CD44 Interaction with c-Src Kinase Promotes Cortactin-mediated Cytoskeleton Function and Hyaluronic Acid-dependent Ovarian Tumor Cell Migration*

Lilly Y. W. Bourguignon‡, Hongbo Zhu, Lijune Shao, and Yue-Wei Chen

From the Department of Cell Biology and Anatomy, School of Medicine, University of Miami, Miami, Florida 33101

In this study we have demonstrated that both CD44 (the hyaluronan (HA) receptor) and c-Src kinase are expressed in human ovarian tumor cells (SK-OV-3.ipl cell line), and that these two proteins are physically associated as a complex in vivo. Using a recombinant cytoplasmic domain of CD44 and an in vitro binding assay, we have detected a specific interaction between CD44 and c-Src kinase. Furthermore, the binding of HA to SK-OV-3.ipl cells promotes c-Src kinase recruitment to CD44 and stimulates c-Src kinase activity, which, in turn, increases tyrosine phosphorylation of the cytoskeletal protein, cortactin. Subsequently, tyrosine phosphorylation of cortactin attenuates its ability to cross-link filamentous actin in vitro. In addition, transfection of SK-OV-3.ipl cells with a dominant active form of c-Src (Y527F) cDNA promotes CD44 and c-Src association with cortactin in membrane projections, and stimulates HA-dependent/CD44-specific ovarian tumor cell migration. Finally, overexpression of a dominant-negative mutant of c-Src kinase (K295R) in SK-OV-3.ipl cells impairs the tumor cell-specific phenotype. Taken together, these findings strongly suggest that CD44 interaction with c-Src kinase plays a pivotal role in initiating cortactin-regulated cytoskeleton function and HA-dependent tumor cell migration, which may be required for human ovarian cancer progression.

The cell adhesion molecule, CD44, is one of the major hyaluronic acid (HA) receptors (1–3). It belongs to a family of transmembrane glycoproteins which contain a variable extracellular domain, a single spanning 23-amino acid transmembrane domain, and a 70-amino acid cytoplasmic domain (4). Nucleotide sequence analyses reveal that many CD44 isoforms (derived from alternative splicing mechanisms) are variants of the standard form, CD44s (4). CD44s (molecular mass ~85 kDa) is the most common isoform of CD44 found in many cell types including human ovarian carcinoma cells (5–9). The presence of high levels of CD44s (often together with CD44v) is emerging as an important metastatic tumor marker in a number of carcinomas, and is also implicated in the unfavorable prognosis of a variety of cancers including human ovarian cancers (5–9).

The invasive phenotype of CD44s-positive epithelial tumor cells has been linked to HA-mediated CD44 signaling and cytoskeletal activation. CD44s contains several HA-binding sites in their extracellular domain (1–3). The binding of HA to CD44s causes cells to adhere to the extracellular matrix (ECM) components (1–3), and has also been implicated in the stimulation of several different biological activities (10–16). The intracellular domain of CD44 binds to signaling proteins such as RhoGTPases (e.g. RhoA) (17); Tiam1, a guanine nucleotide exchange factor for Rac1 (18); and cytoskeletal proteins, including ankyrin (2, 3, 9, 17–21) and the ERM proteins (ezrin, radixin, and moesin) (23). Recent studies indicate that the binding of ECM components (e.g. HA) promote CD44-mediated Tiam1-Rac1 signaling and cytoskeleton function leading to specific structural changes in the plasma membrane and tumor cell migration in metastatic tumor cells (18). These findings strongly suggest that the CD44 molecule provides a direct linkage between the ECM and the cytoskeleton. In particular, the coordinated oncogenic signaling processes contributed by HA-dependent and CD44-mediated cytoskeleton activation is considered to be a possible mechanism underlying tumor cell motility and migration: an obvious prerequisite for metastasis.

The Src family kinases are classified as oncogenic proteins due to their ability to activate cell proliferation (24, 25), spreading (26, 27), and migration (27–30) in many cell types including epithelial tumor cells (30). The amino terminus of Src contains a myristoylation (or palmitoylation) site, which is important for membrane association (31, 32). Src also contains several functional domains including Src homology (SH 3 and SH2 domains, the catalytic protein-tyrosine kinase core, and a conserved regulatory tyrosine phosphorylation site (31, 32). Certain amino acid residues in the c-Src molecule play an important role in modulating its kinase activity. Mutations of specific key amino acids result in either up-regulation or down-regulation of c-Src kinase activity. For example, replacement of tyrosine 527 with phenylalanine (e.g. Y527F, the dominant-active form of c-Src kinase) strongly activates c-Src kinase transactivating capability and enzyme activities (33). Mutation of lysine 295 to arginine (e.g. K295R, the dominant-negative form of c-Src kinase) renders c-Src kinase defective and reduces c-Src kinase-mediated biological activities (33, 34).

In addition, it has been observed that the interaction between Src kinase and membrane-linked molecules regulates receptor signaling and various cellular functions (31, 32). In fact, CD44s-mediated cellular signaling has been suggested to involve Src kinase family members (35). For example, Lck, one of the Src kinase family members, is found to be closely com-
plexed with CD44s during T-cell activation (35). CD44 also selectively associates with active Src family tyrosine kinases (e.g., Lck and Fyn) in glycosphingolipid-rich plasma membrane domains of human peripheral blood lymphocytes (36). Moreover, the cytoplasmic domain of CD44s has been shown to be involved in the recruitment of the Src family (e.g., Src, Yes, and Fyn) in prostate tumor cells during anchorage-independent colony growth (20). Collectively, all these observations support the notion that c-Src kinases participate in CD44-mediated cellular signaling.

The questions of (i) whether CD44s-mediated c-Src kinase signaling plays a direct role in regulating ovarian tumor cell activation and (ii) which cytoskeletal protein(s) is (are) most likely involved in CD44-c-Src kinase-regulated downstream effector function leading to human ovarian cell migration are specifically addressed in this study. We have determined that CD44s (the standard form) and c-Src kinase are physically linked and functionally coupled in human ovarian tumor cells (SK-OV-3-ipl cell line). Furthermore, our data show that the cytoplasmic domain of CD44s is important for the association with c-Src kinase. HTA treatment of ovarian tumor cells recruits c-Src kinase to CD44s and activates c-Src kinase activity. We have also demonstrated that c-Src kinase phosphorylates the cytoskeletal protein cortactin both in vivo and in vitro. Most importantly, cortactin phosphorylation by c-Src kinase attenuates its cross-linking ability of filamentous actin. Overexpression of a dominant-active form of c-Src kinase, by transfecting SK-OV-3-ipl cells with c-Src (Y527F)cDNA in these ovarian tumor cells, promotes the onset of CD44s/c-Src kinase-regulated cortactin function and tumor cell migration. Transfection of SK-OV-3-ipl cells with a dominant-negative mutant of c-Src kinase (K295R-kinase dead) effectively blocks the tumor cell-specific phenotype. Therefore, we believe that CD44-activated c-Src kinase signaling is directly involved in stimulating cortactin-cytoskeleton interaction and HTA-mediated tumor cell migration during the progression of human ovarian cancer.

MATERIALS AND METHODS

Cell Lines and Culture—The SK-OV-3-ipl cell line was established from ascites that developed in a nude mouse given an intraperitoneal injection of SK-OV-3 human ovarian carcinoma cell line (obtained from the American Type Culture Collection) as described previously (37). Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplement (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Cloning, Expression, and Purification of CD44 Cytoplasmic Domain (CD44cyt) from Escherichia coli—The procedure for preparing the fusion protein of CD44’s cytoplasmic domain was the same as described previously (9, 17).

Expression Constructs—The c-Src (Y527F) mutant or the c-Src (K295R) mutant cDNAs (kindly provided by Dr. David Shalloway, Cornell University, Ithaca, NY) was cloned into pEGFPN1 vector (CLONTECH) using PCR-based cloning strategy. Specifically, c-Src (Y527F) mutant cDNA or c-Src (K295R) mutant cDNA was amplified by PCR with two specific primers (left, 5’-GGCTCCGATGGGAGCAGCAAGGGAAGG-3’; right, 5’-GCAAGCTTTTTTCGACTTCC-3’) linked with a specific enzyme digestion site (XhoI or HindIII). PCR product digested with XhoI and HindIII was purified with QiAquick PCR purification kit (Quagen). The cDNA was cloned into pEGFPN1 vector digested with XhoI and HindIII. Subsequently, both c-Src (Y527F) mutant cDNA and c-Src (K295R) mutant cDNA sequences were confirmed by nucleotide sequencing analyses.

Cell Transfection—To establish a transient expression system, SK-OV-3-ipl cells were transfected with various plasmid DNAs (e.g., GFP-tagged c-Src (Y527F) or GFP-c-Src (K295R) or vector alone) using electroporation methods according to those procedures described previously (38). Various transfectants were then analyzed for their protein expression (e.g. c-Src-related proteins) by immunoblot, c-Src kinase activity, and tumor cell migration assays as described below.

Cortactin-cytoskeleton Interaction and HA-mediated tumor cell migration—Purified constitutively activated c-Src kinase recombinant protein (39) was first bound to anti-c-Src kinase-conjugated immunofinity beads. Subsequently, aliquots (10–20 ng of these beads) were incubated with 0.5 ml of a binding buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Triton X-100) in the presence of various concentrations (10–400 ng/ml) of 125I-labeled FLAG-CD44cyt fusion protein (5,000 cpm/ng protein) at 4 °C for 5 h. Specifically, equilibrium binding conditions were determined by performing a time course (1–10 h) of 125I-labeled FLAG-CD44cyt binding to c-Src kinase at 4 °C. The binding equilibrium was found to be established when the in vitro CD44-c-Src kinase binding assay was conducted at 4 °C after 4 h. Following binding, beads were washed extensively in the binding buffer and radioactivity bound to the beads was counted.

As a control, 125I-labeled FLAG-CD44cyt fusion protein was also incubated with uncoupled beads to determine the binding observed due to the nonspecific binding of the ligand. Nonspecific binding, which represented ~15–20% of the total binding, was always subtracted from the total binding. The values expressed under “Results” represent an average of triplicate determinations of three to five experiments with S.D. less than ±5%.

Immunoprecipitation and Immunoblotting Techniques—SK-OV-3-ipl cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rat anti-CD44s antibody or rabbit anti-c-Src kinase antibody followed by goat anti-rat IgG or goat anti-rabbit IgG, respectively. The immunoprecipitated material was solubilized in SDS sample buffer, electrophoresed, and immunoblotted with rabbit anti-c-Src kinase antibody (5 μg/ml) or rat anti-CD44s antibody (5 μg/ml), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-rat IgG (1: 10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Cell lysates were also prepared from SK-OV-3-ipl cells treated with HA at various concentrations (e.g., 5, 10, 25, 50, and 100 μg/ml) for various time intervals (e.g., 0, 2, 5, 10, 15, 20, 30, and 60 min) and immunoprecipitated with anti-CD44s IgG to isolate CD44s-c-Src kinase complex (as described above). Immunoprecipitates were immunoblotted with either anti-Src (Tyr(P)527) (5 μg/ml) or anti-c-Src kinase (5 μg/ml). In some cases, SK-OV-3-ipl cells (treated with HA (50 μg/ml) for 10–60 min) were lysed in the presence of PP2, an inhibitor of P2, and various other tyrosine kinases (purchased form Calbiochem-Novabiochem, San Diego, CA; or untreated) were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer. These materials were then immunoprecipitated by mouse anti-cortactin, followed by immunoblotting with mouse anti-phospho-threonine or reblotting with mouse anti-cortactin plus peroxidase-conjugated goat anti-mouse IgG and ECL chemiluminescence reagent.

In some experiments, SK-OV-3-ipl cells (e.g. untransfected or transfected with GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA or vector alone) were treated with HA (50 μg/ml) for 10–60 min. Cell lysates of these transfectedants were immunoprecipitated by rat anti-CD44s antibody (5 μg/ml). The anti-CD44s-mediated immunoprecipitated material was then immunoblotted with mouse anti-GFP antibody (5 μg/ml) for 1 h at room temperature. Some cell lysate was either immunoblotted with mouse anti-GFP antibody (5 μg/ml) or immunoprecipitated with mouse anti-GFP antibody (5 μg/ml), followed by immunoblotting with rabbit anti-c-Src antibody (5 μg/ml). In some cases, the cell lysate of these cells was immunoprecipitated by mouse anti-cortactin (5 μg/ml), followed by immunoblotting with mouse anti-cortactin antibody plus horseradish peroxidase-conjugated goat anti-mouse IgG (1: 10,000 dilution) and ECL chemiluminescence reagent.

Immunofluorescence Staining—SK-OV-3-ipl cells (untransfected or transfected with various plasmid DNAs such as GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA or vector alone) were first washed with PBS (0.1% phosphate buffer (pH 7.5) and 150 mM
NaCl) buffer and fixed by 2% paraformaldehyde. Subsequently, untransfected/vector-transfected cells or GFP-tagged SK-OV-3.ipi transfecteds were stained with Texas Red-labeled or cyanine (Cy5)-labeled rat anti-CD44s antibody. In some cases, GFP-tagged and cyanine-labeled cells were then rendered permeable by ethanol treatment, followed by anti-CD44s (rat) or anti-c-Src (rabbit) antibody incubation. To detect non-specific antibody binding, cyanine-CD44s labeled cells were incubated with Texas Red-conjugated normal mouse IgG. No labeling was observed in such control samples. These labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA). Cells displaying membrane projections were counted under the microscope. Specifically, every cell in the field was examined for the occurrence of the cell phenotypes (e.g. with or without membrane projections). At least 200–300 cells (in 12 different fields) were examined in each sample. Quantitative values describing the percentage of cells displaying membrane projections in each sample were expressed as “percentage of total cells.”

**Tumor Cell Migration Assays**—Twenty-four transwell units were used for monitoring in vitro cell migration as described previously (9, 17, 18, 21). Specifically, the 5-μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SK-OV-3.ipi cells (untransfected or transfected with GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA or vector alone (−1 × 10^6 cells/well) were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented with 200 μg/ml hyaluronic acid was placed in the lower chamber of the transwell unit. After 18 h of incubation at 37 °C in a humidified 95% air, 5% CO2 atmosphere, vital stain MTT (Sigma) was added for 3 h. The migrative cells at the lower part of the filter were removed by swabbing with small pieces of Whatman filter paper. Both the polycarbonate filter and the Whatman paper were placed in dimethyl sulfoxide to solubilize the crystal. Color intensity was measured in 450 nm.

Cell migration processes were determined by measuring the cells that migrate from the upper chambers to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (9, 17, 18, 21). The CD44-specific cell migration was determined by subtracting nonspecific cell migration (i.e. cells migrate to the lower chamber in the presence of anti-CD44s antibody treatment) from the total migrative cells in the lower chamber. The CD44-specific cell migration in vector-transfected cells (control) is designated as 100%. Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically using Student’s t test, and statistical significance was set at p < 0.01.

**In Vitro c-Src Kinase Assay**—An in vitro c-Src kinase assay using enolase as a substrate was performed as described previously (40). Briefly, lysates from SK-OV-3.ipi cells (treated with HA (50 μg/ml) for 10–60 min; or pretreated with anti-CD44 followed by HA (50 μg/ml) treatment for 10–60 min; or untreated) were prepared and processed for a Src kinase assay (40) and protein A-Sepharose cross-linking experiment to obtain the CD44s-c-Src kinase complexes. For assaying c-Src kinase activity, CD44s-c-Src kinase complexes were incubated with 10 μl of reaction buffer (20 mM PIPES (pH 7.0), 10 mM MnCl₂, 10 μM Na₃VO₄), 1 μl of freshly prepared acid-denatured enolase (Sigma) (5 μg of endolase plus 1 μl of 50 mM HCl incubated at 30 °C for 10 min and then neutralized with 1 μl of 1× PIPES (pH 7.0)), and 20 mM ATP. After 10 min of incubation at 30 °C, reactions were terminated by adding 2× SDS sample buffer. Samples were then electrophoresed and immunoblotted with mouse anti-phosphoryosine antibody plus horseradish peroxidase-conjugated goat anti-mouse IgG and ECL chemiluminescence reagent.

**Phosphorylation of Cortactin by Endogenous c-Src and c-Src (Y527F)/c-Src (K295R) in Vitro**—Untransfected SK-OV-3.ipi cells and various transfecants were treated with HA (50 μg/ml) for various time intervals (0, 2, 5, 10, 15, 20, 30, and 60 min). At each time point, 100 pmol c-Src-related proteins (isolated from HA-treated untransfected cells using anti-c-Src-conjugated immunofinity beads, or SK-OV-3.ipi cells transfected with either c-Src (Y527F) or c-Src (K295R) or vector alone using anti-GFP-conjugated and anti-c-Src kinase antibody) were incubated with 1 μl of the reaction buffer (20 mM PIPES (pH 7.0), 10 mM MnCl₂, 10 μM Na₃VO₄), 1 μl of recombinant cortactin (a gift from Dr. Alan Mak, Queen’s University, Kingston, Ontario, Canada) (5 μg of cortactin dissolved in 2 μl of 1× PIPES (pH 7.0)), and 10 μCi of [γ-32P]ATP (5000 Ci/mmol) at 30°C for 1 h. To quantitate phosphorylated cortactin, aliquots of the reactions were analyzed by SDS-PAGE. The radioactivity associated with GFP-tagged cortactin band was analyzed by autoradiograms quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or scintillating counting methods.

**Results**

**Detection of CD44s and c-Src kinase complex in SK-OV-3.ipi cells**—SK-OV-3.ipi cells (5 × 10^6 cells) were solubilized by 1% Nonidet P-40 buffer followed by immunoprecipitation and/or immunoblot by anti-CD44s antibody or anti-c-Src kinase antibody, respectively as described under “Experimental Procedures.” Lane 1, detection of CD44s with rat anti-CD44s-mediated immunoblot of SK-OV-3.ipi cells. Lane 2, detection of c-Src kinase with rabbit anti-c-Src kinase-mediated immunoblot of SK-OV-3.ipi cells. Lane 3, detection of c-Src kinase in the complex by anti-CD44s-immunoprecipitation followed by immunoblotting with anti-c-Src kinase antibody. Lane 4, detection of CD44s in the complex by anti-c-Src kinase-mediated immunoprecipitation followed by immunoblotting with anti-CD44s antibody. Lane 5, immunoblot of SK-OV-3.ipi cells with preimmune rat IgG. Lane 6, immunoblot of SK-OV-3.ipi cells with preimmune rabbit IgG.

**Immunoblotting with anti-CD44 antibody** (recognizing an epitope located at the NH₂-terminal region of CD44s (the standard form)) indicates that a single CD44s protein (mass ≈ 85 kDa) is expressed in the human ovarian tumor cell line, SK-OV-3.ipi (Fig. 1, lane 1). In addition, we have used anti-c-Src kinase antibody-mediated immunoblot to detect the expression of c-Src kinase as a single polypeptide (mass ≈ 60 kDa) (Fig. 1, lane 2) in SK-OV-3.ipi cells (Fig. 1, lane 2). Both CD44s and c-Src kinase detected in SK-OV-3.ipi cells by anti-CD44s and anti-c-Src kinase-mediated immunoblot is specific since no protein is detected in these cells using preimmune rat IgG or rabbit IgG, respectively (Fig. 1, lane 3).

We then addressed the question of whether there is a physical linkage between CD44s and c-Src kinase in human ovarian tumor cells. First, we carried out anti-CD44s-mediated and anti-c-Src kinase-mediated precipitation followed by anti-c-Src kinase immunoblot (Fig. 1, lane 3) or anti-anti-CD44s immunoblot (Fig. 1, lane 4), respectively. Our results indicate that...
the c-Src kinase band is revealed in anti-CD44s-immunoprecipitated materials (Fig. 1, lane 3), and the CD44s band is detected in the anti-c-Src kinase-immunoprecipitated materials (Fig. 1, lane 4). These findings establish the fact that CD44s and c-Src kinase are closely associated with each other in ovarian tumor cells.

We have also used purified, FLAG-tagged cytoplasmic domain of CD44 fusion protein (FLAG-CD44cyt) and a constitutively activated c-Src kinase recombinant protein to conduct in vitro binding assays. Specifically, the binding of 125I-labeled FLAG-CD44cyt to c-Src kinase under equilibrium binding conditions was tested. Scatchard plot analyses presented in Fig. 2 indicate that c-Src kinase binds to the cytoplasmic domain of CD44 (CD44cyt) at a single site with high affinity (an apparent dissociation constant \( K_d \) of \( \sim 20 \) nM). These findings clearly indicate that the cytoplasmic domain of CD44 interacts directly with c-Src kinase.

**Stimulation of CD44s-associated c-Src Kinase Activity by HA in SK-OV-3.ipl Cells**—Previous studies show that full catalytic activity of c-Src kinase requires phosphorylation of tyrosine 418 (43). Using specific anti-phospho-Src antibody (i.e. anti-Src(Tyr(P)418), designed to detect the activated form of c-Src kinase), we have found that c-Src kinase activation (detected in the CD44s-c-Src kinase complex) is HA-dependent (Fig. 3, panel a). This finding is consistent with our previous studies (42). Tyrosine phosphorylation of cortactin by c-Src kinase occurs as early as 2–5 min and reaches the maximal level \( 30–60 \) min after the addition of HA to SKOV-3 cells treated with HA at various concentrations (e.g., 0, 5, 10, 25, 50, and 100 \( \mu \)g/ml) for 10 min and immunoprecipitated with anti-CD44s antibody (to isolate CD44s-c-Src kinase complex) followed by immunoblotting with various antibodies (e.g. anti-phospho-Src(Tyr(P)418) antibody, anti-Src antibody or anti-phosphotyrosine antibody). A, detection of the activated form of c-Src kinase by anti-phospho-Src(Tyr(P)418)-mediated immunoblot using anti-CD44s-mediated immunoprecipitated materials prepared from SK-OV-3 cells treated with HA at various concentrations (e.g., 0, 5, 10, 25, 50, and 100 \( \mu \)g/ml). B, tyrosine phosphorylation of enolase by c-Src kinase in the CD44s complex detected by anti-phosphotyrosine antibody (panel a). (Note that an equal amount of c-Src kinase (revealed by anti-Src-mediated immunoblot) was used in this kinase assay (panel b).) C, recruitment of c-Src kinase into CD44s complex detected by anti-CD44s-mediated immunoprecipitation followed by anti-c-Src kinase-mediated immunoblot using SK-OV-3.ipl cells treated with 50 \( \mu \)g/ml HA (right lane) or without any HA (left lane).

We have also observed that HA treatment recruits a significant amount of c-Src kinase (Fig. 3C, right lane) into the CD44s-c-Src kinase complex (Fig. 3C, left lanes). Since CD44 does not contain intrinsic catalytic properties, HA must activate and recruit cellular tyrosine protein kinases such as c-Src kinase to regulate tyrosine protein phosphorylation.

The cytoskeletal protein, cortactin, is a known c-Src kinase substrate, which is composed of six and a half 37-amino acid tandem repeats and an SH3 domain at the carboxyl terminus (41, 42). Between the SH3 and the repeat domains, there is an \( \alpha \)-helical structure plus a sequence rich in proline residues (41, 42). Tyrosine phosphorylation of cortactin by c-Src kinase occurs in the region between the proline-rich sequence and the SH3 domain (41, 42, 44). In this study, we have found that HA treatment of ovarian tumor cells (SK-OV-3.ipl cells) stimulates tyrosine phosphorylation of the 85-kDa protein, cortactin, in vitro (Fig. 4, lane 3, a and b). In contrast, the level of cortactin tyrosine phosphorylation appears to be very low in those cells without any HA treatment (Fig. 4, lane 1, a and b) or treated with HA in the presence of PP2 (an inhibitor for the Src family kinases) (Fig. 4, lane 2, a and b). Kinetic analyses show that c-Src kinase activation (measured by c-Src-mediated cortactin phosphorylation) occurs as early as 2–5 min and reaches the maximal level \( 30–60 \) min after the addition of HA to SKOV-3.ipl cells (Fig. 5). These findings clearly indicate that the binding of HA to these CD44s-containing SK-OV-3.ipl cells promotes c-Src kinase activation leading to cortactin phosphorylation.
Our preliminary data indicate that both Yes and Lyn are at least 5-fold less than c-Src kinase in SK-OV-3.1pl cells. Using specific anti-Fyn or anti-Yes-mediated immunoblot on anti-CD44s-precipitated material, we are unable to detect the association of these kinase molecules with CD44s or phosphorylation of cortactin (data not shown). Therefore, c-Src appears to be the major Src kinase family member (not Fyn or Yes) that is associated with CD44s and thus solely responsible for cortactin phosphorylation.

Effects of CD44s-c-Src Kinase Signaling on Cortactin Function and Ovarian Tumor Cell Migration—The invasive phenotype of tumor cells (characterized by membranous projections and tumor cell migration) is closely associated with CD44s-mediated membrane motility and cytoskeleton function (9, 18, 21). To correlate CD44s-c-Src kinase signaling with ovarian tumor cell-specific behaviors, we have transiently transfected the ovarian tumor cells (SK-OV-3.1pl cells) with a dominant active form of GFP-tagged c-Src (Y527F) that is unable to undergo negative feedback by auto-phosphorylation. Unlike c-Src (K295R) (Fig. 6A, lane 3), the active form of GFP-tagged c-Src (Y527F) is associated with CD44s (Fig. 6A, lane 2). The effects of CD44s-c-Src kinase signaling on cortactin localization and ovarian tumor cell motility has been demonstrated in this work.

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Fig. 7. Characterization of c-Src mutant proteins in SK-OV-3.ipl cells transfected with c-Src (Y527F)cDNA or c-Src (K295R)cDNA, and measurement of the F-actin cross-linking activity of cortactin (phosphorylated by c-Src (Y527F) or c-Src (K295R)). SK-OV-3.ipl cells (e.g. untransfected or transfected by GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA alone) were immunoblotted with mouse anti-GFP antibody, or immunoprecipitated with mouse anti-cortactin followed by immunoblotting with mouse anti-phosphotyrosine antibody/reblotting with mouse anti-cortactin antibody as described under "Experimental Procedures." A, detection of GFP protein expression in untransfected cells (lane 1), vector-transfected cells (lane 2), GFP-tagged c-Src (Y527F)cDNA-transfected cells (lane 3), or GFP-tagged c-Src (K295R)cDNA-transfected cells (lane 4) by immunoblotting with mouse anti-GFP antibody. B, panel a, detection of cortactin tyrosine phosphorylation in untransfected cells (lane 1), vector-transfected cells (lane 2), GFP-tagged c-Src (Y527F)cDNA-transfected cells (lane 3), or GFP-tagged c-Src (K295R)cDNA-transfected cells (lane 4) by immunoprecipitating various cell lysates with mouse anti-cortactin followed by immunoblotting with mouse anti-phosphotyrosine antibody. B, panel b, detection of cortactin expression in untransfected cells (lane 1), vector-transfected cells (lane 2), GFP-tagged c-Src (Y527F)cDNA-transfected cells (lane 3), or GFP-tagged c-Src (K295R)cDNA-transfected cells (lane 4) by immunoprecipitating cell lysates with mouse anti-cortactin followed by reblotting with mouse anti-cortactin antibody. C, analysis of cortactin phosphorylation by c-Src-related proteins. Purified cortactin recombinant protein was incubated with c-Src-related proteins isolated from untransfected cells (a) or SK-OV-3.ipl cells (transfected with either GFP-tagged c-Src (Y527F) (c) or GFP-tagged c-Src (K295R) (d) or vector alone (b) using anti-GFP-Sepharose beads) in the kinase reaction buffer at 30 °C for 1 h as described under "Experimental Procedures." To quantitate phosphorylated cortactin, aliquots of the reactions were analyzed by SDS-PAGE. The radioactivity associated with cortactin band were analyzed by Scintillating counting. D, measurement of the F-actin cross-linking activity of cortactin. Purified cortactin recombinant protein treated with c-Src-related proteins was subjected to F-actin cross-linking analysis. Specifically, unphosphorylated cortactin (treated with c-Src-related proteins isolated from untransfected cells (a) or vector-transfected cells (b) or SK-OV-3.ipl cells transfected with c-Src (K295R)cDNA (d) using anti-GFP-conjugated beads); or phosphorylated cortactin (isolated from SK-OV-3.ipl cells transfected with c-Src (K295R)) (Fig. 6C, lanes 5 and 6) into anti-CDC44s-mediated immunoprecipitated materials. No GFP-related protein was observed in anti-CDC44s immunoprecipitates blotted with anti-GFP antibody using vector-transfected cells treated with (lane 2) or without HA (lane 1). These results suggest that the activated c-Src kinase (but not the inactivated c-Src kinase) is involved in the recruitment of c-Src kinase to CDC44s during HA-mediated cellular signaling.

In addition, we have demonstrated that transfection of SK-OV-3.ipl cells with c-Src (Y527F)cDNA stimulates cortactin tyrosine phosphorylation in vivo (Fig. 7B, lane 3, a and b), as compared with cortactin in untransfected (Fig. 7B, lane 1, a and b) or vector-transfected cells (Fig. 7B, lane 2, a and b). Our results also demonstrate that overexpression of c-Src (K295R) does not induce cortactin tyrosine phosphorylation (Fig. 7B, lane 4, a and b). These data support the conclusion that cortactin serves as one of the cellular substrates for c-Src kinase in vivo.

To determine whether there is any kinase activity associated with GFP-tagged c-Src (Y527F) or GFP-tagged c-Src (K295R), we have incubated these c-Src (Y527F)/K295R proteins with purified recombinant cortactin in the presence of [γ-32P]ATP. As shown in Fig. 7C, a significant amount of cortactin phosphorylation occurs in the presence of c-Src (Y527F) (Fig. 7C, a), but not c-Src (K295R) (Fig. 7C, d). In contrast, very little tyrosine phosphorylation of cortactin is detected in control proteins using anti-GFP-conjugated beads prepared from untransfected/vector-transfected cells (Fig. 7C, a and b). Apparently, tyrosine phosphorylation of cortactin activated by c-Src (Y527F) (but not by c-Src (K295R)) can also occur in vitro.

Tyrosine phosphorylation of cortactin by c-Src kinase has been shown to down-regulate its cross-linking with filamentous actin (F-actin) in vitro (41, 42). Here, we have shown that unphosphorylated cortactin (treated by anti-GFP-conjugated beads isolated from untransfected cells (Fig. 7C, a) or vector-transfected cells (Fig. 7C, b) or c-Src (K295R)cDNA-transfected cells (Fig. 7C, d) promotes F-actin cross-linking activity in vitro (Fig. 7D, a, b, and d). In contrast, c-Src (Y527F)-phosphorylated cortactin (Fig. 7C, c) significantly reduces its ability to cross-link F-actin (Fig. 7D, c). These results support the notion that tyrosine phosphorylation of cortactin by c-Src kinase inhibits its interaction with F-actin, which may be required for cytoskeleton-mediated function. Using double immunolabel staining, we have observed that both c-Src kinase (Fig. 8A) and CDC44s (Fig. 8B) are colocalized at the cellular membranes of untransfected SK-OV-3.ipl cells (Fig. 8C). In c-Src (Y527F)cDNA-transfected cells, both GFP-tagged c-Src kinase (Y527F) (Fig. 8D) and CDC44s (Fig. 8E) are closely colocalized in the plasma membranes and long membrane projections (Fig. 8F). In contrast, vector-transfected cells expressing CDC44s on their surface (Fig. 8, inset b) (with no detectable GFP label (Fig. 8, inset a)) are not able to induce long membrane projections (Fig. 8, inset c). It is also noted that overexpression of GFP-(Y527F)cDNA using anti-GFP-conjugated beads (c) was incubated with a TKM buffer (50 mM Tris-HCl (pH7.4), 134 mM KCl, and 1 mM MgCl2). Unphosphorylated or phosphorylated cortactin was then mixed with 125I-labeled F-actin followed by a 30-min incubation at room temperature. Subsequently, the mixture was centrifuged at 25,000 × g for 10 min at room temperature. The supernatant was then collected, and the radioactivity in this fraction was counted. The decrease (or loss) of radioactivity in the supernatant fraction reflects F-actin precipitation due to the cross-linking reaction (41, 42). The F-actin cross-linking reaction in the presence of unphosphorylated cortactin (treated by anti-GFP-conjugated beads prepared from untransfected cells) (control) is designated as 100%. Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically using the Student's t test, and statistical significance was set at p < 0.01.
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Fig. 8. Immunofluorescence staining of c-Src kinase and CD44s in untransfected SK-OV-3.ipl cells or SK-OV-3.ipl transfectants. SK-OV-3.ipl cells (untransfected or transfected with GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA or vector alone) were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immunoreagents as described under “Experimental Procedures.” A–C, FITC-labeled anti-c-Src kinase staining (A), Texas Red-labeled anti-CD44s staining (B), and colocalization of c-Src kinase and CD44s (C) in untransfected SK-OV-3.ipl cells. D–F, GFP-tagged c-Src (Y527F) (D), Texas Red-labeled anti-CD44s staining (E), and colocalization of GFP-tagged c-Src (Y527F) and CD44s (F) in GFP-tagged c-Src (Y527F)cDNA-transfected SK-OV-3.ipl cells. G–I, GFP-tagged c-Src (K295R) (G), Texas Red-labeled anti-CD44s staining (H), and colocalization of GFP-tagged c-Src (K295R) and CD44s (I) in GFP-tagged c-Src (K295R)cDNA-transfected SK-OV-3.ipl cells. Insets a–c, No detectable staining of GFP (a), Texas Red-labeled anti-CD44s (b), and a merged image (c) of a and b in vector-transfected SK-OV-3.ipl cells.

tagged c-Src (K295R) significantly reduces c-Src kinase (Fig. 8G) colocalization (Fig. 8J) with CD44s (Fig. 8H) and the formation of membrane projections (Fig. 8, G–I).

Further analyses indicate that transfection of SK-OV-3.ipl cells with GFP-tagged c-Src (Y527F)cDNA induces a recruitment of cortactin (Fig. 9C) to c-Src kinase (Fig. 9A) and CD44s (Fig. 9B)-linked membrane projections. Our results also indicate that these c-Src (Y527F) transfectants display a significant increase in HA-dependent and a CD44s-specific ovarian tumor cell migration (Table I) as compared with untransfected/vector-transfected tumor cells (Table I). In contrast, overexpression of the dominant-negative form of c-Src (K295R) in SK-OV-3.ipl cells not only blocks the membrane localization of c-Src kinase (Fig. 9D) to CD44s (Fig. 9E) but also inhibits cortactin membrane association (Fig. 9F) with CD44s (Fig. 9E) and the formation of membrane projections (Fig. 9, D–F). Consequently, HA and CD44s-specific tumor cell migration is also inhibited in these c-Src mutant (K295R) transfectants (Table I). These findings suggest that CD44s-mediated c-Src kinase signaling plays a pivotal role in regulating cortactin-cytoskeleton function and HA-mediated tumor cell migration during human ovarian cancer progression.

DISCUSSION

HA is the major glycosaminoglycan of the ECM. It is known to cause cell aggregation of a number of different cell types and has been implicated in the stimulation of cell proliferation, cell migration, cell adhesion, and angiogenesis (9–21, 29). Overexpression of HA often occurs at sites of tumor attachment and invasion (45). The interaction of HA with HA receptors on the surface of a number of different cell types including human ovarian tumor cells (5–9). In the ovary, HA is present in large amounts in the mesothelial lining (7). In fact, it has been postulated that CD44 interaction with HA may be one of the important requirement for the spread of ovarian cancer. Nevertheless, the cellular and molecular mechanisms affecting the ability of CD44-positive ovarian tumor cells to spread and implant at HA-enriched locations within the peritoneal cavity remain unclear.

A number of studies indicate that transmembrane interaction between the cytoplasmic domain of CD44 and cytoskeletal proteins (in particular, ankyrin) plays an important role in CD44-mediated oncogenic signaling (2, 3). In particular, a 15-amino acid sequence located in the cytoplasmic domain of CD44 appears to be required for high affinity ankyrin binding (19–21). Several factors, including protein kinase C (46, 47) and Rho kinase-mediated phosphorylation (17), palmitoylation (48), and GTP binding (49), are required for the up-regulation of CD44-ankyrin interaction. Furthermore, we have found that the S2 subdomain (but not other subdomains) of the ankyrin repeat domain binds to CD44 directly (9). Most recently, Tiam1 (T lymphoma invasion and metastasis), one of the guanine nucleotide (GDP/GTP) exchange factors for Rho-GTPases (e.g. Rac1) has also been found to bind CD44 (18) and to be regulated by the cytoskeletal proteins (e.g. ankyrin) (50) during metastatic tumor cell migration. Therefore, the selective interaction of CD44 with various oncogenic signaling molecules and/or cytoskeletal proteins could be one of the critical steps in promoting abnormal tumor cell motility.

A number of nonreceptor tyrosine kinases (e.g. Src, Abl, Fps, Syk/ZAP70, Jak subfamilies, and several unclassified kinases) are involved in signal transduction pathways by coupling with surface receptors during cellular responses (51). In this study we have found that c-Src kinase and the cell surface adhesion molecule, CD44s are closely associated as a complex in human ovarian tumor cells (Figs. 1, 8, and 9). Abnormal regulation of Src-related enzymes is known to lead to oncogenic cellular transformation. This was first demonstrated by the identification of three Src family members (e.g. c-Src, c-Yes, and c-Fgr) as the transforming elements of acutely transforming retroviruses (52). Several tyrosine phosphoryla-
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Fig. 9. Immunofluorescence staining of c-Src kinase, CD44s and cortactin in SK-OV-3.ipl transfectants. SK-OV-3.ipl cells transfected with GFP-tagged c-Src (Y527F)cDNA or GFP-tagged c-Src (K295R)cDNA were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immunoreagents as described under “Experimental Procedures.” A–C, GFP-tagged c-Src kinase staining (A, green color), cyanine (Cy5)-labeled anti-CD44s staining (B, blue color), and Texas Red-labeled anti-cortactin staining (C, red color) in SK-OV-3.ipl cells transfected with c-Src (Y527F)cDNA. D–F, GFP-tagged c-Src kinase staining (D, green color), Cyanine (Cy5)-labeled anti-CD44s staining (E, blue color), and Texas Red-labeled anti-cortactin staining (F, red color) in SK-OV-3.ipl cells transfected with c-Src (K295R)cDNA.

The intracellular distribution of various proteins in SK-OV-3.ipl transfectants expressing GFP-tagged c-Src mutants (e.g., c-Src (Y527F) or c-Src (K295R)). We have observed that overexpression of the dominant-active form of GFP-tagged c-Src kinase (Y527F) (by transfecting SK-OV-3.ipl cells with c-Src (Y527F)cDNA) promotes colocalization of cortactin (Fig. 9C) with CD44s (Fig. 9B) and c-Src kinase (Fig. 9A) at membrane projections (Fig. 9, A–C). In contrast, transfection of SK-OV-3.ipl cells with the dominant-negative c-Src kinase mutant (K295R)cDNA effectively inhibits the recruitment of cortactin (Fig. 9F) or c-Src kinase (Fig. 9D) to CD44s (Fig. 9E) at the cellular membranes and impairs the formation membrane projections (Fig. 9, D–F) (Table I). These findings suggest that the ability of cortactin to remodel actin filaments (Fig. 9) or become recruited into CD44s-associated membrane projections is tightly regulated by c-Src kinase activity.

There is increasing evidence that the ability of CD44 to transmit information from the cell’s exterior to its interior depends on HA-CD44 interaction and selective downstream molecular switches (1–3). During ovarian tumor cell transformation and spreading, overexpressed CD44s is tightly coupled with at least two different tyrosine kinase-based oncogenic regulators such as p185HER2 (8) and c-Src kinase (20, 35, 36). In SK-OV-3.ipl cells, CD44s and the receptor tyrosine kinase, p185HER2, are physically linked to each other via interchain disulfide bonds, and HA is capable of stimulating CD44s-associated p185HER2 tyrosine kinase activity, causing an increase in the ovarian carcinoma cell growth (8). In this study we have described a CD44s-related nonreceptor c-Src tyrosine kinase signaling pathway in human ovarian tumor cells. Specifically, we have determined that CD44s binds to c-Src kinase both in vivo (Figs. 1, 6, 8, and 9) and in vitro (Fig. 2). In particular, the cytoplasmic domain of CD44s binds to c-Src kinase at a single site with high affinity (an apparent dissociation constant (Kₐ) of ~2.0 nM) (Fig. 2). Preliminary data indicate that the SH3 domain of c-Src kinase may be involved in the binding of a prolinc-containing sequence in the cytoplasmic domain of CD44s (data not shown).

Most Src family kinases are modified with specific lipids that direct them to subdomains of cell membrane that have high cholesterol and glycolipid content, called membrane “rafts”...
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**Table I**

| Cells                                      | Cells displaying membrane projectionsa | % of total cells |
|--------------------------------------------|----------------------------------------|------------------|
| Untransfected cells (control)              | 22                                     | 18–20%           |
| Vector-transfected cells                   | 21                                     | 20–25%           |
| c-Src (Y527F)cDNA-transfected cells        | 63                                     | 85%              |
| c-Src (K295R)cDNA-transfected cells        | 8                                      | 5–10%            |

**Untransfected cells (control)**

- % of control: 100

**Vector-transfected cells**

- % of control: 96

**c-Src (Y527F)cDNA-transfected cells**

- % of control: 280

**c-Src (K295R)cDNA-transfected cells**

- % of control: 49

a The number of cells displaying membrane projections was counted under the microscope. Specifically, every cell in the field was examined for the occurrence of the cell phenotypes (e.g., with or without membrane projections). At least 200–300 cells (in 12 different fields) were examined in each sample. Quantitative values describing the percentage of cells displaying membrane projections in each sample were expressed as “percentage of total cells.” The values expressed in this table represent an average of triplicate determinations of 6 experiments with a standard deviation less than ±5%.

In normal cells, such as human endothelial cells, the level of CD44 expression is relatively low and HA does not bind CD44 very well. In fact, HA binds to RHAMM (another HA binding receptor) better than it does to CD44 in normal cells (75). Consequently, the c-Src kinase activity associated with CD44 in normal cells fails to be activated by HA. In the ovary, large amounts of HA accumulation in the mesothelial lining (7) are involved in tumor attachment/invasion (45). CD44 is overexpressed on the surface of ovarian tumor cells and mediates cell migration in response to HA (9). Therefore, CD44 interaction with HA has been postulated to be one of the important requirements for the spread of ovarian cancer. In this study we have provided new evidence that the binding of HA to CD44 on the surface of ovarian tumor cells not only promotes c-Src kinase recruitment to CD44, but also activates c-Src kinase activity to phosphorylate the cytoskeletal protein, cortactin, leading to tumor cell migration. This new information may establish HA-inducible CD44-c-Src kinase signaling as an important functional indicator to evaluate oncogenic potential, and allow the development of new drug targets to inhibit tumor cell motility during the progression of human ovarian cancer.

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**References**

1. Lesley, J., Hyman, R., and Kincaid, P. W. (1993) _Adv. Immunol._ 54, 271–335
2. Bourguignon, L. Y. W. (1996) _Curr. Top. Membr._ 43, 293–312
3. Bourguignon, L. Y. W. (1998) _Frontiers Biosci._ 3, d637–d649
4. Scrosati, G. R., Bell, M. V., Jackson, D. G., Cornelia, F. B., Gerth, U., and Bell, J. J. (1992) _Proc. Natl. Acad. Sci. U.S.A._ 89, 12160–12164
5. Cannistra, S. A., Kassas, G. S., Nilof, J., DeFranco, B., Kim, Y., and Otzmann, C. (1993) _Cytoskeleton_ 23, 3930–3938
6. Cannistra, S. A., DeFranco, B., Nilof, J., and Otzmann, C. (1995) _Clin. Cancer Res._ 1, 333–342
7. Gardner, M. J., Jones, L. M. H., Caterall, J. B., and Turner, R. A. (1995) _Cancer Letters_ 91, 229–234
8. Bourguignon, L. Y. W., Zhu, H. B., Chu, A., Zhang, L., and Hung, M. C. (1997) _J. Biol. Chem._ 272, 27913–27918
9. Zhu, D., and Bourguignon, L. Y. W. (2000) _J. Cell. Physiol._ 183, 182–195
10. Laurent, T. C., and Fraser, R. E. (1992) _FASEB J._ 6, 2397–404
11. Noble, P. W., McKee, C. M., Cowman, M., and Shin, H. S. (1996) _J. Exp. Med._ 183, 2373–2378
12. Bourguignon, L. Y. W., Lokeswar, B. V., Chen, X., and Kerrick, G. L. (1999) _J. Immunol._ 161, 6634–6644
13. Green, S. J., Tarone, G., and Underhill, C. B. (1988) _Exp. Cell Res._ 178, 224–230
14. West, D. C., and Kumar, S. (1989) _Exp. Cell Res._ 183, 179–196
15. Turley, E. A., Austen, L., Vandefigt, K., and Clary, C. (1991) _J. Cell Biol._ 112, 1041–1047
16. Rooney, P., Kumar, S., and Wang, M. (1995) _Int. J. Cancer_ 60, 632–636
17. Bourguignon, L. Y. W., Zhu, H., Shao, L., Zhu, D., and Chen, Y. W. (1999) _Cell Motil. Cytoskeleton_ 43, 269–267
18. Bourguignon, L. Y. W., Lokeswar, B. V., Chen, X., and Kerrick, G. L. (1999) _J. Immunol._ 161, 6634–6644
19. Green, S. J., Tarone, G., and Underhill, C. B. (1988) _Exp. Cell Res._ 178, 224–230
20. Zhu, D., and Bourguignon, L. Y. W. (1998) _Cell Motil. Cytoskeleton_ 39, 209–222
21. Bourguignon, L. Y. W., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J. T., Muller, W., and Cardiff, R. D. (1998) _J. Cell. Physiol._ 178, 206–215
22. Lokeswar, B. V., and Selzer, M. G. (2000) _J. Biol. Chem._ 275, 27641–27649
23. Tsukita, S., Oishi, K., Sato, N., Sagara, I., Kawai, A., and Tsutaka, S. (1994) _J. Cell Biol._ 126, 391–401
24. Barone, M. V., and Courtnidge, S. A. (1995) _Nature_ 378, 509–512
25. Brome, A. M., and Hunter, T. (1996) _J. Biol. Chem._ 271, 16798–16806
26. Kaplan, K. B., Swedlow, J. R., Morgan, D. O., and Varmus, H. E. (1995) _Genes Dev._ 9, 1565–1571
27. Rodier, J. M., Valles, A. M., Denoueley, M., Thierry, J. P., and Boyer, B. (1995) _J. Cell Biol._ 131, 761–773
28. Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engstrom, U., Wernestad, C., Heldin, C. H., and Heldin, L. (1996) _EMBO J._ 15, 5299–5313
29. Hall, C. L., Lange, L. A., Prober, D. A., Zhang, S., and Turley, E. A. (1996) _Oncogene_ 13, 2213–2224
30. Rahimi, N., Hung, W., Tremblay, E., Saulnier, R., and Elliott, B. (1998) _J. Biol. Chem._ 273, 33714–33721
31. Thomas, S. M., and Brugge, J. S. (1997) _Annu. Rev. Cell Dev. Biol._ 13, 513–609
32. Schlessinger, J. (2000) _Cell_ 100, 293–296
33. Kmiecik, T. E., and Shallowe, D. (1987) _Cell_ 49, 65–73
34. Bagrodia, S., Chakalaparambil, I., Kmiecik, T. E., and Shallowe, D. (1991) _Nature_ 349, 172–175
35. Talbot, T. E., Smut, L., Girison, A. W., Schilder-Tol, E. J., Borst, J., and Pals, S. T. (1996) _J. Biol. Chem._ 271, 2863–2867
36. Bangumar, S., Bird, A., and Hoelsch, D. C. (1998) _Blood_ 92, 3901–3908
37. Yu, D., Wolf, J. K., Scanlon, M., Price, J. E., and Hung, M. C. (1993) _Cancer Res._ 53, 891–896
38. Chu, G., Hayakawa, H., and Berg, P. (1987) _Nucleic Acids Res._ 15, 1311–1326
39. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) _Methods Enzymol._ 201, 130–149
40. Atfi, A., Drobetsky, E., Boissonneault, M., Chapdelaine, A., and Chevalier, S. (1994) _J. Biol. Chem._ 269, 30688–30693
41. Huang, C., Ni, Y., Wang, T., Gao, Y., Haunschild, C. C., and Zhan, X. (1997)
CD44-cSrc in Cytoskeleton Function and SK-OV-3.ipl Migration

42. Huang, C., Liu, J., Haudenschild, C. C., and Zhan, X. (1998) J. Biol. Chem. 273, 13911–13915
43. Osusky, M., Taylor, S. J., and Shalloway, D. (1995) J. Biol. Chem. 270, 25729–25732
44. Wang, K., Knipfer, M., Huang, Q. Q., van Heerden, A., Hsu, L. C., Gutierrez, G., Qui, X. L., and Stedman, H. (1996) J. Biol. Chem. 271, 4304–4314
45. Yeo, T. K., Nagy, J. A., Yeo, K. T., Dvorak, H. F., and Toole, B. P. (1996) J. Biol. Chem. 271, 4304–4314
46. Kalomiris, E. L., and Bourguignon, L. Y. W. (1989) J. Biol. Chem. 264, 8113–8119
47. Bourguignon, L. Y. W., Lokeshwar, V. B., He, J., Chen, X., and Bourguignon, G. J. (1992) Mol. Cell Biol. 12, 4464–4471
48. Bourguignon, L. Y. W., Kalomiris, E. L., and Lokeshwar, V. B. (1991) J. Biol. Chem. 266, 11761–11765
49. Lokeshwar, V. B., and Bourguignon, L. Y. W. (1992) J. Biol. Chem. 267, 22973–22978
50. Bourguignon, L. Y. W., Zhu, H., Shao, L., and Chen, Y. W. (2000) J. Cell Biol., 150, 177–191
51. Boelen, J. B. (1993) Oncogene 8, 2025–2031
52. Cooper, J. A. (1990) in Peptides and Protein Phosphorylation (Kemp, B. E., ed) pp. 85–113, CRC Press, Boca Raton, FL
53. Nishibe, S., Wahl, M. I., and Hernandez-Sotomayor, S. M. T. (1990) Science 250, 1255–1256
54. Bustelo, X. R., Ledbetter, J. A., and Barbacid, M. (1992) Nature 356, 68–71
55. Margolis, B., Hu, P., Katzav, S., Li, W., Oliver, J. M., Ulrich, A., Weiss, A., and Schlessinger, J. (1992) Nature 356, 71–74
56. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) Science 263, 1069–1072
57. Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, and Yamamura, H. (1994) J. Exp. Med. 179, 1725–1729
58. Iwashima, M., Irving, B. A., van Oers, N. S. C., Chan, A. C., and Weiss, A. (1994) Science 263, 1136–1139
59. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) Nature 35, 69–72
60. Okada, M., Nada, S., Yamanishi, Y., Yamamoto, T., and Nakagawa, H. (1991) J. Biol. Chem. 266, 24249–24252
61. Rosen, N., Boelen, J. B., Schwartz, A. M., Cohen, P., DeSeau, V., and Israel, M. A. (1986) J. Biol. Chem. 261, 25770–25776
62. Ottenhoff-Kalf, A. E., Bijlsma, G. A. U., Hennipman, A., Michel, A. A., and Staal, G. E. (1992) Cancer Res. 52, 4773–4778
63. Boyer, B., Roche, S., Denoyelle, M., and Thierry, J. P. (1997) EMBO J. 16, 5904–5913
64. Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R., and Parsons, J. T. (1991) Mol. Cell. Biol. 11, 5113–5124
65. Schuuring, E., van Damme, H., Schuuring-Scholtes, E., Verhoeven, E., Michalidez, R., Geelen, E., de Boer, C., Brok, H., van Buuren, V., and Klein, P. (1998) Cell Adhes. Commun. 6, 185–209
66. Zhan, X., Hu, X., Hampton, B., Burgess, W. H., Friesel, R., and Maciag, T. (1993) J. Biol. Chem. 268, 42427–42431
67. Muthuswamy, S. K., and Muller, W. J. (1995) Oncogene 11, 1801–1810