Integrin $\alpha_6\beta_4$ cooperates with LPA signaling to stimulate Rac through AKAP-Lbc-mediated RhoA activation

Kathleen L. O’Connor, Min Chen, and L. Nicole Towers

Markey Cancer Center and Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky

Submitted 31 March 2011; accepted in final form 1 November 2011

O’Connor KL, Chen M, Towers LN. Integrin $\alpha_6\beta_4$ cooperates with LPA signaling to stimulate Rac through AKAP-Lbc-mediated RhoA activation. Am J Physiol Cell Physiol 302: C605–C614, 2012. First published November 2, 2011; doi:10.1152/ajpcell.00095.2011.—The $\alpha_6\beta_4$ integrin promotes carcinoma invasion through its ability to promote directed migration and polarization of carcinoma cells. In this study, we explore how the $\alpha_6\beta_4$ integrin cooperates with lysophosphatidic acid (LPA) to activate Rho and Rac small GTPases. Through the use of dominant negative Rho constructs, C3 exotransferase, and Rho kinase inhibitor, we find that Rho is critical for LPA-dependent chemotaxis and lamellae formation. However, utilization of specific Rho isoforms depends on integrin $\alpha_6\beta_4$ expression status. Integrin $\alpha_6\beta_4$-negative MDA-MB-435 cells utilize only RhoC for motility, whereas integrin $\alpha_6\beta_4$-expressing cells utilize RhoC but additionally activate and utilize RhoA for LPA-dependent cell motility and lamellae formation. Notably, the activation of RhoA by cooperative LPA and integrin $\alpha_6\beta_4$ signaling requires the Rho guanine nucleotide exchange factor AKAP-Lbc. We also determine that integrin $\alpha_6\beta_4$ cannot activate Rac1 directly but promotes LPA-mediated Rac1 activation that is dependent on RhoA activity and de novo $\beta_1$ integrin ligitation. Finally, we find that the regulation of Rac1 and RhoA in response to LPA is differentially regulated by phosphodiesterases, PKA, and phosphatidylinositol 3-kinase, thus supporting their spatially distinct compartmentalization. In summary, signaling from integrin $\alpha_6\beta_4$ facilitates LPA-stimulated chemotaxis through preferential activation of RhoA, which, in turn, facilitates activation of Rac1.

First published November 2, 2011; doi:10.1152/ajpcell.00095.2011.

Address for reprint requests and other correspondence: K. O’Connor, Markey Cancer Center and Dept. of Molecular and Cellular Biochemistry, Univ. of Kentucky, BBSRB Rm. B369, 741 South Limestone St., Lexington, KY 40506-0509 (e-mail: kloconnor@uky.edu).

http://www.ajpcell.org

0363-6143/12 Copyright © 2012 the American Physiological Society

C605

In normal epithelia, the integrin $\alpha_6\beta_4$ mediates stable adhesion through the formation of hemidesmosomes where it binds to intermediate filaments and can function as a tumor suppressor (44, 59). However, in late stage carcinomas, the $\alpha_6\beta_4$ integrin binds the actin cytoskeleton (43) and is associated with a highly invasive and motile phenotype (25). The $\alpha_6\beta_4$ integrin promotes carcinoma cell motility either through the direct binding of laminin or indirectly through the cooperation with receptors for growth factor, such as hepatocyte growth factor (52), EGF (29), and lysophosphatidic acid (LPA; Ref. 38). Dissecting the signaling pathways enhanced by $\alpha_6\beta_4$ has revealed that this integrin promotes signaling from several proinvasive molecules including Erb-B2 (12), phosphatidylinositol 3-kinase (PI3K; Ref. 51), transcription factors such as nuclear factor of activated T cells (21) and NF-kB (62), cAMP-specific phosphodiesterase (PDE; Ref. 38), and Rac1 (51, 62) and RhoA small GTPases (37). Notably, exogenous expression of this integrin in $\beta$-deficient MDA-MB-435 carcinoma cells facilitates an exaggerated polarization of the cell (i.e., resulting in a distinct front and rear) and stimulates chemotaxis 5- to 20-fold in response to LPA (38).

LPA is a bioactive phospholipid that signals through EDG family of G-protein-coupled receptors and is recognized as an important mediator of tumor progression due to its abundance in tumor tissues and ability to promote mitosis, cell motility, tumor invasion, and angiogenesis. LPA is well documented to signal to the Rho family of small GTPases including the prototypical members RhoA and Rac1 (33).

Rac and Rho GTPases regulate distinct aspects of actin cytoskeletal reorganization that occur in separate regions of a cell during migration. That is, Rac1 has been shown to work at the leading edge where it promotes polymerization of the actin cytoskeleton (17, 18, 23). RhoA activity, in contrast, is required in the body and rear of the cell where it controls contractile and retractile forces needed for translocation (16, 18, 60). However, recent studies (40) using fluorescence resonance energy transfer reporter analyses of Rho family member activities show that RhoA is activated at the leading edge of cells. Moreover, in cells of epithelial origin, RhoA can be utilized in the formation of lamellae (11, 35, 37) rather than simply for the formation of stress fibers as seen in other cell types. One possible reason for this difference may be due to the expression of the $\alpha_6\beta_4$ integrin, which can activate RhoA in carcinoma cells upon binding to laminin (37).

In this study, we explore the cooperation of integrin $\alpha_6\beta_4$ and LPA signaling. We show that the cooperation of LPA and integrin $\alpha_6\beta_4$ signaling can preferentially promote the activation of RhoA downstream of the Rho guanine nucleotide exchange factor (GEF) AKAP-Lbc. We further show that this activation of RhoA plays a major role in promoting cell motility, lamellae formation, and the activation of Rac1.
activation of Rac, however, cannot be achieved by αdβ4-integrin signaling alone as it requires cooperative signaling with β3 integrins and LPA. Despite this dependence between Rho and Rac, we find that Rac and Rho are differentially regulated by PDEs, PKA, and PI3K lending credence to the idea that Rac and Rho require spatial regulation during cell motility in response to LPA.

**MATERIALS AND METHODS**

**Cell treatments.** The culture and characterization of clones of the MDA-MB-435 human carcinoma resulting from stable transfection of the integrin β4 subunit (MDA/β4; clones 5B3 and 3A7) or vector alone (MDA/mock; clones 6d2 and 6d7) have been described previously (38, 51). These clones were originally selected to have comparable levels of αc and β4 integrins on their cell surface. The MDA/β4 clones have both αdβ4 and αβ1 expression, with a slight reduction in overall β4-integrin expression (51). For all experiments, subconfluent cell cultures were harvested with trypsin and rinsed with RPMI medium containing 250 µg/ml heat-inactivated BSA (RPMI/BSA).

To assess the impact of chemoattractant, cells were plated onto collagen-coated dishes in RPMI/BSA and allowed to attach and spread for 2–3 h. Before chemoattractant stimulation, cells were treated with the indicated drug for 30 min or with the β1 integrin-specific mAb Mc13 or control IgG for 5 min. Cells were then treated with 100 nM LPA (18:1 oleoyl-ω-ω-LPA; Sigma) for 5 min before extraction, unless indicated otherwise, and assayed for Rho or Rac activity as described below. Alternatively, serum-starved cells (3 × 10^6) were incubated with 4 µg of anti-β1-mAb Mc13 (mouse anti-β1 integrin mAb, from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC), anti-αc mAb GoH3 (rat mAb; Santa Cruz Biotechnology or Millipore), or anti-β4 rat mAb 439–9B [rat anti-β4 integrin mAb, from Rita Falcioni, Regina Elena Cancer Institute (Rome, Italy) or from BD Biosciences] for 15 min, rinsed, left in suspension, or plated on secondary antibody coated 60-mm dishes at 37°C for 30 min as done previously (37) before being harvested for Rac assay. Where indicated, cells were treated with 30 µM Y27632, 1 mM isobutylmethylxanthine, 15 µM H-89 (Calbiochem), 1 µM protein kinase A inhibitor, 25 µM Ly29002, or 100 nM wortmannin (Sigma) for 15 min before use. H-89 was dissolved in water. All other drugs were solubilized in DMSO. For use of Mc13 as a β1-integrin function blocking antibody, cells were pretreated with 4 µg antibody for 5 min before addition of 100 nM LPA and harvest for Rac assay activities.

cDNAs and transient transfections. Exogenous expression of N19RhoA, N19RhoB, N19RhoC, and N17Rac1 was achieved by electroporation as described previously (37) or by lipid-mediated gene transfer (Lipofectamine 2000; Invitrogen). Cells (10-cm dishes) were transfected with 1 µg of either pCS2-(n)β-gal or pGFP and 4 µg of either control vector or vector containing myc-tagged N19RhoA (36), myc-tagged N19RhoC (see below), or GST-tagged N17Rac1 (obtained from Margaret Chou, University of Pennsylvania). Recombinant protein expression was confirmed by immunoblotting transfected cell extracts for myc-tag expression (for Rho constructs; mouse anti-myc mAb; Invitrogen) or by concentrating expressed GST-Rac based on established protocols (40, 48) and performed as described previously (37). Briefly, RhoA and Rac1 activity assays were performed by incubating cell extracts with glutathione heads (Pharmacia Biotech) coupled with bacterially expressed GST-TRBD fusion protein (provided by Martin Schwartz, University of Virginia) or Rac/cdc42 binding domain of Pak (PBD)-GST fusion protein (obtained from Rick Cerione, Cornell University), respectively, and then electroporated at 450 V and 25 µF. Cells were allowed to recover on ice in the presence of 10 µg of polymyxin B nonapeptide hydrochloride (Sigma) for 15 min. Cells were rinsed with RPMI/BSA three times before use.

**Rho and Rac activity assays.** Rho and Rac activity assays were based on established protocols (45, 48) and performed as described previously (37). Briefly, RhoA and Rac1 activity assays were performed by incubating cell extracts with glutathione beads (Pharmacia Biotech) coupled with bacterially expressed GST-TRBD fusion protein (provided by Martin Schwartz, University of Virginia) or Rac/cdc42 binding domain of Pak (PBD)-GST fusion protein (obtained from Rick Cerione, Cornell University), respectively, and then incubated for 30 min at 4°C. Beads were then rinsed and then eluted in 2× Laemmli sample buffer. Bead eluent and cell extracts (10%) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal protein loading was confirmed by Coomassie blue staining. The bands were visualized using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All plasmid cDNAs used were confirmed by DNA sequencing.

Wild-type RhoC was cloned from MDA-MB-435 cells. RhoC was amplified by RT-PCR using primers 5′-CCA TCG ATA TGG CTA CAA TCC GAA GAA AGA AGC TGG TGA TC-3′ and 5′-CGA ATT CTC AGA GAA TGG GAC ACC TTC GCC GCT GT-3′. The PCR product was confirmed by DNA sequencing and then cloned into the EcoRI and ClaI sites of pcDNA3/myc. N19RhoC was generated using site-directed mutagenesis (Stratagene) based on the primer sequence 5′-TTG TGC CTT GGA AAA CGT CCT CCT CAT CGT CT-3′ and then selecting clones confirmed by sequencing. N19RhoB was similarly constructed based on the sequence 5′-CGG CGC GTG CGG CAA GAA CGT CCT GCT GAT GCT GT-3′ using a myc-tagged, wild-type RhoB obtained from Bing Lim (Beth Israel Deaconess Medical Center, Boston, MA).

**Rho RT-PCR.** RNA was extracted from cells using TRizol reagent (Invitrogen Life Technologies), and purity was confirmed by an optical density 260:280 ratio and analyzed using 0.7% agarose with formaldehyde gel electrophoresis. cDNA was prepared using SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies) before PCR amplification (40 cycles) using the following primer sets: RhoA, forward primer, 5′-CAT GCT TGC TCA TAG TC-3′ and reverse primer, 5′-TCT GCC TTC TTC AGG TT-3′; RhoB, forward, 5′-AGA AGC TGG TGG TGG TG-3′ and reverse, 5′-GAG GTA GTC GTC GGC TT-3′; and Rac, forward, 5′-CTG CAA TTC GAA AG-3′ and reverse, 5′-CTC AGA GAA TGG AG-3′.

**Reduction of gene expression by small interfering RNA.** Suspended cells (3 × 10^6) were electroporated with 200 nM control small interfering (si)RNA (nontargeting; Dharmacon) or individual siRNAs specific for AKAP-Lbc (AKAP13) as reported previously (39). Individual sequences for AKAP-Lbc are 5′-UCAACAGACUCACUA-CAAUAUAU3′ (number 3) and 5′-GGAAGAGCUUGUACGU-GAUU3′ (number 5). Cells were then kept in normal growth medium for 48 h and then assessed for LPA-mediated Rho activation and AKAP-Lbc expression by immunoblot analyses.

**C3 expression and electroporation.** Cells were electroporated with purified, bacterially expressed GST-C3 (cDNA obtained from Keith Burridge, University of North Carolina-Chapel Hill) or GST as described previously (60). Briefly, cells (3 × 10^6) suspended in RPMI were combined with 1 µg of purified GST or GST-C3 protein and then electroporated at 450 V and 25 µF. Cells were allowed to recover on ice in the presence of 10 µg of polymyxin B nonapeptide hydrochloride (Sigma) for 15 min. Cells were rinsed with RPMI/BSA three times before use.

**Migration assays.** Migration assays were performed as described previously (37) using Transwell chamber membranes (6.5-mm diameter; 8-µm pore size; Costar) coated with 10 µg/ml collagen I (Vitrogen; Collagen Biomaterials). LPA (100 nM) was added to the lower chambers. Cells (5 × 10^4) were added to the upper chamber and allowed to migrate for 4 h at 37°C. Nonmigrating cells were removed from the upper chamber with a cotton swab. Migrated cells were fixed, stained with crystal violet, and counted visually.

**Microscopic analyses.** Glass coverslips were coated overnight at 4°C with collagen I (50 µg/ml) or laminin-1 purified from EHS tumor (20 µg/ml; Roche Applied Science) and then blocked with BSA (0.25% in RPMI). Cells were plated on these coverslips, allowed to adhere, treated as indicated, and then rinsed with PBS, fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate phal-
RESULTS

Integrin αβ4 promotes RhoA activation downstream of LPA through RhoGEF AKAP-Lbc. To investigate the role of the αβ4 integrin in LPA-stimulated signaling and chemotaxis, we used clones of the MDA-MB-435 carcinoma cell line that have been stably transfected with the β4 subunit (MDA/β4) or a vector control (MDA/mock). The integrin αβ4 signaling in MDA-MB-435 model has been previously shown to signal in a ligand-independent manner where the integrin can promote motility on nonligand substratum such as collagen (38). In our initial experiments, we assayed RhoA activity in our MDA/β4 and MDA/mock cells stimulated with 100 nM LPA, a concentration we (38) have shown previously to promote a maximal rate of migration. Using the Rhotekin binding assay, we find that activation of RhoA in the MDA/β4 cells peaks within 15 s of LPA treatment. This level of activity is attenuated by 1 min and is maintained at a 2.5-fold level of activation throughout the remainder of this time course (Fig. 1A). In contrast, the MDA/mock cells do not activate RhoA in response to LPA, even when assessed at various times poststimulation (Fig. 1, B and C). These data are quantified in Fig. 1D. These results suggest that the αβ4 integrin is required to effectively couple LPA signaling to the activation of RhoA in these cells.

LPA is known to signal through several Rho GEFs including AKAP-Lbc (9). In our recent analysis of transcriptional changes mediated by integrin αβ4 in these cells, we find that AKAP-Lbc mRNA expression is upregulated in MDA/β4 cells 2.5-fold over control cells (4). To determine whether AKAP-Lbc is the Rho GEF responsible for RhoA activation in the MDA/β4 transfectants, we reduced AKAP-Lbc by two separate siRNAs as done previously (39), plated cells on collagen coated dishes, stimulated cells with 100 nM LPA, and then assessed cell lysates for RhoA activity and AKAP-Lbc expression. As shown in Fig. 2, depletion of AKAP-Lbc dramatically blocks the LPA-induced activation of RhoA, thus suggesting that AKAP-Lbc is the Rho GEF that is activated downstream of cooperative signaling from LPA and αβ4 integrin that leads to RhoA activation.

Integrin αβ4 stimulates chemotaxis and lamellae formation in response to LPA is mediated by RhoA. The contribution of Rho proteins to LPA-stimulated chemotaxis was tested using recombinant C3 exotransferase (which ribosylates and thereby inactivates Rho A, B, and C) and an inhibitor of the Rho effector Rho kinase, Y27632. As shown in Fig. 3, A and B, both treatments suppress LPA-stimulated migration of MDA-MB-435 carcinoma cells by >90%, regardless of integrin αβ4 expression. The specific contributions of RhoA to these processes were assessed by transiently transfecting cells with myc-tagged N19RhoA (Fig. 3C) impeded the chemotaxis of the MDA/β4 cells but not the MDA/mock cells. These data suggest that while Rho signaling is required for LPA-stimulated chemotaxis of MDA-MB-435 carcinoma cells, RhoA specifically contributes only in the presence of the αβ4 integrin. These data are consistent with the observation that RhoA is not activated in the MDA-MB-435 cells in the absence of the αβ4 integrin.

To determine which other Rho isoform may participate in the chemotaxis of MDA/mock cells, we evaluated the expression of Rho isoforms in these cells by RT-PCR. Since C3 treatment is effective in inhibiting both MDA/β4 and MDA/mock, we restricted our survey to the expression of the RhoA-related proteins RhoA, B, and C. Here, we developed an RT-PCR protocol that specifically recognizes these individual Rho isoforms (Fig. 4B). As shown in Fig. 4A, MDA-MB-435 clones express RhoA and RhoC but lack RhoB. The similar expression levels of RhoA and absence of RhoB were confirmed at the protein level by immunoblot analysis (data not

To determine which other Rho isoform may participate in the chemotaxis of MDA/mock cells, we evaluated the expression of Rho isoforms in these cells by RT-PCR. Since C3 treatment is effective in inhibiting both MDA/β4 and MDA/mock, we restricted our survey to the expression of the RhoA-related proteins RhoA, B, and C. Here, we developed an RT-PCR protocol that specifically recognizes these individual Rho isoforms (Fig. 4B). As shown in Fig. 4A, MDA-MB-435 clones express RhoA and RhoC but lack RhoB. The similar expression levels of RhoA and absence of RhoB were confirmed at the protein level by immunoblot analysis (data not
Transient expression of a myc-tagged dominant negative RhoC construct (N19RhoC; Fig. 3D) effectively inhibited both the MDA/αβ4 and MDA/mock cells. The expression of N19RhoB had no affect on cell motility (data not shown) as expected given the lack of RhoB expression. These results suggest that LPA can signal through RhoC to promote chemotaxis but does not need integrin αβ4/H92516/H92524 for this task. Furthermore, these data suggest that integrin αβ4/H92516/H92524 affects RhoA signaling by regulating RhoA at the level of activation rather than expression.

The αβ4 integrin facilitates a morphological polarization of MDA-MB-435 cells in response to LPA, resulting in dramatic membrane ruffling and lamellae formation (38). In cells of epithelial origin, membrane ruffling can be stimulated by the activation of either RhoA or Rac1 (11, 35). To assess the role

Fig. 2. AKAP-Lbc mediates integrin αβ4-facilitated RhoA activation in response to LPA. A: MDA/αβ4 cells were electroporated with nothing (untreated; Un), nontargeting (NT) small interfering (si)RNA or with 1 of 2 individual siRNA targeting AKAP-Lbc (si-Lbc-3 or si-Lbc-5; see Ref. 39). After 48 h, cells were replated on collagen-coated plates (3 h), treated with 100 nM LPA for 4 min, and then harvested for RhoA activity assay. B: lysates from cells in A were immunoblotted for AKAP-Lbc and β-tubulin (loading control) to confirm siRNA-mediated reduction of AKAP-Lbc.

Fig. 3. Rho and Rho kinase are required for LPA-stimulated chemotaxis, but RhoA is necessary only in integrin αβ4-expressing MDA-MB-435 cells. A: MDA/αβ4 mock or MDA/β4 cells were treated with 30 μM of the Rho kinase inhibitor Y27632 and then assayed for LPA-mediated chemotaxis for 4 h in the presence of the inhibitor as described in MATERIALS AND METHODS. B: MDA/αβ4 and MDA/mock cells were electroporated with recombinant GST or GST-C3-exotransferase (C3) protein and then assessed for LPA-mediated chemotaxis as in A. C and D: cells were transiently transfected with empty vector or a myc-tagged N19RhoA (C) or N19RhoC (D) and a β-gal marker gene. After 48 h, cells were assayed for chemotaxis. Migration is reported as the relative number of β-gal staining cells migrated compared with the vector only control. Values are corrected for transfection efficiency. C and D, insets: lysates from transfected cells were immunoblotted for myc-tag to confirm expression of N19RhoA (C) or N19RhoC (D). In all experiments, data represent ≥3 separate experiments and bars represent the mean ± SD. Confidence intervals from paired t-test analyses are represented as follows: *P < 0.01; **P < 0.001; ***P < 0.00001.
of RhoA and Rac1 in the morphological response to LPA, we transiently transfected MDA/β4 cells with either myc-tagged N19RhoA or GST-tagged N17Rac1. Two days after transfection, cells were assayed for lamellae formation and migration in response to LPA (Fig. 5) and expression of ectopic myc-tagged N19RhoA or GST-tagged N17Rac1 was confirmed by immunoblot analysis (data not shown). As shown in Fig. 5, both RhoA and Rac1 contribute significantly to both chemotaxis and the formation of lamellae. It is interesting to note that dominant negative RhoA blocks membrane ruffling, but actin filament bundles persist (Fig. 5C, arrowheads). Also, dominant negative Rac1 does not block membrane ruffling (Fig. 5D, arrow). Notably, LPA does not stimulate lamellae formation in response to LPA in the absence of integrin α6β4 expression (38). These data suggest that Rac1 and RhoA both function to facilitate LPA-stimulated chemotaxis and lamellae formation but that RhoA is specifically involved in membrane ruffling.

LPA-stimulated Rac1 activation is promoted by cooperative signaling between α6β4 and β1 integrins. In MDA-MB-435 cells, the α6β4 integrin stimulates the activation of PI3K and promotes the invasion in a Rac1-dependent manner (51). To assess the role of integrin α6β4 signaling on Rac activation, adherent cells were treated with 100 nM LPA for various times. As shown in Fig. 6A, maximal Rac activation is achieved 4–10 min after LPA stimulation in both the MDA/ mock and MDA/β4 cells; however, Rac1 activation is greater in the MDA/β4 cells where the maximal activation is ~2.5-fold. Interestingly, this activation is blocked by a 5-min pretreatment of cells with the β1-integrin blocking mAb, Mc13 (see Fig. 6A, lanes 6 and 12), thus suggesting that de novo β1-integrin ligation is required for Rac activation. This short-term antibody treatment did not alter cell attachment or morphology compared with nontreated cells (data not shown). Furthermore, β1 integrin antibody treatment did not prevent RhoA activation (data not shown).

Next, we directly assessed Rac1 activation by integrin ligation using antibody cross-linking experiments. For these experiments, MDA/β4 cells were incubated with β1-, β2-, or α6-specific anti-integrin antibodies and then plated on secondary coated plates for 30 min or left in suspension. Here, we find that β1 integrins, but not β2 integrin, are able to activate Rac1 (Fig. 6B). Time-course analysis of integrin clustering shows that Rac activation is maximal at 30 min (data not shown). These data suggest that the α6β4 integrin does not directly activate Rac1 but rather cooperates with β1 integrins and chemoaovattractant signaling to promote Rac activation.

Activation of RhoA precedes Rac1 activation in response to LPA (Fig. 1 vs. Fig. 6), which opens up the possibility that RhoA may function upstream of Rac activation. To assess the role of RhoA in this activation of Rac1, MDA/β4 cells were electroporated with recombinant C3 before LPA stimulation. As shown in Fig. 7A, C3 pretreatment blocks LPA-stimulated Rac activation when cells are plated on collagen. Next, we wanted to determine if RhoA cooperates with β1 integrin engagement to stimulate Rac activation. For these experiments, we electroporated MDA/β4 cells with GST or GST-C3 and then treated cells with 100 nM LPA in suspension or with β1 integrin antibody clustering. As shown in Fig. 7B, C3 pretreatment abrogated the activation of RhoA and Rac1 in the morphological response to LPA, we transiently transfected MDA/β4 cells with either myc-tagged N19RhoA or GST-tagged N17Rac1. Two days after transfection, cells were assayed for lamellae formation and migration in response to LPA (Fig. 5) and expression of ectopic myc-tagged N19RhoA or GST-tagged N17Rac1 was confirmed by immunoblot analysis (data not shown). As shown in Fig. 5, both RhoA and Rac1 contribute significantly to both chemotaxis and the formation of lamellae. It is interesting to note that dominant negative RhoA blocks membrane ruffling, but actin filament bundles persist (Fig. 5C, arrowheads). Also, dominant negative Rac1 does not block membrane ruffling (Fig. 5D, arrow). Notably, LPA does not stimulate lamellae formation in response to LPA in the absence of integrin α6β4 expression (38). These data suggest that Rac1 and RhoA both function to facilitate LPA-stimulated chemotaxis and lamellae formation but that RhoA is specifically involved in membrane ruffling.

LPA-stimulated Rac1 activation is promoted by cooperative signaling between α6β4 and β1 integrins. In MDA-MB-435 cells, the α6β4 integrin stimulates the activation of PI3K and promotes the invasion in a Rac1-dependent manner (51). To assess the role of integrin α6β4 signaling on Rac activation, adherent cells were treated with 100 nM LPA for various times. As shown in Fig. 6A, maximal Rac activation is achieved 4–10 min after LPA stimulation in both the MDA/ mock and MDA/β4 cells; however, Rac1 activation is greater in the MDA/β4 cells where the maximal activation is ~2.5-fold. Interestingly, this activation is blocked by a 5-min pretreatment of cells with the β1-integrin blocking mAb, Mc13 (see Fig. 6A, lanes 6 and 12), thus suggesting that de novo β1-integrin ligation is required for Rac activation. This short-term antibody treatment did not alter cell attachment or morphology compared with nontreated cells (data not shown). Furthermore, β1 integrin antibody treatment did not prevent RhoA activation (data not shown).

Next, we directly assessed Rac1 activation by integrin ligation using antibody cross-linking experiments. For these experiments, MDA/β4 cells were incubated with β1-, β2-, or α6-specific anti-integrin antibodies and then plated on secondary coated plates for 30 min or left in suspension. Here, we find that β1 integrins, but not β2 integrin, are able to activate Rac1 (Fig. 6B). Time-course analysis of integrin clustering shows that Rac activation is maximal at 30 min (data not shown). These data suggest that the α6β4 integrin does not directly activate Rac1 but rather cooperates with β1 integrins and chemoaovattractant signaling to promote Rac activation.

Activation of RhoA precedes Rac1 activation in response to LPA (Fig. 1 vs. Fig. 6), which opens up the possibility that RhoA may function upstream of Rac activation. To assess the role of RhoA in this activation of Rac1, MDA/β4 cells were electroporated with recombinant C3 before LPA stimulation. As shown in Fig. 7A, C3 pretreatment blocks LPA-stimulated Rac activation when cells are plated on collagen. Next, we wanted to determine if RhoA cooperates with β1 integrin engagement to stimulate Rac activation. For these experiments, we electroporated MDA/β4 cells with GST or GST-C3 and then treated cells with 100 nM LPA in suspension or with β1 integrin antibody clustering. As shown in Fig. 7B, C3 pretreatment abrogated the activation
of Rac1. These data suggest that RhoA functions upstream of integrin clustering to activate Rac1. Since C3 has been shown to block Rac activation at high concentrations in vitro (7), we tested the effect of C3 treatment on Rac activation by β1 integrin clustering, which does not require Rho activity (36, 41). We find that C3 treatment does not significantly block activation of Rac by β1 integrin ligation (Fig. 7C). In support of the lack of effect of C3 on Rac at these concentrations, Rac1 displays no mobility shift indicating that it is not ribosylated. Together, these data suggest that Rho activity and de novo β1 integrin ligation and clustering are needed for Rac activation in response to LPA.

RhoA and Rac1 activities downstream of LPA signaling are differentially regulated by cAMP/PKA and PI3K. We have previously shown that the cAMP/PKA pathway differentially regulates Rac1 and RhoA. That is, RhoA is inhibited by elevated cAMP/PKA activity while Rac1 requires PKA for activation. These individual observations have been made in different model systems (36, 37). Here, we analyze the effect of cAMP/PKA pathway on the activity of these GTPases in the same model system, namely the MDA/β4 cells treated with LPA. As shown in Fig. 8, inhibition of PDE activity with isobutylmethylxanthine blocked the activation of RhoA but did not affect Rac1 activity. In contrast, PKA inhibition with H-89 (Fig. 8) or protein kinase A inhibitor (data not shown) prevented Rac1 activation while they potentiate RhoA activation. Similarly, inhibitors of PI3K (wortmannin or Ly294002) have the same effect as the PKA inhibitors. Of note, treatment of cells with each of these inhibitors individually had no effect on basal GTPase activity (data not shown). In total, these results show that Rac and RhoA are reciprocally regulated by the cAMP/PKA and PI3K pathways.

DISCUSSION

The integrin αoβ4 contributes significantly to cell migration, tumor invasion, and metastasis through cooperative signaling with growth factor receptors. More than a decade of work on signaling from the αoβ4 integrin has yielded several main pathways involved in cancer progression including the transcriptional regulation through activation of AP1 (15), NFκB (56), and nuclear factor of activated T cell (21) transcription factors; stimulation of the MAPK pathway (28); PI3K stimulation and subsequent activation of Akt (25, 51); and activation of Rho family GTPases Rac1 (62) and RhoA (37). Of these pathways, RhoA has received the least attention. Our current study on the integrin αoβ4 suggests that the promigratory properties of the αoβ4 integrin are tightly linked to its ability to signal through RhoA. Here, we show that the αoβ4 integrin is needed for MDA-MB-435 cells to activate and utilize RhoA for chemotactic migration in response to LPA, which in turn leads to membrane ruffling and the activation of Rac1.

A major finding of this study is that integrin αoβ4 amplifies RhoA activity in cooperation with LPA signaling through the preferential activation of the GEF activity of AKAP-Lbc, thus identifying the first Rho GEF to be activated by the αoβ4 integrin. Exactly how the αoβ4 integrin promotes the activation of RhoA requires further investigation. However, our results suggest that ligation of αoβ4 integrin results in the activation of RhoA through the activation of PI3K/AKT and cAMP/PKA pathways.
of AKAP-Lbc is not clear. It is known that AKAP-Lbc is regulated by PKA phosphorylation, which, in turn, promotes the 14–3–3-mediated inactivation of its GEF activity (8). We (38) have previously reported that the α5β1 integrin stimulates PDE activity, which subsequently reduces cAMP levels and PKA activity. We find that PDE activity is needed for RhoA activation in the MDA/β3 transfectants (Fig. 8). This observation supports the idea that the α5β1 integrin facilitates AKAP-Lbc activation through its ability to elevate PDE activity. However, this observation does not fully account for the lack of utilization of RhoA and AKAP-Lbc in the absence of integrin α5β3 signaling. It is likely that the α5β3 integrin signals in an alternative manner to activate RhoA-specific GEFs. While PKA phosphorylation of AKAP-Lbc can inactivate this GEF, the mechanisms governing its activation downstream of growth factor signaling have yet to be elucidated. Certainly, the determination of how this integrin precisely cooperates with growth factors to signal to RhoA will give further insight into both integrin α5β3- and LPA-dependent signaling.

LPA has been shown to signal to multiple Rho GEFs including PDZ-RhoGEF, LARG (61), Net1 (34), p115RhoGEF (22), and AKAP-Lbc (9). While the reasons for the use of multiple GEFs is unclear, it is suggested that different Rho GEFs may permit spatial activation of Rho (13, 49). Notably, early work suggested that the primary action of RhoA was to mediate trailing edge retraction in migrating cells (58). In accordance with these concepts, PDZ-RhoGEF operates preferentially at the rear of migrating cells to activate Rho and promote tail retraction in cooperation with ROCKII (20). However, studies using a fluorescence resonance energy transfer reporter for assessing RhoA activity demonstrated that RhoA activity is found at the leading edge (24, 40). This observation supports studies that implicate RhoA in growth factor-mediated membrane ruffling and lamellae formation in epithelial derived cells (11, 24, 35, 37) as well as neutrophils (63). Here, we find that RhoA is activated quickly in β1-expressing MDA-MB-435 cells upon LPA stimulation and is critical for membrane ruffling; this activation of RhoA is suppressed by siRNA-mediated reduction of AKAP-Lbc. Currently, it is not well understood how RhoA signaling contributes to lamellae formation considering the Rho effectors ROCK (11) and mDia1 (24) have been implicated not only in membrane ruffling but also stress fiber formation. It is also unclear why so many GEFs exist for RhoA. One could speculate that AKAP-Lbc might be specifically involved in this membrane ruffling and lamellae formation by affecting where RhoA and subsequently its effectors are activated. More extensive studies will be required to determine the relative contribution of GEF localization and effector choice in RhoA-mediated membrane ruffling.

Here, we find that RhoA activation precedes and is required for Rac1 activation upon LPA stimulation. This observation is in agreement with previous studies (31, 53) suggesting that RhoA is needed for LPA-stimulated Rac1 activation. In further elucidation of this signaling event, we determine that β1-integrin ligation is required for the activation of Rac1 downstream of LPA and α5β3 integrin signaling (Fig. 6). This observation supports the concept that RhoA would be needed for the initial actin protrusions that lead to new β1 integrin contacts and with the extracellular matrix and initial integrin clustering needed for Rac GEF activation. It has also been shown that Rho activity is required for β1 integrin clustering (5). Importantly, these data are in accordance with observations of Rho GTPase dynamics in live mouse embryo fibroblasts. In that study, RhoA activation preceded activation of Rac spatially by 2 μm at the leading edge as well as temporally (26). We find that Rho activity is still required to activate Rac after β1 integrin clustering in response to LPA but not for β1 integrin clustering alone. The difference here is the amount of time the integrins are clustered. With LPA stimulation, the clustering occurs for only 10 min while β1 clustering alone is performed for 30 min. These data suggest that LPA stimulation and RhoA may cooperate with β1 integrin ligation to facilitate a more rapid activation of Rac and supports observations that RhoA activation at the leading edge precedes that of Rac.

The signals that perpetuate Rac are conspicuously inhibitory to RhoA, including PKA and PI3K. Rac and Rho have also been shown to have an antagonistic relationship in which Rac1 can downregulate RhoA activity (3, 47, 48). This observation suggests that cAMP/PKA signaling, as well as PI3K, can control where Rac1 and RhoA are activated and could potentially explain how Rac1 and RhoA activities are compartmentalized. This compartmentalization by PKA is in agreement with our recent studies showing that PKA activity is localized to the leading edge of migrating cells proximal to the plasma membrane on the basal surface (39) where β1 integrin ligation stimulates cAMP accumulation and PKA activation (32, 36). These de novo β1 integrin contacts at the leading edge leading to a transient activation of PKA would facilitate the activation of Rac1 and preferentially exclude RhoA. However, we also find that RhoA leads to the activation of Rac1 by LPA, which appears counterintuitive to this spatial regulation. The activa-
tion of a negative feedback signal shortly after a stimulator signal has been proposed to generate signaling waves and promote spatial signaling (2). These waves of positive and negative signals may permit the initial transient colocalization of these activities, which subsequently leads to the spatial separation of Rac and Rho signals. Alternatively, the contraction of actin filaments by Rho could occur distally from the integrin contacts meaning that Rac activation by integrin clustering does not require RhoA to colocalize with Rac to facilitate its activation.

While we find that the $\alpha_6\beta_4$ integrin can cooperate with LPA receptor signaling to promote Rac activation, it cannot activate Rac1 directly (Fig. 6). The $\alpha_6\beta_4$ integrin has been shown to activate PI3K (12, 51) and dramatically upregulate protein tyrosine kinase activity (50), two activities that are essential for the activation of several Rac GEFs (49). As a result, it has been presumed that $\alpha_6\beta_4$ integrin can activate Rac1 directly. However, the $\alpha_6\beta_4$ integrin signaling also stimulates PDEs that reduce cAMP levels (38), which results in a twofold reduction in PKA activity (unpublished observation) that may impede Rac activation. Interestingly, elevation of cAMP levels with either PDE inhibitors or forskolin treatment does not permit Rac activation by $\alpha_6\beta_4$ integrin clustering (unpublished observation), thus suggesting that integrin $\alpha_6\beta_4$, clustering simply fails to activate a Rac-specific GEF. This would help to explain why the $\alpha_6\beta_4$ integrin cooperates with growth factors such as LPA (Fig. 8), EGF (46, 62), or hepatocyte growth factor (6), which can directly activate Rac GEFs such as Tiam1 (54) or Vav2 (30) to enhance Rac-dependent cell motility and invasion. Interestingly, motility promoted by the $\alpha_6\beta_4$ integrin in the absence of exogenous growth factors does not involve Rac1 (37).

The results from this study show the contributions of RhoA can be discerned from the actions of Rac from the transient transfection of isoform-specific dominant negative constructs, thus suggesting that RhoA and Rac play separate, yet complementary, roles in cell motility. This observation is quite surprising considering that there is an 85% homology between the two proteins on the amino acid level and that these two proteins share multiple regulators and effectors (57). Furthermore, it is believed that these dominant negative constructs function by sequestering GEFs and act nonspecifically, based on previous observations made with Ras (10), although this concept has not been rigorously tested. A recent report by Boulter et al. (1) has shown that overexpression of Rho family proteins controlled by RhoGDI1 (RhoA, RhoC, Rac1, and cdc42) can dramatically affect other Rho members by promoting an imbalance with RhoGDI1 that promotes degradation of most Rho molecules not complexed with RhoGDI and hyperactivation of the GTPases that remain. This group further showed that when specific exogenous Rho proteins are expressed in low abundance, other Rho family members are left unaffected. Notably, we express dominant negative RhoA, RhoC, and Rac1 proteins at low levels by limiting the amount of cDNA construct transfected. Our data suggest that these dominant-inhibitory constructs can indeed work specifically, possibly by specific subcellular targeting of these proteins mediated through the C-terminal hypervariable region, a region that has been utilized to target Rho GAP activity for specific inactivation of distinct Rho isoforms (55). Furthermore, our results show that expression of dominant negative RhoA blocks membrane ruffling but not the formation of actin filament bundles within the cell body that is blocked by C3 treatment (Fig. 4) and Rho kinase inhibition (unpublished observation). These results suggest that RhoA and RhoC may mediate separate functions within a cell. Specifically, in our cell model, RhoA is more involved in membrane ruffling and RhoC appears to be more important for actin filament bundling, which are two events shown to be mediated by Rho kinase (11, 27). The exact mechanisms that govern how these Rho isoforms can mediate these spatially distinct events are important topics for further investigation.

In summary we find that the $\alpha_6\beta_4$ integrin promotes the activation of RhoA downstream of LPA by way of signaling through AKAP-Lbc (Fig. 9). We further demonstrate that the activation of Rac1 downstream of LPA receptor signaling results from the coordinated signaling from RhoA, de novo $\beta_1$ integrin ligation, PKA, and PI3K. Through these data, we show cooperative signaling between the $\alpha_6\beta_4$ and $\beta_1$ integrins. Specifically, clustering of $\beta_1$ integrins in the presence of $\alpha_6\beta_4$, under conditions of costimulation with LPA, promotes a more robust activation of Rac1. This cooperative signaling may help to polarize Rac signaling to the leading edge to promote polarization and directed cell motility.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Steve Akiyama, Keith Burridge, Rita Falcioni, and Bing Lim for reagents. We also thank Drs. Art Mercurio and Sarita Sastry for helpful discussions.

GRANTS

This work was supported by the United States Army Medical Research and Materiel Command Grant DAMD17-98-1-8033 and the National Institutes of Health Grant R01-CA109136.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.L.O. conception and design of research; K.L.O., M.C., and L.N.T. performed experiments; K.L.O., M.C., and L.N.T. analyzed data; K.L.O. and M.C. interpreted results of experiments; K.L.O. prepared figures; K.L.O. drafted manuscript; K.L.O., M.C., and L.N.T. approved final version of manuscript; M.C. edited and revised manuscript.
REFERENCES


