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A kunitz-type protease inhibitor bikunin disrupts ligand-induced oligomerization of receptors for transforming growth factor (TGF)-β and subsequently suppresses TGF-β signalings

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Abstract **We previously found that bikunin (bik), a Kunitz-type protease inhibitor, suppresses transforming growth factor-β1 (TGF-β1)–stimulated expression of urokinase-type plasminogen activator (uPA) in human ovarian cancer cells that lack endogenous bik. In the present study, we tried to elucidate the mechanism by which bik also inhibits plasminogen activator inhibitor type-1 (PAI-1) and collagen synthesis using human ovarian cancer cells. Here, we show that (a) there was an enhanced production of both uPA and PAI-1 in HRA cells in response to TGF-β1; (b) the overexpression of bik in the cells or exogenous bik results in the inhibition of TGF-β1 signaling as measured by phosphorylation of the downstream signaling effector Smad2, nuclear translocation of Smad3, and production of PAI-1 and collagen; (c) bik neither decreased expression of TGF-β receptors (TβRI and TβRII) in either cell types nor altered the specific binding of ^{125}I TGF-β1 to the cells, indicating that the effects of bik in these cells are not mediated by ligand sequestration; (d) TβRI and TβRII present on the same cells exclusively form aggregates in TGF-β1-stimulated cells; (e) co-treatment of TGF-β1–stimulated cells with bik suppresses TGF-β1–induced complex formation of TβRI and TβRII; and (f) a chondroitin-4-sulfate side chain-deleted bik (deglycosylated bik) does not inhibit TGF-β1 signaling or association of type I/type II receptor. We conclude that glycosylated bik attenuates TGF-β1–elicited signaling cascades in cells possibly by abrogating the coupling between TβRI and TβRII and that this probably provides the mechanism for the suppression of uPA and PAI-1 expression.**

Keywords: Bikunin; CD44; Dimerization; Ovarian cancer; TGF-β receptor; Signal transduction

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Abbreviations: bik, bikunin; Bik$^+$, bikunin transfection; BSA, bovine serum albumin; FBS, fetal bovine serum; HA, hyaluronan; LP, Link protein; Luc$^+$, luciferase transfection; PAI-1, plasminogen activator inhibitor type-1; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; uPA, urokinase-type plasminogen activator; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type-I; TβRII, TGF-β receptor type-II

1. Introduction

Bikunin is a secreted Kunitz-type protease inhibitor with a postulated role in protease inhibition [1]. Our laboratory has been studying the function and mechanism of bikunin (bik) on the suppression of the invasive capacity of tumor cells [2–4]. We have proposed that bik is a main participant in the inhibition of tumor cell invasion and metastasis possibly through both direct inhibition of cell-associated plasmin activity and suppression of urokinase-type plasminogen activator (uPA) expression [2].

During the search for novel proteins interacting with bik, we identified at least two types of cell-associated binding proteins, the 40-kDa link protein (LP) and a 45 kDa protein. The former belongs to the hyaluronan (HA)-binding protein. The latter is a membrane-associated putative receptor for bik (bik-R) [3–5]. We postulate that LP is an extracellular matrix-binding protein and acts as a "sink" for bik. The diverse roles proposed for LP can largely be understood as a manifestation of HA stabilization in the pericellular matrix [5,6]. We have established that LP interacts with the NH_2-terminal domain of bik, while bik-R recognizes the chondroitin-4-sulfate side chain of bik [3,4]. Analysis of binding of native bik and chondroitin-4-sulfate-deleted bik (deglycosylated bik) to the cells showed that the low affinity binding site is LP and the high affinity binding site is bik-R [4]. Our previous publications indicated that binding of bik to its binding sites on the cell surface is associated with an inhibition of protein kinase C translocation and activation [2,7,8]. More recently, we reported that bik markedly suppresses the cell invasion possibly through negative regulation of mitogen-activated protein kinase-dependent mechanisms and subsequent suppression of uPA expression, and that bik must bind to both of the bik-binding proteins (LP and bik-R) to effectively suppress the upregulation of uPA expression [2,4,7,8]. Thus, bik could act as a negative modulator of the invasive cells.

CD44 is the major cell-surface receptor for HA [9]. Since one of the bik-binding proteins is LP, which is apparently held at the cell surface by HA [6], it is reasonable to think that CD44 would be involved in this bik-mediated complex at the cell surface. The previous results showed that engagement of bik-R

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by bik and subsequent coupling of bik-R to CD44 may facilitate inhibition of cell stimulation by suppression of CD44 activation (that is CD44 clustering), which finally leads to reduction of uPA expression [10].

To better understand the function of bik, we studied its biological effects in human ovarian cancer HRA and SKOV-3 cells. We have previously demonstrated that these cells have no endogenous bik expression and that increased expression of bik in them inhibits transforming growth factor-β (TGF-β) signaling [11,12]. TGF-β1 is an essential regulator of development, cell proliferation, and matrix deposition [13–15] and is initiated by the binding of TGF-β to TGF-β receptor type II (TβRII). This is followed by the formation and stabilization of TβRI–TβRII complexes, transphosphorylation of TβRI by TβRII, and phosphorylation of the receptor-associated cytoplasmic effector molecules Smad2 and Smad3 by TβRI [13,15]. Studies presented in this report were designed to investigate the effects of bik on TGF-β1-mediated stimulation of uPA, plasminogen activator inhibitor type-1 (PAI-1) and collagen expression in HRA and SKOV-3 human ovarian cancer cells and to elucidate the mechanism(s) involved in this modulation. In addition, human skin fibroblasts were used for co-immunoprecipitation assay.

2. Materials and methods

2.1. Materials

Bikunin purified from human urine was kindly provided by Mochida Pharmaceutical Co., Ltd. (Gotenba, Japan). Characterization of bik and deglycosylated bik (chondroitin-4-sulfate-side chain deleted bik) has been described previously [11]. Ultrapure natural human TGF-β1 was from Genzyme (Cambridge, MA) and R&D Systems (Minneapolis, MN). The antibodies against uPA (#3689 [recognizes the B-chain of uPA] and #3471 [reacts with the A-chain of uPA; interferes with binding of uPA to its receptor]) and PAI-1 (#3785) were obtained from American Diagnostics, Greenwich, CT. Peroxidase-conjugated secondary antibodies were from Dako (Copenhagen, Denmark); antibody to phospho-Smad2 was from Upstate Biotechnology (Lake Placid, NY); antibodies to Smad2 and Smad3 were from Zymed Laboratories (South San Francisco, CA); rabbit anti-TGF-βRI IgG (specific for TGF-βRI p55) was from CosmoBio Co. Ltd., Tokyo, and rabbit anti-TGF-βRII IgG (specific for TGF-βRII p70) was from Santa Cruz Biotechnology, Santa Cruz, CA. Culture media, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Tissue culture plastics were purchased from Costar/Corning (Cambridge, MA) and Falcon (Becton–Dickinson and Co., Bedford, MA). Bovine serum albumin (BSA), Tris–base, dithiothreitol (DTT), phenylmethylsulfonyl fluoride, and ammonium persulfate were from Sigma Chemical Co., St. Louis, MO. Acrylamide, bisacrylamide, and polyvinylidene difluoride (PVDF) membrane were from Bio-Rad. X-ray film was purchased from Kodak Co. The enhanced chemiluminescence (ECL) was purchased from Amersham Pharmacia Biotech, Tokyo, Japan. All other chemicals were, analytical grade.

2.2. Cell culture

The ovarian cancer cell lines, HRA [16] and SKOV-3 (American Type Culture Collection), were grown in RPMI 1640 supplemented with 10% (v/v) FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere with constant humidity. Human skin fibroblast strains were established from explants of human adult skin biopsies obtained from informed healthy volunteers (age 32-40 years). Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2 mM glutamine in the presence of 5% CO_2. Cells at subcultures 2–4 were used. Fibroblasts were grown to subconfluency in 10% serum containing medium. TGF-1 (10 ng/ml) was added either alone or in combination with test compounds in cancer cells preincubated for 30 min to 1 h.

2.3. Bikunin transfection

The bik expression vector pCMV-bik-IRES-bsr (Bik^+) and the control vector pCMV-luciferase-IRES-bsr encoding luciferase (Luc^+) were transfected into HRA cells by the standard calcium phosphate precipitation method as described previously [16].

2.4. Preparation of cytosol and nuclear extracts

Cells were harvested, washed, and lysed in ice-cold lysis buffer (250 mM Tris–HCl, pH 7.5, containing a mixture of protease inhibitors [100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml E-64, 0.5 μg/ml leupeptin, and 1.7 μg/ml pepstatin A]). The debris was removed by centrifugation at $13\,000\times g$ for 20 min at 4 °C. Nuclear extracts were prepared essentially as described [17]. Total protein amount in the extracts was measured using the Bio-Rad Bradford reagent.

2.5. Western blot analysis

Cells were harvested and cell pellets were lysed as described above. Centrifuged lysates (50 μg) from each cell were analyzed by SDS–polyacrylamide gel electrophoresis and transferred to a PVDF 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% BSA. Blots were probed with the following primary antibodies overnight at 4 °C: monoclonal anti-uPA (#3689 plus #3471), anti-PAI-1, anti-phospho-Smad2, anti-Smad2, or anti-Smad3 antibodies. This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 for 1 h. Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to film. Filters were quantitated by scanning densitometry using a Bio-Rad model 620Video Densitometer with a 1-d Analyst software package for Macintosh.

2.6. Northern blot hybridization with cDNA probes

Total RNA isolations were done using the Trizol reagent (Life Technologies, Inc.). Northern blot hybridization was carried out as described previously [10,18]. Samples of total RNA (10 μg) were separated by electrophoresis through denaturing 1.2% agarose gels containing 1% formaldehyde and transferred onto nylon or nitrocellulose membranes using standard molecular biological techniques. Hybridization was carried out with [α-^{32}P]dCTP by random oligonucleotide priming to specific activities of $0.4–0.9\times10^9$ cpm/μg. PAI-1 cDNA was prepared as described [10,18]. Filters were reprobed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase to correct for the amount of RNA loaded onto the filters [10,18]. After hybridization, the membranes were washed and exposed on Kodak BioMax MS-1 film at −70 °C.

2.7. Affinity labeling and immunoprecipitation

Recombinant human TGF-β1 was iodinated using the chloramine T method as described [19]. TGF-1 (1 μg) in 10 μl of 1 M sodium phosphate, pH 7.2, was mixed with 2 μl of $Na^{125}I$ (0.2 μCi). The reaction was initiated by the sequential addition of three 2-μl portions of chloramine T (0.1 mg/ml). The reaction was stopped by the sequential addition of 10 μl of acetyl tyrosine (100 mM), 100 μl of potassium iodide (100 mM), and 100 μl of urea-saturated acetic acid.

For the cross-linking and immunoprecipitation experiments, subconfluent cells in standard medium were rinsed once with KRH binding buffer (50 mM HEPES, pH 7.5, 128 mM NaCl, 1.3 mM $CaCl_2$, and 5 mM $MgSO_4$, 5 mM KCl) containing 50 pM ^{125}I TGF-β1 (HRA and SKOV-3 cells) or 200 pM ^{125}I TGF-β1 (human skin fibroblasts) was added. Plates were incubated at 4 °C with rotation for 2 h and then rinsed three times with ice cold KRH. The plates were prepared in duplicate; one half of the plate was treated with cross-linker and the other half was untreated. The ligand was then cross-linked to the receptor with the addition of 0.5 mg/ml disuccinimidyl suberate (DSS) (Pierce) from a 100-fold concentrated stock in DMSO, followed by incubation at 4 °C for 15 min. Glycine was added to 20 mM and plates were incubated for 10 min at 4 °C. In another plate, no cross-linker was used. Plates were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C for 20–30 min in 1 ml lysis buffer. Cleared lysates were immunoprecipitated at 4 °C overnight with polyclonal antibodies against the TβRI or TβRII. One-twentieth volume of protein A–Sepharose CL-4B beads (Sigma) was added and the lysates were incubated with rotation for 30 min. Protein A–Sepharose beads were rinsed twice with lysis buffer and once with PBS and bound protein was eluted by boiling in 0.5% SDS. Samples were analyzed by

SDS–polyacrylamide gel electrophoresis [19,20] and specific bands were visualized by autoradiography.

2.8. Procollagen deposition analysis by ^3H proline incorporation, SDS–polyacrylamide gel electrophoresis, and autoradiography

The parental and transfected cells were grown to subconfluency in 12-well plates. The medium was changed to serum-free medium supplemented with 50 μg/ml ascorbic acid for the duration of the experiment. Cells were stimulated with 10 ng/ml TGF-β1 and 6 h later, 0.5 μCi/ml ^3H proline (Amersham Biosciences) was added to the medium for 24 h. Medium was harvested from each well, and cells were trypsinized and counted. Cells were lysed and extracellular matrix proteins were fixed in 70% ethanol. Collagen deposition was determined by differential collagenase digestion and liquid scintillation counting [20].

2.9. Cell growth assay: thymidine incorporation

Cells were seeded into 24-well plates in the presence or absence of TGF-β1 (10 ng/ml) for 16 h, as indicated. Cells were pulsed with 0.5 μCi/ml ^3H thymidine (Amersham Bioscience) for 4 h, and incorporation of radioactive label into newly synthesized DNA was analyzed by trichloroacetic acid precipitation and liquid scintillation counting.

2.10. Flow cytometric analysis

A single cell suspension (10^6/ml) was incubated with antibodies or isotype control antibodies on ice for 1 h. Cells were washed three times with washing buffer, and 2 μl of the primary antibody and 3 μl of fluorescein isothiocyanate-conjugated secondary antibody (Dako) were added for 1 h on ice, respectively. Cells were analyzed in a FACScan (Becton–Dickinson). At least 10 000 cells were analyzed per sample in all experiments. All experiments were performed at least twice.

2.11. Statistical analysis

The data presented are means of triplicate determinations in one representative experiment unless stated otherwise. Data are presented as means ± S.D. All statistical analyse were performed using StatView for Macintosh. The Mann–Whitney U test was used for the comparisons between different groups. P less than 0.05 were considered significant.

3. Results

3.1. Effect of bik on TGF-β1-induced expression of PAI-1 mRNA and protein in HRA cells

We have previously studied TGF-β1 signaling in human ovarian cancer HRA cells, which are highly responsive to TGF-β1. Smad2 phosphorylation correlates closely with the TGF-β1-induced PAI-1 expression [21]. In HRA cells, TGF-β1 induces upregulation of both uPA and PAI-1 expression [11,12]. In contrast, SKOV-3 cells do not express PAI-1 as opposed to uPA expression and can be seen in response to TGF-β1.

In the present study, we asked whether the expression of bik can inhibit TGF-β1–mediated biological effects in HRA and SKOV-3 cells. For that purpose, both cells were stably transfected with the full-length wild-type bik cDNA (Bik^+) or with a control luciferase cDNA (Luc^+) as described and then treated with TGF-β1. In a parallel experiment, the cells were pretreated with 1 μM bik or 1 μM deglycosylated bik and then exposed to TGF-β1. TGF-β1 responsiveness in these cell lines was initially analyzed by monitoring the upregulation of PAI-1 mRNA expression. As demonstrated in Fig. 1A and B, treatment of parental HRA cells or Luc^+ cells with 10 ng/ml TGF-β1 for 12 h led to a significant increase in the PAI-1 expression

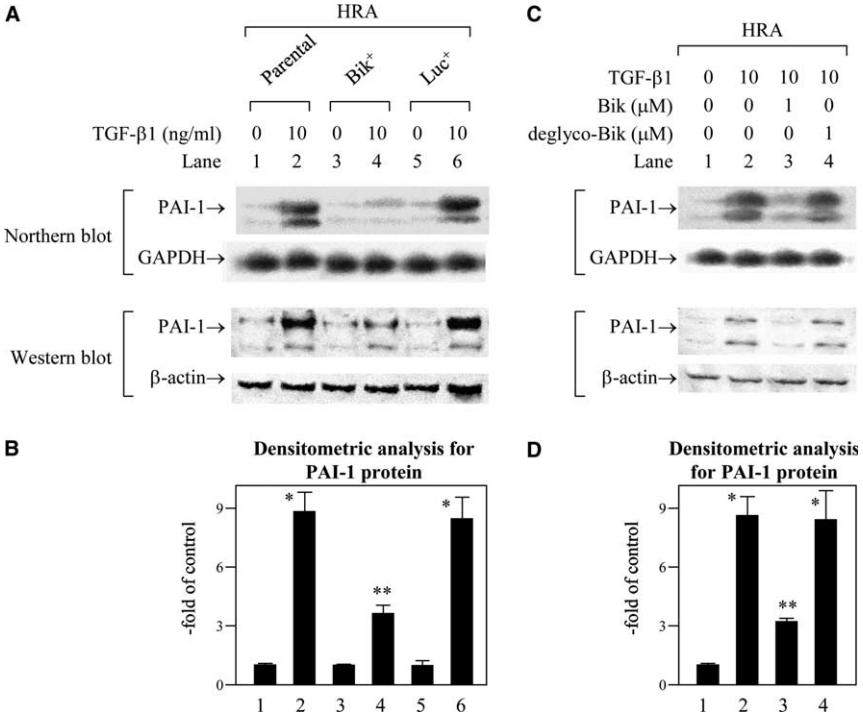


Fig. 1. Bikunin inhibits TGF-β-induced PAI-1 gene expression in HRA cells. (A) and (B) HRA cells stably transfected with bik (Bik^+) or luciferase (Luc^+) were stimulated with 10 ng/ml TGF-β1 for 12 h. (C) and (D) HRA cells pretreated with or without bik or bik lacking chondroitin-4-sulfate side chain (deglyco-Bik) were stimulated with 10 ng/ml TGF-β1 for 12 h. PAI-1 mRNA (upper panel; Northern blot analysis) and protein (lower panel; Western blot analysis) expression. Overexpression of bik gene or exogenous bik reduced PAI-1 mRNA and protein expression in response to TGF-β1. Results presented were obtained in triplicate and are representatives of three independent experiments. (B) and (D) Values of densitometric scan are means ± S.D. of three experiments. $^*P < 0.05$ vs lane 1; and $^{**}P < 0.05$ vs lane 2.

(8.6- or 8.2-fold, $P < 0.05$). On the other hand, it was significantly reduced in the HRA cells transfected with bik (3.2-fold). In a parallel experiment (Fig. 1C and D), 10 ng/ml TGF-β1 produced an increase (peak at 12 h) in the PAI-1 mRNA expression in HRA cells (lane 2). In cells pretreated with bik (lane 3), no TGF-β1–induced increase of PAI-1 mRNA expression was seen. Instead, it was reduced by 65% as compared to TGF-β1-stimulated HRA cells. No such effect was seen when exogenous deglycosylated bik was added to the cells (lane 4). This suggests that chondroitin-4-sulfate side chain is important in mediating the inhibitory effect of bik. In our previous study [16], we have shown that endogenously expressed or exogenously added bik had a similar inhibitory effect on uPA expression in HRA cells.

Next, we studied whether the above-mentioned changes of the PAI-1 gene expression were also associated with changes in the levels of PAI-1 protein. Therefore, we estimated, by Western blot, the ratio of the optical density of the PAI-1 protein, based on the β-actin protein band under control conditions and after the administration of TGF-β1 in the bik transfected cells (Fig. 1A and B) and in HRA cells pretreated with or without bik or deglycosylated bik (Fig. 1C and D). The results showed that TGF-β1 treatment had a significant effect on the expression level of the 60-kDa band corresponding to PAI-1 in TGF-β1-stimulated cells. We observed that bik significantly suppressed the upregulation of PAI-1 in TGF-β1-stimulated cells. On the other hand, no such effect was seen with deglycosylated bik. These data demonstrated that bik can inhibit TGF-β1-dependent upregulation of PAI-1 expression at the mRNA and protein levels.

3.2. Bikunin abrogates TGF-β1-dependent stimulation of collagenous protein synthesis

In the second set of experiments, we tested whether bik regulates extracellular matrix production in HRA cells in the presence of TGF-β1 by utilizing bik transfectants. Lysates of the cells metabolically labeled with ^{3}H proline were analyzed. Parental HRA cells and control cells expressing luciferase and lacking endogenous bik demonstrated enhanced TGF-β1–induced collagen deposition (Fig. 2A). In contrast, abrogation of the stimulatory effect of TGF-β1 on collagenous protein synthesis by ~70% was seen in bik-expressing cells, suggesting suppression of the TGF-β1–mediated collagen deposition by bik (Fig. 2A). Moreover, addition of exogenous, but not deglycosylated bik to the cell culture also led to a diminished TGF-β1-induced collagen deposition (data not shown). Bikunin does not activate the degradative pathways (e.g., matrix metalloproteinases) [16] making it highly unlikely that the lowered amount of collagen would be due to enhanced degradation [19]. Taken together, our results thus far demonstrate that bik is a potent inhibitor of the TGF-β1 signaling as judged by its effect on TGF-1-regulated synthetic events.

HRA cells show no detectable growth response to TGF-β1 as shown in Fig. 2B. Neither did bik have any effect on the growth rate. Thus, overexpression of bik in cells did not affect cell viability or proliferation rate.

3.3. Bikunin inhibits TGF-β1-mediated Smad2 phosphorylation and nuclear translocation of Smad3

In the TGF-β1 signaling pathway, phosphorylation of the cytoplasmic signaling molecules Smad2 by TβRI is the first step in signal transduction. In the present study, we examined whether TGF-β1 induces phosphorylation of Smad2 in HRA (Fig. 3A and B, lanes 1–3) and SKOV-3 (lanes 10–12) cells by Western blotting. In HRA cells, TGF-β1 induced a marked rise in the level of phosphorylated Smad2 protein in a time-dependent manner. As compared with the non-stimulated HRA cells, an 8-fold increase in the phosphorylated Smad2 was observed at 20 min in response to 10 ng/ml TGF-β1. Similar amounts of Smad2 protein were detected by anti-Smad2 antibodies, irrespective of whether cells were stimulated with TGF-β1 or not. On the other hand, TGF-β1 (10 ng/ml, 20 min) did not induce phosphorylation of Smad2 in SKOV-3 cells (lanes 10–12). HRA cells expressing bik demonstrated a clear reduction in the TGF-β1-induced Smad2 phosphorylation (lanes 4–6) compared with the parental HRA cells (lanes 1–3), or HRA cells expressing luciferase (lanes 7–9). No change in the total level of Smad2 was found in response to the expression of bik.

We next investigated the effect of bik and TGF-β1 on the nuclear translocation of Smad3 as an important signaling step in TGF-β1 signaling pathway. An increase in nuclear translocation was observed at 20 min in response to TGF-β1 (Fig. 3C and D, lanes 1–4). HRA cells expressing bik demonstrated a

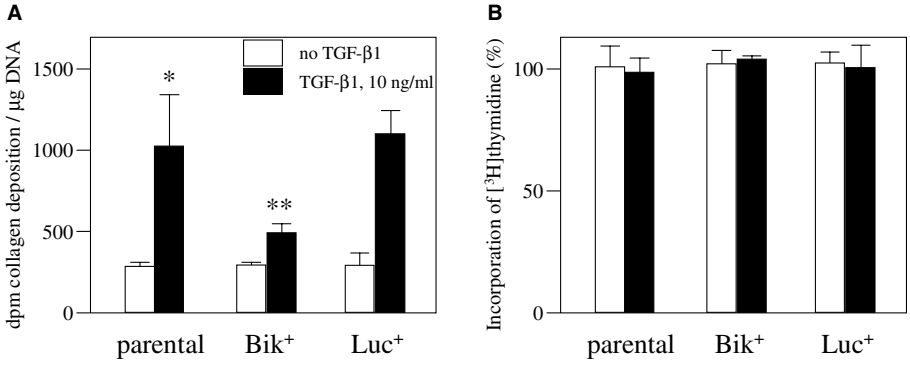


Fig. 2. Bikunin overexpression inhibits TGF-β1–dependent collagenous protein deposition, but not cell proliferation. Newly synthesized collagenous proteins in parental, Bik$^+$ and Luc$^+$ cells were measured in a [^{3}H] proline incorporation assay after 30 h of 10 ng/ml TGF-β1 stimulation. (A) Summary of quantitative analysis of the collagenous proteins expressed by bik transfectants with and without TGF-β1 (10 ng/ml). (B) Cellular proliferation was analyzed by [^{3}H] thymidine incorporation in pools of stably transfected cells expressing bik or luciferase, as indicated. Results are presented as percentage of control (unstimulated proliferation). Experiments were performed in triplicate and data presented are representatives of two independent experiments. Values are means ± S.D. of three independent experiments. * vs **$P < 0.05$.

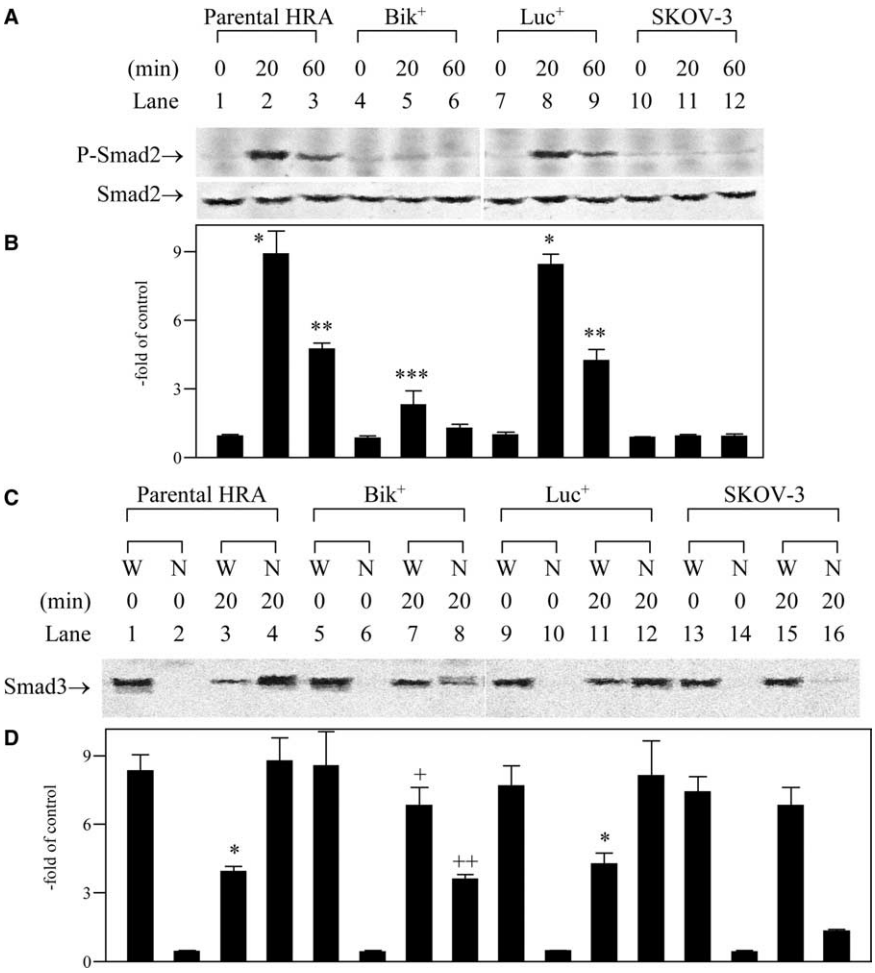


Fig. 3. TGF-β1-induced Smad2 phosphorylation and nuclear translocation of Smad3 are reduced in bik-expressing cells. (A) Pools of HRA (lanes 1–9) and SKOV-3 (lanes 10–12) cells were treated with TGF-β1 (10 ng/ml) for various time points (0, 20 and 60 min) as indicated. Whole cell (W) and nuclear (N) extracts were prepared after the indicated incubation times, and separated by SDS–PAGE. Each sample (50 μg) was resolved by 10% SDS–PAGE and probed with anti-phospho-Smad2 (0.5 μg/ml) to detect the phosphorylated Smad2 protein (top panel). Blots were stripped and reprobed with anti-Smad2 antibody (0.5 μg/ml) (bottom panel). Experiments were repeated three times with essentially identical results. (B) Blots were scanned and analyzed for quantification with the Macintosh software. Band intensities for phospho-Smad2 were normalized to the corresponding band intensities for total Smad2. Data from three experiments were averaged and are represented as means ± S.D., expressed as fold increase with respect to non-stimulated cells (time = 0). $^{*}P < 0.05$ vs lane 1; and $^{**}P < 0.05$ vs lane 2. (C) Nuclear translocation of Smad3 was analyzed by immunoblotting with an antibody specific for total Smad3. Results shown are representatives of three independent experiments. (D) Band intensities for Smad3 were scanned. Data from three experiments were averaged and are represented as means ± S.D., expressed as fold increase with respect to non-stimulated cells (time = 0). $^{*}P < 0.05$ vs lane 1; $^{*}P < 0.05$ vs lane 2; $^{+}P < 0.05$ vs lane 3; and $^{++}P < 0.05$ vs lane 4.

clear reduction in the nuclear translocation of Smad3 (lanes 5–8) when compared with the parental HRA cells (lanes 1–4), or the HRA cells expressing luciferase (lanes 9–12). As expected, no nuclear translocation of Smad3 was seen in SKOV-3 cells upon TGF-β1-treatment (10 ng/ml, 20 min) (lanes 13–16).

3.4. Exogenous bik inhibits TβRI-tβRII oligomerization

In a first set of experiment, we assessed the effect of bik on the expression of TβRI and TβRII on the surface of HRA and SKOV-3 cells by flow cytometry. Cells were incubated with or without TGF-1 (10 ng/ml, 12 h) in the presence or absence of bik (1 μM). Non-immune mouse IgG was used as the control IgG. Bikunin did not have any measurable effect on the expression of TβRI and TRII, irrespective of whether cells were stimulated with TGF-β1 (data not shown) or not.

In order to demonstrate the specificity of the anti-TβRI and anti-TβRII antibodies, we carried out immunoblotting. Cell lysates of HRA (Fig. 4A, upper panel), SKOV-3 cells (Fig. 4A, lower panel) and skin fibroblast cells (data not shown) were probed with anti-TβRI or anti-TβRII antibodies. The 55 kDa and 70 kDa bands were detected by anti-TRI or anti-TβRII antibodies, respectively (Fig. 4A). Following in vitro culture of cells with TGF-β1, there were no significant increases in cell surface expression of TβRI and TβRII compared with the unstimulated cells (data not shown).

We next studied whether bik inhibits TGF-β1 signaling in HRA cells by inhibiting hetero- or oligomerization of TβRs as has been shown to be the mechanism for bik-inhibited CD44-mediated signaling [10]. For that purpose, the cells pretreated with either bik or the vehicle (PBS) were affinity-labeled with ^{125}I TGF-β1. The cell lysates were then immunoprecipitated

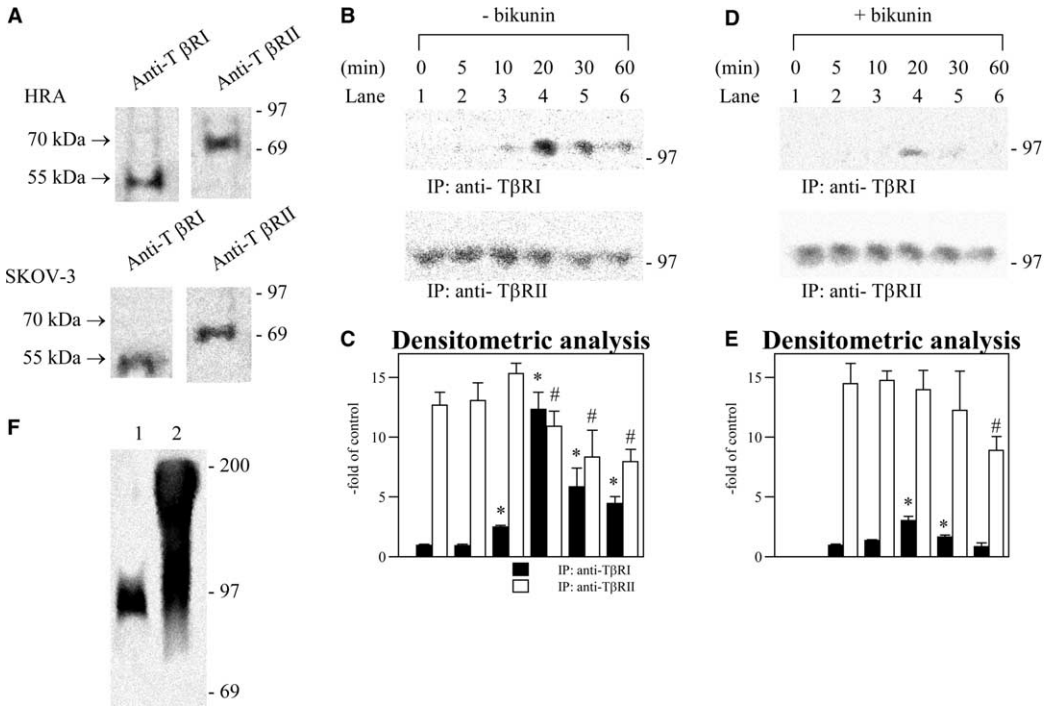


Fig. 4. Bikunin inhibits heterodimer formation between TβRI and TβRII. (A) Cell lysates (50 μl) of HRA (upper panel) and SKOV-3 cells (lower panel) were separated by SDS–PAGE, and specific bands were visualized using antibodies against TGF-β receptor type I or type II. Cells were incubated with 10 ng/ml ^{125}I TGF-β1 in the absence (B and C) or presence (D and E) of bik (1 μM). Cell lysates were immunoprecipitated with anti-TβRI (upper panel) or anti-TβRII (lower panel) antibodies and eluted by boiling in 0.5% SDS. (F) Cells were labeled with ^{125}I TGF-β1 for 20 min and treated with (lane 2) or without (lane 1) the chemical cross-linker DSS. The DSS-treated or non-treated cells were lysed and the lysate was immunoprecipitated with the anti-TβRI antibody. Immunoprecipitates were separated by SDS–PAGE and specific bands were visualized by autoradiography (C and E). Band intensities for TβRII were scanned. Data from three experiments were averaged and are represented as means ± S.D., expressed as fold increase with respect to non-stimulated cells (time = 0). The positions of molecular mass markers (in kDa) are indicated on the right margin. The blots shown are representatives of blots performed on samples from three different experiments. * and $^{\#}P < 0.05$ vs lane 1.

with antibodies against the two TGF-β receptors. Immunoprecipitations using anti-TβRI antibodies (Fig. 4B and C) revealed that TGF–β1 stimulation for more than 10 min induced an increase in the expression of the ~90-kDa band in a time-dependent manner. This protein species is equivalent to 70 kDa, the approximate molecular mass of the type II TGF-β receptor (Fig. 1A), if the molecular mass of the bound monomeric TGF-β1 is subtracted. No TβRI band was seen in the autoradiograms. This is because we did not use cross-linker agent in this study and ^{125}I labeled TGF-β1 directly binds to TβRII but not to TβRI. In a parallel experiment with anti-TβRII antibody, the ~90-kDa band was consistently detected by anti-TβRII antibody in response to ^{125}I TGF-β1. This indicates that the formation of TβRI and TβRII complexes can be seen in cells in response to TGF-β1.

In the presence of bik, the formation of receptor complexes was inhibited as seen when comparing the co-immunoprecipitation results in Fig. 4B and D. The ~90-kDa monomeric forms of TβRII in TGF-β1-stimulated cells migrate similarly to that in unstimulated cells, irrespective of whether bik is used in the experiments, suggesting that bik inhibits dramatically the formation of receptor complexes. These above results suggest that ^{125}I TGF-β1 specifically binds to TβRII and that TβRI and TβRII proteins are able to heteromerize under the TGF-1 stimulation within at least 20 min, and that bik can abrogate TGF-β1-stimulated complex formation of TβRI–TβRII proteins.

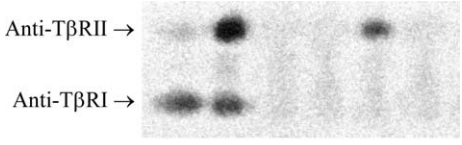
If the complex indeed dissociates into TβRI and TβRII upon lysis of cells, chemical cross-linking may preserve its integrity and thus reveal its presence in intact cells. We therefore labeled cells with ^{125}I TGF-β1 and treated them with the chemical cross-linker DSS. The DSS-treated cells were lysed and the lysate was immunoprecipitated with the anti-TβRI antibody. A smear from 80 to 200 kDa appears as shown in Fig. 4F, lane 2. SDS–PAGE and autoradiography of control samples not subjected to DSS treatment revealed that anti-TβRI antibody brings down only the ^{125}I TGF-β1 bound TβRII protein, which lies within the molecular mass range of 80–90 kDa (Fig. 4F, lane 1). With the DSS pretreatment, however, the same immunoprecipitation procedure recovers many additional smear proteins. The majority of the proteins migrate at the top of the gel, suggesting their association in a large complex. Therefore, we did not use cross-linker agent for further experiments using HRA and SKOV-3 cells.

Some alternative confirmatory experiment would be needed to directly resolve the fact that bik disrupts heterodimerization of the TβRs on the cell surface. We have repeated the experiments on another cell type where visualization of the cell surface receptors is easier to detect. Such a cell type should also be responsive to bik and it may not be an ovarian cell. Our preliminary study demonstrated that ^{125}I labeled TGF-β1 binds to TβRII as well as to TβRI in the human skin fibroblast cells. Interestingly, immunoprecipitations using cross-linker DSS demonstrate the existence of TβRI–TβRII complexes. In

the alternative experiments, therefore, we used the human skin fibroblast cells to easily visualize the cell surface receptors. For double immunoprecipitation experiments, affinity-labeled fibroblasts pretreated with or without bik (1 μM) were immunoprecipitated with antibodies against the TβRI or TβRII (Fig. 5). Immunoprecipitations demonstrate the existence of TβRI–TβRII complexes (lanes 1 and 2) in the absence of bik. In cells treated with bik, however, TβRI and TβRII do not co-immunoprecipitate, regardless of whether anti-type I or anti-type II receptor antibodies are used. No labeled TβRI could be detected by such co-immunoprecipitation experiments (lanes 4 and 5). It is of note that Western blot analysis revealed no change in the total levels of TβRI and TβRII proteins (data not shown).

3.5. *Exogenously expressed bik inhibits association of TβRI–TβRII induced by TGF-β1*

Cells stably transfected with either bik or luciferase were affinity-labeled with ^{125}I TGF-β1, for 2 h at 4 °C, washed, and the cell lysates were then immunoprecipitated with antibodies against the two TGF-β receptors (Fig. 6). In the parental HRA cells (lanes 1 and 2) or in the cells transfected with luciferase (lanes 5 and 6), immunoprecipitation with antibodies against TβRI (Fig. 6A) or TβRII (Fig. 6B) demonstrates co-immunoprecipitation of the two receptors, suggesting the presence of TβRI–TβRII complexes. Using anti-TβRI antibody, ^{125}I TGF-β1-bound TβRII could be detected. In cells expressing bik (lanes 3 and 4), however, TβRI and TβRII do not co-immunoprecipitate. There was ~90% reduction in the TβRI–TβRII complex formation in bik-expressing cells when compared with the parental HRA cells. On the other hand, using anti-TβRII antibody (Fig. 6B), ^{125}I TGF-β1-bound TβRII could be detected either in the presence or absence of bik. Bikunin did not seem to affect ^{125}I TGF-β1 binding to TβRII. As shown in Fig. 6A, lanes 7 and 8, TGF-1 did induce the TβRI–TβRII complex formation in the SKOV-3 cells. We also confirmed that bik inhibits association of TβRI–TβRII induced by TGF-β1 in SKOV-3 cells (lanes 9 and 10). The above results show that there is a significant decrease in the TβRI–TβRII complex formation in bik-expressing cells. This



IP	1	2	3	4	5	6
Anti-TβRI	+	-	-	+	-	-
Anti-TβRII	-	+	-	-	+	-
NI-IgG	-	-	+	-	-	+
bikunin	-	-	-	+	+	+

Fig. 5. Bikunin prevents TβRI/TβRII association in human skin fibroblast cells. Cells were affinity-labeled with ^{125}I TGF-β1 (200 pM) for 2 h and cross-linked as described previously (19). Cell lysates were then prepared and immunoprecipitated with antibodies against TβRI (lanes 1 and 4), TβRII (lanes 2 and 5), or non-immune IgG (lanes 3 and 6). Immunoprecipitants were subjected to SDS–PAGE and autoradiography. TβRI–TRII complexes are only observed when cells were not treated with bik. Lanes 3 and 6 represent the negative control. The data shown are representatives of results obtained in three independent experiments.

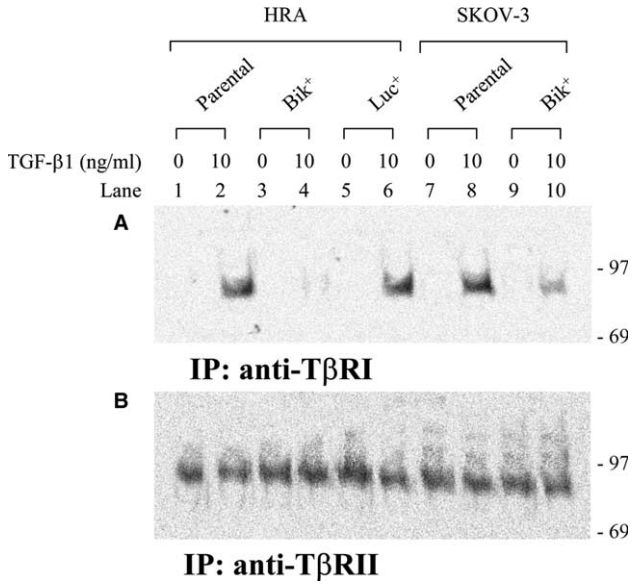


Fig. 6. Bikunin expression prevents TβRI/TβRII association in HRA and SKOV-3 cells. Pools of HRA and SKOV-3 cells stably expressing bik or luciferase were affinity-labeled with ^{125}I TGF-β1 (10 ng/ml) for 2 h at 4 °C. Cell lysates were then prepared and immunoprecipitated with antibodies against TβRI (A) or TβRII (B). Immunoprecipitates were subjected to SDS–PAGE and autoradiography. In both cells, TβRI–TβRII complexes are formed in response to TGF-β1. The complexes are only observed in parental cells and cells expressing luciferase. Bikunin gene transfection abrogates TGF-β1-dependent TβRI–TβRII association. The positions of molecular mass markers (in kDa) are indicated on the right margin. The data shown are representatives of results obtained in three independent experiments.

provides a putative mechanistic explanation for the observed decrease in phosphorylation of Smad2 and nuclear translocation of Smad3 (Fig. 3) and inhibition of TGF-β signaling in these cells (Figs. 1 and 2). Deglycosylated bik lacks the inhibitory effect on TGF-1 signaling (data not shown). These data demonstrate that the high molecular weight chondroitin-4-sulfate modifications of bik render it an inhibitory molecule in TGF-β1 signaling. Immunoprecipitation with antibody against bik demonstrated that there is no co-immunoprecipitation of TβRI or TβRII and bik (data not shown). This suggests that bik does not physiologically interact with TβRI or TβRII.

4. Discussion

We describe for the first time a novel physiological effect of the Kunitz-type protease inhibitor bik against the normal function of the TGF-β pathway in human ovarian cancer HRA and SKOV-3 cells as well as in human skin fibroblasts. We explore the physiological effect first by focusing on the regulation of PAI-1 and collagen synthesis, two well-established physiological responses of many cell types to TGF-β. Then, we aim at exploring and understanding the mechanism by which bik might elicit its cellular effects against the TGF-β pathway. We present two possibilities: (1) bik might directly interfere with the TGF-β pathway. As bik resides extracellularly, it would make sense to explore mechanisms on the cell surface that possibly interferes with proper TGF-β receptor assembly and activation. (2) bik might initiate a signaling

pathway via its elusive bik receptor or alternatively via CD44, whose outcome, intracellularly would be antithetic to normal TGF-β signal propagation.

We previously found that bik inhibits CD44 signaling by preventing coupling CD44 molecules and that it forms membrane complexes with binding protein/receptor and initiates modulation of signal transduction, which results in bik-mediated suppression of cell invasiveness [2–4,7,8,10]. We have clearly demonstrated in the recent studies [10] that exogenous bik forms aggregates (LP–bik–bik-R complex) on the cell surface, that bik can disrupt complex formation of CD44 possibly through coupling between CD44 and bik-R on the plasma membrane (we showed that deglycosylated bik does not have the same effect as the native bik), and that bik-R requires bik for an association with CD44 molecules. This fact suggests that coupling (heterodimer formation) of CD44 proteins and bik-R is important for bik-mediated suppression of signaling. However, a major problem that still remains is that bik-R has not yet been identified [10]. Connection between complex formation of TGF-β receptor proteins and PAI-1 expression is promising [22]. One may estimate that disruption of the CD44-related oligomers with bik may be involved in simultaneous decrease in the TβRI and TβRII coupling.

The present study strongly demonstrated that bik inhibits rather dramatically the formation of TβRI and TβRII complexes, thus explaining the inhibitory effects on signal transduction by this polypeptide factor. In the ovarian cancer cells, however, one cannot directly visualize the TβRI–TβRII complex protein band. We are willing to present the validity of the co-immunoprecipitation data on another cell type where visualization of the cell surface receptors is easier to detect. To better understand the function of bik, human skin fibroblast cells were used for further coimmunoprecipitation experiments. There is increasing evidence [23] that these cells express TβRI and TβRII and TGF-β modulates certain biological functions. We clearly showed that bik disrupts heterodimerization of the TβRs on the fibroblast cell surface.

To more strengthen the evidence for a role of bik in disrupting surface complexes between TGF-β1 and its two signaling receptors, we need further experiments using transient transfection of a constitutively active mutant TGF-β receptor. We have been examining whether bik has an effect on a transfected mutant TβRI that is constitutively active and thus bypasses the need of TGF-1 or TβRII. If the proposed model is correct, bik should have no measurable effect on such a constitutively active mutant receptor.

Although the evidence for disruption of TGF-β receptor complexes by bik is strong, it does not preclude the possibility that bik could also signal via interaction with its own receptor or CD44 in a manner that blocks TGF-β receptor signaling. We have been examining the expression of Smad7 in cells treated with bik or in the stable Bik^+ clone, since Smad7 has been shown to inhibit TGF-β signaling. cDNA microarray analysis was performed on RNA from HRA cells incubated in the presence or absence of TGF-β1 and bik for 24 and 48 h. Bikunin opposed TGF-β1-regulated genes related to fibrosis (PAI-1, collagen, and fibronectin), cell growth (PDGF, FGF, and IGF), and inflammation (COX2 and TNF superfamily member) (data not shown). These analyses also revealed that exogenously added bik or bik gene transfection does not activate Smad7 transcription in unstimulated or TGF-β1-stimulated HRA cells [24]. Furthermore, immunofluorescence analysis revealed that the inhibitory Smad, Smad7, was not exported to the cytoplasm from the nucleus by the treatment with bik (data not shown). Therefore, it is unlikely that bik could mediate signals by direct regulation of an inhibitory Smad like Smad7. Bikunin inhibits TGF-β signal transduction by a mechanism rather than through the regulation of Smad7. We cannot exclude the possibility, however, that bik regulates a protein(s) that interacts with the cytoplasmic domain of the TGF-β receptors or other downstream targets of these receptors, which regulates TGF-β signaling.

We have also clearly demonstrated that bik chondroitin-4-sulfate chains account for its inhibitory activity in cells because deglycosylated bik did not affect TGF-β1 signaling. Correspondingly, deglycosylated bik had no effect on TβRI/TβRII association in co-immunoprecipitation assays. Similar to this function, we have previously observed the inhibition of CD44 signaling in cells treated with bik, but not bik lacking its chondroitin-4-sulfate side chain. Bikunin lacking chondroitin-4-sulfate cannot disrupt the clustering of CD44 proteins, indicating that the chondroitin-4-sulfate side chain of bik is important to display its function [10]. Bikunin has two glycosaminoglycan sites that have been shown to be specific for either O-glycoside (chondroitin sulfate chain) or N-glycoside side chain [25]. We have determined that chondroitin sulfate chain is responsible for the effects we see [25]. We are currently investigating the structural basis for the glycosaminoglycan effect and the relative contributions of chondroitin sulfate chain to bik function.

Besides TGF-β type I and type II receptors, TGF-β has two known co-receptors, betaglycan and endoglin, which are transmembrane glycoproteins with large extracellular regions that bind TGF-β and small cytoplasmic regions without any clearly identifiable signaling motif [26]. Betaglycan is a proteoglycan and is a membrane proteoglycan containing heparan and chondroitin sulfate chains, whose core protein binds all three TGF-β isoforms [27]. Betaglycan is capable of fine tuning the availability of TGF-β to the signaling receptors, thereby determining the outcome of the TGF-β stimulation [28]. In a model that involves TGF-β receptors and CD44, the data at this stage allow an alternative interpretation or model: it may be possible that bik blocks access of betaglycan or endoglin to the signaling receptors. Our preliminary data revealed that by cDNA microarray analysis, HRA cells constitutively express betaglycan [24]. However, binding of bik to the HRA cells was not inhibited by neutralizing antibodies to betaglycan (unpublished data). It is thus unlikely that the role of bik might be needed for complex formation with molecules such as betaglycan.

How general is the observed effect of bik in terms of its cell type specificity? The same inhibitory effect against TGF-β can be observed by addition of exogenous bik on other cell lines, including HCS-2/8 human chondrosarcoma cells (data not shown). These cells also expressed bik-R and CD44 proteins. Thus, bik may be effective in cells expressing these two bik binding proteins.

Nothing is known about the molecular mechanism by which direct bik coupling to TGF-β receptor proteins takes place. Notwithstanding these limitations, this is the first report demonstrating the suppression of TGF-β1-induced expression of PAI-1 mRNA and protein by bik possibly through inhibition of complex formation of TβRI and TβRII proteins on the plasma membrane of tumor cells.

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