Rab-coupling protein coordinates recycling of α5β1 integrin and EGFR1 to promote cell migration in 3D microenvironments

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Here we show that blocking the adhesive function of αvβ3 integrin with soluble RGD ligands, such as osteopontin or cilengitide, promoted association of Rab-coupling protein (RCP) with α5β1 integrin and drove RCP-dependent recycling of α5β1 to the plasma membrane and its mobilization to dynamic ruffling protrusions at the cell front. These RCP-driven changes in α5β1 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 α5β1 integrin did not affect its regulation or ability to form adhesive bonds with substrate fibronectin. Instead, α5β1 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 α5β1 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 an elongated or amoeboid morphology (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 αvβ3 integrin promotes slow/persistent migration, whereas engagement of α5β1 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 αvβ3 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).
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 α5β1 (White et al., 2007). Small GTPases of the Rab11 family, including Rab11α and Rab25, control recycling of internalized α5β1 (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 α5β1 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 p75 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 α5β1 to promote invasive migration (Caswell et al., 2007). Furthermore, Rab11 controls recycling of α6β4 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 chemokine factors such as osteopontin and EGF.

Results

Inhibition of αvβ3 promotes α5β1 recycling in A2780 cells

We have previously shown that perturbation of “short-loop” αvβ3 recycling leads to a marked increase in α5β1 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 α5β1 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 αvβ3 and αvβ5), cRGDfV (a cyclic peptide with selectivity for αvβ5 over αvβ3 integrin; Dechantsreiter et al., 1999), or the soluble αvβ3 ligand osteopontin, α5β1 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 β3 levels by RNAi (Fig. S1, available at available at http://www.jcb.org/cgi/content/fig/jcb.200804140/DC1) increased α5β1 recycling, and treatment of β3 knockdown cells with either cilengitide or osteopontin did not further enhance α5β1 trafficking (Fig. 1 A, right). Cilengitide did not affect the rate at which α5β1 was internalized by A2780 cells (Fig. S1 B). As cilengitide-driven α5β1 recycling was Rab11 dependent (Fig. S2 A), these data indicate that αvβ3 functions constitutively to restrain α5β1 recycling in A2780 ovarian cancer cells and that removal of αvβ3 or its occupation/blockade with soluble, non-ECM ligands such as osteopontin or cilengitide releases this constraint to drive Rab11-dependent trafficking of α5β1 to the plasma membrane.

Inhibition of αvβ3 drives α5β1 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 α5β1 and αvβ3 integrins. This revealed that RCP associated with both α5β1 and αvβ3 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 α5β1 but reduced its association with αvβ3 (Fig. 1 B). Increased association of α5β1 and RCP was detectable within 8 min and was maximal after 20-min exposure to cilengitide (Fig. S3 A, available at availability of http://www.jcb .org/cgi/content/full/jcb.200804140/DC1). To further confirm these associations, we immunoprecipitated α5β1 and αvβ3 using antibodies recognizing α5 and β3 integrin, respectively. Western blots of these immunoprecipitates indicated that both endogenous and overexpressed RCP coprecipitated with α5β1 and αvβ3 (but not with a control antibody) and that addition of cilengitide strongly promoted RCP’s association with α5β1, but correspondingly weakened binding to αvβ3 (Fig. 1 C). Suppression of β3 expression by RNAi also increased association between RCP and α5β1, and treatment of β3 knockdown cells with cilengitide or osteopontin did not further enhance this (Fig. 1 D).

Furthermore, RCP621E, containing a point mutation within the kinase domain (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 α5β1 (White et al., 2007), thus making them potential candidates for components of protein complexes that control α5β1 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 p75 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 α5β1 to promote invasive migration (Caswell et al., 2007). Furthermore, Rab11 controls recycling of α6β4 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 chemokine factors such as osteopontin and EGF.
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 α5β1 was required for the increase in speed (Fig. 2B). In the absence of cilengitide, expression of RCP2145 did not affect cell migration. However, RCP2145 (but not dominant-negative Rab11-FIP2 or Rip11) completely opposed the cilengitide- or osteopontin-driven increase in migration speed (Fig. 2B), 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).

### Table 1

| Treatment | Integrin Recycling | Control | Cilengitide | Osteopontin |
|-----------|--------------------|---------|-------------|-------------|
| BAS       | 60%                | 30%     | 90%         | 70%         |
| CIL       | 90%                | 50%     | 90%         | 70%         |

### Table 2

| Treatment | Integrin Recycling | Control | Cilengitide | Osteopontin |
|-----------|--------------------|---------|-------------|-------------|
| BAS       | 60%                | 30%     | 90%         | 70%         |
| CIL       | 90%                | 50%     | 90%         | 70%         |

### Table 3

| Treatment | Integrin Recycling | Control | Cilengitide | Osteopontin |
|-----------|--------------------|---------|-------------|-------------|
| BAS       | 60%                | 30%     | 90%         | 70%         |
| CIL       | 90%                | 50%     | 90%         | 70%         |

### Figure 1

Inhibition of αvβ3 promotes α5β1 recycling and association of RCP with α5β1. (A) A2780 cells were transfected with a nontargeting shRNA (sh-CON) or a short hairpin-targeting β3 integrin (sh-β3; 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 μM cilengitide, 1 μM cRGDfV, 4.4 μM cRGDfV, or 0.5 μg/ml soluble osteopontin. Cells were then reexposed to MesNa and biotinylated integrin was determined by capture ELISA using microtits wells coated with anti-human α5 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. Values are mean ± SEM; n = 7 (BAS), n = 4 (CIL), and n = 3 (cRGDfV, cRGDfV, and OSP) 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 μM 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 were analyzed for the presence of α5β1 and αvβ3 by immunoblotting with antibodies recognizing the integrin α5 and β3 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 μM cilengitide for 20 min. Lysates were immunoprecipitated using monoclonal antibodies recognizing α5β1 integrin (anti-α5), αvβ3 integrin (anti-β3), or an isotype-matched control antibody (RG-16). The presence of α5 and β3 integrin, GFP-RCP, and endogenous β3 integrin (shRNA-β3), A2780 cells were incubated in the absence (BAS) or presence (CIL) of 1 μM cilengitide or 0.5 μg/ml osteopontin (OSP) for 20 min. Cells were lysed and anti-GFP immunoprecipitates probed for the presence of α5β1 and GFP as in B. (E) A2780 cells were transfected with GFP, GFP-RCP, or GFP-RCP2145, treated with cilengitide, and then lysed. Anti-GFP immunoprecipitates were probed for α5β1 and GFP as in B.

### Figure 2

Unable to drive α5β1 recycling after RCP knockdown or expression of dominant-negative RCP2145 (Fig. 2A). The corresponding dominant-negative mutants of Rip11 or Rab11-FIP2 (Rip11650E and FIP26516) had no effect on cilengitide-induced α5β1 recycling (Fig. S2B), and RCP2145 did not compromise trafficking of the transferrin receptor (Tfn-R) in the presence or absence of cilengitide (Fig. S2C). Internalization of α5β1 was not affected by expression of dominant-negative RCP (Fig. S1B). These data indicate that RCP displays selectivity toward the recycling of specific cargoes (i.e., α5β1 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 α5β1.

As increased α5β1 recycling causes fibroblasts to switch from persistent to random migration (White et al., 2007), we
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 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 α5β1 promote fibronectin (FN)-dependent migration in 3D

The capacity of α5β1 to support migration in 3D matrices is dictated by the way in which it is trafficked. Indeed, α5β1 and FN inhibit invasive migration of Fos-transformed fibroblasts through matrigel (Spence et al., 2006). However, expression of Rab25 changes the way in which α5β1 is trafficked such that α5β1 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 α5β1 trafficking were sufficient to promote migration into 3D matrices. When αvβ3 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 α5β1 integrin (Fig. 4 B). Depletion of β3 integrin with RNAi strongly promoted FN-dependent migration into matrigel (in the absence of FN, β3 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 RCPΔL16 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...
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 αvβ3 was not inhibited, migration on cell-derived matrix had no requirement for RCP (Fig. 5 A), but expression of RCP621E 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-RCP621E) 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 αvβ3 strongly promotes RCP-dependent trafficking of α5β1 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 α5β1 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 α5β1 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 α5β1 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 ($\tau_{50}$) required for cell detachment (Garcia et al., 1998). Because $\tau_{50}$ is proportional to the number of α5β1 and αvβ3 integrins per cell that are occupied in adhesive bonds, this provides a direct measure of integrin adhesive function.
RCP associates with EGFR1 and α5β1 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 α5β1 drove α5β1 and EGFR1 into a coprecipitated 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 function (Boettiger et al., 2001; Shi and Boettiger, 2003; Boettiger, 2007). The generation of τ50 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 ∼14% consistent with the contribution that is typically made to adhesion by αvβ3 in cells expressing both αvβ3 and α5β1. As shown in Fig. 3 A, inhibition of RCP reduces cilengitide-driven α5β1 recycling by at least threefold. Therefore, if recycling were to influence α5β1’s ligand-binding capacity, one would anticipate a large alteration in adhesion strength after inhibition of RCP. However, expression of RCP621E 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 α5β1, it is likely that there are alternative explanations for the ability of this type of recycling to promote cell migration and invasiveness.
with RCP and EGFR1, we sought to determine whether RCP was acting as a molecular bridge to connect α5β1 and EGFR1. First, we found that α5-RNAi prevented coprecipitation of EGFR1 with RCP, whereas EGFR1-RNAi did not reduce association of RCP with α5β1 (Fig. 6 C). Second, we deployed the mutant RCP<sup>379-649</sup> 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 α5β1 integrin but had no capacity to coprecipitate with EGFR1 (Fig. 6 C). Furthermore, dominant-negative RCP<sup>621E</sup> did not coprecipitate with either α5 or EGFR1 (Fig. 6 C) and RNAi of RCP prevented coprecipitation of EGFR1 with α5β1 (not depicted). Collectively, these data indicate that the C terminus of RCP interacts with α5β1 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 α5β1, RCP, and EGFR1 prompted us to investigate the role of RCP in coordination of α5β1 and EGFR1 trafficking. Cilengitide, osteopontin, cRGDFV, and β3-RNAi promoted EGFR1 recycling to a similar extent as was previously observed for α5β1, 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 α5 integrin by RNAi blocked the cilengitide-induced increase in EGFR1 recycling, whereas knockdown of EGFR1 had no effect on recycling of α5β1 (Fig. 7 B). Furthermore, expression of RCP<sup>379-649</sup> (which can associate with α5β1 but not EGFR1) had minimal effect on cilengitide-driven α5β1 recycling, but severely impaired EGFR1 recycling (Fig. 7 C). Collectively, these data indicate that cilengitide-driven recycling of EGFR1 is strongly dependent on α5β1 (but not vice versa) and that effective recycling of EGFR1 requires its association with RCP.

Expression of RCP<sup>379-649</sup> 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 RCP<sup>379-649</sup> (which has little effect on α5β1 recycling but compromises trafficking of EGFR1) on the ability of cilengitide to drive movement of cells into matrigel. Expression of RCP<sup>379-649</sup> 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-RCP<sup>379-649</sup> was localized to vesicular structures toward the cell front (indicating that integrin binding mediates the recruitment of RCP to extending processes), this
levels (Fig. 8, A and B). Because osteopontin or cilengitide-driven EGFR1 recycling required α5β1 and the association of EGFR1 with RCP, we investigated the involvement of α5β1 and RCP in EGFR1 signaling. Either RCP- or α5-RNAi or expression of RCP 379-649 blocked the ability of osteopontin to potentiate EGFR1 autophosphorylation and activation of PKB/Akt (Fig. 8 C). Collectively, these data indicate that after inhibition of αvβ3-mediated cell adhesion, EGFR1 was recruited into a physical complex with α5β1 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 truncated RCP caused pseudopod bifurcation, suggesting that combined recruitment of EGFR1 and α5β1 is necessary for maintenance of pseudopodial dominance, which is in turn required for cilengitide-driven migration in 3D (Fig. 5 C).

αvβ3's modulation of EGFR1 signaling proceeds via RCP and α5β1

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 α5β1 was immunoprecipitated from the lysates using monoclonal antibodies recognizing GFP (anti-GFP), α5 integrin (anti-α5), or an isotype-matched control antibody (RC-16). The presence of α5 integrin, GFP-RCP, and EGFR1 in the immunoprecipitates was then detected by immunoblotting. (D–F) A2780 cells were transfected with a short hairpin–targeting β3 integrin (shRNA-β3; 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 μM cilengitide. The distribution of EGFR1 (green), RCP (red), and F-actin (blue) was visualized by immunofluorescence followed by confocal microscopy. Bar, 30 μm.
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 αvβ3 is the dominant integrin, but when α5β1 dominates cells move more rapidly but with reduced persistence (Danen et al., 2005). We have shown that alterations in the relative rates at which αvβ3 and α5β1 traffic through the endosomal pathway can switch between slow/persistent and rapid/random migration in fibroblasts (White et al., 2007). When αvβ3 is cycling rapidly and is competent to engage ligand, the recycling of α5β1 is slow and cells migrate persistently. Alternatively, when αvβ3 trafficking is disrupted, α5β1 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 αvβ3 increases the rate at which α5β1 is transported from endosomes to the plasma membrane. This increased recycling requires the association of RCP with α5β1 and thus this Rab11 effector represents a key component of an integrin cross talk system that controls a switch between αvβ3- and α5β1-mediated migrational modes. Remarkably, we find that although α5β1-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 α5β1 functions to promote tumor cell invasion and metastasis in vivo, with αvβ3 acting to restrain this.

The role of integrin recycling in this switching between αvβ3- and α5β1-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 α5β1 integrin (and its inhibition by dominant-negative RCP) on α5β1 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 αvβ3 and the small reduction of surface α5β1 levels caused by inhibition of recycling. Unlike the common static adhesion assays, this assay measures the force required for cell

Figure 7. α5β1 is required for recycling of EGFR1, and uncoupling EGFR1 recycling from α5β1 inhibits migration in 3D. (A–C) Cells were transfected with short hairpin vectors targeting β3, α5, β1, 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 α5 integrin being determined by capture ELISA using microtitre wells coated with anti-EGFR1 or α5 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 ± SEM; n = 3 or 4 independent experiments. (D) Migration of A2780 cells expressing GFP, GFP-RCPwt, or GFP-RCP379–649 into matrigel plugs containing 25 μg/ml FN. 1 μM 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 ± SEM for three independent experiments.
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 α5β1 integrin.

The absence of an effect of trafficking on integrin adhesive function led us to examine the influence of α5β1 recycling on cell signaling. Studies on tumor angiogenesis in mice show clearly that knockout of β3 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 α5β1 and EGFR1 together into a physical complex and, although our results do not resolve all of the details of the RCP–α5β1–EGFR1 complex (such as whether these associations involve direct molecular contacts between α5β1 and RCP and EGFR1 and RCP, respectively), we have determined how the trafficking of EGFR1 is coordinated with α5β1. Clearly knockdown of α5β1 opposes the coprecipitation of RCP with EGFR1, indicating that recruitment of RCP’s C-terminal region to α5β1 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 α5β1 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 α5β1 and EGFR1 trafficking. Furthermore,
we find that RTK recycling and signaling is influenced by RCP/α5β1 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–α5β1 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 ciligentitide has elicited some enduring responses in glioma patients (Nabors et al., 2007), but the results for other types of cancer are not encouraging (Friesel et al., 2006; Hariharan et al., 2007). Indeed, the role that αvβ3 plays in cancer-relevant processes is controversial, as some studies implicate αvβ3 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 αvβ3, as knockdown of αvβ3 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 α5β1 and RCP are present (as is the case in many tumor types), inhibition of αvβ3 strongly promotes tumor cell migration, thus overriding the capacity for this integrin to function as an antiangiogenic molecule. We consistently find that cellular responses to ciligentitide, c-RGDfV, small molecule inhibitors of αvβ3 (unpublished data), and osteopontin are indistinguishable from one another (and from β3 knockdown), indicating the possibility that the lack of efficacy of anti-αvβ3 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 αvβ3 leads to increased tumor cell migration in 3D microenvironments, and the elucidation of the RCP–α5β1–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 Nucleoactor 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 RCP[394del] in Damiani et al. (2004); pEGFP-C1 F1P2 in Lindsay and McCaffrey (2002); pEGFP-C1 Rip1, pEGFP-C1 Rip1[153E], pEGFP-C3 RCP[61E], and pEGFP-C1 F1P2[61E] in Lindsay and McCaffrey (2004b).

**Immunoprecipitation and Western blotting**

A2780 cells were lysed in lysis buffer (200 mM NaCl, 75 mM Tris-HCl, pH 7.8, 15 mM Na2EDTA, 0.15% (vol/vol) Tween-20, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 mM 4-2-aminobutyrylbenzenesulfonyl 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-human IgG (Invitrogen) were bound to anti-integrin (anti-α5 or anti-β3; 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-α5 integrin (BD Biosciences), α5β1 integrin (BD Biosciences), α5β3 integrin (BD Biosciences); blots using this antibody were run under nonreducing conditions, anti-RCP (Glenway), 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 injected 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., 2006; 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 (~2 x 104 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 α5β1 integrin and EGFR1 were obtained from BD Biosciences. Ciligentitide, 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 Stoovegol (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 HC1 until 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**

Confocal 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 20x 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 10× objective and an inverted microscope 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 α5 integrin (BD Biosciences), RCP (Genway), and EGFR1 (BD Biosciences) with F-actin being detected using BODIPY FL-phallolidin (Invitrogen). Immunofluorescence was imaged using a 64× 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 64× 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. HP rotation 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 μM cilengitide or 0.5 μg/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-phosphoThr²⁰²/²⁰⁴-ERK (Cell Signaling Technology), and anti-phosphoThr²⁰⁴-ERK (Cell Signaling Technology). The following antibodies were used to assess protein loading: anti-EGFR1 (BD Biosciences), anti-Akt (Cell Signaling Technology), and β-actin (Sigma-Aldrich).

Spinning disc methods
A2780 cells transfected with RCP WT or RCP²⁶²E were plated overnight in complete medium ± 1 μM cilengitide for the last 16 h. Cells were dissociated with 2 mM EDTA and single cell suspensions were added to FN-coated (5 μg/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-μm-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, 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 S3 and α5 integrins, EGFR1, and RCP. Fig. S1 B shows results from internalization assays indicating that addition of cilengitide and expression of dominant-negative RCP²⁶²E do not greatly influence the constitutive endocytosis of α5β1 or EGFR1. Fig. S2 indicates the inability of dominant-negative Rab11²⁴⁴I, Rip11²⁴⁰E, FIP2²⁴⁸E, and RCP²⁶²E to influence migration through FN-containing matrigel, and Fig. S5 B documents the consequences of expression of dominant-negative RCP²⁶²E and/or addition of cilengitide on α5β1 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 RCP²⁶²E. 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 RCP²⁶²E. Video 10 is a high-resolution fluorescence time-lapse sequence showing GFP-RC 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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