GFP-Rabip4 or GFP-Rabip4’, but not cells expressing GFP alone, had cortical F-actin in membrane ruffles at the leading edge (Fig. 3D). Cortical F-actin in membrane ruffles was also observed at the leading edge of PDGF-treated cells expressing GFP-Rabip4s (data not shown).

To study the migration of these cells, we next performed time lapse video microscopy experiments, in which cells were filmed for 4 h in the presence or absence of PDGF. Cells that strongly

FIGURE 2. Rabip4s co-localize with F-actin at the cell periphery. A, cells were trypsinized, plated onto fibronectin-coated coverslips, allowed to adhere for 60 min in the presence of 20 ng/ml PDGF, fixed, and permeabilized. Polyclonal anti-Rabip4s and monoclonal anti-paxillin antibodies were used to detect endogenous Rabip4s and paxillin, respectively. Anti-rabbit FITC and anti-mouse Texas Red conjugates were used as secondary antibodies, whereas Texas Red-conjugated phalloidin was used to detect F-actin. Images were captured using a scanning confocal fluorescence microscope and one confocal section for each labeling is shown. Bar, 10 μm. B, cells plated overnight onto fibronectin-coated coverslips were serum deprived for 30 min, incubated in the presence of PDGF for 60 min, fixed, permeabilized, and stained for endogenous Rabip4s and F-actin, as in A. Bar, 10 μm.

FIGURE 3. Overexpression of Rabip4 leads to constitutive appearance of Rabip4 and actin at the leading edge. Cells transiently transfected with pEGFP, pEGFP-Rabip4, or pEGFP-Rabip4’ cDNA were serum deprived for 30 min, incubated in the presence or absence of PDGF for 60 min, fixed, and permeabilized. Texas Red-conjugated phalloidin was used to detect F-actin. Images were captured using a scanning confocal fluorescence microscope and one confocal section is shown in each image. A, cells were transiently transfected with pEGFP-Rabip4 or pEGFP-Rabip4’ cDNA. Bar, 10 μm. B, quantification of the percentage (%) of cells expressing GFP-Rabip4 with GFP-Rabip4 at the cell periphery in the presence or absence of PDGF. Data are the average of three experiments ± S.E., in which ≥40 transfected cells were observed. C, cells transiently transfected with pEGFP or pEGFP-Rabip4 cDNA were serum deprived for 90 min in absence (basal) or presence of PDGF and subjected to immunofluorescence analysis. One confocal section for each labeling is shown and the profile of GFP or GFP-Rabip4 labeling along the arrow is shown on the right. *, points to an area of a membrane ruffling. Bar, 10 μm. D, quantification of the percentage (%) of cells expressing GFP, GFP-Rabip4, or GFP-Rabip4’ having cortical F-actin in the membrane ruffles at the leading edge in the absence of PDGF treatment. Data are the average of three experiments ± S.E. in which ≥40 transfected cells were counted.
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expressed GFP alone (pointed with arrows in Fig. 4A) or GFP-Rabip4 (pointed with arrows in Fig. 4B) were selected in corresponding phase-contrast time frames. The various frames show the position of cells expressing GFP alone or GFP-Rabip4 at 0, 2, and 4 h of incubation in the presence or absence of PDGF. As expected, the migration tracks of cells expressing GFP alone showed that PDGF treatment stimulated the migration of these cells (Fig. 4C, Table 1, and supplementary Videos 1 and 2). Importantly, the migration tracks of cells expressing GFP-Rabip4 showed that the PDGF treatment stimulated cell migration but also that the cells expressing GFP-Rabip4 displayed an increased mobility under basal conditions compared with cells expressing GFP alone (Fig. 4C, Table 1, and supplementary Videos 3 and 4). This result corroborates our results showing the increased presence of Rabip4 and F-actin at the leading edge of cells expressing GFP-Rabip4, and suggests that overexpression of Rabip4 may induce cell migration independently of PDGF stimulation.

Interaction of Rabip4 with Rab4a Is Required for Efficient PDGF-induced Leading Edge Formation and Cell Migration—We next examined whether the interaction of Rabip4s with Rab4a is required for Rabip4-induced migration by overexpressing a mutated form of Rabip4 or Rabip4’ that is unable to interact with Rab4a, GFP-Rabip4Δ-(507–517), or GFP-Rabip4Δ-(619–629), respectively. Cells expressing GFP-Rabip4Δ-(507–517) or GFP-Rabip4Δ-(619–629) had long extensions and, following PDGF treatment, lacked typical leading edges (Fig. 5A). Compared with control non-transfected cells, only a minority of cells expressing GFP-Rabip4Δ-(507–517) or GFP-Rabip4Δ-(619–629) displayed a typical leading edge following PDGF treatment (Fig. 5B), suggesting that the interaction of Rabip4s with Rab4 may be required for proper PDGF-induced leading edge formation. We next analyzed the migration of cells expressing GFP-Rabip4Δ-(619–629) by time lapse video microscopy. In cells expressing GFP-Rabip4Δ-(619–629), aberrant cell morphology with long extensions and no proper leading edge could also be observed in the phase-contrast time frames (Fig. 6A). The phase-contrast time frames and the subsequently determined migration tracks, furthermore, show that the PDGF treatment did not stimulate the migration of these cells as was the case with control cells expressing GFP alone (Fig. 6B, Table 1, and supplementary Videos 5 and 6). These data indicate that the interaction of Rabip4 with Rab4 may be necessary for PDGF-stimulated migration and further strengthen our hypothesis of a possible role for Rabip4 during migration.

Down-regulation of Rabip4s Expression Impaired PDGF-stimulated Migration of NIH 3T3 Fibroblasts—We next used siRNA silencing to analyze the effects of inhibiting Rabip4s expression on cell migration. Because the sequence of Rabip4 is included within the Rabip4’ sequence, it was not possible to design siRNA specific for Rabip4. Cells were transfected with non-targeting (control) siRNA or with the siRNA against Rabip4s for 72 h. Transfection with the siRNA against Rabip4s led to inhibition of the expression levels of Rabip4s compared with control siRNA (Fig. 6C), that did not modify Rabip4s expression compared with blank transfection. Immunoblotting using antibodies against tubulin or ERK suggested that the expression levels of these two proteins were not altered following transfection with siRNA against Rabip4s. A Transwell

TABLE 1

| Construction   | Basal   | PDGF   |
|----------------|---------|--------|
| GFP            | 1.00 ± 0.14 (n = 6) | 2.15 ± 0.18 (n = 12)* |
| GFP-Rabip4     | 2.60 ± 0.22 (n = 17)* | 2.84 ± 0.35 (n = 15) |
| GFP-Rabip4Δ-(507–517) | 0.72 ± 0.05 (n = 11) | 1.11 ± 0.12 (n = 10)* |

* Significant compared to basal with *p < 0.0001.

* Significant compared with GFP in the same condition with *p < 0.0001.

FIGURE 4. Overexpression of Rabip4 potentiates migration of NIH 3T3 fibroblasts. A, cells transiently transfected with pEGFP cDNA (A) or pEGFP-Rabip4 cDNA (B) were incubated in DMEM containing 1% FCS and filmed for 4 h under constant conditions (5% CO2, 37 °C) before and after the addition of 20 ng/ml PDGF. Cells were observed by phase-contrast optics. Images were recorded at 1 frame/30 s and processed using MetaMorph 2.0 image and QuickTime analysis software. Selected QuickTime phase-contrast images of transfected cells (arrows) are shown at 0, 2, and 4 h in the presence or absence of PDGF treatment. The left panels show the GFP fluorescence of transfected cells. C, migration tracks following video time lapse microscopy cells of expressing GFP or GFP-Rabip4 in the presence or absence of PDGF treatment. Bar, 100 μm.
migratory assay was then performed on cells transfected with control or siRNA against Rabip4s in the presence or absence of PDGF. The migration of cells transfected with control siRNA was increased 2-fold by PDGF (Fig. 6D). Migration of cells transfected with siRNA against Rabip4s was slightly decreased under basal conditions but, more importantly, the effect of PDGF on migration of these cells was markedly hampered suggesting that knocking down Rabip4s expression inhibits PDGF-stimulated migration.

**Role of Rabip4 in Migration**

**Role of Rabip4 in Migration**

Rabip4s Regulate Presentation of Integrins at the Leading Edge of NIH 3T3 Fibroblasts—PDGF treatment of NIH 3T3 fibroblasts has been shown to provoke rapid Rab4a-dependent recycling of αvβ3 integrins from the early endosomes to the leading edge (8). To investigate whether the role of Rabip4s in cell migration may be associated with integrin distribution, we examined whether endogenous Rabip4s are co-localized with αv integrins. Under basal conditions, endogenous Rabip4s and αv integrins were mainly localized at punctuate spots, but the degree of co-localization was very small (Fig. 7A, top). Following PDGF treatment, both αv integrins and Rabip4s appeared at the leading edge of the cells (Fig. 7A). More specifically, some of the positive αv integrins vesicles were also labeled for Rabip4s in the leading edge (supplemental Fig. S1). Furthermore, in cells transfected with GFP-Rabip4, αv integrins were, together with GFP-Rabip4, detected at the leading edge both under basal conditions and following PDGF treatment (Fig. 7B). This result suggests that the increased mobility induced by GFP-Rabip4 overexpression under basal conditions may be partially due to an increase of αv integrins at the leading edge. In contrast, in
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cells expressing GFP-Rabip4Δ-(619–629), αv integrins were, like GFP-Rabip4Δ-(619–629), mostly detected at punctuate perinuclear spots (Fig. 7C), suggesting Rabip4 interaction with Rab4a may be required for proper intracellular trafficking of αv integrins. To further test the possible involvement of Rabip4s in translocation of αv integrins to the leading edge, we inhibited the expression of Rabip4s using siRNA as described above. No change in Rabip4s expression levels was observed when cells were transfected with a non-targeting siRNA (control siRNA), compared with a blank transfection (data not shown). Importantly, when cells were transfected with the Rabip4s siRNA, but not with the control siRNA, reduction in the expression levels of Rabip4s was associated with impaired distribution of αv integrins to the periphery of PDGF-treated cells (Fig. 8A). Quanti-

FIGURE 7. Rabip4s co-localize with integrins at the cell periphery of PDGF-stimulated NIH 3T3 fibroblasts. Cells plated onto fibronectin-coated coverslips were serum deprived for 30 min, incubated in the presence or absence of 20 ng/ml PDGF for 60 min, fixed, and permeabilized prior to immunofluorescence analysis. Images were captured using a scanning confocal fluorescence microscope and one confocal section is shown in each image. Bar, 10 µm. A, polyclonal anti-Rabip4 and rat anti-mouse CD51 antibodies were used to detect endogenous Rabip4s and integrin αv chain, respectively. Anti-rabbit FITC and anti-rat Texas Red conjugates were used as secondary antibodies. Insert shows an enlarged view of the delineated area. B, cells were transiently transfected with pEFP-Rabip4 cDNA. C, cells were transiently transfected with pEFP-Rabip4Δ-(619–629) cDNA.

FIGURE 8. Reduction in Rabip4 expression levels using siRNA leads to impaired PDGF-stimulated presentation of integrins at the leading edge. Cells were transfected with control siRNA or with siRNA against Rabip4s for 72 h. A, cells were serum deprived for 30 min, treated with PDGF for 60 min, fixed, and permeabilized. Polyclonal anti-Rabip4s and rat anti-mouse CD51 antibody were used to detect Rabip4s and integrin αv chain, respectively. Anti-rabbit FITC conjugates and anti-rat Texas Red conjugates were used as secondary antibodies. Images were captured using a scanning confocal fluorescence microscope and one confocal section is shown for each image. Two typical cells from two independent experiments were shown. Bar, 10 µm. B, quantification of the percentage (%) of cells transfected with control siRNA or with siRNA against Rabip4 that have αv integrins at the leading edge following PDGF treatment. Data are the average of three experiments ± S.E. in which ≥100 transfected cells were counted.
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The present study looked at the intracellular localization of endogenous Rabip4s following PDGF stimulation of NIH 3T3 fibroblasts and examined the effects of Rabip4 expression and inhibition. We found that the endogenous Rabip4s, following PDGF treatment, were redistributed to the leading edge where they co-localized with F-actin and αv integrins. In cells expressing GFP-Rabip4s, F-actin, αv integrins, and GFP-Rabip4s were constitutively present at the leading edge, and these cells displayed increased mobility even in the absence of PDGF stimulation. On the contrary, cells expressing mutated Rabip4s that are unable to interact with Rab4a lacked typical leading edges and PDGF treatment did not stimulate migration of cells expressing a mutated Rabip4, suggesting that the interaction of Rabip4s with Rab4a was necessary for PDGF-stimulated cell migration. Furthermore, using siRNA to inhibit Rabip4 expression, we found that the reduced Rabip4 expression levels was associated with impaired presentation of αv integrins at the leading edge of PDGF-treated cells suggesting that Rabip4 may be involved in PDGF-mediated trafficking of αv integrins to the leading edge. In agreement with these results, migration of the Rabip4 knock-down cells was inhibited in response to PDGF. Together, these findings provide evidence for a novel function of Rabip4s in migration of NIH 3T3 fibroblasts. It would be interesting to check whether Rabip4s exert the same function in other cell types.

Due to the lack of tools that could discriminate between Rabip4 and Rabip4’, our observations on the endogenous Rabip4s are in reference to both Rabip4 isoforms. However, using cells that express specifically GFP-Rabip4 or GFP-Rabip4’ or either of the two mutated forms that are unable to bind Rab4, GFP-Rabip4Δ-(507–517) or GFP-Rabip4’Δ-(619–629), respectively, we were able to analyze the effects of expression of each Rabip4 isoform separately. We observed similar effects following expression of either Rabip4 isoform or following the expression of either mutated Rabip4 isoform that is unable to bind Rab4a, in the absence or presence or PDGF stimulation, indicating no striking differences between the two Rabip4 isoforms with respect to their intracellular localization and involvement in PDGF-stimulated migration. Our data suggest that both Rabip4 isoforms play a role in cell migration.

Cells overexpressing Rabip4 had cortical F-actin and αv integrins at the leading edge and migrated both under basal conditions and following PDGF treatment corroborating previously reported results showing that the expression of Rab4a increased the rates of αvβ3 recycling both under basal and PDGF-stimulated conditions (8). Moreover, expression of active Rab5 in fibroblasts was shown to induce lamellipodia formation and cell migration (23). Thus, the high intracellular concentration of Rabip4 in itself may be sufficient to induce fibroblast migration by possibly regulating the vesicular trafficking of integrins to the cell periphery.

Aberrant morphology, absence of normal leading edges, and inability of PDGF to induce migration of cells expressing a mutated Rabip4s form that is unable to bind Rab4a indicated that the interaction of Rabip4 with Rab4a may be important in regulation of trafficking of αv integrins and PDGF-stimulated cell migration. In agreement with these results, increased plasma membrane levels of Glut4 were observed in adipocytes expressing the mutated Rabip4 form that is unable to bind Rab4a, suggesting that uncoupling between Rabip4 and Rab4a leads to defective trafficking of Glut4 (14). Uncoupling of Rabip4 with Rab4a in migrating fibroblasts may thus impair the PDGF-induced redistribution of integrin-containing vesicles toward the plasma membrane or prevent the establishment of polarity and leading edge formation.

We observed nuclear enrichment of phospho-ERK1 and -ERK2, kinases that translocate to nucleus in response to treatment with growth factors (24, 25), following PDGF stimulation of cells expressing GFP-Rabip4 or GFP-Rabip4’Δ-(619–629) (data not shown). This result strongly suggests that PDGF signaling was not altered in the cells expressing GFP-Rabip4 or GFP-Rabip4’Δ-(619–629). PDGF-mediated αvβ3 integrin recycling to the plasma membrane appears to involve PI 3-kinase and PKB/Akt (26) and PKD1 (27). Rabip4 may possibly be a target of the action of these kinases.

Proteins containing FYVE finger domains have been shown to bind PI(3)P (28–30). Most proteins containing a FYVE finger have been found to localize to early endosomes and have been found to play a role in membrane trafficking, cytoskeleton remodeling, and signal transduction (31). However, when expressed alone, the FYVE finger domain of Rabip4 has been localized at the plasma membrane (12, 21). Similarly, whereas PI(3)P has been shown to be enriched in early endosomes (29), a recent study pointed to the presence of PI(3)P at the plasma membrane in response to stimulation with insulin or lysophosphatidic acid and, furthermore, demonstrated a role for PI(3)P during cell migration (32, 33). Thus, in addition to a role at the level of endosomes, interaction of FYVE finger with PI(3)P may be important for the cell processes occurring at the level of plasma membrane. To our knowledge, our study suggests for the first time that a FYVE finger-containing protein can be found at the plasma membrane. It is possible that the FYVE finger domain of Rabip4 may be required for the membrane association of Rabip4 at the plasma membrane.

Integrins play a crucial role in cell adhesion, proliferation, and migration, and have been identified as important regulators of angiogenesis, cancer cell invasion and metastasis (34–36). Integrins are a target for the development of anti-cancer treatment, and a few integrin inhibitors have already been developed (36–39). Our findings demonstrate that the overexpression of Rabip4s leads to constitutive presentation of αv integrins at the leading edge, whereas inhibition of Rabip4s expression is associated with impaired presentation of αv integrins at the cell periphery following PDGF treatment, indicating that Rabip4 is a potential regulator of integrin trafficking.

In conclusion, the present study demonstrates a novel function for Rabip4 in cell migration. Rabip4 is an effector of Rab4a...
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during PDGF-stimulated migration of NIH 3T3 fibroblasts, and furthermore, Rabip4 regulates integrin trafficking. Whereas the precise mechanism by which Rabip4 regulates integrin trafficking remains to be determined, our findings offer an exciting possibility of Rabip4 being a potential new target for the development of anti-cancer treatment.

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