Autotaxin Promotes Cancer Invasion via the Lysophosphatidic Acid Receptor 4: Participation of the Cyclic AMP/EPAC/Rac1 Signaling Pathway in Invadopodia Formation

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Abstract

The ability of cancer cells to invade and metastasize is the major cause of death in cancer patients. Autotaxin (ATX) is a secreted lysophospholipase whose level of expression within tumors correlates strongly with their aggressiveness and invasiveness. ATX is the major enzyme involved in the production of lysophosphatidic acid (LPA), a phospholipid that is known to act mostly through its three first characterized receptors (LPA1, LPA2, and LPA3). Tumor cell invasion across tissue boundaries and metastasis are dependent on the capacity of invasive cancer cells to breach the basement membrane. This process can be initiated by the formation of the actin-rich cell protrusions, invadopodia. In this study, we show that ATX is implicated in the formation of invadopodia in various cancer cell types and this effect is dependent on the production of LPA. We further provide evidence that LPA4 signaling in fibrosarcoma cells regulates invadopodia formation downstream of ATX, a process mediated through the activation of EPAC by cyclic AMP and subsequent Rac1 activation. Results using LPA4 shRNA support the requirement of the LPA4 receptor for cell invasion and metastasis formation. This work presents evidence that blocking the LPA receptor, LPA4, in fibrosarcoma cells could provide an additional tool to improve the efficacy of treatment of metastasis in patients. Because LPA receptors and ATX are currently being targeted in preclinical trials, the current findings should stimulate future studies to evaluate the expression pattern and clinical outcome of LPA4 in various cancer patients. Cancer Res; 70(11); 4634–43. ©2010 AACR.

Introduction

Cancer remains a leading cause of death worldwide despite relentless efforts in basic research and clinical management of the disease. Autotaxin (ATX), a secreted motility-stimulating factor that was originally isolated from the culture supernatant of human melanoma cells (A2058; ref. 1), has been linked with progression of many types of cancer. Uptregulation of ATX in malignancies including breast, lung, colon, ovarian, stomach, and brain cancer was correlated with invasiveness and metastatic potential (2–4). ATX has been found in many biological fluids including blisters, cerebrospinal, peritoneal, and synovial, as well as plasma (5–8). Through its extracellular actions, ATX augments cellular functions closely associated with tumor aggressiveness and metastasis that include proliferation, survival, motility, invasion, and angiogenesis (9, 10). ATX has recently been shown to be molecularly identical to lysophospholipase D, which catalyzes the production of the majority of lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) in vivo (11). Consequently, LPA is thought to be responsible for the majority of ATX effects (12). LPA is a bioactive lipid and a major constituent of serum whose effects are similar to those of ATX (11, 13). LPA is also implicated in many pathophysiologic conditions such as atherosclerosis, hypertension, ischemia reperfusion injury, and, pertinent to this study, cancer (14). LPA mediates cell type-specific responses by engaging high-affinity G protein–coupled receptors (GPCR; ref. 15).

There are currently six known LPA receptors. LPA1, LPA2, and LPA3 are members of the endothelial differentiation gene (edg) receptor family, whereas the more recently deorphaned LPA4, LPA5, and LPA6 belong to the purigenic cluster of GPCRs (16–18). Most research has focused on the first identified LPA receptors, whereas the signaling and physiologic roles of LPA4, LPA5, and LPA6 have received less attention. LPA signals via at least four distinct G protein families, Gα, Gq, G12/13, and G13, and their downstream effectors. The major signaling pathways that become activated include the phosphoinositide 3-kinase (PI3K) and Ras pathways, which depend on Gα, PLC, which depends on Gq, Rho GTPases, which are associated with G12/13 signaling, and cyclic AMP (cAMP) production downstream of Gα (13, 19). Many of these...
signaling routes are involved in tumor survival and invasion (19). Overexpression of ATX and LPA receptors (LPA1, LPA2, and LPA3) has been recently linked to increased tumor invasion and metastasis of breast cancer cells (20). Moreover, pharmacologic inhibition of both ATX and LPA receptors (mostly LPA1, LPA2, and LPA3) has shown to decrease cell migration in vitro and cause tumor regression in mice (21). These studies uncovered an important role for ATX and LPA1, LPA2, and LPA3 receptors in tumor metastasis, a fundamental property of malignant cancer cells and the major cause of death in patients.

Metastatic tumor cells must first degrade the surrounding tissues and reach the bloodstream to travel to distant sites and form new tumors. Cancer cells have been shown to generate protrusions called invadopodia to facilitate their migration and invasion through tumor stroma and the lining of the blood vessels during the process of metastasis (22, 23). Invadopodia are formed by highly invasive cancer cells. They are actin-rich ventral membrane protrusions, which possess extracellular matrix (ECM)–degrading activity (24). The formation of these structures necessitates the convergence of many different signaling pathways and molecules, which are slowly being unraveled. Besides actin, invadopodia contain actin-regulatory proteins, adhesion molecules, membrane remodeling and signaling proteins, and ECM-degrading enzymes (25). The Rho GTPases, including Rac and Cdc42, have repeatedly been shown to promote invadopodia production through their actions on the cytoskeleton (26). Formation of invadopodia is enhanced by ECM rigidity (27), some growth factors such as epidermal growth factor (22), or expression of wild-type or activated forms of the src tyrosine kinase (28). Therefore, despite much research on invadopodia biology, very little is known about specific inducers and receptors during tumor progression.

Because of the implications of ATX and LPA signaling in metastasis and their activation of pathways, which could be implicated in invadopodia formation, we have investigated the involvement of ATX and LPA receptors in invadopodia production. Using the invasive fibrosarcoma cell line HT1080, we observed that ATX through the LPA4 receptor is a strong inducer of invadopodia formation that correlates with the ability of the cells to invade and metastasize. This study also revealed an unexpected signaling pathway for cell invasion involving LPA4-driven cAMP production and subsequent activation of the EPAC-Rap1-Rac1 axis.

Materials and Methods

Reagents

1-Oleoyl-sn-glycerol-3-phosphate sodium salt 18:1 (LPA), L-α-LPC from egg yolk (LPC-egg), 1-oleoyl-sn-glycero-3-phosphocholine 18:1 (LPC-18:1), 2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium (dibutyryl cAMP), 8-(4-chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate monosodium hydrate (8-pCPT), 8-bromoadenosine 3′,5′-cyclic monophosphate sodium (8-bromo cAMP), H-89 dihydrochloride hydrate, LPA receptor antagonist (K166425), forskolin, cholera toxin (CTX), and pertussis toxin were purchased from Sigma-Aldrich. The Rac1 inhibitor GGTI-298 and Rap1 inhibitor GGTI-298 were from Calbiochem (EMD Chemical, Inc.), and myristoylated protein kinase inhibitor (14–22) amide (PKI) was from Biomol International LP. ATX cDNA construct and ATX antibody (84A) were a kind gift from Dr. Tim Clair (Center for Cancer Research, National Cancer Institute, NIH), shRNA against ATX, LPA4, or EPAC was from SABiosciences. The anti-tubulin antibody was from Sigma-Aldrich, the anti-cortactin antibody was from Millipore, the anti-EPAC antibody was from Cell Signaling, and Texas red phalloidin and all secondary antibodies were from Invitrogen (Molecular Probes).

Cell culture and transfections

HT1080 human fibrosarcoma, MDA-MB231 and MCF-7 human breast cancer, A549 human lung cancer, and U87 human glioblastoma were obtained from the American Type Culture Collection. All cell lines were cultured in MEM (Wisent) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 40 μg/mL gentamicin (Shering Canada, Inc.) in a humidified 95% air/5% CO2 incubator at 37°C. Stable transfections with ATX cDNA or shRNA against ATX, LPA4, or EPAC, or scramble shRNA (negative control) were performed with the Fugene reagent from Roche Diagnostics according to the manufacturer’s protocol.

Real-time reverse transcription-PCR

Total RNA was isolated using the TRI-Reagent (Invitrogen) protocol as previously described (29), and quantitative real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research). Primer pairs for LPA4 and human acidic ribosomal phosphoprotein P0 as well as the cycling program are found in Supplementary Materials and Methods.

Western blotting

Cells were lysed on ice in radioimmunoprecipitation assay buffer. Supernatant samples were recovered by centrifugation (13,000 rpm for 30 min at 4°C), and protein concentration was determined using the bicinchoninic acid reagent (Biolynx, Inc.). Immunoblotting was performed as previously described (30).

Invadopodia assays

Coverslips were prepared as previously described (31) using Oregon Green gelatin (Invitrogen). Thirty thousand cells were seeded on each coverslip, allowed to adhere, and incubated in MEM with 0.5% FBS. Following various incubation times as described within the figure legends, cells were fixed with 1% paraformaldehyde for 30 minutes at 4°C and stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 minutes at room temperature. Cells were visualized by fluorescence microscopy, and cells forming invadopodia were counted. Invadopodia were identified by areas of matrix degradation characterized by loss of green fluorescence. Three hundred cells were counted per coverslip.

Fluorescence microscopy

Cells were grown on gelatin-coated coverslips and fixed with 1% paraformaldehyde in PBS at 30 minutes at 4°C,
permeabilized with 0.05% saponin (Sigma-Aldrich) in PBS for 20 minutes, and blocked with 2% bovine serum albumin in PBS for 30 minutes. For calculation of the number of invadopodia per cell, cells were then incubated with anti-cortactin antibody for 2 hours and secondary antibody 488-conjugated anti-mouse for 1 hour at 4°C followed by Texas red phalloidin for 45 minutes and DAPI for 5 minutes. Colocalization of actin and cortactin was visualized using an Axioskop 2 phase-contrast/epifluorescence microscope, and 20 cells were counted per condition (Carl Zeiss, Inc.). Images were taken with a FV1000 scanning confocal microscope (Olympus) coupled to an inverted microscope using a 63× oil immersion objective.

Figure 1. ATX induces invadopodia formation in HT1080 cells. Parental and ATX-overexpressing HT1080 cells were cultured on fluorescent gelatin for 10 h (A and B) or on nonfluorescent gelatin for 4 h (C). A, percentage of invadopodia-producing cells (n = 3–9). Representative images of parental and ATX-transfected cells with associated matrix degradation are shown. Magnification, ×10. B, quantification of ECM degradation area/cell (n = 3), with representative images of degradation by parental and ATX-transfected cells. Magnification, ×40. C, number of F-actin–positive (green) and cortactin-positive (red) invadopodia per cell (n = 2), with representative confocal images showing colocalization of actin and cortactin spots. Magnification, ×60. D, a representative confocal image of ATX-transfected cells showing actin-rich invadopodia that extend into the matrix substratum (F-actin, red; gelatin, green). Columns, mean; bars, SE. ***P < 0.0001.
objective. To quantify the areas of degradation, pictures of fluorescent gelatin were acquired and captured into Image-Pro imaging software (Media Cybernetics), and degradation areas were calculated in pixels for a total of at least 20 cells per coverslip.

Three-dimensional invasion assays
The three-dimensional invasion assay was modified from a previously described technique (32). Details can be found in Supplementary Materials and Methods.

In vivo metastasis assay
Mice were housed and manipulated under pathogen-free conditions in accordance with the guidelines of the local institutional animal care facility. Experimental metastatic potential of HT1080-transfected cells was measured by the lung colonization assay. Briefly, $2 \times 10^5$ cells in 0.1 mL PBS were injected into the tail vein of 5- to 7-week-old female CD1 nude mice. Twenty-nine days later, the mice were sacrificed and the lungs were fixed with Bouin’s solution (Sigma-Aldrich). Metastatic colonies on the lung surface were counted macroscopically.

Results
ATX induces the formation of matrix-degrading invadopodia through the production of LPA from LPC
Because ATX is upregulated in many cancer cell lines and its expression correlates with cell invasion (2–4), we investigated whether ATX is implicated in invadopodia production. As shown in Fig. 1A, ATX overexpression in HT1080 human fibrosarcoma cells induced a significant 5- to 6-fold increase in the percentage of invadopodia-producing cells compared with parental HT1080 cells. Furthermore, the overexpression of ATX resulted in a significant increase of the gelatin degradation area (Fig. 1B), as well as the number of invadopodia.
formed per cell identified by colocalization of actin and cortactin, two known markers of invadopodia (Fig. 1C; ref. 28). Confocal microscopy analysis of ATX degradation areas showed that they were associated with cell membrane protrusions, which were characterized by punctate actin cores that extended from the basal membrane of the cell into the underlying matrix (Fig. 1D). These observations suggested that ATX is involved in both invadopodia formation and function.

To determine whether the effect of ATX was due to the ability of the enzyme to produce LPA from LPC, HT1080 cells were incubated in the presence or absence of LPC or LPA during the invadopodia assay. Results showed concentration-dependent increases in the percentage of invadopodia-producing cells after 10 hours of incubation (Fig. 2A and B), showing that both the substrate and the product of ATX enzymatic activity have the ability to induce the production of invadopodia. Next, depletion of ATX by shRNA abolished LPC- but not LPA-induced invadopodia formation (Fig. 2C), confirming that LPC-derived LPA was a key metabolite in invadopodia production. We further investigated whether the findings observed in the HT1080 cell line applied to other malignant cell lines. LPC induces invadopodia production in MDA-MB231 (breast cancer), A549 (lung cancer), CaCo2 (colon cancer), and U87 (glioblastoma) cell lines. In contrast, MCF-7 (breast cancer), which has an intrinsic defect in cell invasion, failed to respond to LPC (Fig. 2D). These results indicate a role for ATX in the formation of invadopodia by a wide range of neoplastic cells.

**LPA₄ is implicated in the formation of invadopodia through the activation of the Gₛ-cAMP-EPAC pathway**

Because the cellular effects of LPA are due to its binding and activation of various LPA-specific GPCRs (15) and some of these receptors have been linked to tumor invasion (20, 21), we further investigated the role of these receptors and their downstream signaling pathways in mediating the effects of ATX and LPA on invadopodia production. Treatment of ATX-overexpressing cells with a broad-spectrum LPA receptor antagonist (Ki16425) resulted in a significant decrease in invadopodia production (Fig. 3A), suggesting an important role for these receptors in transducing the ATX/LPA-dependent invasive function. We next assessed the relative expression levels of the most characterized LPA receptors (LPA₁, LPA₂, LPA₃, and LPA₄) in HT1080 cells to identify which of the receptors were more likely to mediate the effects of ATX/LPA on invadopodia production. In agreement with previously published results (2), HT1080 cells expressed high levels of LPA₄ with only a minimal expression of LPA₁, LPA₂, and LPA₃ (Fig. 3B). Next, to further assess the role of LPA₄ in invadopodia production, HT1080 cells were transfected with LPA₄ shRNA. Results showed that LPA₄ inhibition abolished invadopodia production induced by LPA or LPC (Fig. 3C), indicating the important contribution of this receptor in ATX-induced invadopodia production.

LPA₄ has been shown to couple to the Gₛ family of G proteins that triggers the activation of adenylyl cyclase, resulting in an increase in cAMP accumulation (33). To verify the

![Figure 3. LPA₄ is implicated in invadopodia production by HT1080 cells. A, ATX-overexpressing cells were treated with Ki16425 (25 μmol/L) for 10 h in an invadopodia assay (n = 3). B, LPA receptors were measured by reverse transcription-PCR (RT-PCR; n = 3). C, effect of LPA₄ shRNA on invadopodia production by unstimulated (ctr), LPA-stimulated (10 μmol/L), or LPC-18:1-stimulated (10 μmol/L) cells (n = 3–4). D, LPA₄ mRNA was measured by RT-PCR (n = 2). Columns, mean; bars, SE. *, P < 0.01; **, P < 0.001; ***, P < 0.0001.](https://cancerres.aacrjournals.org/content/canres/70/11/4638/F3.large.jpg)
involvement of this pathway in invadopodia formation, ATX-overexpressing cells were incubated overnight with CTX that interferes with Gs function. Whereas short-term incubation with CTX is known to activate Gs, prolonged exposure ultimately results in its depletion (34). Depletion of Gs resulted in a drastic reduction of the percent of cells producing invadopodia (Supplementary Fig. S1). Consistent with this result, we next investigated whether increasing intracellular levels of cAMP would result in increases in invadopodia formation.

Exposing parental HT1080 cells to forskolin (an activator of adenyl cyclase) or to dibutyryl cAMP (a stable analogue of cAMP) resulted in 1.5- to 3-fold increases in cells producing invadopodia (Fig. 4A). cAMP exerts its effects by activating the cAMP-dependent protein kinase A (PKA) and the recently discovered exchange protein directly activated by cAMP (EPAC; ref. 35). To determine which of these cAMP effectors was responsible for invadopodia production, we took advantage of cAMP analogues known to specifically activate PKA.

Figure 4. LPA₄ signals through Gₛ-cAMP-EPAC for invadopodia production. HT1080 cells were incubated with or without (ctr) forskolin or dibutyryl cAMP (A), 8-pCPT (B), or 8-bromo cAMP (C) for 10 h in invadopodia assays (n = 3–5). D, parental HT1080 or cells transfected with EPAC shRNA or control shRNA were cultured in the presence or absence (ctr) of LPA (10 μmol/L) or LPC-18:1 (10 μmol/L) and the percent of invadopodia-producing cells was determined (n = 3). Immunoprecipitation of EPAC from equal amounts of protein per sample is also shown. Columns, mean; bars, SE. *, P < 0.01; **, P < 0.001; ***, P < 0.0001.

Figure 5. Rac1 activation downstream of EPAC is required for invadopodia production. A to C, HT1080 cells were incubated with the Rac1 inhibitor (NSC23766) for 30 min before stimulation with LPC-18:1 (10 μmol/L; A), LPA (10 μmol/L; B), or 8-pCPT (50 μmol/L; C) in invadopodia assays. The control (ctr) represents unstimulated HT1080 cells (n = 3–4). Columns, mean; bars, SE. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

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(8-bromo cAMP) or EPAC (8-pCPT). Results indicated that EPAC activation induced a 2.5-fold increase in invadopodia-positive cells, whereas PKA activation had no significant effect (Fig. 4B and C). In addition, the PKA inhibitors H-89 and PKI did not affect ATX-induced invadopodia formation (Supplementary Fig. S2), confirming the lack of involvement of PKA. To further assess the role of EPAC in invadopodia formation, we transfected HT1080 cells with shRNA against EPAC and observed that EPAC inhibition reduced LPA- and LPC-induced matrix degradation (Fig. 4D). Taken together, the results suggested that invadopodia production by ATX is promoted by a Gs-cAMP-EPAC signaling pathway.

**Rac1 activation downstream of EPAC is required for invadopodia production**

EPAC is a nucleotide exchange factor that activates the small G protein Rap1. Selective inhibition of Rap1 using GGTI-298 abolished EPAC-induced invadopodia production (Supplementary Fig. S3), suggesting its involvement downstream of EPAC. Rap1 has many effectors, including the small G protein Rac1 (36), which can induce actin polymerization by the Arp2/3 complex, an event essential to invadopodia formation (37). We therefore used the Rac1 inhibitor NSC23766 to investigate whether Rac1 could be a downstream target of EPAC for invadopodia production. Rac1 inhibition abolished 8-pCPT-induced invadopodia production, whereas the effects induced by LPC and LPA were decreased by ∼50% (Fig. 5A–C). These results suggested that although Rac1 seems to be the essential downstream mediator of EPAC and Rap1 in invadopodia production, LPC and LPA might activate additional pathways that are independent of the EPAC-Rac1 interaction.

**LPA₄ is implicated in three-dimensional invasion and metastasis**

After determining the implication of LPA₄ in invadopodia formation, we further investigated the contribution of this receptor in cell invasion through a three-dimensional matrix and metastasis formation in vivo. For the invasion assay, parental HT1080 cells or cells transfected with LPA₄ shRNA were seeded on top of a type I collagen matrix and incubated with or without LPC and LPA. The results showed that cells transfected with LPA₄ shRNA lose the ability to invade deeply into a three-dimensional matrix in response to LPC or LPA (Fig. 6A and B). In addition, mice injected with LPA₄ shRNA-transfected cells developed significantly less lung metastasis compared with those injected with control shRNA (Fig. 6C). These results indicated an essential role for LPA₄ in HT1080 fibrosarcoma invasion both in vitro and in vivo and further strengthen the

**Figure 6.** LPA₄ is implicated in cell invasion in vitro and in vivo. A and B, parental HT1080 or LPA₄ shRNA-transfected cells were incubated on type I collagen in three-dimensional invasion assays in the presence or absence (ctr) of LPA (10 μmol/L) or LPC-18:1 (10 μmol/L). A, relative intensity of cell staining according to depth of invasion (n = 3). Columns, mean; bars, SE. *, P < 0.01. C, CD1 nude mice were injected with HT1080 cells transfected with control shRNA (n = 12) or LPA₄ shRNA (n = 12). The number of metastasis counted on the lung surface for each mouse is shown. Results are from two separate experiments. Points, mean; bars, SE. ***, P = 0.0008.
relevance of invadopodia formation for cancer cell invasion and metastasis.

**Discussion**

In this study, we showed that ATX is implicated in the production of invadopodia and that this effect is dependent on the production of LPA from LPC, both of which can mediate invadopodia production in various cancer cell types expressing ATX and LPA receptors. We further provide the first evidence that LPA₄ signaling regulates invadopodia formation downstream of ATX and LPA in HT1080 cells, a process mediated through the activation of EPAC by cAMP and subsequent Rac1 activation. RNA interference experiments further support the requirement of LPA₄ for in vitro cell invasion and in vivo metastasis.

ATX and LPA are involved in tumor invasion and metastasis (20, 21), and expression of ATX was correlated with increased invasion of many malignant cell lines (2–4). ATX is also known to augment functions associated with tumor progression and metastasis such as proliferation, survival, motility, invasion, and angiogenesis (9, 10, 12). A very recent study indicated that LPA can induce the formation of actin dots that resemble invadopodia (38). Here, we further showed that ATX, through the production of LPA, regulated the formation and function of the invasive structures, invadopodia, in tumor cells. Invadopodia are implicated in the first steps of mesenchymal invasion due to their ability to make cytoskeletal protrusions enriched in matrix-degrading proteases (22, 23). The role of ATX in invadopodia production may be part of the mechanism to explain how ATX affects tumor cell invasion and metastasis.

LPA receptors have also been implicated in tumorigenesis. LPA has been shown in various studies to stimulate cell motility and to modulate tumor cell invasion, mediated mainly by LPA₁ and Gₛ/α coupling protein (19, 39). LPA₄, which we investigated in this study, has thus far been poorly linked to tumorigenesis. LPA₄ is widely expressed in embryonic tissues, and its mRNA expression is increased at implantation sites in the uterus (33, 40), suggesting a role in matrix degradation and invasion into the uterus wall. Among the few studies of LPA₄ functions in tumor cells, the report of Lee and colleagues (41) suggests that LPA₄ signaling inhibits cell motility and invasion by overactivation of RhoA and inhibition of PI3K, resulting in decreased Rac1 activation. Conversely, LPA₄ has been shown to induce cell transformation and anchorage-independent growth in Myc-transformed cells (42). Our finding that LPA₄ is implicated in the formation of invadopodia through Gₛ, signaling provides a novel role for this LPA receptor and adds an alternative pathway by which ATX and LPA can favor tumor invasion. The discrepancy between our results and those of Lee and colleagues (41), showing a negative role for LPA₄ in the regulation of motility and invasion, may be due to the fact that these authors used a LPA₄-overexpressing cell type in which the LPA₄ response is coupled to Gₛ and G₁₂/₁₃ but does not mediate effects through Gₛ as suggested herein with endogenously LPA₄-expressing HT1080 cells.

In addition to the role of LPA₄ in tumor invasion, one important observation of our study is the finding that cAMP and EPAC are involved in invadopodia production. Although the role of cAMP in various cell functions has been studied for decades, information about its contribution to cell invasion is scarce. Increases in cAMP have previously been correlated with anti-invasiveness of intestinal cancer cells (43). However, many of the effects of cAMP seem to be contradictory and depend on the cell types studied. For example, depending on cell context, cAMP either stimulates or inhibits cell division. It also causes reversion to a normal phenotype in some transformed cells while being important for differentiation of many cell types (44). These observations may be related to the independent or opposing functions of the two main effectors of cAMP: PKA and EPAC (35). Here, we clearly showed that cAMP accumulation led to the production of invadopodia structures and that downstream activation of EPAC and Rap1, but not PKA, was involved in this function. EPAC is an established guanine nucleotide exchange factor for Rap1, a small GTPase, which has been implicated in malignancy (36) mainly through the inside-out activation of integrins that are associated with cell invasion (45). Invadopodia structures are enriched in integrins that interact with the metalloproteinase membrane-type 1 matrix metalloproteinase to trigger cancer cell invasion (46). It is therefore possible that one of the mechanisms by which EPAC affects invadopodia formation is through the inside-out activation of integrins by Rap1.

We also observed that invadopodia formation induced by EPAC was dependent on Rac1 activation. Rap1 is known to activate Rac1 indirectly, through integrin signaling, or more directly due to the activation of the Rap1 effectors Yav2 and Tiam (45). Recently, EPAC and Rap1 have been implicated in translocation of Rac1 to the cell membrane, resulting in the recruitment of the Rac effector cortactin (47). Because Rap1 as well as EPAC activities are spatially regulated in cells (48), it is possible that specific activation of EPAC-Rap1 at sites of invadopodia formation could result in translocation of Rac1 to these sites, leading to the recruitment of cortactin, an essential component of forming invadopodia. Our findings that stimulation of the cAMP-EPAC pathway affects invadopodia production through Rac1 activation identify an alternative pathway for the activation of Rac1 at the sites of invadopodia formation.

Whereas Rac1 inhibition abolished EPAC-induced invadopodia production, the effects of LPC and LPA were only reduced by ~50%. These observations suggested that LPC and LPA triggered the activation of pathways that were independent of the EPAC-Rac1 axis. In support of this, our results further showed a minor role for the Gₛ G protein in invadopodia formation (S1). It is possible that in addition to Gₛ, G₁₂ could also couple to LPA₄ due to Gₛ/G₁₂ switching, a phenomenon whereby phosphorylation of the receptor causes it to switch coupling specificity from Gₛ to G₁₂ (49). In addition, preliminary results from our laboratory indicate that inhibition of Rock,

1 K. Harper et al., unpublished data.
a downstream effector of RhoA, but not PI3K, causes a partial decrease in invadopodia formation induced by ATX. This would implicate a potential involvement of the Rock pathway as well as other LPA receptors such as LPA₁ or LPA₂ in invadopodia formation, which remains to be further elucidated.

To date, studies have indicated that LPA is involved in growth and metastasis of various cancers, including ovarian and breast, mainly through its action on LPA₁, LPA₂, and LPA₃ receptors (20, 21, 39). The expression of LPA receptors is cell specific, and each can elicit different and to some extent overlapping responses on LPA binding (13, 19). Our work presents evidence that inhibition of the LPA receptor LPA₁ could provide an additional tool to improve the efficacy of treatment of metastasis. Because LPA receptors and ATX are currently being targeted in preclinical trials (30), the current findings should stimulate studies to evaluate the expression pattern and clinical outcome of LPA₁, together with other LPA receptors and ATX, in various cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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