Autotaxin promotes motility via G protein-coupled phosphoinositide 3-kinase γ in human melanoma cells

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Abstract Autotaxin (ATX), an exo-nucleotide pyrophosphatase and phosphodiesterase, stimulates tumor cell motility at subnanomolar levels and augments invasiveness and angiogenesis. We investigated the role of G protein-coupled phosphoinositide 3-kinase γ (PI3Kγ) in ATX-mediated tumor cell motility stimulation. Pretreatment of human melanoma cell line A2058 with wortmannin or LY294002 inhibited ATX-induced motility. ATX increased the PI3K activity in p110γ, but not p85, immunoprecipitates. This effect was abrogated by PI3K inhibitors or inhibited by pertussis toxin. Furthermore, stimulation of tumor cell motility by ATX was inhibited by catalytically inactive form of PI3Kγ, strongly indicating the crucial role of PI3Kγ for ATX-mediated motility in human melanoma cells © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Autotaxin; Phosphoinositide 3-kinase; p110γ; Tumor cell motility

1. Introduction

Cell motility is a fundamental process required during normal embryonic development, inflammatory responses, wound healing, and tumor metastasis [1]. Many types of tumor cells have been found to produce 'autocrine motility factors' which stimulate motility of the same tumor cells that make the factor [2]. Autotaxin [ATX; nucleotide-pyrophosphatase phosphodiesterase-2 (NPP-2)] is a 125-kDa glycoprotein initially isolated from the human melanoma cell line A2058. This autocrine motility factor stimulates pertussis toxin (PTx)-sensitive chemotaxis in human melanoma cells at picomolar to nanomolar concentrations [3]. DNA sequence analysis determined 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]. Like other members of the NPP family, ATX has multiple enzymatic activities [5]. The phosphodiesterase (PDE) activity appears to be essential for motility stimulation, since a single point mutation at threonine-210 abolishes the PDE- and tumor cell motility-stimulating activities of ATX [6].

A recent study has revealed that combination of ATX expression with *ras* transformation produced cells with greatly amplified tumorigenesis and metastatic potential compared to *ras*-transformed controls. Thus, ATX appears to augment cellular characteristics necessary for tumor aggressiveness [7]. Furthermore, ATX stimulates HUVECs grown on Matrigel to form tubules, much like vascular endothelial growth factor [8], suggesting that ATX could contribute to the metastatic cascade through multiple mechanisms.

Recently, accumulating evidences suggest that various intracellular signaling molecules such as phosphoinositide 3-kinase (PI3K) [9], phospholipase Cγ (PLCγ) [10], mitogen-activated protein kinase [11], and protein kinase C [12] were differentially involved in the stimulation of motility depending on kinds of chemoattractants and cellular systems. Although ATX has been demonstrated to act as a autocrine motility factor in tumor cells [3], little is known about the signaling mechanism by which ATX stimulates cell motility. Therefore, the clarification of the possible involvement of specific signaling molecule in ATX stimulation of motility would be important for understanding the molecular mechanisms of tumor invasion and metastasis.

In the present study, we investigated the possible involvement of PI3K on ATX-induced tumor cell motility stimulation and found that G protein-coupled PI3Kγ plays a pivotal role in the stimulation of motility by ATX in human melanoma cells.

2. Materials and methods

2.1. Reagents

Wortmannin and LY294002 were from Sigma. PTx was purchased from Calbiochem (San Diego, CA, USA). Anti-PI3K p110γ and p85 antisera were purchased from Santa Cruz Technology (Santa Cruz, CA, USA). [γ-^{32}P]ATP was from Amersham (Piscataway, NJ, USA). All reagents from commercial sources were of analytical grade.

2.2. Cell culture

The human melanoma cell line A2058, originally isolated from To-

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Abbreviations: ATX, autotaxin; PDE, phosphodiesterase; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositols; PtdIn(3)P, phosphatidylinositol 3-phosphate; NPP, nucleotide-pyrophosphatase phosphodiesterase; PTx, pertussis toxin

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daro et al. [9], was maintained as described previously [10]. 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 6 h to induce maximum synchronization.

2.3. Expression plasmid and transfection

pcDNA3 expressing the catalytically inactive mutant of PI3Kγ with isoprenylation signal of K-Ras, PI3KγK832R, was kindly provided by M. Wymann (Institute of Biochemistry, University of Fribourg) and are described elsewhere [11,12]. Human melanoma cells were transiently transfected with pcDNA3 vector or PI3KγK832R plasmid using SuperFact™ transfection system (Qiagen). 25 μl of SuperFact™ transfection reagent was added to 5 μg of plasmid and incubated at room temperature for 10 min. After adding 1 ml of complete medium, DNA and transfection reagent mix were added to 2×10^5 cells per 60 mm dish and incubated for 3 h at 37°C in a humidified 5% CO_2. Cells were washed with PBS and grown in complete medium for 48 h before cell motility assays.

2.4. Purification of recombinant ATX

ATX cDNA, which included the full-length open reading frame, was subcloned into the plasmid vector pcDNA3 (Invitrogen) and then transiently transfected into Cos-1 cells using the DEAE–dextran method [13]. Control for these experiments was Cos-1 cells which 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 both *atx*-transfected and control Cos-1 cells were partially purified by lectin affinity chromatography with concanavalin A-agarose (Vector Laboratories) as described previously in detail [6], and both purified fractions were used as stimulator and control for unstimulated experiment, respectively.

2.5. PI3K activity assay in vitro

PI3K activity was assayed essentially as described previously [14] with some minor modifications. 500 μg of protein from each sample was immunoprecipitated with 1 μg of anti-p110γ or anti-p85 antibody for 90 min at 4°C. Immunoprecipitates were rinsed and then incubated with 0.5 mg/ml phosphoinositols (PtdIns) and 20 μCi of [γ-^{32}P]ATP in reaction buffer (50 μM ATP, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.5) for 30 min at 30°C. The lipid products were extracted by methanol/chloroform and separated in thin-layer chromatography (TLC). Phosphorylated products were detected by Fluorescent Image Analyzer FLA2000 (Fujifilm, Japan).

2.6. Reverse transcription-polymerase chain reaction (PCR) of human melanoma cell p110γ

Total RNA from human melanoma cells, A2058, was isolated using TriZol (Life Technologies, Inc.) following the manufacturer's suggested protocols and reverse transcription-PCR was assayed essentially as described previously [15] with some modification. The primer sequences for p110γ and GAPDH are as follows: sense1, GTGGTGCTGAGAGAGGACAA, antisense1, CTATCAGCAGCAGGTTCACA (1.38-kb fragment); sense2, ACAGATTCTACGAATCATGG, antisense2, GCATTCCTGTCATCAGCATC (0.585-kb fragment); GAPDH sense, GACCCCTTCATTGACCTCAA, GAPDH antisense, CATGGACTGTGGTCATGAGC. The sequence of PCR amplification was one cycle of denaturation at 95°C for 5 min followed by annealing at 56°C for 30 s and extension at 72°C for 1 min. This cycle was followed by 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C, repeated 38 times. The PCR reaction was sampled every five cycles from cycle 25 to 40, inclusively. The samples were separated on a 2% agarose gel, stained with ethidium bromide, and compared for intensity.

2.7. Immunoblotting

A2058 cells were washed with ice-cold PBS and lysed in 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 and leupeptin, 1 mM sodium orthovanadate, and 1 mM NaF). Proteins were separated on SDS–polyacrylamide gel electrophoresis (10%) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in PBS containing 0.1% Tween 20 (PBS-T) and 5% (w/v) dry skim milk powder, and incubated overnight with anti-p110γ. The membranes were then washed with PBS-T and incubated for 1 h with an anti-rabbit secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

2.8. Cell motility assays

Motility assays were performed in triplicate using a 48-well microchemotaxis chamber for 4 h as described previously in detail [3]. In brief, A2058 melanoma cells that were approximately 75–90% confluent were harvested with trypsin-EDTA and allowed to recover at room temperature in DMEM supplemented with 10% heat-inactivated fetal bovine serum for at least 1 h. The cells were then resuspended at 2×10^6 cells/ml in DMEM with 1 mg/ml bovine serum albumin. Gelatin-coated Nuclepore membranes 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 [16].

3. Results and discussion

Dysregulation of motility plays an important role in promoting invasion and metastasis [17]. Cytokines and growth factors stimulate tumor cell motility through various intracellular signaling molecules including PI3K [18], PLCγ [19], mitogen-activated protein kinase [20], and protein kinase C [21]. Recently, ATX was reported as a metastasis-enhancing mito-

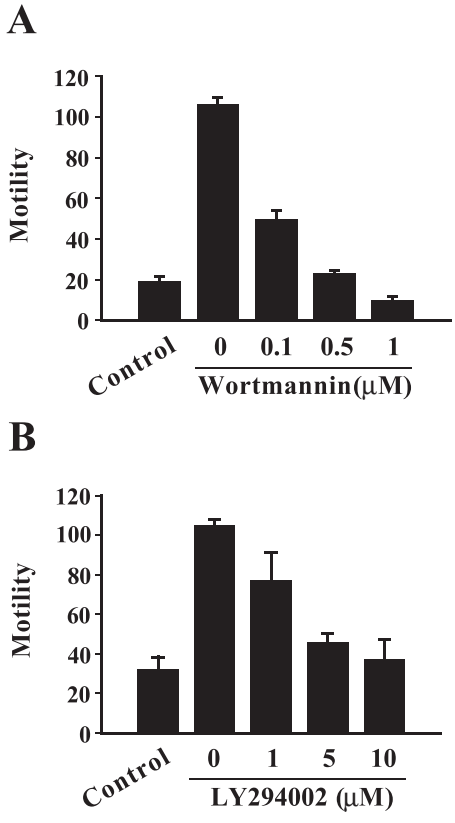

Fig. 1. Effects of PI3K inhibitors on ATX-mediated tumor cell motility. Cells were treated with various concentrations of wortmannin (A) or LY294002 (B) for 30 min before motility assays against ATX or control. Data shown are the densitometric values with the mean ± S.D. of three separate experiments, and motility assay was carried out in triplicate experiments.

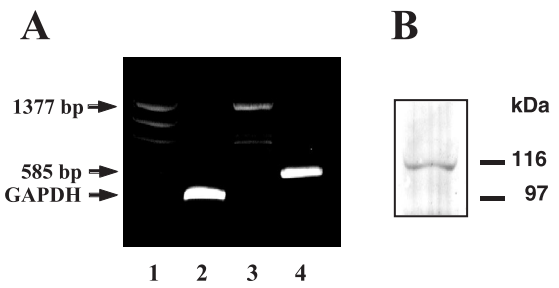

Fig. 2. Presence of p110γ in human melanoma cells. A: 10 μg of total RNA purified from melanoma cells were reverse-transcribed and subjected to PCR using primers designed from the published human p110γ PI3K DNA sequence. GAPDH primers were included as a positive control. Lane 1: Molecular weight markers (ΦX174 DNA-*Hae*III digest); lane 2: GAPDH; lane 3: 1377 bp DNA fragment of p110γ; lane 4: 585 bp DNA fragment of p110γ. B: Cell lysate was immunoblotted with the anti-p110γ antiserum. The results of these experiments are typically representative of three separate experiments.

gen and angiogenic factor without any knowledge about intracellular signaling pathway [7,8]. To identify the factor that mediates the ATX-induced tumor cell motility, we first examined PI3K as a candidate of ATX signaling molecule by utilizing the PI3K inhibitors, wortmannin and LY294002. A2058 cells were incubated with various concentrations of each inhibitors for 30 min, then assayed for their motility response to ATX. As shown in Fig. 1A,B, both PI3K inhibitors blocked the ATX-mediated tumor cell motility stimulation in a dose-dependent manners, indicating the possible involvement of PI3K in the stimulation of tumor cell motility by ATX.

Four mammalian type I PI3K isoforms (p110α, β, γ, δ) have been identified [22]. The type IA PI3Ks, p110α, p110β and p110δ, associate with the p85 family of regulatory subunits, but type IB p110γ binds to a p101 adaptor molecule [12,23]. Whereas type IA PI3Ks are activated by interaction with tyrosine-phosphorylated molecules, p110γ is regulated preferentially by interaction with heterotrimeric G protein subunits [23]. The presence of p110γ was described in human neutrophil [15], NK cells [24], T lymphocytes [25], and vascular smooth muscle cell nuclei [26]. To test whether human melanoma cells express p110γ, we utilized PCR amplification with oligonucleotide primers that are specific for human p110γ. As shown in Fig. 2A, the results revealed fragments of the expected sizes of 1377 and 585 bp. Similarly, immunoblots of cell lysates, performed with polyclonal antibody against p110γ, revealed that this antibody detected a protein band at an approximate molecular mass of 110 kDa. Thus, A2058 cells appear to express both mRNA and protein for p110γ (Fig. 2A,B).

Since G_i protein appears to be involved in ATX-mediated tumor cell motility stimulation [3], we then determined whether p110γ is activated by ATX. A2058 cells were incubated for various times ranging from 15 s to 15 min with 250 ng/ml ATX. The treated cells were lysed and immunoprecipitated with anti-p110γ antibody, and PI3K activities in the immunoprecipitates were assayed. As shown in Fig. 3A, ATX treatment resulted in increased formation of phosphatidylinositol 3-phosphate [PtdIns(3)P] from PtdIns. This increase began as early as 15 s after stimulation with ATX and continued for at least 15 min. The increased production of PtdIns(3)P was abrogated when the cells had been preincubated with either 500 nM wortmannin or 10 μM LY294002 before ATX stimulation (Fig. 3B). Upon preincubation for 90 min with PTx prior to ATX stimulation, ATX-induced PtdIns(3)P production was decreased by 66% (Fig. 3B).

The effect of ATX on type IA PI3Ks was tested in homologous experiments (Fig. 3A). A2058 cells were stimulated with ATX (250 ng/ml) for 15 s to 15 min, lysed, and immunoprecipitated using an anti-p85 antibody. Unlike p110γ, ATX treatment had no significant effect on the production of PtdIns(3)P by anti-p85 immunoprecipitates (Fig. 3A,B). This result is in accordance with the complete inhibition of the chemotactic factor-stimulated generation of PtdIns(3,4,5)P3,

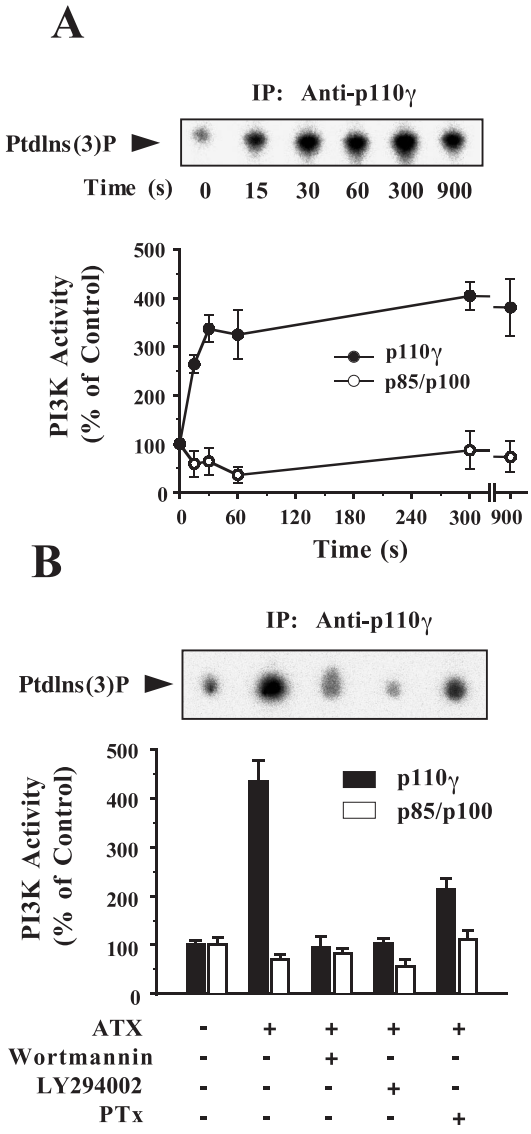

Fig. 3. Activation of p110γ by ATX in A2058 cells. A: Stimulation of the activity of p110γ by ATX in various times. After treatment with 250 ng/ml ATX for the indicate times, cell lysates were subjected to PI3K assay by immunoprecipitation using specific antibody against the p110γ or p85 as described under Section 2. B: Effects of wortmannin, LY294002, and PTx on the stimulation of p110γ activity by ATX. After pretreatment with or without each reagents (0.5 μM wortmannin, 30 min; 10 μM LY294002, 30 min; 200 ng/ml PTx, 90 min), ATX was treated for 300 s. The upper panel depicts the result of a representative TLC plate and the lower panel shows the average densitometric values. Data shown are the mean ± S.D. from triplicate samples and are typically representative of three separate experiments.

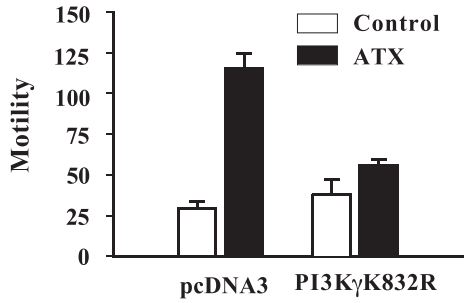

Fig. 4. Involvement of PI3Kγ in ATX-induced tumor cell motility. pcDNA3 or pcDNA3 with PI3KγK832R was transiently transfected into A2058 cells using SuperFact™ system and used for motility assay toward ATX or control. Data shown are the densitometric values with the mean ± S.D. of three separate experiments, and motility assay was carried out in triplicate experiments.

severe defects in migration in murine p110$\gamma^{-/-}$ neutrophils [27], and a reduced migration toward a wide range of chemotactic stimuli in peritoneal PI3Kγ-null macrophages [28]. Fractions purified from the conditioned medium of empty vector-transfected Cos-1 cells did not increase the p110γ activity (data not shown).

To confirm the involvement of PI3Kγ in signal transduction of ATX, pcDNA3 vector alone or pcDNA3 expressing the catalytically inactive mutant of PI3Kγ, PI3KγK832R [11], was transiently transfected into A2058 cells, and their motility against ATX or control was monitored. As shown in Fig. 4, tumor cell motility in cells transfected with empty vector was increased up to a 3.9-fold in response to ATX, which is similar to that of non-transfected cells (compare to Fig. 1). However, overexpression of PI3KγK832R led only to a 1.5-fold increase in tumor cell motility by ATX, indicating that the PI3Kγ signal pathway is closely associated with ATX-mediated tumor cell motility.

In conclusion, the present study provides the first evidence that the G protein-coupled PI3K, p110γ plays an important role in ATX-induced stimulation of motility in human melanoma cells. The role of PI3Kγ in increased tumor cell invasion and angiogenesis by ATX and downstream factors that may be affected by this activated PI3K are subjects of current investigation.

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