Genetic Down-regulation of Phosphoinositide 3-Kinase by Bikunin Correlates with Suppression of Invasion and Metastasis in Human Ovarian Cancer HRA Cells*

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Hiroshi Kobayashi‡, Mika Suzuki, Naohiro Kanayama, and Toshihiko Terao
From the Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, Handayama 1-20-1, Hamamatsu, Shizuoka, 431-3192, Japan

Using a cDNA microarray analysis, we previously found that exposure of a highly invasive ovarian cancer cell line HRA with bikunin, a Kunitz-type protease inhibitor, or bikunin gene overexpression markedly reduced phosphoinositide kinase (PI3K) p85 gene expression, demonstrating that PI3K may be a candidate bikunin target gene. To clarify how reduced levels of PI3K may confer repressed invasiveness, we transfected HRA cells with PI3K p85 antisense-oligodeoxynucleotide (AS-ODN) and compared the properties of the transfected cells with those of parental cells and sense (S)-ODN cells. We have also demonstrated previously that transforming growth factor-β1 (TGF-β1) stimulates urokinase-type plasminogen activator (uPA)-dependent invasion and metastasis of HRA cells. Here, we show that 1) TGF-β1 induced a rapid increase of the PI3K activity that was accompanied by increased expression (5-fold) of the uPA mRNA; 2) pharmacological inhibition of PI3K or AS-PI3K ODN transfection inhibited TGF-β1-stimulated Akt phosphorylation; 3) both PI3K pharmacological inhibitors and forced expression of AS-PI3K ODN reduced TGF-β1-stimulated uPA mRNA and protein expression by ~70% compared with controls; 4) concentrations of PI3K inhibitors, sufficient to inhibit uPA up-regulation, inhibited TGF-β1-dependent HRA cell invasion; 5) the AS-PI3K ODN cells had a decreased ability to invade the extracellular matrix layer as compared with controls; and 6) when the AS-PI3K ODN cells were injected intraperitoneally into nude mice, the mice developed smaller intraperitoneal tumors and showed longer survival. We conclude that PI3K plays an essential role in promoting uPA-mediated invasive phenotype in HRA cells. Our data identify a novel role for PI3K as a bikunin target gene on uPA up-regulation and invasion.

Cell migration and extracellular matrix invasion (ECM)† are some of the major steps in cancer cell metastasis (1). In the past, several investigators have shown that urokinase-type plasminogen activator (uPA) plays critical roles in embryogenesis, tissue remodeling, inflammation, and in the regulation of cancer cell migration, extracellular matrix invasion, and metastasis by degrading the ECM proteins (2). Several reports have indicated that the increased levels of uPA correlate with the invasive properties of certain tumor cell types (3, 4). uPA up-regulation is a major point of convergence for the actions of a variety of effectors that affect cell morphology, adhesion, and locomotion including Src, mitogen-activated protein kinases (MAPKs), and phosphoinositide 3-OH kinase (PI3K) (5).

Bikunin is a Kunitz-type protease inhibitor and a heavily glycosylated protein (6). Bikunin is composed of the N-terminal extension with the O-linked carbohydrate moiety (chondroitin 4-sulfate sugar side chain, Ala-1 to Lys-21 residues), the Kunitz domain I (Lys-22 to Arg-77 residues), and the C-terminal Kunitz domain II, the protease inhibitor domain (6). This glycoprotein has an inhibitory function for trypsin and plasmin. In our previous experiments, several proteins including uPA and its specific receptor, uPAR, known to be involved in invasion and metastasis were significantly suppressed by exogenously added bikunin (7–10). Furthermore, transfection of HRA cells with bikunin gene reduced the expression of uPA and uPAR as well as diminished cellular invasiveness (11). Current investigations have focused on the understanding of molecular mechanism(s) by which bikunin down-regulates uPA and uPAR expression both in vitro and in vivo and controls invasiveness and tumor growth in highly invasive cancer cells (7–11).

Tumor cells expresses two types of bikunin-binding proteins (BPs), a 40-kDa bikunin-BP, which is identical to cartilage link protein (Crtl1), and a 45-kDa bikunin-BP, a putative bikunin receptor (7). Bikunin binds Crtl1 and bikunin receptor on tumor cell surface possibly via the Kunitz domain I and the chondroitin sulfate side chain, respectively. Bikunin must bind directly to both of the cell-associated bikunin-BPs to suppress expression of uPA and uPAR genes. After bikunin interacts directly with cell surface receptors, it efficiently disrupts clustering of CD44 and subsequently suppresses gene expression. Bikunin receptor can physically interact with the CD44 isoform and function as a repressor to down-regulate gene expression by preventing clustering of CD44 isoform (7). This is a putative mechanism by which a secreted protease inhibitor could influence gene expression in HRA cells. We found that several highly metastatic transformed cells synthesize lower levels of bikunin compared with non-tumorigenic cells. Furthermore, we showed that there is a direct correlation between bikunin overexpression and the reduced metastatic potential of primary tumor biopsies (12) and tumor cell lines as reflected by their growth and metastasis.

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‡ To whom correspondence should be addressed. E-mail: hirokoba@hama-med.ac.jp.

† The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; AS, antisense; MAPK, mitogen-activated protein kinase; BP, binding protein; ODN, oligodeoxynucleotides; PI3K, phosphoinositol 3-kinase; S, sense; TGF-β, transforming growth factor-β; bik, bikunin; luc, luciferase; Ab, antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; A0V0, analysis of variance; ERK, extracellular signal-regulated kinase, JNK, c-Jun NH2-terminal kinase; SAPK, stress-activated protein kinase; PKC, protein kinase C.

‡ Y. Hirashima and H. Kobayashi, unpublished observations.
in vitro potential to invade through a MatrigelTM barrier.2 We have shown that bikunin is part of the negative invasive program as evaluated by their invasion and uPA/uPAR synthesis (13–16).

For identifying the full repertoire of bikunin-regulated genes, we recently conducted a cDNA microarray hybridization screening using mRNA from bikunin-treated or bikunin-transfected HRA cells (16). A number of bikunin-regulated genes were identified (16). This screen identified suppression of several genes such as the CDC-like kinase, LIM domain binding, Ets domain transcription factor, Rho GTPase-activating protein, tyrosine phosphorylation-regulated kinase, hyaluronan-binding protein, MT-SP1 (suppression of tumorigenicity 14, 16), pregnancy-associated plasma protein A, and P13K p85, which have previously been implicated in enhancing tumor promotion (16). These results show that bikunin alters the pattern of gene expression in HRA cells leading to a block in cell invasion. Northern blot analysis confirmed that P13K was markedly down-regulated by bikunin. Therefore, P13K is considered to be an important bikunin target gene (16).

Among the cellular signals subject to regulation of proliferation, invasion, and metastasis in tumor cells, members of the MAPK and P13K are perhaps the best characterized. P13K phosphorylates the 3’ position of the inositol ring of phosphoinositides to produce lipids that are now considered as crucial spatio-temporal organizers of various signaling pathways (17). Class I, P13Ks include the catalytic subunits p110α, β, and δ associated with a p85 regulatory subunit and activated by phosphorytrosine-containing motifs encountered on receptors or adapter proteins. For example, phosphatidylinositol 3,4,5-trisphosphate binds to a conserved protein motif called the pleckstrin homology domain, thereby inducing the activation of the serine/threonine kinase Akt/protein kinase B and its upstream activators, the phosphoinositide-dependent kinases (17). It is activated by a large spectrum of cytokines, growth factors, and hormones (18). Activation of P13K in cancer cells is associated with invasion and metastasis (19, 20).

To determine whether P13K plays a role in HRA cell invasion and metastasis, we monitored the amount and the activity of the enzyme in whole cells during the phenotypic change triggered by TGF-β1. To clarify how reduced levels of P13K may confer repressed invasiveness, we transfected a highly invasive HRA cell line with antisense (AS)-P13K p85 oligodeoxynucleotides (ODN) and compared the properties of the transfected clones with those of parental HRA cells. Antisense ODN may offer a potential gene therapy strategy to block transcription or translation of specific genes. Here, we demonstrate that Akt is phosphorylated after TGF-β1 treatment and that specific inhibition of P13K activity and reduced P13K expression prevent the up-regulation and invasion in this cell line.

EXPERIMENTAL PROCEDURES

Materials—Purified human bikunin was obtained from Mochida Pharmaceutical Co. (Gotenba, Japan). Ultrapure natural human TGF-β1 was from Genzyme (Cambridge, MA) and R&D Systems (Minneapolis, MN). LipofectAMINE Plus reagent was purchased from Invitrogen. Boyden-type cell invasion chambers (Bicoat Matrigel invasion chambers) were obtained from Collaborative Biomedical (Franklin Lakes, NJ). The nude mice (BALB/c, nu/nu) were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Culture media, penicillin, streptomycin, and fetal bovine serum were purchased from Life Technologies (Germantown, MD). Tissue culture plastics were purchased from Costar/Corning (Cambridge, MA) and Falcon (BD Biosciences, Tokyo, Japan). Bovine serum albumin, Tris base, dithiothreitol, phenylmethylsulfonyl fluoride, dimethyl sulfoxide (Me2SO), and ammonium persulfate were commercially obtained from Sigma. Protease inhibitor mixture tablets were purchased from Roche Applied Science. Acrylamide, bisacrylamide, and polyvinylidene difluoride membrane were from Bio-Rad, X-ray film was purchased from Eastman Kodak Co. The ECL and [3H]thymidine (80 Ci/mmol) were purchased from Amersham Biosciences. All of the other chemicals were of analytical grade.

Antibodies—The antibodies (Abs) against uPA (Ab3689 recognizes uPA B-chain, and Ab3471 reacts with uPA A-chain and interferes with the binding of uPA to its receptor) were gifts from Dr. R. Hart (American Diagnostics, Greenwich, CT). High molecular weight recombinant uPA and Glu-type plasminogen were also obtained from American Diagnostics. Phospho-Akt (Ser-473) and Akt Abs were purchased from New England Biolabs (Beverly, MA). Anti-β-actin Ab was from Cosmo Bio (Tokyo, Japan). Polyclonal Ab against the p85 subunit of P13K was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The goat anti-P13K polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Antisense—The P13K inhibitors (LY294002 and wortmannin) and p38 MAPK inhibitor (SB202190) were purchased from Calbiochem. The inhibitors were dissolved in Me2SO and used in the following concentrations: LY294002 (10 μM, 30 min), wortmannin (0.1 μM, 1 h), and SB202190 (10 μM, 30 min). TGF-β1 (0.08–10 ng/ml) was added to serum-free medium containing the respective inhibitors and incubated for different periods of time, after which the conditioned media and cells were separately collected. The samples were stored at –80 °C until measured. Me2SO (0.05% v/v) diluted in medium was used as a negative control.

Cell Culture—The ovarian cancer cell line, HRA, was grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin in a 5% CO2 atmosphere with constant humidity (10, 16). Cells were disaggregated routinely with 0.1% trypsin/EDTA solution and replated at a split ratio of 1:10. For all of the experiments in which TGF-β1 was added, cells were incubated in the serum-free medium. The experiments of inhibition of P13K activity were performed as follows. The cells were pretreated with 100 nM wortmannin for 1 h or 10 μM LY294002 for 30 min and then cultured in the presence of TGF-β1 plus wortmannin or LY294002 for the indicated times. Cell viability was judged by trypan blue exclusion and was >90%.

In Vitro P13K Lipid Kinase Assay—A total of 1 × 106 cells/ml were cultured for 48 h and stimulated in Dulbecco’s modified Eagle’s medium as described in the figure legends. After aspiration of the supernatants, reactions were terminated by the addition of 0.5 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 12.5 mM 2-glycerophosphate, 150 mM NaCl, 1.5 mM MgCl2, 2 mM EGTA, 10 mM NaF, 0.5% Triton X-100, 2 mM dithiothreitol, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride). Lysates were rotated at 4 °C for 15 min followed by centrifugation at 14,000 rpm. The supernatants were precleared, and immunoprecipitation was performed using anti-P13K Ab (1 μg/ml). Immunoprecipitates were washed and subjected to in vitro lipid kinase assays using a lipid mixture of 100 μl of 0.1 mg/ml phosphatidylinositol and 0.1 mg/ml phosphorylserine dispersed by sonication in 25 mM HEPES, pH 7.4, and 1 mM EDTA (21). The reaction was initiated by the addition of [γ-32P]ATP (3000 Ci/mmol) and 100 μM ATP to the immunoprecipitates suspended in 80 μl of kinase buffer (5 mM MgCl2, 0.25 mM Tris, 20 mM HEPES, pH 7.4). The reaction was terminated after 15 min, and phospholipids were then separated by TLC (21). The TLC plates were stained with iodine to confirm even extraction of substrate lipid between individual samples, and 32P-labeled phosphorylserine 3,4,5-trisphosphate was visualized by autoradiography (21).

Northern Blot Hybridization with cDNA Probes—Total RNA isolations were done using the TRIzol reagent (Invitrogen). Northern blot hybridization was carried out as described previously (9). Samples of total RNA (10 μg) were separated by electrophoresis through denaturing 1% agarose gels containing 1% formaldehyde and transferred onto a nitrocellulose membrane using standard vacuum techniques. Hybridization was carried out with [32P]dCTP by random oligonucleotide priming to specific activities of 0.4–0.9 × 106 cpm/μg. uPA cDNA was prepared as described previously (22). Filters were reprobed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for the amount of RNA loaded onto the filters (9, 22). After hybridization, the membranes were washed and exposed on Kodak BioMax MS-1 film at ~70 °C. Filters were quantitated by scanning densitometry using a Bio-Rad model 620 video densitometer with one-dimensional Analyst software package for Macintosh.

Bikunin Transfection—The bikunin expression vector pCMV-bikunin-IRE5-her (bik-T) and the control vector pCMV-luciferase-IRE5-her encoding luciferase (her) were transfected into HRA cells by the standard calcium phosphate precipitation method as described previously (11).

Antisense P13K p85 Oligodeoxynucleotides and Cell Transfection—Antisense phosphorothioate oligodeoxynucleotides corresponding to the
human PI3K p85 mRNA were synthesized and consisted of the anti-sense and sense sequences (antisense PI3K, 5'-GTA CGT GTA CCC CTC AGC ACT CAT-3'; and sense PI3K, 5'-ATG AGT GCT GAG GGG TAC CAG TAC-3') (23). Oligonucleotides mixed with Lipofectin reagent were incubated for 15 min at room temperature. Thereafter, the oligonucleotides-liposome complexes were then added to cells and washed twice with medium (24). After 4 h, fresh normal growth medium containing 10% fetal bovine serum was added. The AS-PI3K cell populations (AS number 32 and AS number 34) and S-PI3K cells were used for ECM invasion and in vivo metastatic experiments as described below.

**Western Blot Analysis**—The cell monolayers treated with or without various agents for indicated times were washed with phosphate-buffered saline. 1 x 10^6 cells were lysed in 750 μl of lysis buffer at 4 °C for 15 min and scraped with a rubber policeman. The protein concentrations in the supernatants of cell extracts were measured by the Bio-Rad protein assay. All of the samples were stored at -80 °C until use. In parallel, cells treated in the same condition in different dishes were harvested and counted using a hemocytometer. Centrifuged lysates (50 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by semi-dry transfer. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 2% bovine serum albumin. Blots were probed with the following primary antibodies overnight at 4 °C: monoclonal anti-uPA (number 3471 plus number 3689) (each 1:1,000); anti-phospho-Akt (0.5 μg/ml); anti-Akt (0.5 μg/ml); and anti-β-actin (0.2 μg/ml). This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako) at a dilution of 1:50,000 for 1 h. Detection was achieved by enhanced chemiluminescence (Amersham Biosciences) and exposed to film.

**Cell Proliferation Assay**—To determine the effects of AS-PI3K p85 transfection on HRA cell proliferation, the parental HRA cells and PI3K cells were plated at low density (8 x 10^4/well). After incubation for 7 days, cells were lysed in 1% (w/v) Triton X-100 and naphthol blue-black-stained nuclei were counted in a hemocytometer (25). Alternatively, both cells were cultured in the above conditions. Cells were then labeled with 1 μCi/ml [3H]thymidine for 18 h, and radioactivity incorporated into the DNA was determined by trichloroacetic acid precipitation of the cell lysate (25). In a parallel experiment, the parental HRA cell and AS-PI3K cell monolayers were washed and the serum medium was replaced with starvation medium overnight. The following morning, all of the wells were rinsed with phosphate-buffered saline. Negative control wells received low serum only, and positive control wells received 10 ng/ml TGF-β1. LY294002 or wortmannin was added for 30 min before TGF-β1 to allow for complete blockade. Controls treated with inhibitor alone were also included to determine their effect on proliferation. Cells were allowed to incubate for 48 h and then labeled with thymidine.

**ECM Invasion Assay**—Chemo-invasion assays were carried out in a Boyden chamber as described previously (8). The upper surface of chamber was precoated with a layer of artificial basement membrane, Matrigel. The cell suspension (1 x 10^5 cells/well) was added to the upper chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. To measure invasion, incubation was at 37 °C for 24 h. The invaded cells in the lower side of the filter were stained with hematoxylin. Tripple filters were used for each cell type and assay condition, and 10 random fields were considered. Cells were analyzed by phase (×400) or oil (×600).

**In Vivo Peritoneal Carcinomatosis Model**—To assess survival, three groups were inoculated intraperitoneally with 0.1 ml of tumor cell suspension (5 x 10^6 cells, parent HRA cells, AS-PI3K cells numbers 32 and 34, S-PI3K cells, bik^+ clone, and luc^+ clone) in sterile phosphate-buffered saline. All of the animals receiving tumor cells intraperitoneally were monitored on a daily basis for tumor burden, abdominal distension, weight, cachexia, or other abnormalities (11). Once the life-threatening symptoms became markedly manifested, the animal was killed by cervical dislocation.

**Statistics**—Data are expressed as the mean ± S.D. of at least three independent triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's t test. p < 0.05 was considered statistically significant. The Log-rank test was used to analyze the survival of animals injected with each subline.

**RESULTS**

**TGF-β1-induced Activation of PI3K in HRA Cells: In Vitro**

**PI3K Assay**—There is increasing evidence that autocrine TGF-β signaling may potentially promote tumor cell adhesion, motility, and invasiveness (26, 27). We determined whether immunoprecipitates of PI3K derived from TGF-β1-stimulated HRA cells exhibited enhanced in vitro lipid kinase activity versus that present in immunoprecipitates derived from unstimulated cells. TGF-β1-stimulated an increase in the in vitro activity of PI3K in the parental HRA cells that was extremely rapid and transient, because it was detectable 30 s after stimulation and had returned toward basal levels 10 min after TGF-β1 treatment, indicating a rapid activation of PI3K by TGF-β1 (Fig. 1, left panel).

In a parallel experiment, HRA cells were transiently transfected with AS-PI3K p85. We determined whether immunoprecipitates of PI3K derived from TGF-β1-stimulated AS-PI3K cells exhibited reduced in vitro lipid kinase activity versus that present in immunoprecipitates derived from TGF-β1-stimulated parental HRA cells. Here, we show that AS-PI3K p85 cells significantly exhibited reduced in vitro lipid kinase activity (Fig. 1, right panel).

**TGF-β1-induced Phosphorylation of Akt through PI3K in HRA Cells**—We examined the ability of TGF-β1 to induce Akt phosphorylation by probing Western blots of total cell lysate proteins from TGF-β1-stimulated HRA cells using anti-phospho-Akt antibodies. TGF-β1 (10 ng/ml) stimulated phospho-Akt protein expression from nearly undetectable basal levels. TGF-β1 induced Akt phosphorylation in a time-dependent manner (Fig. 2, A and B), and after 10 min, there was a 2-fold increase in Akt activation (lane 3) with maximum activation (5-fold) observed at 20 min (lane 4) and Akt phosphorylation declined at 60 min (lane 6) after exposure to TGF-β1, indicating a rapid phosphorylation of Akt by TGF-β1. We also observed concentration-dependent phosphorylation of this target protein beginning with only 0.4 ng/ml TGF-β1 (Fig. 2, C and D, lane 3).

We examined whether this increase in Akt phosphorylation can be blocked when HRA cells were preincubated with the PI3K inhibitors wortmannin (0.1 μM, a specific inhibitor of the enzymatic activity of the p110 subunit of PI3K) or LY294002 (10 μM) (Fig. 2, A, B, lanes 8 and 9, C, and D, lanes 6–9). We found that PI3K inhibitors inhibited the entire time course of Akt phosphorylation in response to TFG-β1. Pretreatment (0.1
μM wortmannin or 10 μM LY294002) resulted in 80–90% decrease in Akt phosphorylation.

**Antisense PI3K Inhibits HRA Cell Phospho-Akt Expression**—We examined involvement of the PI3K/Akt pathway in TGF-β signaling through a more specific approach. The role of PI3K in Akt phosphorylation was demonstrated through inhibition with AS-PI3K ODN. For this end, AS-ODN-targeting PI3K and corresponding control S-ODN were used to treat HRA cells with subsequent TGF-β1 (10 ng/ml) stimulation. We evaluate by Western blot analysis on total cell lysate the PI3K p85 protein expression by 80% compared with the corresponding S-ODN, which is consistent with the expectations for Akt as a target of PI3K-activated signals (Fig. 3B).

**Effect of PI3K Pathway Inhibition on TGF-β1-induced uPA Gene Up-regulation**—We have previously shown that TGF-β1 increased uPA mRNA in HRA cells, promoting cell invasion and metastasis, that bikunin reduced the TGF-β1-stimulated uPA mRNA expression, and that uPA stimulation in response to TGF-β1 was dose-dependent with maximal effects being obtained for doses of 10 ng/ml. TGF-β1 increased uPA mRNA expression starting 3 h after stimulation, and the peak effect was observed at 6 h. No effects were observed at the earlier 1-h time point. Furthermore, this effect on uPA up-regulation was mediated through a TGF-β receptor activation because it was completely canceled by a neutralizing anti-TGF-β receptor antibody or TGF-β scavenger, decorin.3 Treatment of cells with actinomycin D before or after stimulation indicated that uPA gene expression was regulated at the transcriptional level.3

The involvement of PI3K in this TGF-β1-stimulated functional response was assessed by the use of the PI3K inhibitors LY294002 (10 μM, 30 min) and wortmannin (0.1 μM, 1 h). As shown in Fig. 4, HRA cell uPA mRNA expression in response to TGF-β1 (10 ng/ml, 6 h) was markedly inhibited by pretreatment of the cells with LY294002 and wortmannin by 92 ± 9 and 85 ± 7%, respectively. The IC50 values for LY294002 and wortmannin inhibition of TGF-β1-stimulated uPA up-regulation were ~2 μM (lane 4) and ~20 nM (lane 7), respectively. In contrast, the addition of SB202190 (p38 MAPK inhibitor) did not alter the TGF-β1-induced increase of uPA gene expression (lane 9).

**Effect of PI3K Pathway Inhibition on TGF-β1-Induced uPA Protein Expression**—As assessed by uPA enzyme-linked immunosorbent assay, day 1 cultured HRA cells spontaneously released detectable amounts of uPA into the culture conditioned medium (28). To verify whether the above-mentioned changes of the uPA gene expression were associated with changes of uPA protein expression, we estimated by Western blot the ratio of optical density of uPA protein over β-actin protein under control conditions and after administration of TGF-β1 in the presence or absence of the PI3K inhibitors wortmannin (lane 3) or LY294002 (lane 4) or p38 MAPK inhibitor SB202190 (lane 5). As shown in Fig. 5, TGF-β1 induced a 4.5-fold increase of uPA protein expression in HRA cells (lane 2). As was the case with the uPA mRNA expression, these PI3K inhibitors but not p38 MAPK inhibitor blunted the stimulatory effect of TGF-β1 on uPA protein expression.

**Antisense PI3K ODN Inhibits TGF-β1-stimulated uPA Gene Expression in HRA Cells**—HRA cells were transfected with bikunin (bik), AS-PI3K, S-PI3K, or a luciferase (Luc) control.

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3 H. Kobayashi, M. Suzuki, N. Kanayama, and T. Terao, unpublished data.
TGF-β-ODN were incubated with or without corresponding control sense (HRA cells transiently transfected with AS-PI3K ODN or the corresponding control sense construct; lane AS versus lane S, transfection with sense construct). TGF-β1 (10 ng/ml, 20 min). Total cell lysates were prepared and separated by Western blot. Phospho-Akt and total Akt were detected with specific antibodies to anti-phospho-Akt or anti-Akt, respectively. AS-PI3K ODN transfection inhibited HRA cell phospho-Akt expression. AS-PI3K cell 32 and AS-PI3K ODN (Fig. 6A, lanes 3 and 4) after TGF-β1 treatment overlapped that of luc+ cells (lanes 1 and 2). On the contrary, TGF-β1 treatment of AS-PI3K ODN resulted in abrogation of uPA mRNA expression (Fig. 6A, lanes 5 and 6). Furthermore, AS-PI3K ODN cells 32 and 34 (data not shown) reduced up-regulation of uPA protein in response to TGF-β1 stimulation (Fig. 6B, lanes 5 and 6). Northern and Western blot analysis showed 70 and 90% inhibition of uPA mRNA and protein expression, respectively, by AS-PI3K ODN, whereas S-PI3K ODN was unable to change uPA expression in TGF-β1-stimulated cells (Fig. 6B, lanes 3 and 4). Under our conditions, AS-PI3K transfection did not induce any death signaling (data not shown). In a parallel experiment, we found that bik− marked reduced the expression of uPA mRNA and protein (Fig. 6B, lanes 7 and 8).

**Fig. 3.** Inhibition of TGF-β1-induced HRA cell Akt phosphorylation by antisense PI3K ODN. A, evaluation by Western blot analysis on total cell lysate of p85 PI3K expression after transfection experiments. Lane C, unstimulated HRA cells (control); lane S, transfection with sense construct; lane AS, transfection with antisense construct. B, HRA cells transiently transfected with AS-PI3K ODN or the corresponding control sense (S)-ODN were incubated with or without TGF-β1 (10 ng/ml, 20 min). Total cell lysates were prepared and separated by Western blot. Phospho-Akt and total Akt were detected with specific antibodies to anti-phospho-Akt or anti-Akt, respectively. AS-PI3K ODN transfection inhibited HRA cell phospho-Akt expression. C, data from four experiments were scanned and analyzed for quantification with the Macintosh software. Band intensities for phospho-Akt and total Akt were detected with anti-phospho-Akt or anti-Akt, respectively. AS-PI3K ODN transfection inhibited HRA cell phospho-Akt expression. AS-PI3K ODN (Fig. 6A, lanes 3 and 4) after TGF-β1 treatment overlapped that of luc+ cells (lanes 1 and 2). On the contrary, TGF-β1 treatment of AS-PI3K ODN resulted in abrogation of uPA mRNA expression (Fig. 6A, lanes 5 and 6). Furthermore, AS-PI3K ODN cells 32 and 34 (data not shown) reduced up-regulation of uPA protein in response to TGF-β1 stimulation (Fig. 6B, lanes 5 and 6). Northern and Western blot analysis showed 70 and 90% inhibition of uPA mRNA and protein expression, respectively, by AS-PI3K ODN, whereas S-PI3K ODN was unable to change uPA expression in TGF-β1-stimulated cells (Fig. 6B, lanes 3 and 4). Under our conditions, AS-PI3K transfection did not induce any death signaling (data not shown). In a parallel experiment, we found that bik− markedly reduced the expression of uPA mRNA and protein (Fig. 6B, lanes 7 and 8).

**Fig. 4.** A and B, effect of PI3K pathway inhibition on TGF-β1-induced uPA gene up-regulation. Northern blot analysis is shown. HRA cells pretreated with or without pharmacological inhibitors were stimulated with or without TGF-β1 (10 ng/ml) for 6 h. PI3K antagonism blocked the TGF-β1-induced stimulation of uPA mRNA expression (mean values ± S.D. of three separate experiments for each condition). *, p < 0.05 versus control (lane 1); #, p < 0.05 versus lane 2.

**Fig. 5.** Effect of pharmacological inhibition of PI3K on up-regulation of uPA protein in HRA cells stimulated with TGF-β1. A, HRA cells pretreated with or without pharmacological inhibitors were stimulated with or without TGF-β1 (10 ng/ml) for 24 h. Cell lysates were prepared with lysis buffer and separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. uPA was detected with anti-uPA antibodies. B, composite uPA expression determined by densitometric analysis normalized to 0 ng/ml TGF-β1. Results represent mean ± S.D. from three independent experiments. *, p < 0.05 versus control (lane 1); #, p < 0.05 versus lane 2.
plays an active role in HRA cell invasion. To this end, we used the conventional transfilter invasion assay. We previously reported (28) that TGF-β1 could potentiate HRA cell invasion through Matrigel and that HRA cell invasion was uPA-dependent. As expected, the bik− clone resulted in ∼50% reduction in cell invasion (11). The involvement of PI3K in this TGF-β1-stimulated functional response was assessed by the use of the PI3K inhibitors wortmannin and LY294002 (Fig. 7). HRA cell invasiveness in response to 10 ng/ml TGF-β1 was significantly inhibited by pretreatment of the cells with wortmannin and LY294002 in a dose-dependent manner (70 ± 13% (10 μM LY294002) and 78 ± 10% (0.1 μM wortmannin). Thus, specific PI3K inhibitors inhibited invasion by >70%. The IC50 values for wortmannin and LY294002 inhibition of TGF-β1-stimulated invasiveness were ∼20 nm and ∼4 μM, respectively. These two inhibitors did by themselves suppress invasiveness even in the absence of TGF-β1. These findings demonstrate that PI3K/Akt promotes HRA cell invasiveness in a manner that depends on its kinase activity. Taken together, our results demonstrated a critical role of PI3K in the invasive signaling of TGF-β1 in HRA cells.

**HRA Cells Transfected with Bikunin, AS-PI3K, or S-PI3K Exhibit Distinct Invasive Properties in Matrigel Invasion Assay**—We evaluated the biological effect of reduced PI3K expression by analyzing the ability of AS-PI3K ODN cells to invade Matrigel. Fig. 8 shows significant decreases in cell invasion by 70–85% when AS-PI3K cells were used in the upper chamber, irrespective of whether cells were stimulated with TGF-β1. Indeed, cell populations expressing low levels of PI3K had a decreased ability to invade the Matrigel layer as compared with luc+ clone or parental cells that had not been transfected at all. Thus, cell-invasive ability was not affected by transfection procedures. Our results support the notion that PI3K mediates cell invasion through cell-associated uPA up-regulation. We speculate that bikunin-dependent suppression of ECM invasion may occur, at least in part, through suppression of PI3K-dependent up-regulation of uPA.

**In Vitro Growth Regulation of HRA Cells by AS-PI3K Transfection**—We studied the effect of AS-PI3K p85 transfection on proliferation rates of HRA cells in culture using a standard [3H]thymidine incorporation assay. There was no statistically significant difference in cell proliferation between the parental HRA cells and AS-PI3K cells. This was confirmed by a direct cell-counting assay for 7 days. In addition, stimulation of parental HRA cell and AS-PI3K cell monolayers with TGF-β1 produced no increase in cell proliferation. Administration of PI3K inhibitors alone or coadministration of TGF-β1 and PI3K inhibitors did not affect cell proliferation. The ratios of viable to non-viable cells were identical between parental HRA cells and
AS-PI3K cells, indicating that AS-PI3K ODN does not affect proliferation or viability of the HRA cells.

**PI3K Down-regulation Inhibits Intraperitoneal HRA Tumor Growth in Nude Mice**—The *in vitro* results prompted us to examine whether PI3K/Akt has any role in tumor growth and regulating the expression of uPA in an *in vivo* system. To further demonstrate the anti-tumoral activity of AS-PI3K in ovarian cancer, we evaluated the effect of reduced PI3K expression on the tumor development of HRA cells intraperitoneally injected in nude mice. Cells were inoculated intraperitoneally into mice, and tumor size was evaluated at day 9 (Fig. 9A). No significant tumor reduction or growth was observed for luc- and S-PI3K cells. The weights of the S-PI3K-transfected tumors were almost identical to the tumors of the non-transfected mice. When AS-PI3K cells were injected into mice, the size of the tumors was reduced drastically compared with luc- or S-PI3K-transfected mice. The intraperitoneal tumor growth of bik- and AS-PI3K clones was significantly inhibited as compared with non-transfected HRA cells (55% inhibition for bik-, 80% inhibition for AS number 32, and 60% inhibition for AS number 34). Expression of uPA was confirmed by Western blot analysis of the intraperitoneal tumors obtained at day 9, demonstrating that bik- clones and AS numbers 32 and 34 showed a weak signal for uPA (data not shown). In addition, immunoblot analysis revealed that 3 days after tumor cell plating, PI3K expression was still reduced in cells derived from AS numbers 32 and 34 (data not shown). This inhibition was transient, and PI3K expression returned after 9 days (data not shown).

We next examined the effects of PI3K inhibitor LY294002 on tumor growth and survival. Nude mice bearing HRA cells were randomly assigned to one of two groups, control or LY294002. The LY294002 was delivered intraperitoneally to the mice for 7 days. Down-regulation of Akt was documented by Western blot analysis of tumor lysates. Although a dose of 100 mg/kg LY294002 eliminated the phosphorylation of Akt, this inhibition was transient and Akt activity returned after 1 h (data not shown). The LY294002 by itself had minimal antitumor effect (reduced tumor growth (−30% reduction)). We could not perform a dose-escalation study, because doses of greater than 150 mg/kg LY294002 resulted in mechanism-based or idiosyncratic severe side effects. These doses resulted in severe respiratory depression and lethargy.

**Survival of Tumor-bearing Mice**—We ascertained the relative survival times of nude mice after intraperitoneal transfer of each cell (Fig. 9B). The mean survival times of nude mice receiving 5 × 10⁶ parental HRA cells (n = 7) and the luc- clones (n = 7) were 8 and 8 days, respectively. The mean survival time of animals receiving the bik- clone (n = 7), AS-PI3K cells number 32 (n = 9), AS-PI3K cells number 34 (n = 7), and S-PI3K cells (n = 8) was increased and/or decreased to 12, 15, 17, and 8 days, respectively. There was a significant increase in the mean survival times of the group receiving bik- clone, AS number 32, and AS number 34 cells compared with the luc- clone and the parental cells (p < 0.05). Consistent with the delayed growth of the intraperitoneal tumors, the survival time was prolonged.

**DISCUSSION**

We previously showed that bikunin inhibits *in vivo* metastasis of the mouse and human cancer cell lines possibly via suppression of uPA and uPAR expression without affecting *in vitro* cell proliferation (28). Exogenously added bikunin or bikunin gene transfection markedly suppressed intraperitoneal disseminated metastasis of ovarian cancer HRA cells (11). TGF-β1 (10 ng/ml) markedly stimulates up-regulation of uPA mRNA and protein in HRA cells that results in promoting cell invasion and metastasis (28). Indeed, bikunin is able to repress TGF-β1-dependent uPA up-regulation at the mRNA and protein levels (28). In addition, a previous microarray study identified the PI3K p85 gene as a target for down-regulation in HRA cells treated with bikunin, suggesting that PI3K may be a candidate bikunin target gene (16).

In this study, we show that TGF-β1 induced PI3K activation in HRA cells by an *in vitro* lipid kinase assay (Fig. 1) and that PI3K-dependent Akt phosphorylation (Figs. 2 and 3) correlated closely with TGF-β1-induced up-regulation of uPA mRNA and protein by Northern blot (Fig. 4) and Western blot (Fig. 5) analyses. PI3K inhibition with wortmannin or LY294002 sig-

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**Fig. 8.** Effects of reduced PI3K expression on the unstimulated and TGF-β1-stimulated invasive response in HRA cells. A total of 1 × 10⁶ HRA cells transfected with bik-, AS-PI3K, S-PI3K, or luc- were incubated with TGF-β1 (10 ng/ml) in a Boyden chamber at 37 °C for 24 h. Cell invasion was assessed as described under "Experimental Procedures." Results are expressed as a mean invasion cells (±S.D.). The data are representative of at least three separate experiments. *, p < 0.05 versus HRA; #, p < 0.05 versus HRA + TGF-β1.

**Fig. 9.** A, reduced PI3K expression specifically inhibits intraperitoneal tumor growth in nude mice. Cells (5 × 10⁶ cells/200 μl) parental HRA cells, bik- clone, AS numbers 32 and 34, S-PI3K cells, and luc- clone) were implanted intraperitoneally into the abdomen of female athymic nude mice. Four mice were used in each set of experiments. Mice were followed for tumor growth for 9 days. Data represent mean ± S.D. tumor volumes at day 9. This experiment is representative of two independent experiments. *, p < 0.05 versus HRA. B, survival of nude mice after intraperitoneal transfer of tumor cells. These groups of female nude mice were injected intraperitoneally with 5 × 10⁶ HRA cells on day 0 and monitored daily for morbidity. Survival of the animals was monitored, and the data were analyzed statistically by the Log-rank test. Survival of bik- clone and AS numbers 32 and 34 transfectant mice was significantly longer than the parent HRA mice and luc- transfectant mice. There was no significant difference between the S-PI3K cell group and the parental cell group. Results are representative of two separate experiments. *, p < 0.05 versus HRA.
significantly but not totally attenuated TGF-β1-induced uPA up-regulation. One of the principal findings of this study is that TGF-β1 up-regulates uPA expression in HRA cells, at least in part, through PI3K activation. To further determine the function of PI3K in ECM invasion, peritoneal disseminated metastasis, and tumor growth and to check the status of uPA, several experimental approaches were used including modulation of PI3K expression by transfection with AS-PI3K, S-PI3K, or bikunin as a comparable experiment. We transfected a highly invasive HRA cell line with AS-PI3K and compared the properties of the transfected cells with those of parental cells. We selected two cell populations (AS number 32 and AS number 34) that expressed significantly reduced levels of PI3K. Here, we show that the level of uPA was reduced dramatically in AS-PI3K cells or its tumor-bearing mice, that decrease in uPA activity in AS-PI3K cells correlates with the level of uPA expression (data not shown), and that reduced PI3K expression may confer repressed invasiveness and peritoneal disseminated metastasis possibly through a constitutive down-regulation of uPA mRNA and protein, irrespective of whether cells were stimulated with TGF-β1. We provide evidence that AS-PI3K transfection may reduce uPA up-regulation by abrogating PI3K-dependent signaling pathway. Therefore, reduced PI3K expression in bikunin-transfected clones may reflect decreased uPA up-regulation, implying that reduced PI3K activity facilitates a shift in balance toward down-regulation of uPA. In addition, thymidine incorporation assay revealed that the AS-PI3K ODN does not affect proliferation or viability of the HRA cells. This result indicates that the slower growth in an in vivo tumor assay is because of the effects on reduced cell invasiveness but not reduced cell proliferation.

In recent years, the PI3K pathway has been suggested to be highly involved in tumor cell invasion (19, 20). The possible PI3K effectors might be linked to uPA induction and invasiveness. It has been established that up-regulation of uPA by insulin-like growth factor-I depends upon PI3K and ERK in breast cancer cell line MDA-MB-231 (29), so that MAPK pathways ERK, JNK/SAPK, and p38-MAPK represent a significant component in the regulation of uPA expression in human prostate cancer cell line PC3 (30), and that MAPK, PI3K, small Rho GTPases such as Cdc42 and Rac1 are key effectors that regulate dynamic changes in cell migration (31). The downstream targets of PI3K products also include Akt, p70S6 kinase, cytoskeletal proteins, and PKC (32). The link between PI3K and the regulation and functional significance of the PI3K pathway in modulating the cancer cell phenotype is not completely clear, up-regulation of uPA induced by MAPK cascade phosphorylation of the activation loop sites of PKC has been recently elucidated and involves a PDK1 phosphorylation of uPA expression by transfection with TGF-β1. We provide evidence that down-regulation of uPA induced by MAPK cascade phosphorylation was inhibited by not only MAPK inhibitor PD98059 but also by PI3K inhibitor LY294002, and that inhibition of PI3K by LY294002 or AS-PI3K ODN transfection did not abrogate TGF-β1-mediated ERK1/2 activation. In contrast, TGF-β1-induced Akt phosphorylation was inhibited by PD98059 (data not shown). These data may mean that PI3K as an essential downstream mediator of the MAPK signaling pathway involved in uPA expression and invasion in response to TGF-β1. It is possible that PI3K may be a key signaling molecule responsible for TGF-β1-induced uPA synthesis and, subsequently, ovarian cancer cell invasion and metastasis.

In conclusion, we tested the hypothesis that down-regulation of PI3K expression by bikunin plays a role in inhibition of TGF-β1-dependent induction of uPA and associated increases in tumor invasiveness. We have demonstrated for the first time that blocking the expression or functional activity of PI3K by its antisense strategy or pharmacological inhibition, respectively, abrogates TGF-β1-dependent uPA up-regulation in human ovarian cancer cells. Decreased invasiveness, tumor growth, and peritoneal disseminated metastasis correlate with reduced PI3K expression, which is a putative bikunin target gene. We provide evidence showing that bikunin decreases the uPA up-regulation, at least in part, through suppression of PI3K expression. These findings are particularly important because PI3K signaling pathway is considered to be a potential target for inhibiting tumor invasion and metastasis. A role for PI3K in TGF-β1-mediated invasiveness is novel. Bikunin will have additional global effects on cancer cells by modulating the expression of a large number of cellular genes (16). Clearly, the genomic response to bikunin signaling is complex. Future studies focused on the regulation and functional significance of the target genes should increase our knowledge of the biological activity of bikunin in non-neoplastic and neoplastic cells.

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