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Bikunin Down-Regulates Heterodimerization Between CD44 and Growth Factor Receptors and Subsequently Suppresses Agonist-Mediated Signaling

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Abstract We provided evidence previously that bikunin, a Kunitz-type protease inhibitor, can disrupt dimerization of CD44 proteins, which may result in suppression of receptor-mediated MAP kinase signaling. However, to what extent dimerization may alter ligand-induced signaling has not been documented. Given the recent recognition that some growth factor receptors can form heterodimers with CD44, the present study was undertaken to determine whether the CD44 and growth factor receptors (e.g., EGFR, FGFR, HGFR, VEGFR, TGF-βRI, or TGF-βRII) can form heterodimers in cancer cells and, if so, to investigate the potential functional consequences of such heterodimerization. We also examined whether bikunin can abrogate these heterodimerizations and inhibit CD44/growth factor-dependent signaling. Here, we show direct evidence for heterodimerization of CD44–FGFR and CD44-TGF-βRI in human chondrosarcoma HCS-2/8 cells, CD44–EGFR complex in human glioma U87MG cells, and CD44–TGF-βRI heterodimer in human ovarian cancer HRA cells. Coupling of CD44 and growth factor receptor may be selective, depending on a cell type. Bikunin does not alter the ligand binding, whereas functionally reduces heterodimerization between CD44 and growth factor receptors. The disruption of heterodimerization substantially reduces receptor-induced tyrosine phosphorylation and ERK1/2 activation. Taken together, our data suggest that bikunin-mediated suppression of heterodimerization between CD44 and growth factors may inhibit the agonist-promoted activation of the signaling pathway. J. Cell. Biochem. 94: 995–1009, 2005.

Key words: bikunin; CD44; growth factor receptors; dimer formation; signal transduction; urokinase

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; HA, hyaluronan; HGFR, hepatocyte growth factor receptor; LP, link protein; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; TGF-β, transforming growth factor-beta; TGF-βRI, TGF-β type I receptor; TGF-βRII, TGF-β type II receptor; uPA, urokinase-type plasminogen activator; VEGFR, vascular endothelial cell growth factor receptor.

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We have shown that bikunin, a Kunitz-type protease inhibitor, is proposed as a main participant in inhibition of tumor cell invasion and metastasis possibly through both direct inhibition of cell-associated plasmin activity [Kobayashi et al., 1994a,b,c; Kobayashi et al., 1995a,b,d] and suppression of uPA expression [Kobayashi et al., 1995c; Kobayashi et al., 1996a,b; Kobayashi et al., 1998a,b; Sugino et al., 1998]. We have been studying the function and mechanism of bikunin on suppression of the invasive capacity of tumor cells. Our previous publications indicated that binding of bikunin to its binding sites on the cell surface has been implicated in inhibition of protein kinase C translocation and activation [Kobayashi et al., 1998b; Kobayashi et al., 2001]. More recently we reported that bikunin markedly suppresses cancer cell invasion possibly through negative regulation of mitogen-activated protein kinase (MAPK)-dependent mechanisms and subsequently suppression of uPA expression [Kobayashi et al., 1996a; Kobayashi et al., 2000a; Hirashima et al., 2001; Kobayashi et al., 2001]. During the search for novel proteins interacting with bikunin, we identified at least two types of cell-associated binding protein; the 40 kDa link protein (LP), one of the hyaluronan (HA)-binding proteins, as a cell-associated bikunin-binding protein, and a 45 kDa specific receptor for bikunin (bikunin-R), which is a membrane-associated unidentified molecule, as a putative bikunin receptor (bikunin-R) [Kobayashi et al., 1994d; Kobayashi et al., 1998c; Kobayashi et al., 2000b,c; Hirashima et al., 2001]. We postulate that LP may function in capturing bikunin for presentation to the signaling receptor, bikunin-R. Since LP is apparently held at the cell surface by HA [Kohda et al., 1996], it is reasonable to think that CD44 would be involved in this bikunin-mediated complex at the cell surface.

CD44 is the major cell-surface receptor for HA [Aruffo et al., 1990] with a postulated role in matrix adhesion [Carter and Wayner, 1998] and tumor invasion [Knudson et al., 1984; Pauli and Knudson, 1988] and metastasis [Guo et al., 1994]. CD44 couples with tyrosine kinases (e.g., c-Src kinase and $p185^{HER2}$ kinase) [Bourguignon et al., 1997], serine/threonine kinesis (e.g., protein kinase C and Rho-binding kinase) [Kalomiris and Bourguignon, 1989; Bourguignon et al., 1999] and ankyrin/ezrin-radixin-moesin (ERM) [Bourguignon et al., 1992]. Activation-induced clustering followed by homodimerization and oligomerization of CD44 represents a ligand-mediated signal transduction mechanism [Underhill et al., 1983]. It is likely that bikunin-R is a candidate for functional receptor for bikunin and may be an accessory receptor for CD44 proteins [Suzuki et al., 2002]. Engagement of bikunin-R by its ligand bikunin and subsequent coupling of bikunin-R to CD44 may facilitate inhibition of agonist stimulation by suppression of CD44 activation (that is CD44 clustering and homodimerization), which finally leads to reduction of CD44-mediated up-regulation of uPA expression [Suzuki et al., 2002].

Oligomerization of CD44 may play important roles in receptor trafficking and signaling [Liu and Sy, 1997]. In addition to forming homodimers, several receptors have been shown to heterodimerize with CD44 [Sherman et al., 2000; Wobus et al., 2001; Bourguignon et al., 2002; Bertotti and Comoglio, 2003]. In some cases, heterodimerization between CD44 and growth factor receptors was found to be essential for the formation of functional receptors such as TGF-β type I receptor (TGF-βRI) [Bourguignon et al., 2002] or epidermal growth factor receptor (EGFR) [Zhang et al., 1996, 1997; Jo et al., 2000; Wobus et al., 2001, 2002]. These findings support the notion that a strong binding interaction occurs between CD44 and growth factor receptors. Moreover, HA and TGF-β bind to their own specific receptors (e.g., CD44 or TGF-βRI), but their respective downstream signaling pathway(s) appear to be tightly linked [Bourguignon et al., 2002]. Therefore, formation of heterodimers has been found to modulate the signaling of both receptors.

The potential regulatory influences that these heteromeric assemblies may have on the function of receptors co-existing in the same cell led us to investigate whether growth factor receptors that are ubiquitously expressed in cell types could function as heterodimers. Several such widely distributed receptors are the EGFR, fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR) (c-Met), vascular endothelial cell growth factor receptor (VEGFR), TGF-βRI, and TGF-β type II receptor (TGF-βRII), which may be co-expressed with CD44 in cell types. Although we have been clearly documented that bikunin can disrupt homodimerization of CD44 [Suzuki et al., 2002], suppression of heterodimerization and

subsequent inhibition of agonist-induced phosphorylation and signal transduction has not been examined. In the present study, we tried to answer the mechanism by which bikunin efficiently inhibits heteromerization of CD44 and growth factor receptors, which results in suppression of agonist-induced MAPK activation.

MATERIALS AND METHODS

Materials

Bikunin was kindly provided by Mochida Pharmaceutical Co. Ltd. (Gotenba, Shizuoka, Japan). Human recombinant TGF-β1 and EGF were purchased from R & D systems, Inc., Minneapolis, MN. The radioligand [^{125}I]TGF-β1 was prepared as described [Frolik et al., 1984]. Unless otherwise stated, all chemicals were of reagent grade or higher and were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Antibodies

Monoclonal antibodies (MAbs) against CD44 molecules (anti-CD44 mAb and anti-CD44v9 mAb) were obtained from Seikagaku Kogyo Co. Ltd., Tokyo. Anti-CD44 mAb recognizes the epitope involved in HA binding and anti-CD44v9 mAb recognizes the CD44v isoforms containing epitope v9 alone. Therefore, anti-CD44 mAb, but not anti-CD44v9 mAb, inhibits HA binding to the CD44 proteins on their cell surface. Of note that anti-CD44 mAb reacts with both standard form of CD44 (CD44s) and all types of CD44 variant isoforms. Monoclonal antibodies raised against CD44v isoforms containing epitope v3, v4, v5, and v6 were obtained from Novocastra Laboratories Ltd. (Benton Lane, Newcastle, UK). Rabbit anti-human CD44 polyclonal antibody (pAb) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The other antibodies used were: anti-phosphotyrosine mouse monoclonal antibody 4G10, anti-human EGFR antibody (06–129) and neutralizing anti-EGFR antibody (05–101) (Upstate Biotechnology, Inc., Lake Placid, NY); rabbit anti-TGF-βRI IgG (specific for the ALK-5 form of TGF-βRI p55), rabbit anti-TGF-βRII IgG (specific for TGF-βRII p70), neutralizing anti-TGF-βRII antibody, anti-c-Met antibody (h-met, sc-161), and polyclonal rabbit anti-FGFR-1 C terminus (Santa Cruz Biotechnology); mouse monoclonal phospho-specific anti-ERK1/2 antibody clone E10 (New England Biolabs, Beverly, MA); anti-rabbit antibody conjugated with horseradish peroxidase and anti-mouse antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Tokyo, Japan); anti-VEGFR was from CosmoBio Co., Tokyo, Japan.

Cell Culture

Human ovarian cancer HRA cells [Kobayashi et al., 2000; Suzuki et al., 2003], human glioblastoma U87MG cells (American Type Culture Collection, Tokyo, Japan) and human chondrosarcoma HCS-2/8 cells [Takigawa et al., 1997; Kobayashi et al., 2000] were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere with constant humidity [Kobayashi et al., 1996; Kobayashi et al., 2000]. Cell lines were cultured with serum-containing DMEM, then subcultured overnight on serum-free DMEM. We have used the specific growth factor-depleted serum when treating with each growth factor. For example, preincubation of TGF-β-depleted serum was used to test this growth factor specificity. To prepare TGF-β-depleted serum, serum was incubated with anti-TGF-β antibody and protein G-Sepharose at 4°C for 4 h. After a brief centrifugation, a supernatant was recovered and used as TGF-β-depleted serum.

In some experiments for flow cytometric analysis, at the end of the incubation, cells were then dissociated with 0.25% trypsin and 0.05M EDTA solution supplemented with *Streptomyces* hyaluronidase (10 μg/ml). Cells were used for measurement of CD44s and CD44v isoforms by flow cytometry, immunoblotting, and immunoprecipitation [Liu and Sy, 1996].

Flow Cytometric Analysis

Since anti-CD44 mAb does not effectively recognize HA-bound CD44 and the amount of HA present around the cells can greatly influence the binding of this antibody, cells were pretreated with hyaluronidase and used for further experiments. A single cell suspension (10^6/ml) was incubated with affinity purified antibodies or isotope 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 second antibody (Dako, Copenhagen, Denmark) were added for 1 h on ice, respectively. Cells were analyzed in a FACScan (Beckton Dickinson, Tokyo, Japan). At least 10,000 cells were analyzed per sample in all experiments. All experiments were performed at least twice.

Membrane Preparation

Cells were washed twice with ice-cold PBS. They were then disrupted by homogenization with a Polytron homogenizer in 10 ml of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor. Lysates were centrifuged at 500*g* for 5 min at 4°C to remove nuclei and unbroken cells. The supernatant was then centrifuged at 45,000*g* for 20 min, and the pellet was washed twice in the same buffer. Membrane preparations were used immediately for ligand binding assays.

Immunoblotting and Immunoprecipitation Techniques

Cells were solubilized in buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1.0% Nonidet P-40, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin) or buffer A containing 0.5% SDS. Note that CD44-growth factor receptor heterodimers were not dissociated under the buffer A condition, whereas 0.5% SDS abrogated CD44-growth factor receptor heterodimers in the cell lines used in this study. Quantitation of protein was performed using the bicinchoninic acid protein assay kit (Sigma). For immunoprecipitation of the total cell lysates, only protein G-Sepharose was added. Protein G-Sepharose-antibody-antigen complexes were then collected by centrifugation at 15,000*g*. Cell lysates were incubated overnight at 4°C with additional antibodies (1:200 dilution) before the addition of protein G-Sepharose for 3 h. The immunoprecipitates were washed four times with cold buffer A and resuspended in sample buffer. The sample was then centrifuged at 15,000*g* for 15 min, and the supernatant was analyzed by SDS–PAGE in a polyacrylamide gel. Separated polypeptides were then transferred onto PVDF filters. After blocking nonspecific sites with 2% BSA, the polyvinylidene difluoride (PVDF) filters were incubated with each of the specific antibodies (0.5–1 μg/ml) followed by incubating with horseradish peroxidase-labeled antibodies The blots were then developed by the ECL system (Amersham Bioscience). Filters were quantitated by scanning densitometry using a Bio-Rad model 620Video Densitometer with one dimensional Analyst software package for Macintosh.

In some experiments, cells were solubilized by buffer A and immunoprecipitated with mouse (or rabbit) anti-CD44 IgG followed by immunoblotting with rabbit (or mouse) anti-CD44 IgG or rabbit anti-growth factor receptor IgG.

Furthermore, cells treated with various reagents (e.g., HA (50 μg/ml; Sigma) or TGF-β1 (10 ng/ml) or pre-treated with anti- TGF-β antibody (10 μg/ml) or bikunin (1 μM) followed by HA (50 μg/ml) or ligand (10 ng/ml) treatment or without any treatment) were solubilized by buffer A and immunoprecipitated with mouse (or rabbit) anti-CD44 antibody or rabbit anti-TGF-βRI, respectively. The immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with anti-phosphothreonine antibody, 4G10.

In some experiments, buffer A-solubilized cell lysates (isolated from cells treated with or without TGF-β1 (10 ng/ml) in the presence or absence of bikunin (1 μM)) were analyzed by SDS–PAGE followed by immunoblotting with anti-phospho-ERK1/2 or anti-total ERK1/2 antibody, respectively.

TGF-β1 Binding Assay and Co-Immunoprecipitation

The ligand binding of [^{125}I]TGF-β1 to HRA cells was performed according to previously published protocols [Beall and Pearce, 2001]. Cells were treated with 10 ng/ml [^{125}I]TGF-β1 for 3 h at 4°C, washed extensively, and then treated with 0.5 mg/ml of disuccinimidyl suberate (Pierce, Rockford, IL) for 30 min at 4°C to cross-link the ligand to the receptors. Cell lysates were subjected to immunoprecipitation with anti-TGF-βRII antibody. Phosphorimaging analysis of all dried gels was performed using the Macintosh Image System.

Statistical Analysis

The data presented are the mean of triplicate determinations in one representative experiment unless stated otherwise. Data are presented as mean ± SD. All statistical analysis was 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.

RESULTS

Expression of CD44 and Growth Factor Receptors on Tumor Cells

Binding of specific antibodies directed against CD44 and different CD44 variant isoforms and growth factor receptors was initially assessed by flow cytometry using human ovarian cancer HRA cells, human glioma U87MG cells, and human chondrosarcoma HCS-2/8 cells (data not shown). Flow cytometric analysis was performed using anti-CD44 mAb that binds to a HA-binding epitope common to all CD44 proteins as well as five anti-CD44v mAbs that selectively recognizes CD44v isoforms containing epitope v3, v4, v5, v6, or v9 on cells. Nonimmune mouse IgG was used as the control IgG. The results demonstrated that these cells express CD44 molecules: HRA cells are positive for a standard form of CD44 and CD44v isoforms containing epitope v3 and v6, HCS-2/8 cells produce CD44 and CD44v isoforms containing epitope v9 [Suzuki et al., 2002], and U87MG cells are positive for CD44 but negative for CD44v isoforms containing epitope v3, v4, v5, v6, and v9. In a parallel experiment, we show that HRA cells expressed TGF-βRI, TGF-βRII, and EGFR; U87MG cells expressed EGFR; and HCS-2/8 cells expressed TGF-βRI, TGF-βRII, and FGFR (data not shown).

In order to further identify the presence of CD44 and growth factor receptors in each cell line, we immunoblotted cell lysates with specific antibodies against CD44 and CD44v isoforms as well as each growth factor receptor (Fig. 1). Here, we show that a single band of the CD44 protein is expressed in U87MG cells which correspond to the 90 kDa CD44 polypeptide (lane 2). HRA cells are positive for CD44 (85 kDa; lane 2), CD44v3 (140 kDa; lane 3) and v6 (140 kDa; lane 6), HCS-2/8 cells are positive for CD44 (85 kDa; lane 2) and CD44v9 (100 kDa; lane 7). No CD44-containing immunoreactive material is observed in control samples when normal mouse IgG or pre-immune rabbit IgG is used in these experiments (lane 1). In addition, HRA cells predominantly expressed TGF-βRI (55 kDa; lane 12), TGF-βRII (70 kDa; lane 13) and EGFR (170 kDa; lane 8); U87MG cells expressed EGFR (170 kDa; lane 8); and HCS-2/8 cells expressed TGF-βRI (55 kDa; lane 12), TGF-βRII (70 kDa; lane 13) and FGFR (140 kDa; lane 9), respectively. Thus, Western blot data are consistent with those from flow cytometry.

We next assessed whether bikunin can influence expression of CD44 and growth factor receptors on their cell surface by flow cytometry (data not shown). Bikunin did not inhibit the expression of CD44 and CD44v isoforms in these cells. Cells pretreated with bikunin were stimulated with respective agonists: HCS-2/8 cells were incubated with 100 nM PMA, U87MG cells were incubated with EGF (10 ng/ml), and HRA cells were incubated with TGF-β1 (10 ng/ml), respectively. Bikunin did not inhibit the expression of new CD44 proteins. In addition, it failed to modulate expression of growth factor receptors, irrespective of whether cell lines were stimulated with either ligand or agonist (data not shown).

Characterization of Heterodimerization of CD44 and Growth Factor Receptors by Co-Immunoprecipitation

We examine the direct physical association between CD44 and growth factor receptors in HRA cells (Fig. 2A,B), U87MG cells (Fig. 2C), and HCS-2/8 cells (Fig. 2D). In a first attempt to determine whether CD44 could form a heterodimer with the growth factor receptors (e.g., EGFR, FGFR, TGF-βRI, or TGF-βRII), a co-immunoprecipitation experiment between CD44 and each growth factor receptor was designed. The ability of the CD44 to form heterodimers with the growth factor receptors was initially assessed in HRA cells co-expressing the CD44, CD44v3, CD44v6, TGF-βRI, TGF-βRII, and EGFR. In a parallel experiment, we also examine whether bikunin affects the heterodimer formation. HRA cells pretreated without (Fig. 2A, left panel) or with bikunin (Fig. 2B, right panel) (1 μM, 60 min) were incubated with TGF-β1 (10 ng/ml). After cell lysates from HRA cells were prepared with buffer A and immunoprecipitated using the mouse or rabbit anti-CD44 antibody, the presence of CD44-growth factor receptor heterodimer complex in the immunoprecipitates was probed using a rabbit or mouse anti-CD44 antibody and antibodies to EGFR, FGFR, HGFR, VEGFR, TGF-βRI, or TGF-βRII. As shown in Figure 2A, left panel, several major species corresponding to monomeric forms of CD44 (85 kDa; lane 1), CD44v3 (140 kDa; lane 2), and CD44v6 (140 kDa; lane 3),

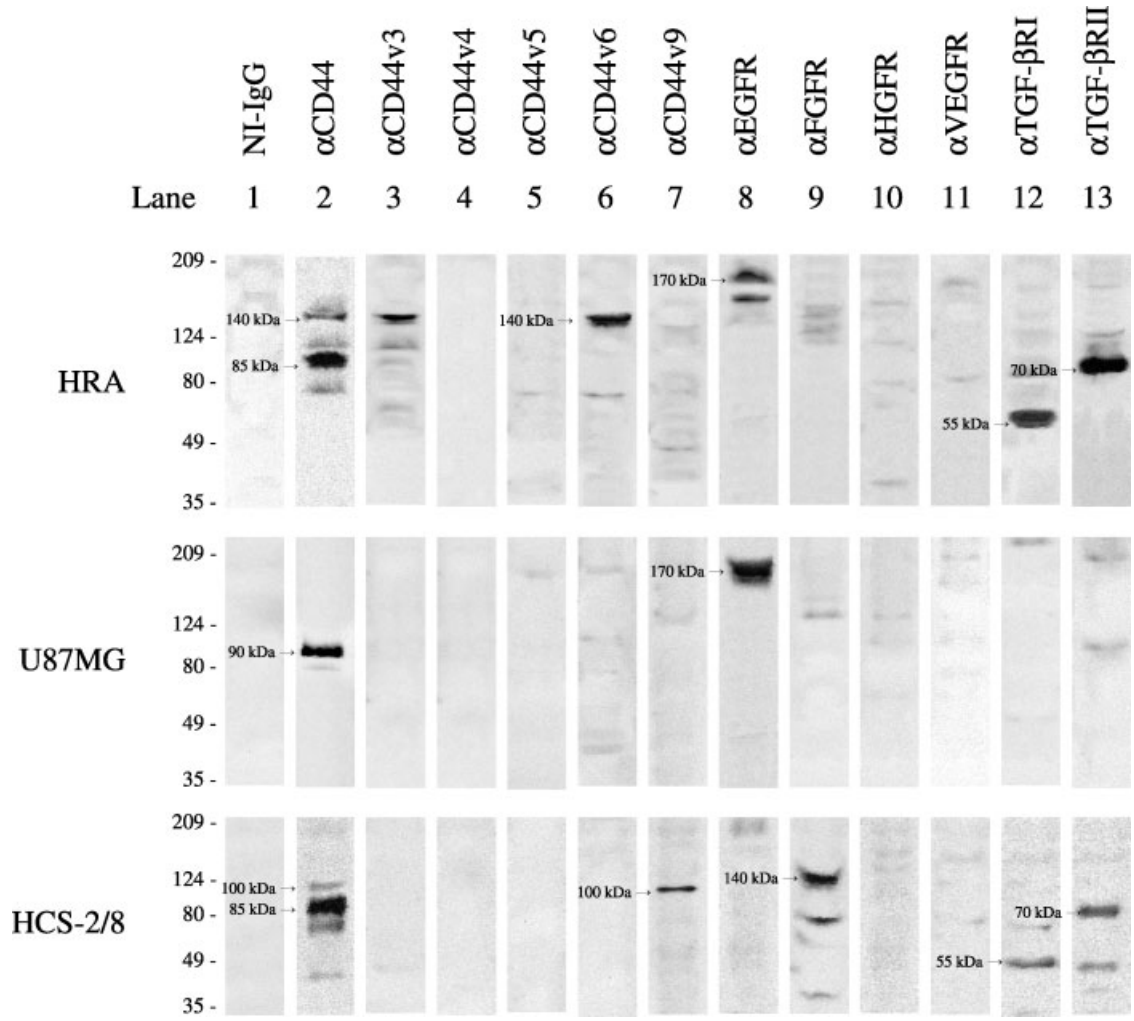

Fig. 1. Expression of CD44 and growth factor receptors on HRA, U87MG, and HCS-2/8 cells by Western blot. Western blot analysis was performed to confirm expression of CD44 and growth factor receptors. A representative result of two independent experiments is shown. The positions of molecular mass markers are indicated on the left (in kDa).

TGF-βRI (55 kDa; lane 4), and EGFR (170 kDa; lane 6) were detected. However, specific immunoreactivities for FGFR, HGFR, VEGFR (data not shown), and TGF-βRII (lane 5) were not detected at the HRA cell lysates. In a parallel experiment, we examined whether bikunin can modulate heterodimerization of CD44 and growth factor receptors (Fig. 2A, right panel). Interestingly, exogenous bikunin abrogated heterodimerization of CD44 and growth factor receptors (e.g., TGF-βRI and ERGF). Note that bikunin did not suppress expression of CD44 and growth factor receptor proteins.

To test the stability of CD44-TGF-βRI heterodimers in HRA cells, the cells were lysed in buffer A containing 0.5% SDS, immunoprecipitated using anti-CD44 antibody, and detected with anti-TGF-βRI or anti-EGFR antibody. As shown in Figure 2B, it was apparent that cell lysis in buffer A containing 0.5% SDS in the absence of cross-linking agents leads to nearly complete dissociation of CD44-TGF-βRI (lane 4) and CD44-EGFR heterodimers (lane 5). Thus, immunoreactive bands for TGF-βRI and EGFR were not detectable under the SDS-containing condition. Taken together, these results allow us to conclude that the CD44 and co-expressing growth factor receptors can form the SDS-sensitive heterodimers and indicate that stable and selective intermolecular interactions can also occur between the two receptors as well.

In a second experiment, specific immunoreactivities for monomeric form of CD44 (90 kDa) and EGFR (170 kDa) was detected at the cell

extract of U87MG cells pretreated with EGF (10 ng/ml), when the cell lysates were immunoprecipitated with anti-CD44 antibody (Fig. 2C, left panel). In a third experiment, specific immunoreactive bands of FGFR (lane 3) and TGF-βRI (lane 4) was detected at the cell extract of PMA (100 nM)-stimulated HCS-2/8 cells (Fig. 2D, left panel). These results confirm the selectivity of the immunoprecipitation approach and are possibly due to the differences of co-expressing CD44 and growth factor receptors.

We also examined whether bikunin can modulate heterodimerization of CD44 and growth factor receptors in the stimulated U87MG (Fig. 2C, right panel) and HCS-2/8 cells (Fig. 2D, right panel). Similar to HRA cells, exogenous bikunin could abrogate heterodimerization of CD44–EGFR complex in U87MG cells as well as CD44–FGFR complex and CD44–TGF-βRI complex in HCS-2/8 cells. Note that dimerization of CD44 and CD44 variant isoforms is SDS-resistant [Suzuki et al., 2002].

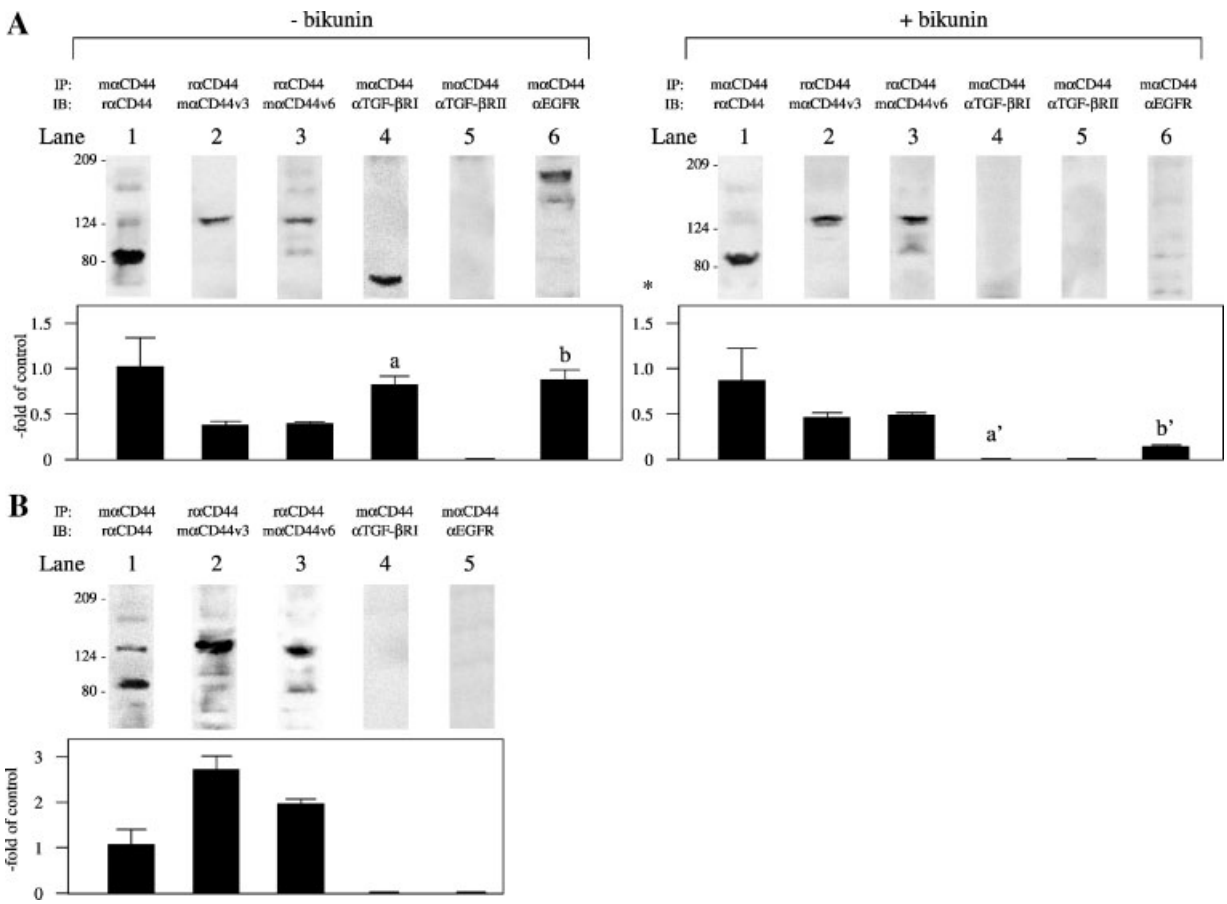

Fig. 2. Immunodetection and characterization of CD44-growth factor receptor heterodimers by co-immunoprecipitation. **A** and **B**: HRA cells co-expressing CD44, CD44v3, CD44v6, TGF-βRI, TGF-βRII, and EGFR pretreated without (**left panel**) or with bikunin (**right panel**) (1 μM, 60 min) were lysed in buffer A (A) or buffer A containing 0.5% SDS (B) and subjected to immunoprecipitation using mouse (m) or rabbit (r) anti-CD44 antibody. The coimmunoprecipitates were immunoblotted using antibodies to CD44 (**lane 1**), CD44v3 (**lane 2**), CD44v6 (**lane 3**), TGF-βRI (**lane 4**), TGF-βRII (**lane 5**), and EGFR (**lane 6**), respectively. Note that CD44-growth factor receptor heterodimers were nearly completely dissociated under the condition containing SDS. **C**: EGF (10 ng/ml)-stimulated U87MG cells co-expressing CD44 and EGFR pretreated without (**left panel**) or with bikunin (**right panel**) (1 μM, 60 min) were lysed in buffer A and subjected to immunoprecipitation using mouse anti-CD44 antibody. The coimmunoprecipitates were immunoblotted using antibodies to CD44 (**lane 1**) and EGFR (**lane 2**), respectively. **D**: PMA (100 nM)-stimulated HCS-2/8 cells co-expressing CD44, CD44v9, FGFR, TGF-βRI, and TGF-βRII pretreated without (**left panel**) or with bikunin (**right panel**) (1 μM, 60 min) were lysed in buffer A and subjected to immunoprecipitation using anti-CD44 antibody. The coimmunoprecipitates were immunoblotted using antibodies to CD44 (**lane 1**), CD44v9 (**lane 2**), FGFR (**lane 3**), and TGF-βRI (**lane 4**), respectively. The positions of molecular mass markers are indicated on the left (in kDa). Two additional experiments gave similar results. a versus a′ and b versus b′, $P < 0.05$.

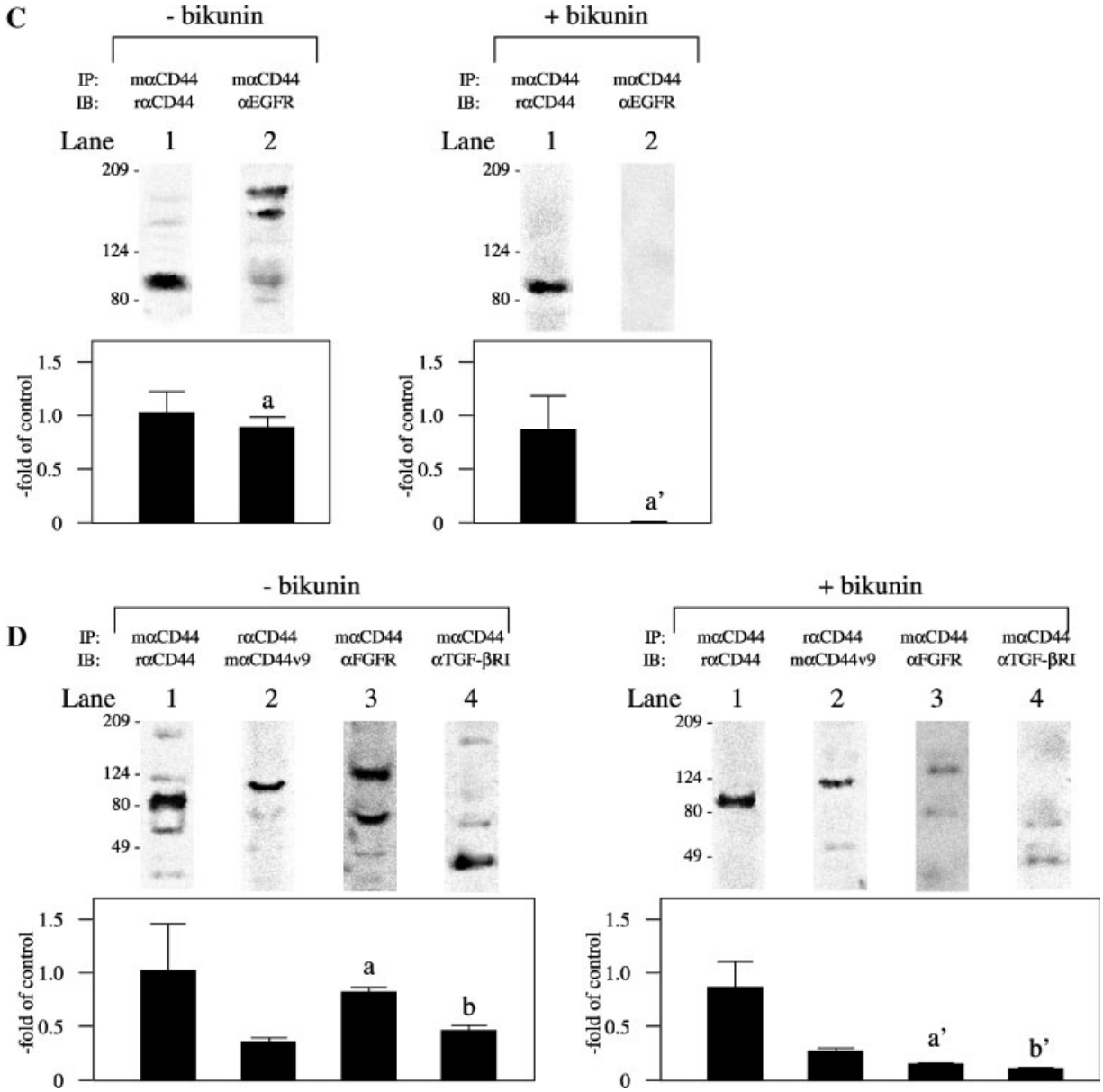

Fig. 2. (*Continued*)

In order to further identify the presence of CD44–growth factor receptor complex formation in HRA cells, we immunoblotted the anti-TGF-βRI antibody-precipitated proteins with a specific mouse mAb against CD44, CD44v3, or CD44v6. We show that two bands of the immunoreactive CD44 proteins (85 and 140 kDa) (Fig. 3, lane 1), a single band of the CD44v3 (140 kDa) (Fig. 3, lane 2), or CD44v6 (140 kDa) (Fig. 3, lane 3) protein are expressed in cells which corresponds to the 85 kDa CD44 or the 140 kDa CD44v3 or CD44v6 polypeptide. Furthermore, the anti-EGFR antibody-precipitated proteins were immunoblotted with anti-CD44 antibodies. We confirmed the physical association of CD44 protein with EGFR (Fig. 3, lanes 7–9). As expected, CD44 does not interact with TGF-βRII (lanes 4–6).

Ligand Binding of the CD44-TGF-βRI Heterodimer

We compared the ligand binding properties of HRA cells pretreated with or without bikunin. Because the ligand binding and TGF-βRII

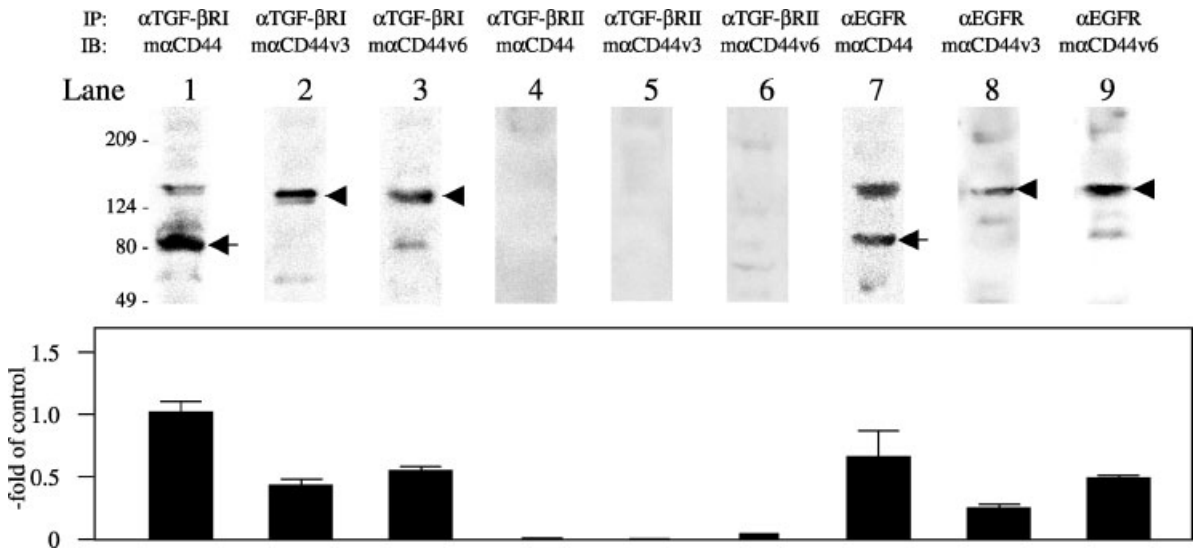

Fig. 3. Immunodetection of co-immunoprecipitated CD44-growth factor receptors. HRA cells were harvested and immunoprecipitations were carried out on cell extracts using anti-TGF-βRI (**lanes 1–3**), anti-TGF-βRII (**lanes 4–6**), or anti-EGFR (**lanes 7–9**) antibody. Immunocomplexes were analyzed by SDS–PAGE. Cell lysates were immunoblotted with a mouse monoclonal anti-CD44, anti-CD44v3, or anti-CD44v6 antibody. Western blots are representative of three independent experiments. Arrow, CD44 molecule; arrowhead, CD44v3 or CD44v6 molecule. IP, immunoprecipitation; IB, immunoblot. a versus a′ and b versus b′, $P < 0.05$.

interaction studies were performed in the presence or absence of bikunin, HRA cells were tested for its ability to bind TGF-β1 and form a complex with TGF-βRII in vivo. HRA cells were labeled with [^{125}I]TGF-β1 for 30 min at 37°C. After cross-linking and cell lysis, TGF-βRII was immunoprecipitated from the lysates with the anti-TGF-βRII antibody. As shown in Figure 4, ligand binding assays revealed that HRA cells pretreated with or without bikunin had similar high affinities for TGF-βRII. Thus, bikunin did not alter TGF-β1 binding to the cells.

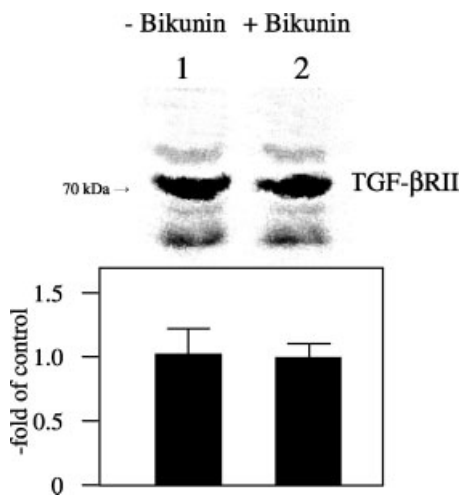

Fig. 4. TGF-β1 binding to TGF-βRII in the presence or absence of bikunin. Radioligand binding studies were carried out as described under ''Materials and Methods'' section. Co-immunoprecipitation of [^{125}I]TGF-β1-bound TGF-βRII in HRA cells pretreated with (**lane 2**) or without (**lane 1**) 1 μM bikunin. HRA cells pretreated with or without bikunin were labeled with [^{125}I]TGF-β1. Paired samples were used for total cell lysate and Western blot analysis. The positions of molecular mass markers are indicated on the left (in kDa). Two additional experiments gave similar results.

Functional Characterization of CD44–Growth Factor Receptor Interactions

We next determined the functional consequences of CD44-growth factor receptor heterodimerization by measuring receptor-mediated stimulation of several effector pathways. It has been established that, in HRA cells, we could detect stimulation of ERK1/2 by TGF-β1 [Hirashima et al., 2003; Kobayashi et al., 2003], and that bikunin significantly suppressed TGF-β1-mediated ERK1/2 phosphorylation [Hirashima et al., 2003; Kobayashi et al., 2003]. The efficacy of bikunin to modulate the MAPKs p38 and phosphoinositide-3-kinase (PI3K) was assessed in the previously published data [Kobayashi et al., 2002].

In an expanded series of experiments, the ability of TGF-β1 to stimulate the ERK1/2 phosphorylation was tested in HCS-2/8 cells (Fig. 5A) and U87MG cells (Fig. 5B) pretreated with or without bikunin. In the absence of

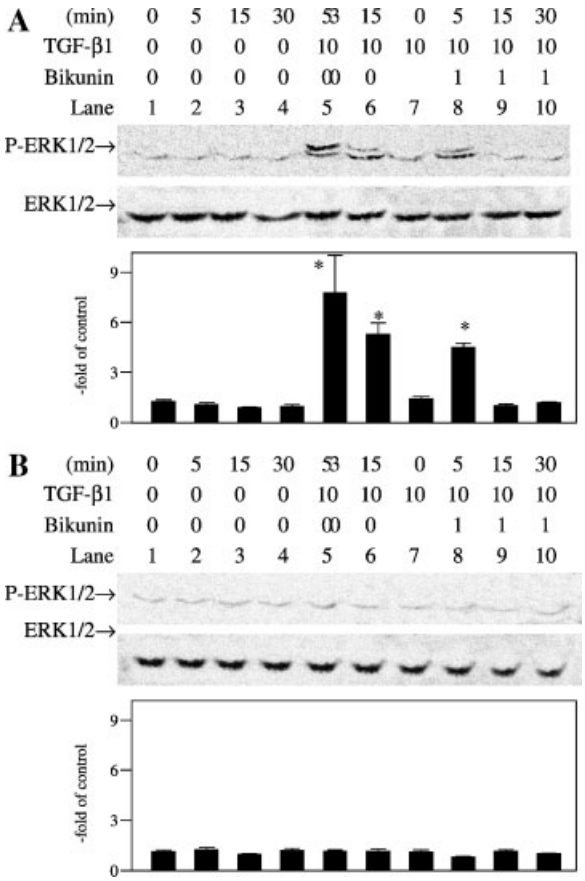

Fig. 5. Suppression by bikunin of TGF-β1 stimulation of ERK1/2 phosphorylation. HCS-2/8 cells expressing FGFR and TGF-βRI/TGF-βRII together (**A**) or U87MG cells expressing EGFR (**B**) were assayed for TGF-β1 (10 ng/ml)-dependent ERK1/2 phosphorylation. ERK1/2 activation is shown in the **upper part** of each panel (as measured by anti-phospho-ERK1/2 antibodies) in response to stimulation by 10 ng/ml TGF-β1 for 0, 5, 15, and 30 min of serum-starved HCS-2/8 cells pretreated without (**lanes 1–7**) or with exogenous bikunin (**lanes 8–10**; 1 μM). As a loading control, the total ERK1/2 pool in the lysate was measured using anti-ERK1/2 antibodies (**lower panel**), and the anti-phospho-ERK data are normalized with respect to the total ERK pool. Fifty micrograms of cell lysate was loaded into each lane. SDS–PAGE was followed by immunoblot, developed with enhanced chemiluminescence. **Lower panel**: Densitometric analysis of total ERK2 and phospho-ERK1/2 levels on Western blots exposed in the linear range was performed. The bar graph was derived from the ratio of p-ERK1/2 and ERK1/2 densitometric measurements for each condition. Experiments were performed twice with similar results. *, $P < 0.05$ versus lane 1.

bikunin, TGF-β1 significantly promoted a dose-dependent increase in ERK1/2 phosphorylation in HCS-2/8 cells (Fig. 5A, lanes 5–7), but not U87MG cells (Fig. 5B, lanes 5–7). EC_{50} and maximal stimulated values were ~2 and 10 ng/ml for TGF-β1, respectively, in HCS-2/8 cells (data not shown). Indeed, agonist stimulation of the TGF-β receptors results in a consistent activation of ERK1/2, which peaked at 5 min and returned to almost basal levels by 30 min. In contrast, bikunin markedly suppressed TGF-β1-dependent phosphorylation of ERK1/2 proteins in HCS-2/8 cells (Fig. 5A, lanes 8–10). Given that the TGF-βRI and TGF-βRII were expressed at equivalent levels in HCS-2/8 cells, these results suggest that abrogation of heterodimerization by bikunin may suppress the TGF-β1-promoted ERK1/2 activation. These results allow us to speculate that suppression of TGF-β1–induced signaling by bikunin may result from abrogation of heterodimerization between CD44 and TGF-β receptors.

Phosphorylation of the CD44-TGF-βRI Heterodimer

To delineate a mechanistic basis for the observed cross-activation of the CD44-TGF-βRI heterodimer complex, we assessed whole cell receptor phosphorylation in response to both HA and TGF-β1. We tested whether HA or TGF-β1 treatment induces CD44 or TGF-βRI phosphorylation in HRA cells as measured by immunoblotting with anti-phosphotyrosine antibody of immunoprecipitated CD44 or TGF-βRI. To determine whether HA or TGF-β1 activates CD44 or TGF-βRI, HRA cells were treated with 50 μg/ml of HA or 10 ng/ml EGF. As depicted in Figure 6A, left panel, lane 2, HA produced a rapid and robust phosphorylation of the TGF-βRI (55 kDa), CD44 (85 kDa), CD44v3 or CD44v6 (140 kDa), EGFR (170 kDa), and some unidentified bands. Similar to HA stimulation, TGF-β1 induced a rapid phosphorylation of the TGF-βRI (55 kDa), CD44 (85 kDa), and CD44v3 or CD44v6 (140 kDa), but not ERGR (170 kDa) (lane 4). As shown in Figure 6A, right panel, lanes 2 and 4, HA significantly increased phosphorylation of the TGF-βRI and vice versa. These results indicated that activation of the TGF-βRI of the CD44-TGF-βRI heterodimer resulted in cross-phosphorylation of CD44 and vice versa. This cross-phosphorylation is not simply due to cross-reactivity of the agonists.

To elucidate the selectivity of the observed CD44-TGF-βRI cross-phosphorylation, we examined agonist-induced phosphorylation of CD44-EGFR heterodimer with different functional properties in U87MG cells. Cells were immunoprecipitated with anti-CD44 (left panel) or anti-EGFR (right panel) antibodies. As shown in Figure 6B, EGF stimulates a robust

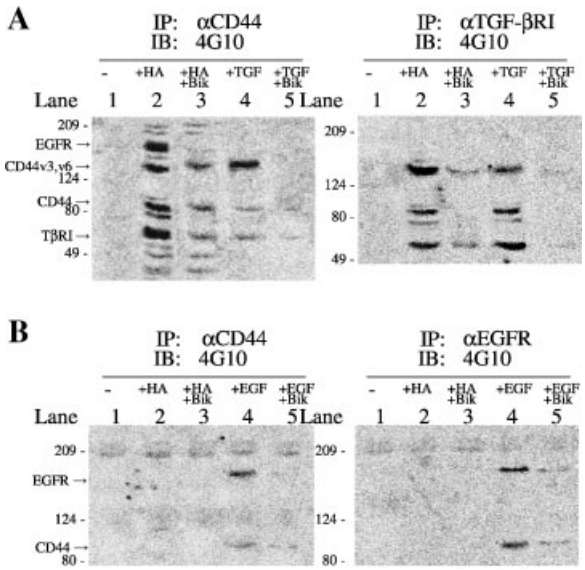

Fig. 6. Agonist-induced cross-phosphorylation of CD44-growth factor receptor heterodimer. **A**: HRA cells co-expressing CD44, EGFR, and TGF-βRI/TGF-βRII were pretreated with or without bikunin (1 μM) and subsequently exposed to 50 μg/ml hyaluronan (HA) or 10 ng/ml TGF-β1 for 20 min, and whole cell receptor phosphorylation was determined as described under ''Experimental Procedures.'' **Left panel**: CD44 was immunoprecipitated with anti-CD44 antibody; and **right panel**, TGF-βRI was immunoprecipitated with rabbit anti-TGF-βRI antibodies. Immunocomplexes were analyzed by SDS–PAGE and immunoblotted with antiphosphotyrosine antibody, 4G10. Western blots are representative of at least two independent experiments. IP, immunoprecipitation; IB, immunoblot. Autoradiographs from representative experiments are shown. Note that phosphorylation of CD44 of the CD44-TGF-βRI heterodimer was significantly increased above basal levels in the presence of HA (**lane 2**) or TGF-β1 (**lane 4**). Bikunin significantly suppressed agonist-induced phosphorylation of CD44 or TGF-βRI (**lanes 3** and **5**). **B**: U87MG cells pretreated with or without bikunin were exposed to 50 μg/ml HA or 10 ng/ml TGF-β1 for 20 min, and whole cell receptor phosphorylation was determined. **Left panel**: CD44 was immunoprecipitated with anti-CD44 antibodies; and **right panel**, cell lysates were immunoprecipitated with anti-TGF-βRI antibodies. Immunocomplexes were analyzed by SDS–PAGE and immunoblotted with antiphosphotyrosine antibody. Phosphorylation of the EGFR of the CD44-EGFR heterodimer was significantly increased above basal levels in the presence of EGF (**lane 4**), but not HA (**lane 2**). Bikunin significantly suppressed agonist-induced phosphorylation of CD44 or EGFR (**lanes 3** and **5**). The positions of molecular mass markers are indicated on the left (in kDa). Two additional experiments gave similar results.

phosphorylation of EGFR (170 kDa; lane 4) and CD44 (90 kDa; lane 4), whereas HA neither increase phosphorylation of CD44 nor EGFR in U87MG cells co-expressing CD44 and EGFR (lane 2). The loss of HA-mediated phosphorylation is not due to a nearly complete dissociation of CD44-EGFR heterodimer, since EGFR forms stable heterodimers with CD44.

Interestingly, bikunin can markedly reduce agonist-induced phosphorylation of CD44 proteins and TGF-βRI (or EGFR) in both cell lines (Fig. 6A,B, lanes 3 and 5). These findings suggest that the specific pattern of agonist-induced phosphorylation of heterodimeric receptors may largely depend on their functional properties. Diminished agonist-induced phosphorylation of these receptors by bikunin may be associated with the loss of heterodimerization and subsequent suppression of receptor phosphorylation and downstream signaling.

HA-Induced TGF-βRI Phosphorylation Is Independent of Autocrine TGF-β1

We determined whether HA-induced TGF-βRI phosphorylation is associated with increased TGF-β1 levels in HRA cells. As shown in Figure 7, preincubation of cells with neutralizing antibodies to TGF-β1 did not affect HA-induced TGF-βRI phosphorylation (lane 3), whereas it completely prevented the phosphorylation induced by TGF-β1 (lane 5).

DISCUSSION

In the present study, we try to answer the mechanism, by which bikunin abrogates heterodimerization of CD44 and growth factor receptors, which may lead to suppress receptor phosphorylation and receptor-mediated signaling, including MAPK activation. For this, the occurrence of agonist/ligand-induced heterodimerization between the CD44 and growth factor receptors in cell types was initially assessed by co-immunoprecipitation in living human cancer cells co-expressing at least the two receptors. The potential functional consequences of the heterodimerization were also assessed by determining the influence of receptor co-expression on the ability of the receptors to stimulate MAPK pathways. The present results allow us to conclude that the CD44 and co-expressing growth factor receptors can form heterodimers and indicate that stable intermolecular interactions (SDS-sensitive) can occur between the two receptors as well. Heterodimerization may represent a regulatory cross-talk process arising through the creation of a receptor form that has distinct functional properties. Interestingly, exogenous bikunin can, for the first time, abrogate heterodimerization of CD44-growth factor receptor complex, although it does not alter receptor expression and ligand binding to

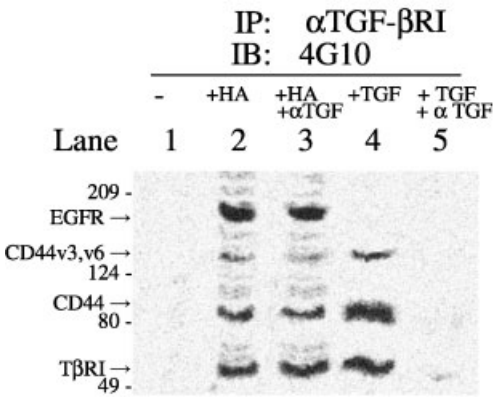

Fig. 7. HA-induced TGF-βRI phosphorylation is independent of autocrine TGF-β1. Serum-starved HRA cells pretreated for 1 h with anti-TGF-β1 neutralizing antibody (10 μg/ml) and treated for 20 min with HA (50 μg/ml) or TGF-β1 (10 ng/ml). TGF-βRI was immunoprecipitated from cell lysates, and samples from each lysate were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibody, 4G10. Two additional experiments gave similar results.

the cells. Diminished agonist-induced phosphorylation of these receptors by bikunin may be associated with the loss of heterodimerization and subsequent reduction of signaling.

The existence of homo- and heterodimers has been demonstrated for several growth factor receptors using co-immunoprecipitation [Zhang et al., 1996, 1997; Jo et al., 2000; Sherman et al., 2000; Wobus et al., 2001, 2002; Bourguignon et al., 2002; Bertotti and Comoglio, 2003]. We have previously shown that members of the CD44 family exist as constitutive homodimers when expressed alone [Kobayashi et al., 1998]: agonist stimulation is associated with dimerization of CD44 proteins, that agonist-induced clustering of CD44 proteins, but not simple up-regulation of CD44 proteins, is necessary to allow up-regulation of uPA expression, and that suppression of clustering of CD44 proteins is required for bikunin-mediated down-regulation of uPA expression in human chondrosarcoma HCS-2/8 cells [Suzuki et al., 2002]. Furthermore, activation of growth factor receptor, for example EGFR, triggers the induction of receptor dimerization, which enables cross-phosphorylation to occur between two receptor molecules (CD44 and EGFR). This dimerization model may provide a universal mechanism to activate the type I receptor family for growth factors and subsequent transformation [Bouloughouris and Elder, 2002]. In addition, heterodimers can be formed between CD44 and members of growth factor receptor families [Zhang et al., 1996; Jo et al., 2000; Wobus et al., 2001, 2002; Bourguignon et al., 2002; Bertotti and Comoglio, 2003]. In the present study, we explored the functional consequences of a physical interaction between CD44 and the growth factor receptors including EGFR, FGFR, and TGF-βRI, although these receptors share no sequence homology. Thus, it appears that heterodimerization differentially affects the properties of these closely related receptors, and this is unique for each heterodimeric complex. Heterodimerization could have functional relevance in vivo: CD44 and growth factor receptors functionally interact in tumor invasion [Jo et al., 2000; Wobus et al., 2001, 2002; Bourguignon et al., 2002; Bertotti and Comoglio, 2003]. Studies have shown extensive cross-talk between CD44- and growth factor receptor-mediated tumorigenic responses [Jo et al., 2000; Wobus et al., 2001, 2002; Bourguignon et al., 2002; Bertotti and Comoglio, 2003].

Previous studies have reported effects of dimerization on the signaling properties of CD44 [Liu and Sy, 1997]. Agonist-induced phosphorylation of intracellular serine and threonine residues within the cytoplasmic portion is the initial step in the receptors [Bouloughouris and Elder, 2002]. After phosphorylation, CD44 is rapidly recruited to the plasma membrane where it facilitates CD44-dependent signalings, including Tiam1, Vav2, RhoA-activated ROK, c-Src, p185HER2, and tyrosine kinase [Bourguignon et al., 2001]. The present study shows that activation of the CD44 of the CD44-growth factor receptor heterodimer resulted in cross-phosphorylation of growth factor receptor and vice versa. The simplest explanation for these findings is that these heterodimers exist in a physically restrained conformation as proposed in the three-dimensional dimer model. Cross-phosphorylation may provide a plausible explanation for enhanced ERK1/2 signaling of the CD44-growth factor receptor heterodimer. Conversely, lack of cross-phosphorylation by bikunin could explain increased abrogation of heterodimerization, which results in increased resistance to agonist-induced signaling.

A major prerequisite for the physiological assembly of heterodimeric complex is their coexpression in the same cells. A particularly high degree of colocalization between the CD44 and the growth factor receptors was observed in

the leading edge of tumor invasion [Murai et al., 2004]. However, future co-immunoprecipitation studies from cancer tissue are necessary to elucidate whether physical interaction between CD44 and growth factor receptors may also occur in vivo.

We propose the following theory regarding the role of bikunin in suppression of agonist-stimulated signal transduction. Clustering of CD44 proteins and co-expressing growth factor receptors may be able to function in the membrane environment. This might, in turn, stimulate the phosphorylation of two receptors and subsequently activate MAPK-dependent signaling. Exogenous bikunin could bind to bikunin-R with high affinity [Suzuki et al., 2002]. The bikunin–bikunin-R complex formation might bring an easy access of bikunin-R to CD44 proteins, because bikunin-R is a putative CD44 accessory protein [Suzuki et al., 2002]. The subsequent complex suppresses heterodimerization of CD44 proteins and growth factor receptors possibly by steric hindrance or allosteric change, which would inhibit signal transduction involved in MAPK activation. That is bikunin may promote the disaggregation of CD44-growth factor receptor heterodimer clustering through the formation of a putative multimeric structure containing bikunin-R. These results allow us to speculate that bikunin-R functions by the inhibitory mechanism of clustering of CD44-growth factor receptor heterodimer complex [Suzuki et al., 2002]. Unfortunately, we could not detect CD44–growth factor receptor heterodimer complex or multimeric complex containing bikunin by biochemical techniques in the cell lines used in the present study, since these complexes may be sensitive to SDS.

Blockade of growth factor receptor activity would provide a novel strategy for the treatment of cancer. Therefore, new agents developed to inhibit EGFR function include monoclonal antibodies to EGFR and small-molecule receptor tyrosine kinase inhibitors. Preclinical studies showed that additive or synergistic growth inhibition resulted from the combination of either type of inhibitor with chemotherapy and/or radiotherapy [Bunn and Franklin, 2002].

In conclusion, we provide biochemical and functional evidence for heterodimerization of CD44 and growth factor receptor. We show that formation of heterodimers selectively cross-modulates receptor phosphorylation and MAPK activation. Direct intramembrane protein–protein interactions may thus provide a novel regulatory mechanism that could enhance the activation of growth factor receptors. This is manifested by the bikunin-dependent loss of ERK1/2 MAPK stimulation by HA or growth factor possibly through abrogation of heterodimerization of CD44 and growth factor receptors.

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