Heregulin (HRG)-induced cell responses are mediated by the ErbB family of tyrosine kinase receptors. In this study we have investigated HRG activation of ErbB2, extracellular signal-regulated kinase (ERK) signaling, and their role in regulating hyaluronan synthase (HAS) activity in human ovarian tumor cells (SK-OV-3.ipl cells). Immunological and biochemical analyses indicate that ErbB2, ErbB3, and ErbB4 are all expressed in SK-OV-3.ipl cells and that ErbB4 (but not ErbB3) is physically linked to ErbB2 following HRG stimulation. Furthermore, our data indicate that the HRG-induced ErbB2-ErbB4 complexes stimulate ErbB2 tyrosine kinase, which induces both ERK phosphorylation and kinase activity. The activated ERK then increases the phosphorylation of HAS1, HAS2, and HAS3. Consequently, all three HAS isozymes are activated resulting in hyaluronan (HA) production. Because HRG-mediated HAS isozyme phosphorylation/activation can be effectively blocked by either AG825 (an ErbB2 inhibitor) or thiazolidinedione compound (an ERK blocker), we conclude that ErbB2-ERK signaling and HAS isozyme expression/activation are functionally coupled in SK-OV-3.ipl cells. HRG also promotes HA- and CD44-dependent oncogetic events (e.g. CD44-Cdc42 association, p21-activated kinase 1 activation, and p21-activated kinase 1-filamin complex formation) and tumor cell-specific behaviors in an ErbB2-ERK signaling-dependent manner. Finally, we have found that the down-regulation of HAS isozyme expression (by transfecting cells with HAS1/HAS2/HAS3-specific small interfering RNAs) not only inhibits HRG-mediated HAS phosphorylation/activation and HA production but also impairs CD44-specific Cdc42-PAK1/filamin signaling, cytoskeleton activation and tumor cell behaviors. Taken together, these findings clearly indicate that HRG activation of ErbB2-ERK signaling modulates HAS phosphorylation/activation and HA production leading to CD44-mediated oncogetic events and ovarian cancer progression.

Ovarian cancer has the highest mortality rate of any gynecological malignancy. The first step in the spread of ovarian cancer is the shedding of cells from the primary tumor followed by rapid tumor cell growth, migration, and invasion throughout the peritoneal cavity (1, 2). A number of studies have aimed at identifying specific adhesion molecule(s) that are expressed in ovarian carcinomas, accumulate in the peritoneal mesothelium, and correlate with tumor cell invasive behaviors. Among such candidate molecules is hyaluronan (HA),2 which is a nonsulfated, unbranched glycosaminoglycan consisting of the repeating disaccharide units, D-glucuronic acid and N-acetyl-d-glucosamine (3, 4). HA is found in the extracellular matrix of most mammalian tissues (3–7). Furthermore, HA has been shown to accumulate at sites of ovarian tumor cell attachment in the mesothelial lining of the peritoneum and appears to play an important role in promoting tumor cell-specific behaviors (5–7).

The biosynthesis of HA is regulated by three mammalian HA synthase isozymes, HA synthase 1 (HAS1), HA synthase 2 (HAS2), and HA synthase 3 (HAS3) (8–11). Although the three HAS genes are located on different chromosomes, they share a great deal of sequence homology (12–14). HAS1 synthesizes a low level of a large size HA (~1 × 106 to 1 × 107 Da) (12, 13). HAS2 produces relatively high amounts of large size HA (~1 × 106 to 1 × 107 Da) and is definitely involved in cell proliferation, angiogenesis, as well as embryonic and cardiac development (14). HAS3, which synthesizes a small size HA (~1 × 105 Da), is one of the most biologically active HAS molecules and is known to contribute to the malignant phenotype in many different cell types (11, 12). Recent studies indicate that the expression of HAS1 restores the metastatic potential of mouse mammary carcinoma mutant cells that have low HA production and low metastatic capability (15). Furthermore, overexpression of HAS2 and HAS3 stimulates both tumorigenicity and tumor progression (16, 17). In ovarian cancer patients, all three HAS molecules are detected in ovarian carcinoma tissues (18). In particular, HAS1 expression in ovarian cancer patients appears to be closely associated with tumor angiogenesis and disease.

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1 A Veterans Affairs Research Career Scientist. To whom correspondence should be addressed: Endocrine Unit (111N), Dept. of Medicine, University of California at San Francisco and Veterans Affairs Medical Center, 4150 Clement St., San Francisco, CA 94121. Tel.: 415-221-4810 (ext. 3321); Fax: 415-383-1638; E-mail: lilly.bourguignon@ucsf.edu.

2 The abbreviations used are: HA, hyaluronan; HRG, heregulin; ErbB2, neu or p185HER2; ERK, extracellular signal-regulated kinase; HAS, hyaluronan synthase; thiazolidinedione, 3-(2-aminoethyl)-5-((4-ethoxyphenyl)methyl-ene)-2,4-thiazolidinedione; PAK1, p21-activated kinase 1; GlcA, gluconic acid; EGF, epidermal growth factor; siRNA, small interference RNA; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; S-400HR, Hiprep Sephacryl HR 400 HR; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ANOVA, analysis of variance.
progression and is also an important predictor of patient survival (18). Thus, dysregulation of HAS expression and activities often results in abnormal production of HA and directly contributes to aberrant cellular processes such as transformation and metastasis (19).

The HER2 oncogene (also called ErbB2 or neu) encodes a 185-kDa membrane protein (p185HER2), which contains a single transmembrane-spanning region and a tyrosine kinase-associated cytoplasmic domain (20, 21). This protein, initially discovered as an activated oncogenic variant (neu) is overexpressed in many epithelial tumors, including mammary and ovarian carcinomas (22, 23). Overexpression or amplification of HER2 oncogenes ErbB2 in ovarian cancer is associated with a poor prognosis (22, 23). However, the cellular and molecular mechanisms by which ErbB2 enhances the growth and survival of ovarian cancer cells are not completely understood. HA has been shown to constitutively regulate ErbB2 tyrosine kinase activities and to influence ErbB2 interaction with CD44 (an HA receptor) signaling in tumor cells (24). Previously, we have determined that CD44 and ErbB2 are physically linked to each other via interchain disulfide bonds in human ovarian tumor cells (25). Most importantly, HA binding to a CD44-associated ErbB2 complex activates the ErbB2 tyrosine kinase activity and promotes ovarian carcinoma cell growth (25). HA-mediated CD44 association with ErbB2 signaling complexes in ovarian tumor cells is also mediated by molecular scaffolds and adaptors such as Vav2 (an Rac-specific guanine nucleotide exchange factor, GEF) and Grb2 (26). In addition, endogenous Vav2 and Grb2 are physically associated with CD44 and ErbB2 in the signaling complex, and HA treatment induces the recruitment of both Vav2 and Grb2 into CD44v3-ErbB2-containing multicomplexes leading to the coactivation of Rac1 and Ras signaling and ovarian tumor cell growth and migration (26).

Most recently, we have found that HA-mediated CD44-ErbB2 interaction (mediated by N-WASP) promotes β-catenin signaling and actin-cytoskeleton functions leading to certain tumor-specific behaviors (e.g. transcriptional activation and tumor cell migration) and ovarian cancer progression (27). Clearly, there are direct signaling interactions among ErbB2, HA, and CD44 during ovarian tumor cell activation. The question of whether other stimuli such as growth factors might also participate in activating ErbB2 tyrosine kinase, influence HA-CD44 signaling, and stimulate metastatic behaviors of ovarian tumor cells has not been fully addressed.

Heregulin (HRG) (or neuregulin) belongs to the epidermal growth factor (EGF)-like ligand-binding family originally identified in a search for activators of ErbB2 (28). Components of the HRG signaling pathways interact with the human ErbB/Her receptor family of proteins, which is composed of four tyrosine kinase receptors (Her/ErbB1 or EGF receptor, Her2/ErbB2, Her3/ErbB3, and Her4/ErbB4) and plays an important role in promoting ovarian tumor cell growth and migration (28–30). Although HRG activates ErbB2 tyrosine kinase, it does not bind ErbB2 directly or induce ErbB2 dimerization. In fact, ErbB2 activation is often mediated by HRG binding to ErbB3 or ErbB4 and the subsequent recruitment of ErbB2 into ErbB3 or ErbB4 heterodimer complexes (31–33). Overexpression of HRG and the ErbB tyrosine kinases is closely associated with tumorigenic esis and ovarian cancer progression (22, 30, 34–36). Accumulating evidence indicates that HRG induces ErbB tyrosine kinase activation leading to the onset of intracellular signaling events involving the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase cascades (37–39). In particular, it is known that ERK phosphorylation of certain target proteins is directly involved in cellular activation by coordinat ing extracellular cues and intracellular signals (40–42).

Although ErbB activation is closely associated with HRG and ErbB tyrosine kinases, the one or more specific ERK-regulated downstream targets that are capable of mediating both growth and migration responses in HRG/ErbB-stimulated ovarian cancer progression have not been fully identified.

In this study we present new evidence indicating that HRG activation of ErbB2-ERK signaling plays a pivotal role in regulating HAS activities and HA production/size modifications required for CD44-mediated signaling, ovarian tumor cell growth, and migration. Our findings also indicate that direct “cross-talk” between HRG-mediated ErbB2-ERK signaling and HA-CD44 activation may be one of the most important signaling events in human ovarian carcinoma development.

MATERIALS AND METHODS

Cell Culture—The SK-OV-3.1pl cell line was established from ascites that developed in a nu/nu mouse given an intraperitoneal injection of SK-OV-3 human ovarian carcinoma cell line (obtained from the American Type Culture Collection) as described previously (25, 26, 42). Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum. Cells were routinely serum-starved (and therefore deprived of serum HRG) before adding HRG.

Antibodies and Reagents—Both rabbit anti-ErbB2 antibody and rabbit anti-phospho-ErbB2 antibody were obtained from Upstate Biotechnology (Charlottesville, VA). Rabbit anti-ErbB3 antibody, rabbit anti-ErbB4 antibody, rabbit anti-phospho-PAK1 antibody, and rabbit anti-phospho-ErbB2 antibody were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-phospho-threonine antibody and rabbit anti-phospho-serine antibody were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-phospho-HER2 antibody and rabbit anti-phospho-HER2 antibody were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-phospho-threonine antibody and rabbit anti-phospho-serine antibody were obtained from Zymed Laboratories Inc. (South San Francisco, CA). Other immunoreagents such as rabbit anti-ERK antibody and rabbit anti-phospho-ERK antibody were purchased from Oncogene Research Products (San Diego, CA) and Cell Signaling Technology, respectively. For the preparation of polyclonal rabbit antibodies against HAS1, HAS2, and HAS3, KLH-conjugated 15–18 amino acids unique for each of the HAS isozymes (e.g. the HAS1 sequences: [C]-VAHERADWSG-PSRAAE-amide, or HAS2 sequences: [C]-KESKRPFSESQKTV-amide, or HAS3 sequences: CGKKEPQSSLFAE-1acid) were first synthesized by the Peptide Laboratory (Cambridge Research Biochemicals, United Kingdom) using an Advanced Chemtech automatic synthesizer (model ACT350). All polyclonal antibodies were prepared by inoculating specific HAS isozyme peptides (with Freund’s Complete Adjuvant/Freund’s Incomplete Adjuvant) into rabbits intradermally and/or subcutaneously according to standard antibody production procedures (Covance Research Products, Denver, PA). Subsequently,
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rabit anti-HAS1/HAS2/HAS3 IgG and preimmune IgG were obtained by conventional DEAE-cellulose chromatography and tested for specificity (by enzyme-linked immunosorbent assay and immunoblot assays). Monoclonal anti-CD44 antibody (Clone: 020; Isotype: IgG2b; obtained from CMB-TECH, Inc., San Francisco, CA) recognizes a common determinant of the HA-binding region of CD44 isoforms, including CD44s, CD44E, and CD44 variant species. This rat anti-CD44 antibody was routinely used for HA-related blocking experiments. HRG-1α was purchased from Sigma. Both AG825 (an ErbB2 inhibitor) and thiazolidinedione compound (3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione) (an ERK inhibitor) were obtained from Calbiochem. Heregulin was purchased from EMD, Chemical Inc. (San Diego, CA).

Preparations of HAS1/HAS2/HAS3siRNA and CD44siRNA—The siRNA sequence targeting human HAS1/HAS2/HAS3 or CD44 (from mRNA sequence, Gene Bank™, accession number NM_001523 (for HAS1), NM_005328 (for HAS2), AF234839 (for HAS3), and AJ251595 (for CD44)) corresponds to the coding region relative to the first nucleotide of the start codon. Target sequences were selected using the software developed by Ambion Inc., UK. As recommended by Ambion, HAS1/HAS2/HAS3- or CD44-specific targeted regions were selected beginning 50–100 nucleotides downstream from the start codon. Sequences close to 50% G/C content were chosen. Specifically, HAS1 target sequence (5'-AATAACCTCTTGCAGCAGTTT-3'), HAS2 target sequence (5'-AAGGCTTTGTGTGCTTTTTTG-3'), HAS3 target sequence (5'-AAAGCTTTGTTGCTTTTTTG-3'), and CD44 target sequence (5'-AATAAGCAGCTTGGCAGCAG-3'), were used. HAS1/HAS2/HAS3- and CD44-specific target sequences were then aligned to the human genome database in a BLAST search to eliminate sequences with significant homology to other genes. Sense and antisense oligonucleotides were provided by the University of California at San Francisco Biomolecular Research Unit. For the construction of the siRNA a transcription-based kit was used (Silencer™ siRNA construction kit, Ambion, Austin, TX).

Plasmid Construction—The human HAS2 expression plasmid was prepared by inserting its coding sequence (Gene Bank™ accession number NM_005328) obtained by reverse transcription-PCR to the vector pcDNA 3.1/CT-GFP-TOPO (Invitrogen). Briefly, cells were plated at a density of 2 × 10⁴ cells per 100-mm dish and transfected with 25 µg/dish plasmid cDNA using LipofectAMINE 2000. Transfected cells were grown in the culture medium for at least 24–48 h. Various transfectants were then analyzed for their protein expression by anti-HAS2 and anti-GFP-mediated immunoblotting as described below.

Immunoblotting and Immunoprecipitation Techniques—SK-OV-3-ipl cells were serum-starved for 24 h followed by incubation with 20 ng/ml HRG (or no HRG) for various time intervals (e.g., 0, 1, 3, 5, 7, 10, or 15 min) at 37 °C. Some cells were pre-treated with various inhibitors (e.g., 25 µM AG825 (an ErbB2 inhibitor), or 1 µM thiazolidinedione (an ERK inhibitor)) for 1 h in the presence or absence of 20 ng/ml HRG. Subsequently, cells were solubilized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 0.5% Nonidet P-40, 0.2 mM Na₃VO₄, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 5 µg/ml aprotinin and immunoblotted using various immunoreagents (e.g., anti-ErbB2, anti-ErbB3, anti-ErbB4, anti-phospho-ErbB2, anti-ERK, or anti-phospho-ERK antibodies) (1 µg/ml).

In addition, immunoblot analyses of HAS1, HAS2, HAS3, CD44, or actin in cells (untransfected or transfected with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA/HAS2siRNA/HAS3siRNA, 50 pmol CD44siRNA- or HAS/CD44-related scrambled sequences, or no siRNA) were carried out. In some cases, SK-OV-3-ipl cells were incubated with 20 ng/ml HRG (or no HRG) for various time intervals (e.g., 0, 1, 3, 5, 7, 10, or 15 min). These cells were extracted with Nonidet P-40 (as described above) and subjected to immunoprecipitation using rabbit anti-ErbB4 antibody followed by adding goat anti-rabbit IgG beads. The immunoprecipitated material was solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with various antibody (e.g., rabbit anti-ErbB2 antibody (5 µg/ml) or rabbit anti-ErbB4 antibody, respectively) for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 h. The blots were then developed using ECL chemiluminescence reagent (Amersham Biosciences) according to the manufacturer’s protocols.

Nonidet P-40-solubilized cell lysates were also immunoprecipitated by anti-HAS1-conjugated beads, anti-HAS2-conjugated beads, or anti-HAS3-conjugated beads or preimmune IgG-conjugated beads followed by SDS-PAGE (4–12% gradient gels) and silver staining analyses. In some experiments, Nonidet P-40-solubilized cell lysates were immunoprecipitated by anti-HAS1-conjugated Immunobeads, anti-HAS2-conjugated Immunobeads, or anti-HAS3-conjugated Immunobeads. The supernatant fraction of anti-HAS1-immunoprecipitated materials was then immunoblotted with anti-HAS2 antibody or anti-HAS3 antibody. Similarly, the supernatant fraction of anti-HAS2-immunoprecipitated materials was immunoblotted with anti-HAS1 antibody or anti-HAS3 antibody. The supernatant fraction of anti-HAS3-immunoprecipitated materials was immunoblotted with anti-HAS1 antibody or anti-HAS2 antibody.
In some experiments, SK-OV-3.ipl cells (pretreated with various inhibitors such as 25 μM AG825 (an ErbB2 inhibitor), or 1 μM thiazolidinedione compound (an ERK inhibitor), or no drug for 1 h followed by incubating with 20 ng/ml HRG (or no HRG) for 5 min at 37 °C) were processed for immunoprecipitation using rabbit anti-HAS1 antibody, rabbit anti-HAS2 antibody, or rabbit anti-HAS3 antibody-conjugated beads followed by immunoblotting with mouse anti-phospho-tyrosine antibody or rabbit anti-HAS1 antibody/anti-HAS2 antibody/anti-HAS3 antibody, respectively according the procedures described above.

**ERK-mediated Protein Phosphorylation in Vitro**—ERK was prepared by anti-ERK-conjugated immunoaffinity column chromatography. The ERK kinase reaction was then carried out in 50 μl of the kinase buffer containing 25 mM Tris–HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10 mM MgCl2, 0.1% CHAPS, 0.1 μM calycin A, 200 μM [32P]ATP, 100 ng ERK (isolated from SK-OV-3.ipl cells pretreated with various inhibitors such as 25 μM AG825 (an ErbB2 inhibitor), or 1 μM thiazolidinedione (an ERK inhibitor), or no drug for 1 h followed by incubating with 20 ng/ml HRG (or no HRG) for 5 min at 37 °C) and 1 μg of HAS1 bound to anti-HAS1 Immunobeads (or 1 μg of HAS2 bound to anti-HAS2 Immunobeads or 1 μg of HAS3 bound to anti-HAS3-conjugated Immunobeads). After incubation for 30 min at 30 °C, the reactions were terminated by adding 20% cold trichloroacetic acid; 2 mg/ml bovine serum albumin was then added as a carrier. Trichloroacetic acid-precipitated proteins were spotted on 3MM filter papers followed by extensive wash with 10% trichloroacetic acid. The radioactivity associated with unactivated HAS isozymes (in an unphosphorylated form by incubating with ERK from untreated cells), activated HAS isozyme (in a phosphorylated form by incubating with ERK from HRG-treated cells), and unactivated HAS2 (in an unphosphorylated form by incubating with ERK from HRG-treated cells), and unactivated HAS3 (in an unphosphorylated form by incubating with ERK from HRG-treated cells). For autoradiographic analyses, gels were vacuum-dried and exposed to Kodak x-ray film at −70 °C.

**Measurement of HA Concentrations**—The HA content of the conditioned medium in SK-OV-3.ipl cell culture was measured by an enzyme-linked immunosorbent assay-like assay as described previously (10, 43). Briefly, the conditioned medium obtained from SK-OV-3.ipl cells (untreated or treated with HRG or pretreated with 25 μM AG825 (an ErbB2 inhibitor) or 1 μM thiazolidinedione compound (an ERK inhibitor) plus HRG or transfected (with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA/HAS2siRNA/HAS3siRNA, or 50 pmol of HAS-related scrambled sequences followed by 20 ng/ml HRG addition) was collected. A mixture of conditioned medium and the biotinylated HA-binding region of aggrecan (b-HABP, Seikagaku Corp., Tokyo, Japan) was added to 96-well plates precoated with HA-bovine serum albumin and then incubated for 1 h at 37 °C. Horseradish peroxidase-conjugated streptavidin was then used as a probe followed by enzymatic activity measurement using α-phenylenediamine dihydrochloride (Sigma) as a substrate. The HA concentrations were calculated from a standard curve obtained using serial dilution of standard HA.

**HA Synthase Assay**—HAS activity in the crude membranes was monitored using UDP-[14C]GlcA (320 mCi/mmol, ICN Biomedicals, Inc., Irvine, CA) as described previously (10, 43). Briefly, the crude membrane fractions isolated from cells ((i) untreated, (ii) treated with 20 ng/ml HRG, (iii) pretreated with 25 μM AG825 (an ErbB2 inhibitor) or 1 μM thiazolidinedione compound (an ERK inhibitor) plus HRG, or (iv) transfected with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA/HAS2siRNA/HAS3siRNA, or 50 pmol of HAS-related scrambled sequences followed by 20 ng/ml HRG addition) were resuspended and incubated at 37 °C for 1 h in 0.2 ml of 25 mM Hepes–NaOH (pH 7.0), 5 mM dithiothreitol, 15 mM MgCl2, 0.1 mM UDP-GlcNAc, 2 μM UDP-GlcA, and 2 μCi of UDP-[14C]GlcA. Reactions were terminated by adding SDS at 2% (w/v). Radioactivity incorporated into high molecular mass HA was measured by descending paper chromatography using Whatman 3MM paper developed in 1M ammonium acetate (pH 5.5) and ethanol (35:65, v/v). The amount of radioactivity at the origins was measured by liquid scintillation counting. The HAS activity was determined by calculating the amounts of GlcA incorporated using known specific radioactivities. We routinely assessed the incorporation of radioactivity incorporation into “HA” made by HAS-containing membrane using Streptomyces hyaluronidase-treated or untreated procedures. We also used radiolabeled UDP-sugar without the second UDP-sugar as a control. The amount of non-HA materials (representing <10–12% of the total radioactivity) was then subtracted from the total radioactivity incorporation.

In some cases, the ERK kinase reaction was carried out first in 50 μl of the kinase buffer containing unlabeled ATP, ERK (isolated from SK-OV-3.ipl cells treated with 20 ng/ml HRG (or no HRG) for 5 min at 37 °C) and HAS1 bound to anti-HAS1 Immunobeads (or HAS2 bound to anti-HAS2 Immunobeads or HAS3 bound to anti-HAS3-conjugated Immunobeads). After incubation for 30 min at 30 °C, the reactions were terminated by adding 20% cold trichloroacetic acid; 2 mg/ml bovine serum albumin was then added as a carrier. Trichloroacetic acid-precipitated proteins were spotted on 3MM filter papers followed by extensive wash with 10% trichloroacetic acid. The radioactivity associated with trichloroacetic acid-precipitated materials was analyzed by liquid scintillation counting.

In some experiments, the radioactively labeled HAS isozyme-conjugated Immunobeads were extensively washed. The polypeptide banding pattern of the HAS isoforms was then determined by SDS-PAGE (4–12% gradient gels) and silver staining. For autoradiographic analyses, gels were vacuum-dried and exposed to Kodak x-ray film at −70 °C.

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(an ERK inhibitor), or no drug) or transfected (with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA/HAS2siRNA/HAS3siRNA, 50 pmol of siRNA containing scrambled sequences, or no siRNA) were metabolically labeled with 100 μCi/ml [3H]glucosamine (Amersham Biosciences) for 18 h and subsequently rinsed with Hanks’ balanced salt solution two to three times. The medium and washes were combined and collected. Radioactively labeled cells were then treated with the trypsin-EDTA followed by washing with Hanks’ balanced salt solution for 10 min at 37 °C. Both the trypsin-EDTA solution and cell pellets were collected.

After adding 100 μg/ml unlabeled HA (Healon™, Amersham Biosciences) to various fractions containing medium, trypsin, and cell pellet materials, samples were digested with proteinase K (50 μg/ml) at 60 °C for 4 h, heated at 100 °C for 10 min, and centrifuged at 15,000 × g for 15 min. The supernatants were then precipitated with ethanol, analyzed for HA size distribution using 1 × 30-cm Hiprep™ Sephacryl™ 400 HR (S-400HR) column chromatography (Amersham Biosciences) and eluted with a solution containing 0.15M sodium acetate, 0.1% CHAPS (pH 6.8) as described previously (57, 58). Fractions of 0.5 ml were collected. The amount of radioactivity associated with each fraction was determined by scintillation counting. Some fractions were also used to test for their ability to bind to HA-binding protein-coated beads and/or to be digested by PH20 hyaluronidase. In the Hiprep™ Sephacryl™ 400 HR (S-400HR) gel-filtration analyses, four protein markers, such as bovine serum albumin (67 kDa), catalase (230 kDa), ferritin (400 kDa), and thyroglobulin (669 kDa) (purchased from Amersham Biosciences UK), plus two polysaccharide size standards, including Healon™ HA (500 kDa) (prepared according to the procedures described by McKee et al. (95)) and purified HA fragment (280 kDa) (purchased from ICN Biomedicals), were used. The sizes of certain HA fragments (e.g. the 400-kDa HA or the 80-kDa isolated from the S-400HR column) were further verified by 0.5% (w/v) agarose gel electrophoresis and Stains-all stain (44, 73) using both Select-HA™ LoLadder (in the range of ~27 to ~500 kDa) and Select-HA™ HiLadder (in the range of ~500 to 1,500 kDa) obtained from Hyalose (Oklahoma City, OK) as HA standards.

Tumor Cell Growth Assays—Exponentially growing SK-OV-3.ipl cells were harvested by trypsinization and plated in 96-well plates at a density of 2.5 × 10^3 cells/well in Dulbecco’s modified Eagle medium/F-12 medium supplemented with 10% fetal bovine serum. These cells were then transfected with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA HAS1/HAS2/HAS3-related scrambled sequences, CD44siRNA- or CD44-related scrambled sequences, or no siRNA) for 24 h at 37 °C. After fetal bovine serum was removed from the medium, these siRNA-treated were incubated with 20 ng/ml HRG (or no HRG) for an additional 24 h at 37 °C. In some experiments, untransfected cells were treated with rat anti-CD44 or various drugs, 25 μM AG825 (an ErbB2 inhibitor), 1 μM thiazolidinedione compound (an ERK inhibitor), or no drug for 1 h followed by incubating with 20 ng/ml HRG (or no HRG) for 24 h at 37 °C. The in vitro growth of these cells was analyzed by measuring increases in cell number using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (CellTiter 96™ non-radioactive cell proliferation assay according to the procedures provided by Promega, Madison, WI). Subsequently, viable cell-mediated reaction products were recorded by using a Molecular Devices (Spectra Max 250) enzyme-linked immunosorbent assay reader at a wavelength of 450 nm.

Tumor Cell Migration Assays—Twenty-four Transwell units were used for monitoring in vitro cell migration as described previously (26, 45–52). Specifically, the 8-μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SK-OV-3.ipl cells, which were (i) transfected with 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1/HAS2/HAS3-related scrambled sequences, 50 pmol CD44siRNA, 50 pmol CD44-related scrambled sequences, or no siRNA, (ii) transfected with 50 pmol of scrambled siRNA or no siRNA, or (iii) treated with various inhibitors (such as 25 μM AG825 (an ErbB2 inhibitor), or 1 μM thiazolidinedione compound (an ERK inhibitor), or no drug for 1 h), were placed in the upper chamber of the Transwell unit. The medium containing 20 ng/ml HRG or no HRG was placed in the lower chamber of the Transwell unit. After 18-h incubation at 37 °C in a humidified 95% air/5% CO2 atmosphere, cells on the lower side of the filter were removed by wiping with a cotton swap. Cell-migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (26, 45–52). The CD44-specific cell migration was determined by subtracting non-specific cell migration (i.e. cells migrate to the lower chamber in the presence of anti-CD44 antibody treatment). The number of ovarian tumor cells migrating in untreated SK-OV-3.ipl cells (control) or in SK-OV-3.ipl cells treated with scrambled siRNA is designated as 100%.

RESULTS

HRG-stimulated ErbB2 Association with ErbB4/ErbB3 in Ovarian Tumor Cells

The binding of HRG to ErbB family tyrosine kinases, such as ErbB3 and ErbB4, is known to induce important changes in cellular signaling (37–39). Immunoblot analyses using a panel of anti-ErbB family tyrosine kinases indicate that the three ErbB receptors such as ErbB2, ErbB3 and ErbB4 are expressed at different levels in SK-OV-3.ipl cells (Fig. 1A, lane 1–3). The amount of ErbB3 expression is low (Fig. 1A, lane 1, lane 2), whereas the expression of ErbB2 and ErbB4 are relatively high in SK-OV-3.ipl cells (Fig. 1A, lane 1 and 3). We have also provided an anti-actin-specific immunoblot of cell lysates from SK-OV-3.ipl cells (Fig. 1A, lane 1 and 3) as loading controls. These observations are consistent with previous findings showing variations in the levels of ErbB receptor expression in ovarian tumor cells (53). Several lines of evidence have indicated that HRG is capable of promoting heterodimerization of ErbB2 with other ErbB family of receptors (31–33). Here, we have addressed the question of whether there is a physical linkage between ErbB2 with ErbB4 (or ErbB3) in ovarian tumor cells (SK-OV-3.ipl cell line) following HRG stimula-
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SK-OV-3.ipll cells. These findings clearly establish that ErbB2 and ErbB4 are constitutively associated with each other in SK-OV-3.ipll cells, and there is an additional accumulation of ErbB2 into ErbB4 complexes following HRG treatment of the ovarian tumor cells.

HRG-mediated ErbB2-ERK Signaling: ERK-mediated HAS Phosphorylation and Activation in SK-OV-3.ipll Cells

Components of HRG signaling pathways have been shown to interact with ErbB receptors and stimulate ErbB2 tyrosine kinase and ERK activity (37–39). In this study we have observed that HRG treatment of SK-OV-3.ipll cells activates ErbB2 tyrosine kinase activity (Fig. 2A, panels a and b, lane 2), which, in turn, stimulates a downstream kinase cascade, including the mitogen-activated protein kinase (in particular ERK1 and ERK2) pathway (Fig. 2B, panels a and b, lane 2). In contrast, very little tyrosine phosphorylation of ErbB2 (Fig. 2A, panels a and b, lane 1) and ERK phosphorylation (Fig. 2B, panels a and b, lane 1) was detected in those SK-OV-3.ipll cells not treated with HRG. SK-OV-3.ipll cells treated with various drugs (e.g. the ErbB2 inhibitor (AG825) or an ERK inhibitor (thiazolidinedione compound) underwent a significant inhibition of HRG-mediated activation of ErbB2 (Fig. 2A, panels a and b, lanes 3 and 4) and ERK (Fig. 2B, panels a and b, lanes 3 and 4). These observations strongly suggest that HRG interaction with the ErbB family of tyrosine kinases is capable of activating ErbB2 kinase activity and influencing its downstream effectors such as ERK.

In ovarian cancer, high levels of HA are associated with tumor progression (54). Several growth factors have been
HAS Expression

Molecular cloning data and immunological analyses have shown that there are at least three HAS synthases, designated HAS1, HAS2, and HAS3, which are derived from three distinct genes (8–11). To analyze the expression of HAS molecules in SK-OV-3.ipl cells, we have developed a panel of polyclonal antibodies, which are specifically raised against HAS isozyme-specific sequences. Using these newly developed HAS subtype-specific antibodies, we have identified the presence of all three HAS isozymes that display molecular masses of ~62 kDa for HAS1 (Fig. 3A, lane 2), ~60 kDa for HAS2 (Fig. 3A, lane 4), and ~60 kDa for HAS3 (Fig. 3A, lane 6), respectively. The purity and specificity of these HAS isozyme-related immunoreagents were further assessed by anti-HAS1/anti-HAS2/anti-HAS3-mediated immunoprecipitation followed by silver-staining analyses (Fig. 3B). Our results confirmed that the HAS isozymes correspond to a single polypeptide at ~62 kDa (for HAS1) (Fig. 3B, lane 2), ~60 kDa (for HAS2) (Fig. 3B, lane 4), and ~60 kDa (for HAS3) (Fig. 3B, lane 6). We conclude that these HAS isozymes detected by specific anti-HAS1/anti-HAS2/anti-HAS3-mediated immunoblot and immunoprecipitation are specific, because no protein was detected with those cells incubated with preimmune IgG (Fig. 3A, lanes 1, 3, and 5; Fig. 3B, lanes 1, 3, and 5). In addition, we observed that both the anti-HAS2 antibody (Fig. 3C, panel a, lane 1) and the anti-GFP antibody (Fig. 3C, panel b, lane 1) recognized the same band isolated from HEK293 cells transfected with GFP-tagged HAS2cDNA (not vector-transfected control sample (Fig. 3C, lanes 1 and 2). Similarly, both anti-HAS1 and anti-HAS3 also recognized recombinant HAS1 and HAS3 in HEK293 cells transfected with GFP-tagged HAS1cDNA and HAS3cDNA, respectively (data not shown). Therefore, we believe that our anti-HAS antibodies recognize their targeted HAS isozymes.

HRG-ErbB2-ERK Signaling-mediated HAS Activation

Previous studies have indicated that the impact of HRG-ErbB2-activated ERK signaling on oncopogenesis highly depends on the ability of ERK to phosphorylate certain cellular substrates (40–42). In this study we have measured the kinase activity associated with the ERK molecules isolated from HRG-stimulated cells (Table 2). Specifically, the kinase activity was measured in the presence of the ErbB2-activated ERK, and the results are shown in Table 2. Our results indicate that the appropriate removal of the targeted HAS did not cause the loss of the other two HAS isozymes (Fig. 3D, (I) lanes 1 and 2, (II) lanes 1 and 2, and (III) lanes 1 and 2). Although the expression of HAS molecules has been detected in many tumor cell types, the cellular and molecular mechanisms involved in regulating HAS activity have not been fully investigated.

| TABLE 1 |
| Measurement of HA production in SK-OV-3.ipl cells |
| The procedures for measuring HA production in the medium of SK-OV-3.ipl cells treated with various reagents (e.g., 25 μM AG825, 1 μM thiazolidinedione compound, 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA + HAS2siRNA + HAS3siRNA, or 50 pmol of siRNA-scrambled sequences) in the presence or absence of 20 ng/ml HRG were described under “Materials and Methods.” The amount of HA production in the medium of cells treated with no drug (in the absence of HRG) (control) or treated with scrambled sequence (in the absence of HRG) (control) is designated as 100%. |

| Cells | Amount of HA production |
|---|---|
| | −HRG | +HRG |
| Untreated cells (control) | 860 ± 26 (100) | 2,100 ± 98 (244) |
| AG825 treatment | 805 ± 32 (94) | 850 ± 24 (99) |
| Thiazolidinedione treatment | 816 ± 30 (95) | 947 ± 28 (110) |
| Scrambled sequence treatment (control) | 854 ± 27 (100) | 2,218 ± 95 (260) |
| HAS1siRNA treatment | 683 ± 21 (80) | 820 ± 23 (96) |
| HAS2siRNA treatment | 615 ± 15 (72) | 800 ± 19 (94) |
| HAS3siRNA treatment | 695 ± 18 (81) | 876 ± 21 (103) |
| HAS1siRNA + HAS2siRNA + HAS3siRNA treatment | 214 ± 5 (25) | 265 ± 7 (31) |

*Data represent mean ± S.E. of HA production (ng/mg total protein) in each sample. *Significantly different (p < 0.001; ANOVA; n = 6) as compared with untreated (no drug treatment and no HRG addition) (control) samples. *Significantly different (p < 0.001; ANOVA; n = 5) as compared with scrambled sequence treated (in the absence of HRG) (control) samples.
tionally active and that all three HAS molecules serve as specific cellular substrates for ERK.

Furthermore, we have found that phosphorylation of HAS (e.g. HAS1, HAS2, and HAS3) (Fig. 4A, panels a and b, lane 2; Fig. 4BII, panels a and b, lane 2; and Fig. 4BIII, panels a and b, lane 2) at the serine residues was significantly enhanced in SK-OV-3.1ipl cells treated with HRG. In contrast, HRG-induced HAS phosphorylation at the serine residues was relatively low in SK-OV-3.1ipl cells without any HRG treatment (Fig. 4I).

The low amount of HAS phosphorylation by ERK (isolated from cells with no HRG treatment or pretreated with various inhibitors followed by HRG treatment) results in a significant reduction of HA production and HAS activity (Tables 1A and 3A, respectively). The low amount of HAS phosphorylation by ERK (isolated from cells with no HRG treatment or pretreated with various inhibitors followed by HRG treatment) results in a significant reduction of HA production and HAS activity (Tables 1A and 3A, respectively). It is possible that the concentration ratio between ERK and its substrate(s) (e.g. HAS isozymes) in the in vitro reaction condition (using HRG-ErbB2-stimulated ERK with purified HAS protein) (Fig. 4A) may be different from the ERK:HAS ratio that exists in HRG-ErbB2-stimulated cells in vivo (Fig. 4B).

This may explain the apparent discrepancy in the extent of HAS phosphorylation by ERK detected in Fig. 4A versus Fig. 4B. Importantly, ERK inhibitor (thiazolidinedione compound) appears to block HRG-ErbB2-mediated HAS phosphorylation measured by either in vivo or in vitro methods. These findings suggest that ERK is closely involved in

![Image](49x360 to 408x618)

**TABLE 2**

Effects of various drugs on HRG-mediated ERK phosphorylation of HAS isozymes and activities

In these experiments, the activity of ERK isolated from SK-OV-3.1ipl cells (treated with 25 μM AG825 or 1 μM thiazolidinedione compound) (in the presence or absence of 20 ng/ml HRG) was measured by the incorporation of [32P]ATP into HAS1, HAS2, and HAS3 (bound to the anti-HAS1 or anti-HAS2 or anti-HAS3-conjugated beads, respectively) as described under “Materials and Methods.” The amount of [32P]ATP incorporation into HAS isozymes (e.g. HAS1, HAS2, or HAS3) by ERK isolated from cells treated with no drug (in the absence of HRG) (control) is designated as 100%.

| Experiments                  | ERK-mediated [32P]ATP incorporation |     |
|------------------------------|------------------------------------|-----|
|                              | into HAS1          | into HAS2          | into HAS3          |
| −HRG                         | +HRG                | −HRG                | +HRG                |
| Untreated cells (control)    | 33 ± 1.1 (100)      | 150 ± 6.2 (455)    | 37 ± 1.1 (100)     | 217 ± 0.8 (586)  |
| AG825-treated cells          | 32 ± 1.4 (97)a      | 45 ± 1.3 (136)a    | 35 ± 1.6 (95)b     | 48 ± 1.0 (130)b  |
| Thiazolidinedione-treated cells | 31 ± 1.2 (94)b      | 47 ± 1.2 (142)b    | 36 ± 1.1 (97)b     | 47 ± 1.2 (127)b  |

a Data represent mean ± S.E. of [32P]ATP incorporation (cpm) into HAS isozymes (e.g. HAS1, HAS2, or HAS3) in each sample.

b Significantly different (p < 0.005; ANOVA; n = 6) as compared with ERK isolated from cells treated with no drug (in the absence of HRG) (control) cells.

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Effects of HRG-ErbB2-ERK Activation on HA Size Distribution

There is now compelling evidence that the one or more sizes of HA accumulated in the extracellular matrix of tumor cells are linked to HA modification and tumor cell behaviors (5–7, 54). However, the cellular and molecular mechanisms involved in HRG-activated HA metabolism and HA-mediated ovarian tumor progression are not well understood. To analyze the effects of HRG-ErbB2-ERK signaling on HA-size distribution, SK-OV-3.ipl cells were metabolically labeled with [3H]glucosamine for 18 h. HA was then recovered from the medium and analyzed using Sephacryl S-400HR gel-filtration methods similar to the procedures described previously (58, 59). Most of the HA (with a molecular mass ~400 kDa) accumulated in the medium of untreated cells (Fig. 5A). Furthermore, our results indicate that externally added HRG caused a decrease in the polymer size of endogenous HA from ~400 to ~80 kDa (Fig. 5B). These findings indicate that HRG treatment was involved in the modification of endogenous HA polymers. Impairment of ErbB2 activation and ERK signaling by treating cells with AG825 (an ErbB2 inhibitor) (Fig. 5C) or thiazolidinedione compound (the ERK blocker) (Fig. 5D) favored the formation of 400-kDa HA polymers and blocked the production of ~80-kDa endogenous HA polymer in the medium of cells treated with HRG. These results regulating HRG-ErbB2-mediated HAS phosphorylation and activity.

FIGURE 4. Measurement of HAS phosphorylation in SK-OV-3.ipl cells.

A, detection of ERK-mediated HAS phosphorylation and HAS activity. The ERK kinase reaction was first carried out in the kinase buffer containing [32P]ATP, ERK (isolated from SK-OV-3.ipl cells either untreated or treated with 20 ng/ml HRG for 5 min at 37 °C), and HAS1 bound to anti-HAS1 Immunobeads (or HAS2 bound to HAS3 Immunobeads) as described under “Materials and Methods.” After the kinase reaction, the HAS isozyme-conjugated Immunobeads were extensively washed. The polypeptide banding pattern of the HAS isozymes was then determined by SDS-PAGE (4%–12% gradient gels) and silver staining followed by autoradiographic analyses (lanes 1, 3, and 5; autoradiogram of HAS1 (lane 1) or HAS2 (lane 3) or HAS3 (lane 5) incubated with HRG (isolated from untreated cells); and lanes 2, 4, and 6; autoradiogram of HAS1 (lane 1) or HAS2 (lane 3) or HAS3 (lane 5) incubated with ERK (isolated from HRG-treated cells)). The ERK kinase reaction was also carried out in the kinase buffer containing non-radioactive ATP, ERK (isolated from SK-OV-3.ipl cells either untreated or treated with 20 ng/ml HRG for 5 min at 37 °C), and HAS1 bound to anti-HAS1 Immunobeads (or HAS2 bound to anti-HAS2 Immunobeads or HAS3 bound to anti-HAS3-conjugated Immunobeads) as described above. After the kinase reaction, the HAS isozyme-conjugated Immunobeads were extensively washed. The HAS activity of the HAS1 bound to anti-HAS1 Immunobeads (or HAS2 bound to anti-HAS2 Immunobeads or HAS3 bound to anti-HAS3-conjugated Immunobeads) was then assessed by using UDP-[14C]GlcA as described under “Materials and Methods.” The activity level of HAS isozymes was compared between unactivated HAS isozymes (in an unphosphorylated form by incubating with ERK from untreated cells) (lanes 1, 3, and 5) and activated HAS isozymes (in a phosphorylated form by incubating with ERK from HRG-treated cells) (lanes 2, 4, and 6). The values expressed in Fig. 4A represent an average of triplicate determinations in four experiments with a standard deviation <±5%. (*, there was a significant difference (p < 0.05; Student’s t test) in HAS activities between the ERK (from HRG-treated cells) treated and the ERK (from untreated cells) treated (control) samples. In B, (I), detection of HAS1 phosphorylation by anti-HAS1-mediated immunoprecipitation followed by immunoblotting with anti-phospho-serine (a) or reblotting with anti-HAS1 as a loading control (b) in cells treated with no HRG (lane 1), treated with HRG for 5 min (lane 2), pretreated with thiazolidinedione compound (an ERK blocker) for 1 h followed by HRG treatment for 5 min (lane 4). (II), detection of HAS2 phosphorylation by anti-HAS2-mediated immunoprecipitation followed by immunoblotting with anti-phospho-serine (a) or reblotting with anti-HAS2 as a loading control (b) in cells treated with no HRG (lane 1), treated with HRG for 5 min (lane 2), pretreated with thiazolidinedione compound (an ERK blocker) for 1 h followed by HRG treatment for 5 min (lane 4). (III), detection of HAS3 phosphorylation by anti-HAS3-mediated immunoprecipitation followed by immunoblotting with anti-phospho-serine (a) or reblotting with anti-HAS3 as a loading control (b) in cells treated with no HRG (lane 1), treated with HRG for 5 min (lane 2), pretreated with AG825 (an ErbB2 inhibitor) for 1 h followed by HRG treatment for 5 min (lane 3), or pretreated with thiazolidinedione compound (an ERK blocker) for 1 h followed by HRG treatment for 5 min (lane 4).
support the notion that HRG-mediated ErbB2 activation and ERK signaling are required for HA modification.

Finally, we have transfected SK-OV-3.ipl cells with specific siRNA sequences targeting human HAS1, HAS2, and HAS3, respectively. These HAS1siRNA/HAS2/HAS3 siRNA target sequences effectively down-regulated HAS1/HAS2/HAS3 expression in SK-OV-3.ipl cells (Fig. 6A, panels a (lane 2), b (lane 4), and c (lane 6)). No significant reduction of HAS1 or HAS2 or HAS3 was observed in cells treated with transfection reagents containing scrambled sequences (Fig. 6A, panels a (lane 1), b (lane 3), and c (lane 5)). We have also provided an anti-actin-specific immunoblot of cell lysates from cells treated with transfection reagents containing HAS1siRNA or HAS2siRNA or HAS3siRNA target sequences or scrambled

### TABLE 3

**Measurement of HAS activity**

The procedures for measuring HAS activity using SK-OV-3.ipl cells treated with various reagents (e.g. 25 μM AG825, 1 μM thiazolidinedione compound, 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA + HAS2siRNA + HAS3siRNA, or 50 pmol of HASsiRNA-scrambled sequence) in the presence or absence of 20 ng/ml HRG were described under “Materials and Methods.”

| Treatments | HAS activity |
|------------|--------------|
|             | −HRG         | +HRG         |
|             | pmol/h/mg protein (% of control) | pmol/h/mg protein (% of control) |
| **A) Effects of various drugs on HRG-mediated HAS activity** | | |
| No treatment (control) | 1.40 ± 0.04 (100) | 3.76 ± 0.09 (269) |
| AG825 treatment | 1.33 ± 0.03 (95) | 1.54 ± 0.05 (110) |
| Thiazolidinedione treatment | 1.38 ± 0.03 (99) | 1.58 ± 0.06 (113) |
| **B) Effects of HASsiRNA treatment on HRG-mediated HAS activity** | | |
| Scrambled sequence treatment (control) | 1.42 ± 0.041 (100) | 3.50 ± 0.013 (246) |
| HAS1siRNA treatment | 1.05 ± 0.015 (74) | 1.49 ± 0.032 (105) |
| HAS2siRNA treatment | 1.02 ± 0.021 (72) | 1.36 ± 0.051 (96) |
| HAS3siRNA treatment | 1.13 ± 0.042 (80) | 1.50 ± 0.040 (100) |
| HAS1siRNA + HAS2siRNA + HAS3siRNA treatment | 0.31 ± 0.006 (22) | 0.37 ± 0.013 (20) |

* Data represent mean ± S.E. of HAS Activity (pmol/h/mg protein) in each sample.

* Significantly different (p < 0.001; ANOVA; n = 6) as compared with untreated (no drug treatment and no HRG addition) (control) samples.

* Significantly different (p < 0.001; ANOVA; n = 5) as compared with scrambled sequence treated (in the absence of HRG) (control) samples.

**FIGURE 5. Analyses of HRG-mediated HA size distribution in SK-OV-3.ipl cells.** SK-OV-3.ipl cells were metabolically labeled with [3H]glucosamine. HA was then collected from the medium and analyzed by Hiprep™ Sephacryl™ 400 HR (S-400HR) gel filtration as described under “Materials and Methods.” A and B, HA size distribution detected in the medium of cells treated with no drug in the presence (b) or absence (a) of externally added HRG (inset: HA size distribution detected in the medium of cells treated with a combination of HAS1siRNA, HAS2siRNA, and HAS3siRNA in the presence of externally added HRG (b)). C and D, HA size distribution detected in the medium of cells pretreated with AG825 (an ErbB2 inhibitor) followed by HRG treatment (C), or pretreated with thiazolidinedione compound (an ERK blocker) followed by HRG treatment (D). (Markers used in the S-400HR column chromatography include 669, 500, 400, 280, 230, and 67 kDa, indicated by arrowheads and/or arrows.)
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sequences (Fig. 6A, panels a–d, lanes 1–6) as loading controls. Therefore, we conclude that the selective “knockdown” of HAS1 or HAS2 or HAS3 expression by these HAS1/HAS2/HAS3 siRNAs is specific. Further analyses indicate that transfection of SK-OV-3.ipl cells with the individual HAS1siRNA, HAS2siRNA, and HAS3siRNA, respectively, partially inhibited HAS activity (Table 3B) and HA production (Table 1B). Moreover, transfection of SK-OV-3.ipl cells with a combination of all three HASsiRNAs (HAS1siRNA, HAS2siRNA, and HAS3siRNA) significantly blocked both the basal level and the HRG-induced HAS activity (Table 3B) and HA production (Table 1B). In addition, we have found that down-regulation of all three HASs by treating cells with HAS1siRNA, HAS2siRNA, and HAS3siRNA not only inhibited HRG-induced production of ~80 kDa endogenous HA polymer in the medium but also allowed the retention of the endogenous HA with a molecular mass of ~400 kDa size (Fig. 5B, panel b, inset). In contrast, HRG is capable of promoting the formation of ~80 kDa endogenous HA polymer in the medium of cells treated with HASsiRNA-scarred sequence (Fig. 5B, panel a, inset). These results suggest that HAS molecules are required for HRG-stimulated HA size modification.

Effects of HRG-ErbB2-ERK Activation on CD44 Signaling and Ovarian Tumor Cell Functions

It has been postulated that HAS activation, plus HA production and modification, are involved in ovarian tumor cell attachment, growth, and migration through the formation of the HA-containing extracellular matrix (18). It is well established that HA is one of the ligands recognized by surface CD44 molecules. Specific HA binding motifs have been identified and localized in the extracellular domain of CD44 (7, 61, 62). CD44 interaction with HA is one of the important requirements for the spread of ovarian cancer. In this study, we have investigated whether HRG-mediated signaling events (e.g. ErbB2 activation, ERK function, HAS activation, hyaluronidase expression, and HA production/modification) are directly involved in CD44-mediated oncogenic signaling and ovarian tumor cell activation.

It is now increasingly evident that RhoGTPases such as Cdc42 regulate a wide variety of cellular activities mediated by HA-CD44 interaction (42). One class of important cellular targets of Cdc42GTPase activity is the p21-activated kinases (PAKs) (e.g. PAK1) (63, 64). PAK1 belongs to the family of serine/threonine protein kinases and plays an important role in the interaction of cytoskeletal proteins such as filamin A (an actin-binding protein known to induce the cross-linking processes of actin filaments) (65, 66). Our present results indicate that HRG activates CD44 association with Cdc42 (Fig. 7A, panels a and b, lanes 1 and 2) and stimulates PAK1 activation (Fig. 7A, lanes 3; 7B, lane 3; and 7C, lane 3) or thiazolidinedione compound (an ERK inhibitor) (Figs. 7A, lane 4; 7B, lane 4; and 7C, lane 4) or HAS1siRNA/HAS2siRNA/HAS3siRNA (Figs. 7A, lane 5; 7B, lane 5; and 7C, lane 5) causes a significant inhibition of HRG-mediated CD44-associated signaling (e.g. CD44-Cdc42 association, PAK1 activation, and PAK1-filamin complex formation) plus tumor cell growth and migration (Table 4, A and B). Various tumor cell-specific properties (e.g. HRG-induced CD44-Cdc42 association, PAK1 activation, and PAK1-filamin complex formation and tumor cell growth/migration) were also significantly down-regulated in anti-CD44 (an HA-blocking agent)-treated cells (Figs. 7A, lane 6; 7B, lane 6; and 7C, lane 6) and Table 4C). These observations support the notion that both ErbB2-ERK signaling events and HA-CD44 interaction are functionally linked in HRG-mediated ovarian tumor cell activation.

To further confirm that CD44 is needed for HRG-stimulated ovarian tumor cell activation, we next transfected SK-OV-3.ipl cells with a specific siRNA sequence targeting CD44. As shown in Fig. 6B, this siRNA successfully suppressed CD44 expression in SK-OV-3.ipl cells (Fig. 6B, panel a, lane 2). In control experiments, SK-OV-3.ipl cells were treated with transfection reagents containing scrambled sequences, and no CD44 down-regulation was observed (Fig. 6B, panel a, lane 1). Because other cellular proteins, such as actin, continued to be expressed at comparable levels in the CD44siRNA-treated cells (Fig. 6B, panel b, lane 2) as well as in control cells containing scrambled sequences (Fig. 6B, panel b, lane 1), we conclude that the selective down-regulation of CD44 expression by CD44siRNAs is target-specific. Further analyses indicate that HRG-activated ErbB2- and ERK-signaling events (e.g. PAK1 activation, PAK1-filamin complex formation, and tumor cell growth/migration) in CD44siRNA-treated cells were signifi-
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In recent years, there has been considerable interest in the ErbB receptors (e.g. ErbB1, ErbB2, ErbB3, and ErbB4) and their interactions with specific growth factors in ovarian cancer progression. For example, ErbB1 interacts with EGF (67) and a number of other growth factors (e.g. transforming growth factor-α, betacellulin, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, and epigen, etc.) and forms heteromers with other members of ErbB family members (28–30). Both ErbB3 and ErbB4 bind to HRG, which belongs to a group of EGF-like ligands, and is often expressed in ovarian cancer tissues (28–30). However, ErbB4 can also associate with betacellulin, heparin-binding EGF-like growth factor, amphiregulin, and epiregulin (68–70). The one or more specific ligands for ErbB2 have not been identified at this time. In this study we have found that at least three ErbB receptors (ErbB2, ErbB3, and ErbB4) are expressed in ovarian tumor cells (SK-OV-3.ipl cells). The level of ErbB2 expression appears to be greater than ErbB3 and/or ErbB4 in SK-OV-3.ipl cells (Fig. 1). Overexpression of the ErbB receptor family of tyrosine kinases (in particular, ErbB2) often correlates with poor survival rates of many known cancers, including ovarian cancer (42, 51). Upon HRG activation, we have noted that a complex formation between ErbB2 and ErbB4 occurs in SK-OV-3.ipl cells (Fig. 1). These observations are consistent with previous studies showing that ErbB2 serves as a coreceptor for other ErbB receptors such as ErbB3 and/or ErbB4 to form heteromers following HRG stimulation (31–33). The question of whether HRG-ErbB receptor interaction plays a role in facilitating peritoneal metastatic dissemination of ovarian cancer by increasing cell growth and migration has not been fully investigated.

One of the important features of HRG-ErbB receptor interactions is their ability to stimulate signal transduction that enhances tumor cell growth, migration, and malignant transformation (22, 30, 34–39). Various growth factors (e.g. HRG) have been shown to stimulate ErbB2 kinase activity and promote Ras-mediated stimulation of a downstream kinase cascade, which includes the Raf-1/MEK/mitogen-activated protein kinase (ERK) pathway leading to tumor cell growth and migration (37–39). Here, we have demonstrated that HRG-ErbB signaling is capable of inducing marked phosphorylation of ERK (in particular, ERK2) in ovarian tumor cells (Fig. 2). Moreover, both ERK phosphorylation and kinase activity (Fig. 2) can be blocked by treating cells with an ErbB2 kinase inhibitor during HRG stimulation (Table 2 and Fig. 2). Thus, it appears that HRG-activated ERK2 phosphorylation and activity are closely coupled with ErbB2 activation in ovarian tumor cells. Activated ERK can either phosphorylate a number of cytoplasmic targets or migrate to the nucleus, where they phosphorylate and activate certain transcriptional factors such as c-fos and Elk-1 (50, 71, 72). Despite widespread interest in HRG-mediated
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TABLE 4
Analyses of HRG-mediated tumor cell growth and migration

Procedures for measuring tumor cell growth in SK-OV-3.1pl cells (untreated or treated with 25 μM AG825, 25 μM thiazolidinedione compound, 50 pmol of HAS1siRNA, 50 pmol of HAS2siRNA, 50 pmol of HAS3siRNA, 50 pmol of HAS1siRNA + HAS2siRNA + HAS3siRNA, 50 pmol of HAS1siRNA-scrambled sequence, 50 pmol of CD44siRNA or CD44siRNA-scrambled sequence, or 10 μg/ml anti-CD44 antibody) in the presence or absence of 20 ng/ml HRG for 24 h were described under “Materials and Methods.” The CD44-specific cell migration in cells without any treatment (A and C), treated with HASsiRNA-scrambled sequences (B), or treated with CD44siRNA-scrambled sequences (D) is designated as 100%. We have found that all three HASs are expressed in ovarian tumor cells (SK-OV-3.1pl cells) (Fig. 3). A number of studies have shown that manipulation of specific HAS isozone expression effectively influences tumorigenesis and metastasis (15–19). The function of HAS1, HAS2, and HAS3 appears to be cell- and tissue-specific (8–11). Analyses of HAS2 knock-out mice indicate that the signaling interaction between HA and ErbB2 is tightly coupled (76, 77). HA is also known to influence tumor cell behaviors in a manner dependent on ErbB2 receptor signaling (24–27). Several growth factors (e.g. EGF, transforming growth factor-β, and Osteopodin) have been shown to control HA synthesis and HAS expression (55–57, 78). However, the regulatory processes involved in controlling HAS activities required for HA production in ovarian tumor cells are unknown presently. Here, we have demonstrated that HAS phosphorylation by ERK (isolated from SK-OV-3.1pl cells in the presence of HRG treatment) significantly up-regulates HAS activities and HA production (Fig. 4 and Tables 1 and 2). In contrast, the reduction of HAS phosphorylation by pretreating cells with ErbB2 tyrosine kinase inhibitor (AG825) or ERK inhibitor (thiazolidinedione compound) in the presence or absence of HRG treatment, abolishes HAS activities and HA production. Thus, HRG-ErbB2-mediated ERK signaling plays a role in regulating HAS phosphorylation and the subsequent HA production during ovarian cancer progression.

Currently, the Weigel group reported that the activity of streptococcal HAS is lipid-dependent and Nonidet P-40-sensitive (50). If the Nonidet P-40-solubilized HAS is inactivated and can be phosphorylated by ERK in vitro (Fig. 4), this does not mean that the one or more phosphorylation sites are the same as those detected in vivo or that the same sites are also accessible in the native protein in live cells. The fact that ERK phosphorylation of HAS isozone restores some HAS activity in vitro suggests that HAS phosphorylation plays a role in modulating HAS conformation and activity in a lipid-independent manner.

An abnormal accumulation of HA matrix induced by HAS overexpression and activation often causes cellular transformation, tumorigenesis, and malignant progression (15–19, 79). Our results indicate that down-regulation of all three HAS isozones effectively reduces HRG-mediated HA production.
(Table 1) and tumor cell-specific behaviors (e.g. ovarian tumor cell growth and migration) (Table 4). These findings are consistent with a previous study showing antisense-mediated suppression of HAS2 inhibits the tumorigenesis and breast cancer progression (80). The size distribution of HA also appears to be closely associated with tumor progression (58). In this study we have noted that HRG treatment of cells induces the formation of a small sized HA (~80 kDa) (Fig. 5). When HRG-mediated ErbB2 activation or ERK signaling is inhibited (by treating cells with AG825 (the ErbB2 inhibitor) or thiazolidinedione compound (an ERK inhibitor)), the HA size decrease is also inhibited (Fig. 5). Both hyaluronidases (81) and reactive oxygen species (82) have been implicated in the partial degradation or modifications of HA polymers. The question of which regulatory mechanisms (hyaluronidases versus reactive oxygen species) are involved in HRG-mediated HA modifications is currently under investigation in our laboratory. Specific fragments of HA (large HA versus small HA) have been shown to play important roles in regulating a variety of cellular functions, including cell proliferation, migration, gene expression, angiogenesis, and tumor progression (93, 94). Size modification of HRG-activated HA fragments (shifting 80-kDa sizes into 400-kDa fragments) caused by treating cells with various signaling perturbation agents (AG825 (the ErbB2 inhibitor) or thiazolidinedione compound (an ERK inhibitor)) (Fig. 5) could significantly alter the proper HA configuration required for cellular signaling and functions. The fact that the loss of 80-kDa HA fragments correlates well with the reduction of tumor-specific properties (Table 4) suggests that this 80-kDa species is critically important for ovarian tumor functions.

Preliminary data indicate that HRG also activates hyaluronidases such as Hyal-2 and PH20 (Spam1) in SK-OV-3.ipl cells. Although the three HAS isozymes are thought to make HA of different sizes, ranging from 0.1 to 10 million Da, the active hyaluronidases detected in ovarian tumor cells (e.g. SK-OV-3.ipl cells) may participate in the degradation of added large radiolabeled HA polymers resulting in smaller HA sizes (400 and 80 kDa) during the course of the experiments shown in Fig. 5. Different HA sizes have been shown to influence a variety of biological activities (93, 94). In our study, we only measured net HA accumulation and did not correct for actual HA biosynthesis or degradation. Thus, the change in HA size from 400 to 80 kDa after HRG treatment (Fig. 5A versus 4B) could be due to either an increase in HA degradation or to an altered regulation/modification of HAS isozyme activities. The possible "cross-talk" between HAS isozymes and hyaluronidases during HRG signaling in SK-OV-3.ipl cells is currently under investigation in our laboratory. This may also explain the apparent discrepancy that each of the HASsiRNAs has approximately the same level of activity against various parameters measured in Tables 1, 3, and 4, but their combined activities are not additive. The possible "coordinated interaction," between down-regulation of HAS isozymes and changes in hyaluronidase activities/HA sizes during HRG-mediated tumor behaviors, awaits further investigation.

Recently, we have observed that up-regulation of both growth and migration occurs in ovarian tumor cells treated with small size HA (~80 kDa) (and to a lesser extent with ~400 kDa HA). Because anti-CD44 antibody inhibits HA fragment-mediated biological responses, it is possible that a CD44-related HA receptor is involved in HA-mediated ovarian tumor functions. Similar results showing selective HA fragment effects on tumor cell activation and function have been observed previously (58). Investigations into the cellular mechanisms underlying the relationship of HA/CD44 and metastatic behavior of tumor cells have demonstrated that the CD44 cytoplasmic domain selectively interacts with certain cytoskeletal proteins (e.g. ankyrin and ERM) (7, 83–88) and a variety of signaling components (e.g. the Src family tyrosine kinases (46), p185HER2 (25, 26), transforming growth factor-β receptors (52), Rho-kinase (48, 49), the guanine nucleotide exchange factors (e.g. Tiam1 (47) and Vav2 (26)), and Gab-1-linked phosphatidylinositol 3-kinase (49)) during tumor progression. In addition, CD44 has been shown to be involved in the production of certain cytokines (e.g. interleukin 8 (89) and FGF-2 (86)) and hormones (e.g. parathyroid hormone-related protein (52)) in tumor cells. These findings clearly indicate that CD44 plays a pivotal role in activating oncogenic signaling and HA-mediated tumor cell functions.

Members of the Rho subclass of the Ras superfamily (small molecular weight GTPases (e.g. RhoA, Rac1, and Cdc42)) are known to be associated with changes in the membrane-linked cytoskeleton (90). For example, activation of RhoA, Rac1, and Cdc42 has been found to produce specific structural changes in the plasma membrane-cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (90). Furthermore, overexpression of RhoGTPases in human tumors often correlates with a poor cancer prognosis (91, 92). Consequently, coordinated RhoGTPase signaling is considered to be important in the mechanism underlying cell growth and migration, both of which are obvious prerequisites for metastasis (90–92). Our recent studies indicate that HA-CD44 interaction stimulates Cdc42 binding to IQGAP1 and mediates the activation of actin cytoskeleton (50) as well as ovarian tumor cell migration/invasion (50). In the search for additional cellular targets of Cdc42 that correlate with metastatic behavior in ovarian tumor cells, the PAK family of kinases (e.g. PAK1) documented to be one of the downstream effectors of Cdc42, has been identified (63, 64). PAK1 is known to be involved in cortical actin remodeling induced by RhoGTPases such as Rac or Cdc42 and plays a significant role in a variety of biological activities (63, 64). In this study our results indicate that HRG-ErbB2-mediated ERK signaling stimulates both Cdc42-CD44 association and PAK1 phosphorylation (Fig. 7). The fact that filamin (a 280-kDa actin-binding protein) known to bind the p21-binding domain of PAK1 (amino acids 52–132) (65, 66) forms a complex with PAK1 during HRG-ErbB2-mediated ERK signaling suggests that a PAK1-filamin interaction could contribute significantly to cytoskeletal regulation. Our results are consistent with the previous findings that PAK1 activation is closely involved in filamin-mediated motility (65, 66).
Heregulin Stimulates ErbB2/ERK Signaling and HAS Activation

FIGURE 8. A proposed model for HRG-mediated ErbB2-ERK signaling and HAS activation in CD44-dependent ovarian tumor cell activation. The binding of HRG to the ErbB2-ErbB4 complex (step 1) in SK-OV-3.ipl cells stimulates ErbB2 tyrosine kinases (step 2) and ERK activity (step 3) resulting in tumor cell growth and migration. At the same time, HRG-mediated ErbB2-ERK signaling also induces HAS phosphorylation, activation, and HA production (step 4). Subsequently, the binding of HA to CD44 (step 5) promotes Cdc42-CD44 association (step 6), PAK1 phosphorylation/activation and PAK1-filamin complex formation (step 7) leading to cytoskeleton activation and tumor cell migration. Taken together, these results indicate that HRG-mediated ErbB2-ERK signaling plays a pivotal role in up-regulating HAS-mediated HA production required for CD44-dependent ovarian tumor progression.

to CD44) or down-regulation of CD44 by transfecting cells with CD44siRNA prevents HRG-mediated ovarian tumor cell growth and migration (Table 4). These findings indicate that CD44 serves as a downstream effector for HRG signaling and that CD44 interaction with HRG-induced HA plays an important role in ovarian tumor cell-specific behaviors. Taken together, we propose that the binding of HRG to ErbB receptors (in particular, the ErbB2-ErbB4 complex) stimulates ErbB2 tyrosine kinase and ERK activities resulting in HAS phosphorylation/activation and HA production/size modifications. Subsequently, HA promotes CD44 signaling leading to Cdc42-PAK and filamin-associated cytoskeleton activation and ovarian tumor cell growth and migration (Fig. 8).

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