

Rab-coupling protein coordinates recycling of $\alpha 5 \beta 1$ integrin and EGFR1 to promote cell migration in 3D microenvironments

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Here we show that blocking the adhesive function of $\alpha v \beta 3$ integrin with soluble RGD ligands, such as osteopontin or cilengitide, promoted association of Rab-coupling protein (RCP) with $\alpha 5 \beta 1$ integrin and drove RCP-dependent recycling of $\alpha 5 \beta 1$ to the plasma membrane and its mobilization to dynamic ruffling protrusions at the cell front. These RCP-driven changes in $\alpha 5 \beta 1$ trafficking led to acquisition of rapid/random movement on two-dimensional substrates and to a marked increase in fibronectin-dependent migration of tumor cells into three-dimensional matrices. Recycling of $\alpha 5 \beta 1$ integrin did not

affect its regulation or ability to form adhesive bonds with substrate fibronectin. Instead, $\alpha 5 \beta 1$ controlled the association of EGFR1 with RCP to promote the coordinate recycling of these two receptors. This modified signaling downstream of EGFR1 to increase its autophosphorylation and activation of the proinvasive kinase PKB/Akt. We conclude that RCP provides a scaffold that promotes the physical association and coordinate trafficking of $\alpha 5 \beta 1$ and EGFR1 and that this drives migration of tumor cells into three-dimensional matrices.

Introduction

Cell migration is essential to processes such as embryonic development and wound healing but also drives the pathology of diseases such as cancer. Indeed, one of the features of malignant cells, and one that makes cancer so difficult to treat, is their capacity to migrate invasively through the stroma to form metastases (Sahai, 2005). Cell migration can be random or persistent. In 2D, on stiff substrates, cells tend to adopt a polar form with a leading lamellipodium, whereas in 3D they can assume elongated or amoeboid morphologies (Sahai, 2005). A cell's ability to switch between these migrational modes is likely dictated by the way in which it interacts with and responds to the surrounding ECM. Integrins are heterodimeric transmembrane receptors that physically link the ECM to the intracellular actin cytoskeleton but are also signaling molecules that transduce signals bidirectionally across the plasma membrane (Hynes, 2002). The signaling capacity of certain integrin heterodimers

has been linked with particular modes of cell migration. For example, a study on epithelial cells has concluded that activation of Rac downstream of $\alpha v \beta 3$ integrin promotes slow/persistent migration, whereas engagement of $\alpha 5 \beta 1$ that acts via RhoA-ROCK signaling drives rapid/random cell movement (Danen et al., 2005).

Osteopontin is a secreted matrix molecule that becomes incorporated into mineralized bone matrix and a ligand for several cell surface receptors including CD44 and $\alpha v \beta 3$ integrin (Rangaswami et al., 2006). Increased secretion of osteopontin is associated with a range of malignancies and correlates strongly with tumor progression and metastasis (Rittling and Chambers, 2004; Khodavirdi et al., 2006). Some tumors secrete large quantities of soluble osteopontin, which does not become incorporated into immobile ECM and is, therefore, unlikely to mediate adhesive interactions between cells and the matrix (Rittling et al., 2002). Nevertheless, osteopontin promotes chemotactic cell migration of several cancer cell lines (Tuck et al., 1999).

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Abbreviations used in this paper: FIP, family of interacting protein; FN, fibronectin; RBD, Rab11-binding domain; RCP, Rab-coupling protein; Rip11, Rab11-interacting protein; RTK, receptor tyrosine kinase; shRNA, short hairpin RNA; Tfn-R, transferrin receptor.

The online version of this article contains supplemental material.

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Although it has been shown that osteopontin-driven migration may involve modulation of EGF receptor and c-Src signaling (Tuck et al., 2003; Das et al., 2004), the relationship between the binding of osteopontin to αv integrins and the acquisition of tumor cell invasive capacity is not known.

Surface integrins are continuously endocytosed, and then efficiently returned (or recycled) back to the plasma membrane (Caswell and Norman, 2006; Jones et al., 2006; Pellinen and Ivaska, 2006). The way in which integrins are recycled dictates the migrational mode of fibroblasts, and this is largely owing to the influence of recycling pathways on $\alpha 5 \beta 1$ (White et al., 2007). Small GTPases of the Rab11 family, including Rab11a and Rab25, control recycling of internalized $\alpha 5 \beta 1$ (Roberts et al., 2001; Caswell et al., 2007), and this is likely to require the contribution of at least one Rab11 effector protein. The Rab11 family of interacting proteins (Rab11-FIPs) bind to GTP-bound Rab11s and have been shown to be effectors of Rab11 (Hales et al., 2001; Lindsay et al., 2002; Lindsay and McCaffrey, 2002; Wallace et al., 2002; Prekeris, 2003; Peden et al., 2004; Horgan et al., 2007), thus making them potential candidates for components of protein complexes that control $\alpha 5 \beta 1$ recycling. The Rab11-FIPs share a highly homologous C-terminal Rab11-binding domain (RBD) and are categorized into two subfamilies according to their domain structures (Wallace et al., 2002; Prekeris, 2003). The three identified class I FIPs (Rab-coupling protein [RCP; aka Rab11-FIP1], Rab11-FIP2, and Rab11-interacting protein [Rip11; aka pp75 and Rab11-FIP5]) possess an N-terminal phosphatidic acid/phosphatidylinositol 3,4,5-trisphosphate-binding C2 domain (Lindsay and McCaffrey, 2004a), whereas the two class II FIPs (Rab11-FIP3 and Rab11-FIP4) can bind to Arf6 as well as Rab11s and are thus considered to be effectors for both these classes of GTPase (Hickson et al., 2003; Horgan et al., 2004). It is now becoming clear that Rab11-regulated processes can be linked to cancer (Goldenring et al., 1999). Indeed, Rab25 contributes to the aggressiveness of breast and ovarian cancers (Cheng et al., 2004) and likely achieves this in part by altering the trafficking of $\alpha 5 \beta 1$ to promote invasive migration (Caswell et al., 2007). Furthermore, Rab11 controls recycling of $\alpha 6 \beta 4$ integrin in breast cancer cells in a way that may contribute to hypoxia-induced invasive migration (Yoon et al., 2005). These findings, in combination with the fact that the gene for RCP is located within a small amplicon (8p11-12) prevalent in breast cancer (Gelsi-Boyer et al., 2005), led us to investigate a potential role for class I Rab11-FIPs in coordinating the trafficking of integrins and other proinvasive receptors as tumor cells migrate in 3D microenvironments in response to tumor-relevant chemokinetic factors such as osteopontin and EGF.

Results

Inhibition of $\alpha v \beta 3$ promotes $\alpha 5 \beta 1$ recycling in A2780 cells

We have previously shown that perturbation of “short-loop” $\alpha v \beta 3$ recycling leads to a marked increase in $\alpha 5 \beta 1$ recycling (White et al., 2007), so we sought to determine whether such reciprocal cross talk between these two integrins also occurs in cancer cells. The basal recycling rate of $\alpha 5 \beta 1$ integrin A2780

ovarian cancer cells was similar to fibroblasts, with 25–30% of internalized integrin returned to the plasma membrane within 15 min (Fig. 1 A, left). However, in cells challenged with either cilengitide (a cyclic RGD peptide known to bind to and inhibit $\alpha v \beta 3$ and $\alpha v \beta 5$), cRGDFV (a cyclic peptide with selectivity for $\alpha v \beta 3$ over $\alpha v \beta 5$ integrin; Dechantreiter et al., 1999), or the soluble $\alpha v \beta 3$ ligand osteopontin, $\alpha 5 \beta 1$ recycling increased by at least twofold, whereas a control cyclic peptide (cRADfV) was ineffective in this regard (Fig. 1 A, left). Consistent with this, suppression of $\beta 3$ levels by RNAi (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>) increased $\alpha 5 \beta 1$ recycling, and treatment of $\beta 3$ knockdown cells with either cilengitide or osteopontin did not further enhance $\alpha 5 \beta 1$ trafficking (Fig. 1 A, right). Cilengitide did not affect the rate at which $\alpha 5 \beta 1$ was internalized by A2780 cells (Fig. S1 B). As cilengitide-driven $\alpha 5 \beta 1$ recycling was Rab11 dependent (Fig. S2 A), these data indicate that $\alpha v \beta 3$ functions constitutively to restrain $\alpha 5 \beta 1$ recycling in A2780 ovarian cancer cells and that removal of $\alpha v \beta 3$ or its occupation/blockade with soluble, non-ECM ligands such as osteopontin or cilengitide releases this constraint to drive Rab11-dependent trafficking of $\alpha 5 \beta 1$ to the plasma membrane.

Inhibition of $\alpha v \beta 3$ drives $\alpha 5 \beta 1$ and RCP into a coprecipitable complex

Because physical associations may be formed between Rab effectors and their cargoes (Fan et al., 2004), we immunoprecipitated members of the class I family of Rab11 effectors from A2780 cells and probed for the presence of $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins. This revealed that RCP associated with both $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins (Fig. 1 B) and pretreatment of the cells with cilengitide (Fig. 1, B and D) or osteopontin (Fig. 1 D) strongly promoted coprecipitation of RCP with $\alpha 5 \beta 1$ but reduced its association with $\alpha v \beta 3$ (Fig. 1 B). Increased association of $\alpha 5 \beta 1$ and RCP was detectable within 8 min and was maximal after 20-min exposure to cilengitide (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>). To further confirm these associations, we immunoprecipitated $\alpha 5 \beta 1$ and $\alpha v \beta 3$ using antibodies recognizing $\alpha 5$ and $\beta 3$ integrin, respectively. Western blots of these immunoprecipitates indicated that both endogenous and overexpressed RCP coprecipitated with $\alpha 5 \beta 1$ and $\alpha v \beta 3$ (but not with a control antibody) and that addition of cilengitide strongly promoted RCP's association with $\alpha 5 \beta 1$, but correspondingly weakened binding to $\alpha v \beta 3$ (Fig. 1 C). Suppression of $\beta 3$ expression by RNAi also increased association between RCP and $\alpha 5 \beta 1$, and treatment of $\beta 3$ knockdown cells with cilengitide or osteopontin did not further enhance this (Fig. 1 D). Furthermore, RCP^{G21E}, containing a point mutation within the RBD of RCP rendering it unable to interact with Rab11 (and therefore to be considered a dominant-negative inhibitor of RCP function), did not associate with $\alpha 5 \beta 1$ (Fig. 1 E), indicating that Rab11 likely contributes to $\alpha 5 \beta 1$ –RCP complex formation.

RCP regulates integrin recycling and $\alpha 5 \beta 1$ -dependent cell movement

Although cilengitide promoted $\alpha 5 \beta 1$ recycling in cells expressing control RNAi or those overexpressing RCP^{wt}, it was completely

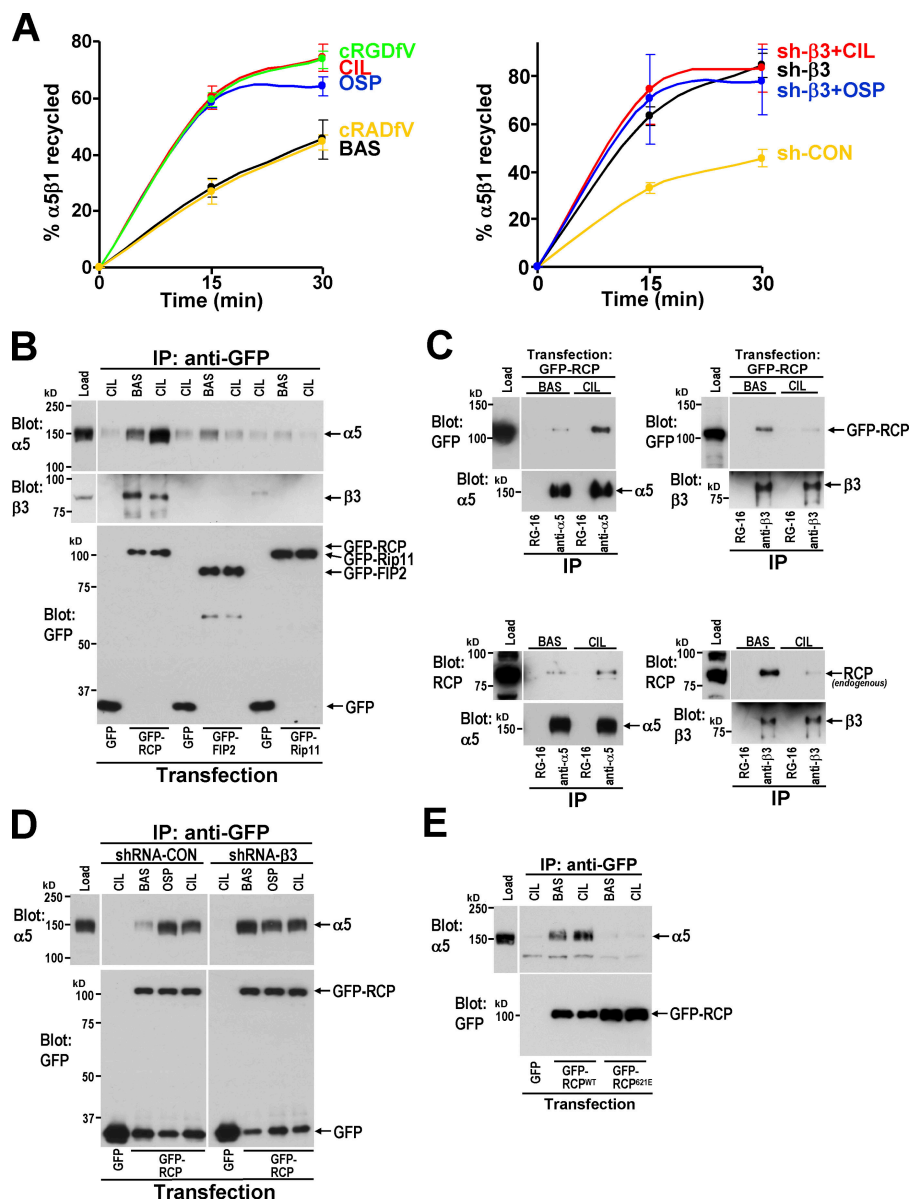


Figure 1. Inhibition of $\alpha v\beta 3$ promotes $\alpha 5\beta 1$ recycling and association of RCP with $\alpha 5\beta 1$. (A) A2780 cells were transfected with a non-targeting shRNA (sh-CON) or a short hairpin-targeting $\beta 3$ integrin (sh- $\beta 3$; right) or were left untransfected (left). Cells were surface labeled with 0.2 mg/ml NHS-S-S-Biotin at 4°C and internalization was then allowed to proceed for 30 min at 37°C. Biotin remaining at the cell surface was removed by exposure to MesNa at 4°C, and internalized integrin was chased back to the cell surface at 37°C for the indicated times in the absence (BAS) or presence (CIL) of 1 μ M cilengitide, 1 μ M cRADFV, 4.4 μ M cRGDFV, or 0.5 μ g/ml soluble osteopontin. Cells were then reexposed to MesNa and biotinylated integrin was determined by capture ELISA using microtitre wells coated with anti-human $\alpha 5$ integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as a percentage of the pool of integrin labeled during the internalization period. (left) Values are mean \pm SEM; $n = 7$ (BAS), $n = 4$ (CIL), and $n = 3$ (cRADFV, cRGDFV, and OSP) independent experiments. (right) Values are mean \pm SEM; $n = 3$ independent experiments. (B) A2780 cells were transfected with plasmids encoding GFP or GFP fused to the class I FIPs (GFP-RCP, GFP-FIP2, and GFP-Rip11). Transfected cells were incubated in the absence (BAS) or presence (CIL) of 1 μ M cilengitide for 20 min and lysed in a buffer containing 0.15% Tween-20. GFP and GFP fusion proteins were immunoprecipitated from lysates by incubation with beads coupled to a monoclonal antibody recognizing GFP, and these immunoprecipitates analyzed for the presence of $\alpha 5\beta 1$ and $\alpha v\beta 3$ by immunoblotting with antibodies recognizing the integrin $\alpha 5$ and $\beta 3$ chains, respectively. The loading of GFP and GFP fusions was confirmed by immunoblotting with an antibody recognizing GFP (bottom). (C) Cells were transfected with GFP-RCP (top) or left untransfected (bottom), and then incubated in the absence (BAS) or presence (CIL) of 1 μ M cilengitide for 20 min. Lysates were immunoprecipitated using monoclonal antibodies recognizing $\alpha 5\beta 1$ integrin (anti- $\alpha 5$), $\alpha v\beta 3$ integrin (anti- $\beta 3$), or an isotype-matched control antibody (RG-16). The presence of $\alpha 5$ and $\beta 3$ integrin, GFP-RCP, and endogenous

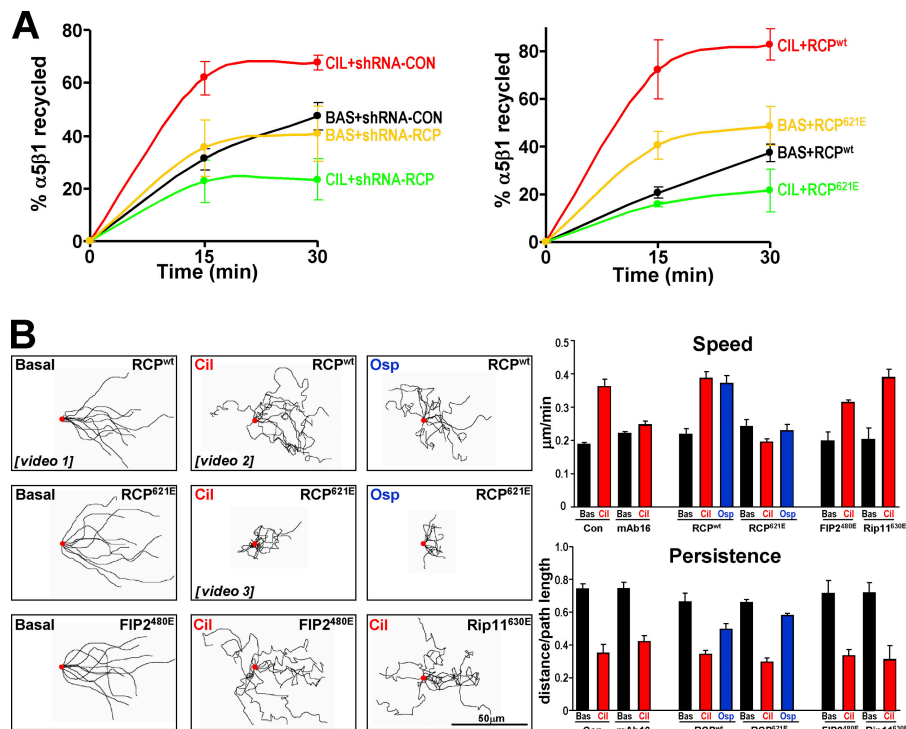
RCP were detected by immunoblotting. (D) After cotransfection of GFP or GFP-RCP with either non-targeting shRNA (shRNA-CON) or a short hairpin targeting $\beta 3$ integrin (shRNA- $\beta 3$), A2780 cells were incubated in the absence (BAS) or presence (CIL) of 1 μ M cilengitide or 0.5 μ g/ml osteopontin (OSP) for 20 min. Cells were lysed and anti-GFP immunoprecipitates probed for the presence of $\alpha 5\beta 1$ and GFP as in B. (E) A2780 cells were transfected with GFP, GFP-RCP^{WT}, or GFP-RCP^{D21E}, treated with cilengitide, and then lysed. Anti-GFP immunoprecipitates were probed for $\alpha 5\beta 1$ and GFP as in B.

unable to drive $\alpha 5\beta 1$ recycling after RCP knockdown or expression of dominant-negative RCP^{D21E} (Fig. 2 A). The corresponding dominant-negative mutants of Rip11 or Rab11-FIP2 (Rip11^{630E} and FIP2^{481E}) had no effect on cilengitide-induced $\alpha 5\beta 1$ recycling (Fig. S2 B), and RCP^{D21E} did not compromise trafficking of the transferrin receptor (Tfn-R) in the presence or absence of cilengitide (Fig. S2 C). Internalization of $\alpha 5\beta 1$ was not affected by expression of dominant-negative RCP (Fig. S1 B). These data indicate that RCP displays selectivity toward the recycling of specific cargoes (i.e., $\alpha 5\beta 1$ not Tfn-R) that follow Rab11-dependent routes to the cell surface and that RCP is likely to be the only class I FIP that has a role in trafficking $\alpha 5\beta 1$.

As increased $\alpha 5\beta 1$ recycling causes fibroblasts to switch from persistent to random migration (White et al., 2007), we

analyzed the movement of A2780 cells as they migrated into scratch wounds. A2780 cells migrated persistently after wounding (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>), but treatment with either cilengitide (Video 2) or osteopontin induced a significantly more rapid but less persistent mode of migration, and $\alpha 5\beta 1$ was required for the increase in speed (Fig. 2 B). In the absence of cilengitide, expression of RCP^{D21E} did not effect cell migration. However, RCP^{D21E} (but not dominant-negative Rab11-FIP2 or Rip11) completely opposed the cilengitide- or osteopontin-driven increase in migration speed (Fig. 2 B), such that in the combined presence of the cyclic peptide and dominant-negative RCP the cells appeared to wobble on the spot and were largely unable to move away from their original positions (Video 3).

Figure 2. Cilengitide and osteopontin drive alterations in $\alpha 5 \beta 1$ recycling and cell migration that are dependent on RCP. (A) A2780 cells were transfected with a nontargeting shRNA (shRNA-CON), a short hairpin targeting RCP (shRNA-RCP), GFP-RCP^{wt}, or dominant-negative GFP-RCP^{621E}. Recycling of $\alpha 5 \beta 1$ integrin was determined in the presence and absence of 1 μ M cilengitide as for Fig. 1 A. Values are mean \pm SEM; $n = 3$ independent experiments. (B) Cells were transfected with GFP-RCP^{wt} or GFP-RCP^{621E} and allowed to grow to confluence. Confluent monolayers were wounded with a plastic pipette tip and the cells were allowed to migrate into the wound in the absence (Basal) or presence of 1 μ M cilengitide (Cil) or 0.5 μ g/ml osteopontin (Osp). The cells were observed by time-lapse video microscopy, the movement of individual cells followed using cell tracking software, and this is presented as overlays of representative trajectories described by cells during the first 9 h of their migration into the wound. The starting position of each cell is denoted by the red dot. Examples of time-lapse movies that correspond to these experiments are included as Videos 1–3 as indicated (available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>). The persistence and speed of migration were extracted from the track plots. Persistence is defined as the ratio of the vectorial distance traveled to the total path length described by the cell. Values are mean \pm SEM; $n = 3$ independent experiments. Bar, 50 μ m.



Fluorescence time-lapse imaging revealed that migrating A2780 cells adopted a fanlike morphology in which the actin-rich lamellipodium advanced in an organized, processive fashion, with retrograde movement of filaments back from the leading edge being clearly visible (Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>). Furthermore, 3D reconstruction of serial z stack confocal images indicated that the F-actin-rich lamellipodial tip of migrating A2780 cells did not rise much above the surface of the substratum (Fig. 3 A, left). However, upon challenge with cilengitide or osteopontin, or when $\alpha v \beta 3$ was suppressed by RNAi, the morphology of migrating A2780 cells was drastically altered, with an apparent collapse of the lamellipodium and the presence of anterior protrusions containing $\alpha 5 \beta 1$ integrin, RCP, and F-actin (Fig. S4). 3D reconstructions indicated that these protrusions were composed of numerous actin-rich ruffles that extended upward from the cell front (Fig. 3 A, middle and right). Fluorescence time-lapse imaging showed these cilengitide- and osteopontin-driven structures to be very actively protrusive and always anterior to the direction of migration (Videos 4–6), indicating that they were neither dorsal ruffles nor retracting lamellae, but likely locomoting structures contributing to the cell's forward motion. Moreover, expression of dominant-negative RCP (but not dominant-negative FIP2 or Rip11) blocked the ability of A2780 cells to extend these anterior ruffling protrusions (Fig. 3 B), indicating a requirement for RCP (but not other class I Rab11-FIPs) in this type of cell migration.

RCP and $\alpha 5 \beta 1$ promote fibronectin (FN)-dependent migration in 3D

The capacity of $\alpha 5 \beta 1$ to support migration in 3D matrices is dictated by the way in which it is trafficked. Indeed, $\alpha 5 \beta 1$ and

FN inhibit invasive migration of Fos-transformed fibroblasts through matrigel (Spence et al., 2006). However, expression of Rab25 changes the way in which $\alpha 5 \beta 1$ is trafficked such that $\alpha 5 \beta 1$ supports migration of Fos-transformed fibroblasts through 3D FN-containing matrices (Caswell et al., 2007). We, therefore, sought to determine whether cilengitide-driven, RCP-dependent changes in $\alpha 5 \beta 1$ trafficking were sufficient to promote migration into 3D matrices. When $\alpha v \beta 3$ was not inhibited, migration of A2780 cells was unaffected by addition of FN to the matrigel (Fig. 4 A). However, addition of cilengitide or osteopontin strongly promoted FN-dependent migration of A2780 cells into matrigel plugs (Fig. 4 A). Experiments using blocking antibodies and RNAi of integrin subunits indicated that this component of migration was dependent on the presence and RGD ligand-binding capacity of $\alpha 5 \beta 1$ integrin (Fig. 4 B). Depletion of $\beta 3$ integrin with RNAi strongly promoted FN-dependent migration into matrigel (in the absence of FN, $\beta 3$ knockdown suppressed migration), and neither cilengitide nor osteopontin were able to further increase this (Fig. 4 A). Although basal migration of A2780 cells did not require RCP, the cilengitide-driven increase in migration through matrigel was suppressed by dominant-negative RCP^{621E} or short hairpin RNA (shRNA) of RCP (Fig. 4 C), but was not affected by the corresponding mutants of Rab11-FIP2 or Rip11 (Fig. S5 A, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>).

To determine how cilengitide promotes cell migration in 3D, while minimizing the technical difficulties in imaging cell migration within dense 3D microenvironments, we analyzed the migration of cells plated onto cell-derived matrix. We have previously found that expression of Rab25 induced A2780 cells to be more invasive and to switch their mode of migration to one that is

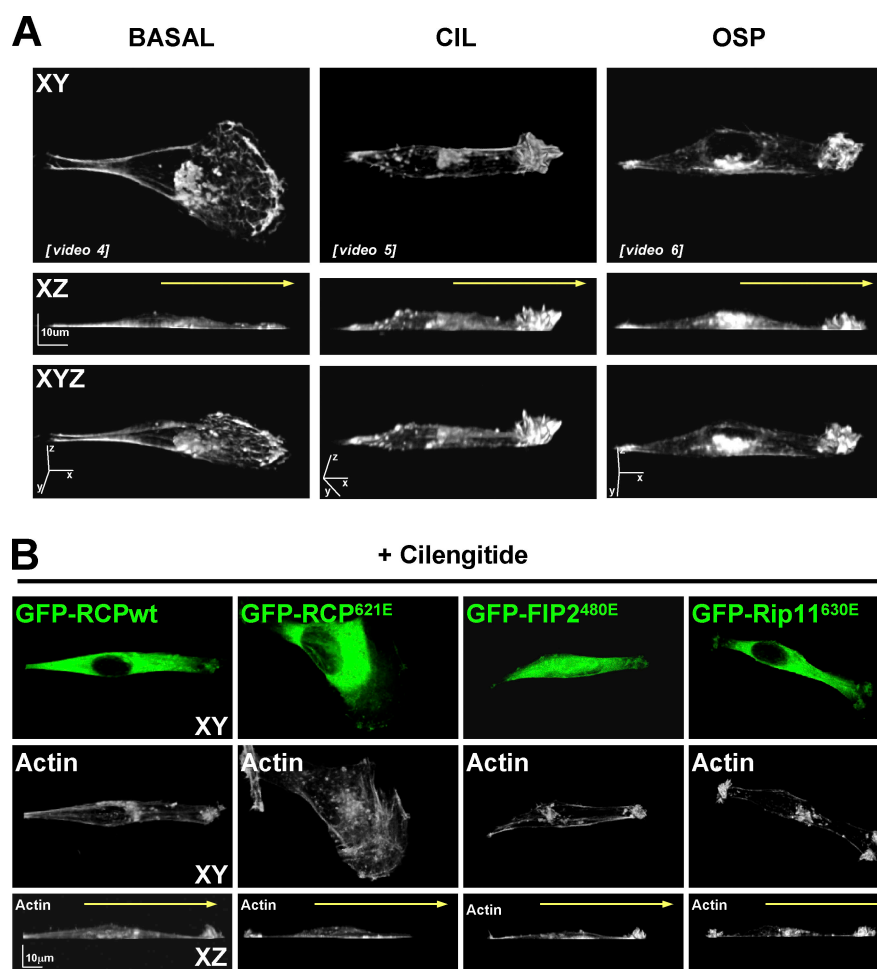


Figure 3. Inhibition of $\alpha v \beta 3$ promotes the formation of dynamic actin-rich protrusions at the cell front. (A) Confluent monolayers were wounded with a plastic pipette tip and the cells were allowed to migrate for 2 h into the wound in the absence (BASAL) or presence of 1 μ M cilengitide (CIL) or 0.5 μ g/ml osteopontin (OSP). Cells were fixed and permeabilized and F-actin was visualized using fluorescently conjugated phalloidin. 3D reconstructions were generated from serial Z sections captured using a confocal microscope. Yellow arrow denotes the direction of cell migration. Bar, 10 μ m. Time-lapse movies indicating the dynamics of GFP-actin under conditions corresponding to the experiments presented in A are included as Videos 4–6, respectively (available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>). (B) A2780 cells were transfected with GFP-RCP^{wt}, GFP-RCP^{621E}, GFP-FIP2^{480E}, or GFP-Rip1^{1630E}. Confluent monolayers were wounded with a plastic pipette tip and the cells were allowed to migrate for 2 h into the wound in the absence or presence of 1 μ M cilengitide. Cells were fixed and permeabilized and GFP and F-actin was visualized by fluorescence microscopy. 3D reconstructions were generated from serial Z sections captured using a confocal microscope. Yellow arrow denotes the direction of cell migration. Bar, 10 μ m.

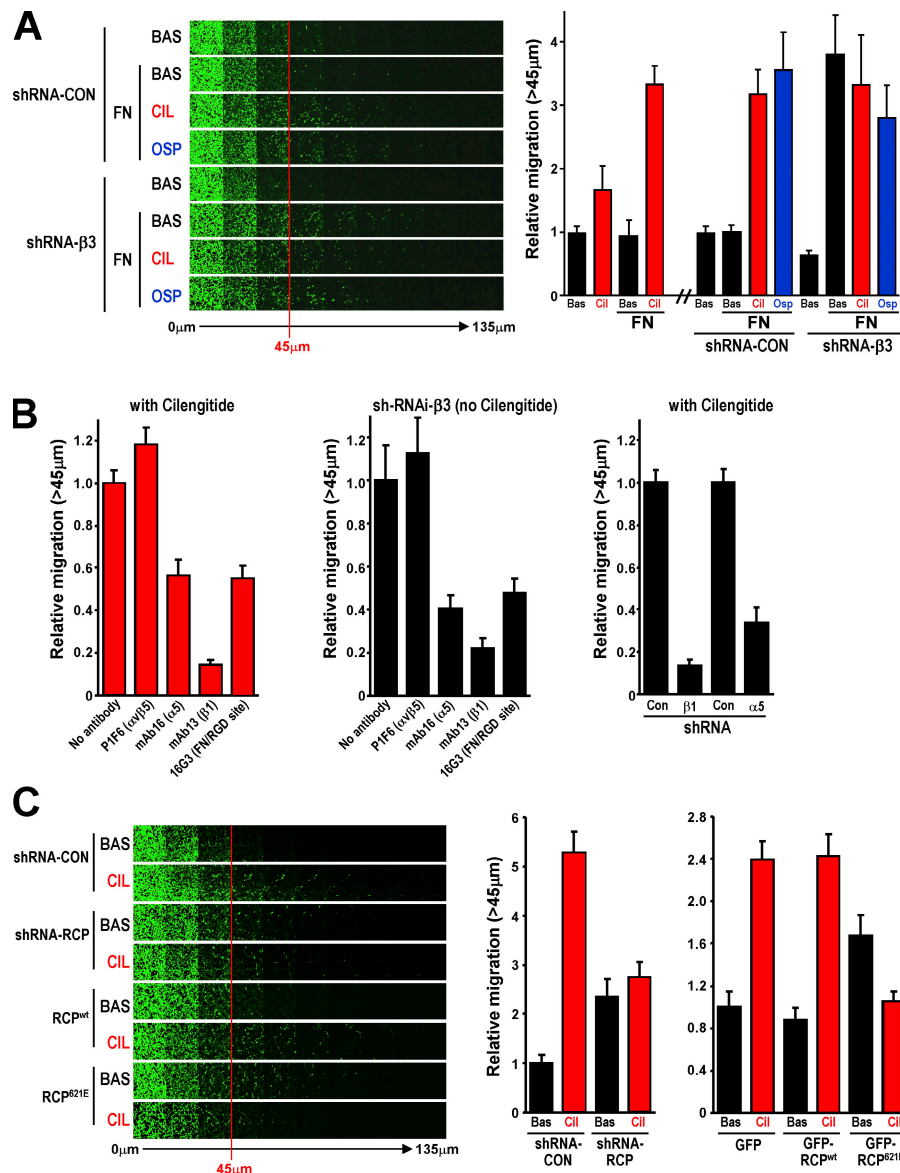
characterized by the extension of long pseudopods (Caswell et al., 2007). As previously observed, A2780 cells migrated rapidly across cell-derived matrices and maintained their overall sluglike morphology as they moved (Fig. 5 A and Video 7, available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>). Addition of cilengitide altered the morphology of cells migrating on 3D matrices to extend long pseudopods in the direction of migration (Fig. 5, A and B; and Video 8). When $\alpha v \beta 3$ was not inhibited, migration on cell-derived matrix had no requirement for RCP (Fig. 5 A), but expression of RCP^{621E} completely abrogated cilengitide-driven pseudopodial extension and markedly decreased both the speed and persistence of cell movement (Fig. 5, A and B; and Video 9). The dynamic localization of RCP within migrating cells was also strikingly altered by treatment with cilengitide. In untreated cells, GFP-RCP localized to the perinuclear region and to vesicles distributed fairly uniformly throughout the cytoplasm (Fig. 5 C). However, after addition of cilengitide, GFP-RCP (but not dominant-negative GFP-RCP^{621E}) was recruited to the tip of the extending pseudopod (Fig. 5 C). Although there was considerable movement of RCP-positive tubulovesicular structures in and out of the pseudopodial region, the RCP-containing structures at the distal pseudopod tip appeared to be relatively immobile and closely associated with the plasma membrane. Collectively, these data indicate that inhibition of $\alpha v \beta 3$ strongly promotes RCP-dependent trafficking of $\alpha 5 \beta 1$ integrin, which

alters cellular morphology to favor rapid/random movement on plastic surfaces and a pseudopodial-driven mode of migration on a 3D matrix.

RCP-regulated recycling does not affect the ability of $\alpha 5 \beta 1$ to bind to FN

Regulation of integrin internalization and recycling may influence cell adhesion and migration by controlling the level of heterodimer exposed at the plasma membrane. Addition of cilengitide and expression of mutant RCPs influence the amount of $\alpha 5 \beta 1$ expressed at the cell surface (Fig. S5 B), but do so only modestly and to a degree that is consistent with the way that they alter recycling from a relatively small (~ 15 – 20% of the quantity of surface receptor [not depicted]) intracellular pool of $\alpha 5 \beta 1$ integrin. We had, therefore, previously hypothesized that endosomal pathways may not function primarily to control the quantity of surface receptors, but that they regulate integrin function in other ways, for example, by “cleaning” internalized integrins of bound ligand, thus maintaining ligand-binding competency (Caswell and Norman, 2006; White et al., 2007). To analyze the effect of recycling on integrin function we used a spinning disc approach, which measures the mean shear stress (τ_{50}) required for cell detachment (Garcia et al., 1998). Because τ_{50} is proportional to the number of $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins per cell that are occupied in adhesive bonds, this provides a direct measure of integrin adhesive

Figure 4. Inhibition of $\alpha v \beta 3$ promotes migration into matrigel that is dependent on RCP and engagement of $\alpha 5 \beta 1$ with FN. (A) Migration of A2780 cells expressing a nontargeting shRNA (shRNA-Con) or a short hairpin targeting $\beta 3$ integrin (shRNA- $\beta 3$) into a matrigel plug in the presence and absence of 25 μ g/ml FN was determined using an inverted matrigel plug assay. 1 μ M cilengitide (CIL) and 0.5 μ g/ml osteopontin (OSP) were added as indicated to the matrigel and to both the upper and lower chambers of the inverted matrigel plug assay. Invading cells were stained with Calcein AM and visualized by confocal microscopy. Serial optical sections were captured at 15- μ m intervals and are presented as a sequence in which the individual optical sections are placed alongside one another with increasing depth from left to right as indicated. Migration was quantitated by measuring the fluorescence intensity of cells penetrating the matrigel to depths of 45 μ m and greater and expressing this as a percentage of the total fluorescence intensity of all cells within the plug. Data represents mean \pm SEM from three independent experiments. (B) The involvement of $\alpha 5 \beta 1$ integrin in migration driven by addition of cilengitide (left and right) or induced by suppression of $\alpha v \beta 3$ levels (middle) was determined by addition of the indicated integrin- and FN-blocking antibodies (left and middle) and also by RNAi of the $\alpha 5$ and $\beta 1$ subunits of $\alpha 5 \beta 1$ (right). Migration was quantified as for A. Values are mean \pm SEM from three independent experiments. (C) The migration of A2780 cells expressing nontargeting shRNA (shRNA-Con), a short hairpin targeting RCP integrin (shRNA-RCP), GFP, GFP-RCP^{wt}, or GFP-RCP^{621E} into FN-containing matrigel was determined and quantified as for A.



function (Boettiger et al., 2001; Shi and Boettiger, 2003; Boettiger, 2007). The generation of τ_{50} from curve fits of the spinning data (Fig. 6 A) was used to generate a comparison of integrin bond levels (Fig. 6 B). Addition of cilengitide reduced the total adhesion, as measured by the spinning disc, by $\sim 14\%$ consistent with the contribution that is typically made to adhesion by $\alpha v \beta 3$ in cells expressing both $\alpha v \beta 3$ and $\alpha 5 \beta 1$. As shown in Fig. 3 A, inhibition of RCP reduces cilengitide-driven $\alpha 5 \beta 1$ recycling by at least threefold. Therefore, if recycling were to influence $\alpha 5 \beta 1$'s ligand-binding capacity, one would anticipate a large alteration in adhesion strength after inhibition of RCP. However, expression of RCP^{621E} effected only a small change (10–15% reduction) in adhesion strength (Fig. 6 B), indicating that the quantity and strength of integrin–ligand adhesive bonds is proportional to the level of surface expression (Fig. S5 B) and not to the proportion of integrin recycled. Because RCP does not influence the ligand-binding capacity of $\alpha 5 \beta 1$, it is likely that there are alternative explanations for the ability of this type of recycling to promote cell migration and invasiveness.

RCP associates with EGFR1 and $\alpha 5 \beta 1$ to coordinate their recycling

As the endosomal pathway is known to have profound influence on the way that many receptors communicate with their downstream effectors (von Zastrow and Sorkin, 2007), it is possible that integrin recycling alters cell migration by influencing the assembly of signalosomes that mediate receptor tyrosine kinase (RTK) signaling. To investigate this possibility we probed RCP immunoprecipitates with antibodies recognizing phosphotyrosine. Although cytoskeletal phosphotyrosine-containing proteins such as paxillin were not present in RCP immunoprecipitates (Fig. S3 B), we consistently observed a high molecular mass phosphotyrosine-rich band with the approximate molecular mass of EGFR1 (not depicted). Indeed, addition of cilengitide, osteopontin, or RNAi of $\beta 3$ drove $\alpha 5 \beta 1$ and EGFR1 into a coprecipitable complex with endogenous or overexpressed RCP (Fig. 6 C), and this correlated closely with recruitment of EGFR1 to F-actin-rich protrusions at the cell front and its colocalization with RCP at these structures (Fig. 6, D–F). Given this coprecipitation of

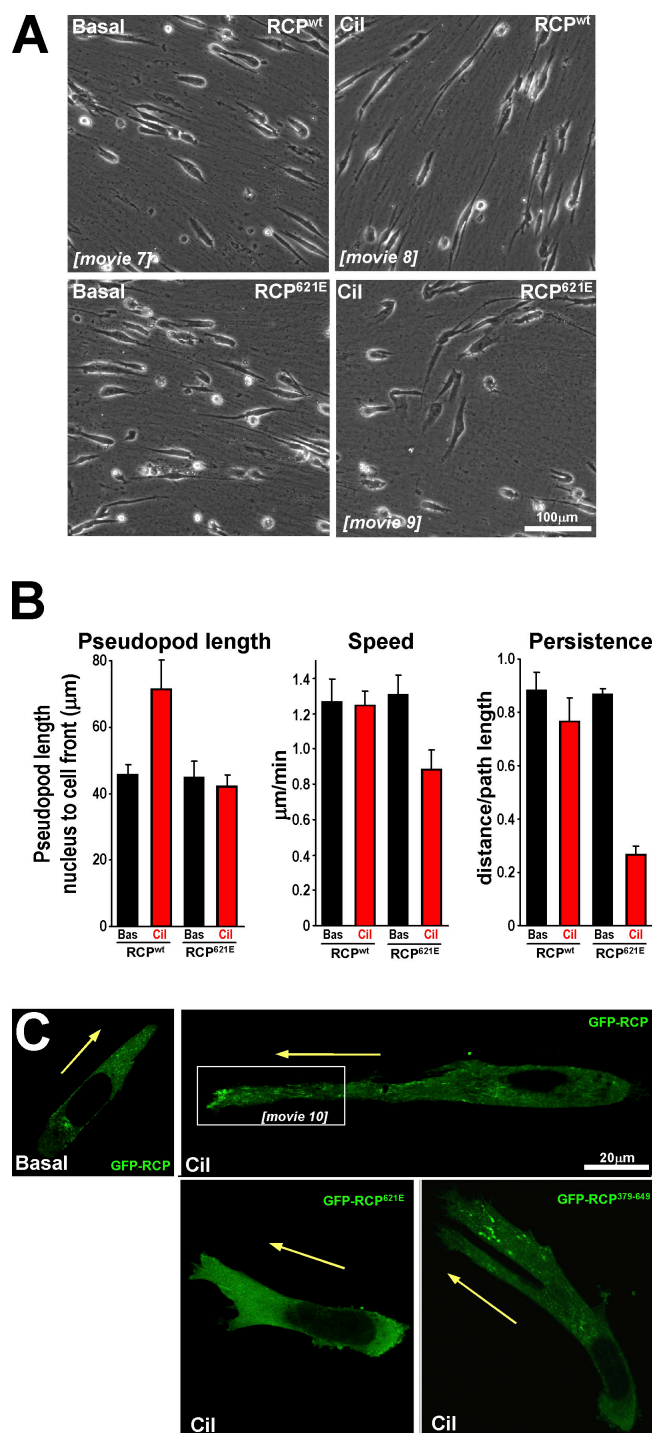


Figure 5. Cilengitide drives RCP to the tips of extending pseudopods. (A and B) Cells were transfected with GFP-RCP^{wt} or GFP-RCP^{621E} and plated onto cell-derived matrix in the presence and absence of 1 μ M cilengitide \sim 4 h before time-lapse microscopy. Images were captured every 5 min over a 6-h period and movies were generated from these (Videos 7–9) and stills from these movies are presented. Bar, 100 μ m. (B) Speed and persistence of migration were determined as for Fig. 3 B, values are mean \pm SEM; $n = 3$ independent experiments. To obtain a measure of pseudopod length, the distance between the center of the nucleus and the cell front (with respect to the direction of migration) was measured using ImageJ. Data represents mean \pm SEM; $n = 3$ independent experiments. (C) Cells were transfected with GFP-RCP, GFP-RCP^{621E}, or GFP-RCP³⁷⁹⁻⁶⁴⁹ and plated onto cell-derived matrix in the presence and absence of 1 μ M cilengitide and imaged by confocal microscopy. Images were captured at 1 frame/second over a period of 100 s and movies were generated

α 5 β 1 with RCP and EGFR1, we sought to determine whether RCP was acting as a molecular bridge to connect α 5 β 1 and EGFR1. First, we found that α 5-RNAi prevented coprecipitation of EGFR1 with RCP, whereas EGFR1-RNAi did not reduce association of RCP with α 5 β 1 (Fig. 6 C). Second, we deployed the mutant RCP³⁷⁹⁻⁶⁴⁹ that lacks an N-terminal portion of the protein, including the C2 domain, but retains the C terminus and Rab11-binding domain. Interestingly, this truncated RCP associated with α 5 β 1 integrin but had no capacity to coprecipitate with EGFR1 (Fig. 6 C). Furthermore, dominant-negative RCP^{621E} did not coprecipitate with either α 5 or EGFR1 (Fig. 6 C) and RNAi of RCP prevented coprecipitation of EGFR1 with α 5 β 1 (not depicted). Collectively, these data indicate that the C terminus of RCP interacts with α 5 β 1 to permit an association between the N terminus of RCP with EGFR1, thus bringing these two receptors together into a coprecipitable complex.

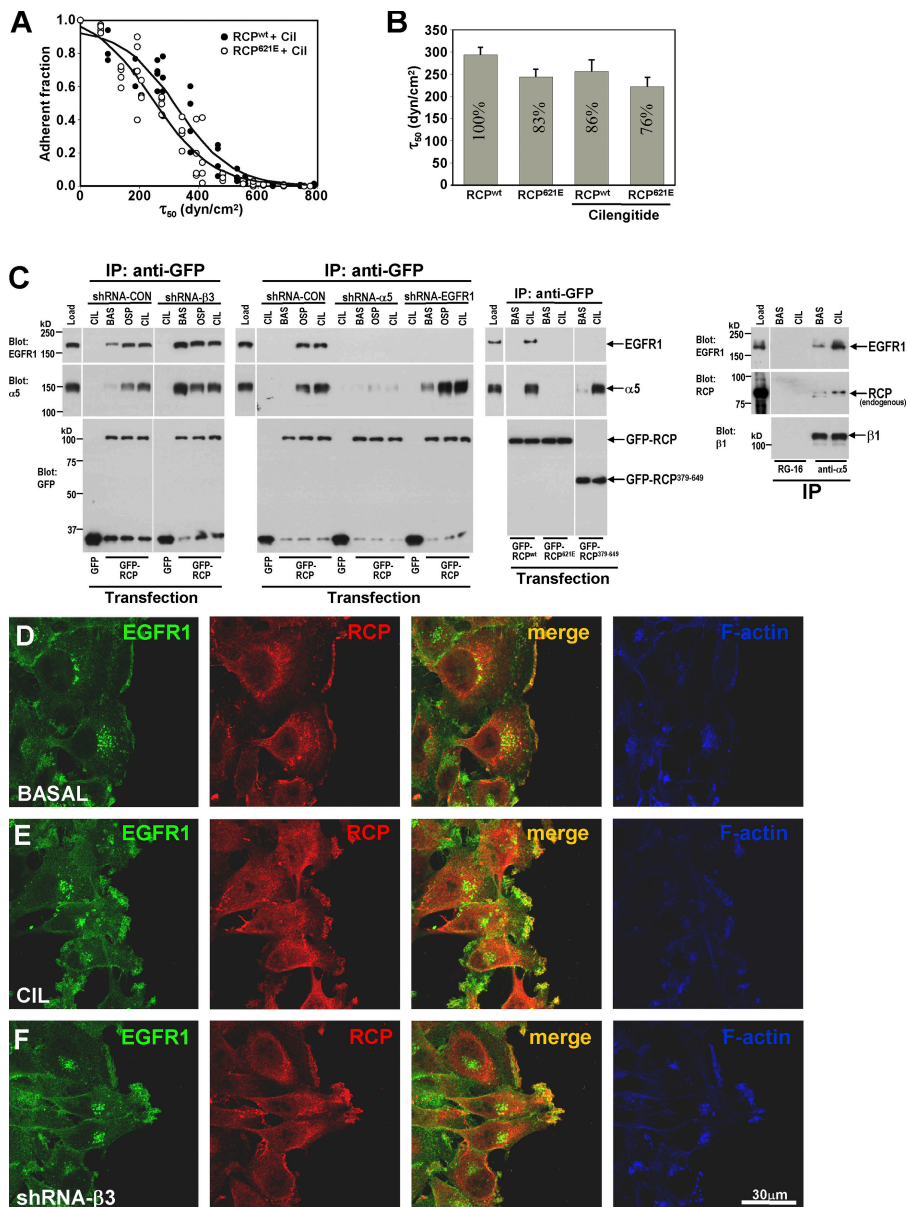
The presence of such a physical association between α 5 β 1, RCP, and EGFR1 prompted us to investigate the role of RCP in coordination of α 5 β 1 and EGFR1 trafficking. Cilengitide, osteopontin, cRGDFV, and β 3-RNAi promoted EGFR1 recycling to a similar extent as was previously observed for α 5 β 1, and knockdown of RCP prevented this (Fig. 7 A). Furthermore, addition of cilengitide and/or expression of dominant-negative RCP did not affect internalization of EGFR1 by A2780 cells (Fig. S1 B). Suppression of α 5 integrin by RNAi blocked the cilengitide-induced increase in EGFR1 recycling, whereas knockdown of EGFR1 had no effect on recycling of α 5 β 1 (Fig. 7 B). Furthermore, expression of RCP³⁷⁹⁻⁶⁴⁹ (which can associate with α 5 β 1 but not EGFR1) had minimal effect on cilengitide-driven α 5 β 1 recycling, but severely impaired EGFR1 recycling (Fig. 7 C). Collectively, these data indicate that cilengitide-driven recycling of EGFR1 is strongly dependent on α 5 β 1 (but not vice versa) and that effective recycling of EGFR1 requires its association with RCP.

Expression of RCP³⁷⁹⁻⁶⁴⁹ opposes cilengitide-driven migration into FN-containing matrigel

EGFR1-RNAi inhibited the ability of A2780 cells to migrate into matrigel under all circumstances investigated (unpublished data), indicating a requirement for EGFR1 in the invasive process. To determine the role of trafficking to EGFR1's contribution to migration in 3D, we investigated the consequences of expressing RCP³⁷⁹⁻⁶⁴⁹ (which has little effect on α 5 β 1 recycling but compromises trafficking of EGFR1) on the ability of cilengitide to drive movement of cells into matrigel. Expression of RCP³⁷⁹⁻⁶⁴⁹ did not affect the basal migration of A2780 cells but inhibited the ability of cilengitide to drive cells into FN-containing matrix (Fig. 7 D). Moreover, although GFP-RCP³⁷⁹⁻⁶⁴⁹ was localized to vesicular structures toward the cell front (indicating that integrin binding mediates the recruitment of RCP to extending processes), this

from these. Single section confocal image stills corresponding to individual frames from these movies are presented. Bar, 20 μ m. The yellow arrows indicate the direction of migration and the portion of the cell within the white square is presented as Video 10. Videos are available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>.

Figure 6. RCP does not affect integrin ligand-binding ability but mediates an association between $\alpha 5 \beta 1$ and EGFR1. (A and B) A2780 cells were transfected with either RCP^{wt} or the dominant-negative RCP^{G21E} and incubated for 24 h \pm 1 μ M cilengitide for the last 16 h. Adhesion to FN was analyzed using the spinning disc. (A) The analysis of cell density at 61 points for each treatment as a function of wall shear stress (τ_{50}) is shown. The associated curve fit (solid lines) R^2 values for the fits are 0.95 (RCP^{wt} with cilengitide) and 0.92 (RCP^{G21E} with cilengitide). The curve fits were used to extract a mean shear stress for cell detachment (τ_{50}) that is proportional to the number of adhesive integrin-ligand bonds (Boettiger, 2007). (B) The combined analyses for τ_{50} from three independent experiments run in triplicate are shown; normalized percentages are on the bars. Values are mean \pm SEM ($n = 8$ or 9). (C) Cells were transfected with hairpin vectors targeting $\beta 3$ integrin, $\alpha 5$ integrin, or EGFR1 in combination with GFP, GFP-RCP^{wt}, or GFP-RCP^{G21E} (left), or were left untransfected (right). After incubation with 1 μ M cilengitide (CIL) or 0.5 μ g/ml osteopontin (OSP), cells were lysed and GFP-RCP or $\alpha 5 \beta 1$ was immunoprecipitated from the lysates using monoclonal antibodies recognizing GFP (anti-GFP), $\alpha 5$ integrin (anti- $\alpha 5$), or an isotype-matched control antibody (RG-16). The presence of $\alpha 5$ integrin, GFP-RCP, endogenous RCP, and EGFR1 in the immunoprecipitates was then detected by immunoblotting. (D–F) A2780 cells were transfected with a short hairpin-targeting $\beta 3$ integrin (shRNA- $\beta 3$; F) or were left untransfected (D and E). Confluent monolayers were wounded and the cells were allowed to migrate into the wound in the absence (D and F) or presence (E) of 1 μ M cilengitide. The distribution of EGFR1 (green), RCP (red), and F-actin (blue) was visualized by immunofluorescence followed by confocal microscopy. Bar, 30 μ m.



truncated RCP caused pseudopod bifurcation, suggesting that combined recruitment of EGFR1 and $\alpha 5 \beta 1$ is necessary for maintenance of pseudopodial dominance, which is in turn required for cilengitide-driven migration in 3D (Fig. 5 C).

α v β 3's modulation of EGFR1 signaling proceeds via RCP and α 5 β 1

Cilengitide addition and expression of dominant-negative RCP does not greatly affect the exposure of EGFR1 at the cell surface (Fig. S5 B). However, the way in which receptors are trafficked is now established to modulate the downstream signaling profile of several RTKs (von Zastrow and Sorkin, 2007). Indeed, we found that pretreatment of A2780 cells with osteopontin or cilengitide or β 3-RNAi (all of which enhance EGFR1 recycling) strongly potentiated the ability of EGF to drive EGFR1 autophosphorylation (Fig. 8, A and B). Pretreatment of cells with either osteopontin or cilengitide enhanced downstream signals to PKB/Akt but phosphorylation of ERK1/2 remained at basal

levels (Fig. 8, A and B). Because osteopontin or cilengitide-driven EGFR1 recycling required $\alpha 5\beta 1$ and the association of EGFR1 with RCP, we investigated the involvement of $\alpha 5$ and RCP in EGFR1 signaling. Either RCP- or $\alpha 5$ -RNAi or expression of RCP³⁷⁹⁻⁶⁴⁹ blocked the ability of osteopontin to potentiate EGF-driven EGFR1 autophosphorylation and activation of PKB/Akt (Fig. 8 C). Collectively, these data indicate that after inhibition of $\alpha \nu \beta 3$ -mediated cell adhesion, EGFR1 was recruited into a physical complex with $\alpha 5\beta 1$ and RCP, which enhances EGFR1 recycling to the plasma membrane and its ability to autophosphorylate and to signal via PKB/Akt.

Discussion

Fibroblasts and some tumor cells can move upon plastic or glass substrates in different ways (Pankov et al., 2005). Cells can migrate with persistence, allowing their translocation for considerable distances from the starting point. Alternatively, some

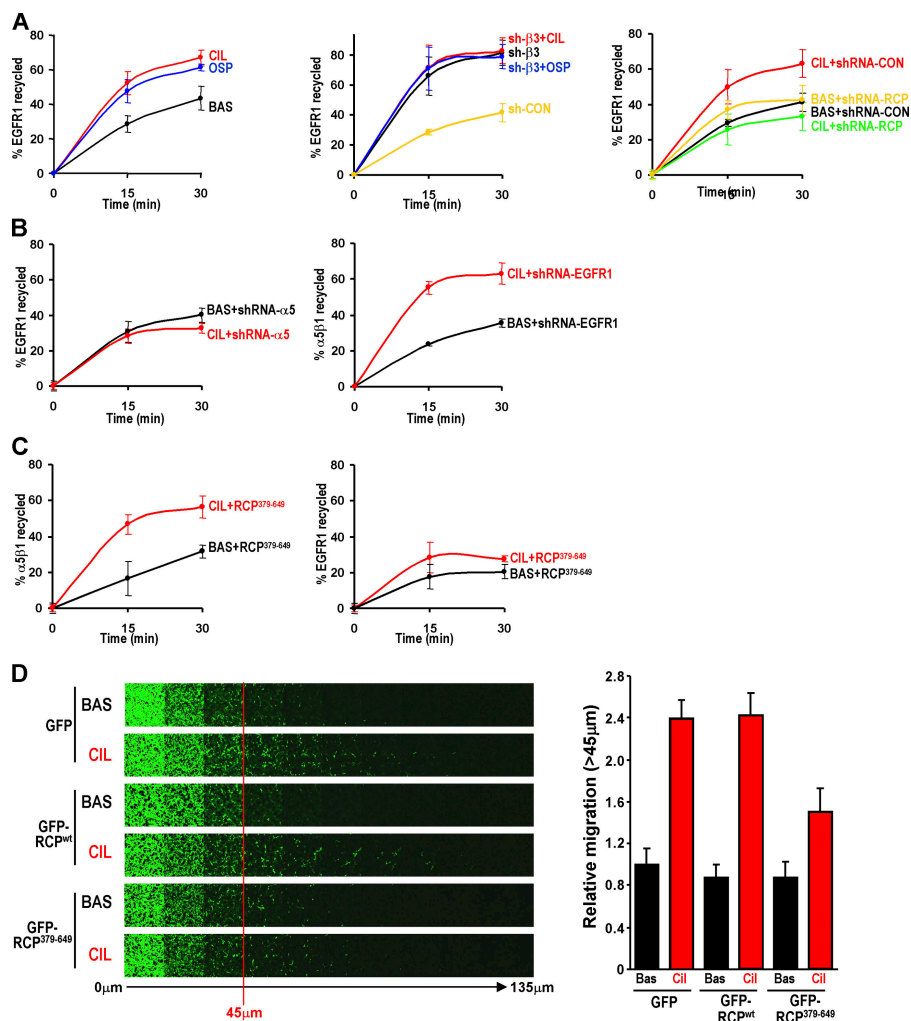


Figure 7. $\alpha 5\beta 1$ is required for recycling of EGFR1; and uncoupling EGFR1 recycling from $\alpha 5\beta 1$ inhibits migration in 3D. (A–C) Cells were transfected with short hairpin vectors targeting $\beta 3$, $\alpha 5$, $\beta 1$, RCP, or EGFR1 or with truncated GFP-RCP³⁷⁹⁻⁶⁴⁹ as indicated. The receptor recycling protocol was then followed as for Fig. 1 A but with biotinylated EGFR1 or $\alpha 5\beta 1$ integrin being determined by capture ELISA using microtitre wells coated with anti-EGFR1 or $\alpha 5$ integrin monoclonal antibodies as indicated by the y-axis labeling of the graphs. The proportion of EGFR1 or integrin recycled to the plasma membrane is expressed as a percentage of the pool of integrin labeled during the internalization period. Values are mean \pm SEM; $n = 3$ or 4 independent experiments. (D) Migration of A2780 cells expressing GFP, GFP-RCP^{wt}, or GFP-RCP³⁷⁹⁻⁶⁴⁹ into matrigel plugs containing 25 μ g/ml FN. 1 μ M cilengitide (CIL) was added as indicated to the matrigel and to both the upper and lower chambers of the inverted invasion assay. Migrating cells were detected and quantified as for Fig. 4. Values are mean \pm SEM for three independent experiments.

cells move rapidly but with low persistence, thus remaining nearer their starting point. Previous analyses have shown that persistent migration occurs on FN substrates when $\alpha v\beta 3$ is the dominant integrin, but when $\alpha 5\beta 1$ dominates cells move more rapidly but with reduced persistence (Danen et al., 2005). We have shown that alterations in the relative rates at which $\alpha v\beta 3$ and $\alpha 5\beta 1$ traffic through the endosomal pathway can switch between slow/persistent and rapid/random migration in fibroblasts (White et al., 2007). When $\alpha v\beta 3$ is cycling rapidly and is competent to engage ligand, the recycling of $\alpha 5\beta 1$ is slow and cells migrate persistently. Alternatively, when $\alpha v\beta 3$ trafficking is disrupted, $\alpha 5\beta 1$ recycling is increased and this reduces migrational persistence. Here, we report that treatment of tumor cells with endogenous (soluble osteopontin) and pharmacological (cilengitide and c-RGDfV) antagonists of $\alpha v\beta 3$ increases the rate at which $\alpha 5\beta 1$ is transported from endosomes to the plasma membrane. This increased recycling requires the association of RCP with $\alpha 5\beta 1$ and thus this Rab11 effector represents a key component of an integrin cross talk system that controls a switch between $\alpha v\beta 3$ - and $\alpha 5\beta 1$ -mediated migrational modes. Remarkably, we find that although $\alpha 5\beta 1$ -driven migration has little persistence on plastic or glass surfaces (2D), it corresponds

to increased migration into a FN-rich 3D gel. Moreover, this type of cell movement was characterized by the extension of long pseudopods at the cell front as cells migrate on a 3D matrix, and RCP was concentrated at the tips of these invasive structures. This implies that $\alpha 5\beta 1$ functions to promote tumor cell invasion and metastasis in vivo, with $\alpha v\beta 3$ acting to restrain this.

The role of integrin recycling in this switching between $\alpha v\beta 3$ - and $\alpha 5\beta 1$ -mediated migration suggested that the recycling process itself was affecting integrin function. Although differences in the total levels of surface integrin could not explain the differences, it was possible that integrins required recycling to reactivate or to remove bound ligand fragments (Caswell and Norman, 2006). To test this hypothesis, we have measured the effect of cilengitide-induced recycling of $\alpha 5\beta 1$ integrin (and its inhibition by dominant-negative RCP) on $\alpha 5\beta 1$ surface expression and its ability to form adhesive bonds with substrate FN. The formation and the strength of the adhesive bonds was affected only to the extent expected for the contributions of $\alpha v\beta 3$ and the small reduction of surface $\alpha 5\beta 1$ levels caused by inhibition of recycling. Unlike the common static adhesion assays, this assay measures the force required for cell

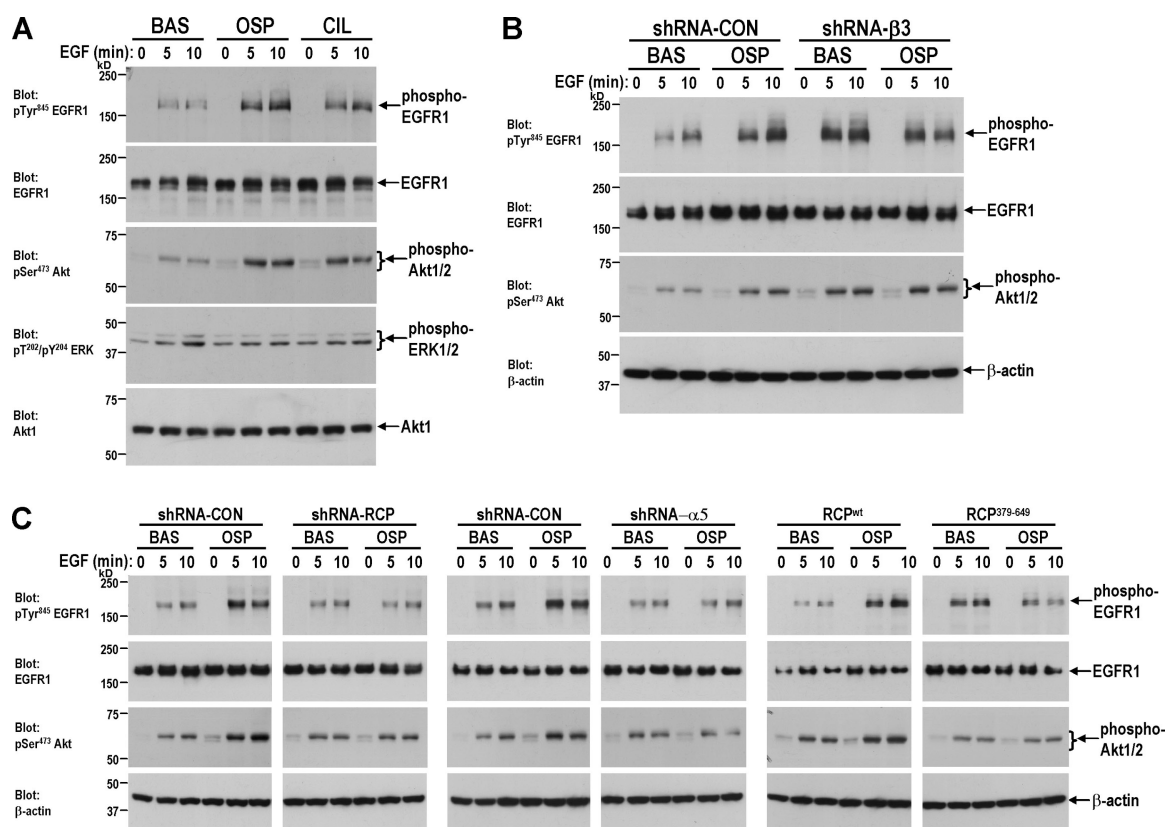


Figure 8. Osteopontin enhances EGFR1 signaling via a mechanism that requires $\alpha 5\beta 1$, RCP, and association of EGFR1 with RCP. Cells were transfected with short hairpin vectors targeting $\beta 3$, $\alpha 5$, $\beta 1$, RCP or EGFR1 or with truncated GFP-RCP³⁷⁹⁻⁶⁴⁹ as indicated. Cells were serum starved overnight followed by a 40-min treatment with either 1 μ M cilengitide or 0.5 μ g/ml osteopontin. The cells were then challenged with EGF for the indicated times and lysed. Levels of active phosphoTyr⁸⁴⁵-EGFR1, phosphoSer⁴⁷³-Akt, and phosphoThr²⁰²/phosphoTyr²⁰⁴-ERK were determined by Western blotting. Protein loading of β -actin, Akt1, and EGFR1 was confirmed by Western blotting.

detachment, which is directly proportional to the number of adhesive integrin bonds (Shi and Boettiger, 2003; Boettiger, 2007). Therefore, recycling had no effect on ligand binding and adhesive function of $\alpha 5\beta 1$ integrin.

The absence of an effect of trafficking on integrin adhesive function led us to examine the influence of $\alpha 5\beta 1$ recycling on cell signaling. Studies on tumor angiogenesis in mice show clearly that knockout of $\beta 3$ leads to increased endothelial cell proliferation and migration owing to increased VEGFR2 signaling (Reynolds et al., 2004). Other studies have described physical complexes formed by integrins and RTKs, but the way in which these associations may regulate signaling is not clear (Schneller et al., 1997; Soldi et al., 1999). Here we have found that RCP is required to bring $\alpha 5\beta 1$ and EGFR1 together into a physical complex and, although our results do not resolve all of the details of the RCP- $\alpha 5\beta 1$ -EGFR1 complex (such as whether these associations involve direct molecular contacts between $\alpha 5\beta 1$ and RCP and EGFR1 and RCP, respectively), we have determined how the trafficking of EGFR1 is coordinated with $\alpha 5\beta 1$. Clearly knockdown of $\alpha 5\beta 1$ opposes the coprecipitation of RCP with EGFR1, indicating that recruitment of RCP's C-terminal region to $\alpha 5\beta 1$ may likely be a prerequisite for subsequent association of the N terminus of RCP with EGFR1 and for subsequent enhanced EGFR1 recycling. In this regard, it is possible that $\alpha 5\beta 1$ is acting as a "receptor" for RCP to guide

this Rab effector to a membrane subdomain, whereupon a secondary association with EGFR1 is formed.

Efficient signaling from EGFR1 to the Ras-MEK-ERK axis requires receptor internalization, and a close association between EGFR1 and components of the MAPK cascade has been observed to occur at late endosomes (von Zastrow and Sorkin, 2007). Conversely, it appears that EGFR1-stimulated activation of phosphoinositide-3 kinase most likely occurs at or near the plasma membrane and that communication between EGFR1 and PKB/Akt is terminated after receptor endocytosis (Haugh and Meyer, 2002). In view of this, we propose that RCP-dependent recycling acts to slow the rate at which EGFR1 reaches later endosomes where sustained activation of MAPKs occurs and favors its localization to the plasma membrane or recycling compartments where the receptor can recruit phosphoinositide-3 kinase and thereby activate PKB/Akt. Indeed a recent study has found that c-Src promotes the trafficking of FGF receptor from endosomes to the plasma membrane and that this favors activation of PKB/Akt over signaling to the MEK-ERK axis (Sandilands et al., 2007). We find that c-Src binds to RCP and that this kinase is required for integrin and RTK recycling (unpublished data). Osteopontin enhances EGFR1 signaling in a way that involves c-Src (Das et al., 2004), which in light of the data presented here is likely to be mediated by RCP-dependent effects on $\alpha 5\beta 1$ and EGFR1 trafficking. Furthermore,

we find that RTK recycling and signaling is influenced by RCP/ $\alpha 5 \beta 1$ in other cell types, including VEGFR2 in endothelial cells and FGF receptor in fibroblasts (unpublished data). It is interesting to note that the genes for RCP and FGF receptor 1 are located close to one another within the 8p11-12 chromosomal region that is amplified in several cancers, including some breast tumors (Gelsi-Boyer et al., 2005). In this regard, it will be necessary to determine the molecular details of this apparently promiscuous association of RTKs with the RCP- $\alpha 5 \beta 1$ complex and how this influences their trafficking and signaling in cancer types where the 8p11-12 chromosomal locus is amplified.

In phase I and II clinical trials cilengitide has elicited some enduring responses in glioma patients (Nabors et al., 2007), but the results for other types of cancer are not encouraging (Friess et al., 2006; Hariharan et al., 2007). Indeed, the role that $\alpha v \beta 3$ plays in cancer-relevant processes is controversial, as some studies implicate $\alpha v \beta 3$ as a proangiogenic integrin (Brooks et al., 1994), whereas other work contradicts this (Reynolds et al., 2002). Our findings support both pro- and antiinvasive roles for $\alpha v \beta 3$, as knockdown of $\alpha v \beta 3$ can reduce A2780 cell migration but only in the absence of FN or when RCP-dependent processes are compromised. Conversely, when FN is abundant and $\alpha 5 \beta 1$ and RCP are present (as is the case in many tumor types), inhibition of $\alpha v \beta 3$ strongly promotes tumor cell migration, thus overriding the capacity for this integrin to function as an antimigratory molecule. We consistently find that cellular responses to cilengitide, c-RGDFv, small molecule inhibitors of $\alpha v \beta 3$ (unpublished data), and osteopontin are indistinguishable from one another (and from $\beta 3$ knockdown), indicating the possibility that the lack of efficacy of anti- $\alpha v \beta 3$ compounds in cancer may be owing to the capacity of these molecules to mimic the prometastatic effects of osteopontin.

In conclusion, our surprising finding that inhibition of $\alpha v \beta 3$ leads to increased tumor cell migration in 3D microenvironments, and the elucidation of the RCP- $\alpha 5 \beta 1$ -EGFR1-dependent mechanisms that drive this, highlight the need to fully understand how integrin trafficking regulates cell migration if the therapeutic potential of anti-integrin agents is to be maximized.

Materials and methods

Cell culture and transfection

A2780 cells were maintained in RPMI supplemented with 10% (vol/vol) serum at 37°C and 10% CO₂. All constructs, shRNAi vectors, and RNAi oligonucleotides were transfected into cells using the Nucleofector system (solution T, program A-23; Amaxa) according to the manufacturer's instructions. All plasmids were purified by CsCl banding before transfection by nucleofection.

Plasmid constructs

The Rab11-FIP constructs have been previously described as follows: pEGFP-C3 RCP and pEGFP-C1 RCP³⁷⁹⁻⁶⁴⁹ in Damiani et al. (2004); pEGFP-C1 FIP2 in Lindsay and McCaffrey (2002); pEGFP-C1 Rip11, pEGFP-C1 Rip11^{630E}, pEGFP-C3 RCP^{621E}, and pEGFP-C1 FIP2^{481E} in Lindsay and McCaffrey (2004b).

Immunoprecipitation and Western blotting

A2780 cells were lysed in lysis buffer (200 mM NaCl, 75 mM Tris-HCl, pH 7, 15 mM NaF, 1.5 mM Na₃VO₄, 7.5 mM EDTA, 7.5 mM EGTA, 0.15% (vol/vol) Tween-20, 50 µg/ml leupeptin, 50 µg/ml aprotinin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride). Lysates were passed

three times through a 27-gauge needle and clarified by centrifugation at 10,000 g for 10 min at 4°C. Magnetic beads conjugated to sheep anti-mouse IgG (Invitrogen) were bound to anti-integrin (anti- $\alpha 5$ or anti- $\beta 3$; BD Biosciences), anti-GFP (Abcam), or control monoclonal (RG-16; Sigma-Aldrich) antibodies. Antibody-coated beads were incubated with lysates for 2 h at 4°C with constant rotation. Unbound proteins were removed by extensive washing in lysis buffer, and specifically associated proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blotting as described previously (Roberts et al., 2001). Antibodies used for Western blotting were as follows: anti- $\alpha 5$ integrin (BD Biosciences), anti- $\beta 1$ integrin (BD Biosciences), anti- $\beta 3$ integrin (BD Biosciences; blots using this antibody were run under nonreducing conditions), anti-RCP (Genway), anti-EGFR1 (BD Biosciences) and anti-GFP (Abcam).

Inverted matrigel plug assay

Inverted matrigel assays were performed as described previously (Hennigan et al., 1994). In brief, matrigel supplemented with 25 µg/ml FN as indicated was allowed to polymerize in transwell inserts (Corning) for 1 h at 37°C. Inserts were then inverted and cells were seeded directly onto the opposite face of the filter. Transwell inserts were finally placed in serum-free medium, and medium supplemented with 10% FCS and 30 ng/ml EGF was placed on top of the matrix, providing a chemotactic gradient. Where appropriate, 1 µM cilengitide, 0.5 µg/ml osteopontin, and 1.0 µg/ml of integrin/FN-blocking antibodies were added to the matrigel before plug polymerization and also to the medium throughout the system. 48 h after seeding, migrating cells were stained with Calcein AM and visualized by confocal microscopy with serial optical sections being captured at 15-µm intervals.

Generation of cell-derived matrix

Cell-derived matrix was generated as described previously (Cukierman et al., 2001; Bass et al., 2007). In brief, gelatin-coated tissue culture-ware was cross-linked with glutaraldehyde, quenched, and equilibrated in DME containing 10% FCS. Primary cultured human dermal fibroblasts were seeded at near confluence ($\sim 2 \times 10^4$ cells/cm²) and grown for 10 d in DME containing 10% FCS and 50 µg/ml ascorbic acid. Matrices were denuded of living cells by incubation with PBS containing 20 mM NH₄OH and 0.5% Triton X-100, and DNA residue was removed by incubation with DNaseI. Matrices were blocked with 0.1% heat-denatured BSA before seeding of cells.

Receptor internalization and recycling assays

Internalization and recycling assays were performed after 30–45 min of serum starvation and subsequently conducted, including the internalization and recycling periods, in the absence of serum. Integrin and EGFR1 internalization and recycling assays were performed as described previously (Roberts et al., 2001). Internalization assays were performed in the presence of 0.6 mM of primaquine as described in Roberts et al. (2001). For measurement of recycling, the receptor internalization conditions were 30 min at 37°C for all experiments. The antibodies used for capture ELISA detection of $\alpha 5 \beta 1$ integrin and EGFR1 were obtained from BD Biosciences. Cilengitide, osteopontin, cRADfV, and cRGDFV were added to the cells only during the recycling period.

¹²⁵I-labeled transferrin recycling assays were performed essentially as described in van Dam and Stoorvogel (2002) with some modifications. In brief, serum-starved cells were incubated with ¹²⁵I-labeled transferrin (0.1 µCi/well; PerkinElmer) for 1 h at 4°C in PBS with 1% (wt/vol) BSA. The tracer was allowed to internalize for 30 min at 37°C (to label the recycling compartment). Tracer remaining at the cell surface was removed by incubation with acid PBS (corrected to pH 4.0 by the addition of HCl) at 4°C for 6 min, and the tracer was allowed to recycle at 37°C in serum-free DME supplemented with 1% BSA and 50 µM desferoxamine (Sigma-Aldrich). The quantity of ¹²⁵I recycled into the medium is expressed as a percentage of the number of counts incorporated during the internalization period.

Microscopy, track-plot analysis, and 3D reconstruction

Confluent monolayers were wounded with a plastic pipette tip and placed on the stage of an inverted microscope (Axiovert S100; Carl Zeiss, Inc.) in an atmosphere of 5% CO₂ at 37°C. Cells were observed using a 20× phase-contrast objective and images were collected every 20 min using a digital camera (C4742-95; Hamamatsu Photonics). Movies were generated and cell tracks were analyzed using Bioimaging software (Andor). For fluorescence time-lapse imaging, cells were plated onto 6-well plates or glass-bottomed 3-cm plates (coated with cell-derived matrix as appropriate)

and incubated at 37°C until cells adhered and began migrating (4 h). Cells were imaged using a 10x objective and an inverted microscope in an atmosphere of 5% CO₂ at 37°C or a 64x objective and an inverted confocal microscope (Fluoview FV1000; Olympus) in an atmosphere of 5% CO₂ at 37°C. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 10 min and then permeabilized in PBS containing 0.2% Triton X-100. Primary antibodies for immunofluorescence were $\alpha 5$ integrin (BD Biosciences), RCP (Genway), and EGFR1 (BD Biosciences) with F-actin being detected using BODIPY_{650/665}-phalloidin (Invitrogen). Immunofluorescence was imaged using a 64x objective and an inverted confocal microscope. Images from the confocal microscope were imported into Photoshop (Adobe) and linear-only adjustments made to their brightness and contrast.

To generate 3D reconstructions, serial z-stack images were captured using a 64x objective and an inverted confocal microscope and imported into Velocity Visualization (Improvision). Individual cells were cropped in the XY plane from fields of view containing more than one cell. HR opacity rendering was used to create a 3D image, which was rotated along the x axis to create XZ, XZ, and XYZ projections.

Assessment of EGF signaling

Cells were serum starved overnight. On the day of the experiment, cells were treated with either 1 μ M cilengitide or 0.5 μ g/ml osteopontin for 40 min, immediately before a challenge with 30 ng/ml EGF (Sigma-Aldrich) for 5 or 10 min as indicated. Western blotting was then used to determine levels of active signaling moieties using the following antibodies: anti-phosphoTyr⁸⁴⁵-EGFR1 (Cell Signaling Technology), anti-phosphoSer⁴⁷³-Akt (Cell Signaling Technology), and anti-phosphoThr²⁰²/phosphoTyr²⁰⁴-ERK (Cell Signaling Technology). The following antibodies were used to assess protein loading: anti-EGFR1 (BD Biosciences), anti-Akt1 (Cell Signaling Technology), and β -actin (Sigma-Aldrich).

Spinning disc methods

A2780 cells transfected with RCP^{wt} or RCP^{621E} were plated overnight in complete medium \pm 1 μ M cilengitide for the last 16 h. Cells were dissociated with 2 mM EDTA and single cell suspensions were added to FN-coated (5 μ g/ml) glass coverslips and incubated at 25°C for 60 min. Coverslips were placed on the spinning disc device and exposed to a linear hydrodynamic shear gradient for 5 min (Boettiger, 2007). Coverslips were removed from the spinning device, fixed in formalin, permeabilized, and stained with DAPI. 61 fields at 15 different radial positions plus the center were counted for 1- μ m-diameter fields and plotted as a function of shear stress. Curve fits were performed using SigmaPlot 7.0 using the formula $f = 1/\{a - \exp[b(t - c)]\}$, where f is the fraction of adherent cells, t is the corresponding shear stress, and a , b , and c are fit parameters; c is the shear stress for 50% detachment.

Online supplemental material

Fig. S1 A displays Western blots indicating the efficacy of shRNA vectors targeting $\beta 3$ and $\alpha 5$ integrins, EGFR1, and RCP. Fig. S1 B shows results from internalization assays indicating that addition of cilengitide and expression of dominant-negative RCP^{621E} do not greatly influence the constitutive endocytosis of $\alpha 5\beta 1$ or EGFR1. Fig. S2 indicates the inability of dominant-negative Rab11^{124L}, Rip1^{163OE}, FIP2^{480E}, and RCP^{621E} to influence recycling of $\alpha 5\beta 1$ integrin and Tfn-R. Fig. S3 shows the time course over which physical association of GFP-RCP and $\alpha 5\beta 1$ integrin is established after the addition of cilengitide and shows the lack of coprecipitation of paxillin with GFP-RCP. Fig. S4 shows confocal immunofluorescence images illustrating the influence of cilengitide/osteopontin or knockdown of $\beta 3$ integrin on cellular morphology and the distribution of $\alpha 5\beta 1$ and RCP. Fig. S5 shows data indicating that dominant-negative Rip1^{163OE} and FIP2^{480E} do not influence migration through FN-containing matrigel, and Fig. S5 B documents the consequences of expression of dominant-negative RCP^{621E} and/or addition of cilengitide on $\alpha 5\beta 1$ and EGFR1 surface expression. Videos 1–3 show that addition of cilengitide promotes a rapid/random mode of cell migration on 2D surfaces that is opposed by expression of dominant-negative RCP^{621E}. Videos 4–6 show that whereas A2780 cells expressing GFP-actin extend an organized fan-like lamellipodium as they move, after treatment with cilengitide or osteopontin their movement is characterized by the formation of highly dynamic actin-rich protrusions at the cell front. Videos 7–9 show that cilengitide promotes a pseudopodial mode of cell migration on cell-derived matrix that is opposed by expression of RCP^{621E}. Video 10 is a high-resolution fluorescence time-lapse sequence showing GFP-RCP localization to the tip of the extending pseudopod as A2780 cells migrate on a 3D matrix. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200804140/DC1>.

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