Cdc42 and Rac1 are necessary for autotaxin-induced tumor cell motility in A2058 melanoma cells

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Abstract Autotaxin (ATX) is a strong motogen that can increase invasiveness and angiogenesis. In the present study, we investigated the signal transduction mechanism of ATX-induced tumor cell motility. Unlike N19RhoA expressing cells, the cells expressing N17Cdc42 or N17Rac1 showed reduced motility against ATX. ATX activated Cdc42 and Rac1 and increased complex formation between these small G proteins and p21-activated kinase (PAK). Furthermore, ATX phosphorylated focal adhesion kinase (FAK) that was not shown in cells expressing dominant negative mutants of Cdc42 or Rac1. Collectively, these data strongly indicate that Cdc42 and Rac1 are essential for ATX-induced tumor cell motility in A2058 melanoma cells, and that PAK and FAK might be also involved in the process.

Key words: Autotaxin; Rac1; Cdc42; Focal adhesion kinase

1. Introduction

Active tumor cell motility is involved in many stages of the metastatic cascade, including the transition from in situ to invasive carcinoma [1]. Dysregulation of motility plays an important role in promoting invasion and metastasis [2]. Autotaxin [ATX; nucleotide-pyrophosphatase phosphodiesterase-2 (NPP-2)] is a 125-kDa autocrine motility factor which stimulates motility of the same tumor cells that make the factor [3]. ATX was initially isolated from A2058 human melanoma cells and DNA sequence analysis revealed that ATX was homologous to a family of exo/ecto NPPs that includes the B cell activation marker, PC-1, and the neural differentiation antigen, B10 [4]. ATX contains two adjacent somatomedin B regions, the loop region of an EF-hand, and a type I phosphodiesterase catalytic site, and possesses 5′-nucleotide pyrophosphatase/ATPase activities [5,6]. Recently, ATX was reported to augment cellular characteristics necessary for tumor aggressiveness [7]. ATX expression in *ras*-transformed NIH3T3 cells showed amplified tumorigenesis and metastatic potential compared to *ras*-transformed control. Furthermore, ATX was reported as an angiogenic factor that stimulates HUVECs grown on Matrigel to form tubules [8]. Accumulating evidence suggests that various intracellular signaling molecules such as phosphatidylinositide 3-kinase (PI3K), phospholipase Cγ, mitogen-activated protein kinase, and protein kinase C were differentially involved in the stimulation of motility, depending on kinds of chemoattractants and cellular systems [2]. Small G proteins of the Rho family also have various effects on the actin cytoskeleton and focal adhesions that may actively participate in chemoattractant-induced motility. While Cdc42 controls the formation of filopodia, Rac1 and RhoA are involved in the formation of lamellipodia and actin stress fibers, respectively [9–11]. Activated Cdc42 and Rac1 bind and activate p21-activated kinase (PAK) which in turn targets cytoskeletal movements [12].

Focal adhesion kinase (FAK) is a cytoplasmic protein-tyrosine kinase that localizes to focal adhesions [13]. FAK can be phosphorylated by growth factors and during formation of focal adhesions and spreading [14]. Activated FAK in turn recruits other proteins, including paxillin, $p130^{Cas}$, vinculin, and talin in focal adhesions. These protein complexes anchor the actin cytoskeleton and provide structural integrity to cells [15].

Although ATX has been demonstrated to stimulate tumor cell motility and to augment characteristics necessary for tumor aggressiveness, little is known about the intracellular signaling mechanism of ATX except its link to G protein-coupled unknown receptor [16]. Recently, we have shown that G protein-coupled PI3Kγ is involved in ATX-induced tumor cell motility stimulation [17]. In the present study, we continued to investigate the cellular signal transduction mechanism of ATX-induced tumor cell motility and found that Cdc42 and Rac1 mediate in ATX-induced motility of A2058 melanoma cells.

2. Materials and methods

2.1. Reagents

C3 exoenzyme and pertussis toxin (PTx) were purchased from Calbiochem (San Diego, CA, USA). Anti-Rac1, anti-Cdc42, anti-FAK, and anti-phosphotyrosine (PY20) antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Anti-PAK1 was purchased from Santa Cruz Technology (Santa Cruz, CA, USA). Y27632 was from Tocris (Bristol, UK), and Sepharose 4B was from

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Abbreviations: ATX, autotaxin; PI3K, phosphatidylinositide 3-kinase; lysoPLD, lysophospholipase D; LPA, lysophosphatidic acid; FAK, focal adhesion kinase; PAK, p21-activated kinase; NPP, nucleotide-pyrophosphatase phosphodiesterase; PTx, pertussis toxin

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Amersham Bioscience (Seoul, Korea). LY294002, Wortmannin, and MBP were from Sigma (Seoul, Korea). All reagents from commercial sources were of analytical grade.

2.2. Cell culture

The human melanoma cell line A2058, originally isolated from Todaro et al.[18], was maintained as described previously [19]. The cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (complete medium) in a humidified 5% CO_2. For analysis of ATX-induced signals, cells in exponential growth were washed extensively to remove growth factors and resuspended in serum-free media (basal media) for 16 h to induce maximum synchronization.

2.3. Expression plasmid and transfection

Vectors (pEBG) expressing the catalytically inactive mutants N17Rac1 and N17Cdc42 were obtained from Dr. John Blenis of Harvard Medical School. N19RhoA in pcDNA3 was from Dr. Seung-Kil Park of Chungnam University, Korea, and GST-P21-binding domain (GST-PBD) of PAK was from Dr. Dongeun Park of Seoul University, Korea. A2058 melanoma cells were transiently transfected with each vector using the SuperFect™ transfection system (Qiagen) as described previously in detail [17]. Cells were washed with phosphate-buffered saline (PBS) and grown in complete medium for 48 h before cell motility, immunoprecipitation, and immunoblotting analysis.

2.4. Purification of recombinant ATX (rATX)

Teratocarcinoma ATX cDNA and its T210A mutant form (A210ATX) were subcloned into the plasmid vector pcDNA3 (Invitrogen) and then transiently transfected into Cos-1 cells using the DEAE–dextran method [20]. A210ATX mutant is an ATX mutant without phosphodiesterase and tumor cell motility-stimulating activities [21]. Control for these experiments was Cos-1 cells that had been transfected with empty pcDNA3 vector. After overnight recovery in complete medium, DMEM containing 0.1 mg/ml bovine serum albumin was added to the cells, harvested after 48 h, and concentrated. The concentrated supernatants from *ATX*-, A210ATX-transfected, and control Cos-1 cells were partially purified by lectin affinity chromatography with concanavalin A–agarose (Vector Laboratories) as described previously in detail [21].

2.5. Immunoprecipitation and immunoblotting

Cell lysates were prepared with lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, and 1 mM NaF). FAK and PAK were precipitated from the cell lysates (400 μg of total protein) with specific antibodies for 3 h at 4°C. Protein–antibody complexes were collected with protein A–Sepharose beads for 3 h at 4°C. The precipitates were washed three times with ice-cold lysis buffer, resolved by 7.5% SDS–PAGE. Tyrosine phosphorylation and protein levels of FAK, PAK, Cdc42, and Rac1 were assessed by immunoblotting with anti-phosphotyrosine (PY20) or the specific antibodies as used for precipitations. Immunolabeling was detected by ECL (Amersham Life Science) according to the manufacturer's instructions.

2.6. Small G protein pull-down assays

The Cdc42-GTP and Rac1-GTP pull-down assay was modified from Benard et al. [22]. In brief, Cells were washed with ice-cold PBS and lysed with lysis buffer (150 mM NaCl, 0.8 mM $MgCl_2$, 5 mM EGTA, 1% NP-40, 50 mM HEPES, pH 7.5, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After centrifuged at 14 000×*g* for 5 min, 20 μl of a 50% slurry of GST-PAK-PBD glutathione-Sepharose 4B added to cell lysate and incubated for 45 min at 4°C. Proteins bound to beads were washed with 50 mM Tris–HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and eluted in Laemmli sample buffer. Proteins were analyzed by Western blotting using antibodies against Cdc42 or Rac1.

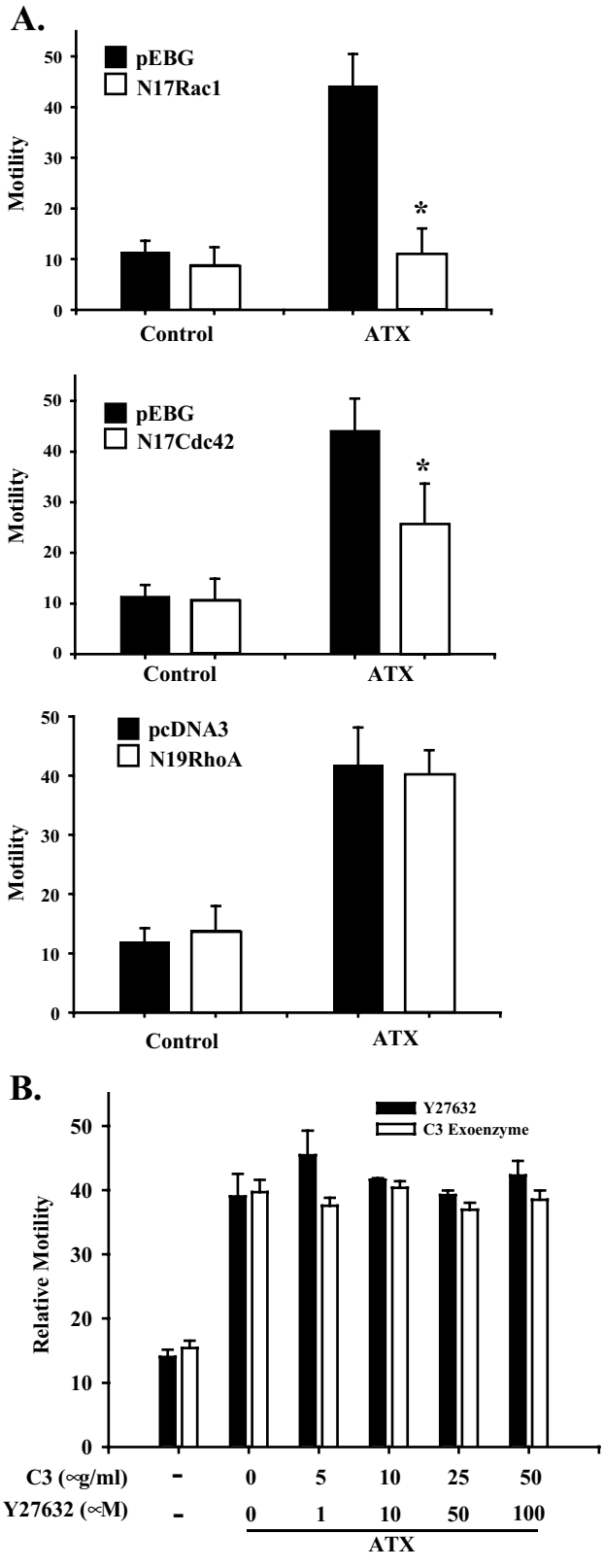

Fig. 1. Cdc42 and Rac1 are involved in ATX-induced tumor cell motility. A: A2058 melanoma cells were transiently transfected with each of N17Rac1, N17Cdc42, N19RhoA, and the corresponding control vectors. B: After preincubation with various concentrations of RhoA or Rho kinase inhibitors, cells were further incubated with or without 250 ng/ml ATX. Motility was analyzed by using modified Boyden chamber assay. Data shown are the densitometric values with the mean ± S.D. of three separate experiments, and motility assay was carried out in triplicate. $*P < 0.05$ in comparison with vector-transfected cells.

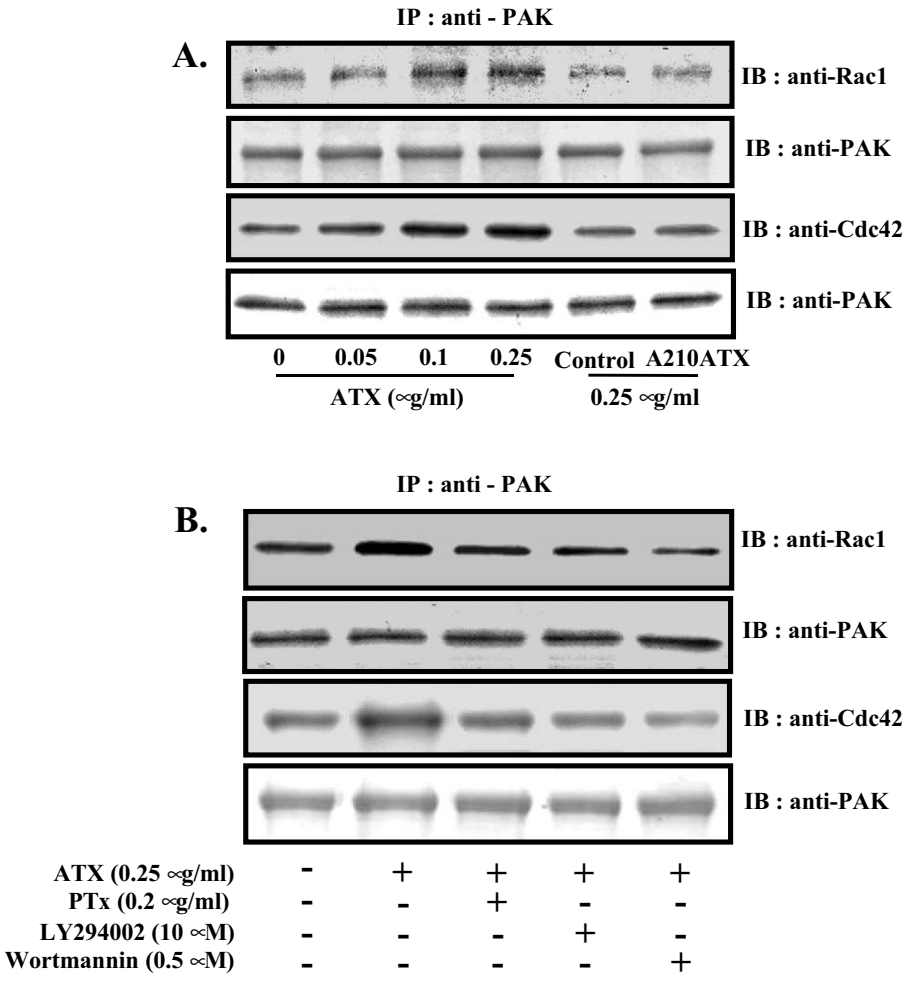

Fig. 2. ATX induces Rac1-PAK and Cdc42-PAK complex formation. A: A2058 melanoma cells were serum-deprived and then treated with vehicle, ATX, and A210ATX for 30 min. B: Cells were treated with or without each reagents (0.2 μg/ml PTx, 90 min; 0.5 μM wortmannin, 30 min; 10 μM LY294002, 30 min) before incubating with ATX. Cell lysates were immunoprecipitated (IP) with an anti-PAK antibody following by anti-Rac1 or anti-Cdc42 immunoblotting (IB). Data shown are representative of three experiments with similar results.

2.7. Cell motility assays

Motility assays were performed in triplicate using a 48-well microchemotaxis chamber for 4 h as described previously in detail [16]. Cells were harvested using a trypsin/EDTA solution and resuspended in DMEM with 1 mg/ml bovine serum albumin. The bottom wells were filled with varying concentrations of purified ATX and A210ATX mutant. Gelatin-coated polyvinyl pyrrolidine-free polycarbonate filters with 8 μm pores membranes (Neuroprobe) used in these modified Boyden chambers were fixed and stained using Diff-Quik reagents (American Scientific Products). Chemotaxis was quantified densitometrically using EagleSight Software v. 3.2 (Stratagene) for data analysis as described previously [17].

2.8. Statistical analysis

Results are expressed as the mean ± S.D., and an analysis was done by one-way Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

3. Results and discussion

ATX has been shown to augment the cellular characteristics necessary for tumor invasiveness [7] and angiogenesis [8]. However, little is known about the signaling pathway of ATX-induced tumor cell motility. Recently, we have shown that the G protein-coupled isoform of PI3K (PI3Kγ) is necessary for ATX-induced tumor cell motility in A2058 melanoma

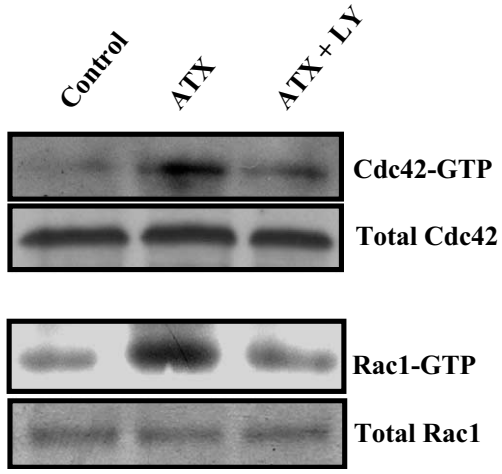

Fig. 3. ATX activates Cdc42 and Rac1. A2058 melanoma cells were serum-deprived and treated with or without 10 μM LY294002 (LY) for 30 min before incubating with ATX (0.25 μg/ml) for 30 min. Cellular extracts were incubated with GST-PAK-PBD beads. The bound proteins (Cdc42-GTP or Rac1-GTP) were analyzed by Western blot analysis using specific anti-Cdc42 and Rac1 antibodies. Data shown are representative of four experiments with similar results.

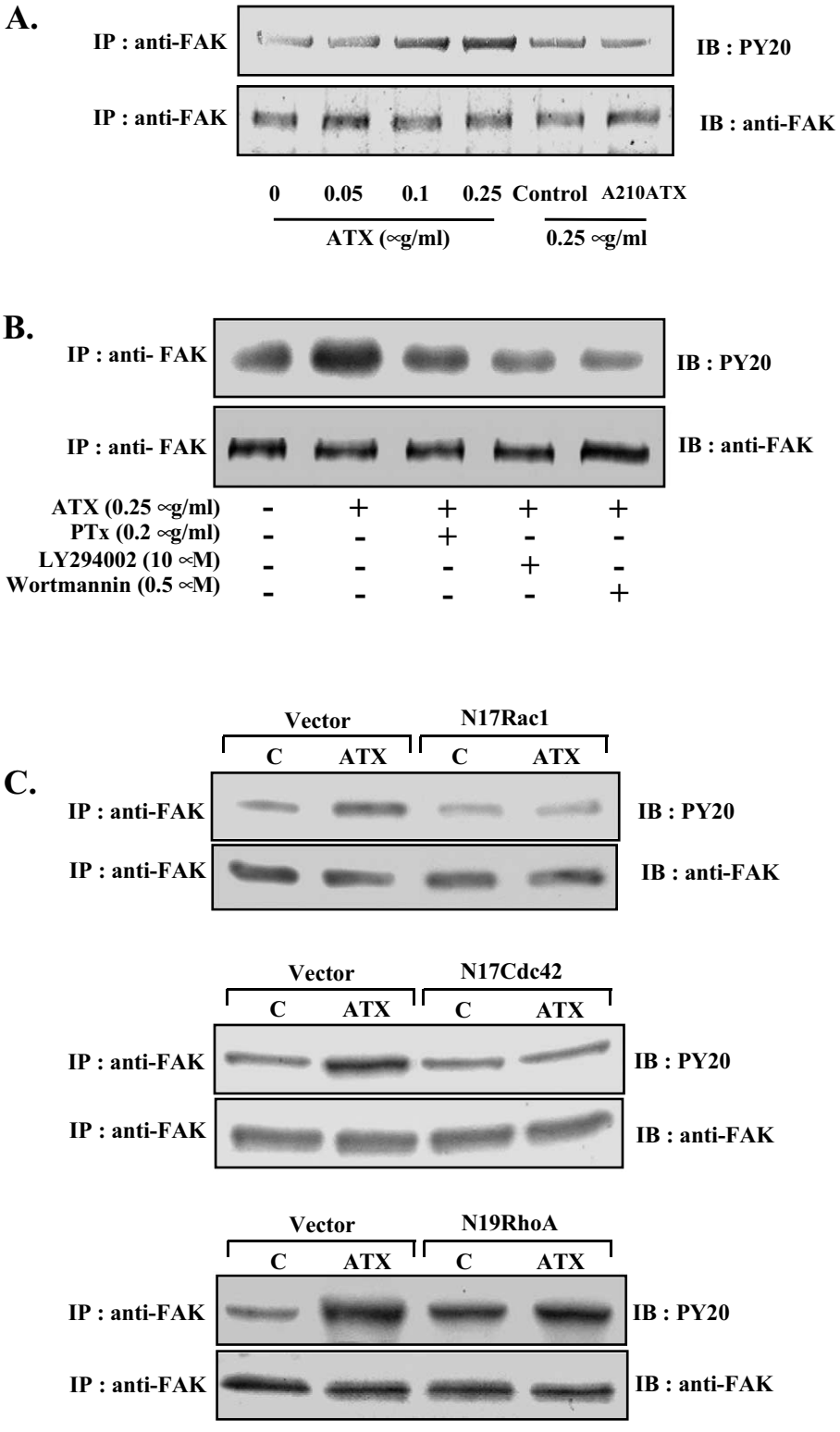

Fig. 4. FAK is phosphorylated by ATX. A: A2058 melanoma cells were serum-deprived and then treated with control, ATX, and A210ATX for 30 min. B: Cells were treated with or without each reagent (0.2 μg/ml PTx, 90 min; 0.5 μM wortmannin, 30 min; 10 μM LY294002, 30 min) before ATX treatment. C: After transfection with each of N17Rac1, N17Cdc42, N19RhoA, and the corresponding control vectors, cells were incubated with either control or ATX for 30 min. Cell lysates were immunoprecipitated (IP) with an anti-FAK antibody and followed by immunoblotting (IB) with anti-phosphotyrosine antibody (PY20). Data shown are representative of four experiments with similar results.

cells [17]. ATX increased PI3Kγ activity that was abolished by pretreatment of A2058 melanoma cells with PTx or PI3K inhibitors. In the present study, we investigated the involvement of Rho family GTPases in ATX-induced tumor cell motility since Cdc42, Rac1, and RhoA located downstream of PI3K have been recognized as key regulators for cell migration [23]. ATX-induced cell motility was measured in A2058 melanoma cells transfected with pEBG vector,

pcDNA3 vector, and inactive mutants of Rac1 (N17Rac1), Cdc42 (N17Cdc42), and RhoA (N19RhoA). As shown in Fig. 1A, overexpression of dominant negative Rac1 or Cdc42 completely or partially inhibited ATX-induced motility, respectively. In contrast, overexpression of dominant negative RhoA had no effects on ATX-induced cell motility. Consistent with the result, C3 exoenzyme or Y27632, which inhibits RhoA and Rho kinase, respectively, did not inhibit cell motility by ATX (Fig. 1B), confirming that RhoA was not involved in the signal transduction of ATX-induced tumor cell motility. These results suggested the possible involvement of Cdc42 and Rac1, but not RhoA, in ATX-induced tumor cell motility. These observations are in accordance with Cdc42/Rac dependent- and RhoA independent-enhancement of cell motility by Vav3 [24] and VEGF [25]. One of the well-characterized downstream target molecules of Rac/Cdc42 is PAK [26]. By direct interaction of its p21-binding domain with GTP-bound active Cdc42 or Rac, group I PAKs (PAK1–3) undergoes a conformational change that allows its autophosphorylation and activation [27]. To test the effects of ATX on PAK1, A2058 melanoma cells were treated with increasing concentrations of ATX and A210ATX mutant. As shown in Fig. 2, ATX increased association of Rac1 and Cdc42 with PAK in a dose-dependent manner, while control or A210ATX did not. This complex formation was completely blocked by PTx or PI3K inhibitors, wortmannin or LY294002, (Fig. 2B), suggesting that Rac1 and PAK were located downstream of PI3Kγ in ATX-induced tumor cell motility.

To verify that activated Rac1 and Cdc42 form a complex with PAK, direct pull-down experiments were performed by measuring the binding of GTP-bound GTPase to the p21-binding domain of PAK1 fused to glutathione S-transferase (GST-PBD) [28]. As shown in Fig. 3, ATX increased the levels of Cdc42-GTP and Rac1-GTP that were inhibited by the pre-incubation with LY294002, confirming that ATX activates Cdc42 and Rac1 via the activation of PI3Kγ.

FAK is a non-receptor protein-tyrosine kinase that is localized at focal contact sites and plays a critical role in controlling cell migration [29,30]. An increased level of FAK expression is highly correlated with the invasion potential of human tumors [31]. FAK phosphorylation was increased by increasing concentrations of ATX but not by vector control or A210ATX mutant (Fig. 4A). ATX-induced phosphorylation of FAK was completely inhibited by PTx or PI3K inhibitors (Fig. 4B). In addition, FAK phosphorylation was also inhibited by dominant negative mutants of Rac1 and Cdc42 but not by RhoA (Fig. 4C). The data suggest that FAK phosphorylation is the consequence of ATX-induced activation of PI3K, Rac1, and Cdc42. At present, we do not have direct evidence showing that FAK phosphorylation is necessary for ATX-induced cell motility. However, one might be able to speculate that FAK phosphorylation could be involved in ATX-induced cell motility because inhibition of the same upstream molecules inhibits both FAK phosphorylation and cell motility. Furthermore, A210, an ATX mutant without motility-stimulating activity, did not phosphorylate FAK phosphorylation.

Recently, ATX was reported to have a lysophospholipase D (lysoPLD) activity [32]. LysoPLD catalyzes the conversion of lysophosphatidylcholine to lysophosphatidic acid (LPA), which mediates multiple biological functions, including cytoskeletal reorganization, chemotaxis, and cell growth through activation of specific G protein-coupled receptors [33,34]. It is very possible that ATX mediates its biological effects through the generation of LPA as suggested [32]. Our findings, which demonstrate that ATX-induced cell motility is sensitive to PTx treatment and dependent on the activation of Rac1 and Cdc42, appear to agree with the signaling pathways demonstrated with activation of Edg receptors by LPA [35]. LPA was also shown to increase phosphorylation of FAK in human osteosarcoma cells [36] and in preosteoblastic cells [37]. Furthermore, RhoA was demonstrated to activate FAK phosphorylation [38], and ROCK inhibitor Y27632 suppressed LPA-induced tyrosine phosphorylation of FAK [39]. Our data showed that RhoA was not involved in ATX-induced FAK phosphorylation in A2058 melanoma cells (Fig. 4C). It is possible that different small G proteins are involved in ATX/LPA-induced cell motility in different cell types.

The present study provides the evidence that ATX activates small G proteins and induces Cdc42/Rac1-PAK1 complex formation. Furthermore, ATX increased the phosphorylation of FAK which is located downstream of PI3K and small G proteins in ATX-induced tumor cell motility. At present we do not know how Cdc42 and Rac1 regulate FAK phosphorylation. Since FAK was coimmunoprecipitated with both NCK and PAK in vascular endothelial growth factor-activated endothelial cell [40], NCK might be a mediator that connects between PAK and FAK in ATX-induced tumor cell motility.

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