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Glypican-1 Is Overexpressed in Human Breast Cancer and Modulates the Mitogenic Effects of Multiple Heparin-binding Growth Factors in Breast Cancer Cells[1]

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ABSTRACT

Glypicans are a family of glycosylphosphatidylinositol-anchored cell surface heparan sulfate proteoglycans implicated in the control of cellular growth and differentiation. Here we show that glypican-1 is strongly expressed in human breast cancers, whereas expression of glypican-1 is low in normal breast tissues. In contrast, the expression of glypican-3 and -4 is only slightly increased in breast cancers by comparison with normal breast tissues, and glypican-2 and -5 are below the level of detection by Northern blotting in both normal and cancer samples. Treatment of MDA-MB-231 and MDA-MB-468 breast cancer cells with phosphoinositide-specific phospholipase-C abrogated the mitogenic response to two heparin-binding growth factors, heparin-binding epidermal growth factor-like growth factor and fibroblast growth factor 2. Stable transfection of these cells with a glypican-1 antisense construct markedly decreased glypican-1 protein levels and the mitogenic response to the same heparin-binding growth factors, as well as that to heregulin α, heregulin β, and hepatocyte growth factor. Syndecan-1 was also expressed at high levels in both breast cancer tissues and breast cancer cells when compared with normal breast tissues. There was a good correlation between glypican-1 and syndecan-1 expression in the tumors. However, clones expressing the glypican-1 antisense construct did not exhibit decreased syndecan-1 levels, indicating that loss of responsiveness to heparin-binding growth factors in these clones was not due to altered syndecan-1 expression. Furthermore, 8 of 10 tumors with stage 2 or 3 disease exhibited high levels of glypican-1 by Northern blot analysis. In contrast, low levels of glypican-1 mRNA were evident in 1 of 10 tumors with stage 2 or 3 disease and in 9 of 10 tumors with stage 1 disease. Taken together, these data suggest that glypican-1 may play a pivotal role in the ability of breast cancer cells to exhibit a mitogenic response to multiple heparin-binding growth factors and may contribute to disease progression in this malignancy.

INTRODUCTION

The mortality of breast cancer in the United States has recently leveled off and even decreased slightly (1, 2). However, it remains the second leading common cause of cancer death in women in the United States (1, 2). Breast cancer is expected to account for 192,200 new cancer cases in the United States in 2001, and more than 40,000 women are projected to die this year from this disease (1). A variety of molecular alterations have been reported in breast cancer. These include loss of heterozygosity (1p, 3p, 7q, 11p, 17p, 17, and 18q), mutations (*BCRA1*,*2*, *p53*, and c-H-*ras*-1), and gene amplifications [c-*myc* and c-*erb*B-2 (3–10)]. In the case of c-*erb*B-2, overexpression has been correlated with aggressive disease and decreased patient survival. Furthermore, anti-erbB-2 antibodies can suppress breast cancer cell growth *in vitro* and decrease tumor burden *in vivo* (11, 12), thereby prolonging patient survival (13).

To date, a ligand that binds to c-*erb*B-2 has not been identified (14, 15). Instead, c-*erb*B-2 is capable of heterodimerizing with the other members of the EGF[5] receptor family once these receptors bind their ligands (14–17). These ligands are either members of the EGF family that bind directly to the EGF receptor or members of the HRG family that bind erbB-3 and erbB-4 (18). In addition to EGF, the EGF family of ligands includes transforming growth factor α, HB-EGF, betacellulin, amphiregulin, and epiregulin (16). HB-EGF, amphiregulin, and HRG are heparin-binding factors. In addition, breast cancers overexpress FGF-2 and type 1–4 FGF receptors (19) as well as HGF and its receptor (c-Met; Ref. 20). Both FGF-2 and HGF are heparin-binding factors. Together, these observations suggest that multiple heparin-binding growth factors have the potential to contribute to the pathobiology of breast cancer in humans.

The binding of heparin-binding growth factors to their cell surface receptors often requires the presence of cell surface HSPGs (19, 21, 22). There are two main families of such molecules, syndecans and glypicans, which differ significantly in core protein domain structure (23, 24). Six members of the glypican family (glypican-1–6) and four members of the syndecan family (syndecan-1–4) have been reported to date (25–28). They have important functions with respect to cell behavior, including cell-cell and cell-extracellular matrix adhesion (29, 30), growth factor signaling (23, 31), and protection of growth factors such as FGF-2 from thermal denaturation and proteolytic attack (32, 33). They also regulate the interaction of several heparin-binding growth factors with their receptors and, consequently, their biological activity (34).

In view of the potential importance of heparin-binding growth factors in breast cancer and the requirement of these factors for HSPGs, in the present study we investigated the expression and action of glypicans and syndecan-1 in breast cancer. We now report that glypican-1 and syndecan-1 are overexpressed in human breast cancer and that stable expression of a glypican-1 antisense mRNA in breast cancer cells results in reduced glypican-1 protein expression, leading to an attenuated mitogenic response to FGF-2, HB-EGF, HRG-α, HRG-β, and HGF.

MATERIALS AND METHODS

Materials. The following materials were purchased from the manufacturer indicated: (*a*) fetal bovine serum, Leibovitz's medium, trypsin solution, penicillin-streptomycin solution, and Geneticin (G418), Irvine Scientific (Santa Ana, CA); (*b*) Genescreen membranes, New England Nuclear (Boston, MA); (*c*) restriction enzymes, pMH vector, the random primed labeling kit, Genius 3 nonradioactive nucleic acid detection kit, and Genius 4 RNA labeling kit, Boehringer Mannheim (Indianapolis, IN); (*d*) PI-PLC, Oxford Glycosciences Inc. (Bedford, MA); (*e*) Sequenase version 1.0 DNA Sequencing,

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[5] The abbreviations used are: EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol; HSPG, heparan sulfate proteoglycan; PI-PLC, phosphoinositide-specific phospholipase C; HB-EGF, heparin-binding epidermal growth factor-like growth factor; FGF, fibroblast growth factor; HRG, heregulin; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; nt, nucleotide(s); PMSF, phenylmethylsulfonyl fluoride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

USB Specialty Biochemicals (Cleveland, OH); (*f*) [α-^{32}P]dCTP and [α-^{32}P]CTP, Amersham (Arlington Heights, IL); (*g*) DNA molecular weight markers and LipofectAMINE, Life Technologies, Inc. (Gaithersburg, MD); (*h*) nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stock solution, Roche Molecular Biochemicals (Indianapolis, IN); (*i*) monoclonal mouse anti-CD138 (syndecan-1) antibody, Serotec Inc. (Raleigh, NC); (*j*) horseradish peroxidase-conjugated antirabbit antibody, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); (*k*) Cy3-conjugated antirabbit IgG antibody, Jackson ImmunoResearch (West Grove, PA); (*l*) pBluescript-IISK+, Stratagene (La Jolla, CA); (*m*) DEAE-Sephacel, Pharmacia Biotech (Piscataway, NJ); (*n*) enhanced chemiluminescence blotting kit, Pierce (Rockford, IL); (*o*) pCDNA3.1 Myc-His, Invitrogen (Carlsbad, CA); (*p*) Centriprep Concentrators, Amicon Inc. (Naperville, IL); (*q*) HRG-α, HRG-β, and HGF, R&D Systems (Minneapolis, MN); and (*r*) heparitinase (Heparinase III), chondroitinase ABC, and all other reagents, Sigma Chemical Co. (St. Louis, MO). Human recombinant EGF was a gift from Chiron Inc. (Emmeryville, CA). Human recombinant HB-EGF (residues 73–149 of mature human HB-EGF) and human recombinant FGF-2 were gifts from Dr. J. Abraham (Scios Nova Inc., Mountain View, CA). IGF-I was a gift from Genentech Inc. (South San Francisco, CA). MDA-MB-231 and MDA-MB-468 human breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA).

Tissue Samples. Breast cancer tissues (20 female patients; median age, 60.8 years; age range, 38–76 years) were obtained from surgical specimens from patients with breast cancer. Normal human breast tissue samples were obtained from the same (matched pairs) mastectomy samples, but at a distance of at least 5 cm from the cancer area. According to the tumor-node-metastasis (TNM) classification of the Union Internationale Contre le Cancer, 10 tumors were stage 1, 7 tumors were stage 2, and 3 tumors were stage 3 breast carcinoma. Freshly removed tissue samples were fixed in 10% formaldehyde solution for 12–24 h and embedded in paraffin for histological analysis. In addition, tissue samples were frozen in liquid nitrogen immediately after surgical removal and maintained at −80°C until use for RNA extraction. All studies were approved by the Ethics Committee of the Yamanashi-Medical University and by the Human Subjects Committee at the University of California, Irvine.

Construction of Vectors. A 599-bp human glypican cDNA probe (nt 920-1518) was isolated as described previously (35) and subcloned into Bluescript-IISK+ vector. For *in situ* hybridization, a 210-bp cDNA fragment (nt 1280–1489) of human glypican was subcloned into Bluescript-IISK+ vector. Authenticity was confirmed by sequencing. Glypican-2, -3, -4, and -5 constructs were prepared as described previously (36–38). A glypican-1 antisense construct was prepared by reverse transcription-PCR amplification of human placenta cDNA, as described previously (36). Briefly, the 1751-bp fragment (nt 123-1873; GenBank accession number X54232), which covered from 100 bp downstream of the start codon to 25 bp downstream of the end of the coding region, was subcloned in the antisense orientation into the pMH expression vector. The primers used for the glypican-1 preparation contained a *Eco*RI and *Hin*dIII site, respectively, attached to the 5′- end and preceded by a 3-bp overhang: sense primer, 5′-GTA*GAATTC*GGACCTTGGCTCTGCCCTTC; and antisense primer, 5′-AGT*AAGCTT*GTAAGGGCCAGGAAGAGGAG. A 400-bp human syndecan-1 cDNA probe (nt 502–902) was isolated and subcloned into Bluescript-IISK+ vector.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted by the single-step acid guanidinium thiocyanate phenol chloroform method. RNA was size-fractionated on 1.2% agarose/1.8 M formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation. Blots were prehybridized and hybridized with cDNA probes or riboprobes and washed under high stringency conditions as reported previously (39). Blots were then exposed at −80°C to XAR-5 films (Eastman Kodak, Rochester, NY), and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. A *Bam*HI 190-bp fragment of mouse 7S cDNA that cross-hybridizes with human cytoplasmic RNA was used to confirm equal RNA loading and transfer (39).

Immunohistochemistry. A highly specific, affinity-purified rabbit antirat glypican-1 antibody that recognizes human glypican-1 was used for immunohistochemistry (25, 36). We have demonstrated previously that preabsorption of the anti-glypican-1 antibody with the glypican-1 peptide antigen completely abolished immunoreactivity (25, 36). Paraffin-embedded sections (4 μm) from breast cancer and normal breast tissues were subjected to immunostaining using the streptavidin-peroxidase technique (Kirkegaad & Perry Laboratories Inc., Gaithersburg, MD). Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were first incubated for 15 min at room temperature with 10% normal goat serum and then incubated for 16 h at 4°C with glypican antibody (2.5 μg/ml) in PBS containing 1% BSA. Bound antibodies were detected with biotinylated goat antirabbit IgG secondary antibodies and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer's hematoxylin. Also, sections incubated with nonimmune rabbit IgG or without primary antibodies did not yield positive immunoreactivity.

Immunoblotting. Cells were washed with PBS (4°C) and solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 μg/ml pepstatin A, 1 mM PMSF, and 1% Triton X-100. Glypican-1 digestion with heparitinase (1 unit/ml) was performed by incubating samples (30 μl) at 37°C for 6 h. Incubations were terminated by the addition of 7.5 μl of 5× SDS sample buffer and heating at 95°C for 10 min. To prepare membranes, cells were homogenized in 20 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and 2 mM benzamidine. After centrifugation at 1,500 × *g* for 10 min, supernatants were collected and centrifuged at 25,000 × *g* for 30 min. The resulting pellets were resuspended in 20 mM HEPES (pH 7.4) containing 10 mM leupeptin and solubilized in 5× SDS sample buffer and heated at 95°C for 10 min. For reduction and alkylation with iodoacetamide, protein lysates were incubated at 95°C for 4 min in the presence of 10 mM DTT before the addition of iodoacetamide (50 mM, final concentration). Samples were then incubated at 95°C for 2 min. For syndecan-1 (40), total lysates were brought to 6 M urea and 50 mM sodium acetate (pH 4.5). They were boiled for 10 min and centrifuged to remove cell debris. DEAE-Sepharose beads were added to the supernatants, and the mixtures were rotated overnight at 4°C. The DEAE beads were washed with PBS containing 0.1% Triton X-100, and the bound proteoglycans were eluted from the beads with 1 M NaCl in PBS containing 0.1% Triton X-100 and then diluted with 20 mM Tris (pH 7.0) containing 5 mM $CaCl_2$ to a final concentration of 0.1 M NaCl. For syndecan-1, digestion with chondroitinase Tris-hydrochloride (0.2 unit/sample) was performed at 37°C for 2 h and terminated by the addition of 5× SDS sample buffer and heating at 95°C for 10 min, subjected to SDS-PAGE and transferred to Immobilon P membranes. Membranes were incubated for 90 min with an affinity-purified rabbit antirat glypican-1 antibody or a highly specific monoclonal mouse antihuman syndecan-1 antibody, washed, and incubated with a secondary antibody against rabbit IgG or mouse IgG for 60 min. Visualization was performed by enhanced chemiluminescence.

Glypican-1 and Syndecan-1 Purification. GAG-containing forms of glypican-1 and syndecan-1 were purified by anion exchange chromatography on DEAE-Sephacel equilibrated in buffer A [50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.1% Triton X-100], as reported previously (36). Cell lysates in buffer B [50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 μg/ml pepstatin A, and 1 mM PMSF] were loaded directly onto columns containing the gel using column volumes of 0.5 ml of packed gel per milligram of protein. Columns were eluted stepwise with buffer A, buffer C [50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, and 0.1% Triton X-100], buffer D [50 mM Tris-HCl (pH 8.0), 6 M urea, 0.25 M NaCl, and 0.1% Triton X-100], and buffer E [50 mM sodium formate (pH 3.5), 6 M urea, 0.2 M NaCl, and 0.1% Triton X-100]. After restoring the pH with 50 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100, gypican-1 or syndecan-1 was eluted from the column with buffer F [50 mM Tris-HCl (pH 8.0), 0.75 M NaCl, and 0.1% Triton X-100]. The eluted material was diluted 5-fold with 50 mM Tris (pH 8.0) and 0.1% Triton X-100 detergent, concentrated, and clarified by filtration (36). Samples were then resuspended in buffer B and analyzed by immunoblotting.

***In Situ* Hybridization.** To carry out *in situ* hybridization, tissue sections (4-μm thick) were placed on 3-aminopropyl-methoxysilane-coated slides, deparaffinized, and incubated at room temperature for 20 min with 0.2 N HCl and for 15 min with 50 μg/ml proteinase K at 37°C (36, 39). The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde and incubated briefly twice with PBS containing 2 mg/ml glycine and once in 50% (v/v) formamide/2× SSC for 1 h before initiation of the hybridization reaction by the addition of 100 μl of hybridization buffer. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 μg/ml yeast tRNA, 1× Denhardt's solution, 10% dextran sulfate, 40% formamide, and 100 ng/ml of the indicated digoxigenin-labeled riboprobe (36, 39). Hybridization was performed in a moist chamber for 18 h at 42°C. The sections were then washed sequentially with 50% formamide/2× SSC for 30 min at 42°C, 2× SSC for 20 min at 42°C, and 0.2× SSC for 20 min at 42°C.

For immunological detection, the Genius 3 nonradioactive nucleic acid detection kit was used as reported previously (36) with a 1:2000 dilution of alkaline phosphatase-conjugated polyclonal sheep antidigoxigenin Fab fragment antibody. Sections were incubated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution in a dark box for 3 h. After the reaction was stopped with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, the sections were mounted in aqueous mounting medium.

Cell Culture and Growth Assay. Human breast cancer cells were routinely grown in Leibovitz's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). To perform growth assays, MDA-MB-231 and MDA-MB-468 cells were plated overnight at a density of 10,000 cells/well in 96-well plates, washed in HBSS, and subsequently incubated in serum-free medium (Leibovitz's medium containing 0.1% BSA, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and antibodies) in the absence or presence of various growth factors. For experiments with PI-PLC, cells were incubated with the indicated concentrations of PI-PLC for 1 h. Subsequently, the medium was removed, and serum-free medium supplemented with PI-PLC and growth factors were added. Incubations were continued for 48 h before adding MTT (62.5 μg/well) for 4 h (41). Cellular MTT was solubilized with acidic isopropanol, and absorbance was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA). The results of the MTT assay correlated with results obtained by cell counting with a hemocytometer.

Stable Transfection. Stable transfection of G1-AS-1751 into MDA-MB-231 and MDA-MB-468 cells was performed using the lipofection method as described previously (34). Briefly, after reaching confluence, cells were split 1:10 into selection medium (complete medium supplemented with 1.5 mg/ml G418 on MDA-MB-231 cells and 0.5 mg/ml G418 on MDA-MB-468 cells), and single clones were isolated after 3–4 weeks. After expansion of individual clones, cells from each individual clone were screened for expression of glypican-1 sense and antisense mRNA by Northern blot analysis and for glypican-1 protein expression by immunoblotting. Parental MDA-MB-231 cells were also transfected with an empty expression vector carrying the neomycin resistance gene as a control. Positive clones were routinely grown in selection medium.

Statistics. Student's *t* test was used for statistical analysis of the experiments. $P < 0.05$ was taken as the level of significance. Results of MTT cell growth assays are expressed as the SE of at least three separate experiments.

RESULTS

Expression of Members of the Glypican Family and Syndecan-1 in Human Breast Tissue. Northern blot analysis was performed on total RNA isolated from 20 normal breast tissues and 20 breast cancer samples. The 3.7-kb glypican-1 mRNA transcript was of relatively low abundance in 6 of 20 normal breast tissue samples and below the level of detection in the other 14 samples. In contrast, 10 of 20 breast cancer samples exhibited moderate to high levels of glypican-1 mRNA. The glypican-3 mRNA transcript was expressed at moderate to high levels in 5 of 20 normal breast tissue samples. In the breast cancers, it was expressed at moderate to high levels in 6 of 20 samples. The glypican-4 mRNA transcripts were below the level of detection in the normal samples and present at low levels in 7 of 20 breast cancer samples. The 3.4- and 2.6-kb syndecan-1 mRNA transcripts were present at low levels in all 20 normal breast tissue samples. In contrast, in the breast cancers, both syndecan-1 transcripts were expressed at moderate to high levels in 9 of 20 samples. A representative Northern blot is shown in Fig. 1. Glypican-2 and -5 mRNA transcripts were below the level of detection in both normal and cancer samples. The same cDNAs used in present study are able to detect the presence of the corresponding glypican-2 and -5 mRNA transcripts in human brain RNA (36), indicating that the failure to detect these transcripts in breast tissues was not due to technical difficulties with these cDNAs.

Densitometric analysis of all of the autoradiographs indicated that in comparison with normal breast tissues, there was a 6.4-fold increase ($P < 0.005$) in glypican-1 mRNA levels in the breast cancer tissues (Fig. 2). There was also a 4.0-fold increase in glypican-3 mRNA levels, but this difference failed to achieve statistical significance ($P = 0.0583$). However, a subgroup of patients had relatively high levels of glypican-1 (10 cases; $P < 0.001$) or glypican-3 (6 cases; $P < 0.01$). There was also a 3.5-fold increase ($P < 0.005$) in syndecan-1 mRNA levels in the breast cancer samples (Fig. 2). Furthermore, a subgroup of patients had relatively high levels of syndecan-1 (nine cases; $P < 0.001$). Interestingly, 8 of 10 tumor samples that exhibited high glypican-1 mRNA levels (Fig. 2, ■) also exhibited high syndecan-1 mRNA levels (Fig. 2, ♦). Furthermore, 8 of 10 tumors with stage 2 or 3 disease exhibited high levels of glypican-1 and syndecan-1 by Northern blot analysis. In contrast, low levels of glypican-1 mRNA were evident in only 1 of 10 tumors with stage 2 or 3 disease, but in 9 of 10 tumors with stage 1 disease.

Immunohistochemistry and *in Situ* Hybridization. Immunohistochemical analysis of five normal tissues using the same highly specific anti-glypican-1 antibody did not reveal any glypican-1 immunoreactivity (Fig. 3*A*). In contrast, five of five cancer tissues exhibited moderate to strong glypican-1 immunoreactivity in the intraductal carcinoma cells. This immunoreactivity was most marked in cancer cells that had a distorted morphology with prominent nuclei and abundant cytoplasm (Fig. 4*A*, outlined by *arrowheads*). In contrast, the small cancer cells that had a more differentiated lobular architecture exhibited faint glypican-1 immunoreactivity (Fig. 4*A*). Moderate to strong glypican-1 immunoreactivity was also present in the fibroblasts surrounding the cancer cells.

In situ hybridization analysis using a highly specific riboprobe was next carried out on serial sections in the same tissue samples to delineate the sites of expression of glypican-1. Ductal cells in the normal breast tissue did not exhibit an *in situ* hybridization signal (Fig. 3*B*). In contrast, a weak to moderate glypican-1 mRNA *in situ* hybridization signal was evident in the intraductal carcinoma cells (Fig. 4*B*). The surrounding fibroblasts also exhibited a faint glypican-1 mRNA signal, whereas the cancer cells that exhibited the more differentiated lobular architecture exhibited a more intense glypican-1 mRNA *in situ* hybridization signal (Fig. 4*B*). A second example of a well-differentiated cancer sample (H&E staining) is shown in Fig. 4*D*. *In situ* hybridization of serial sections revealed moderate glypican-1 mRNA signal in the cancer cells, whereas the surrounding fibroblasts exhibited a weak glypican-1 mRNA signal (Fig. 4*E*). A glypican-1 sense probe did not reveal any specific signal (Fig. 4, *C* and *F*).

Glypican Expression in Human Breast Cancer Cell Lines. To determine whether cultured breast cancer cells express any glypicans,

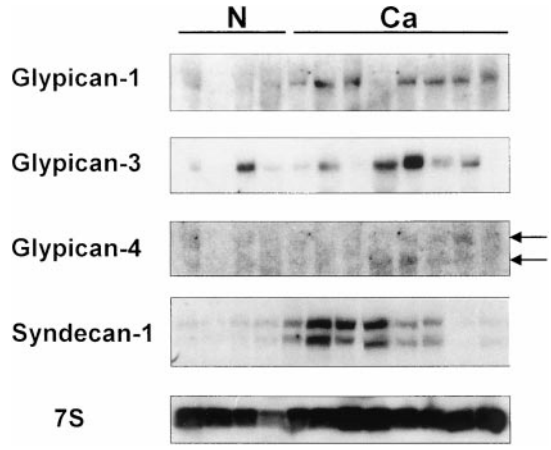

Fig. 1. Northern blot analysis of glypicans and syndecan-1 in breast tissues. Total RNA (20 μg/lane) from four normal breast tissues (*N*) and eight breast cancers (*Ca*) was subjected to Northern blot analysis using ^{32}P-labeled glypican-1, glypican-3, glypican-4, and syndecan-1 cDNA probes (500,000 cpm/ml). A 7S ribosomal cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure times were 1 day for glypican-1, glypican-3, and syndecan-1; 2 days for glypican-4; and 6 h for 7S. *Arrows* indicate the two glypican-4 mRNA transcripts (3.4 and 4.6 kb).

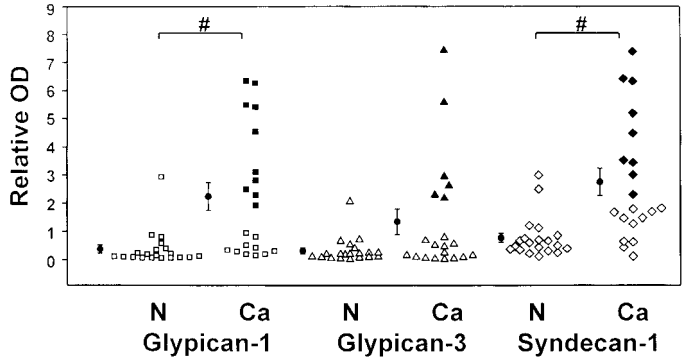

Fig. 2. Relative expression of glypican-1, glypican-3, and syndecan-1 mRNA. Autoradiographs of Northern blots for glypican-1, glypican-3, syndecan-1, and 7S RNA from 20 normal and 20 cancerous breast tissue samples were analyzed by densitometry, and the level of glypican-1, glypican-3, or syndecan-1 expression was calculated as the ratio of glypican-1 (*square*), glypican-3 (*triangle*) or syndecan-1 (*diamond*) and the corresponding 7S RNA. *Closed symbols* represent groups of breast cancer tissues with high expression of glypican-1 (■), glypican-3 (▲), and syndecan-1 (◆). Data are expressed as the mean scores ± SD. The mean glypican-1 and syndecan-1 scores of the cancerous samples were significantly greater than the respective mean values from normal breast tissue samples (#, $P < 0.005$).

total RNA was isolated from two breast cancer cell lines. Northern blot analysis revealed relatively high levels of glypican-1 mRNA in both cell lines (MDA-MB-231 and MDA-MB-468; Fig. 5*A*). In contrast, glypican-2, -3, -4, and -5 were below the level of detection by Northern blot analysis in both cell lines. Immunoblotting with a highly specific anti-glypican-1 antibody revealed the presence of a 55-kDa band corresponding to glypican-1 in total lysates from both cell lines (Fig. 5*B*). Heparitinase treatment of the lysates was not required for demonstrating the 55-kDa protein. To assess whether there was a membrane-bound component, membrane preparations (30 μg) were digested with heparitinase and subjected to SDS-PAGE followed by immunoblotting. The 55-kDa band was also observed in the membrane samples (Fig. 5*B*). To determine whether glypican-1 is released by breast cancer cells, conditioned serum-free medium from MDA-MB-231 and MDA-MB-468 cells was collected during a 48-h incubation and subjected to anion exchange chromatography to isolate HSPGs. Immunoblotting revealed the presence of the 55-kDa band representing the glypican-1 core protein (Fig. 5*B*). Because glycanated glypican-1 migrates as a more compact band in the presence of heparitinase, the signal was more intense in heparitinase-digested samples of both cells. These results confirm that both cells release glycanated glypican-1 into the culture medium.

To further confirm the identity of the 55-kDa protein as glypican-1, membrane samples (30 μg) were subjected to heparitinase digestion and analyzed by SDS-PAGE under reducing and nonreducing conditions. The 55-kDa band that was observed under reducing conditions migrated as a band of ~48 kDa under nonreducing conditions (Fig. 6). This shift is characteristic of the core proteins of glypicans because these proteins have many disulfide bonds and migrate more rapidly under nonreducing conditions than under reducing conditions (40).

Syndecan-1 Expression in Human Breast Cancer Cell Lines. Next we determined whether cultured breast cancer cells express syndecan-1. Northern blot analysis revealed relatively high levels of syndecan-1 mRNA in MDA-MB-468 cells and lower levels of both transcripts in MDA-MB-231 cells (Fig. 7*A*). Immunoblotting of total lysates from both cell lines with a highly specific anti-syndecan-1 antibody revealed the presence of a very faint 200-kDa band corresponding to syndecan-1 (data not shown). Immunoblotting of serum-free conditioned medium subjected to anion exchange chromatography also revealed the presence of the 200-kDa syndecan-1 for both cell lines (Fig. 7*B*). However, syndecan-1 was more abundant in conditioned medium for MDA-MB-468 cells by comparison with medium from MDA-MB-231 cells.

Effects of PI-PLC on Growth Factor Action in Breast Cancer Cells. To determine whether glypicans regulate growth factor action in breast cancer cells, MDA-MB-231 and MDA-MB-468 cells were incubated in the absence or presence of PI-PLC. This enzyme cleaves glypicans and other proteins that associate with membranes via a covalent GPI lipid linkage. In both cells (Fig. 8), IGF-I, HB-EGF, and FGF-2 induced cell proliferation. Preincubation of either cell line with PI-PLC (0.5 unit/ml) and subsequent incubation with the same concentrations of each growth factor in the presence of PI-PLC (0.1 unit/ml) completely blocked the stimulatory effect of HB-EGF and FGF-2. In contrast, in both cell lines, PI-PLC had no significant effect on the growth-stimulatory actions of IGF-I, which is not a heparin-binding growth factor (Fig. 8).

Effects of Glypican-1 Antisense Levels on Growth Factor Responsiveness. Because PI-PLC can remove many GPI-anchored proteins from the cell surface, we next sought to determine whether it is possible to modulate responsiveness to heparin-binding growth factors by altering endogenous glypican-1 protein levels. Accordingly, we transfected MDA-MB-231 and MDA-MB-468 cells with a glypican-1 antisense construct (G1-AS-1751). Northern blot analysis of total RNA using a glypican-1 sense riboprobe revealed high levels of glypican-1 antisense mRNA in MDA-MB-231 and MDA-MB-468 clones, whereas the parental cells and sham-transfected MDA-MB-231 cells did not exhibit a glypican-1 antisense mRNA transcript (Fig. 9*A*). Analysis with the antisense probe revealed that glypican-1 mRNA was expressed in parental and sham-transfected cells but was present at very low levels in the antisense-transfected clones (Fig. 9*B*). There was also a marked decrease in the 55-kDa glypican-1 protein in these clones, as determined by immunoblotting (Fig. 9*C*). On the other hand, the transfection of glypi-

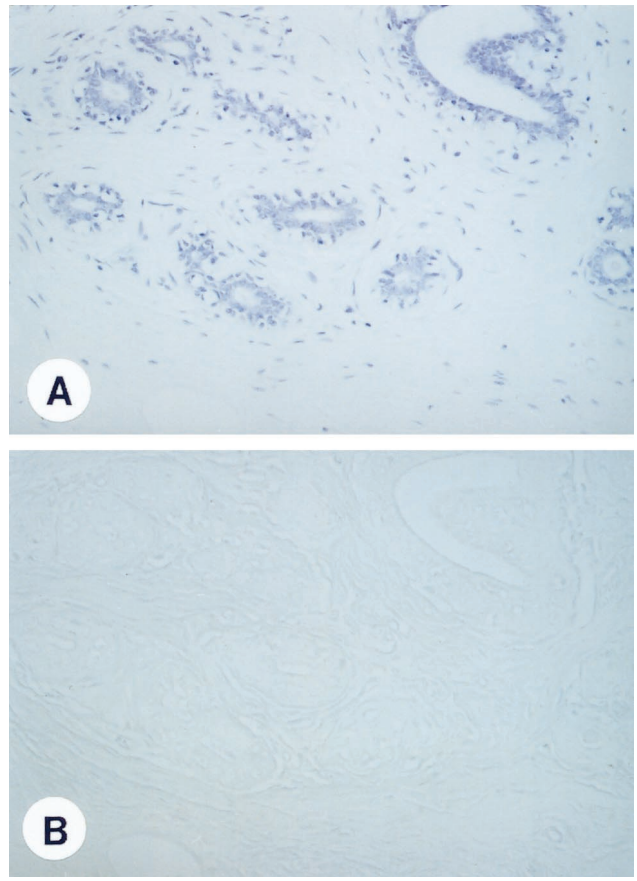

Fig. 3. Glypican-1 expression in the normal breast tissue. *A*, immunostaining did not yield any glypican-1 immunoreactivity in the connective tissue cells or in the terminal ductal-lobular unit. *B*, *in situ* hybridization analysis of serial section did not yield any specific signals. Magnification, ×200.

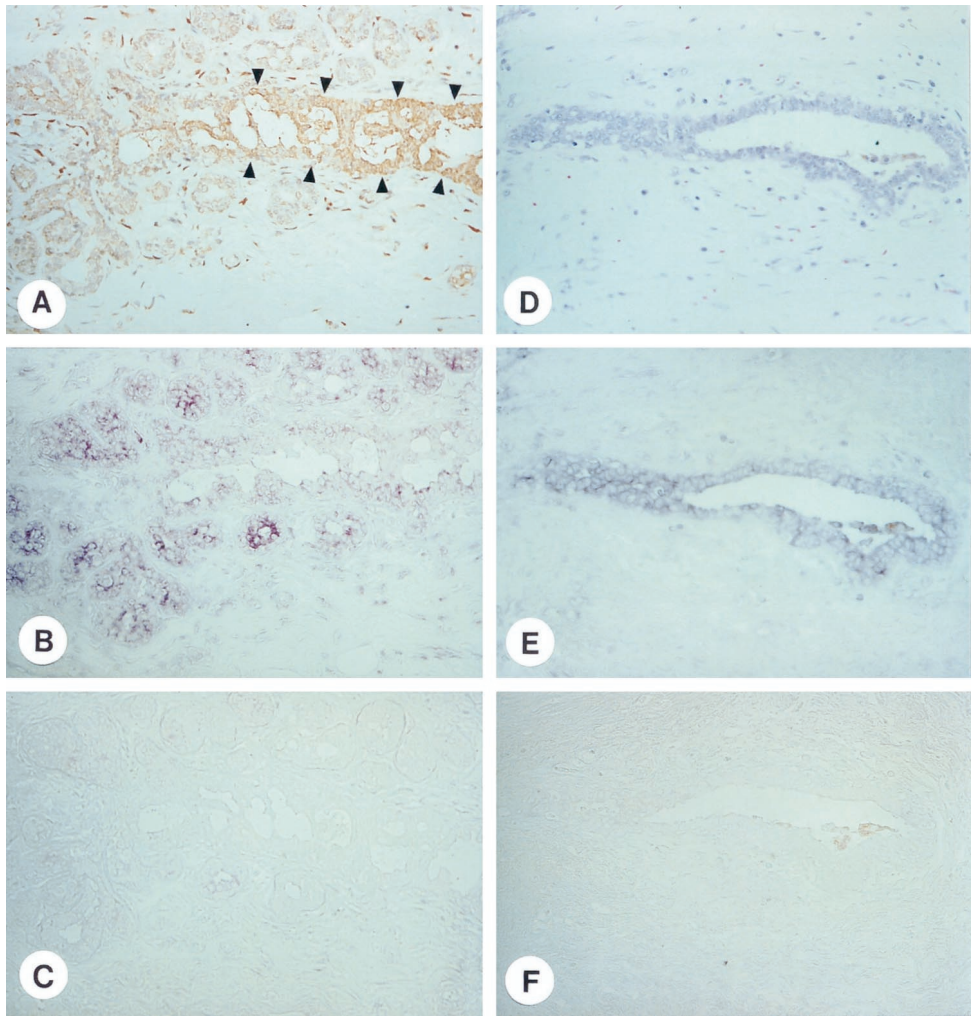

Fig. 4. Glypican-1 localization in human breast cancer tissue. *A*, immunostaining revealed moderate to strong glypican-1 immunoreactivity in the intraductal carcinomas and adjacent fibroblasts. *B*, *in situ* hybridization analysis of serial sections revealed a moderate to strong glypican-1 mRNA signal in the small cancer cells that had a lobular architecture. *D*, H&E-stained section. *E*, *in situ* hybridization analysis of serial sections revealed a moderate glypican-1 mRNA signal in the cancer cells. *C* and *F*, hybridization with the glypican-1 sense probe did not yield any specific signals. Magnification, ×200.

can-1 antisense did not change the level of syndecan-1 on Northern blot analysis (Fig. 9*D*). Other glypican family members were below the level of detection by Northern blot analysis in the parental cells, sham-transfected cells, and all clones. The decreased expression of glypican-1 was associated with a marked attenuation of the growth-stimulatory effects of HB-EGF, HRG-α, HRG-β, FGF-2, and HGF (Fig. 10). In contrast, the growth-stimulatory action of IGF-I was similar in parental, sham-transfected, and glypican-1 antisense-transfected MDA-MB-231 and MDA-MB-468 cells (Fig. 10).

DISCUSSION

HSPGs are present on the surfaces of nearly all adherent cells, where they bind a number of heparin-binding proteins, including growth factors, extracellular matrix molecules, cell-cell adhesion molecules, and molecules involved in several degradative pathways (42–44). HSPGs belong primarily to two families of molecules, syndecans, which are transmembrane proteins, and glypicans, which are attached to the plasma membrane via GPI anchors (25). In breast cancer, there are several reports that HSPGs could be responsible for differences in their proliferative and invasive properties (45, 46). HSPGs are known to interact with FGF-2, keratinocyte growth factor, vascular endothelial growth factor, HB-EGF, and HGF (47–53). HGF and FGF are known to regulate the morphogenesis and differentiation of mammary epithelial cells, and HSPGs are likely to be important regulators of the development of the gland (54, 55). Moreover, HSPGs exhibit a differential pattern of distribution in normal and malignant breast epithelial cells, and this difference in HSPG distribution correlates with differences in sensitivity to FGF-2 (45, 56). Together, these observations suggest that altered expression and function of HSPGs may contribute to the aberrant growth of breast cancer cells.

In this study, we determined that a significant percentage of human breast cancers express relatively high levels of several glypicans and syndecan-1. By Northern blot analysis, there was a significant 6.4-fold increase in glypican-1 mRNA levels in the cancer tissues by comparison with the normal controls. There was also a slight increase in glypican-3 and -4 mRNA levels in the cancer samples. The overall increase in glypican-3 mRNA levels in the cancer samples almost achieved statistical significance. Furthermore, a subgroup of the breast cancer samples exhibited a significant increase in glypican-3 mRNA levels by comparison with the mean level in the normal samples. Glypican-2 and -5 mRNA transcripts were below the level of detection in both the normal and cancer samples. Syndecan-1 mRNA levels were also significantly increased (3.5-fold) in the cancer tissues by comparison with the normal controls. There was a remarkably good correlation between glypican-1 and syndecan-1 expression in the tumors. Thus, 8 of 10 tumors with high glypican-1 mRNA levels also exhibited high syndecan-1 mRNA levels. Furthermore, 8 of 10 tumors with stage 2 or 3 disease exhibited high levels of glypican-1 and syndecan-1 by Northern blot analysis. In contrast, low levels of glypican-1 and syndecan-1 mRNA were evident in only 1 tumor with stage 2 disease, but in 9 of 10 tumors with stage 1 disease. Taken together, these observations suggest that glypican-1 and

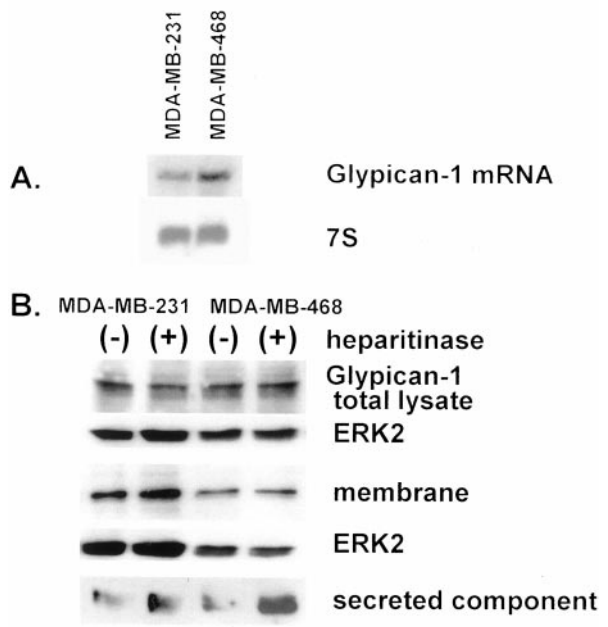

Fig. 5. Glypican-1 expression in breast cancer cell lines. *A*, Northern blots of total RNA (20 μg/lane) isolated from MDA-MB-231 and MDA-MB-468 cells were hybridized with ^{32}P-labeled glypican-1 cDNA probe (500,000 cpm/ml) and with a 7S cDNA probe (50,000 cpm/ml). Exposure times were 1 day for glypican-1 and 6 h for 7S. *B*, immunoblotting with anti-glypican-1 antibody (250 ng/ml) was performed using 30 μg of total lysates, 30 μg of membrane proteins, or 50 μl of serum-free medium (48 h incubation) subjected to anion exchange chromatography. All samples were incubated in the absence (−) and presence (+) of heparitinase. Equal loading was verified by immunoblotting for extracellular signal-regulated kinase 2.

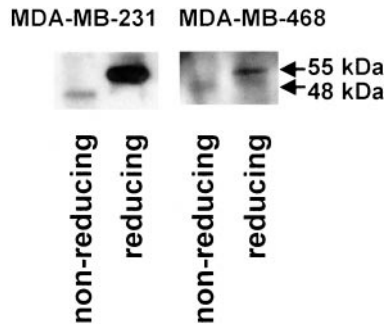

Fig. 6. Effects of reducing condition on glypican-1 migration pattern. Immunoblotting with anti-glypican-1 antibody was performed using 30 μg of total cell lysates prepared from MDA-MB-231 and MDA-MB-468 cells. Lysates were subjected to heparitinase digestion and analyzed by SDS-PAGE under nonreducing and reducing conditions as described in "Materials and Methods."

syndecan-1 may have the potential to contribute to the growth advantage of breast cancer cells in patients with a more advanced stage of this disease.

By immunohistochemistry, moderate to strong glypican-1 immunoreactivity was present in the poorly differentiated intraductal cancer cells and the adjacent fibroblasts, whereas faint glypican-1 immunoreactivity was present in the more well-differentiated lobular carcinoma cells. In sharp contrast with the immunostaining results, a moderate to strong glypican-1 mRNA *in situ* hybridization signal was present in the well-differentiated lobular cancer cells, whereas a weak *in situ* hybridization signal was present in the more poorly differentiated cancer cells and the adjacent fibroblasts. These observations suggest that glypican-1 synthesis is decreased in the more poorly differentiated breast cancer cells by comparison with the more well-differentiated cells and that breast cancer cells are able to secrete glypican-1 *in vivo*. However, the released glypican-1 may preferentially associate with the poorly differentiated cancer cells and the neighboring fibroblasts. In support of this hypothesis, both breast cancer cell lines examined in the present study were found to express and secrete glypican-1. Alternatively, glypican-1 degradation by these cell types may be markedly attenuated. Syndecan-1 may also be secreted by the breast cancer cells *in vivo*, because both cell lines also secreted syndecan-1 and because syndecan-1 immunoreactivity is abundant in the stroma surrounding infiltrating ductal breast carcinomas (46). Thus, the present findings are somewhat different from our previous results with pancreatic ductal adenocarcinomas, in which the cancer cells often exhibit strong glypican-1 immunoreactivity as well as a strong *in situ* hybridization signal (36).

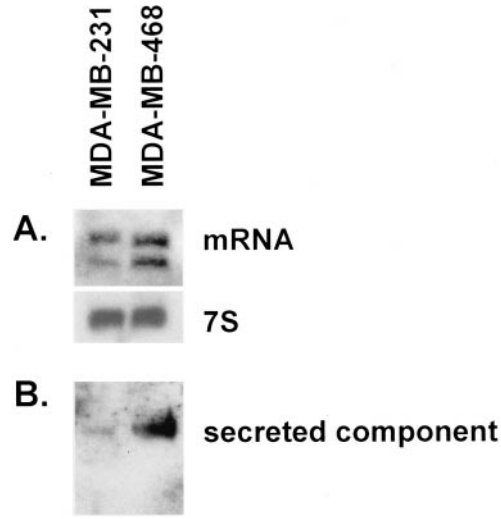

Fig. 7. Syndecan-1 expression in breast cancer cell lines. *A*, Northern blots of total RNA (20 μg/lane) isolated from MDA-MB-231 and MDA-MB-468 cells were hybridized with ^{32}P-labeled syndecan-1 cDNA probe (500,000 cpm/ml) and with a 7S cDNA probe (50,000 cpm/ml). Exposure times were 1 day for syndecan-1 and 6 h for 7S. *B*, MDA-MB-231 and MDA-MB-468 cells were incubated for 48 h in serum-free medium. The medium (100 ml) was then subjected to anion exchange chromatography as described in "Materials and Methods." Immunoblot analysis was carried out with an anti-syndecan-1 antibody.

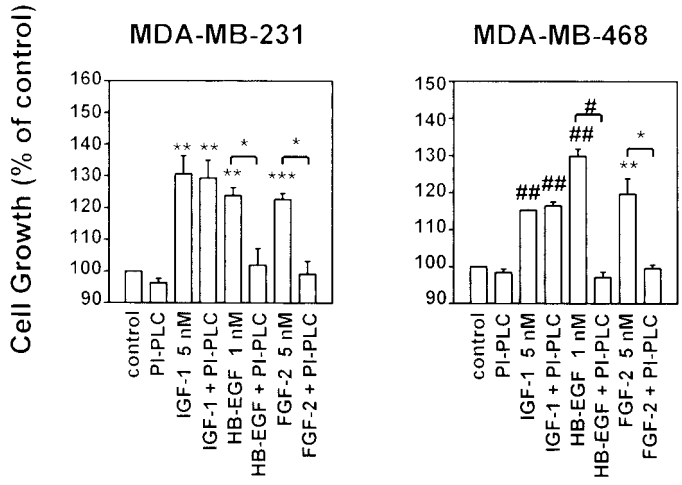

Fig. 8. Effects of cleavage of GPI anchors on growth factor action in breast cancer cells. MDA-MB-231 and MDA-MB-468 cells were incubated with the indicated concentrations of IGF-I, HB-EGF, and FGF-2 in the absence or presence of PI-PLC as described in "Materials and Methods." Cell growth was determined by the MTT assay. Data are expressed as the percentage of increase or decrease of the respective untreated controls and are the means ± SE of eight determinations per experiment from three separate experiments (#, $P < 0.001$; *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.01$; ##, $P < 0.005$, as compared with control).

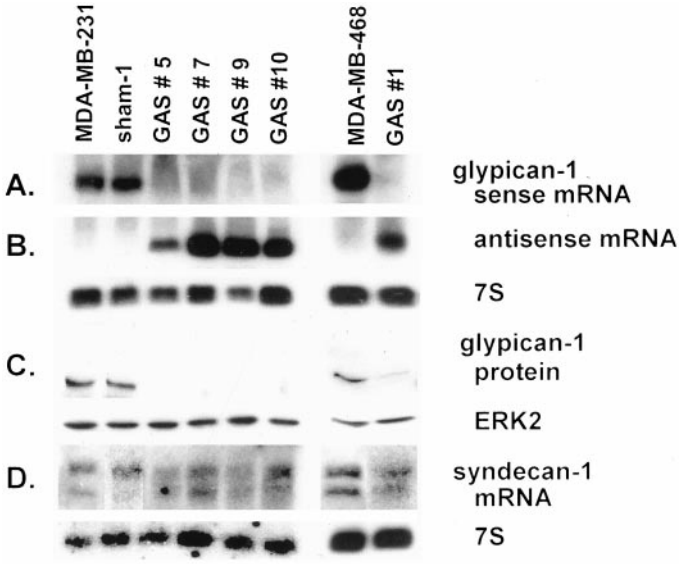

Fig. 9. Expression of glypican-1 and syndecan-1 in MDA-MB-231 and MDA-MB-468 clones. Total RNA (20 μg/lane) isolated from the indicated cell lines was hybridized with a ^{32}P-labeled glypican-1 sense riboprobe (*A*) and with a glypican-1 antisense riboprobe (*B*). Equal loading was verified by hybridizing with a 7S cDNA probe. Exposure times were 10 h for glypican-1 antisense and sense riboprobe and 6 h for 7S. *C*, immunoblotting of total cell lysates (30 μg/lane) of parental and glypican-1 antisense-transfected MDA-MB-231 and MDA-MB-468 clones was performed with the glypican-1 antibody (250 ng/ml). Equal loading was verified by immunoblotting for extracellular signal-regulated kinase 2. *D*, total RNA (20 μg/lane) isolated from MDA-MB-231 and MDA-MB-468 cells and clones was hybridized with a ^{32}P-labeled syndecan-1 cDNA probe. Equal loading was verified by hybridizing with a 7S cDNA probe. Exposure times were 1 day for syndecan-1 and 6 h for 7S.

MDA-MB-231 and MDA-MB-468 breast cancer cell lines expressed glypican-1 on the cell surface, as determined by immunoblotting of solubilized membrane preparations. The presence of glypican-1 on the cell surface suggested that it may enhance the interaction of heparin-binding growth factors with their high-affinity receptors. Indeed, treatment of cells with PI-PLC, an enzyme that removes surface-bound glypicans by cleaving GPI-anchored proteins, abrogated the mitogenic effects of HB-EGF and FGF-2 in both cell lines. These observations suggest that the mitogenic effects of these growth factors are dependent on the presence of surface-bound glypican-1. However, PI-PLC might also remove other GPI-anchored proteins from the cell surface. Therefore, we also generated clones of stably transfected MDA-MB-231 and MDA-MB-468 cells expressing a glypican-1 antisense construct. These clones exhibited a marked decrease in endogenous glypican-1 mRNA and protein level and a marked attenuation of the mitogenic response to several heparin-binding growth factors (HB-EGF, HRG-α, HRG-β, FGF-2, and HGF) that act through four distinct tyrosine kinase receptors (57). In contrast, IGF-I, which is not a heparin-binding growth factor, exerted similar mitogenic effects in parental, sham-transfected, and glypican-1 antisense-transfected cells.

Two lines of evidence suggest that syndecan-1 is not as crucial as glypican-1 for the activation of mitogenic signaling by heparin-binding growth factors in breast cancer cells. First, syndecan-1 is not a GPI-anchored molecule and is therefore not removed from the cell surface by PI-PLC treatment. However, the presence of syndecan-1 after treatment with PI-PLC was not capable of conferring responsiveness to HB-EGF and FGF-2 in either MDA-MB-231 or MDA-MB-468 cells. Second, MDA-MB-231 and MDA-MB-468 clones expressing the glypican-1 antisense construct did not exhibit a decrease in syndecan-1, yet they were no longer responsive to multiple heparin-binding growth factors. The extracellular domain of syndecan-1 directly regulates cell motility and invasiveness (40), and induction of stromal syndecan-1, coupled with decreased syndecan-1 expression in malignant cells, may promote the metastatic phenotype of infiltrating ductal breast carcinoma (46). Together, these observations suggest that syndecan-1 may play a critical role in motility and invasiveness in breast cancer, whereas glypican-1 may act to enhance the growth-promoting effects of heparin-binding growth factors in breast cancer cells.

Our findings raise the possibility that pharmacological or molecular interventions that interfere with glypican-1 and syndecan-1 function or expression may have a therapeutic role in breast cancer. In addition, the ability of breast cancer cells to synthesize and secrete glypican-1 and syndecan-1 at high levels raises the possibility that glypican-1 and

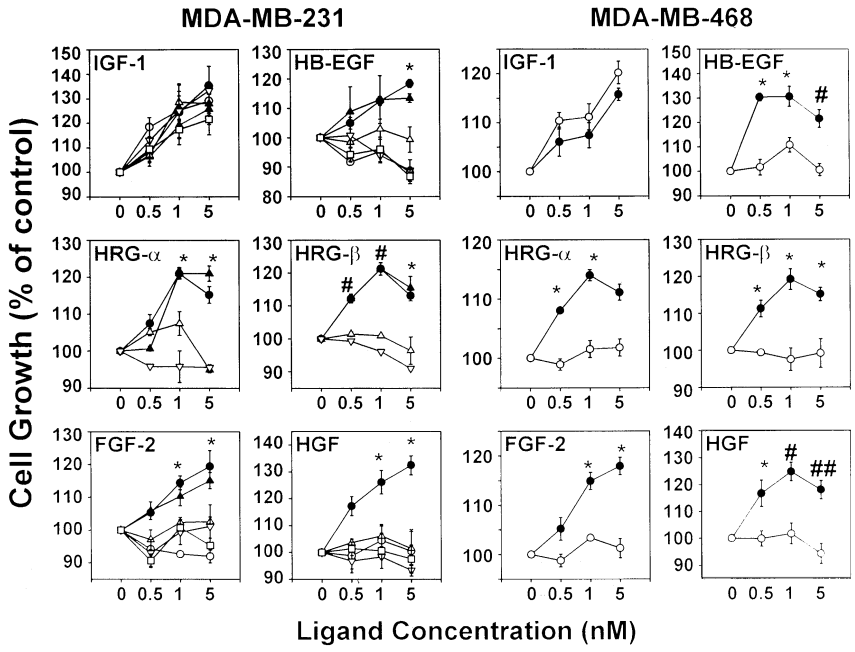

Fig. 10. Effects of decreased endogenous glypican-1 levels on growth factor responsiveness. Parental (●), sham-transfected (▲), and glypican-1 antisense mRNA-expressing MDA-MB-231 and MDA-MB-468 clones (*open symbols*) were incubated for 48 h with the indicated concentrations of IGF-I, HB-EGF, HRG-α, HRG-β, FGF-2, and HGF. Cell growth was determined by the MTT assay. Data are expressed as the percentage of change from unstimulated controls and are the means ± SE of eight determinations per experiment from three separate experiments (##, $P < 0.001$; #, $P < 0.005$; ∗, $P < 0.05$, as compared with the respective controls).

syndecan-1 may be present in the serum of breast cancer patients and may serve as a tumor marker for this malignancy.

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