Hyaluronan Constitutively Regulates ErbB2 Phosphorylation and Signaling Complex Formation in Carcinoma Cells*

Shibnath Ghatak‡§, Suniti Misra‡, and Bryan P. Toole¶

From the Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425

Hyaluronan is enriched in many types of human cancers, and manipulations of hyaluronan expression or interactions have a major influence on tumor progression in animal models. Increased ErbB2 activity is characteristic of several cancers and is responsible for many aspects of malignant cell behavior in these cancers. In this study we show that constitutively high levels of active, i.e. autophosphorylated, ErbB2 in HCT116 colon carcinoma cells and TA3/St mammary carcinoma cells are dependent on endogenous hyaluronan-CD44 interaction. Dependence on hyaluronan-CD44 interaction was demonstrated by the administration of hyaluronan oligomers, experimentally induced expression of soluble CD44, and small interfering RNA knockdown of CD44 expression. On the other hand, increasing hyaluronan production by overexpression of hyaluronan synthase 2 or emmprin causes elevated ErbB2 phosphorylation in MCF-7 mammary carcinoma cells, which normally exhibit low levels of ErbB2 activity. Furthermore, in HCT116 and TA3/St cells, inhibition of endogenous hyaluronan-CD44 interaction causes disassembly of a constitutive, lipid raft-associated, signaling complex containing phosphorylated ErbB2, CD44, ezrin, phosphoinositide 3-kinase, and the chaperone molecules Hsp90 and cdc37. Stimulation of hyaluronan production in MCF-7 cells induces assembly of this complex. We conclude that hyaluronan regulates ErbB2 activity and its interactions with other signaling factors in carcinoma cells.

Hyaluronan is a multifunctional polysaccharide that is present in the extracellular and pericellular matrices of most tissues. Hyaluronan is enriched in matrices surrounding cells that are proliferating or migrating and influences cell behavior during embryonic development (1) and in cancer (2). Like most extracellular matrix macromolecules, hyaluronan has important structural and signaling functions. The signaling functions of hyaluronan are mediated by cell surface receptors, especially CD44 and RHAMM (3). Analysis of the Has2-1 null mouse has shown that hyaluronan is essential for ErbB2 (HER2) signaling during endocardial-cushion mesenchyme transformation and for the onset of mesenchymal cell invasiveness (4, 5). Likewise, biochemical studies have demonstrated that exogenously added hyaluronan promotes ErbB2 signaling in ovarian carcinoma cells via interactions between CD44, Grb2, Vav2, and ErbB2 (6). In previous studies we have shown that interference with endogenous hyaluronan-CD44 interactions inhibits constitutive PI 3-kinase/Akt signaling in mammary carcinoma, colon carcinoma, and glioma cells (7–9). Also, studies from several laboratories have shown that treatment of cells with exogenous hyaluronan (10, 11) or experimental up-regulation of hyaluronan synthesis (8, 12) promotes this signaling pathway. Because ErbB2 activity is elevated in many malignant cancer cell types and because it regulates the PI 3-kinase/Akt pathway (13, 14), we investigated whether hyaluronan constitutively regulates ErbB2 activity and influences the interactions of ErbB2 with other signaling components.

EXPERIMENTAL PROCEDURES

Materials—Hyaluronan oligosaccharides (oligomers) (Anika Therapeutics, Inc., Woonsocket, MA) that were used in this study were a mixed fraction of average molecular weight ∼2.5 × 10^5 composed of 3–10 disaccharide units. No contaminants were detected in these oligomers by high pressure liquid chromatography or capillary electrophoresis. Assays for other glycosaminoglycans, protein, nucleic acids, and endotoxins were also negative (7, 15). Antibodies against total ErbB2 (extracellular domain), phosphorylated ErbB2, p85 subunit of PI 3-kinase, ezrin, and Hsp90 were from Upstate Biotechnology, Lake Placid, NY. Antibodies against human CD44 were from Calbiochem (catalog number 217594) or from Santa Cruz Biotechnology, Inc.; antibody against p110α subunit of PI 3-kinase was from Santa Cruz Biotechnology, Inc., and antibody against mouse CD44 (KM21) was from ATCC (Manassas, VA). Antibody against cdc37 was a gift from Dr. Nicholas Grammatikakis (16). The secondary antibodies used were ECL anti-rabbit IgG (peroxidase-linked species-specific F(ab')2 fragment from donkey) and ECL anti-mouse IgG (peroxidase-linked species-specific F(ab')2 fragment from sheep) from Amersham Biosciences. Enhanced chemiluminescence reagents (Western Lightning Chemiluminescence Reagent Plus) were from PerkinElmer Life Sciences. Unless specified, all other reagents were the highest grade from Sigma.

Cell Culture—HCT 116 colon carcinoma cells were obtained from Dr. B. Vogelstein, Johns Hopkins Medical School. TA3/St mammary carcinoma cells were provided by Dr. H. F. Dvorak, Harvard Medical School, and maintained in our laboratory (17). Stable transfectants of TA3/St cells, overexpressing soluble CD44, mutated soluble CD44, or vector only, were prepared as described previously (18). The soluble CD44 does not contain transmembrane or cytoplasmic domains (19). MCF-7 cells were purchased from ATCC. All cell lines were routinely maintained in complete medium, i.e. Dulbecco’s modified Eagle’s medium-high glucose (Invitrogen), containing 10% fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml, and an additional 1 μM glutamine (Invitrogen). The cell lines were maintained at 37 °C in 5% CO₂ and passed every 3–4 days.

Adenoviruses and Cell Transfection—Recombinant adenoviruses, tritin; MOPS, 4-morpholinepropanesulfonic acid; o-HA, hyaluronan oligomers; pErbB2, phosphorylated ErbB2.
driving expression of β-galactosidase, Has2, emmprin, or soluble CD44, were prepared as described previously (9, 12, 20, 21). Cells were plated in six-well plates and allowed to grow under routine conditions until 70% confluent. The growth medium was then replaced with serum-free medium. The cells were infected with the adenovirus constructs at 200–300 multiplicity of infection per cell by incubation for 90 min with intermittent gentle rocking. The virus-containing medium was removed and replaced with 3 ml per well of complete serum-containing medium. The cultures were then incubated at 37 °C overnight and transferred to 150-mm dishes for a further 24-h incubation before processing for lysis, immunoprecipitation, and Western blotting.

RNA Silencing—siRNA for human CD44 was designed from sequences of human CD44 obtained from the NCBI data base using programs available on-line (NCBI accession numbers AH003670, AY101193, U40373, M59040, and M24915). We chose the sequence, 5'-GAACGAAUCCUGAAGACAUCU-3' (sense strand), after a BLAST search of the data base. The double-stranded siRNA was prepared by Dharmacon, Inc. (Lafayette, CO). The nonspecific control IX RNA (cat.

FIG. 1. Hyaluronan oligomers inhibit constitutive ErbB2 phosphorylation. HCT116 human colon carcinoma and TA3/St mouse mammary carcinoma cells were incubated for 24 h with 100 µg/ml hyaluronan oligomers (o-HA). The cell layer was lysed and immunoprecipitated with antibodies against total ErbB2 (A), CD44 (B), and the p85 subunit of PI 3-kinase (C). Aliquots of the redissolved immunoprecipitates were subject to SDS-PAGE and Western blotting using antibodies against phosphorylated ErbB2 (pErbB2) and total ErbB2 (ErbB2). IP, immunoprecipitate; WB, Western blot.

FIG. 2. Antagonists of endogenous hyaluronan interactions inhibit constitutive ErbB2 phosphorylation. A, HCT116 and TA3/St cells were treated with and without o-HA in the same fashion as described in Fig. 1 in three separate experiments. The cells were lysed and immunoprecipitated with antibodies against ErbB2 or p85 subunit of PI 3-kinase and then electrophoresed and immunoblotted with antibody against pErbB2. The bands were analyzed by densitometry, and the data are presented as means of the three experiments ± standard error. B, HCT116 cells were treated with a recombinant adenovirus driving expression of β-galactosidase (as control, bar 1) or soluble CD44 (bar 2) as described in the text. TA3/St cells were stably transfected with vector only (bar 3) or with cDNA constructs for soluble CD44 mutated in the hyaluronan-binding domain (bar 4) or intact soluble CD44 (bar 5) as described previously (18). The cells were then processed for immunoprecipitation with antibody against ErbB2, Western blotting with antibody against phosphorylated ErbB2, and densitometry, as in A. Similar results were obtained when immunoprecipitation was carried out with antibody against the p85 subunit of PI 3-kinase (data not shown). C, HCT116 cells were transfected with siRNA against CD44 or with control RNA and then processed for immunoprecipitation with antibody against ErbB2, Western blotting with antibody against phosphorylated ErbB2, and densitometry. Similar results were obtained when immunoprecipitation was carried out with antibody against the p85 subunit of PI 3-kinase (data not shown). No significant changes in total ErbB2 levels were observed in any of the experiments described in A, B, or C. D, diagram showing the postulated action of each of the reagents used in A, B, and C.
HCT116 cells were transfected with the siRNA in 2 ml of complete medium in six-well plates with cells at 70–90% confluence. Transfections were performed with 200 pmol siRNA using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. The cells were then incubated at 37 °C in 5% CO2 for 24 h, replated in 150-mm dishes, and allowed to grow for 48 h in complete medium. The cells were then harvested and processed for lysis, immunoprecipitation, and Western blotting.

**Cell Lysis, Immunoprecipitations, and Western Blotting**—Cells were treated with versene solution (Invitrogen) at 37 °C for 15 min and then collected as a pellet by centrifugation at 4 °C. After washing the cells twice in ice-cold phosphate-buffered saline, they were lysed in buffer containing 1% (v/v) Nonidet P-40, 0.5 mM EGTA, 5 mM sodium orthovanadate, 10% (v/v) glycerol, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, and 50 µM HEPES, pH 7.5. Lysis was carried out by vortexing for three cycles of 15 s at high speed followed by cooling on ice. The lysate was clarified by centrifugation at 12,000 × g for 15 min at 4 °C in an Eppendorf 5415R centrifuge. An aliquot was kept for protein determination; the rest was flash-frozen in liquid nitrogen and stored at −80 °C until use.

For immunoprecipitations, all operations were done at 4 °C unless otherwise mentioned. Cell lysates were diluted to 1 µg protein/µl using the lysis buffer above. Aliquots of 500 µl of lysate were mixed with 5 µl of antibody against ErbB2, p85 subunit of PI 3-kinase, or CD44 and incubated for 2 h while rotating on a wheel. The immune complex was captured by adding 80 µl of 1:1 (v/v) protein A-Sepharose 4B suspension and incubated for another 1 h. The Sepharose 4B beads were collected by brief centrifugation followed by washing three times in ice-cold lysis buffer, three times with lithium chloride buffer (5 mM LiCl, 0.1 mM sodium orthovanadate, 0.1 mM Tris-Cl, pH 7.4), and three times with buffer containing 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM Tris-Cl, pH 7.4. Finally, the immune complexes were recovered from the beads in 50 µl of SDS-containing denaturing buffer and heated to 65 °C for 5 min.

For SDS-PAGE, the denatured immunoprecipitates were loaded onto a 10% polyacrylamide gel at 15–30 µg of protein per lane in an Invitrogen minigel apparatus. Proteins were transferred to nitrocellulose membranes and blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 followed by washing in the same Tris-Tween buffer. The membranes were probed with the appropriate antibody diluted in Tris-buffered saline containing 5% bovine serum albumin (for polyclonal antibodies) or 5% nonfat dry milk (for monoclonal antibodies) followed by treatment with peroxidase-linked ECL secondary antibodies and chemiluminescence reagents. Sizes of proteins were estimated from prestained molecular weight standards electrophoresed in the same blot as the samples. Immuno-reactive bands were quantified by densitometry.

**RESULTS AND DISCUSSION**

**Inhibition of Constitutive ErbB2 Phosphorylation by Antagonists of Endogenous Hyaluronan Interactions**—HCT116 colon carcinoma cells and TA3/St mouse mammary carcinoma cells exhibit relatively high levels of constitutive ErbB2 phosphorylation, and so these cells were used to determine the effects of endogenous hyaluronan on constitutive ErbB2 phosphorylation. Small hyaluronan oligomers (6–20 monosaccharide length) compete for multivalent binding of endogenous hyaluronan to cell surface receptors such as CD44 (23) and consequently attenuate cell survival signaling (7) (also see Fig. 2D). Therefore we incubated the HCT116 and TA3/St cells for 24 h with and without 100 µg/ml hyaluronan oligomers in complete medium for 24 h. Cells (~2 × 10^7 cells) were harvested using ice-cold phosphate-buffered saline and pelleted at 1000 × g at 4 °C. The cell pellet was then lysed in 1 ml of lysis buffer, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM MgCl2, 2 mM NaF, 10 mM Na-PPi, 10 mM Na3VO4, 20 µg/ml aprotinin, 20 µg/ml phenylmethylsulfonyl fluoride, 25 mM MOPS, pH 6.5. The viscous lysate was then sheared 10 times using a 26.5 needle. For the gradient, sucrose solutions (90, 35 and 5%) were prepared in the lysis buffer. One ml of lysate was mixed with an equal volume of 90% sucrose solution in an ultracentrifuge tube. This solution was overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose solution. The gradient was centrifuged in a swinging bucket rotor for 16 h at 110,000 × g at 4 °C in a Sorvall Superspin 630 centrifuge. Fractions (300 µl) were collected from the bottom up using a minipump and then stored at −20 °C until use.

**Lipid-rich Microdomains**—Separation of lipid raft fractions was carried out in a sucrose step gradient (22). HCT116 cells were grown to 90% confluence in complete medium and divided into three groups in 150-mm dishes: untreated, treated with 5 mM MBCD (Sigma) in phosphate-buffered saline for 1 h at 37 °C, or treated with 100 µg/ml hyaluronan oligomers in complete medium for 24 h. Cells (~2 × 10^7 cells) were harvested using ice-cold phosphate-buffered saline and pelleted at 1000 × g at 4 °C. The cell pellet was then lysed in 1 ml of lysis buffer, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM MgCl2, 2 mM NaF, 10 mM Na-PPi, 10 mM Na3VO4, 20 µg/ml aprotinin, 20 µg/ml phenylmethylsulfonyl fluoride, 25 mM MOPS, pH 6.5. The viscous lysate was then sheared 10 times using a 26.5 needle. For the gradient, sucrose solutions (90, 35 and 5%) were prepared in the lysis buffer. One ml of lysate was mixed with an equal volume of 90% sucrose solution in an ultracentrifuge tube. This solution was overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose solution. The gradient was centrifuged in a swinging bucket rotor for 16 h at 110,000 × g at 4 °C in a Sorvall Superspin 630 centrifuge. Fractions (300 µl) were collected from the bottom up using a minipump and then stored at −20 °C until use.

**RESULTS AND DISCUSSION**

**Inhibition of Constitutive ErbB2 Phosphorylation by Antagonists of Endogenous Hyaluronan Interactions**—HCT116 colon carcinoma cells and TA3/St mouse mammary carcinoma cells exhibit relatively high levels of constitutive ErbB2 phosphorylation, and so these cells were used to determine the effects of endogenous hyaluronan on constitutive ErbB2 phosphorylation. Small hyaluronan oligomers (6–20 monosaccharide length) compete for multivalent binding of endogenous hyaluronan polymer to cell surface receptors such as CD44 (23) and consequently attenuate cell survival signaling (7) (also see Fig. 2D). Therefore we incubated the HCT116 and TA3/St cells for 24 h with and without 100 µg/ml hyaluronan oligomers in complete medium for 24 h. Cells (~2 × 10^7 cells) were harvested using ice-cold phosphate-buffered saline and pelleted at 1000 × g at 4 °C. The cell pellet was then lysed in 1 ml of lysis buffer, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM MgCl2, 2 mM NaF, 10 mM Na-PPi, 10 mM Na3VO4, 20 µg/ml aprotinin, 20 µg/ml phenylmethylsulfonyl fluoride, 25 mM MOPS, pH 6.5. The viscous lysate was then sheared 10 times using a 26.5 needle. For the gradient, sucrose solutions (90, 35 and 5%) were prepared in the lysis buffer. One ml of lysate was mixed with an equal volume of 90% sucrose solution in an ultracentrifuge tube. This solution was overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose solution. The gradient was centrifuged in a swinging bucket rotor for 16 h at 110,000 × g at 4 °C in a Sorvall Superspin 630 centrifuge. Fractions (300 µl) were collected from the bottom up using a minipump and then stored at −20 °C until use.
complete serum-containing medium. After removing the media, the cells were washed and lysed in 1% Nonidet P-40 lysis buffer followed by immunoprecipitation with antibody that recognizes total ErbB2, i.e. active phosphorylated plus inactive non-phosphorylated ErbB2. Aliquots of the immunoprecipitates were then Western blotted with antibodies that recognize total ErbB2 or only phosphorylated ErbB2 as described under “Experimental Procedures.” Typical blots are shown in Fig. 1A, where it can be seen that treatment of the tumor cells with hyaluronan oligomers causes inhibition of ErbB2 phosphorylation without significantly affecting total ErbB2 expression.

Previously published evidence indicates that in some tumor cells ErbB2 interacts with the hyaluronan receptor, CD44, and that hyaluronan-CD44 interaction stimulates ErbB2 activation (6, 24, 25). Thus we determined whether antibody against CD44 co-immunoprecipitates ErbB2 from HCT116 and TA3/St cell lysates and whether hyaluronan oligomers affect the amount of phosphorylated ErbB2 in these immunoprecipitates. We found that phosphorylated ErbB2 was present in the CD44 immunoprecipitates from untreated cells, but as expected it was greatly diminished in the immunoprecipitates from hyaluronan oligomer-treated cells; no effect on total ErbB2 was observed (Fig. 1B). Because it is known that ErbB2/ErbB3 forms a complex with the p85 regulatory subunit of PI 3-kinase (26), we also analyzed total and phosphorylated ErbB2 in immunoprecipitates obtained with antibody against the p85 subunit. Again we found that ErbB2 was co-immunoprecipitated with p85 and that treatment of the cells with hyaluronan oligomers suppressed the amount of ErbB2 phosphorylation in these immunoprecipitates (Fig. 1C).

The immunoprecipitations with antibody against ErbB2

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Antagonists of endogenous hyaluronan interactions inhibit assembly of a constitutive ErbB2-containing signaling complex. HCT116 cells were treated with a recombinant adenovirus driving expression of β-galactosidase (as control) or soluble CD44 (solCD44) as described in the text. These cells were also transfected with siRNA against CD44 (siRNA) or with control RNA. TA3/St cells were stably transfected with vector only or with cDNA constructs for soluble CD44 mutated in the hyaluronan-binding domain (msolCD44) or intact solCD44. The cells were then processed for immunoprecipitation with antibody against ErbB2 and Western blotting with antibodies against phosphorylated ErbB2, CD44, ezrin, cdc37, p110α subunit of PI 3-kinase, and total ErbB2.

![Figure 6](http://www.jbc.org/)

**FIG. 6.** Antagonists of endogenous hyaluronan interactions inhibit assembly of a constitutive ErbB2-containing signaling complex. The experiment described in Fig. 5 was repeated three times and processed for immunoprecipitation with antibody against ErbB2, Western blotting with the various antibodies, and densitometry. HCT116 cells: bars 1, control RNA; bars 2, siRNA to CD44; bars 3, β-galactosidase adenovirus (as control); bars 4, soluble CD44 adenovirus. TA3/St cells: bars 5, vector-transfected; bars 6, mutated soluble CD44-transfected; bars 7, soluble CD44-transfected. The results obtained for Western blotting of phosphorylated ErbB2 are shown in Fig. 2, B and C. Similar results were obtained when immunoprecipitation was carried out with antibody against the p85 subunit of PI 3-kinase (data not shown). No significant changes in total ErbB2 levels were observed in these experiments.
were repeated three times, and the effect of hyaluronan oligomers was quantitated by densitometry. It was found that the oligomers inhibit ErbB2 phosphorylation by 84% in the HCT116 cells and by 94% in the TA3/St cells (Fig. 2A). A similar analysis was performed with three sets of p85 immunoprecipitates. In similar fashion, 86% inhibition of ErbB2 phosphorylation was seen in hyaluronan oligomer-treated HCT116 cells and 90% inhibition in TA3/St cells (Fig. 2A). However, no significant effect on the amounts of p85 or total ErbB2 in these immunoprecipitates was observed.

A plausible interpretation for the inhibition of ErbB2 phosphorylation by hyaluronan oligomers is that these oligomers inhibit endogenous hyaluronan-receptor binding and consequent signaling (7). If this were the case, other antagonists of hyaluronan-receptor interactions should have a similar effect. Thus we determined the effect of experimentally induced expression of soluble CD44, which acts as competitive decoy for binding of endogenous hyaluronan (Fig. 2D). First we examined the effect of infection of HCT116 cells with an adenovirus driving expression of soluble CD44, using an adenovirus expressing b-galactosidase as a control (9). Soluble CD44 caused 72% inhibition of ErbB2 phosphorylation (Fig. 2B). We also examined the levels of ErbB2 phosphorylation in TA3/St cells that were stably transfected with soluble CD44 or soluble CD44 mutated in the hyaluronan-binding domain as compared with vector-transfected cells (18, 27). Soluble CD44 caused 54% inhibition, whereas the mutated soluble CD44 did not have a significant effect (Fig. 2B). Similar results were obtained when ErbB2 was co-immunoprecipitated with the p85 subunit of PI 3-kinase (data not shown).

Inhibition of Assembly of a Constitutive, ErbB2-containing Signaling Complex by Antagonists of Endogenous Hyaluronan Interactions—The data presented in Fig. 1B indicate that phosphorylated ErbB2 and CD44 are present as a complex and that phosphorylation of ErbB2 within this complex is dependent on hyaluronan. Therefore we blotted the ErbB2 immunoprecipitates obtained in the previous section with antibodies that recognize CD44 and found that CD44 co-immunoprecipitates with ErbB2 from lysates of both HCT116 and TA3/St cells. However, the amount of CD44 in the complex was greatly decreased after treatment of the tumor cells with hyaluronan oligomers (Fig. 3). Another important signaling factor that interacts with CD44 is Ezrin (28), and so we probed the immunoprecipitates with antibody that recognizes Ezrin and found that it is also present in the complex. As with CD44 and phosphorylated ErbB2, the levels of Ezrin in the immunoprecipitates were diminished by treatment of the tumor cells with hyaluronan oligomers (Fig. 3).

We found above that phosphorylated ErbB2 co-immunoprecipitates with the p85 regulatory subunit of PI 3-kinase and that treatment of the tumor cells with hyaluronan oligomers decreases the amount of phosphorylated ErbB2 in this complex (Figs. 1C and 2A). However, we found that the amount of p85 in the ErbB2 immunoprecipitates (and vice versa) is not affected by the oligomers (Fig. 3). We also examined the effect of hyaluronan oligomers on the level of p110y catalytic subunit of PI 3-kinase in this complex because it is known to form a complex with CD44 via interaction with Gab-1 (11). Unlike the p85 subunit, the amount of p110y subunit in the complex was decreased by treatment of the tumor cells with hyaluronan antagonists (Fig. 3). We then probed the p85 immunoprecipitates obtained as in the previous section with the antibodies against CD44, Ezrin, and p110y and found the same pattern, i.e. these three components were present in the immunoprecipitates, but the amounts present after hyaluronan oligomer treatment of the cells was decreased (data not shown).

To ensure that ErbB2, CD44, Ezrin and PI 3-kinase are specifically present in the immunoprecipitated complex, we probed for several other cell surface-associated molecules, i.e. emmprin (29), the Na+-H+ exchanger, NHE1 (30), and transferrin receptor (31), but did not detect significant amounts in the immunoprecipitates. We also probed for the chaperone molecules Hsp90 and cdc37 because Hsp90 is required for ErbB2 activity (32, 33), and cdc37 is known to act cooperatively with Hsp90 in stabilizing several kinases (16, 34). We found that both of these chaperones are present in the complex but...
they are diminished in amount by treatment of the cells with hyaluronan oligomers (Fig. 3).

The results obtained for ErbB2 immunoprecipitates from three experiments with HCT116 and TA3/St cells were quantitated, and the results are shown in Fig. 4. As can be seen from this figure, treatment of the cells with hyaluronan oligomers inhibits assembly of phosphorylated ErbB2, CD44, ezrin, Hsp90/cdc37, and the p110α subunit of PI 3-kinase into the complex. However, the total amount of ErbB2 and the amount of p85 subunit of PI 3-kinase remain constant. Very similar results were obtained on analysis of p85 immunoprecipitates from these three experiments (data not shown). To obtain a semiquantitative estimate of what proportion of each component is present in the complex, we performed densitometry on Western blots of each component in the ErbB2 immunoprecipitate and in the supernatant after immunoprecipitation from HCT116 cells. We found that 80% of total ErbB2 appeared in the immunoprecipitate. About 30–50% of each of the other components was also co-immunoprecipitated along with ErbB2. Thus, this complex contains a major proportion of the cellular pool of each of these signaling components.

Then we examined immunoprecipitates from lysates of HCT116 and TA3/St cells overexpressing soluble CD44 as described in the previous section (Fig. 2B). We found that this treatment also greatly diminishes the amounts of phosphorylated ErbB2, CD44, ezrin, Hsp90/cdc37, and p110α subunit of PI 3-kinase in the immunoprecipitates (Figs. 5 and 6). However, this treatment did not significantly change the total amounts of ErbB2 in the complex (Fig. 5). In the TA3/St cells, we found that overexpression of soluble CD44 mutated in the hyaluronan-binding site had no significant effect (Figs. 5 and 6).

Inhibition of Constitutive ErbB2 Phosphorylation and Assembly of the Signaling Complex by Inhibition of CD44 Expression—The most likely explanation for the effects of hyaluronan oligomers or soluble CD44, as described above, is that they act as antagonists of endogenous hyaluronan-receptor interaction. Because the hyaluronan receptor CD44 is present in the signaling complex, we tested the role of CD44 by siRNA silencing of CD44 expression. The siRNA that we chose to use causes 70–90% inhibition of CD44 expression in HCT116 cells. Treatment of the cells with this siRNA diminished phosphorylation of ErbB2 by 80% (Fig. 2C) and greatly inhibited assembly of phosphorylated ErbB2, CD44, ezrin, p110α subunit of PI 3-kinase, and Hsp90/cdc37 into the complex (Figs. 5 and 6).

Stimulation of ErbB2 Phosphorylation and Assembly of the Signaling Complex by Increased Hyaluronan Production—We also determined whether increased hyaluronan synthesis causes an increase in ErbB2 activity in cells with low constitutive ErbB2 activity. We found that MCF-7 human mammary carcinoma cells exhibit 5–20% of the constitutive ErbB2 activity of that in HCT116 or TA3/St cells under the conditions of our experiments. Therefore we treated MCF-7 cells with an adenovirus driving expression of the hyaluronan synthase, Has2. This causes a 2–5-fold increase in hyaluronan production in these cells, whereas infection with the control adenovirus expressing β-galactosidase has little or no effect on hyaluronan production (8, 12). Using these cells, we found that ErbB2 phosphorylation is increased in parallel with the increase in hyaluronan production (Fig. 7). In addition, we found that this
Augmentation of ErbB2 phosphorylation by increased endogenous hyaluronan is reversed by co-treatment of the Has2 adenovirus-infected cells with hyaluronan oligomers (data not shown). In previous studies we showed that emmprin, a cell surface glycoprotein that is highly up-regulated in malignant cancer cells (29, 35), stimulates hyaluronan synthesis (21). Thus we also determined the effect of up-regulation of emmprin in MCF-7 cells using a recombinant emmprin adenovirus previously shown to increase hyaluronan production in MCF-7 cells by 2–8-fold (8, 21). This treatment also induces ErbB2 phosphorylation, and the emmprin-stimulated phosphorylation is dependent on hyaluronan because it is reversed by co-treatment of the cells with hyaluronan oligomers (Fig. 7).

Quantitation of the results from three experiments is shown in Fig. 8. ErbB2 phosphorylation was stimulated by 8- to 13-fold by up-regulation of Has2 or emmprin expression and was completely reversed by co-treatment with hyaluronan oligomers. Very similar results were observed for immunoprecipitates obtained with antibodies against ErbB2 (Fig. 8A) and the p85 subunit of PI 3-kinase (Fig. 8B).

Next, we examined the effect of increased hyaluronan production on complex formation in MCF-7 cells, which we found to have low amounts of constitutive complex. As above, we used recombinant Has2 and emmprin adenoviruses to increase hyaluronan production in these cells. As expected, increased hyaluronan causes increased complex assembly, and co-treatment with hyaluronan oligomers reverses this increase (Figs. 7 and 9).

A previous study by Bourguignon et al. (6) has shown that addition of 50 μg/ml exogenous, high molecular weight, hyaluronan polymer to SKOV-3 ovarian carcinoma cells promotes ErbB2 activity and interaction with CD44. We treated MCF-7 cells with exogenous hyaluronan polymer in a similar fashion but did not detect any significant increase in ErbB2 activity or complex assembly (data not shown). Because increased hyaluronan synthesis induces ErbB2 activity and complex assembly in MCF-7 cells, we conclude that addition of exogenous hyalu-
Hyaluronan and ErbB2 Signaling

Fig. 12. Hypothetical model for the effects of hyaluronan on ErbB2 signaling. Endogenous hyaluronan-CD44 interaction is required for formation of a constitutive signaling complex containing CD44, activated ErbB2, ezrin, PI 3-kinase, and the chaperone partners, Hsp90/cdc37. Previous studies have demonstrated the interaction of ErbB2 with CD44 via interactions with Vav2 and Grb2 (6). Likewise, interaction of PI 3-kinase with CD44 is mediated by Gab-1 (11). Direct interactions between ezrin and CD44 (28), between ezrin and PI 3-kinase (41), and between Hsp90 and ErbB2 (32, 33) have also been demonstrated. Antagonists of hyaluronan-CD44 interaction, e.g. hyaluronan oligomers, soluble hyaluronan-binding proteins or siRNA against CD44, cause disassembly of the constitutive complex present in tumor cells with high levels of ErbB2 activity and consequently inhibit this activity. On the other hand, increased hyaluronan production induces assembly of this complex in cells with low levels of ErbB2 activity and consequently stimulates ErbB2 activity. Hyaluronan-stimulated ErbB2 activity is essential for correct embryonic morphogenesis and epithelial-mesenchymal transition (EMT) (5), whereas increased, deregulated ErbB2 activity promotes the progression of malignant properties such as invasiveness, increased cell survival, and drug resistance in cancer cells (13, 14, 43).

Assembly of the ErbB2-containing Signaling Complex in Lipid Rafts—Because signaling complexes frequently assemble within lipid-rich microdomains or “rafts” in the plasma membrane (36) and because ErbB2 is associated with rafts (37), we prepared raft fractions by sucrose gradient centrifugation. This treatment caused all of the CD44 to concentrate at the bottom of the gradient (Fig. 10B), whereas most of the rest became concentrated in the bottom fractions of the gradient (Fig. 10A). We then pooled fractions 3–5 from the gradients because these roughly correspond to the putative rafts. We immunoprecipitated these pooled fractions with antibody against ErbB2 or CD44 and Western blotted the immunoprecipitates with antibodies against phosphorylated ErbB2 and CD44. Phosphorylated ErbB2 and CD44 co-immunoprecipitated from pooled fractions 3–5 obtained from untreated cells (Fig. 11). As another marker for the signaling complex described above, we probed the immunoprecipitates with antibody against ezrin and found that it was also present. However, these components were much reduced in amount in the immunoprecipitates from the pooled fractions obtained from hyaluronan oligomer-treated cells (Fig. 11). Thus we conclude that the phosphorylated ErbB2- and CD44-containing complex is present in lipid raft-like structures in the untreated cells and that treatment of the cells with hyaluronan oligomers causes this raft-associated complex to disassemble.

Conclusions—The results obtained in this study indicate that hyaluronan constitutively promotes formation of a signaling complex that contains CD44, phosphorylated ErbB2, PI 3-kinase, ezrin, and the chaperones, Hsp90 and cdc37, and that this complex assembles within a lipid raft-like structure. Previous studies using SKOV-3 ovarian carcinoma cells have shown that ErbB2 interacts with CD44 via Grb2 and Vav2 and that addition of exogenous hyaluronan promotes this interaction as well as ErbB2 activity (6). We did not detect any significant increase in ErbB2 activity or complex assembly on treatment of MCF-7 breast carcinoma cells with exogenously added hyaluronan, implying that increased endogenous synthesis of hyaluronan is not equivalent to the addition of exogenous hyaluronan in this system. Hyaluronan is synthesized by transmembrane syntheses with their active sites situated on the cytoplasmic side of the plasma membrane (38). Newly synthesized hyaluronan is extruded through the plasma membrane while still attached to the synthase. Subsequent to synthesis it can be retained at the plasma membrane by sustained interaction with the synthase (39) or by concomitant interaction with CD44 (40). Alternatively, it can be released from the synthase and then bind back to receptors or assemble into extracellular matrices (1). Simple addition of exogenous hyaluronan cannot duplicate all of these configurations. We believe that hyaluronan produced by endogenous mechanisms results in more accurate replication of compartmentalization of hyaluronan and resultant signaling than a simple addition to culture medium. However, in some cases it is likely that treatment with exogenous hyaluronan results in interactions with available cell surface receptors and other binding proteins, leading to physiologically relevant consequences (5, 6, 10, 11, 30). Another possibility is that the hyaluronan synthases have signaling properties independent of hyaluronan. This seems an unlikely explanation for our results because hyaluronan antagonists reverse the effects of increased hyaluronan synthesis expression (Figs. 7–9).

Previous work has shown that PI 3-kinase interacts with CD44 via Gab-1 (11), whereas ezrin binds directly to CD44 (28) and to PI 3-kinase (41). Hsp90 binds to and stabilizes ErbB2 (32, 33) and usually requires interaction with cdc37 for its activity (16, 34). Interestingly, vertebrate cdc37 was originally...
cloned in our laboratory as an intracellular hyaluronan-binding protein (42), but the function of hyaluronan-cdc37 interaction is still not understood. Our work demonstrates that all of these factors assemble into a single complex. Inhibition of hyaluronan-CD44 interaction in cells that contain a constitutively high level of active ErbB2 causes this complex to disassemble (Figs. 4 and 6) and leads to loss of ErbB2 activity (Fig. 2). This in turn would lead to decreased PI 3-kinase/Akt cell survival pathway activity (7) and to attenuated activity of other signaling pathways, including the mitogen-activated protein kinase and focal adhesion kinase pathways (8). On the other hand, increased hyaluronan production leads to increased complex formation (Fig. 9) and ErbB2 activity (Fig. 8) and thus to increases in multiple cell signaling activities (6, 8, 11, 12). These interactions are shown diagrammatically in Fig. 12. During embryonic development, hyaluronan is essential for ErbB2 signaling involved in epithelial-mesenchymal transition and mesenchymal cell invasion during formation of cushion mesenchyme from endocardial epithelium (5). In breast and ovarian cancers, increased constitutive levels of ErbB2 activity have been linked to malignancy and drug resistance (13, 14, 43). Thus, in the latter case, inhibition of hyaluronan synthesis or of hyaluronan interactions with cell surface receptors may provide novel approaches to treatment of cancers exhibiting high ErbB2 activity.

REFERENCES
1. Toole, B. P. (2001) Semin. Cell Dev. Biol. 12, 79–87
2. Toole, B. P. (2004) Nat. Rev. Cancer 4, 528–539
3. Turbey, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) J. Biol. Chem. 277, 4589–4592
4. Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeld, J., Augustine, M. L., Calabro, A. Jr., Kubalak, S., Klewer, S. E., and McDonald, J. A. (2000) J. Clin. Investig. 106, 349–360
5. Camenisch, T. D., Schroeder, J. A., Bradley, J., Klewer, S. E., and McDonald, J. A. (2002) Nat. Med. 8, 850–855
6. Bourguignon, L. Y., Zhu, H., Zhou, B., Diedrich, F., Singleton, P. A., and Hung, M. C. (2001) J. Biol. Chem. 276, 48679–48692
7. Ghatak, S., Misra, S., and Toole, B. P. (2002) J. Biol. Chem. 277, 38013–38020
8. Misra, S., Ghatak, S., Zoltan-Jones, A., and Toole, B. P. (2003) J. Biol. Chem. 278, 25285–25288
9. Ward, J. A., Huang, L., Guo, H., Ghatak, S., and Toole, B. P. (2003) Am. J. Pathol. 162, 1403–1409
10. Sohara, Y., Ishiguro, N., Machida, K., Kurata, H., Thant, A. A., Senga, T., Matsuda, S., Kimata, K., Iwata, H., and Hamaguchi, M. (2001) Mol. Biol. Cell 12, 1859–1868
11. Bourguignon, L. Y., Singleton, P. A., Zhu, H., and Diedrich, P. (2003) J. Biol. Chem. 278, 29420–29434
12. Zoltan-Jones, A., Huang, L., Ghatak, S., and Toole, B. P. (2003) J. Biol. Chem. 278, 45801–45810
13. Yarden, Y., and Sliwkowski, M. X. (2001) Nat. Rev. Mol. Cell. Biol. 2, 127–137
14. Zhou, B. P., and Hung, M. C. (2003) Semin. Oncol. 30, 38–48
15. Zeng, C., Toole, B. P., Kinney, S. D., Kuo, J. W., and Stamenkovic, I. (1998) Int. J. Cancer 77, 396–401
16. Grammatikakis, N., Lin, J. H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) Mol. Cell. Biol. 19, 1661–1671
17. Yeo, T. K., Nagy, A. J., Yeo, K. T., Dvorak, H. F., and Toole, B. P. (1996) Am. J. Pathol. 148, 1733–1740
18. Yu, Q., Toole, B. P., and Stamenkovic, I. (1997) J. Exp. Med. 186, 1985–1996
19. Yu, Q., and Toole, B. P. (1996) J. Biol. Chem. 271, 20603–20607
20. Li, R., Huang, L., Guo, H., and Toole, B. P. (2001) J. Cell. Physiol. 186, 371–379
21. Marieb, E. A., Zoltan-Jones, A., Li, R., Misra, S., Ghatak, S., Cao, J., Zucker, S., and Toole, B. P. (2004) Cancer Res. 64, 1229–1232
22. Claas, C., Stipp, C. S., and Hemler, M. E. (2001) J. Biol. Chem. 276, 7974–7984
23. Lesley, J., Hascaill, V. C., Tammi, M., and Hyman, R. (2000) J. Biol. Chem. 275, 26967–26975
24. Wobus, M., Rangwala, R., Sheyn, I., Hennigan, R., Coila, B., Lower, E. F., Yassin, R. S., and Sherman, L. S. (2002) Appl. Immunohistochem. Mol. Morphol. 10, 34–39
25. Tnatas, D., Kanagasundaram, V., Kaye, A., and Novak, U. (2002) J. Clin. Neurosci. 9, 282–288
26. Helyer, N. J., Kim, M. S., and Koland, J. G. (2001) J. Biol. Chem. 276, 42153–42161
27. Peterson, R. M., Yu, Q., Stamenkovic, I., and Toole, B. P. (2000) Am. J. Pathol. 156, 2159–2167
28. Legg, J. W., Lewis, C. A., Parsons, M., Ng, T., and Isacke, C. M. (2002) Nat. Cell Biol. 4, 399–407
29. Biswas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nabeshima, K. (1998) Cancer Res. 58, 434–439
30. Bourguignon, L. Y., Singleton, P. A., Diedrich, F., Stern, R., and Gilad, E. (2004) J. Biol. Chem. 279, 26991–27007
31. Hommelgaard, A. M., Lerdrup, M., and van Deurs, B. (2004) Mol. Biol. Cell 15, 1557–1567
32. Blagosklonny, M. V. (2002) Leukemia 16, 455–462
33. Citri, A., Kochupurakkal, B. S., and Yarden, Y. (2004) Cell Cycle 3, 51–60
34. Pratt, W. B., Silverstein, A. M., and Galigniana, M. D. (1999) Cell. Signal. 11, 839–851
35. Zucker, S., Hymowitz, M., Rollo, E. E., Mann, R., Conner, C. E., Cao, J., Foda, H. D., Tompkins, D. C., and Toole, B. P. (2001) Am. J. Pathol. 158, 1921–1928
36. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
37. Nagy, P., Verheg, G., Sebestyen, Z., Harvath, G., Lockett, S. J., Damjanovic, S., Park, J. W., Jovin, T. M., and Szollosi, J. (2002) J. Cell Sci. 115, 4251–4262
38. Weigel, P. H., Hascaill, V. C., and Tammi, M. (1997) J. Biol. Chem. 272, 13979–14000
39. Heldin, P., and Pertzoff, H. (1993) Exp. Cell Res. 208, 422–429
40. Luke, H. J., and Pehrm, P. (1999) Biochem. J. 343, 71–75
41. Gautreau, A., Poulet, P., Louard, D., and Arpin, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7300–7305
42. Grammatikakis, N., Grammatikakis, A., Yoneda, M., Yu, Q., Banerjee, S. D., and Toole, B. P. (1995) J. Biol. Chem. 270, 16189–16205
43. Chen, J. S., Lan, K., and Hung, M. C. (2003) Drug Resist. Updates 6, 129–136
