Suppression of Urokinase Expression and Invasion by a Soybean Kunitz Trypsin Inhibitor Are Mediated through Inhibition of Src-dependent Signaling Pathways*

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A soybean Kunitz trypsin inhibitor (KTI) interacts with cells as a negative modulator of the invasive cells. Using complementary pharmacological and genetic approaches, we provide novel findings regarding mechanisms by which KTI inhibits signaling pathways in ovarian cancer cells leading to invasion. Transforming growth factor-β1 (TGF-β1) directly activates Src kinase, which in turn activates ERK-phosphatidylinositol 3-kinase/Akt, the downstream targets of Src, for urokinase-type plasminogen activator (uPA) up-regulation in human ovarian cancer HRA cells. Preincubation of the HRA cells with KTI reduced the ability of TGF-β1 to trigger the uPA expression at the gene level and at the protein level. To further elucidate the mechanism of the KTI-dependent suppressive effect of TGF-β1-induced uPA expression and invasion, we investigated which signaling pathway transduced by KTI is responsible for this inhibitory effect. Here, we show that 1) KTI suppressed TGF-β1-induced phosphorylation of Src, ERK1/2, and Akt by 40–60%; 2) KTI was insensitive to suppress the phosphorylation of ERK1/2 and Akt in the constitutively active (CA)-c-Src (Y529F) cells; 3) uPA expression was upregulated in TGF-β1-stimulated HRA cells and in unstimulated Y529F cells; 4) the addition of KTI reduced the TGF-β1-induced increase of uPA gene and protein expression in the wild-type c-Src-transfected cells (in contrast, KTI could not inhibit uPA expression in the Y529F cells); and 5) CA-c-Src transfection resulted in a 2-fold increase in invasiveness, whereas KTI did not reduce invasion of the Y529F cells. Using additional complementary genetic approaches (CA-MEK1, CA-Akt, or kinase-dead-Akt), we conclude that KTI may suppress uPA expression and promotion of invasion possibly through one or more upstream targets of Src.

We have previously reported in a series of reports that a Kunitz-type protease inhibitor, bikunin derived from human urine (also known as urinary trypsin inhibitor), suppresses expression of uPA, phosphorylation of ERK1/2, and cancer cell invasion in vitro and peritoneal disseminated metastasis and lung metastasis in vivo (1–5). More recently, we investigated the effects of soybean trypsin inhibitor on the uPA expression, signal transduction involved in the expression of uPA, and invasion in human ovarian cancer HRA cells (4, 7). Soybean trypsin inhibitor contains a Kunitz trypsin inhibitor (KTI)1 and a Bowman-Birk inhibitor (BBI) (8). We previously showed (6, 7) that 1) uPA expression observed in HRA cells is inhibited by preincubation of the cells with KTI with an IC50 of ~2 μM, whereas BBI failed to repress uPA expression; 2) cell invasiveness is inhibited by treatment of the cells with KTI with an IC50 of ~3 μM, whereas BBI failed to suppress cell invasion; 3) KTI suppresses HRA cell invasion by blocking uPA up-regulation, which may be mediated by one or more binding proteins other than a bikunin-binding protein and/or its receptor; and 4) TGF-β1-mediated activation of ERK1/2 is significantly reduced by preincubation of the cells with KTI. We conclude that KTI, but not BBI, could inhibit cell invasiveness at least through suppression of the MAPK-dependent uPA signaling cascade (6, 7).

It has been reported that there appeared to be a specific interaction between bikunin and the tumor cell surface (9–12). Tumor cells express two types of bikunin-binding proteins; a 40-kDa bikunin-binding protein, which is identical to cartilage link protein, and a 45-kDa bikunin-binding protein, a putative bikunin receptor (12, 13). Bikunin binds link protein and bikunin receptor on tumor cell surface possibly via the N-terminal Kunitz domain I and the chondroitin sulfate side chain, respectively (13). Bikunin must bind directly to both of the cell-associated bikunin-binding proteins to suppress expression of uPA and uPA receptor genes (14). However, KTI

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has no chondroitin 4-sulfate glycosaminoglycan side chain. Our recent data (6) showed that neither KTI nor BBI inhibits binding of radiolabeled-bikunin to HRA cells or bikunin does not inhibit KTI binding to the cells, suggesting that their binding molecule(s) in the plasma membranes is(are) different from those of bikunin. These data allow us to hypothesize that the mechanisms of KTI will be different from those of bikunin.

These findings lead to many questions about the difference in the mechanisms between KTI and bikunin. We therefore investigated the effects of KTI on the TGF-β-induced uPA up-regulation and invasiveness of wild-type and transfected cells in relation to the status of Src, ERK, and PI3K/Akt in HRA cells, because TGF-β1 can stimulate uPA expression through the Src-dependent, ERK-specific signaling cascade (15). We show for the first time that KTI suppresses uPA up-regulation and promotion of invasion possibly through one or more upstream targets of Src.

**EXPERIMENTAL PROCEDURES**

**Materials—**A soybean KTI was obtained from Fuji Oil Co. Ltd., Osaka, Japan (6, 15). Lipofectamine Plus reagent was purchased from Invitrogen. High molecular weight recombinant uPA and Glu-type plasminogen were obtained from American Diagnostics (Greenwich, CT). Boyden-type cell invasion chambers (BioCoat Matrigel invasion chambers) were obtained from Collaborative Biomedical (Franklin Lakes, NJ). Ultrapure natural human TGF-β1 was from Genzyme (Cambridge, MA) and R&D Systems (Minneapolis, MN). Genistein and PD98059 (50 μM, p38 kinase inhibitor), and PD98059 (50 μM, specific inhibitor of MEK) were obtained from Calbiochem. Culture media, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen. Tissue culture plastics were purchased from Costar/Corning (Cambridge, MA) and Falcon (BD Biosciences). Bovine serum albumin, Tris-base, phosphate-buffered saline. Lipofectamine Plus reagent was purchased from Invitrogen. Acrylamide, bisacrylamide, and polyvinylidene difluoride membrane were from Bio-Rad. X-ray film was purchased from Hyperfilm (Amersham Biosciences). Protein estimation reagents (BCA kit) were from Pierce. All other chemicals were of analytical grade.

**Pharmacological Inhibitors—**The inhibitors were dissolved in Me2SO (cell culture grade, Sigma) and used in the following concentrations: wortmannin (100 nM, specific inhibitor of PI3K), LY294002 (10 μM, specific inhibitor of the p110 catalytic subunit of PI3K), herbimycin A (30 μM, tyrosine kinase inhibitor), the mek inhibitor, and PD98059 (50 μM, specific inhibitor of MEK). All of the inhibitors except wortmannin (Sigma) were obtained form Calbiochem. The inhibitors diluted in normal growth medium were added to wells containing confluent cells and incubated for 30 min to 1 h. TGF-β1 (10 ng/ml) was added to serum-free medium containing the respective inhibitor for the indicated times. Western blot analyses were performed using the TRIzol reagent (Invitrogen). Lipofectamine Plus reagent was purchased from Invitrogen. uPA and uPA cDNA was prepared as described (14, 21). Samples of total RNA were separated by electrophoresis through denaturant gel, transferred onto nylon or nitrocellulose membranes using standard molecular biological techniques. Hybridization was carried out as described previously (14, 21).

**Chemoinvasion assays—**Extracellular Matrix Invasion Assay—

**Statistics—**Data are expressed as mean ± S.D. of at least three independent triplicate experiments. All statistical analysis was performed using the Bio-Rad protein assay. Total RNA isolations were done using the TRIzol reagent (Invitrogen).

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**Experimental Procedures**

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irrespective of whether cells were stimulated with TGF-β. Immunoblotted similar amounts of Src protein in HRA cells, 31430 band intensities for total Src. Data from three experiments were averaged and are represented as the mean were scanned and analyzed for quantification with Macintosh software. Band intensities for phospho-c-Src were normalized to the corresponding

pendent manner (lanes 10–12) of response to TGF-β tration-dependent phosphorylation of ERK1/2 and Akt in re-

In the previous study (15), we observed concen-

tation-dependent Akt phosphorylation at 20 min in response to 10 ng/ml TGF-β. Cells were lysed with lysis buffer, and the protein content of each sample was quantitated. Each sample (50 μg) was resolved by 10% SDS-PAGE and probed with anti-phospho-c-Src (p-Src, 0.5 μg/ml, upper panel) to detect the phosphorylated Src protein. Blots were stripped and reprobed with anti-c-Src antibody (0.5 μg/ml, lower panel). Experiments were repeated three times with essentially identical results. B and D, blots were scanned and analyzed for quantification with Macintosh software. Band intensities for phospho-c-Src were normalized to the corresponding band intensities for total Src. Data from three experiments were averaged and are represented as the mean ± S.D., expressed as -fold increase with respect to lane 1. Different letters (a–d) represent statistical differences (p < 0.05).

RESULTS

KTI Suppressed TGF-β1-induced Src Phosphorylation—Previously, we have investigated signaling pathways involved in TGF-β1 activation in human ovarian cancer cell lines, HRA and SKOV-3 (15). We showed that TGF-β1 induced a marked rise in the level of phosphorylated Src protein in a time-dependent manner (15). In the present study, we examined whether a soybean KTI suppressed TGF-β1-induced phosphorylation of Src in HRA and SKOV-3 cells by Western blot. As compared with nonstimulated HRA cells, a 7-fold increase in phosphorylated Src was observed at 20 min in response to 10 ng/ml TGF-β1 (Fig. 1, lane 1 versus lane 3). The anti-Src antibodies immunoblotted similar amounts of Src protein in HRA cells, irrespective of whether cells were stimulated with TGF-β1. Here, we showed that KTI suppressed TGF-β1-stimulated Src phosphorylation in a dose-dependent manner (lanes 4–6). Furthermore, KTI also suppressed TGF-β1 (10 ng/ml, 20 min)-induced phosphorylation of Src in SKOV-3 cells in a dose-depen-
dent manner (lanes 10–12).

KTI Suppressed TGF-β1-induced Phosphorylation of Akt through PI3K—In the previous study (15), we observed concentration-dependent phosphorylation of ERK1/2 and Akt in re-
sponse to TGF-β1 beginning with only 0.4–1 ng/ml TGF-β1. Src activation has been linked to the activation of MAPK and PI3K in HRA cells (15). The facts that a MEK inhibitor, PD98059, inhibited the TGF-β1-activated Akt activation and that PI3K inhibitors, LY294002 and wortmannin, had no effect on the TGF-β1-induced ERK1/2 activity suggest a presence of cross-talk between the ERK and Akt cascades and that PI3K is a downstream target of MAPK (15). Here, we show the ability of KTI to TGF-β1-induced phosphorylation of Akt and ERK1/2 by Western blot using antibodies to phosphorylated Akt (or phosphorylated ERK1/2) or total Akt (or total ERK1/2), respectively.

As shown in Fig. 2A, the increase in Akt phosphorylation in response to TGF-β1 could be reduced when HRA cells were preincubated with KTI (10 μM, 1 h; lane 2 versus lane 3), wortmannin (100 nM, 1 h; lane 4), or LY294002 (10 μM, 30 min; lane 6) by 50%, 80%, or 80%, respectively. Pretreatment of HRA cells with 50 μM PD98059 (lane 8) also markedly abrogated the TGF-β1-stimulated Akt phosphorylation by 80%. In contrast, TGF-β1-dependent Akt phosphorylation was not suppressed by p38 MAPK inhibitor, SB202190 (lane 10). The combined treatment of KTI together with pharmacological inhibitors (lanes 4–9) did not provide a further suppression of TGF-β1-induced Akt phosphorylation.

The role of PI3K in Akt activation was more specifically demonstrated through inhibition with PI3K antisense ODN. We used antisense ODN targeting of the gene for PI3K (lanes 12 and 13) and corresponding control ODNs (S PI3K ODN (lanes 14 and 15) and inverted antisense (iAS) PI3K ODN (data not shown)). PI3K p85 protein expression was reduced by the antisense strategy (15). Previously, we showed that antisense PI3K ODN transfection abrogated Akt phosphorylation in HRA cells, irrespective of whether cells were stimulated with TGF-β1 (15). Note that TGF-β1-stimulated Akt phosphoryla-
tion was not impaired by S PI3K ODN or iAS ODN (15). In this study, KTI inhibited the TGF-β1-induced phosphorylation of Akt in the S PI3K ODN cells (lanes 14 and 15). However, KTI did not affect phospho-Akt level in AS PI3K ODN transfected cells (lanes 12 and 13), because TGF-β1 could not induce Akt phosphorylation.

To confirm that KTI-dependent suppression of TGF-β1-stimulated Akt phosphorylation is mediated by ERK, HRA cells expressing CA-MEK1 or control MEK1 were pretreated with KTI, and then stimulated with TGF-β1. We found that overexpression of CA-MEK1, but not control MEK1, increased basal ERK phosphorylation (Fig. 2C, lane 1 versus lane 4). KTI could not significantly suppress the TGF-β1-induced phosphorylation of ERK in HRA cells transfected with CA-MEK1 (Fig. 2C, lane 5 versus lane 6). On the other hand, KTI inhibited the ERK phosphorylation in the control MEK1 cells (Fig. 2C, lane 8 versus lane 9).

In a parallel experiment, phospho-Akt band was strongly
detected in cells expressing CA-MEK1 (Fig. 2E, lanes 4–6), but not in cells expressing control MEK1 (Fig. 2E, lanes 7–9), irrespective of whether cells were stimulated with TGF-β1. Furthermore, pretreatment for 1 h with 10 μM KTI could not significantly diminish the TGF-β1-induced phosphorylation of Akt band in the CA-MEK1 cells (Fig. 2E, lane 5 versus lane 6), supporting that KTI may be involved in one or more upstream targets of ERK.

As shown in Fig. 2G, the increase in ERK1/2 phosphorylation in response to TGF-β1 could be blocked when HRA cells were preincubated with KTI (10 μM, 1 h, lane 3) or PD98059 (50 μM, lane 8) by 60 and 70%, respectively. In contrast, pretreatment of HRA cells with wortmannin (100 nM, 1 h, lane 4) or LY294002 (10 μM, 30 min, lane 6) did not abrogated the TGF-β1-stimulated ERK1/2 phosphorylation, because PI3K/Akt is a downstream target of ERK (15). In addition, TGF-β1-dependent ERK1/2 phosphorylation was not suppressed by SB202190 (lane 10). KTI failed to strengthen the PD98059-induced suppression of TGF-β1-induced ERK1/2 phosphorylation (lane 9 versus lane 8). We also showed that antisense PI3K ODN transfection did not abrogate ERK1/2 phosphorylation in HRA cells in response to TGF-β1 (lane 12). As expected, KTI inhibited the TGF-β1-induced phosphorylation of ERK1/2 in the AS PI3K ODN cells (lanes 12 and 13) or S PI3K ODN cells (lanes 14 and 15).

**KTI Suppressed TGF-β1-stimulated Phosphorylation of ERK1/2 or Akt via an Src-dependent Mechanism**—The pharmacological Src inhibitor PP2 inhibited the ERK1/2 (Fig. 3A, lane 4) and Akt (Fig. 3C, lane 4) activation by 65–75%. KTI did not enhance the suppression by PP2 of TGF-β1-induced phosphorylation of ERK1/2 (Fig. 3A, lane 5) and Akt (Fig. 3C, lane 5). Furthermore, general tyrosine kinase inhibition with genistein (lane 6) or herbimycin A (lane 7) also significantly attenuated TGF-β1-induced ERK1/2 or Akt phosphorylation. Suppression of TGF-β1-induced activation of Src by KTI supports that the critical position of KTI may not be a downstream target(s) of Src pathway.

**Effects of KTI on the Phosphorylation of ERK1/2 and Akt in Cells Expressing CA-c-Src**—Our biochemical approach clearly showed that KTI may be correlated with inhibition of the Src-MAPK-Akt signaling cascade. But it left open the question, functional significance of this finding. To address that KTI...
FIG. 4. Effect of KTI on phosphorylation of ERK1/2 and Akt in cells expressing wild-type c-Src (WT) or constitutively active c-Src (Y529F). HRA cells expressing wild-type c-Src (WT) or constitutively active c-Src (Y529F) were serum-starved for 16 h before treatment with KTI (10 μM, lane 3), PP2 (25 μM, lane 4), genistein (50 μM, lane 6), herbimycin A (10 μM, lane 7), or vehicle (lane 2) for 30 min prior to stimulation with or without 10 ng/ml TGF-β1 for 20 min. Cell lysates were prepared and subjected to Western blot analysis for the activation of ERK1/2 (p-ERK1/2 (A and B)) or Akt (p-Akt (C and D)). The same blots were stripped and reprobed with anti-human ERK1/2 or Akt antibody. B and D, blots were scanned and analyzed for quantification with Macintosh software. Composite densitometric analysis of phospho-ERK1/2 (B) and phospho-Akt (D) normalized to control are shown, and data from three experiments were averaged and are represented as the mean ± S.D., expressed as -fold increase with respect to nonstimulated cells. Different letters (a–f) represent statistical differences (p < 0.05).
functions at the upstream target(s) of Src, the phosphorylation state of ERK1/2 and Akt was examined in cells expressing either wild-type c-Src (WT) or CA-c-Src (Y529F) (Fig. 4). Immunoblot analysis showed that both cell lines expressed similar amounts of total ERK1/2 and Akt.

Phospho-ERK1/2 bands were strongly detected in the Y529F cells (Fig. 4A, lane 5), but not in the WT cells (Fig. 4A, lane 1), even when the cells were not stimulated with TGF-β1. Further, pretreatment for 1 h with 10 μM KTI could not diminish the TGF-β1-induced phosphorylation of ERK1/2 bands in the Y529F cells (Fig. 4A, lane 8 versus lane 6), supporting that KTI may be involved in the upstream target(s) of c-Src. In a parallel experiment, CA-c-Src increased basal Akt phosphorylation (Fig. 4C, lane 1 versus lane 5). KTI could not suppress the TGF-β1-induced phosphorylation of Akt in HRA cells transfected with CA-c-Src (Fig. 4C, lane 8 versus lane 6). On the other hand, KTI could inhibit the ERK1/2 and Akt phosphorylation in the WT cells (lane 2 versus lane 4).

Effect of KTI on TGF-β1-induced Expression of uPA mRNA and Protein—Our previous data showed that Src and ERK1/2 phosphorylation correlated closely with TGF-β1-induced uPA expression by enzyme-linked immunosorbent assay, Western blot, and Northern blot analyses in HRA cells (15). The involvement of Src in this TGF-β1-stimulated functional response was assessed by the use of KTI and the pharmacological inhibitors (PP2, PD98059, LY294002, or SB202190) in the WT cells or in the Y529F cells. As shown in Fig. 5A, 10 ng/ml TGF-β1 produced an increase (peak at 6 h) of uPA mRNA expression in the WT cells (lane 2). The concomitant addition of PP2 (lane 4), PD98059 (lane 5), or LY294002 (lane 6) canceled the TGF-β1-induced increase of uPA mRNA expression by 60–70%, suggesting involvement of the Src-MAPK-Akt pathway in the TGF-β1-induced uPA synthesis. Further, the addition of KTI (lane 3) reduced the TGF-β1-induced increase of uPA gene expression by 45%. In contrast, the addition of SB202190 (P38 inhibitor) did not alter the TGF-β1-induced increase of uPA gene expression (lane 7).

In a parallel experiment, in the Y529F cells, uPA mRNA expression was enhanced even in unstimulated cells (lane 8 versus lane 1). TGF-β1 markedly produced an increase (peak at 6 h) of uPA mRNA expression in the Y529F cells (lane 9). The concomitant addition of PP2 (lane 11), PD98059 (lane 12), or LY294002 (lane 13) reduced the TGF-β1-induced up-regulation of uPA mRNA expression by 45–55%. In contrast, the addition of KTI (lane 10) or SB202190 (lane 14) did not alter the TGF-β1-induced increase of uPA gene expression. Thus, these data allow us to speculate that TGF-β1-dependent Src-MAPK-Akt pathway is involved in uPA production and that KTI inhibits the upstream target(s) of Src.

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 KTI, PP2, PD98059, or LY294002 (Fig. 5C). We found that TGF-β1 treatment had a significant effect on expression of the 50-kDa band corresponding to uPA in TGF-β1-stimulated cells. We verified that the above mentioned changes of the uPA gene expression were associated with changes of uPA protein expression. These data strongly demonstrate suppression by KTI of the upstream targets of Src-MAPK-PI3K signaling cascade, leading to synthesis of uPA mRNA and protein, in response to TGF-β1 in HRA cells.

**Effects of KTI on the TGF-β1-induced Up-regulation of uPA Expression in Cells Expressing CA-MEK1, Control MEK1, CA-Akt, or KD-Akt**—To further confirm that KTI-dependent suppression of unstimulated and TGF-β1-stimulated uPA expression is mediated by an upstream target of Src, HRA cells expressing CA-MEK1 or CA-Akt were pretreated with KTI, and then stimulated with TGF-β1. HRA cells expressing control MEK1 or KD-Akt were used as controls. It has been speculated that if KTI functions at the upstream target(s) of Src, KTI has no ability to inhibit basal uPA expression in cells expressing CA-MEK1 or CA-Akt. We estimated by Western blot the ratio of optical density of uPA protein over β-actin protein (Fig. 5C). Seventy-two hours after transfection, serum-deprived cells pretreated with or without KTI were incubated in the presence or absence of 10 ng/ml TGF-β1 for 24 h, cell lysates were prepared, and then uPA expression was measured by Western blot.

First, we determined the effects of overexpression of CA-MEK1, CA-Akt, or KD-Akt on basal and TGF-β1-stimulated uPA expression. In the cells transfected with CA-MEK1 or CA-Akt, uPA expression was enhanced by 250% (lane 1) and 175% (lane 2), respectively, even in unstimulated cells. TGF-β1 did not produce an increase of uPA protein expression in the CA-MEK1 cells (4-fold, lane 7) and CA-Akt cells (2-fold, lane 11), compared with control MEK1 cells (lane 3). In contrast, in the cells transfected with KD-Akt, uPA expression was reduced by one-half even when cells were stimulated with TGF-β1 (lane 15). Thus, the overexpression of CA-MEK1 and CA-Akt enhanced the basal, but not TGF-β1-induced, uPA expression, as compared with control MEK1 and the overexpression of KD-Akt abrogated the basal and stimulated uPA expression. Each transfected cell line was preincubated with or without 10 μM KTI and then exposed to 10 ng/ml TGF-β1. In the CA-MEK1 cells, KTI failed to inhibit basal (lane 7) and TGF-β1-stimulated (lane 8) uPA expression. In the CA-Akt cells, KTI also failed to inhibit TGF-β1-stimulated uPA expression (lane 12), and basal uPA expression was not affected (lane 10). The expression of KD-Akt suppressed TGF-β1-induced expression of uPA (lane 16). In the control MEK1 cells, KTI significantly reduced TGF-β1-induced uPA expression (lane 4). These data suggest that KTI does not inhibit the downstream target(s) of ERK and Akt.

**Effects of KTI on TGF-β1-stimulated Invasive Response in Cells Expressing Either Wild-type c-Src (WT), CA-c-Src (Y529F), CA-MEK1, Control MEK1, CA-Akt, or KD-Akt**—We reported previously increased cell invasion and elevated uPA production as components of TGF-β1-induced ovarian cancer cell invasion (15). TGF-β1-stimulated HRA cell invasion through modified basement membrane matrix (Matrigel) requires uPA activity (15). As shown in Fig. 6, in cells expressing WT-c-Src, TGF-β1 significantly stimulates cell invasiveness. KTI treatment resulted in suppression of invasion by 40–50%, irrespective of whether cells were stimulated with TGF-β1. PD98059 significantly reduced cell invasion by 75%.

In a parallel experiment, the CA-c-Src-transfected cells stimulated with or without TGF-β1 (10 ng/ml) were analyzed for invasion in vitro (Fig. 7). CA-c-Src transfection resulted in a 1.5-fold increase in invasiveness. Interestingly, inhibition by KTI did not reduce invasion, whereas treatment of cells with PD98059 markedly suppressed (75%). These data indicate that Src-dependent pathways contribute to an invasive response in HRA cells. CA-c-Src was not impaired by KTI. In agreement with the results of CA-c-Src overexpression, transfection of CA-MEK1 or CA-Akt increased cell invasion—250% relative to empty vector, irrespective of whether cells were stimulated with TGF-β1, but the increase was not abrogated by pretreatment with KTI. KTI did not suppress invasion in cells express-
ing CA-MEK1 or CA-Akt, supporting a role for KTI upstream of Src in the suppression of TGF-β1-induced uPA expression and invasion. In a parallel experiment, we confirmed that transfection of cells with KD-Akt results in a marked suppression of invasion due to a lack of uPA production.

**DISCUSSION**

The previous data (15) support a role for TGF-β1 activation of at least two distinct pathways (Src-MAPK-PI3K-NF-κB-dependent and Src-MAPK-AP-1-dependent) for TGF-β1-dependent uPA up-regulation and promotion of invasion, and that the Src-MAPK-PI3K is a main pathway for this response in HRA cells (Fig. 8). To examine the KTI-mediated action, we initially tested the effect of pharmacological inhibitors of Src, ERK, and Akt on TGF-β1-induced uPA expression and invasion. Because pharmacological inhibitors have nonspecific effects that could be misleading, complementary genetic approaches have been performed to confirm the findings from the use of small molecules. For this, we further examined the effect of inhibiting or activating these signaling pathways by transfecting the cells with antisense ODNs to block gene translation/transcription directly through targeting PI3K or with CA-c-Src, CA-MEK1, CA-Akt, or KD-Akt. The treated cells were analyzed for phosphorylation of target proteins as well as uPA expression and invasiveness.

In the present study we have identified a novel mechanism of a soybean KTI regulation in the TGF-β1 signaling pathways in human ovarian cancer cells. KTI inhibits TGF-β1-stimulated activation of Src, ERK, and Akt in HRA cells, which results in suppression of uPA expression and invasion. Note that TGF-β1 stimulates Src kinase, which will activate the MAPK and PI3K/Akt (15). The MEK-1 inhibitor PD98059 and PI3K inhibitors wortmannin and LY294002, at concentrations that abolished respective kinase activity, masked the effects of KTI upon uPA production. KTI inhibited the TGF-β1-induced uPA up-regulation in cells expressing WT c-Src. In cells expressing the CA-c-Src and expressing CA-MEK1 or CA-Akt, however, we found that KTI failed to reverse TGF-β1-induced activation of ERK and PI3K/Akt as well as TGF-β1-induced uPA up-regulation and invasion. Thus, complementary genetic approaches confirmed the findings from the use of small molecules. To our knowledge this is the first study to identify an inhibitory action of KTI upon the upstream target(s) of Src in response to TGF-β1.

Accumulating evidence indicates that activated Src may in-
duce cell transformation through Ras-ERK-dependent and Rac1-dependent pathways (23). Active Src can potently stimulate the Ras-ERK pathway, primarily by the tyrosine phosphorylation of Shc by Src, followed by the recruitment to the membrane of the adapter protein Grb2 and the Ras guanine nucleotide exchange factor Sos, thereby promoting Ras activation (24). The Ras-Raf-MEK-ERK and the Ras-P13K-Akt routes independently or dependently form the two major branches of intracellular signaling. These two pathways have central roles in the regulation of cell survival, proliferation, and invasion. Due to their importance, the cross-talk between these two pathways has been investigated by many investigators (25, 26). It should be noted that Ras causes the activation of the ERK and P13K, and P13K might be upstream/downstream of Ras, thus regulating ERK. Furthermore, P13K contributes to the activation of phospholipase C-γ. Phospholipase C-γ causes the mobilization of calcium, which is also required for the activation of ERK. Thus, signals from Src, Ras, P13K, and phospholipase C-γ may converge on the activation of ERK. As for TGF-β receptor, however, it has been demonstrated that P13K-Akt, in HRA cells, is a downstream target of ERK. The facts that a MEK inhibitor inhibited the TGF-β1-induced Akt activation and that P13K inhibitors had no effect on the TGF-β1-induced ERK1/2 activation suggests a presence of cross-talk between the ERK and Akt cascades and that P13K-Akt may be a downstream cascade of ERK. These results allow us to speculate that TGF-β receptor may induce Src-Ras through the assembly of the Shc-Grb2-Sos complex as well as through other mechanisms (Rac1-dependent pathways), whereas Src-Ras signaling does not directly connect with P13K-Akt cascade. Our findings are generally consistent with the existence of this linear pathway, but all of the components of the pathway under investigation can be activated by other upstream components that are part of other pathways. Thus, one cannot conclude from these findings that the effects observed are in fact due to a linear pathway.

We sought to identify the site(s) and mechanism(s) responsible for the inhibitory action of KTI. We found that the site of action of KTI is an upstream of Src, at the level of Src itself or above. Inhibition of TGF-β1-stimulated Src activation by KTI may imply that dephosphorylation at tyrosine of Src or uncoupling of the pathway further upstream is a far more likely mechanism of action. One possibility is a role for an inducible MAPK phosphatase, in particular MAPK phosphatase-1 (27). We examined whether KTI exposure leads to both the rapid induction of MAPK phosphatase-1 mRNA and its sustained expression. Target genes in ovarian cancer cells were identified by microarray analysis (4). Unfortunately, induction of MAPK phosphatase-1 protein was not seen when cells were treated with bikunin (4) or KTI (data not shown).

TGF-β family members signal through transmembrane Ser-Thr kinase receptors that directly regulate the intracellular Smad pathway. Increased levels of Smad3 or Smad4 can induce apoptosis (28). In the present study, however, TGF-β1 neither induced apoptosis (data not shown) nor cell growth inhibition in HRA cells. Therefore, the Smad pathway may not be involved in TGF-β1-dependent signaling cascade in these cell lines. At present we cannot rule out that other signals are also involved. Supporting this possibility is our finding that KTI does not completely suppress the Src-MAPK-P13K/Akt signaling cascade. If another signal is involved in the inhibitory actions of KTI, then this may explain why KTI is a less effective inhibitor of the Src than pharmacological inhibitor PP2. Alternatively, it may be that other Src-independent mechanisms are also involved and Src only plays a conditional role in the inhibitory effects of KTI. Although the present studies have strongly revealed one such potential mechanism for KTI, involving the upstream target(s) of Src, the cell-specific expression of Src may dictate which effects are manifest upon uPA expression.

KTI is the potent inhibitor of trypsin. KTI inhibited cell invasiveness at least through suppression of MAPK-dependent uPA signaling cascade, whereas Bowman-Birk inhibitor (BBI), another type of soybean trypsin inhibitor, was inactive (6, 7). Therefore, it is unlikely that trypsin inhibitory activity is specific for suppression of signaling cascades. The specific molecular structure of Kunitz-type protease inhibitor may be important for its action.

There are some conflicting data on biological functions of TGF-β. TGF-β is also a potent inducer of growth inhibition in several cell types, and the TGF-β signaling pathway has been implicated in tumor suppression (29). It has been reported that TGF-β inhibited MAPK activity in the rat fibroblast cell line 3Y1 and in v-Src-transformed 3Y0 (SR-3Y1), suggesting that TGF-β1 specifically induces degradation of activated Src kinase (30). Additionally, the previous study provides evidence that TGF-β in HRA and SKOV-3 cells (15) as well as HaCaT and Madin-Darby canine kidney cells (30) induces a rapid and transient increase in Src kinase activity. On the other hand, TGF-β has been reported to negatively regulate Src kinases in HepG2 and PC3 carcinoma cells (30). Although the reason for the differing results remains unknown, these data are inconsistent with our present results, which may depend on the stage of differentiation, culture conditions, and a cell type.

We have no information whether TGF-β can directly induce activation of Src in ovarian cancer cells. We are examining whether Shc will work as an adaptor molecule to mediate phosphorylserine-dependent signaling events. In fact, several receptor protein-tyrosine kinases have tyrosine autophosphorylation sites, which bind to Shc, and the receptor-bound Shc becomes tyrosine-phosphorylated by the receptor and recruits another Src homology 2-containing adaptor protein to the phosphorylated residues. Further studies are therefore warranted to define the role of KTI and each signaling pathway in modulating the sensitivity of ovarian cancer cells to TGF-β.

In conclusion, using complementary pharmacological and genetic approaches, we have identified a novel mechanism of regulation between KTI and TGF-β signaling pathways in human ovarian cancer cells, involving the upstream target(s) of Src. These findings identify anti-invasive properties of KTI at the level of tumor cells and may be relevant to the use of KTI in modulating cancer cell invasion and metastasis.
Suppression of Urokinase Expression and Invasion by a Soybean Kunitz Trypsin Inhibitor Are Mediated through Inhibition of Src-dependent Signaling Pathways
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