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Cell Migration Is Regulated by Platelet-Derived Growth Factor Receptor Endocytosis▽†

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Cell migration requires spatial and temporal processes that detect and transfer extracellular stimuli into intracellular signals. The platelet-derived growth factor (PDGF) receptor is a cell surface receptor on fibroblasts that regulates proliferation and chemotaxis in response to PDGF. How the PDGF signal is transmitted accurately through the receptor into cells is an unresolved question. Here, we report a new intracellular signaling pathway by which DOCK4, a Rac1 guanine exchange factor, and Dynamin regulate cell migration by PDGF receptor endocytosis. We showed by a series of biochemical and microscopy techniques that Grb2 serves as an adaptor protein in the formation of a ternary complex between the PDGF receptor, DOCK4, and Dynamin, which is formed at the leading edge of cells. We found that this ternary complex regulates PDGF-dependent cell migration by promoting PDGF receptor endocytosis and Rac1 activation at the cell membrane. This study revealed a new mechanism by which cell migration is regulated by PDGF receptor endocytosis.

Chemoattractants bind to cell surface receptors, resulting in the cytoskeletal reorganization that permits the migration of cells toward a stimulus. In fibroblasts, the platelet-derived growth factor receptor β (PDGFRβ) is a cell surface receptor tyrosine kinase (RTK) that regulates cell proliferation and chemotaxis in response to PDGF. PDGF binding activates PDGF receptor autophosphorylation, which in turn mediates a series of intracellular signaling cascades initiated by the association of SH2 domain-containing adaptor proteins (25). The adaptor protein Grb2 at the plasma membrane binds to Ras exchange factor Sos1, activating mitogen-activated protein kinase (MAPK) and cell proliferation signals (19). Grb2 also plays a critical role in receptor internalization via its interaction with dynamin, an exchange factor that facilitates receptor entry into endocytic vesicles (32). Grb2 regulates ubiquitination and the degradation of the receptor via its interaction with Cbl, an E3 ubiquitin ligase (33). While the role of Grb2 in modulating receptor levels and facilitating growth factor-dependent mitogenic signals is defined, its role in coordinating receptor-dependent chemotaxis has not been elucidated.

The small GTPase Rac1 plays a crucial role in PDGF-mediated chemotaxis by regulating cortical actin at the leading edge of cells. PDGF receptor activation promotes GTP loading and the translocation of Rac1 to the cell membrane via guanine exchange factors (GEFs). The DOCK family of Rac1 GEFs, also called CDM proteins (for *Caenorhabditis elegans ced-5*, vertebrate DOCK180, and *Drosophila* myoblast city), are regulators of cell migration and have been implicated in various biological processes, such as lymphocyte migration, phagocytosis, and cancer progression (6, 10, 30, 35). In migrating fibroblasts, DOCK proteins localize to the cell's leading edge via their interaction with the phospholipid PIP3, but a direct molecular link to PDGF has not been established (5). Biochemical studies show that Rac activation requires the DHR2/docker domain of DOCK proteins and the expression of the PH domain-containing protein Ced-12/ELMO. Previously we identified DOCK4 in a screen for novel tumor suppressor genes using representational difference analysis on mouse tumor cell lines (35). DOCK4, like other CDM proteins, binds ELMO and exerts its biochemical effects on the small GTPases Rac and Rap1 (30, 35). An interesting observation is that the amino acid sequence toward the C terminus is not conserved among individual DOCK family members. The alternate splicing of the DOCK4 gene has been reported, but how amino acid sequence variation alters the signaling properties of DOCK4 for the regulation of cell migration is unknown.

Members of the Nck family of adaptor proteins, CrkII and Nck, have been reported to bind to the C terminus of DOCK180 (12, 29). Here, we show that the third member of the family of Nck adaptors, namely Grb2, binds to wild-type DOCK4. We found that a ternary complex formed by Grb2-DOCK4-Dynamin2 interacts with PDGF-activated PDGFβ receptor and promotes growth factor-dependent migration without altering cell proliferation. PDGF-dependent migration requires receptor endocytosis and is regulated by the formation of a DOCK4-Grb2-Dynamin2-PDGFRβ complex at the cell's leading edge. These studies provide novel mechanistic insights into PDGFRβ regulation and cell migration.

MATERIALS AND METHODS

Cell lines, reagents, and tissue samples. NIH 3T3 cells were obtained from the American Type Culture Collection (Manassas, VA). Recombinant PDGF was

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obtained from Cell Signaling Technology (Beverly, MA), phenylarsine oxide (PAO) from MP Biomedicals (Solon, OH), and PD98059 and LY294002 from Calbiochem (San Diego, CA). Anti-DOCK4 and anti-isoform (DOCK4-Ex49)-specific antibodies (Abs) were generated as described previously (37). Anti-Grb2, anti-PDGFRβ, anti-CrkII, anti-Rap1, anti-Dynamin, anti-DOCK180, anti-Sos1, and anti-GST Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag M2 monoclonal Ab was from Sigma-Aldrich (St. Louis, MO); anti-Rac1, anti-phosphotyrosine-PY20, anti-Cbl, and anti-Dynamin2 Abs were from BD Pharmingen (San Diego, CA); anti-AKT, anti-phospho-AKT (Ser473), anti-p44/42, and anti-phospho-p44/42 MAPK Abs were from Cell Signaling Technology; and anti-green fluorescent protein (anti-GFP) Ab was from Covance (Berkeley, CA). Anti-Elmo Ab was kindly provided by K. S. Ravichandran (University of Virginia). The Alexa Fluor 594 protein labeling kit was from Molecular Probes (Eugene, OR). Discarded surgical specimens free of patient identifiers but with confirmed diagnosis were obtained from the Massachusetts General Hospital tumor bank.

Expression analysis and transfection studies. Wild-type, isoform DOCK4, and truncated DOCK4 cDNAs were cloned into the retroviral plasmid pBabe-puro with an N-terminal Flag epitope or N-terminal GFP epitope. All constructs were confirmed by sequencing. Grb2 plasmid was kindly provided by D. Bar-Sagi (State University New York), CrkII plasmids by B. Mayer (University of Connecticut), Raichu-Rac1/KRasCT plasmids by M. Matsuda (Kyoto University), and Dynamin plasmids and GFP-tagged Rab4, Rab5, and Rab11 plasmids by V. Hsu (Brigham and Women's Hospital). Recombinant retroviruses were produced by the transient transfection of 293T cells with pMD.MLV gag-pol (a helper plasmid), pMD.G (vesicular stomatitis virus G pseudotype), and the pBabe-puro of interest using FuGene6 (Roche Diagnostic Corporation, Indianapolis, IN). Stable transfectants were selected with puromycin (2.5 μg/ml) for DOCK4 constructs as a pool to minimize clonal variation. Hereafter, the pBabe-puro DOCK4-transduced cells are referred to as 3T3-DOCK4. For transient transfection studies, cDNA constructs or small interfering RNA (siRNA) duplexes were transfected with lipid reagents, and cell lysates were prepared 40 h after transfection. siRNAs for DOCK4 were prepared as described previously (35), while Grb2 and CrkII were acquired from Santa Cruz Biotechnology.

Chemotaxis assay. Migration was assayed in 24-well transwell cell culture chambers with 8 μm-pore membranes (Coster, Cambridge, MA), as described previously (26). Membranes were precoated with fibronectin (20 μg/ml) for the chemotaxis assay. PDGF (30 ng/ml) or soluble fibronectin (20 μg/ml; see Fig. S4b in the supplemental material) was added to the lower chamber as the chemoattractant. After cells were added to the upper chamber (1×10^5 cells/well) and incubated for 12 h, cells attached to the lower surface of the membrane were detached by trypsin and counted using a hemocytometer. The migration index was defined as the ratios of migrating cell numbers in the experimental groups divided by those in the controls. Each assay was performed in triplicate, and at least three experiments were performed for each set. Chemokinesis was tested in checkerboard assays and was negative. For studies using PAO, PD98059 and LY294002 cells were preincubated with various concentrations of these inhibitors and then subjected to chemotaxis assay.

Biochemical methods, microscopy, live cell imaging, and fluorescent resonance energy transfer-based (FRET) studies are described in detail in the supplemental material.

Statistical analysis. Statistical analysis was performed using Student's *t* test for unpaired data considered significant at the level of $P < 0.05$.

RESULTS

Formation of a PDGFRβ-Grb2-DOCK4 ternary complex at the cell's leading edge. An alternately spliced isoform of DOCK4 differs from the wild-type protein by the absence of exon 49 (Fig. 1a) and its limited expression in the stereocilia of the cochlea and the retina, whereas the wild-type DOCK4 protein is present in diverse tissues (37). Exon 49 encodes the motif PPψPPR (ψ denotes a hydrophobic residue) that is conserved in evolution (see Fig. S1a in the supplemental material) and is identical to the Grb2 binding site of Sos1 (19). The restricted tissue expression of the DOCK4 isoform and the absence of the Grb2 binding motif suggest that an interaction between the adaptor protein Grb2 and DOCK4 is functionally important for DOCK4 function. We tested this hypothesis by comparing the capacity of both wild-type and isoform DOCK4 to interact with Grb2 by coimmunoprecipitation assays between endogenous Grb2- and GST-fused DOCK4 proteins. We observed that Grb2 binds significantly more to wild-type DOCK4 than to its isoform (see Fig. S1b in the supplemental material). Grb2 consists of a single SH2 domain flanked by two SH3 domains. Using a GST pulldown assay with GST-Grb2 fusion proteins, we observed that endogenous DOCK4 binds to a Grb2 mutant with an N-terminal mutant SH3 domain, Grb2P49L, but is not capable of interacting with a Grb2 mutant with a C-terminal defective SH3 domain, Grb2G203R (see Fig. S1c in the supplemental material). These results indicate that DOCK4 binds to the C-terminal SH3 domain of Grb2, and this interaction is virtually abolished in a naturally occurring DOCK4 splice variant.

We next explored the possibility that the DOCK4 and Grb2 complex interacts with the PDGF receptor. We used NIH 3T3 fibroblasts in which the mitogenic and chemotactic responses mediated by PDGFRβ in response to PDGF are well established (25). First, we observed that endogenous DOCK4 coimmunoprecipitated with endogenous Grb2, and this interaction is increased by PDGF stimulation (Fig. 1c). DOCK4-Grb2 binding also was confirmed in HeLa, IEC6, and HCT116 cells (see Fig. S1f in the supplemental material). Second, we found that endogenous DOCK4 coimmunoprecipitated with endogenous PDGFRβ following PDGF stimulation (Fig. 1c). These data indicate that in cells, the DOCK4/Grb2 complex interacts with activated PDGFRβ.

To further elucidate the function of this interaction, we used N-terminal Flag-tagged DOCK4 cloned in the retroviral vector pBabe-puro (Fig. 1b). The DOCK4 wild type and isoform both are potent Rac activators (37). DOCK4 ΔDHR2 (an internal deletion of 77 amino acids) is null for Rac GEF, while ΔSH3 exhibits enhanced Rac GEF function (20, 37). In ΔC, the serine-proline-rich C terminus anchoring SH3 domains of DOCK4 interacting proteins, like Grb2, is deleted. NIH 3T3 cells expressing these constructs were established by retroviral infection, followed by the selection of a pool of puromycin-resistant cells to minimize clonal variation. Moreover, as judged by Western blot analysis, comparable protein expression among these cellular pools was observed (see Fig. S1d in the supplemental material). We then tested the ability of isoform DOCK4 to bind to the PDGFRβ and found that wild-type DOCK4 binds significantly more to Grb2 and PDGFRβ than its isoform (see Fig. S1e in the supplemental material). These results suggest that exon 49 of DOCK4 is critical for the recruitment of the Grb2-DOCK4 complex to the PDGFRβ. DOCK4 also binds to ELMO and CrkII through its SH3 domain and C-terminal proline-rich domain, respectively (20), and we found that PDGF stimulation increased DOCK4-CrkII binding modestly, while DOCK4-ELMO binding was unaffected (see Fig. S2a in the supplemental material). Using GST pulldown assays on HeLa cell lysates, we found that more endogenous DOCK180 bound to GST-CrkII than either GST-Grb2P49L or GST-Grb2G203R. In contrast, comparable amounts of endogenous DOCK4 bound to GST-CrkII and GST-Grb2P49L (see Fig. S2b in the supplemental material). These observations are not surprising, as the sequence motif PPVPPR is not present in DOCK180. It is likely that the

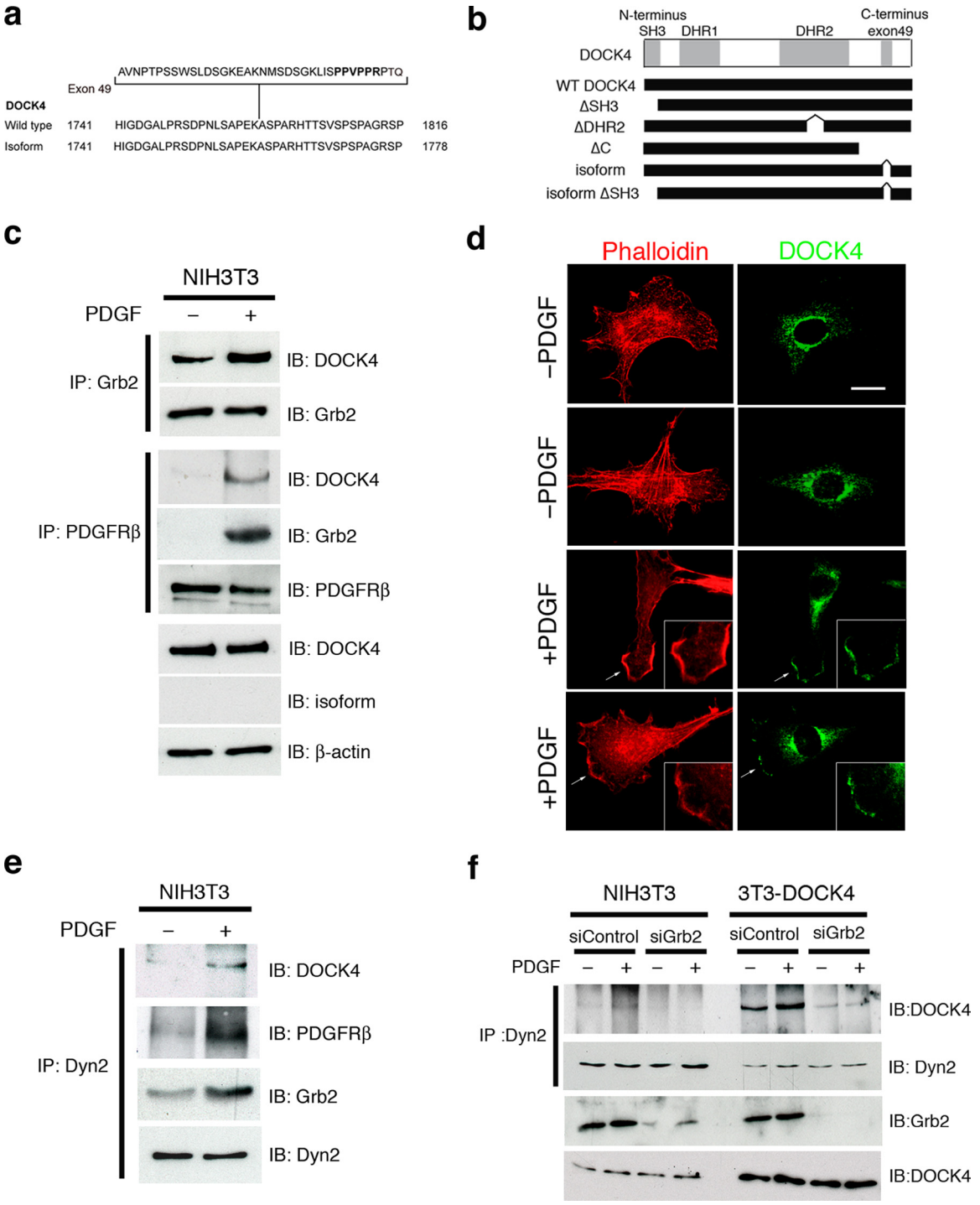

FIG. 1. DOCK4 forms PDGFRβ-Grb2-DOCK4 ternary complex upon PDGF stimulation. (a) Sequence of wild-type (WT) DOCK4 and a splice variation around exon 49 is shown. (b) Schematic representation of the DOCK4 constructs used in the manuscript. DHR, DOCK homology region. ΔSH3 lacks the N-terminal 76 amino acids, ΔDHR2 lacks the internal deletion of 77 amino acids in the DHR2 domain (residues 1357 to 1433), and ΔC lacks the C-terminal 380 amino acids. The N-terminal Flag epitope was tagged to these constructs. (c) After NIH 3T3 cells were left untreated or treated with PDGF (30 ng/ml) for 5 min, cell lysates were immunoprecipitated (IP) with either anti-Grb2 or anti-PDGFRβ Ab, followed by immunoblotting (IB) with the indicated Abs. Control blots of lysate for DOCK4, its isoform, and β-actin are shown. (d) Fluorescence staining of phalloidin (red) and DOCK4 (green) after incubation with or without PDGF (30 ng/ml) for 5 min. Arrows show cortical ruffles at the leading edge. The insets show higher magnifications of the area indicated by the arrow. Bar, 10 μm. (e) After NIH 3T3 cells were left untreated or treated with PDGF (30 ng/ml) for 5 min, cell lysates were immunoprecipitated with anti-Dynamin Ab, followed by immunoblotting with the indicated Abs. (f) Transient transfection of control siRNA and Grb2 siRNA into NIH 3T3 and 3T3-DOCK4 cells. After treatment with or without PDGF (30 ng/ml) for 5 min, cell lysates were immunoprecipitated with anti-Dynamin Ab, followed by immunoblotting with the indicated Abs. Control blots of lysate for DOCK4 and Grb2 are shown.

C-terminal region of DOCK4 provides molecular specificity toward adaptor protein Grb2 and the phosphorylated PDGFRβ.

In resting NIH 3T3 cells, endogenous DOCK4 protein is expressed in the cytoplasm, specifically in perinuclear and cytoplasmic vesicles. However, upon PDGF stimulation DOCK4 is recruited to the membrane ruffles at the leading edge, which is rich in cortical actin (Fig. 1d). Similarly, when GFP-tagged DOCK4 is expressed in NIH 3T3 cells, it also is recruited to the cortical ruffles of the leading edge after PDGF stimulation, where it colocalizes with PDGFRβ (see Fig. S2c and d in the supplemental material).

DOCK4 binds to Dynamin2 via Grb2 at the leading edge of migrating cells. We examined whether DOCK4 expression can affect Grb2 binding with other proteins, such as Sos1, Dynamin2, and Cbl, and we found that only Grb2-Dynamin2 binding is significantly increased by the overexpression of wild-type DOCK4 (see Fig. S3a in the supplemental material). After PDGF stimulation, Grb2-Cbl binding was not observed at 5 min but was evident 2 h later (data not shown).

Dynamin is a regulator of receptor endocytosis, as it is a GTPase that functions to pinch off vesicles from the plasma membrane (23). Since Dynamin preferentially binds to the N-terminal SH3 domain of Grb2 (32) and DOCK4 preferentially binds to the C-terminal SH3 domain of Grb2 (see Fig. S1c in the supplemental material), we hypothesized that DOCK4 and Dynamin formed a complex via Grb2. We observed that, after PDGF stimulation, Dynamin2 coimmunoprecipitated with endogenous DOCK4 and PDGFRβ in NIH 3T3 cells (Fig. 1e). We then found that Dynamin2 binds significantly more to wild-type DOCK4 than to its isoform (see Fig. S3b in the supplemental material). To confirm that Grb2 bridges Dynamin2 to DOCK4, we silenced Grb2 expression by using siRNA and found that DOCK4-Dynamin2 binding was significantly reduced even in the presence of PDGF (Fig. 1f). Since PDGF stimulation resulted in the association of Dynamin2 with PDGFRβ (Fig. 1e; see Fig. S3b in the supplemental material), we then analyzed whether DOCK4 expression altered the kinetics of Dynamin2-PDGFRβ binding after PDGF stimulation. In control 3T3-EV cells, Dynamin2-PDGFRβ binding reached its maximum 10 min after stimulation. In 3T3-DOCK4 cells, Dynamin2-PDGFRβ binding reached its maximum 5 min after stimulation, while in 3T3-isoform cells it was delayed until 30 min after stimulation (see Fig. S3c in the supplemental material).

It has been reported that Dynamin2 is present in membrane ruffles at the leading edge of PDGF-stimulated fibroblasts and directly interacts with cortactin (21). Confocal immunofluorescence microscopy showed that GFP-tagged wild-type DOCK4 colocalized with Dynamin2 at the membrane ruffles as early as 1 min after PDGF stimulation (see Fig. S3d in the supplemental material). After 5 min of stimulation, the accumulation of Dynamin2 and DOCK4 at the membrane ruffle is sustained. Taken together, these data suggest that DOCK4-Dynamin2-PDGFRβ complex formation precedes the receptor internalization. Importantly, GFP-tagged isoform DOCK4 rarely was localized at the membrane ruffles (see Fig. S3e in the supplemental material). Taken together, these observations indicate that the DOCK4-Grb2-Dynamin2 complex is formed at membrane ruffles at the leading edge of migratory cells.

DOCK4 Rac GEF function and DOCK4-Grb2 binding are important for PDGF-dependent migration. PDGF stimulates both cell proliferation and migration in fibroblasts. No appreciable difference in cell proliferation rates was observed among several DOCK4 stable cell lines (see Fig. S4a in the supplemental material). We then examined the role of DOCK4 in PDGF-dependent migration using Boyden chamber assays. PDGF-dependent migration was significantly increased by the expression of wild-type DOCK4 and ΔSH3, while it was significantly decreased by the expression of ΔC, ΔDHR2, and isoform DOCK4 (Fig. 2a). Migration toward soluble fibronectin was not affected by DOCK4 expression (see Fig. S4b in the supplemental material), indicating that DOCK4 specifically regulates PDGF-dependent migration. We further examined the effect of silencing DOCK4 by siRNA in PDGF-dependent migration (Fig. 2b). Silencing DOCK4 effectively reduced the PDGF-dependent migratory response of NIH 3T3 cells. The DOCK4 sequence targeted by the siRNA is located at the N-terminal SH3 domain. To test whether this effect of siRNA was specific against endogenous DOCK4, we transfected the ΔSH3 construct into the NIH 3T3 cells treated with the siRNA and noted that the PDGF-dependent migration defect induced by the siRNA was significantly recovered. Importantly, the rescue of cell migration after DOCK4 silencing was not observed when we transfected the isoform ΔSH3 construct, in which the siRNA targeting site is removed from isoform DOCK4 (Fig. 1b).

To investigate the molecular mechanism of cell migration, we examined various intracellular pathways downstream of the PDGF receptor. Consistently with previous reports (3, 34), we first confirmed that both phosphatidylinositol 3-kinase and MAPK pathways are involved in PDGF-dependent migration (see Fig. S4c in the supplemental material). However, DOCK4 expression or silencing DOCK4 had no effect on the PDGF-dependent phosphorylation of ERK1/2 and Akt/PKB (see Fig. S4d in the supplemental material). Regarding the activation of small GTPases, the GTP-bound form of Rac but not Rho, Cdc42, or Rap was observed (data not shown). GTP-Rac levels were high in 3T3-DOCK4, 3T3-ΔSH3, and 3T3-isoform cells, while ΔDHR2 and ΔC expression had a dominant-negative effect on GTP-Rac levels (Fig. 2c). The decrease of GTP-Rac levels in 3T3-ΔDHR2 and 3T3-ΔC cells can explain the observed migration defect, which is consistent with the published studies of the role of Rac1 in PDGF-dependent migration (25). However, in 3T3-isoform cells, GTP-Rac levels cannot account for the observed migration defect, suggesting that DOCK4-Grb2 binding is essential for cell migration, not GTP-Rac levels. In these cells, the formation of PDGF-dependent PDGFRβ-Grb2-DOCK4 complex is inefficient (see Fig. S1e in the supplemental material). We also observed that GTP-Rac levels were significantly reduced when endogenous DOCK4 was silenced by the siRNA (Fig. 2d), suggesting that DOCK4 is important for PDGF-induced Rac activation.

To further study the role of Grb2 and CrkII in cell migration, we transiently transfected dominant-negative constructs of Grb2 and CrkII into NIH 3T3 cells, and we noted that PDGF-dependent migration was significantly suppressed by the expression of the dominant-negative Grb2 (P49L/G203R) but not the dominant-negative CrkII (K170) (Fig. 2e, left). We also confirmed that silencing Grb2 but not CrkII by siRNAs

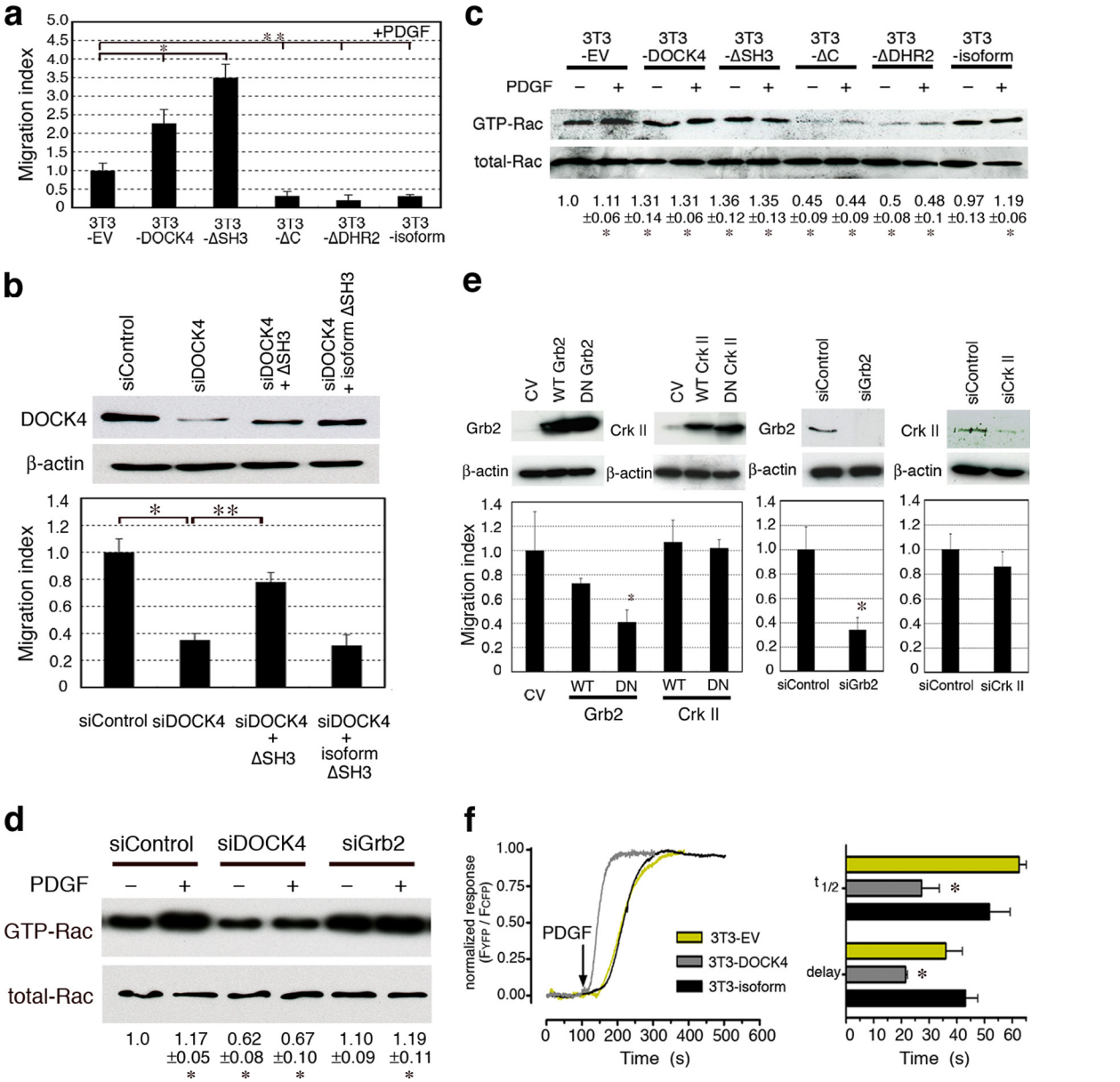

FIG. 2. DOCK4 Rac GEF function and DOCK4-Grb2 binding are required for PDGF-dependent migration. (a) Chemotactic responses to 30 ng/ml PDGF (top) or 20 μg/ml soluble fibronectin (bottom) of NIH 3T3 cells stably expressing DOCK4 constructs are shown. Means are shown, and bars represent standard deviations. (∗ and ∗∗, $P < 0.05$ by Student's t test). (b) Transient transfection of siRNA for DOCK4 along with control, nontargeting oligonucleotides. Western blot photographs (top) and chemotactic responses to 30 ng/ml PDGF (bottom) are shown. Means are shown, and bars represent standard deviations. (∗ and ∗∗, $P < 0.05$ by Student's t test). (c) Activation of Rac1 after PDGF stimulation (30 ng/ml) for 5 min. Active GTP-bound Rac was isolated from cellular lysates by incubation with the GST-linked CRIB domain of Pak, followed by Western blotting with anti-Rac Ab. The relative amounts of Rac GTPase from three independent experiments are shown by quantitative analysis normalized to total Rac expression (∗, $P < 0.05$ compared to values for the nonstimulated 3T3-EV control; Student's t test). (d) Transient transfection of siRNAs for DOCK4 and Grb2 along with siControl, nontargeting oligonucleotides. Rac activation after PDGF stimulation (30 ng/ml) for 5 min was determined. The relative amounts of Rac GTPase levels are shown by quantitative analysis normalized to total Rac expression (∗, $P < 0.05$ compared to values for the nonstimulated control; Student's t test). (e) On the left is shown the transient transfection of control vector (CV), wild-type (WT) Grb2, dominant-negative (DN) Grb2 (P49L/G203R), wild-type CrkII, and dominant-negative CrkII (K170). (e) On the right is shown the transient transfection of siRNAs for Grb2 and CrkII along with siControl, nontargeting oligonucleotides. Western blot photographs (top) and chemotactic responses to 30 ng/ml PDGF (bottom) are shown. Mean results are shown, and the bars show standard deviations (∗, $P < 0.05$ by Student's t test). (f) FRET analysis of Rac activation in 3T3 cells. Cells expressing the Raichu-Rac sensor alone (3T3-EV) and with the wild-type DOCK4 (3T3-DOCK4) or its isoform (3T3-isoform) were used to assess the kinetics of Rac activation in response to PDGF. In the left panel, the traces show the FRET ratio (F_{YFP}/F_{CFP}; normalized and corrected for bleedthrough and cross talk, where YFP is yellow fluorescent protein and CFP is cyan fluorescent protein) obtained in cells superfused with buffer alone or with buffer containing 10 nM PDGF (black arrow). Data are from a single experiment; identical results were obtained in at least three others. Bars in the right panel represent the half-time of Rac activation and delay times in response to PDGF. (∗, $P < 0.05$ compared to results for the 3T3-EV control; Student's t test).

effectively reduced the PDGF-dependent migratory response of NIH 3T3 cells (Fig. 2e, right). We further observed that GTP-Rac levels were unaltered by silencing Grb2 (Fig. 2d). Taken together, these observations suggest that PDGFRβ coupling to DOCK4 via Grb2 along with GTP-Rac plays a role in PDGF-dependent migration.

We next investigated whether DOCK4 regulates the activation of Rac at the cell membrane. We used a FRET-based approach using the sensor Raichu-Rac1, which was previously described (13), to record in live cells and in real time the activation switch of Rac1 at the membrane (see Fig. S4e in the supplemental material). We found that cells expressing the isoform differentiated themselves from cells expressing DOCK by a significantly delayed time and half-time of Rac1 activation (Fig. 2f). These observations suggest that DOCK4 has the capacity to regulate Rac1 at the cell membrane. Taken together, our data indicate that the interaction of DOCK4 and PDGFR is a critical event mediating Rac1 activation.

DOCK4 regulates ligand-induced PDGFRβ endocytosis. After stimulation, RTKs are internalized and then either recycled to the cell surface or degraded in the lysosome (18). Receptor endocytosis requires the adaptor protein Grb2 and is thought to contribute to the downregulation of growth factor signaling (8). We observed that total and cell surface PDGFRβ levels were unaltered by DOCK4 expression (see Fig. S5a and b in the supplemental material). Furthermore, we observed that the ligand-dependent degradation of PDGFRβ also was unaltered by DOCK4 expression (see Fig. S5c in the supplemental material). In all cell lines, the total PDGFRβ level was not changed for up to 60 min after stimulation, and then at 120 min it decreased by ~25% of the basal level, suggesting that Grb2-DOCK4 interaction does not regulate PDGFRβ degradation. In addition, we examined the rate of turnover of PDGFRβ using pulse-chase analysis, and we found that DOCK4 expression did not alter the receptor turnover (see Fig. S5d in the supplemental material).

It has been observed recently that in *Drosophila melanogaster* oogenesis, border cell migration triggered by RTKs, epidermal growth factor receptor, and PVR (analogous to PDGF/vascular endothelial growth factor) requires receptor endocytosis signals (14). Therefore, to investigate whether receptor endocytosis is required for PDGF-dependent migration, we transiently transfected dominant-negative dynamin (K44A) into NIH 3T3 cells and observed a significant suppression of PDGF-dependent migration (Fig. 3a). Similarly, treatment with the inhibitor of endocytosis, PAO, led to a dramatic suppression of PDGF-dependent migration even at a 1 μM dose (Fig. 3a), although GTP-Rac levels were unaltered by Dynamin K44A or PAO (see Fig. S4f in the supplemental material). We confirmed that both PAO and Dynamin K44A blocked ligand-induced PDGFRβ endocytosis (see Fig. S5e in the supplemental material). We then studied whether DOCK4-Grb2 binding could regulate the ligand-induced internalization of PDGFRβ using flow cytometry (Fig. 3b). The decrease of surface PDGFRβ was observed in PDGF-treated cells but not in EGF-treated cells (data not shown). In control 3T3-EV cells, the surface expression of PDGFRβ was decreased 10 min after stimulation and returned to about 80 to 90% of the basal level by 30 min. However, in 3T3-DOCK4 cells, the surface expression of PDGFRβ was decreased only 2.5 min after stimulation, returned to about 80 to 90% of the basal level by 10 min, and then decreased again after 30 min. In contrast, the decrease of surface PDGFRβ expression was not observed until 30 min after stimulation in 3T3-isoform cells (Fig. 3b, left). Notably, Rac GEF null 3T3-ΔDHR2 cells showed a pattern similar to that of 3T3-EV cells (data not shown). Cell surface biotinylation also was performed to investigate receptor internalization, which also gave comparable results (Fig. 3c). PDGF stimulation induced an approximately 50% decrease of PDGFRβ surface expression in each cell line as calculated by both assays, suggesting that DOCK4 does not regulate the amount of the receptors internalized. We used a transient siRNA approach and observed that, by silencing DOCK4 or Grb2, surface PDGFRβ expression was not decreased until 30 min after stimulation (Fig. 3b, right). Taken together, these observations indicate that DOCK4-Grb2 binding accelerates the ligand-induced PDGFRβ internalization.

We next quantified the level of endosomal PDGFRβ using trypsin treatment to facilitate the direct analysis of endosomal receptor (Fig. 3d). Consistently with a publication (2) and our flow cytometry and biotinylation results, endosomal PDGFRβ reached its maximum level 10 min after stimulation and then decreased by 30 min in 3T3-EV cells. In contrast, in 3T3-DOCK4 cells, endosomal PDGFRβ was observed 2.5 min after stimulation, decreased by 10 min, and then increased again by 30 min. In 3T3-isoform cells, the accumulation of endosomal PDGFRβ was delayed until 30 min after stimulation. In addition, Rac GEF null 3T3-ΔDHR2 cells showed a pattern similar to that of 3T3-EV cells (data not shown).

We further examined the subcellular distribution of the internalized PDGFRβ by confocal immunofluorescence microscopy (see Fig. S6a in the supplemental material). The diffuse staining of PDGFRβ at the plasma membrane was seen in all cells prior to PDGF stimulation. Consistently with our data, PDGFRβ expression in endosomal vesicles was observed 10 min after stimulation in 3T3-EV cells. In contrast, in 3T3-DOCK4 cells, PDGFRβ was observed in endosomal vesicles only 2.5 min after stimulation, and then, at 10 min, a portion of PDGFRβ was observed at the plasma membrane in addition to endosomal vesicles. In 3T3-isoform cells, endosomal vesicles were not observed until 30 min after stimulation. In addition, siRNA study indicated that, by silencing DOCK4, endosomal vesicles were not observed until 30 min, as was the case in 3T3-isoform cells (see Fig. S6b in the supplemental material).

To get further insight into the role of DOCK4 in the PDGFRβ recycling pathway, we examined the subcellular distribution of PDGFRβ along with three regulatory components of the endocytic/recycling pathway, namely, Rab4, Rab5, and Rab11. GFP-tagged Rab fusion proteins have been shown to localize to subcellular compartments similarly to their respective endogenous counterparts (28). After GFP-tagged Rab4, Rab5, and Rab11 were transfected, images were acquired and the overlap of fluorescence signal was quantified (Fig. 3e; also see methods described in the supplemental material). Prior to PDGF stimulation, PDGFRβ expression rarely was observed in endosomal vesicles in all cells (data not shown). Ten minutes after stimulation, many PDGFRβ-labeled endosomal vesicles were observed in 3T3-EV and 3T3-DOCK4 cells but not 3T3-isoform cells. The majority of PDGFRβ-labeled vesicles (>95%) colocalized with Rab5, a marker of early endosomes,

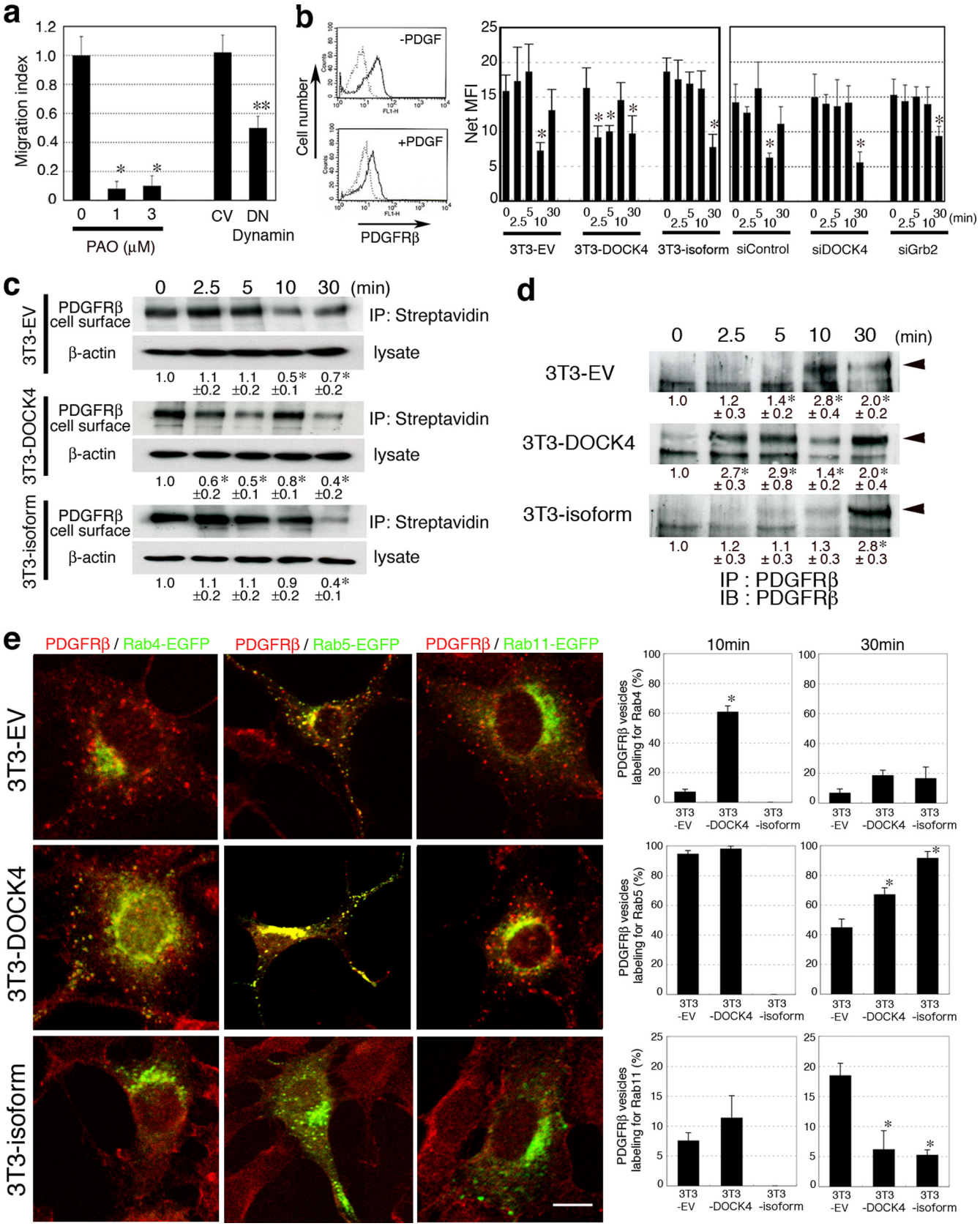

FIG. 3. DOCK4 regulates ligand-induced PDGFRβ endocytosis. (a) Chemotactic responses to PDGF (30 ng/ml) with the endocytosis inhibitor PAO or transient expression of dominant-negative (DN) Dynamin (K44A) are shown. CV, control vector. Mean results are shown, and the bars show standard deviations. (∗ and ∗∗, $P < 0.05$ by Student's t test). (b) Surface expression of PDGFRβ was determined by fluorescence-activated

in both cell types. In 3T3-EV cells, a small portion (~7%) of PDGFRβ-labeled vesicles colocalized with either Rab4 or Rab11, the markers for recycling endosomes. In contrast, in 3T3-DOCK4 cells, about 60% of PDGFRβ-labeled vesicles contained Rab4, a marker for rapid recycling. However, the number of PDGFRβ-labeled vesicles containing Rab11, a marker for slow recycling, is similar (~10%) to that for 3T3-EV cells. These colocalization studies suggest that in 3T3-DOCK4 cells, internalized PDGFRβ is recycled back to the plasma membrane at 10 min, probably via the Rab4-driven recycling pathway. Thirty minutes after stimulation, many PDGFRβ-labeled vesicles were observed in all three cell lines. In 3T3-EV cells, the number of PDGFRβ-labeled vesicles containing Rab11 but not Rab4 was increased to about 20%. In 3T3-DOCK4 cells, the number of PDGFRβ-labeled vesicles containing Rab4 was decreased to <20%. Approximately 40 and 60% of PDGFRβ-labeled vesicles colocalized with Rab5 in 3T3-EV and 3T3-DOCK4 cells, respectively, but in 3T3-isoform cells, more than 90% colocalized with Rab5, supporting the finding that the kinetics of internalization in these cells was delayed. Taken together, our results suggest that Grb2-DOCK4 binding regulates PDGFRβ internalization, and the overexpression of DOCK4 alters PDGFRβ traffic as it is recycled back to the plasma membrane, probably via Rab4-positive endosomes.

DOCK4 cooperates with receptor endocytosis at the leading region of migrating cells following PDGF stimulation. To investigate the relationship between cell migration and endocytosis, we used live cell imaging. We first analyzed the distribution of GFP-tagged wild-type DOCK4 and isoform DOCK4 after PDGF stimulation. These experiments showed that PDGF stimulation induces cell migration and lamellipodia formation in 3T3-GFP-DOCK4 cells, and a fraction of DOCK4 is redistributed toward membrane ruffles of the leading edge (Fig. 4a; also see the movie in Fig. S4a in the supplemental material). In contrast, cell migration or isoform DOCK4 localization to the membrane rarely was observed in 3T3-GFP isoform cells, although they formed membrane ruffles and lamellipodia (Fig. 4b; also see the movie in Fig. S4b in the supplemental material). We next examined the entry of fluorescent PDGF in both migrating and nonmigrating cells. After the addition of Alexa Fluor 594-labeled PDGF, fluorescence was monitored to elucidate the amount and location of PDGF entry with respect to the cell body. These experiments showed that the majority of 3T3-GFP-DOCK4 cells started moving after the addition of Alexa Fluor 594-labeled PDGF, and PDGF entry into the migrating cells was occurring frequently at the ruffling front and extending lamellipodia rather than at the lagging back region, indicating the polarization of receptor endocytosis (Fig. 4c; also see the movie in Fig. S4c in the supplemental material). Consistently with our data presented in Fig. 1 and 3, the entry of Alexa Fluor 594-labeled PDGF rarely occurred in 3T3-GFP isoform cells, in contrast to the neighboring non-GFP control NIH 3T3 cells (Fig. 4d; also see the movie in Fig. S4d in the supplemental material). In addition, we examined the distribution of the internalized PDGF by quantifying the relative fluorescence per unit of area within the three regions of a migrating cell. We divided the cells into leading, middle, and lagging regions and counted 15 cells at 7.5 min after PDGF stimulation (Fig. 4c, right). We confirmed that Alexa Fluor 594-labeled PDGF signal accumulation was highest at the leading region and lowest at the lagging region. These observations are consistent with results obtained from studies of clathrin-mediated endocytosis in migrating cells that show clathrin polarization toward the leading edge (24). These data demonstrated that PDGF induces the subcellular redistribution of DOCK4 in the direction of cell migration, where it facilitates PDGFRβ endocytosis.

DISCUSSION

In this report, we delineate a novel signaling pathway nucleated by adaptor protein Grb2 that specifically regulates PDGF-dependent migration and receptor endocytosis. After PDGF stimulation, the phosphorylated PDGFRβ-bound Grb2 links the receptor to membrane pinchase Dynamin2 via its N-terminal SH3 domain and Rac GTPase regulator, DOCK4, via its C-terminal SH3 domain (Fig. 4e). Dynamin2 previously has been shown to bind cortactin in lamellipodia and potentially to regulate cell migration (21). The recruitment of Rac to cortical actin also requires Dynamin2 expression, suggesting a function association between these proteins (27). Our microscopic studies suggest that the DOCK4-Grb2-Dynamin2 complex is recruited to membrane ruffles at the polarized leading edge of PDGF-stimulated fibroblasts. These subcellular compartments could be platforms for the rapid endocytosis of RTKs that would result in spatially restricted RTK signaling. Our observations reveal the biochemical basis for how membrane-

cell sorting at the indicated times after PDGF stimulation (30 ng/ml) and by the net mean fluorescence intensity (MFI) from at least three independent experiments. Transient transfection of siRNAs for DOCK4, Grb2, and the control nontargeting sequence into NIH 3T3 cells (right). Mean results are shown, and the bars show standard deviations (*, $P < 0.05$ compared to results for the control [0 min] of each cell line; Student's *t* test). (c) Cell surface expression of PDGFRβ was determined at the indicated times after PDGF stimulation (30 ng/ml). Cell surface PDGFRβ proteins were biotinylated and streptavidin affinity precipitated prior to immunoblot (IB) analysis for PDGFRβ and for control β-actin expression in the lysate. The relative amounts of surface PDGFRβ levels from three independent experiments are shown by quantitative analysis. (*, $P < 0.05$ compared to results for the control at 0 min; Student's *t* test). IP, immunoprecipitation. (d) Time course of the PDGFRβ internalization using endosomal protection assays. Arrows show the 180-kDa mature form of PDGFRβ. Total PDGFRβ was evaluated in parallel in nontrypsinized cells, and the relative amounts of mature PDGFRβ from three independent experiments are shown by quantitative analysis (*, $P < 0.05$ compared to results for the control at 0 min; Student's *t* test). (e) Confocal analysis for subcellular localization of PDGFRβ and GFP-Rab4-labeled, GFP-Rab5-labeled, and GFP-Rab11-labeled endosomes. Cells transiently expressing GFP-Rab4-Rab5 and GFP-Rab11 (green) were stimulated for 10 and 30 min with PDGF (30 ng/ml), fixed, and stained with anti-PDGFRβ Ab (red). Images were analyzed for fluorescence signal overlap (see Materials and Methods). Representative merged images at 10 min are shown (left). The percentages of PDGFRβ endosome labeling for GFP-Rab proteins at the indicated time points are shown (right) ($n = 20$ cells) (*, $P < 0.05$ compared to results for the 3T3-EV control; Student's *t* test). Bar, 10 μm.

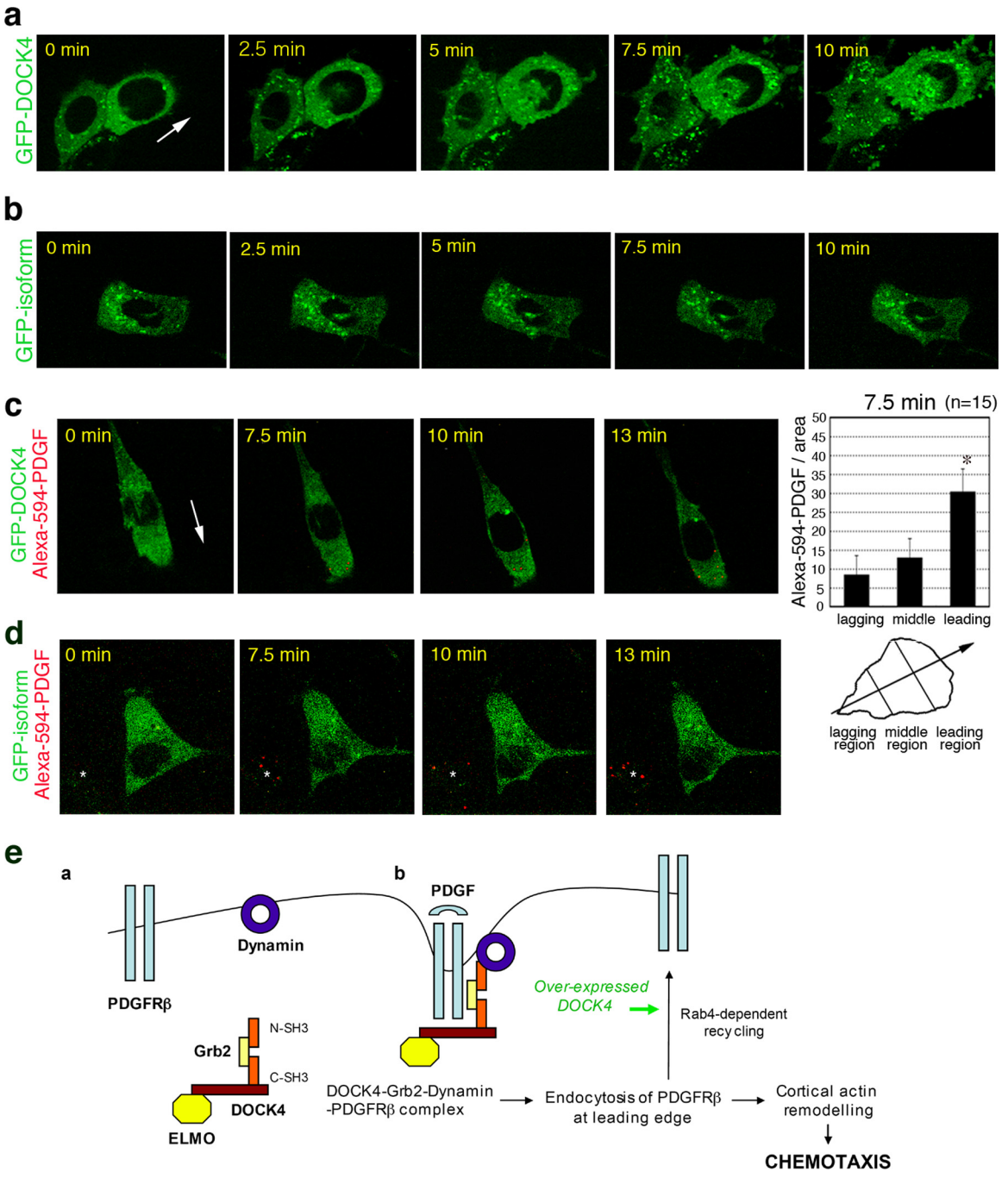

FIG. 4. Live imaging of cell migration and PDGF internalization. (a and b) Confocal images of living NIH 3T3 cells expressing GFP-DOCK4 (a) and GFP-isoform (b) visualized after 30 ng/ml PDGF stimulation. (c and d) Dual-color confocal images of living NIH 3T3 cells expressing GFP-DOCK4 (c) and the GFP isoform (d) visualized after 30 ng/ml Alexa Fluor 594-labeled PDGF (red). The quantification of the relative Alexa Fluor 594-PDGF fluorescence intensity per unit of area within three regions along the migrating axis of the cells is shown on the right ($n = 15$ cells). Mean results are shown, and the bars show standard deviations (*, $P < 0.05$ by Student's t test). Corresponding supplemental movies are available for panels a to d (see the movies in Fig. S4a to d in the supplemental material). Arrows show the migrating axis of the cells. Asterisks show the non-GFP control NIH 3T3 cells. Time is indicated in minutes in the upper left corner. (e) A model for PDGF-dependent chemotaxis. Part a of the model shows that in resting conditions, PDGFRβ, Dynamin2, and DOCK4 are not in complex, but ELMO-bound DOCK4 interacts with Grb2 via its C-terminal SH3 domain and is present in the cytoplasm. Part b shows that after PDGF stimulation, the receptor is tyrosine phosphorylated and binds to the SH2 domain of Grb2, bringing ELMO-DOCK4 into the complex. The N-terminal SH3 domain of Grb2 binds to Dynamin2 together with DOCK4, which results in the endocytosis of PDGFRβ at the leading edge. In addition, DOCK4 overexpression activates the Rab4-regulated rapid recycling pathway of PDGFRβ toward the cell membrane.

triggered chemotactic cues directly couple to the small GTPase Rac.

Our studies elucidate two properties of DOCK4 in cell migration. First, the Rac-GEF function mediated by the DHR2 domain is necessary, since the expression of DOCK4 ΔDHR2 leads to a drastic shutdown of cell migration. DOCK4 silencing significantly reduces GTP-Rac levels in fibroblasts without altering basal and fibronectin-guided cell migration. Second, C-terminal amino acid residues with sites for adaptor proteins CrkII and Grb2 are required for chemotaxis. Unlike Grb2, CrkII has an established role in cell migration (9), but observations of isoform DOCK4, together with silencing studies, support the idea that Grb2, but not CrkII, plays a crucial role in PDGF-dependent chemotaxis. Our data agree with genetic evidence from *C. elegans* that the Grb2 ortholog *Sem-5* is required for sex myoblast migration (4). Therefore, we reason that both Grb2 and CrkII have key roles in cell migration, and as for PDGF-dependent migration in fibroblasts, Grb2 and DOCK4 are indispensable regulators.

An emerging theme in receptor endocytosis research is that the internalized receptor enters distinct subcellular compartments where it is actively signaling and is not necessarily down-regulated (22). Our results revealed that the DOCK4-Grb2-Dynamin2 protein complex regulates the internalization of PDGFRβ and maintains it in an endosomal but active phosphorylation state. Recent *Drosophila* studies of border cell migration show that the absence of endocytosis regulators Cbl and small GTPase Rab5 GEF Sprint lead to migration defects via delocalized guidance signaling (14). We observe that the blocking of receptor endocytosis with either chemical inhibitor or by the expression of Dynamin K44A results in the failure of PDGF-dependent migration. We also show that DOCK4 expression positively regulates chemotaxis and receptor internalization in a Grb2-dependent manner. Grb2 regulates the internalization of RTKs through clathrin-dependent and clathrin-independent pathways (15, 36). However, the current study does not distinguish which pathway is regulated by the DOCK4-Grb2 complex.

Receptor traffic regulated by the Rab GTPases has been shown to be essential for cell migration (16). Internalized receptors are recycled to the plasma membrane via one of at least two distinct recycling pathways: the Rab4-driven rapid recycling pathway and Rab11-driven slow recycling pathway. Unlike the epidermal growth factor receptor, the pathways that control endogenous PDGFRβ trafficking, including receptor internalization, recycling, and degradation, have not been studied extensively. The overexpression of PDGFRβ in PAE or HepG2 cells does not appear to show receptor recycling to the membrane after PDGF stimulation (31). However, these studies may not reflect the physiological behavior of endogenously expressed PDGFRβ. In this study, we have used NIH 3T3 cells, which proliferate and migrate in response to PDGF and have high levels of endogenous PDGFRβ expression. We observe that PDGFRβ is internalized efficiently and recycled back to the surface without significant receptor degradation. These observations of internalization and recycling are similar to the endogenous PDGFRβ trafficking in mouse embryonic fibroblasts (MEFs), but the kinetics of ligand-induced receptor degradation in NIH 3T3 cells is slower compared to that of MEFs, as previously described (2, 17). We reason that the kinetics of PDGFRβ endocytosis depend on the cell type and the experimental design. In this study, the overexpression of DOCK4 induced the rapid recycling pathway of PDGFRβ via the Rab4-driven pathway, while in control 3T3-EV cells, receptor recycling via the Rab11 pathway was observed at 30 min. After PDGF stimulation, we observe DOCK4 expression at the leading edge, where it colocalizes with PDGFRβ. However, colocalization is not sustained after the receptor is internalized and after trafficking in endosomal vesicles, and therefore we reason that DOCK4, via its interaction with Grb2 and Dynamin, regulates PDGFRβ at the leading edge. How DOCK4 expression enhances receptor traffic via the Rab4-driven recycling pathway has not been elucidated in this investigation. The PDGF stimulation of NIH 3T3 cells has been shown to activate the Rab4 pathway for the transport of the integrin receptor, which is essential for cell spreading and attachment (1). The expression of dominant-negative Rab4 or Rab11 in either 3T3-EV or 3T3-DOCK4 cells has no influence on PDGF-dependent migration (see Fig. S8 in the supplemental material). Thus, elucidating how DOCK4 influences the two recycling pathways during cell migration represents the next challenge.

DOCK4 was isolated as a homozygous deletion in the NF2 and p53 null mouse osteosarcoma cell line 3081 (35). The expression of DOCK4 in the 3081 tumor cell line decreased cell growth, suggesting a potential tumor suppressor role for DOCK4. However, to date no genetic evidence of inactivating mutations in DOCK4 have been observed in mammalian tumors. The current study shows that in early-passage NIH 3T3 fibroblasts, DOCK4 specifically regulates cell migration without altering cell proliferation. This important distinction in DOCK4 function may be a reflection of the tumor cell genotype. While no direct biochemical interactions have been described between NF2 and DOCK4, it has been shown that ERM proteins bind ELMO, regulating the function of DOCK proteins (11). Given the reciprocal relationship between ERM proteins and NF2, the loss of DOCK4 may enhance the tumor phenotype induced by NF2 deficiency. In addition, NF2 has been shown to regulate cell contact-dependent cell proliferation by blocking EGF receptor endocytosis (7). Our current study shows that DOCK4 is a positive regulator of PDGF receptor endocytosis, thus it is possible that the loss of DOCK4 in NF2-deficient cells enhances the tumor phenotype.

The novel signaling pathway described here gives novel insights into fibroblast cell migration with direct relevance to wound healing. Recently, we defined the role of DOCK4 and Rac1 in Wnt–β-catenin signaling and in Wnt ligand-mediated chemotaxis, suggesting that its expression enhances tumor progression (30). Thus, the association between Grb2, Dynamin2, DOCK4, and growth factor receptors represents a potential molecular target for both wound healing and invasive cancer therapy.

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We declare no competing financial interests.

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