Bikunin Target Genes in Ovarian Cancer Cells Identified by Microarray Analysis

Received for publication, January 9, 2003, and in revised form, February 3, 2003
Published, JBC Papers in Press, February 5, 2003, DOI 10.1074/jbc.M300239200

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Bikunin, a Kunitz-type protease inhibitor, could potentially suppress tumor cell invasion and metastasis. Our previous study revealed that overexpression of bikunin in a human ovarian cancer cell line, HRA, resulted in a down-regulation in uPA and uPAR gene expression. For identifying the full repertoire of bikunin-regulated genes, a cDNA microarray hybridization screening was conducted using mRNA from bikunin-treated or bikunin-transfected HRA cells. A number of bikunin-regulated genes were identified, and their regulation was confirmed by Northern blot analysis. Our screen identified 11 bikunin-stimulated genes and 29 bikunin-repressed genes. The identified genes can indeed be classified into distinct subsets. These include transcriptional regulators, oncogenes/tumor suppressor genes, signaling molecules, growth/cell cycle, invasion/metastasis, cytokines, apoptosis, ion channels, extracellular matrix proteins, as well as some proteases. This screen identified suppression of several genes such as CDC-like kinase, LIM domain binding, Ets domain transcription factor, Rho GTPase-activating protein, tyrosine phosphorylation-regulated kinase, hyaluronan-binding protein, matriptase, and pregnancy-associated plasma protein-A (PAPP-A), which have previously been implicated in enhancing tumor promotion. Northern blot analysis confirmed that several genes including matriptase and PAPP-A were down-regulated by bikunin by ~9-fold. Further, genetic inhibition of matriptase or PAPP-A could lead to diminished invasion. These results show that bikunin alters the pattern of gene expression in HRA cells leading to a block in cell invasion.

Cancer metastasis is a complex multistep process involving sequential interactions between the disseminating tumor cells and a continuously changing host microenvironment. These interactions include cell-cell and cell-extracellular matrix (ECM) communication, which regulate tumor cell attachment, spreading, and migration, the dissolution of tissue barriers through the degradative activity of enzymes such as metallo and serine proteinases, and growth modulation by autocrine or paracrine growth factors (1).

Although bikunin was originally identified as a Kunitz-type protease inhibitor essential for inhibition of trypsin and plasmin, there is now increasing evidence to indicate a role for this glycoprotein in the regulation of cell biology (2). Bikunin may be important in inhibiting the inflammatory response: it has been shown to inhibit the induction of pro-inflammatory cytokines in several types of cells (2, 3). In animal models of inflammatory status, mice treated with bikunin exhibit decreases in several indices of inflammation (3, 4).

Interestingly, in the case of neoplastic cells, exposure of bikunin to cancer cells induces suppression of invasion and metastasis (5, 6). The bikunin molecule has effects independent of its ability to inhibit trypsin and plasmin (7). A number of high affinity specific receptors and binding proteins exist for bikunin on the tumor cell surface (8). It binds cartilage link protein (Crtl1), one of the hyaluronan-binding proteins (8). Bikunin also interacts with the 45-kDa bikunin receptor, a putative CD44 accessory protein (8). Bikunin may cause inhibition of ECM invasion by interacting both with its receptor and Crtl1 in various cell types (8). It is possible that bikunin is associated as a heterotrimer with the bikunin receptor and Crtl1.

It has been established that uPA and uPAR have been shown to modulate the invasion of tumor cells derived from several organs (9). We have previously reported that bikunin down-regulates genes involved in tumor aggressiveness (6, 7). For example, bikunin target genes uPA and uPAR are central components of the tumor invasion program (9). We provide evidence that bikunin repressed uPA and uPAR expression by inhibiting the MAP kinase pathway (10). Further, transfection of HRA cells with the bikunin gene caused pronounced inhibition of uPA expression and drastic suppression of ECM invasion (11). Exogenously added bikunin could suppress peritoneal disseminated metastasis and intraperitoneal ovarian tumor growth, and expression of uPA in tumors of bikunin-injected nude mice was significantly lower than in controls (5). Similarly, when bikunin-transfected cells were intraperitoneally injected into the mice, the size of the intraperitoneal tumors and the levels of uPA were also suppressed compared with luciferase-negative controls (5).

* This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to H. K. and Y. H.) and by a grant from the Yamashina Foundation for Research on Metabolic Disorders. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MT-SP1, membrane type serine protease 1; luc, luciferase; PAPP-A, pregnancy-associated plasma protein-A; IGF, insulin-like growth factor; Crtl1, cartilage link protein 1.

‡ M. Suzuki and H. Kobayashi, unpublished data.
transfected control cells (11). These data strongly support that uPA is mechanistically involved in the regulation of these processes. Why bikunin treatment is capable of inducing inhibition of cancer cell invasion is not fully known. Presumably, there are select target genes that bikunin is capable of regulating. The physiological function of bikunin still remains to be established.

To understand better how bikunin regulates different facets of tumor cell biology, we sought to identify bikunin-regulated genes in a human ovarian cancer cell line, HRA, using microarray technology. There has not been any attempt to systematically define the full repertoire of bikunin-regulated genes. Identification of these genes is required not only for revealing the nature of all signaling pathways used by bikunin but also for defining the set of proteins that are induced or repressed by this inhibitor. In the current study, we started this investigation using a cDNA microarray hybridization assay of RNA isolated from bikunin-treated and untreated HRA cells. In other experiments, we used HRA cells transfected with the bikunin gene. Here we show, using two different approaches, that we have identified at least 40 bikunin target genes, and that bikunin-selective targets include genes involved in the regulation of transcription factors, oncogenes/tumor suppressor genes, signal transduction modifiers, cell growth, cell cycle, inflammation, invasion/metastasis, apoptosis, ion channels, extracellular matrix proteins, and several proteases. The encoded proteins are involved in a broad range of cellular functions and signaling pathways. Interestingly, two different members of the protease (matrilysin and PAPPA-A) were markedly repressed by bikunin. We confirm that, consistent with this, exposure of ovarian cancer cells to bikunin or bikunin gene transfection suppresses a matrilysin/PAPPA-A-dependent invasion, suggesting a potential role for bikunin in modulating tumor biology.

EXPERIMENTAL PROCEDURES

Materials—Purified human bikunin was obtained from Mochida Pharmaceutical Co. (Gotenba, Japan). LipofectAMINE Plus reagent was purchased from Invitrogen. Mouse monoclonal anti-PAPPA antibodies (IgG2b) were obtained from CosmoBio Inc. (Tokyo, Japan). Boyden-type cell invasion chambers (BioCoat Matrigel™ Invasion chambers) were obtained from Collaborative Biomedical (Franklin Lakes, NJ). The nude mice (Balb-c, nu/nu) were obtained from SLC (Hamamatsu, Japan). All other chemicals were analytical grade.

Cell Culture—The ovarian cancer cell line, HRA, was grown in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) under a 5% CO2 atmosphere with constant humidity (6). For all experiments in which bikunin was added, cells were incubated in serum-free medium. Cells were disaggregated routinely with 0.1% trypsin/EDTA solution and replated at a split ratio of 1:10. Total RNA isolations were performed using the Trizol reagent (Invitrogen). Protein concentrations in the supernatants of cell extracts were measured by the Bio-Rad protein assay.

Microarray Probe Labeling and Hybridization—The cDNA microarray probe synthesis reaction in which some of the dTTP was replaced with either Cy3-labeled dUTP or Cy5-labeled dUTP. In some experiments, the bikunin sample was labeled with Cy5, and in others, it was labeled with Cy5, with essentially identical results. Hybridization of both Cy5- and Cy5-labeled probes to the same microarray was carried out in a sealed, humid hybridization cassette for about 14 h at 65 °C. Microarrays were washed and then dried by centrifugation at room temperature. Nine genes were tested for confirmation by Northern blot hybridization.

Microarray Data Analysis—To determine the fluorescent intensities of the two dyes for each spot, the fluorescence signals of Cy3- and Cy5-tagged cDNA spots on arrays at 532 nm (Cy3) and 655 nm (Cy5) simultaneously were scanned immediately with an Affymetrix 428 Aray Scanner and quantitated using a BioDiscovery ImaGene Ver. 4.2 (TaKaRa Bio Inc.). The background-subtracted median ratio value was calculated for each spot, and replicate spots on each slide were averaged. The fluorescence intensities were normalized by applying a scaling factor so that the median fluorescence ratio of all spots with detectable signals above background on each microarray was 1.0. The spots that displayed a 2-fold or greater difference in fluorescence intensities between the two dyes were used to generate gene clusters. Poor quality spots were removed if they were very small, irregularly shaped, or with pixels that were not uniformly distributed throughout the spot.

Northern Blot Hybridization with cDNA Probes—Northern blot hybridization was carried out as described previously (5). Samples of total RNA (25 μg) were separated by electrophoresis through denaturing 1.2% agarose gels containing 1% formaldehyde and transferred onto nylon or nitrocellulose membranes (Hybond N+, Amersham Biosciences) using standard molecular biological techniques. Hybridization was carried out with [α-32P]dCTP by random oligonucleotide priming to specific activities of 0.4–0.9 × 106 cpm/μg. The following cDNA sequences were used as probes: DFBK2, bp 1544–2154 of the human cDNA (12); E74-like factor 2 (new Ets-related factor; NERF), NERF cDNA (13); triple functional domain (PTPRF-interacting), Trio cDNA (encoding amino acids 2249–2861 plus 140 bp of 3’ nontranslated sequence) (14); suppression of tumorigenicity 14 (MT-SP1-matrilysin), the full-length MT-SP1 cDNA (pcDNA3/MT-SP1) ligated into the PCR fragment under the control of a cytomegalovirus promoter (15); a partial human PAPPA-A cDNA (553 bp), a PCR product (GenBank™[D28727; nucleotides 5593–6145] generated from human ovary using PAPPA-A-specific primers (F: 5’-TGA GTC TCT GAC CAT TTG GTT GCT GCA GAA AAG GGA GCA G-3’). uPA cDNA and uPAR cDNA were prepared as described previously (5). Filters were reprobed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for the amount of RNA loaded onto the filters (5). After hybridization, the membranes were washed and exposed on Kodak BioMax MS-1 film at ~70 °C. Filters were quantitated by scanning densitometry using a Bio-Rad model 620 Video Densitometer with a 1-d Analyst software package from Macintosh.

Bikunin Transfection—A human ovarian carcinoma cell line, PCMV-bikunin-IREs-bsr and the control vector pCMV-luciferase-IREs-bsr (16) encoding luciferase (luc) were transfected into HRA cells by the standard calcium phosphate precipitation method (17) as described previously (11). The cells were selected in the presence of 10 mg/ml blasticidin S hydrochloride (Funakoshi Co Ltd., Tokyo, Japan). Resistant clones were obtained after 4 weeks, and bik* transfectants and a luc* transfectant were obtained. The cells were subsequently maintained in the presence of 10 mg/ml blasticidin S hydrochloride. The initial bik* mass cultures were subjected to at least two rounds of subcloning in order to obtain stable bik* clones (Clones 1–5). DNA sequencing verified the correct insertion of the bik cDNA. Finally, HRA bik* tumor cell clones with inducible bik protein expression were confirmed by immunocytochemical staining and Western blot analysis (18). luc* transfectants were used as a control (11).

Antisense MT-SP1 and PAPPA-A Oligodeoxynucleotides and Cell Transfection—Antisense 18-base phosphorothioate oligodeoxynucleotides corresponding to the human MT-SP1 and PAPPA-A mRNA were synthesized and consisted of the antisense sequences of 5’-AGC TGC TCA TCC TAG GCA-3’ (AS-MT-SP1) and 5’-GCC CAA CTC CTG CTC GAA-3’ (AS-PAPPA-A), respectively. Oligonucleotides mixed with lipofectin reagent were incubated for 15 min at room temperature. Thereafter, the oligonucleotide-liposome complexes were then added to cells and washed twice with medium (19). After 4 h, fresh normal growth medium containing 10% fetal bovine serum was added. Eight hours later the cells were analyzed for PAPPA protein expression by Western blot, and the cells were used for ECM invasion experiments as described below.

Western Blot Analysis—HRA cells transiently transfected with either AS-PAPPA-A as well as stably transfected with bikunin were harvested by treatment with 1× phosphate-buffered saline, and cell pellets were resuspended in radiolimmune precipitation assay buffer. Centrifuged lysates (50 μg) from each cell line were analyzed by SDS-polyacrylamide gel electrophoresis on 15% gels and transblotted to a polyvinylidene difluoride membrane by semi-dry transfer. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and then incubated with primary antibodies overnight at 4 °C; rabbit polyclonal anti-bikunin (1:1000) or monoclonal anti-PAPPA-A (1:1000). This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Copenhagen, Denmark) at a dilution of 1:4000.
Bikunin Target Genes Identified by Microarray

The primary goal of this study was to identify genes regulated at the transcriptional level by bikunin signaling in human ovarian cancer cells. HRA cells were evaluated for their suitability in studying gene expression changes in response to bikunin stimulation. The cell line was found not to express mRNA and protein for bikunin (11). Exogenously added bikunin induced a suppression of tumor cell invasive ability in a modified Boyden chamber assay compared with control cells (5–8, 10). Furthermore, transfection of bikunin gene in these cells also induces a specific and significant decrease in cell invasiveness (11). Bikunin had negligible effects on the in vitro growth of the HRA cells (5–8, 10, 11). Previous work has shown that bikunin could down-regulate uPA and uPAR expression in some ovarian cancer cell lines (5–8, 10, 11). Thus, this decrease in cell invasiveness was not due to a decrease in cell proliferation but rather a suppression of uPA and uPAR gene expression. Therefore, two different methods (bikunin exposure and bikunin gene transfection) were used to identify genes induced or repressed in the HRA cells in microarray technologies.

Microarray Analysis of HRA Cells, Identification of Target Genes in HRA Cells Transfected with the Bikunin Gene—In the first set of experiments, we tried to achieve the overexpression of the bikunin gene in HRA cells (Exp. 1, Table I). To test whether bikunin transfection could activate or inactivate potential target genes, HRA cells were stably transfected with bikunin or luciferase control (luc–). Of the stable bikunin transfectants, the clone 1 (C1) cells were selected for the microarray analysis because it showed the greatest bikunin expression (11). Total RNA was purified from these samples, labeled with either Cy3 or Cy5 fluorescence, and then hybridized to the TaKaRa Human 3K CHIP. Each slide contained duplicate sets of samples, and the colors of the two cDNA probes were reversed in duplicate assays. Quantification of the signals produced two kinds of information: the intensity of the signal was proportional to the abundance of the corresponding mRNA, and the degree of redness or greenness indicated the fold induction or repression of mRNA by bikunin transfection of the cells. The average hybridization intensity across all probe sets from C1 cells was normalized to that obtained from a control luc–. For each gene, the intensity ratio of C1/luc– was calculated. Ratios of 1 indicate equal intensities and, therefore, no change in gene expression between the two cell lines. Ratios below 0.5 indicate down-regulation of gene expression in C1 cells. In contrast, ratios above 2.0 indicate an up-regulation of gene expression in C1 cells. Using this criterion, we found that 12 and 31 genes were up- and down-regulated in C1 cells, respectively. Further, our analyses revealed that 3 mRNAs (D30612, BC012816, and NM005399) were induced by 3.0-fold or more, and 7 mRNAs (NM030941, NM021978, NM003992, AB028983, NM006482, NM002375, and U28727) were repressed by 3.0-fold or more (Table I, Exp. 1).

Identification of Target Genes in HRA Cells Exposed to Bikunin—Many extracellular stimuli are known to activate or suppress multiple and dependent signaling pathways leading to transcriptional activation or suppression of different families of genes. Bikunin transfection experiments in our studies have effects on gene expression (see Table I, Exp. 1). We examined whether each of the identified target genes might also be regulated by exogenously added bikunin. Thus, in the second setting of experiments, for undertaking a systematic analysis of bikunin-regulated genes, HRA cells were exposed to a control serum-free medium or the serum-free medium supplemented with purified bikunin (40 μg/ml or 1 μM) for 6 h, and total RNA was isolated from treated and untreated cells (Table I, Exp. 2). We chose the length of treatment to be 6 h, because our previous studies have shown that this is the optimum time for suppression of uPA and uPAR (5–8, 10). The two sets of RNA from bikunin-treated and untreated cells were then used for microarray analysis. For each gene, HRA cells treated with bikunin (HRA(+)) was compared with untreated cells (HRA(−)), and the intensity ratio of HRA(+/−) was calculated. Using the above criterion, we found that 11 and 29 genes were up- and down-regulated in HRA(+), respectively. If ±3.0 is a conservative estimate for determining the minimum magnitude of real ratios, we found up-regulation of 7 genes (3.14–8.57-fold higher in bikunin treatment), whereas 17 genes were down-regulated upon bikunin treatment (3.01–7.12-fold lower).

Taken together, there were 40 gene targets commonly identified by both methods (bikunin exposure and bikunin transfection), in which 11 or 29 genes were induced or repressed, respectively. The majority of target genes observed in the C1 cells were also affected by exogenous bikunin (Table I). This suggests that the ability of bikunin to regulate the genes identified in this study are likely caused by their ability to bind to cells via specific bikunin receptors and not via a nonspecific target. The putative function of genes selectively regulated by bikunin may correlate with the biological phenotype affected by bikunin in ovarian cancer cells.

Summary of Genes Induced or Repressed by Bikunin—Analysis of the results identified several genes that were commonly altered in response to both bikunin exposure and bikunin overexpression (Table I), most of which had not previously been identified as bikunin target genes. Cellular functions of many but not all of the bikunin-regulated genes identified by our screen are known. In Table I, the proteins encoded by these genes are grouped according to their functions. The identified bikunin-stimulated or repressed genes can indeed be classified into distinct subsets, each of which is probably induced by a distinct bikunin-elicited signaling pathway. These include transcriptional regulators, oncogenes/tumor suppressor genes, signaling molecules, growth, proliferation, and cell cycle, invasion/metastasis, cytokines, apoptosis, ion channels, extracellular matrix proteins, as well as several proteases.

In the previous experiments, several proteins (for examples, uPA and uPAR) known to be involved in invasion and metastasis were significantly suppressed by bikunin. It is interesting to note that, in this microarray analysis, uPA and uPAR were at levels 1.87- and 1.79-fold lower in bikunin-transfected cells than in control cells. A repeat of the cDNA microarray with mRNA samples obtained in cells exposed to bikunin for 6 h confirmed the down-regulation of both uPA (~1.74-fold) and uPAR (~1.95-fold) but not significantly.

In general, bikunin is able to repress several genes related to tumor aggressiveness. For example, TAF11 (20), GA-binding
are the Rho GTPase-activating protein 5 (25), dual-specificity to transcription molecules. The repressed signaling molecules protein (21), GTF2H2 (22), E74-like factor 2 (13), LIM domain binding 2 (23), and CDC-like kinase 3 (CLK3) (24) are related to transcription molecules. The repressed signaling molecules are the Rho GTPase-activating protein 5 (25), dual-specificity tyrosine phosphorylation-regulated kinase 2 (12), and four-and-a-half LIM domain proteins (FHL) (26). Examples of oncogenes/tumor suppressor genes are Rho GTPase-activating protein 5 (25), zinc finger protein 282 (27), and RAB11A (28).

| GenBank™ accession no. | Fold change* | Description |
|------------------------|--------------|-------------|
| NM005399               | -3.56        | CDC-like kinase 3 |
| NM005643               | -2.70        | TAF11 RNA polymerase II, TATA box-binding protein (TBP)-associated factor, 28 kDa, transcription factor |
| NM001290               | -2.60        | LIM domain binding 2 |
| NM002040               | -2.54        | GA-binding protein transcription factor, α subunit (60 kD) |
| D42046                 | -2.46        | DNA2 (DNA replication helicase, yeast, homology)-like |
| NM033334               | -2.44        | Nuclear receptor subfamily 6, group A, member 1 |
| NM001515               | -2.36        | Homo sapiens general transcription factor IIH, polypeptide 2, 44 kDa (GTF2H2), mRNA |
| NM006874               | -2.28        | E74-like factor 2 (cts domain transcription factor) |
| NM004663               | -2.06        | RAB11A member RAS oncogene family |
| AB028983               | -3.50        | Adenylate cyclase 2 (brain) |
| NM006482               | -3.44        | Dual-specificity tyrosine-(Tyr)-phosphorylation regulated kinase 2 |
| NM004468               | -2.86        | Four and a half LIM domains 3 |
| NM001173               | -2.82        | Rho GTPase activating protein 5 |
| NM006509               | -2.90        | v-rel avian reticuloendotheliosis viral oncogene homolog B (nuclear factor of Kappa light polypeptide gene enhancer in B-cells 3) |
| NM001173               | -2.82        | Rho GTPase activating protein 5 |
| NM0014221              | -2.44        | Homo sapiens mature T-cell proliferation 1 (MTPC1), mRNA |
| NM007116               | -2.39        | Triple functional domain (PTPRF interacting) |
| NM002444               | -2.24        | Moesin |
| D30612                 | +8.04        | Zinc finger protein 282, apoptosis and tumor suppression |
| NM002375               | -3.34        | Microtubule-associated protein 4 |
| NM004132               | -2.70        | Hyaluronad-binding protein 2 |
| NM0014221              | -2.44        | Homo sapiens mature T-cell proliferation 1 (MTPC1), mRNA |
| NM003165               | -2.44        | Homo sapiens syntaxin binding protein 1 (STXBP1), mRNA |
| NM003872               | -2.24        | Neurophilin 2 |
| NM004336               | +2.51        | CD61 antigen (target of antiproliferative antibody 1) |
| NM004102               | +2.62        | CD61 antigen (target of antiproliferative antibody 1) |
| NM004336               | +2.51        | CD61 antigen (target of antiproliferative antibody 1) |
| NM001569               | -2.94        | Interleukin-1 receptor-associated kinase 1 |
| BC012816               | +5.49        | TGFβ-induced factor 2 (TALE family homobox) |
| NM013979               | -2.10        | BCL2/adenovirus E1B 19kD-interacting protein 1, anti-apoptotic activity |
| NM007217               | +2.26        | Programmed cell death 10 |
| NM032183               | +2.64        | Selenium binding protein 1 |
| D30612                 | +8.04        | Zinc finger protein 282, apoptosis and tumor suppression |
| NM002249               | -2.54        | Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 |
| NM001286               | -2.44        | Chlorid channel 6 |
| NM002997               | -2.82        | Syndecan 1 |
| NM005567               | -2.70        | Lectin galactoside-binding, soluble, 3 binding protein |
| NM001855               | +2.48        | Collagen type XV, α 1 |
| NM030441               | -6.66        | Exonuclease NKF-sp |
| U28727                 | -3.04        | Pregnancy-associated plasma protein A |
| NM004132               | -2.70        | Hyaluronan-binding protein 2 |
| NM006219               | -2.64        | Phosphoinositide 3-kinase catalytic, β polypeptide |
| NM003837               | -2.64        | Glutathione transferase 1 (maleylacetate isomerase) |
| NM004529               | +2.71        | Phospholipase A2, group V |
| NM005399               | +4.66        | Protein kinase, AMP-activated, β 2 non-catalytic subunit |
| NM005166               | +2.62        | Amyloid β (A4) precursor-like protein 1 |

* The numbers indicate the -fold induction of each gene as determined by the microarray analysis software. + and – are increased and decreased in mRNA after bikunin treatment, respectively. Some genes (parentheses) are listed in more than one category.
Bikunin Target Genes Identified by Microarray

**Fig. 1. Northern analysis of selected bikunin-repressed genes.** The regulation of uPA and uPAR mRNA as well as five bikunin target genes identified in the microarray screen was independently tested using Northern blot analysis. Blots were probed with GAPDH to normalize for differences in RNA loading. All reactions were performed in duplicate, and the average fold repression is shown. 10 μg of total RNA from luciferase transfectant control cells (−) or bikunin transfectant C1 cells (+) were analyzed. The numbers on the top compare the folds of induction/repression of each mRNA as measured by microarray and Northern blot analysis. Panel 1, uPA; panel 2, uPAR; panel 3, E74-like factor 2; panel 4, dual-specificity tyrosine phosphorylation-regulated kinase 2; panel 5, triple functional domain; panel 6, suppression of tumorigenicity 14; panel 7, PAPP-A; panel 8, GAPDH.

addition, bikunin modulates a large number of cellular regulatory proteins affecting cell growth such as hyaluronan-binding protein 2, CD81 antigen, and fatty acid-binding protein 3. An example of the invasion/metastasis-related group is suppression of tumorigenicity 14 (matritpin, epithin). This protease has been shown to activate uPA. Bikunin also represses pro-inflammatory cytokine-associated signaling and enhances apoptosis. Two genes encode a voltage-independent calcium-activated channel and a voltage-dependent chloride channel (29). Syndecan has a role in cell adhesion, maturation, proliferation, and prognosis (30). PAPP-A is the insulin-like growth factor-binding protein protease (31). Therefore, most of the bikunin target genes are involved in modulation of cell proliferation, invasion, and tumor metastasis.

**Validation of Microarray Results Using Northern Blot Analysis**—To validate the data obtained in the microarray analysis, we have subjected the RNA samples from the cells that were originally used for microarray analysis to Northern blot analysis. Fold induction calculated from the microarray data was compared with that obtained using Northern blot analysis (Fig. 1). We initially measured expression levels of two bikunin target genes, uPA and uPAR, that have been identified in other systems (5–8, 10, 11). The hybridization signals for uPA and uPAR were significantly higher for the RNA isolated from the control-treated cells, and the intensity of hybridization signal was very faint for the RNA isolated from bikunin-transfected cells. Quantitation of the signal revealed that the expression of uPA and uPAR was 6.3- and 2.1-fold lower in cells transfected with bikunin over the control cells. Thus, Northern blot analysis revealed that uPA and uPAR were both reduced in response to bikunin expression.

The other six genes were selected for secondary confirmation based on a combination of cDNA probe availability and putative gene function because of interesting properties of the encoded proteins. As shown in Fig. 1, all of the five candidate bikunin-repressed genes were strongly reduced by bikunin, although the level of GAPDH mRNA was unchanged. For all of the genes examined, the actual fold induction by Northern blot analysis was significantly greater than that derived from the microarray analysis. Quantitation of the Northern signals revealed that the fold changes observed in the microarray analysis were in general underestimates. Thus, even a relatively small difference noted in the microarray analysis may be physiologically significant. This analysis cannot exclude the possibility that stimulation/repression occurs indirectly through activation/inactivation of one or more intermediary molecules.

**Functions of Bikunin-repressed Genes, PAPP-A and MT-SP1 (Matritpin)—**It is clear that an exposure of cells to bikunin or transfection of cells with the bikunin gene profoundly changes the cellular abundance of a large number of mRNAs whose products are essential to every aspect of cell physiology. Because PAPP-A and MT-SP1 (matritpin) were identified as the strongly repressed genes in response to treatment with bikunin (Table I and Figs. 1 and 2), we selected them for further functional studies. We determined whether genetic inhibition of PAPP-A or MT-SP1 (matritpin) could lead to diminished invasion that is dependent on PAPP-A or MT-SP1 (matritpin) protein. The AS-PAPP-A or AS-MT-SP1 constructs were transfected into HRA cells for transient down-expression by using the LipofectAMINE Plus reagent. The conditioned medium was collected after transfection, and the level of PAPP-A was analyzed by Western blot. As expected, PAPP-A protein expression was reduced when the cells were transfected with AS-PAPP-A (Fig. 3, A and B). Since we could not obtain anti-MT-SP1 antibody, down-regulation of MT-SP1 expression was not determined in this study. Cells that down-express PAPP-A or possibly MT-SP1 were cultured for determining their invasive ability in a modified Boyden chamber assay. Wild-type HRA cells, LipofectAMINE Plus-alone cells, and luciferase-transfected cells (luc−) served as controls. As shown in Fig. 3C, cell invasion was significantly reduced in AS-MT-SP1 cells or to a lesser degree in AS-PAPP-A cells. Cell growth was down-regulated in AS-PAPP-A cells by −20%, whereas MT-SP1 down-expression failed to affect cell proliferation. These results dem-
shown that MT-SP1 and PAPP-A have a specific effect on the cell invasiveness in HRA cells.

**DISCUSSION**

Current investigations have focused on the understanding of the molecular mechanism(s) by which bikunin controls invasiveness in human ovarian cancer HRA cells. To address the mechanisms that explain these functions, we sought to identify bikunin-regulated genes using microarray analysis in cultured cells. Here we show that either overexpression of bikunin or treatment of HRA cells with bikunin-altered gene expression in a similar fashion, suggesting that the actions of bikunin occur extracellularly or at the plasma membrane. Altered gene expression was confirmed by Northern blot analysis. Most significantly, antisense for either of two proteins (PAPP-A and MT-SP1) down-regulated by bikunin, mimics the loss of invasive ability caused by bikunin.

Of the 43 bikunin-regulated genes reported in this study, 12 genes were induced or 31 genes were repressed. The genes selectively regulated by bikunin have a role in functions such as tumor biology. All of the transcriptional factor- and signaling molecule-related genes were repressed by bikunin. Bikunin also modulates a large number of oncogenes/tumor suppressor genes, cytokines, and (extra)cellular regulatory proteins affecting cell adhesion, growth, and apoptosis (32–34). In addition, the NM002249 encoded protein is an integral membrane protein that forms a voltage-independent calcium-activated channel with three other calmodulin-binding subunits (35). The chloride channel 6 encodes a voltage-dependent chloride channel gene (29). These data may be related to the fact that bikunin can inhibit cytokine-induced calcium influx in several types of cells (6, 36).

An example of the invasion/metastasis-related group is suppression of tumorigenicity 14 (37). The protein encoded by suppression of tumorigenicity 14 (matriptase, epithin) gene is strongly repressed. Epithin was originally identified as a mouse type II membrane serine protease, which plays important roles in cell migration and tumor cell metastasis (37). The human orthologues of epithin, MT-SP1 and its N-terminal-truncated form, matriptase, have also been reported (38, 39). Matriptase is an 80-kDa matrix-degrading protease (40), and is complexed with a Kunitz-type serine protease inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1) (41). The isolated cDNA for matriptase appears to be a truncated form of MT-SP1, lacking the N-terminal 172 amino acids of the coding sequence (39). Matriptase can convert hepatocyte growth factor (HGF)/scattering factor to its active form, and can activate c-Met tyrosine phosphorylation (42). Further, protease-activated receptor 2 (PAR2) and single-chain uPA (sc-uPA) were identified as substrates of matriptase (43). Studies with MT-SP1/matriptase suggest that epithin and its human orthologue may play important roles in cell migration as well as cancer invasion and metastasis. In the previous experiments, several proteins including uPA and uPAR known to be involved in invasion and metastasis were significantly suppressed by bikunin (5–8, 10, 11). Although the changes in mRNA levels detected by microarray hybridization were generally moderate, in each case where the changes were confirmed by Northern analysis, they were shown to be much more dramatic than corresponding microarray results (Fig. 1). A similar underestimate of the degree of changes of mRNA expression by microarray analysis has been reported in other studies (44).

It would be particularly interesting to know whether the two genes, *MT-SP1* (matriptase) and *PAPP-A*, are strongly repressed by bikunin treatment. In this study we showed that MT-SP1 down-expression by an antisense strategy can specifically reduce cell invasion (Fig. 3). Therefore, we speculate that bikunin can inhibit tumor cell invasion possibly via suppression of MT-SP1 (matriptase)-dependent activation of HGF, c-Met, and uPA. PAPP-A recently has been identified as an insulin-like growth factor (IGF)-binding protein (IGF-BP)-4 protease (31). IGFs are mitogenic peptides that regulate cell proliferation. IGF-BP-4 is an inhibitor of IGF action (45), and
proteolysis of IGF-BP-4 enhances IGF bioavailability (46). Thus, it is likely that PAPP-A plays an important role in regulating the availability of IGFs and regulating tumor cell functions. In this study we also showed that HRA cells transiently transfected with an AS-PAPP-A are less invasive than control cells. Altogether, these results suggest that MT-SF1 (matriptase) and PAPP-A may have a direct pro-invasive effect in HRA cells.

A certain gene (PAPP-A) is affected as early as 3 h following bikunin treatment. It seems likely that PAPP-A is an early response gene among putative bikunin target genes. Another gene (MT-SF1, matriptase) is a late response gene (Fig. 2). The molecular basis for this type of specificity may be due to some combination of bikunin-dependent interactions with other cofactors or pathways. There may also be unique cell cofactors that play a role in dictating bikunin specificity. Bikunin is a protease inhibitor rather than a transcription factor. Thus the changes in gene expression are probably caused by some form of altered signaling.

Taken together, most of the bikunin down-regulated genes are shown to inhibit cell proliferation, invasion, and tumor metastasis, whereas most of the up-regulated genes promote apoptosis and tumor suppression. Therefore, bikunin may negatively regulate possible cross-talk between tumor aggressiveness and extracellular signaling actions. Experiments reported here support our previous data (5–8, 10, 11). It is not known, however, how many of these genes identified in this study contain functional response elements. It is possible that bikunin may repress these genes via a transrepression mechanism that involves competition for limiting amounts of co-activators.

In conclusion, we have delineated for the first time the biochemical mechanism by which bikunin reduces ECM invasion by modulating specific gene expression. Decreased expression of specific genes such as matriptase by bikunin may reflect down-regulation of uPA activation, implying that bikunin facilitates a shift in balance toward decreasing proteolytic activity of uPA. The results presented here should alert us to the fact that bikunin will have additional global effects on neoplastic cells by modulating the expression of a large number of cellular genes. Clearly, the genomic response to bikunin signaling is complex. Future studies focused on the regulation and functional significance of the target genes reported here should increase our knowledge of the biological activity of bikunin in non-neoplastic and neoplastic cells.

Acknowledgments—We thank Drs. M. Fujie, K. Shibata, T. Noguchi, and A Suzuki (Equipment center and Photo center, Hamamatsu University School of Medicine) for helping with the biochemical analysis. We are also thankful to Drs. H. Morishita, Y. Kato, K. Kato, and H. Sato (BioResearch Institute, Mochida Pharmaceutical Co., Gotenba, Shizuoka), Drs. Y. Tanaka and T Kondo (Chugai Pharmaceutical Co. Ltd., Tokyo), and Drs. S. Miyauchi and M. Ikeda (Seikaguag Kogyo Co. Ltd., Tokyo) for their continuous and generous support of our work.

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