uPAR promotes formation of the p130Cas–Crk complex to activate Rac through DOCK180

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The urokinase-type plasminogen activator receptor (uPAR) drives tumor cell membrane protrusion and motility through activation of Rac; however, the pathway leading from uPAR to Rac activation has not been described. In this study we identify DOCK180 as the guanine nucleotide exchange factor acting downstream of uPAR. We show that uPAR cooperates with integrin complexes containing \( \beta_3 \) integrin to drive formation of the p130Cas–CrkII signaling complex and activation of Rac, resulting in a Rac-driven elongated-mesenchymal morphology, cell motility, and invasion. Our findings identify a signaling pathway underlying the morphological changes and increased cell motility associated with uPAR expression.

Introduction

The urokinase-type plasminogen activator receptor (uPAR) is overexpressed in many human cancers, its expression often correlating with poor prognosis (Memarzadeh et al., 2002; Kaneko et al., 2003; El-Kott et al., 2004; Salajegheh et al., 2005; Meng et al., 2006; for review see Bene et al., 2004). It is expressed as a glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein and in a soluble form that is secreted or shed from the cell surface (Pedersen et al., 1993; Pyke et al., 1993; Blasi and Carmeliet, 2002). Through binding to its ligands, the protease uPA and the extracellular matrix glycoprotein vitronectin, uPAR may be involved in several processes related to tumor progression, including growth factor signaling (Liu et al., 2002; Chaurasia et al., 2006; Jo et al., 2006), release of sequestered growth factors from the ECM (Saksela and Rifkin, 1990; Sato et al., 1990; Ribatti et al., 1999), and reemergence from tumor cell dormancy (for review see Aguirre-Ghiso, 2007). Importantly, expression of uPAR is associated with the acquisition of a motile, invasive tumor cell phenotype, a process thought to be crucial for cancer metastasis (Vial et al., 2003; Lester et al., 2007; Madsen et al., 2007).

GPI-anchored uPAR localizes to the leading edge of migrating cells, and complexes of uPA–uPAR are thought to promote cell motility by activating the plasminogen system to degrade ECM (Blasi and Carmeliet, 2002; Dano et al., 2005). In addition to its roles in the regulation of pericellular proteolysis, a large body of evidence has identified uPAR as a signaling receptor that activates intracellular pathways. Activation of the Rho family small GTPase Rac has emerged as an important event in the promotion of motility and invasion by uPAR (Kjoller and Hall, 2001; Vial et al., 2003). Ectopic uPAR expression results in Rac-dependent lamellipodial protrusion and cell motility (Kjoller and Hall, 2001; Jo et al., 2003), and inhibiting endogenous uPAR expression inactivates Rac and strongly inhibits lamellipodial protrusion and cell motility (Ma et al., 2002; Vial et al., 2003). Rac activation by uPAR can occur in the absence of uPA, but depends on binding to vitronectin (Kjoller and Hall, 2001; Ma et al., 2002; Madsen et al., 2007). However, uPA binding may contribute to signaling by increasing the affinity of uPAR for vitronectin (Sidenius et al., 2002; Madsen et al., 2007). Because the vitronectin-binding site is located on the opposite side of the molecule from the uPA-biding cleft, multimeric complexes containing all three molecules may form (Linas et al., 2005; Madsen et al., 2007).

Being GPI anchored and lacking transmembrane and cytoplasmic domains, uPAR relies on transmembrane coreceptors for intracellular signaling. Potential coreceptors for uPAR include G protein–coupled receptors (Resnati et al., 2002), tetraspanins (Bass et al., 2005), low density lipoprotein receptor-related protein (Czekay et al., 2001), and Endo180/UPARAP (Behrendt et al., 2000). In particular, several studies suggest that integrins...
are involved in uPAR signaling. Expression of uPAR results in integrin-associated signaling events such as phosphorylation of FAK and Src family kinases (Aguirre Ghiso, 2002; Zhang et al., 2003; Wei et al., 2007). uPAR–integrin interactions have been shown by communoprecipitation of uPAR with leukocyte integrin Mac-1 (Simon et al., 1996), fibronectin receptors αβ1 and αβ2 (Wei et al., 2001; Wei et al., 2005), and vitronectin receptors αβ1, αβ2, and αβ3 (Carriero et al., 1999; Degryse et al., 2005). The formation of these uPAR–integrin interactions may depend both on integrin subunit expression and composition of the ECM (Xue et al., 1997). Association of uPAR with integrins has been proposed to alter integrin conformation (Wei et al., 2005). However, the existence of direct uPAR–integrin binding remains controversial, as a recent study has shown that the putative integrin-binding residues in uPAR are dispensable (Madsen et al., 2007). These authors proposed that uPAR interacts indirectly with integrins by increasing cell matrix adhesion through uPAR–vitronectin binding, therefore facilitating integrin binding to ligands.

Of particular interest in the context of cell motility is how uPAR signals to Rac activation. Cycling of small GTPases between active GTP-bound and inactive GDP-bound forms is regulated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP (Bos et al., 2007), and GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity (for review see Jaffe and Hall, 2005). Because many studies link uPAR to integrin signaling, we used a candidate approach to identify integrin-associated GEFs that might be required for Rac activation in uPAR-expressing cell lines. These studies identified DOCK180 as the GEF involved in uPAR-mediated Rac activation. We then investigated how uPAR influences signaling to DOCK180.

**Results**

**DOCK180 is required for uPAR-driven Rac activation and invasion**

Because uPAR may signal together with integrins, we examined the role of GEFs that have been linked to integrin signaling to identify GEFs that may function downstream of uPAR. A literature search identified α-PIX, β-PIX, DOCK180, Sos1, Tiam1, Tiam2, Vav1, Vav2, and Vav3 as potential Rac GEFs downstream of integrins (Kiyokawa et al., 1998; Moeres et al., 2000; Marignani and Carpenter, 2001; Matsuo et al., 2003; Rosenberger et al., 2003; Arthur et al., 2004; Gakidis et al., 2004; Faccio et al., 2005; Hamelers et al., 2005). We used RNAi to silence expression of these GEFs (apart from α-PIX, Vav1, and Vav3 for which no expression was detected) in the colon carcinoma cell line BE. BE cells endogenously express uPAR and exhibit a bipolar mesenchymal morphology with abundant membrane ruffling and lamellipodia shown by phalloidin staining to be F-actin rich (Fig. 1A). This characteristic morphology, together with extensive random migration, is abrogated by silencing uPAR or Rac (Vial et al., 2003). We used this easily scorable phenotype to search for GEFs whose silencing mimicked the effects of silencing uPAR. DOCK180 was the only GEF for which silencing resulted in similar effects to abrogating uPAR expression, resulting in flattening, loss of ruffles and lamellipodia, more pronounced cortical actin staining, and occasional stress fibers (Fig. 1A and Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1).

To show that uPAR signals through DOCK180 to activate Rac, we used ectopic expression in human embryonic kidney (HEK) 293T cells that lack endogenous uPAR. Transfection with a uPAR expression construct activates Rac approximately twofold (Fig. 1, B and C). Strikingly, this stimulation was lost when uPAR was expressed in HEK 293T cells in which DOCK180 expression had been abrogated with either of two different siRNAs (Fig. 1, B and C). Significantly, in empty vector controls, DOCK180 silencing had no effect on Rac activity, showing basal Rac activity in HEK cells that lack endogenous uPAR. In contrast, transfection with uPAR expression construct activated Rac to a similar extent (Fig. 1, A and C). Therefore, in this system DOCK180 is required for uPAR-driven Rac activation rather than basal levels of Rac activity.

To examine whether DOCK180 is required for Rac activation in tumor cell lines expressing uPAR, we used BE, MDA-MB-231 breast carcinoma cells, and SNB19 glioblastoma cells in which
endogenous uPAR signaling is required for cell motility or invasion (Mohan et al., 1999; Sturgeon et al., 2002; Vial et al., 2003). We silenced DOCK180 expression in these cell lines using a panel of siRNA oligonucleotides, including an ON-TARGET SMART pool that incorporates technology designed to reduce “off-target” effects. Each siRNA treatment abrogating DOCK180 expression significantly reduced Rac activation (Fig. 2 A). The degree of inhibition of Rac activation resulting from silencing DOCK180 was very similar to that from silencing uPAR (~50–60%; Fig. 2 A). As well as reducing Rac activation, siRNA treatments against either DOCK180 or uPAR elicited similar morphological changes in the three cell lines with reduced membrane ruffling and lamellipodial protrusion (Fig. S1 B and not depicted), demonstrating that the effects are a true consequence of silencing these genes rather than a nonspecific or off-target effect.

Because uPAR-driven Rac activation has been shown to promote invasion (Vial et al., 2003), BE and MDA-MB-231 cells were assayed for invasion of a three-dimensional collagen matrix in response to a chemotactic gradient of serum. Fig. 2 B shows that in both cell lines silencing DOCK180 or uPAR inhibited invasion to a comparable degree (40–50%). Confirming that loss of Rac reduced cell motility, time-lapse phase-contrast microscopy revealed a severe defect in random cell motility when DOCK180 or uPAR was silenced (Videos 1–3 [BE], available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1; and not depicted for MDA-MB-231 and SNB19).

These studies show that in an ectopic uPAR expression system and in three different tumor cell lines expressing endogenous uPAR, silencing DOCK180 results in reduced Rac activation. This suggests that uPAR signals through DOCK180 for uPAR-driven Rac activation and membrane protrusion, resulting in cell motility and invasion.

**uPAR drives tyrosine phosphorylation of p130Cas and formation of the Cas–Crk complex**

Because uPAR signals through DOCK180 to activate Rac, the roles of known upstream regulators of DOCK180 were examined to characterize the pathway linking uPAR and DOCK180. The regulation of DOCK180 by integrin signaling involves protein–protein interactions where the N-terminal SH3 domain of the adaptor protein Crk binds to a proline-rich region in the substrate domain (SD) of the adaptor p130Cas (Sakai et al., 1994). The p130Cas–Crk–DOCK180 module associates with integrins via binding of p130Cas to FAK (Polte and Hanks, 1995). To investigate whether uPAR influences p130Cas SD tyrosine phosphorylation and recruitment of Crk, we first examined the effects of ectopic uPAR expression in HEK 293T cells. Expression of uPAR results in an ~50% increase in tyrosine phosphorylation of the p130Cas SD (Fig. 3 A). Increased tyrosine phosphorylation of p130Cas was associated with a dramatic induction of the p130Cas–Crk complex, as determined by coimmunoprecipitation of Crk and p130Cas (Fig. 3 B). These results show that ectopic expression of uPAR drives formation of the p130Cas–Crk complex. As the p130Cas SD has been shown to be phosphorylated by Src family kinases (Vuori et al., 1996), and as uPAR has been linked to c-Src activity (Zhang et al., 2003), we investigated whether uPAR-dependent Rac activation required c-Src activity. Ectopic expression of uPAR in HEK 293T cells led to increased c-Src phosphorylation on the Y416 activation site and treatment with the Src inhibitors PP1 or PP2 or the structurally unrelated SU6656 blocked Rac activation, whereas PP3, the inactive stereoisomer of PP2, had no effect (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1).

To examine whether endogenously expressed uPAR signals through p130Cas and Crk, we silenced uPAR expression in the tumor cell lines. Silencing uPAR with each of three different siRNA oligonucleotides reduced p130Cas SD tyrosine phosphorylation by up to 40% in each cell line (Fig. 3 C). Similar results were observed in SNB19 and MDA-MB-231 cells (unpublished data). In all three tumor cell lines, the formation of the p130Cas–Crk complex was also strongly inhibited by silencing uPAR (Fig. 3 D).
To confirm that p130Cas and Crk are required for uPAR signaling to Rac activation, we used the ectopic uPAR expression system. Silencing p130Cas or Crk abrogated the 2–2.5-fold stimulation of Rac-GTP loading on uPAR expression in HEK 293T cells (Fig. 4, A and B). As with DOCK180 silencing (Fig. 1, B and C), silencing p130Cas or Crk did not affect basal Rac-GTP loading, demonstrating the specific role of p130Cas and Crk in uPAR signaling to Rac. Consistent with the findings from the ectopic expression studies, silencing the expression of either adaptor using three different siRNA oligonucleotides, including ON-TARGET SMART pools, in the endogenous uPAR-expressing tumor cell lines BE and SNB19 resulted in ~50–60% inhibition of Rac activity (Fig. 5 A).

These results argue that uPAR activates Rac through driving the formation of p130Cas–Crk complexes that could potentially recruit DOCK180 through association with Crk. To confirm that signaling through uPAR can recruit DOCK180 to complexes containing p130Cas, we immunoprecipitated DOCK180 and

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Figure 2. Requirement for \(\beta_3\) integrin in uPAR-driven Rac activation

Our data show that uPAR expression drives tyrosine phosphorylation of the p130Cas SD, promoting the formation of the p130Cas–Crk complex that recruits DOCK180. This leads to Rac activation and acquisition of a motile, invasive phenotype in tumor cell lines. Because the p130Cas–Crk–DOCK180 pathway is known to be activated by integrin-mediated adhesion (Kiyokawa et al., 1998), we investigated which integrins are involved.

Figure 4. p130Cas and Crk are required for uPAR-stimulated Rac activation in HEK 293T cells.

Figure 5. p130Cas and Crk are required for Rac activation and invasion in uPAR-expressing tumor cell lines.

To demonstrate that p130Cas–Crk complex signaling to Rac contributes to tumor cell invasion, we studied whether abrogating expression of p130Cas or Crk affects BE cell invasion of a three-dimensional collagen matrix. Fig. 5 C shows that silencing p130Cas or Crk inhibited invasion by ∼40–50%. Silencing p130Cas or Crk has similar effects on Rac activation (Fig. 1 C) or invasion (Fig. 2 B) to when either uPAR or DOCK180 is silenced (Fig. 2, A and B).

Requirement for \(\beta_3\) integrin in uPAR-driven Rac activation

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BE, MDA-M231, and SNB19 cells express αβ3, αβ5, and β1 integrin (see Fig. 7 D; Fig. S5 D, available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1; and not depicted), which have been reported to interact with uPAR (Carriero et al., 1999; Degryse et al., 2005; Chaurasia et al., 2006). Blocking antibody and siRNA experiments showed that αβ3 and αβ5, but not uPAR, are required for adhesion to vitronectin (Fig. S5, A–C). To investigate the involvement of integrins in uPAR signaling to Rac activation, β integrin subunits were silenced using panels of siRNA oligonucleotides consisting of two individual oligonucleotide duplexes and one ON-TARGET SMART pool for each target (ITGB1#1, ITGB1#2, and ITGB1 OT for targeting β1 integrin; ITGB3#1, ITGB3#2, and ITGB3 OT for targeting β3 integrin; and ITGB5#1, ITGB5#2, and ITGB5 OT for targeting β5 integrin). Only silencing of β1 integrin mimicked the phenotype of uPAR or DOCK180 silencing in BE cells (Fig. S4, A and B). Similar effects were observed in MDA-MB-231 and SNB19 cells (unpublished data). Silencing αβ3 resulted in loss of polarity and delocalization of membrane ruffling whereas silencing αβ5 resulted in defects in adhesion and rear retraction (Fig. S4, A and B).

Rac pull-down assays showed that silencing β1 integrin but not β1 integrin in BE, MDA-MB-231, and SNB19 cells decreased Rac activation (Fig. 6 A). Consistent with the lack of an effect on membrane ruffling, silencing β1 integrin had no effect on Rac activation (Fig. S4 C). The effect of silencing β1 integrin...
is very similar in magnitude to that observed when uPAR, DOCK180, Crk, or p130Cas is silenced. Consistent with the fact that αβ3 is a major vitronectin receptor (Cheresh and Spiro, 1987), we found that silencing uPAR or β3 integrin only affected Rac activity in BE cells plated on vitronectin and not on collagen or fibronectin, which are major β1 integrin ligands (Fig. S5 E). Similarly, ectopic expression of uPAR in HEK 293T cells led to Rac activation if the cells were plated in serum-free medium on vitronectin or in serum as a source of vitronectin, but there was no Rac activation if the cells were plated in serum-free medium on fibronectin or collagen-1 (Fig. S5 F).

The contributions of signaling through β1 and β3 integrin subunits to invasion were examined by testing β1- and β3-silenced BE and MDA-MB-231 cells for invasion of a three-dimensional collagen-1 matrix (Fig. 6 B). In both BE and MDA-MB-231 cells, silencing β3 integrin inhibited invasion by ∼40%. Invasion in these assays was dependent on vitronectin present in serum as no invasion took place in the absence of serum, and the addition of a vitronectin-blocking antibody (Zanetti et al., 1994) blocked serum-dependent invasion (Fig. 6 C). This was consistent with the observed effects on invasion of silencing uPAR, DOCK180, Crk, and p130Cas in these cells. Although it did not affect Rac activation, silencing β3 integrin decreased invasion by ∼90% in BE cells and 40–50% in MDA-MB-231 cells. This is not unexpected because all collagen-binding integrins contain the β3 subunit, and adhesion to the substratum is essential for the elongated/mesenchymal mode of migration (Pollard and Borisy, 2003).

To investigate whether β1 integrin was required for uPAR-driven Rac activation in HEK 293T cells, integrin subunits were silenced with siRNA; Fig. 6 (D and E) shows that silencing β1 in HEK 293T cells blocked Rac activation. However, unlike the tumor cell lines, β1 silencing did reduce Rac activation in uPAR-transfected HEK 293T cells. Significantly, flow cytometry showed that αβ3 was not expressed at the surface of control or empty vector–transfected HEK 293T cells but was expressed at the surface of uPAR-transfected cells (Fig. 7 A). Previous work has shown that sustained extracellular signal–regulated kinase (ERK) activation leads to surface expression of β3 integrin (Woods et al., 2001), and as uPAR-mediated ERK activation has been shown to be β1 dependent (Aguirre Ghiso et al., 1999), we examined whether β1 integrin expression and ERK activation were required for surface expression of β3. Fig. 7 A shows that silencing β1 integrin or treatment with the MAPK/ERK kinase (MEK) inhibitors PD184352 or U0126 (unpublished data) blocked surface expression of αβ3 in uPAR-transfected HEK 293T cells. Silencing β3 integrin but not β1 integrin blocked uPAR-dependent ERK activation, showing that ERK activation by uPAR requires β3 but not β1 integrin (Fig. 7 B). Consistent with the observations that β1 integrin signals to ERK activation and surface expression of αβ3, inhibition of ERK activation with MEK inhibitors PD184352 or U0126 blocked Rac activation in uPAR-transfected HEK 293T cells (Fig. 7 C). These results show that signaling via uPAR and β1 integrins to ERK activation can provide the surface localization of αβ3 required for uPAR-dependent Rac activation. Although β1 integrin–dependent ERK activation was required for surface expression of β3 integrin, immunoblotting showed that uPAR expression, β1 integrin knockdown, or inhibition of ERK activation did not affect total cell levels of β1 integrin (Fig. 7 A).

In contrast to HEK 293T cells, uPAR or β3 integrin silencing in BE colon carcinoma cells did not affect the surface localization of αβ3 integrin (Fig. 7 D); however, surface expression of αβ3 integrin in BE, MDA-MB231, and SNB19 was partially dependent on ERK activation (Fig. 7 D and Fig. S5 D). In none of the tumor cell lines was β1 integrin or uPAR required for ERK activation (Fig. 7 E). BE and MDA-MB231 harbor activating mutations in KRAS and BRAF or KRAS alone (Vial et al., 2003) that presumably uncouple ERK activation from a requirement for β1 integrin and uPAR. These results therefore argue that uPAR signals through αβ3 for Rac activation, but that uPAR signaling through β1 integrins can provide an ERK signal for surface localization of αβ3.

β3 integrin is required for p130Cas SD tyrosine phosphorylation and formation of the p130Cas-CrkII complex

Because β3 integrin–silenced cells had defects in morphology, Rac activation, and invasion similar to those observed in cells where components of the uPAR–DOCK180 pathway had been silenced, we examined the roles of β3 integrin subunits in signaling through the p130Cas–CrkII adaptor complex. Silencing β3 integrin in BE, MDA-MB231, and SNB19 tumor cells strongly reduced tyrosine phosphorylation of the p130Cas SD (Fig. 8 A). Conversely, silencing β1 integrin did not affect p130Cas SD phosphorylation. In keeping with the p130Cas SD tyrosine phosphorylation data, coimmunoprecipitation of p130Cas with CrkII was also inhibited by silencing β3 integrin, whereas silencing of β1 integrin had no effect (Fig. 8 B). These data show that expression of uPAR promotes signaling through β3 integrin to drive tyrosine phosphorylation of the p130Cas SD and formation of the p130Cas–CrkII adaptor complex. Consistent with the requirement for Src in Rac activation driven by uPAR, silencing β3 integrin blocked Src activation driven by uPAR (Fig. S3, B and D) but did not affect FAK Y397 phosphorylation (Fig. S3 C). Silencing β3 integrin also abrogated the stimulation of p130Cas SD tyrosine phosphorylation by ectopically expressed uPAR in HEK 293T cells (Fig. 8 C). However, as observed for uPAR-driven Rac activation, silencing β3 integrin in HEK 293T cells also abrogated uPAR-driven p130Cas SD tyrosine phosphorylation. This is consistent with the role of β3 integrin in promoting cell surface expression of β3 integrin by cooperating with uPAR to activate ERK, a function of β3 integrin that is not required in the tumor cells where ERK activity does not require β1 integrin (Fig. 7 E).

Discussion

In this study we have identified a mechanism of Rac activation by uPAR. We show for the first time that in both ectopic and endogenous systems uPAR expression results in activation of Rac via the GEF DOCK180. DOCK180 has been shown to have a role in cell motility (Klemke et al., 1998) and developmental processes such as myoblast fusion, dorsal closure, and phagocytosis.
Figure 7. Cooperation between uPAR signaling with \( \beta_1 \) integrin to ERK-MAPK activation and \( \beta_3 \) integrin–dependent Rac activation. (A) Cell surface expression of \( \alpha_\beta_3 \) in uPAR- or vector-transfected HEK 293T cells. Top three left panels, siRNA-transfected cells; top right panel, 2 \( \mu \)M PD184352 or vehicle (DMSO) treatment. Red, IgG control; blue, \( \alpha_\beta_3 \) vector transfected; green, uPAR – \( \alpha_\beta_3 \) transfected. (bottom) \( \beta_3 \) integrin immunoblot of siRNA-transfected or PD184352-treated HEK 293T cells. (B) HEK 293T cells were transfected as in A and ERK1/2 activation was measured (mean ± SEM; \( n = 6 \)). *, \( P < 0.05 \); unpaired Student’s \( t \) test. (C) Rac activation in HEK 293T cells transfected with uPAR or vector and treated with MEK inhibitors or vehicle (DMSO)
of apoptotic cells (Nolan et al., 1998; Wu and Horvitz, 1998; Moore et al., 2007). Our data show that uPAR signaling to DOCK180 results in the induction of tumor cell motility and invasion. In several systems, ELMO has been linked to DOCK180 function possibly through acting as a cofactor for GEF activity (Gumieny et al., 2001; Brugnera et al., 2002). Whether it is involved in uPAR-driven Rac activation will be an interesting topic for future investigation.

Having identified DOCK180 as a Rac GEF regulated by uPAR, we examined how DOCK180 is activated downstream of uPAR. Previous work in other systems shows that integrin signaling recruits DOCK180 to the plasma membrane via the formation of a p130Cas–Crk–DOCK180 complex. Key to the formation of this complex is tyrosine phosphorylation of the p130Cas SD that recruits Crk–DOCK180 complexes via the SH2 domain of Crk. We show that p130Cas, expressed endogenously by tumor cells or ectopically in HEK 293T cells, drives the tyrosine phosphorylation of the p130Cas SD and formation of the p130Cas–Crk complex. For Rac activation by uPAR, uPA does not seem to be essential (Kjoller and Hall 2001) and is not expressed by HEK 293T cells (Wei et al., 1994). However, uPA–uPAR interactions may play an important role in other systems or in tumor cell invasion in vivo, whether by enhancing uPAR binding to vitronectin or through mechanisms such as focused ECM proteolysis at the leading edge or enhancing local availability of growth factors. Consistent with uPAR signaling through the p130Cas–Crk complex, we find that p130Cas and Crk are required for Rac activation by uPAR and for the invasion of uPAR-expressing tumor cells.

p130Cas is a multifunctional adaptor protein required for embryonic development and oncogenic signal transduction in tumor cells (Auvinen et al., 1995; Niewers et al., 1997; Honda et al., 1998; Kirsch et al., 2002; Cabodi et al., 2006). It is also an important regulator of cell migration, and in particular its association with Crk constitutes a molecular switch vital for cell motility by recruiting DOCK180 to integrin-containing adhesion complexes (Klemke et al., 1998). These complexes also serve a mechanosensory function allowing the cell to sense the physical properties, such as rigidity, of the ECM. Consistent with uPAR signaling through the p130Cas–Crk complex, interestingly, in vitro data suggests that p130Cas can function as a transducer of mechanical signals, with the SD adopting an extended conformation permissive for phosphorylation in response to increased physical force (Sawada et al., 2006). This could promote Rac-driven migration in response to physical cues in the extracellular environment. As we have shown that uPAR stimulates the tyrosine phosphorylation of the p130Cas SD, the role of uPAR in integrin-mediated mechanotransduction is an interesting subject for future investigation. In addition, it is well known that p130Cas and Crk can interact with other partners besides DOCK180. Therefore, promotion of p130Cas SD tyrosine phosphorylation and p130Cas–CrkII complex assembly by uPAR may regulate a variety of other signaling pathways.

The identification of DOCK180, an integrin-associated GEF, in uPAR-Rac signaling is in keeping with a large body of evidence implicating integrins as the signaling partners of uPAR. A relatively large array of integrin heterodimers have been shown to interact with uPAR, but whether any of these are specifically required for uPAR to activate Rac in tumor cells had not been previously investigated. In the tumor cell lines we examined, β3 but not β1 integrin was required for uPAR–DOCK180 signaling to Rac activation. In HEK 293T cells, β1 as well as β3 integrin are required for uPAR-stimulated Rac activation, but in these cells the role of β1 integrin appears to be to provide the ERK activation (Aguirre-Ghiso et al., 1999) necessary for surface expression of αvβ3. Thus, in some cells uPAR–β1 integrin signaling to ERK-dependent surface expression of αvβ3 cooperates with uPAR–αvβ3 signaling for Rac activation, whereas in other cells uPAR–β3 integrin drives Rac activation but ERK activation does not seem to require uPAR or β1 integrin signaling.

Several papers have emphasized the importance of vitronectin in membrane protrusion and cell motility induced by uPAR expression, and both integrin signaling and direct binding of uPAR to vitronectin were recently shown to be required for stimulation of membrane ruffling and lamellipodial protrusion by ectopic uPAR expression in HEK 293T cells (Kjoller and Hall, 2001; Madsen et al., 2007). We have shown that uPAR and β3 integrin are required for Rac activation in tumor cells cultured on vitronectin or in the presence of serum, which is an abundant source of vitronectin with concentrations in the range of 200 to 400 μg/ml (Schwartz et al., 1999). In the collagen-I–based three-dimensional invasion assay we have used, a function-blocking antivitronectin antibody inhibits serum-stimulated invasion, demonstrating that invasion is dependent on vitronectin. Consistent with its role in uPAR-driven Rac activation in the presence of vitronectin, silencing β3 integrin expression also inhibits serum-stimulated invasion.

These data suggest that uPAR and β3 integrin engage vitronectin to promote Rac activity and tumor cell invasion. The nature of uPAR–integrin interactions is controversial. Although many studies have shown uPAR–integrin communoprecipitation, this does not prove the existence of direct binding. Immunoprecipitation under gentle conditions may result in the detection of many proteins associated with detergent-resistant lipid rafts, including uPAR and integrins. The study of Madsen et al. (2007) has cast doubt on the role of specific uPAR residues in mediating binding to integrins, although it does not rule out direct interactions involving multiple residues over a large binding surface. Our data are consistent with a model where both uPAR and β3 integrin coordinately engage vitronectin. This could affect signaling in several ways, for example, by facilitation of integrin–ligand interaction, effects on integrin clustering, or modification of integrin...
which is a major regulator of polarized migration (Etienne-Manneville, 2004). Also by inhibiting internalization of lipid rafts, which contain both uPAR and binding sites for activated Rac (del Pozo et al., 2004), and by regulating interactions with RhoGDI (Del Pozo et al., 2002), \( \beta_1 \) integrin may affect spatial control of Rac-driven protrusion and motility.

Determination of the molecular mechanisms underlying uPAR signaling, such as the Rac activation pathway described here, is essential to provide insight into the well-established role of uPAR in tumor cell invasion. Understanding these pathways will provide new therapeutic targets for the prevention of human tumor metastasis.

Figure 8. \( \beta_3 \) integrin is required for p130Cas SD tyrosine phosphorylation and formation of the p130Cas–CrkII complex. (A) p130Cas SD tyrosine phosphorylation in siRNA-transfected cells. Left, representative immunoblot (BE); right, quantitation (mean + SEM; \( n = 5 \)). *, \( P < 0.05 \); **, \( P < 0.01 \); unpaired Student’s t test. Closed bars, BE; open bars, MDA-MB-231; shaded bars, SNB19. Irrelevant lanes were removed (represented by vertical black lines). (B) Analysis of p130Cas–Crk complexes. Left, representative immunoblots (BE); right, quantitation (mean + SEM; \( n = 4 \)). *, \( P < 0.05 \); **, \( P < 0.01 \); unpaired Student’s t test. Closed bars, BE; open bars, MDA-MB-231; shaded bars, SNB19. (C) Analysis of p130Cas SD tyrosine phosphorylation in HEK 293T cells transfected with siRNAs and uPAR or empty vector was performed as described in Fig. 3 (mean + SEM; \( n = 4 \)). *, \( P < 0.05 \); unpaired Student’s t test. Immunoblots showing knockdown are in Fig. S2 E, available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1.
Materials and methods

Antibodies and reagents

The following antibodies were used: anti-Rac1 (clone 23AB; Fitzgerald), anti-uPAR (R&D Systems), anti-GAPDH (Novus Biologicals), anti-DOCK180, anti–β integrin, anti–β integrin (clone P5D2; Santa Cruz Biotechnology, Inc.), anti–β integrin (clone J10A), anti–β integrin rabbit polyclonal, anti–αv,β3 (LM609), anti-α5,β1 (PI16), antivitronectin (clone BV2; Millipore), anti-p130Cas, anti-Crk, anti-FAK (BD Biosciences), anti–α tubulin, anti-thapsigargin (Sigma-Aldrich), anti-p130Cas phosphotyrosine 410 (Cell Signaling Technology), anti–α–Src (clone GD11; Millipore), anti-Src phosphoY416 (Invitrogen), anti-FAK phosphoY939 (Affinity Bioreagents), and mouse IgG isotype controls (R&D systems). Vitronectin and fibronectin (purified from human serum) were purchased from Sigma-Aldrich. Bovine Type I collagen solution was purchased from Invitrogen. PD184352 was obtained from C. Springer (Institute of Cancer Research, Sutton, England, UK) and U0126 was obtained from Sigma-Aldrich. PKP1, PP2, PP3, and SU6656 were purchased from EMD). Texas red–labeled phalloidin was purchased from Invitrogen. pCMV-MbPA was provided by A. Hall (Memorial Sloan-Kettering Cancer Center, New York, NY). HRP-coupled secondary antibodies were from purchased Sigma-Aldrich and fluorescent-coupled secondary antibodies were from purchased Li-COR Biosciences.

Cell culture

BE colon carcinoma cells were obtained from the Institute of Cancer Research Tissue Resource Laboratory; HEK 293T and MDA-MB-231 breast carcinoma cells from the American Type Culture Collection; and SNB19 glioblastoma cells from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. All cells were maintained in DME, supplemented with 10% FCS purchased from PAA Laboratories, 100 μg/ml streptomycin, and 60 μg/ml penicillin. Cells were maintained at 37°C and 10% CO2. siRNA transfections in tumor cell lines were performed using InterferIN (Polyplus) according to the manufacturer’s instructions. The final concentration of siRNA in the transfection was 2 nM. For siRNA transfection of HEK 293T cells, HiPerfect (QIAGEN) was used with 50-nM final concentration of siRNA according to the manufacturer’s instructions. Sequences of siRNA were made against the nontargeting control and ON-TARGET SMART Pools were compared with ON-TARGET nontargeting controls. Transfection of HEK 293T cells with plasmid DNA (6 μg DNA per 10-cm cell culture dish) was performed using GeneJuice (EMD) and incubated at 4 °C overnight or for 3 h on a rotating wheel with antibody

24 h at a rate of one frame per site per 4 min. Movies were exported from Simple PCI software as uncompressed AVI files with a frame rate of 15 frames per second. Premiere (v6.0, Adobe) was used to compress movie files using the MPEG codec, which were then converted to MOV (Quicktime) format using iMovie HD with a frame rate of 15 frames per second (dimensions: 640 x 512).

Confocal sections were obtained with a laser-scanning confocal imaging system (MRC 1024; Bio-Rad Laboratories) mounted on an upright fluorescence microscope (E600; Nikon) with Plan Apo 60x oil immersion objective (NA 1.4) at 21°C, and using LaserSharp acquisition software (Bio-Rad Laboratories). Images were exported as PIC files and processed for brightness and contrast using Photoshop, supplemented with PIC file recognition plug-in (Bio-Rad Laboratories).

Analysis of Rac activation

A GST fusion of the CRIB domain of PAK1 was used to pull down the activated form of Rac (Benard et al., 1999). The PAK1-CRIB domain GST fusion protein was bound to glutathione-Sepharose beads (GE Healthcare). 5 x 105-106 cells in a 10-cm dish were washed in Rac wash buffer (50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT [Sigma-Aldrich], and EDTA-free complete protease inhibitors [Roche]) and lysed on ice for 3 min in ice-cold Rac lysis buffer (50 mM Tris-HCl, 10% glycerol, 1% NP-40, 5 mM MgCl2, 100 mM NaCl, and EDTA-free complete protease inhibitors). Lysates were cleared by centrifugation at 13,200 rpm for 5 min in a centrifuge (5810R; Eppendorf) at 4°C and an aliquot was kept for determination of total Rac levels by Western blotting. The remainder of the lysate was incubated with 30 μl PAK-CRIB-Sepharose beads for 45 min at 4°C, rotating wheel at 4°C. Beads were collected by brief centrifugation and washed three times in 500 μl of ice-cold Rac lysis buffer and re-suspended in 20 μl of LDS sample buffer (Invitrogen), and electrophoresis (NOVEX NuPAGE Midi gel system; Invitrogen) was performed with the total volume of each Rac pull down and equivalent volumes of total cell lysate for determination of total Rac. Fluorescent immunoblotting of Rac in pull downs and total lysate used anti-Rac clone 23AB (Fitzgerald) and the Odyssey (Li-COR Biosciences). Data from the Odyssey were exported into Excel (Microsoft) and the ratio of signals for Rac GTP/Total Rac was calculated for each sample. For tumor cell lines, data are normalized to values from mock-transfected cells. Statistical comparisons for each siRNA were made against the nontargeting control and ON-TARGET SMART Pools were compared with ON-TARGET nontargeting controls. For HEK 293T cells, data are shown as fold stimulation of Rac activation, obtained by dividing the Rac activation in uPAR-transfected cells by that of the vector control for each condition.

Invasion assay

Cells were suspended in 2.3 mg/ml of serum-free liquid bovine collagen at 106 cells/ml. 100-μl aliquots were dispensed into black 96-well View-Plates (PerkinElmer) coated with bovine serum albumin. Plates were centrifuged at 300 g and incubated in a 37°C/10% CO2 tissue culture incubator. Once collagen had polymerized, FCS was added on top of the collagen to a final concentration of 5%. For vitronectin-blocking studies, 20 μg/ml antivitronectin antibody or isotype-matched IgG control were preincubated with FCS for 30 min at room temperature. After 24 h incubation at 37°C in 1% CO2, cells were fixed and stained for 2 h in 4% formaldehyde solution (Sigma-Aldrich) containing 5 μg/ml Hoechst 33258 nuclear stain (Invitrogen). Confocal z slices were collected from each well at 50 μm to count invaded cells, and at the bottom (3 μm) to count total cells using a high content microscope (INCELL3000; GE Healthcare) with a 40x PlanFluo ELWD objective (0.6 N.A.; Nikon). Nuclear staining in each slice was quantified automatically using INCELL3000 Object Intensity module to determine the percentage of invaded cells. Samples were run in quadruplicate and averaged. Data analysis was performed using Excel. Invasion index was calculated at number of cells at 50 μm per total number of cells. Data are presented as a percentage of the invasion index of mock-transfected cells. Statistical comparisons for siRNAs were made against the nontargeting control, and ON-TARGET SMART Pools were compared with ON-TARGET nontargeting controls.

Immunoprecipitation and immunoblotting

Cells were grown in 10- or 15-cm plates and lysed in 1% NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 25 mM sodium β-glycerophosphate, 1 mM sodium vanadate, 5 mM NaF, and complete protease inhibitors). A minimum of 1 mg of total cellular protein was incubated at 4°C overnight or for 3 h on a rotating wheel with antibody

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and complexes, and were then precipitated with 25 μl of protein G-Agarose (Thermo Fisher Scientific) for 20 min on a rotating wheel at 4°C. Beads were washed at least three times in 500 μl of lysis buffer and resuspended in NuPAGE LDS sample buffer before SDS-PAGE. Fluorescent immunoblotting was conducted using the Odyssey infrared scanner according to the manufacturer’s protocols (LI-COR Biosciences). Fluorescence data from the Odyssey were exported into Excel. For phosphorylation analysis, signals from phosphospecific antibodies were divided by that of antibodies recognizing total protein (e.g., PY410/Total p130Cas). For p130Cas-Crk complexes, p130Cas signal was divided by Crk signal. For siRNA experiments in tumor cells, data were normalized to values from mock-transfected cells. For HEK 293T cells, data are presented as fold stimulation of phosphorylation, obtained by dividing the phospho/total ratio in upAR-transfected cells by the vector control. In all cases, statistical comparisons for each siRNA were made against the nontargeting control, and ON-TARGET SMART Pools were compared with ON-TARGET nontargeting controls.

Quantitative PCR
Total cellular RNA was isolated from cultured cells using Trizol (Invitrogen) or the RNeasy Mini kit (QIAGEN). Real-time RT-PCR amplifications were performed using the Brilliant II SYBR Green QRT-PCR Master Mix kit (Stratagene). A standard curve was constructed using a range of 0.01 to 10 ng RNA from BE cells for each set of primers used. Relative quantitation was performed using the ∆∆Ct method. All primers used were Quantitect SYBR green primer assays (QIAGEN). Reactions were performed in triplicate in 50 μl volumes containing 25 μl of 2x Brilliant II mastermix, 5 μl of 10x Quantitect SYBR green primer assay, 1 μl of RT- RNase-block enzyme mixture (Stratagene), and the appropriate amount of RNA with remaining volume made up with nuclease-free water (Ambion). PCR was performed in a Fast Real-Time PCR cycler (7900HT, Applied Biosystems). Data were analyzed using SDS software (Applied Biosystems).

Flow cytometry
Detached cells (5 × 10^6) were stained on ice for 45 min using 10 μg/ml LM609, 10 μg/ml P1F6, or 1 μg/ml PDS2 to detect αβ3, αβ5, and β1, respectively. Alexa flour 488-conjugated goat anti-mouse F(ab)2 fragment used for detection (at 1:250) was obtained from Invitrogen. Cells were analyzed on an LSR II flow cytometer (BD Biosciences).

Adhesion assays
Adhesion assays were performed according to the method of Cunningham et al. (2003). Cells were detached by short incubation with trypsin, counted, and washed in serum-free medium. 3 × 10^4 cells were allowed to attach to plates (7900HT; Applied Biosystems). Data were analyzed using SDS software (Applied Biosystems).

Online supplemental material
Fig. S1 shows a morphological screen of integrin-associated Rac GEFs. Fig. S2 shows siRNA-mediated knockdown of upAR, DOCK180, p130Cas, Crk, and integrin subunits. Fig. S3 shows that upAR-driven Rac activity in 293T cells requires β3 integrin–dependent Src activation. Fig. S4 shows that silencing of αβ3 integrin subunits in BE colon carcinoma cells affects cell morphology. Fig. S5 shows that upAR-driven Rac activation is vitronectin dependent but adhesion to vitronectin requires αvβ3 or αvβ5, but not upAR. Video 1 shows control BE colon carcinoma cells. Video 2 shows BE colon carcinoma cells transfected with siRNA-targeting upAR. Video 3 shows BE colon carcinoma cells transfected with siRNA-targeting DOCK180. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712050/DC1.

References

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