Hyaluronan-CD44 Interaction with Leukemia-associated RhoGEF and Epidermal Growth Factor Receptor Promotes Rho/Ras Co-activation, Phospholipase Cε-Ca\(^{2+}\) Signaling, and Cytoskeleton Modification in Head and Neck Squamous Cell Carcinoma Cells

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In this study we have examined the interaction of CD44 (a major hyaluronan (HA) receptor) with a RhoA-specific guanine nucleotide exchange factor (leukemia-associated RhoGEF (LARG)) in human head and neck squamous carcinoma cells (HNSCC-HSC-3 cell line). Immunoprecipitation and immunoblot analyses indicate that CD44 and the LARG protein are expressed in HSC-3 cells and that these two proteins are physically associated as a complex. HA-CD44 binding induces LARG-specific RhoA signaling and phospholipase Cε (PLCε) activity. In particular, the activation of RhoA-PLCε by HA stimulates inositol 1,4,5-triphosphate production, intracellular Ca\(^{2+}\) mobilization, and the up-regulation of Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMkII), leading to phosphorylation of the cytoskeletal protein, filamin. The phosphorylation of filamin reduces its interaction with filamentous actin, promoting tumor cell migration. The CD44-LARG complex also interacts with the EGFR receptor (EGFR). Most importantly, the binding of HA to the CD44-LARG-EGFR complex activates the EGFR receptor kinase, which in turn promotes Ras-mediated stimulation of a downstream kinase cascade including the Raf-1 and ERK pathways leading to HNSCC cell growth. Using a recombinant fragment of LARG (the LARG-PDZ domain) and a binding assay, we have determined that the LARG-PDZ domain serves as a direct linker between CD44 and EGFR. Transfection of the HSC-3 cells with LARG-PDZcDNA significantly reduces LARG association with CD44 and EGFR. Overexpression of the LARG-PDZ domain also functions as a dominant-negative mutant (similar to the PLC/CA\(^{2+}\)-calmodulin-dependent kinase II (CaMkII) and EGFR/MAPK inhibitor effects) to block HA/CD44-mediated signaling events (e.g., EGFR kinase activation, Ras/RhoA co-activation, Raf-ERK signaling, PLCε-mediated inositol 1,4,5-triphosphate production, intracellular Ca\(^{2+}\) mobilization, CaMkII activity, filamin phosphorylation, and filamin-actin binding) and to abrogate tumor cell growth/migration. Taken together, our findings suggest that CD44 interaction with LARG and EGFR plays a pivotal role in Rho/Ras co-activation, PLCε-Ca\(^{2+}\) signaling, and Raf/ERK up-regulation required for CaMkII-mediated cytoskeleton function and in head and neck squamous cell carcinoma progression.
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HNSCC cancers (29–31). The overall survival rate and time of relapse for these HNSCC cancer patients with EGFR overexpression is significantly shorter than those without EGFR overexpression. Consequently, EGFR overexpression may be a useful prognosis marker (29). EGFR is a transmembrane glycoprotein that possesses intrinsic tyrosine kinase activity (32). Previous studies have found that guanidine nucleotide (GDP/GTP) exchange on Ras is significantly stimulated by tyrosine phosphorylation of EGFR (33, 34). Thus, it appears that EGFR activation mediates Ras-mediated stimulation of a downstream kinase cascade, which includes the Raf-1/MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase)/MAPK pathway leading to tumor cell growth (33, 34).

Members of the Rho subclass of the Ras superfamily (small molecular weight GTPases, e.g. RhoA, Rac1, and Cdc42) are involved in transducing a variety of signals regulating many different cellular processes (35). Overexpression of certain RhoGTPases in human tumors often correlates with a poor prognosis (36, 37). In particular, coordinated RhoGTase signaling is considered to be a possible mechanism underlying cell proliferation and motility, an obvious prerequisite for metastasis (25–39). Presently, very little information is available regarding how the RhoGTPases are activated by cell surface receptors. To date, at least 30 different guanine nucleotide exchange factors (GEFs) have been identified (40).

The leukemia-associated Rho guanine nucleotide exchange factor (LARG) was originally identified as a fusion partner with mixed-lineage leukemia in a patient with acute myeloid leukemia (41). Structurally, LARG contains a number of functional domains and structural motifs found in signal transduction proteins and oncogenes. These motifs include the N-terminal PDZ domain, a RGS (regulator of G protein signaling) domain, a dbl homology domain (DH), and a pleckstrin homology domain (PH) (41). The DH domain of all Dbl family proteins interacts with the inactive, GDP-bound GTPase and promotes the release of GDP and its subsequent exchange for GTP, thereby activating specific members of the Ras superfamily of GTP-binding proteins (41, 42). The LARG has been clearly shown to act as a GDP/GTP exchange protein for the Rho subfamily of GTPases, including RhoA (41, 43). The questions of whether HA/CD44 is capable of inducing LARG-mediated RhoA activation and which RhoA-targeted downstream effector(s) is involved in regulating head and neck cancer progression remain to be answered.

Intracellular Ca2+ mobilization is known to be regulated by phosphoinositide-specific phospholipases Cs (PLCs) (44–47). At least three different guanine nucleotide exchange factors (GEFs) have been identified (40).

The DH domain of all Dbl family proteins interacts with the inactive, GDP-bound GTPase and promotes the release of GDP and its subsequent exchange for GTP, thereby activating specific members of the Ras superfamily of GTP-binding proteins (41, 42). The LARG has been clearly shown to act as a GDP/GTP exchange protein for the Rho subfamily of GTPases, including RhoA (41, 43). The questions of whether HA/CD44 is capable of inducing LARG-mediated RhoA activation and which RhoA-targeted downstream effector(s) is involved in regulating head and neck cancer progression remain to be answered.

Intracellular Ca2+ mobilization is known to be regulated by phosphoinositide-specific phospholipases Cs (PLCs) (44–47). At least three major PLC families (PLCβ, PLCγ, and PLCε) exist, and each family type has a number of subtypes (48, 49). Activation of PLCβ isoforms requires the presence of heterotrimeric G protein subunits Go (50–52) and Gβγ (53–55). The activity of PLCγ isoforms can be up-regulated via phosphorylation and translocation as a consequence of the activation of tyrosine kinase receptors such as EGF receptor (56–58). Recently, PLCε has been identified as a possible downstream target for RhoGTPases (e.g. RhoA) involved in the regulation of Ca2+ signaling (59, 60). Specifically, PLCε interacts with RhoA in a GTP-dependent manner and promotes inositol 1,4,5-triphosphate (IP3)-mediated Ca2+ mobilization (59, 60). Although the cellular and molecular mechanisms involved in HA/CD44-mediated Ca2+ signaling are currently not well understood, one of the likely pathways may involve the phosphatidylinositol cascade that leads to Ca2+ release from intracellular stores.

One of the mechanisms by which intracellular Ca2+ may trigger early signal transducing events during HA- and CD44-mediated cellular activation involves its interaction with calmodulin, a ubiquitous Ca2+-binding protein (44–46; 61,62). Ca2+-dependent calmodulin is known to be involved in the activation of several important enzymes including Ca2+-calmodulin-dependent kinase II (CaMKII), a serine/threonine protein kinase that phosphorylates diverse substrates including cytoskeletal proteins such as filamin (63, 64). Filamin is a phosphoprotein dimer comprising two subunits of molecular mass 280,000 kDa (M, 280), each of which contains 24 tandem repeats of about 96 amino acids (65, 66). Recent studies indicate that filamin interacts with several proteins (e.g. Smad 2/5 and p21-activated kinase 1(Pak1)) involved in signal transduction (67, 68). However, one of the best characterized properties for filamin is its ability to cross-link the actin cytoskeleton into orthogonal networks and to modulate cell shape changes and cell motility (69). Biochemical analyses indicate that filamin phosphorylation by CaMKII increases filamin critical actin filament gelling concentration and reduces the binding of filamin to filamentous actin (F-actin) (63). Thus, phosphorylation by CaMKII is implicated in the regulation of filamin interaction with F-actin (63). Previous studies have identified that the cytoplasmic domain of CD44 also serves as a cellular substrate for CaMKII. Furthermore, CD44 phosphorylation by CaMKII enhances its interaction with the cytoskeleton and stimulates cell migration (71). Thus, CaMKII appears to be directly involved in HA-CD44-mediated signaling and cellular activities. However, the mechanism by which HA/CD44 mediates CaMKII activity in cytoskeleton-mediated HNSCC migration has not been addressed.

In this paper we have investigated CD44 interaction with LARG and EGFR in promoting RhoA/Ras activation and Raf/ERK-PLC-Ca2+ signaling in HSC-3 cells. In addition, we have examined the involvement of these interactions in regulating HA-activated CaMKII and its downstream effectors required for HNSCC-specific tumor behaviors (e.g. tumor cell growth and migration).

MATERIALS AND METHODS

Cell Culture—An HSC-3 cell line derived from human squamous carcinoma cells of mouth was kindly provided from Dr. Randy Kramer (University of California, San Francisco, CA). HSC-3 cells were grown in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Cells were routinely serum-starved (and therefore deprived of serum HA) before adding HA.

Antibodies and Reagents—Monoclonal rat anti-CD44 antibody (Clone 020, isotype IgG2b, obtained from CMB-TECH, Inc., San Francisco, CA.) recognizes a determinant of the HA binding region common to CD44 and its principal variant isoforms (17, 19–24). This rat anti-CD44 was routinely used for HA-related blocking experiments. For the preparation of polyclonal rabbit anti-LARG antibody, specific synthetic peptides (≈15–17 amino acids unique for LARG sequences) were prepared by ImmunoVision Technologies Co. (Daly City, CA) using an Advanced Chemtech automatic synthesizer (model ACT350). Conjugated LARGs (to polylysine) were injected into rabbits to raise the antibodies. The anti-LARG sera was collected from each bleed and stored at 4 °C containing 0.1% azide. Rabbit anti-LARG IgG was prepared using conventional DEAE-cellulose chromatography and was tested to be monospecific (by immunoblot assays).

Mouse anti-CaMKII antibody and goat anti-phospho-Raf-1 antibody were obtained from Transduction Laboratories and Santa Cruz Biotechnology (Santa Cruz, CA, respectively. Mouse anti-ERK antibody, rabbit anti-phospho-ERK antibody, rabbit anti-PLCε, mouse anti-EGFR antibody, mouse anti-phospho-EGFR antibody, the Ras activation assay kit, and the CaMKII assay kit were purchased from Upstate Biotechnology (Lake Placid, NY). GST-tagged RhoA and inhibitors (e.g. KN93, U73122, 2-APB, AG1478, and U0126) were obtained from Calbiochem. Clostridium botulinum C3 toxin (C3-toxin) was obtained.

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from List Biological Laboratories, Inc. Rabbit anti-His antibody was purchased from Amersham Biosciences. Human recombinant epidermal growth factor (EGF) was from Invitrogen.

Both Healon HA (Mw 500,000) and rooster comb HA (Mw 500,000) (Sigma) were purified by gel filtration chromatography using a Sephacryl S1000 column. The purity of the high molecular weight HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography. Both HA preparations are free from pro-inflammatory or other contaminants (105) and were used interchangeably in many biological studies in our laboratory. Sigma HA was used for those experiments described below. The expression of His-tagged LARG-PDZcDNA fragment was confirmed by SDS-PAGE and immunoblot/immunoprecipitation analyses. LARG was isolated from HSC-3 cells as described below. The expression of His-tagged LARG-PDZcDNA fragment was confirmed by SDS-PAGE and immunoblot/immunoprecipitation analyses. LARG was isolated from HSC-3 cells pretreated with anti-CD44 antibody for 1 h followed by 50 μg/ml HA treatment for 2 min (or no HA) at 37 °C as described under “Materials and Methods.” Data represent an average of triplicates from 3–5 experiments. The S.D. was less than 5%.

Procedures for conjugating purified Healon HA or purified Sigma HA to fluorescein isothiocyanate (FITC) were essentially the same as described by deBilly and Wik (100). Radiodination of FITC-Healon HA (or FITC-Sigma HA), performed with iodobeads (Pierce) according to the manufacturer’s instructions resulted in a specific radioactivity of ~5 × 10^6 cpm/μg.

**TABLE 1**

| Treatments                        | 125I-labeled FITC-Healon HA binding (cpm) (%) | 125I-labeled FITC-purified Sigma HA binding (cpm) (%) |
|-----------------------------------|----------------------------------------------|----------------------------------------------------|
| No treatment (control)            | 22,000 ± 1,100 (100%)                        | 20,500 ± 820 (100%)                                |
| Normal Rat IgG treatment          | 20,960 ± 840 (98%)                           | 20,700 ± 621 (101%)                                |
| Anti-CD44 IgG treatment           | 10,750 ± 565 (49%)                           | 9,840 ± 492 (48%)                                  |

**B. HA/CD44-mediated LARG-RhoA activation**

| Treatments                        | Amount of [35S]GTPγS bound to RhoA<sup>b</sup> |
|-----------------------------------|-----------------------------------------------|
|                                  | pmol | % of control |
| No treatment (control)            | 1.45 | 100          |
| HA treatment                      | 2.48 | 241          |
| Anti-CD44 IgG                     | 1.38 | 95           |
| Anti-CD44 IgG + HA treatment      | 1.42 | 98           |

**C. Effect of LARG-PDZ overexpression on HA-dependent LARG-mediated RhoA activation**

| Cells                             | Amount of [35S]GTPγS bound to RhoA<sup>b</sup> |
|-----------------------------------|-----------------------------------------------|
| Vector-transfected cells          | 1100 (245)                                    |
| LARG-PDZcDNA-transfected cells    | 58 (55)                                       |

<sup>a</sup> HSC-3 cells (untreated or pretreated with either normal rat IgG or rat anti-CD44 antibody) followed by incubating with 125I-labeled FITC-Healon HA or 125I-labeled FITC-purified Sigma HA as described under “Materials and Methods.”

<sup>b</sup> Purified Escherichia coli-derived GST-tagged RhoA was preloaded with GDP. Subsequently, LARG (bound to anti-LARG-conjugated Sepharose beads) isolated from HSC-3 cells (transfected with LARG-PDZcDNA vector or vector alone) followed by 50 μg/ml HA treatment for 2 min (or no HA) at 37 °C was added to the reaction buffer containing 2.5 μM [35S]GTPγS (~1250 Ci/mmol) (in the presence or absence of 2.25 μM GTPγS) for 10 min followed by adding 2.5 pmol of GDP-loaded GST-tagged RhoA. After 10 min the reaction of each sample was terminated. The radioactivity associated with GST-tagged RhoA was measured by scintillation counting. In some experiments LARG-catalyzed GDT/GTP exchange on RhoA was determined by the same procedures using LARG isolated from HSC-3 cells pretreated with anti-CD44 antibody for 1 h followed by 50 μg/ml HA treatment for 2 min (or no HA) at 37 °C as described under “Materials and Methods.” Data represent an average of triplicates from 3–5 experiments. The S.D. was less than 5%.

Procedures for conjugating purified Healon HA or purified Sigma HA to fluorescein isothiocyanate (FITC) were essentially the same as described by deBilly and Wik (100). Radiodination of FITC-Healon HA (or FITC-Sigma HA), performed with iodobeads (Pierce) according to the manufacturer’s instructions resulted in a specific radioactivity of ~5 × 10^6 cpm/μg.

**Binding of 125I-labeled FITC-Healon HA (or FITC-Sigma HA) to HSC-3 Cells—HSC-3 cells (~2 × 10^6 cells) (untreated or treated with rat anti-CD44 IgG (1 μg/ml) or normal rat IgG (1 μg/ml) for 1 h at 37 °C) were incubated with either 1 μg of 125I-labeled FITC-Healon HA or FITC-Sigma HA at 4 °C for 30 min. After binding, labeled cells were washed twice. Cell-associated radioactivity was estimated using γ counting. All binding studies were carried out in triplicate. FITC-Healon HA-labeled cells were also examined with a confocal laser scanning microscope.

**LARG-mediated GDP/GTP Exchange for RhoA Protein—**Purified E. coli-derived GST-tagged RhoA (20 pmol) was preloaded with GDP (30 μM) in 10 μl of buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 4.7 mM EDTA, 0.16 mM MgCl₂, and 200 μg/ml bovine serum albumin (BSA) at 37 °C for 7 min. To terminate preloading procedures, additional MgCl₂ was then added to the solution (reaching a final concentration of 9.16 mM) as described previously (17). Subsequently, 2 pmol of LARG (bound to anti-LARG-conjugated Sepharose beads) isolated from HSC-3 cells (transfected with LARG-PDZcDNA vector or vector alone pretreated with HA (50 μg/ml)) for 2 min at 37 °C) was incubated with 2.5 μM [35S]GTPγS (~1250 Ci/mmol) (in the presence or absence of 2.25 μM GTPγS) for 10 min followed by adding 2.5 pmol of GDP-loaded GST-tagged RhoA (or GDP-treated GST). At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl₂ followed by filtering through nitrocellulose filters. The radioactivity associated with the filters was measured by scintillation fluid as described previously (17). The amount of [35S]GTPγS bound to LARG (bound to anti-LARG-Sepharose beads) or control sample (preimmune serum-conjugated Sepharose beads) in the absence of GST-tagged RhoA was subtracted from the original values. In some cases, LARG-catalyzed GDT/GTP exchange on RhoA was measured using HSC-3 cells preincubated with anti-CD44 antibody (10 μg/ml) for 1 h followed by 2 min of HA (50 μg/ml) or no HA treatment.

**Cloning and Expression of the PDZ Domain of LARG—**The LARG-PDZcDNA fragment (bp 954–1326; accession number AF180681) was amplified by RT-PCR using PDZ-specific primers (upper primer 5'-GCCATGGGTCAGCGTTGCGTAAT-3' and 5'-CACCTCAGGTCGGCAAGTGGAATC-3'). The LARG-PDZcDNA fragment was cloned into pcDNA3.1-V5/His TOPO vector (Invitrogen). The inserted PDZ sequence was confirmed by nucleotide sequencing analyses (University of California at San Francisco Biomolecular Resource Center). This V5/His-tagged LARG-PDZ fragment cDNA was then used for transient expression in COS-7 cells or HSC-3 cells as described below. The expression of His-tagged LARG-PDZ domain in COS-7 or HSC-3 cells was detected by SDS-PAGE and immunoblot/immunoprecipitation analyses. LARG was isolated from
HSC-3 cells using anti-LARG-conjugated immuno-beads. LARG-PDZ fragments were isolated from HSC-3 cells transfected with His-tagged LARG-PDZcDNA and prepared by anti-His-conjugated immunoaffinity column chromatography.

In Vitro Binding of LARG-PDZ Domain to CD44 or EGFR—Aliquots (0.5–1 ng of protein) of purified CD44-conjugated or EGFR-conjugated Sepharose beads were incubated in 0.5 ml of binding buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) containing various concentrations (10–800 ng/ml) of 125I-labeled His-tagged LARG-PDZ fragment (isolated from COS-7 or HSC-3 cells) (5000 cpm/ng of protein) at 4 °C for 4 h. After binding, the CD44- or EGFR-conjugated beads were washed extensively in binding buffer, and the bead-bound radioactivity was counted. Nonspecific binding was determined using a 50–100-fold excess of unlabeled LARG-PDZ fragment in the presence of the same concentration of 125I-labeled LARG-PDZ. Nonspecific binding, which was ~20% of the total binding, was always subtracted from the total binding.

Immunoblotting and Immunoprecipitation Techniques—HSC-3 cells were serum-starved for 24 h followed by incubation with 50 μg/ml HA (or no HA or 15 ng of EGF) for various time intervals (e.g. 0, 1, 2, 3, 5, 7, 10, or 15 min) at 37 °C. Subsequently, cells were solubilized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40 (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 immuno-reagents (e.g. rabbit anti-LARG antibody or anti-EGFR or anti-phospho-EGFR or anti-Ras, anti-Raf-1, anti-ERK antibody, or anti-phospho-ERK) (5 μg/ml). In some experiments, immunoblot analyses of LARG, EGFR, phosphorylated EGFR, ERK, or phosphorylated ERK or CD44 in cells (transfected with LARG-PDZcDNA or vector alone) were also carried out. In some cases, HSC-3 cells (transfected with LARG-PDZcDNA or vector alone) were incubated with 50 μg/ml HA (or no HA) for various time intervals (e.g. 0, 1, 2, 3, 5, 7, 10, or 15 min). These cells were extracted with Nonidet P-40 (as described above) and subjected to immunoprecipitation using rat anti-CD44 antibody (1 μg/ml) followed by goat anti-rat IgG-conjugated bead incubation. The materials associated with anti-CD44-immuno-beads were solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% BSA, the nitrocellulose filter was incubated with various antibodies (e.g. anti-LARG antibody (5 μg/ml) or anti-CD44 antibody or anti-EGFR or anti-phospho-EGFR or anti-Ras, anti-Raf-1, anti-ERK antibody, or anti-phospho-ERK) (5 μg/ml). In some experiments, immunoblot analyses of LARG, EGFR, phosphorylated EGFR, ERK, or phosphorylated ERK or CD44 in cells (transfected with LARG-PDZcDNA or vector alone) were also carried out. In some cases, HSC-3 cells (transfected with LARG-PDZcDNA or vector alone) were incubated with 50 μg/ml HA (or no HA) for various time intervals (e.g. 0, 1, 2, 3, 5, 7, 10, or 15 min). These cells were extracted with Nonidet P-40 (as described above) and subjected to immunoprecipitation using rat anti-CD44 antibody (1 μg/ml) followed by goat anti-rat IgG-conjugated bead incubation. The materials associated with anti-CD44-immuno-beads were solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% BSA, the nitrocellulose filter was incubated with various antibodies (e.g. anti-LARG antibody (5 μg/ml) or anti-CD44 antibody or anti-EGFR or anti-phospho-EGFR antibody (5 μg/ml) or anti-anti-Raf-1 (5 μg/ml) or anti-ERK or anti-phospho-ERK (5 μg/ml), respectively) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse/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.

CaMKII Activity Assay—HSC-3 cells were first lysed by a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerol-phosphate, and protease inhibitor mixture (Calbiochem) for 30 min. Cell debris was removed by 10 min of centrifugation at 15,000 × g, and the supernatants were incubated with 2.5 mg of mouse anti-CaMKII-conjugated beads to isolate CaMKII at 4 °C for 1 h. CaMKII activity was then determined by the incorporation of [32P]ATP into its specific substrate peptide (KKALR-RQETVDAL) (70, 106) in the presence of CaMKII (associated with anti-CaMKII-conjugated beads) and the CaMKII assay kit or purified filamin (isolated from HSC-3 cells using anti-filamin-immuno column). In some cases HSC-3 cells (untransfected or transfected with LARG-PDZcDNA; untreated or pretreated with anti-CD44 antibody (10 μg/ml) or various drugs such as C-3 toxin (1 μM), U73122 (1 μM), or KN93 (5 μM) for 1 h followed by 10 min of HA (50 μg/ml) treatment) were lysed, and CaMKII was isolated using anti-CaMKII beads. The activity of CaMKII was then measured by the incorporation of [32P]ATP into filamin using the CaMKII assay kit described above.

In some experiments Ca2+ (2.0 mM) was added to the CaMKII (isolated from HSC-3 cells using the anti-CaMKII-conjugated beads). The activity of CaMKII was then measured by the incorporation of [32P]ATP into its specific substrate peptide (KKALR-RQETVDAL) (70, 106). In addition, 5 μM KN93 (a CaMKII inhibitor) was added directly to the CaMKII for 1 h followed by adding 2.0 mM Ca2+ to determine [32P]ATP incorporation into the peptide (KKALR-RQETVDAL) or purified filamin (isolated from HSC-3 cells using anti-filamin-immuno beads). Reactions were then terminated by adding 20% cold trichloroacetic acid, and 2 mg/ml BSA was then added as a carrier. Trichloroacetic acid precipitated proteins were spotted on Whatman No. 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. A reversed sequence (LAD-VTEQRRLKK) was used as a nonspecific substrate to compare with the specific substrate (KKALR-RQETVDAL) for CaMKII activity. The radioactivity associated with preimmune-IgG-associated beads (nonspecific substrate for filamin) was also measured. The amount of nonspecific substrate phosphorylation by CaMKII was always subtracted from CaMKII-specific substrate phosphorylation.

F-actin Cross-linking Assay—The procedures for conducting F-actin cross-linking experiments were the same as those described previously (63, 64) with some modifications. Unphosphorylated filamin or CaMKII phosphorylated filamin (as described above) (50–100 nM) in 50 μl of TKM buffer (50 mM Tris-HCl (pH7.4), 134 mM KCl, and 1 mM MgCl2) was mixed with an equal volume of 8 μM 125I-labeled F-actin (~106 daltons) 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 (63, 64) with some modifications. Unphosphorylated filamin or CaMKII phosphorylated filamin (as described above) (50–100 nM) in 50 μl of TKM buffer (50 mM Tris-HCl (pH7.4), 134 mM KCl, and 1 mM MgCl2) was mixed with an equal volume of 8 μM 125I-labeled F-actin (~106 daltons) 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 (63, 64). The F-actin cross-linking reaction in the presence of unphosphorylated filamin (control) is designated as 100%. In some cases unphosphorylated filamin isolated from vector-transfected cells using His-conjugated Sepharose beads (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.
HA-CD44 Interaction with LARG and EGFR Promotes Signaling

Measurement of Intracellular Ca\(^{2+}\) Mobilization—HSC-3 cells (untreated or pretreated with anti-CD44 antibody (1 \(\mu\)g/ml) or various inhibitors (U73122 (a PLC inhibitor, 1 \(\mu\)M), C3-toxin (a RhoA inhibitor, 1 \(\mu\)M), 2APB (an IP\(_3\) receptor inhibitor, 1 \(\mu\)M)) at 37 °C for 1 h) or transfected with LARG-PDZcDNA or vector alone) were first incubated with 10 \(\mu\)M Fura-2/AM (Calbiochem) for 1 h at room temperature in a buffer solution containing 145 mM NaCl, 5 mM KCl, 0.1 mM MgCl\(_2\), 5 mM glucose, and 15 mM HEPES (pH 7.3) in the presence or absence of 1 mM CaCl\(_2\). Cells were subsequently washed three times with the same buffer. Cells (10\(^6\) cells/ml) resuspended in 0.1 mM phosphate saline buffer (pH 7.0) were incubated simultaneously with an equal volume of 0.1 M phosphate saline buffer (pH 7.0) containing HA (50 \(\mu\)g/ml) into a 20-\(\mu\)l chamber alternately illuminated with 200-ms flashes of 340 and 380 nm at every 10 ms (monitoring the emission wavelength of 510 nm) using a dual-wavelength fluorescence imaging system (Intracel Imaging Inc., Cincinnati, OH). The concentration of intracellular Ca\(^{2+}\) was determined by the equation Ca\(^{2+}\) = \(K_D \times ((R - R_{\text{min}})/(R_{\text{max}} - R)) \times F/B\), where Ca\(^{2+}\) is the intracellular Ca\(^{2+}\). \(K_D\) is the dissociation constant of Fura-2 for Ca\(^{2+}\), \(R\) is the ratio of the Fura-2 fluorescence excited at 340 nm divided by the fluorescence excited at 380 nm, and \(R_{\text{max}}\) and \(R_{\text{min}}\) are minimal and maximal fluorescence ratios, respectively, obtained in ionomycin in the presence of 7 mM EGTA or 2 mM Ca\(^{2+}\). \(F\) and \(B\) are the fluorescence voltage signals at 380 nm in 5.0 \(\mu\)M ionomycin in the presence of 7 mM EGTA and 2 mM Ca\(^{2+}\), respectively.

PLCe Activity Assay—The procedures for analyzing the activity of PLC\(_e\) were similar to those described previously (59, 60). Briefly, HSC-3 cells were solubilized in Nonidet P-40 buffer in the presence of protease inhibitors plus 1 \(\mu\)M NaF, 1 \(\mu\)M sodium orthovanadate, and 1 \(\mu\)M okadaic acid. Lysates were spun at 5000 \(\times\) g, and the supernatants were collected. PLC\(_e\) was immunoprecipitated from the supernatants using anti-PLCe-conjugated beads. These PLC\(_e\)-conjugated beads were then incubated with GDP-/GTP-bound RhoA. Subsequently, RhoA-PLCe-immunobeads were incubated with vesicles containing 100 \(\mu\)M phosphatidyethanolamine, 10 \(\mu\)M phosphatidylserine, 10 \(\mu\)M phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), and 0.028 \(\mu\)M \(^{3}\)H]PIP\(_2\) in PLC activity buffer (25 mM HEPES, 80 mM KCl, 3 mM EGTA, 0.5 mM dithiothreitol (pH 7.0) at 37 °C. In some cases PLC\(_e\) isolated from HSC-3 cells (untreated or pretreated with LARG-PDZcDNA or vector alone) that were treated with HA (50 \(\mu\)g/ml) at 37 °C for 2 min (or pretreated with 10 \(\mu\)g/ml anti-CD44 antibody, 1 \(\mu\)M C-3 toxin for 1 h followed by 2 min incubation of HA (50 \(\mu\)g/ml) or no HA incubation) was also incubated in this enzyme assay mixture. Reactions were carried out for 10 min and terminated by adding 10% trichloroacetic acid and 2 mg/ml BSA. Precipitated proteins were removed by centrifugation. Radioactive \(^{3}\)H]PIP\(_2\) (liberated from \(^{3}\)H]PIP\(_2\) in the trichloroacetic acid-soluble supernatant fraction was analyzed using scintillation counting. PIP\(_2\) hydrolysis was linear for 15 min under the conditions used.

Tumor Cell Growth Assays—HSC-3 cells (untreated or transfected with LARG-PDZcDNA or vector alone) (5 \(\times\) 10\(^4\) cells/well) were treated with HA (50 \(\mu\)g/ml) or pretreated with rat anti-CD44 or various drugs, AG1478 (an EGFR inhibitor, 1 \(\mu\)M), and U0126 (a MAPK inhibitor, 1 \(\mu\)M) for 1 h followed by HA (50 \(\mu\)g/ml) treatment for 24 h or no HA treatment. These cells were then plated in 96-well culture plates in 0.2 ml of Dulbecco’s modified Eagle’s medium/F-12 medium (a 50:50 mixture) supplemented (Invitrogen) containing either 0.5% fetal bovine serum or no serum for 24 h at 37 °C in 5%CO\(_2\), 95% air. In each experiment a total of 5 plates (10 wells/treatment/plate) was used. Experiments were repeated three times. The growth of these cells were analyzed by measuring increases in cell number using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole (bromide) assays (Cell-Titer 96 nonradioactive cell proliferation assay according to the procedures provided by Promega). Subsequently, viable cell-mediated reaction products were recorded by a Molecular Devices (Spectra Max 250) enzyme-linked immunosorbent assay reader at a wavelength of 450 nm.

RESULTS

HA-mediated CD44 Interaction with LARG in HSC-3 Cells—The primary tumor type in head and neck cancer is squamous cell carcinoma (HNSCC). These tumor cells display persistent cell migration, invasion, and growth leading to local recurrence and distant lymph node metastasis (1–6). Previously, it was found that genetic alterations in certain oncogenes (e.g., Ras, Myc, EGFR, and Cyclin D1) are closely associated with HNSCC progression (73). Some studies indicate that RhoGTPase expression is abnormal in premalignant and HNSCC cell lines (74). These findings suggest that motility-related proteins (e.g., RhoGTPases) may be important in defining HNSCC metastatic phenotype. The HSC-3 cells used in the present study were isolated from an invasive/migrative human oral squamous cell carcinoma (75). They were found to be poorly differentiated with a scattered morphology and to have invasive/migrative properties (75, 76). Both RhoGTPase signaling and EGFR activation have been shown to play an important role in modulating cell motility and survival in the HSC-3 cells (77, 78). Presently, only limited information is available concerning the specific signaling pathways in HSC-3 cells that regulate tumor behaviors (e.g., migration and growth). Thus, identifying the specific extracellular matrix-
CD44 antibody inhibits about 50% of 125I-labeled FITC-HA binding to HA and FITC-Sigma HA on HSC-3 cell surface are very comparable staining also demonstrates that the binding intensities of FITC-Healon (Table 1A). Confocal microscopic examination of immunofluorescence HA interaction with CD44 in HSC-3 cells (Table 1A). Our data indicate immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting with anti-LARG antibody (a) or reblotting with anti-CD44 antibody (b) as a loading control.

In this study, we have established a binding assay using either 125I-labeled FITC-conjugated Healon HA or purified Sigma HA to measure HA interaction with CD44 in HSC-3 cells (Table 1A). Our data indicate that both Healon HA and Sigma HA bind HSC-3 cells to a similar extent (Table 1A). Confocal microscopic examination of immunofluorescence staining also demonstrates that the binding intensities of FITC-Healon HA and FITC-Sigma HA on HSC-3 cell surface are very comparable (data not shown). Most importantly, pretreatment of cells with rat anti-CD44 antibody inhibits about 50% of 125I-labeled FITC-HA binding to the cells (Table 1A). However, normal rat IgG treatment does not appear to block HA binding (Table 1A). These results indicate that CD44 is one of the major HA receptors on HSC-3 cells.

A number of studies have determined that CD44 and RhoGEF are structurally and functionally coupled in tumors (36, 37). As part of our continued effort to identify CD44 isomorphically-linked RhoGEFs, which correlate with certain metastatic behaviors, a candidate molecule, named LARG, has been identified. To determine whether LARG protein expression occurs in the human HSC-3 cells, immunoblot analysis utilizing an anti-LARG antibody designed to recognize a LARG-specific sequence reveals a single polypeptide (molecular mass \( \approx 175 \) kDa) (Fig. 1A, lane 2). We demonstrated that the LARG protein detected by anti-LARG-mediated immunoblot is specific since no protein is detected in those cells incubated with preimmune rabbit IgG (Fig. 1A, lane 1). In addition, we have carried out anti-CD44-mediated immunoprecipitation followed by anti-LARG immunoblot (Fig. 1Aa) or anti-CD44 immunoblot (Fig. 1Bb) using untreated HSC-3 cells. Our results indicate that a low level of LARG (Fig. 1Ba, lane 1) is present in the anti-CD44-immunoprecipitated materials (Fig. 1Bb, lane 1). Subsequently, we have determined that HA treatment causes the recruitment of a significant amount of LARG (Fig. 1Ba, lane 2) into the CD44-LARG complex (Fig. 1Bb, lane 2). Pretreatment of HSC-3 cells with anti-CD44 antibody followed by HA treatment results in a reduction of LARG (Fig. 1Ba, lane 3) accumulation in the anti-CD44-immunoprecipitated materials (Fig. 1Bb, lane 3). These findings clearly establish that CD44 and LARG are closely associated with each other in vivo, particularly after HA treatment of the HNSCC cells such as HSC-3 cells.

**HA/CD44 Stimulation of LARG-RhoA Activation in HSC-3 Cells**—It has been reported that the dbl DH domain of LARG functions as an exchange factor for Rho-like GTPases such as RhoA (41–43). To confirm that the LARG molecule in HSC-3 cells functions as a GDP/GTP exchange factor for RhoGTPases, we first isolated LARG from cells using anti-LARG-conjugated Sepharose beads. As indicated in Table 1B, the isolated LARG activates GDP/GTP exchange on RhoA. Most importantly, we have observed that the addition of HA to CD44-expressing HSC-3 cells stimulates the maximal amount of bound [35S]GTP\( \gamma \)S to GST-RhoA (at least a 2.4-fold increase) as compared with LARG isolated from untreated HSC-3 cells (Table 1B). It is also noted that the amount of [35S]GTP\( \gamma \)S-bound RhoA detected in samples using LARG isolated from HSC-3 cells pretreated with anti-CD44 antibody followed by HA treatment was significantly reduced (Table 1B). In control samples a basal level of [35S]GTP\( \gamma \)S-bound RhoA was observed using LARG isolated from HSC-3 cells pretreated with anti-CD44 antibody alone (Table 1B). Therefore, we believe that HA binding to CD44 is directly involved in LARG-mediated RhoA activation in HSC-3 cells.

**PLC<sub>e</sub> Serves as a Novel Downstream Target for LARG-RhoA Signaling and Regulates Ca<sup>2+</sup>** **Signaling in HSC-3 Cells**—HA/CD44 interaction has been shown to be tightly coupled with intracellular Ca<sup>2+</sup> mobilization pathways in many different cell types (44–46). During these signaling events, PLCs first hydrolyze PIP<sub>2</sub> into IP<sub>3</sub>, which leads to Ca<sup>2+</sup> release from intracellular stores (47–49). In searching for a possible linkage between HA/CD44-mediated LARG-RhoA signaling and intracellular Ca<sup>2+</sup> regulation, we incubated one of the PLCs, PLC<sub>e</sub> (isolated from HSC-3 cells), with GDP- or GTP-loaded forms of RhoA-GST-conjugated beads. Proteins associated with RhoA-GST-beads were then analyzed by immunoblotting with anti-PLCe (Fig. 2A). Our results indicate that a large amount of PLC<sub>e</sub> is associated with GTP-bound RhoA-GST beads (Fig. 2Ab), whereas a small amount of PLC<sub>e</sub> is bound to GDP-bound RhoA (Fig. 2Aa). These results are consistent with previous findings which determined that PLC<sub>e</sub>-RhoA interaction is GTP-dependent.

Next, we examined the potential impact of LARG-activated RhoA on the regulation of PLC<sub>e</sub> activity (measured by PLC<sub>e</sub>-mediated IP<sub>3</sub> production). Our results indicate that the amount of IP<sub>3</sub> production generated by PLC<sub>e</sub> activated by GTP-RhoA (Fig. 2Bb) is significantly higher than PLC<sub>e</sub> activated by GDP-bound RhoA (Fig. 2Ba). Therefore, we conclude that the GTP-bound form of RhoA is directly involved in the activation of PLC<sub>e</sub>. Moreover, we have determined that the stimulation of PLC<sub>e</sub> activity occurs immediately after HA addition to HSC-3 cells (Fig. 2C, a and b). Preincubation with anti-CD44 antibody (Fig. 2Cc) or C3 toxin (a RhoA inhibitor) (Fig. 2C, d and e) blocks PLC<sub>e</sub> activation in HSC-3 cells treated with HA (or no HA), indicating that HA induces PLC<sub>e</sub> activity in a CD44-dependent and RhoA-specific manner in HNSCC cells.

It is well documented that PLC-mediated IP<sub>3</sub> production triggers intracellular Ca<sup>2+</sup> mobilization (47–49). In this study we have used the fluorescence indicator, Fura-2, to measure the intracellular free Ca<sup>2+</sup> concentration after HA binding to HSC-3 cells. The ratio of the fluorescence signal from Fura-2 at 340- and 380-nm excitation was monitored and used to determine the intracellular Ca<sup>2+</sup> concentration. Our results clearly indicate that the intracellular Ca<sup>2+</sup> concentration is elevated...
after the addition of HA to HSC-3 cells followed by a period of continuous Ca\(^{2+}\)/H\(_{11001}\) influx (Fig. 3A). These data demonstrate that intracellular Ca\(^{2+}\)/H\(_{11001}\) mobilization is one of the early signaling events that occurs after HA binding to HSC-3 cells. Pretreatment of HSC-3 cells with anti-CD44 antibody (Fig. 3B) or various inhibitors (e.g., U73122 (a PLC inhibitor) (Fig. 3C) or 2-APB (a membrane-permeable blocker of IP\(_3\) receptor-mediated Ca\(^{2+}\)/H\(_{11001}\) release) (Fig. 3D)) or C-3 toxin (a RhoA inhibitor) (Fig. 3E) effectively inhibits HA-mediated intracellular Ca\(^{2+}\) elevation. These findings strongly suggest that Ca\(^{2+}\) signaling in HSC-3 cells involves both HA/CD44-dependent and RhoA/PLC/IP\(_3\) receptor-regulated processes.

**Detection of CaMKII in HSC-3 Cells**—HA-stimulated intracellular Ca\(^{2+}\) mobilization mediates important components of the CD44 signaling pathways by controlling cellular functions, such as adhesion, migration, and secretion (44–46). Some Ca\(^{2+}\) signals may be mediated by CaMKII, which is a ubiquitous serine/threonine protein kinase. CaMKII phosphorylates diverse substrates including cytoskeletal proteins (61–64). In this study we have measured the kinase activity associated with the CaMKII molecule isolated from HSC-3 cells (Table 2). Specifically, the kinase activity was determined by the ability of CaMKII to phosphorylate a purified substrate peptide (KKALKRRQETVDAL). Our results

![FIGURE 2. Demonstration of RhoA-mediated PLC\(_e\) activity. A, anti-PLC\(_e\)-mediated immunoblot of PLC\(_e\) (isolated from HSC-3 cells) associated with GDP-bound RhoA beads (lane a) or GTP-bound RhoA beads (lane b). B, detection of IP\(_3\) production generated by GDP-RhoA-stimulated PLC\(_e\) (a) or GTP-RhoA-stimulated PLC\(_e\) (b). C, detection of IP\(_3\) production generated by PLC\(_e\) isolated from HSC-3 cells (untreated (a), treated with HA (50 μg/ml) for 2 min (b), pretreated with anti-CD44 (10 μg/ml) for 1 h followed by 2 min HA (50 μg/ml) incubation (c), pretreated with C-3 toxin (1 μM) for 1 h and no HA treatment (d), or pretreated with C-3 toxin (1 μM) for 1 h followed by 2 min of HA (50 μg/ml) incubation (e).](image1)

**TABLE 2**

**Detection of CaMKII activity in vitro**

In these experiments, 2.0 mM Ca\(^{2+}\) (or no Ca\(^{2+}\)) was added to the CaMKII (isolated from HSC-3 cells using the anti-CaMKII-conjugated bead). The activity of CaMKII was then measured by the incorporation of \([\text{\textsuperscript{32}}P]ATP\) into its specific substrate peptide (KKALKRRQETVDAL). In addition, 5 μM KN93 (a CaMKII inhibitor) was added directly to the CaMKII for 1 h followed by adding 2.0 mM Ca\(^{2+}\) to determine \([\text{\textsuperscript{32}}P]ATP\) incorporation into the peptide (KKALKRRQETVDAL) as described under “Materials and Methods.” Each assay was set up in triplicate and repeated at least three times. The values expressed in this table represent an average of triplicate determinations of 3–5 experiments.

| Treatments        | \([\text{\textsuperscript{32}}P]\) incorporation into the substrate peptide (KKALKRRQETVDAL) | cpm |
|-------------------|------------------------------------------------------------------------------------|-----|
| No treatment (control) | 3500 ± 175                                                                         |     |
| Ca\(^{2+}\) treatment | 7800 ± 268                                                                         |     |
| Ca\(^{2+}\) + KN93 treatment | 4125 ± 180                                                                       |     |
indicate that CaMKII isolated from HSC-3 cells is clearly capable of phosphorylating this peptide (KKALRRQETVDAL) in the presence of Ca\(^{2+}\) using a kinase assay containing calmodulin (Table 2). It is noted that the amount of peptide (KKALRRQETVDAL) phosphorylation is low when CaMKII was incubated with no Ca\(^{2+}\) (Table 2). Preincubation of CaMKII with KN-93 (a CaMKII inhibitor) also blocks Ca\(^{2+}\)-mediated CaMKII phosphorylation of the peptide (KKALRRQETVDAL) (Table 2). These results confirm that CaMKII (stimulated by Ca\(^{2+}\)) isolated from HSC-3 cells is functionally active.

*Filamin Serves as a Cellular Substrate for CaMKII and Interacts with F-actin Promoting HNSCC Cell Migration*—Although CaMKII has been shown to regulate actin cytoskeleton organization (63, 64), the specific cytoskeletal components regulated by CaMKII in HA-mediated CD44 signaling in HSC-3 cells have yet to be clearly defined. In this study we have observed that CaMKII isolated from HSC-3 cells is capable of phosphorylating filamin (an actin binding protein) in the presence of Ca\(^{2+}\) (Fig. 4A1b). Our data indicate that the level of filamin phosphorylation is relatively low when CaMKII was incubated without Ca\(^{2+}\) (Fig. 4A1a) or treated with KN-93 (a CaMKII inhibitor) plus Ca\(^{2+}\) (Fig. 4A1c). These results demonstrate that filamin can be phosphorylated by Ca\(^{2+}\)-activated CaMKII. Ca\(^{2+}\) signaling plays an important role in activating several Ca\(^{2+}\)-dependent and cytoskeleton-regulated events. The fact that a CaMKII inhibitor (KN93) can effectively block Ca\(^{2+}\)-induced function suggests that Ca\(^{2+}\) signals in these cells were largely mediated by CaMKII. Identification of other Ca\(^{2+}\) effectors in HSC-3 cells awaits further investigation.

Moreover, our data show that filamin with a low level of phosphorylation by CaMKII in the absence of Ca\(^{2+}\) (Fig. 4A2a) or in the presence of a CAMKII inhibitor (KN-93) plus Ca\(^{2+}\) (Fig. 4A2c) is capable of crosslinking the actin filaments into bundles. However, phosphorylation of filamin by Ca\(^{2+}\)-activated CaMKII down-regulates its ability to crosslink filamentous actin (F-actin) (Fig. 4A2b). These results are consistent with previous findings suggesting filamin plays an important role as a filamentous actin (F-actin) modulator required for cytoskeleton reorganization (63, 64).

Further analyses indicate that the level of filamin phosphorylation by CaMKII isolated from HSC-3 cells treated with HA is significantly enhanced (Fig. 4B1c). In contrast, filamin phosphorylation by CaMKII prepared from HSC-3 cells without any HA treatment is relatively low (Fig. 4B1a). It is also noted that filamin phosphorylation using CaMKII isolated from those HSC-3 cells pretreated with anti-CD44 antibody (Fig. 4B1b) or various drugs (C-3 toxin (a RhoA inhibitor) (Fig. 4B1d), U-73122 (a PLC inhibitor) (Fig. 4B1e), or KN93 (a CaMKII inhibitor) (Fig. 4B1f)) followed by HA treatment is greatly reduced. Our data also indicate that filamin with a low level of phosphorylation (by CaMKII isolated from untreated HSC-3 cells (Fig. 4B2a) or those HSC-3 cells pretreated with anti-CD44 antibody (Fig. 4B2b) or various drugs (C-3 toxin (a RhoA inhibitor) (Fig. 4B2d), U-73122 (a PLC inhibitor) (Fig. 4B2e), or KN93 (a CaMKII inhibitor) (Fig. 4B2f)) in the presence of HA binds to the filamentous actin (F-actin). However, phosphorylation of filamin by CaMKII isolated from HA-treated HSC-3 cells reduces its ability to interact with F-actin (Fig. 4B2c). These observations strongly support the conclusion that HA-mediated filamin phosphorylation and filamin-F-actin binding are CD44-dependent and RhoA/PLC/CaMKII-sensitive. Presently, filamin phosphorylation by CaMKII is the only cytoskeletal protein we have studied. The question of whether other cytoskeletal proteins regulated by CaMKII is also involved in HA/CD44-mediated head and neck cancer progression is currently under investigation in our laboratory.

![Figure 4](image-url)

**Figure 4. Detection of CaMKII-mediated phosphorylation and measurement of the F-actin cross-linking activity of filamin.** A1, these experiments 2.0 mM Ca\(^{2+}\) (or no Ca\(^{2+}\)) was added to the CaMKII (isolated from HSC-3 cells using the anti-CaMKII-conjugated beads). The activity of CaMKII was then measured by the incorporation of \(^{32}\)P-ATP into filamin as described under "Materials and Methods" (detection of filamin phosphorylation by CaMKII treated with no Ca\(^{2+}\) (a), treated with 2.0 mM Ca\(^{2+}\) (b), or treated with KN93 plus Ca\(^{2+}\) (c)). A2, measurement of the F-actin cross-linking activity of filamin. Unphosphorylated filamin (prepared as A1a) (a), phosphorylated filamin (prepared as A1b) (b), or unphosphorylated filamin (prepared as A1c) (c) was mixed with \(^{125}\)I-labeled F-actin as described under "Materials and Methods." The F-actin cross-linking reaction in the presence of unphosphorylated filamin (using CaMKII and no Ca\(^{2+}\)) (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. B1, the activity of CaMKII (isolated from HSC-3 cells) was measured by \(^{32}\)P-ATP incorporation into filamin using the kinase assay described under "Materials and Methods." a-f, filamin phosphorylation by CaMKII isolated from HSC-3 cells (untreated (a), pretreated with anti-CD44 for 4 h followed by 10 min of HA treatment (b), treated with HA for 10 min (c), pretreated with C-3 toxin for 1 h followed by 10 min of HA treatment (d), pretreated with U-73122 for 1 h followed by 10 min of HA treatment (e), or pretreated with KN93 for 1 h followed by 10 min of HA treatment (f)). B2, measurement of the F-actin cross-linking activity of unphosphorylated or phosphorylated filamin using CaMKII isolated from HSC-3 cells (untreated (prepared as B1a) (a), pretreated with anti-CD44 for 1 h followed by 10 min of HA treatment (prepared as B1b) (b), treated with 10 min of HA treatment (prepared as B1c) (c), pretreated with C-3 toxin for 1 h followed by 10 min of HA treatment (prepared as B1d) (d), pretreated with U-73122 for 1 h followed by 10 min of HA treatment (prepared as B1e) (e), or pretreated with KN93 for 1 h followed by HA treatment (prepared as B1f) (f).
TABLE 3

Measurement of HSC-3 cell migration

Procedures for measuring tumor cell migration in HSC-3 cells (untreated or pretreated with anti-CD44 antibody (10 μg/ml) for 1 h or various inhibitors such as C-3 toxin (a RhoA inhibitor, 1 μM), U73122 (a PLC inhibitor, 1 μM), or 2APB (an IP₃ receptor inhibitor, 1 μM) for 1 h or transfected with LARG-PDZcDNA or vector alone) in the presence or absence of HA (200 μg/ml) for 18 h were described under “Materials and Methods.” Each assay was set up in triplicate and repeated at least three times. The values expressed in this table represent an average of triplicate determinations of 3–5 experiments with a S.D. less than ±5%.

A. HA/CD44-mediated HSC-3 cell migration

| Treatments                | Cell migration | % of control |
|---------------------------|----------------|--------------|
| No treatment (control)    | 100            | 100          |
| HA treatment              | 248            | 124          |
| Anti-CD44 lgG             | 92             | 46           |
| Anti-CD44 lgG + HA treatment | 95        | 47.5         |

B. Effects of various drugs on HA-dependent HSC-3 cell migration

| Cells                     | Cell migration | % of control |
|---------------------------|----------------|--------------|
| No drug treatment         | 100            | 100          |
| C-3 toxin treatment       | 58             | 62           |
| U-73122 treatment         | 55             | 61           |
| 2-APB treatment           | 56             | 59           |

C. Effect of LARG-PDZ overexpression on HA-dependent HSC-3 cell migration

| Cells                                                                 | Cell migration | % of control |
|-----------------------------------------------------------------------|----------------|--------------|
| Vector-transfected cells (control)                                    | 100            | 249          |
| LARG-PDZcDNA-transfected cells                                        | 57             | 62           |

Furthermore, using in vitro tumor cell migration assays, we have found that HSC-3 cells undergo active cell migration (Table 3). HA activates HNSCC cell migration (Table 3A), but when HSC-3 cells were pretreated with anti-CD44 antibody followed by HA addition, their ability to undergo cell migration is greatly reduced (Table 3A). In control samples a basal level of cell migration was detected in HSC-3 cells pretreated with anti-CD44 antibody alone (Table 3A). These observations demonstrate that HA-mediated HNSCC cell migration is CD44-dependent. Treatment of HSC-3 cells with various agents (e.g. the RhoA inhibitor (C-3 toxin), the PLC inhibitor (U73122), or the CaMKII blocker (KN-93)) causes a significant inhibition of HA-mediated HSC-3 cell migration (Table 3B). Thus, it appears that the addition of HA enhances cell migration that is mediated by RhoA/PLC/IP₃-regulated Ca²⁺ signaling (Fig. 3 and Table 3) and CaMKII (Table 3).

HA-mediated CD44-LARG Interaction with EGFR in HSC-3 Cells—To examine the possible relationship between the CD44-LARG complex and other important signaling molecules such as the EGFR in HSC-3 cells, we have analyzed the anti-CD44-mediated immunoprecipitates from cell lysates by immunoblotting with anti-EGFR (Fig. 5Aa), anti-phosphorylated EGFR (Fig. 5Ab), or anti-LARG (Fig. 5Ac) antibody, respectively. Our results demonstrate that EGFR (Fig. 5Aa) is complexed with both CD44 (Fig. 5Ad) and LARG (Fig. 5Ac). In HSC-3 cells, we do not detect CD44 association with other GEFs (e.g. p115RhoGEF, Vav2, and Tiam1) as shown previously in breast and ovarian cancer cells (17–19). These GEFs (e.g. p115RhoGEF, Vav, and Tiam1) appear to be present in very low levels. Therefore, HA/CD44 interaction with these signaling molecules is not readily detectable. Both EGFR and LARG are the two major proteins co-immunoprecipitated with CD44. However, we cannot rule out the possibility of a CD44 association with other minor proteins in these cells. Furthermore, we have observed that HA treatment of HSC-3 cells stimulates EGFR tyrosine kinase activity (Fig. 5Ab) and causes a significant increase in the amount of EGFR (Fig. 5Aa, lane 2) and LARG (Fig. 5Ac, lane 2) recruited into the CD44-associated (Fig. 5Ad, lane 2) signaling complex. These events activate Ras signaling (Fig. 5Ba, lane 2), which in turn stimulates a downstream kinase cascade, the Raf-1 (Fig. 5Bb, lane 2) and MAPK (in particular ERK1 and ERK2) pathways (Fig. 5Bb, c and d, lane 2) and HSC-3 cell growth (Table 4A). The amount of HA-mediated Ras activation and Raf-1/ERK phosphorylation (Fig. 5Bb, a, b, and c, lane 2) is similar to EGFR-induced Ras signaling and Raf-1/ERK phosphorylation (Fig. 5Bb, a, b, and c, lane 4). In contrast, very little tyrosine phosphorylation of EGFR and EGFR/phosphorylated EGF-R-LARG recruitment is detected in untreated HSC-3 cells (Fig. 5Aa, a, b, and c, lane 1) or cells pretreated with anti-CD44 antibody followed by HA treatment (Fig. 5Aa, a, b, and c, lane 3). Consequently, a significantly reduced Ras signaling and Raf-1/ERK phosphorylation was detected in those HSC-3 cells treated with no HA (Fig. 5Bb, a, b, c, and d, lane 1) or pretreated with anti-CD44 antibody followed by HA addition (Fig. 5Bb, a, b, c, and d, lane 3). HSC-3 cells treated with various drugs (e.g. the EGFR inhibitor (AG1478) or the MAPK blocker (U0126)) causes a significant inhibition of HA-mediated HSC-3 cell growth (Table 4B). These observations strongly suggest that HA interaction with CD44-LARG complex is capable of activating EGFR kinase activity (similar to EGF treatment) and assembling EGFR into a large complex.
required for the activation of multiple signaling pathways including both RhoA and Ras pathways.

Effects of LARG-PDZ Overexpression on CD44-LARG-EGFR Complex Formation and RhoA/Ras Signaling in HSC-3 Cells—Previous studies have indicated that the PDZ domain of LARG is involved in membrane binding during growth factor receptor-mediated cellular signaling (43). To determine whether there is a direct interaction between LARG and certain membrane proteins such as CD44 and EGFR, we prepared a purified recombinant PDZ fragment of LARG and two membrane proteins (e.g. CD44 and EGFR) for a binding analysis. Specifically, we incubated $^{125}$I-labeled LARG-PDZ fragment (containing the sequence between amino acids 80–148 of the LARG PDZ domain) with CD44 (or EGFR) under equilibrium binding conditions. Scatchard plot analyses presented in Fig. 6, A and B, indicate that the PDZ fragment of LARG binds to CD44 (or EGFR) at a single site with high affinity (an apparent dissociation constant ($K_d$) of $0.26 \text{ nM}$ (LARG-PDZ-CD44 binding) (Fig. 6A) or $0.1 \text{ nM}$ (LARG-PDZ-EGFR binding) (Fig. 6B)). These observations support the conclusion that the PDZ domain of LARG is responsible for the recognition of both CD44 and EGFR.

To further analyze the interaction between the PDZ domain of LARG and CD44 and EGFR in HSC-3 cells, we have prepared a LARG-PDZ fragment construct that was then cloned into a His-tagged expression vector followed by transient transfection of the His-tagged PDZcDNA (or vector alone) into HSC-3 cells. When these transfectants were immunoprecipitated by anti-CD44 antibody followed by immunoblotting with anti-CD44 and anti-His, we determined that the His-tagged PDZ fragment of LARG (Fig. 7Ac, lanes 3 and 4) is co-precipitated with CD44 (Fig. 7Ad, lanes 3 and 4) from cells transfected with the His-LARG-PDZcDNA (in the presence or absence of HA) (Fig. 7A, B).
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lanes 3 and 4). However, no detectable cellular protein (Fig. 7Ac, lanes 1 and 2) was found in anti-CD44-mediated immunoprecipitated/immunoblotted materials (Fig. 7Ad, lanes 1 and 2) isolated from vector-transfected cells treated with or without HA (Fig. 7A, lanes 1 and 2). These results indicate that the PDZ domain of LARG is closely complexed with CD44 in HSC-3 cells.

Moreover, we have demonstrated that HA is capable of promoting the recruitment of endogenous LARG (Fig. 7Aa, lanes 1 and 2) together with EGFR (Fig. 7Ab, lanes 1 and 2) into a complex with CD44 (Fig. 7Ad, lanes 1 and 2) in vector-transfected cells (Fig. 7A, lanes 1 and 2). In contrast, transfection of HSC-3 cells with LARG PDZcDNA not only causes significant reduction in endogenous LARG (Fig. 7Aa, lanes 3 and 4) association with CD44 (Fig. 7Ad, lanes 3 and 4) but also exhibits a marked inhibition of HA-mediated recruitment of LARG-linked EGFR (Fig. 7Aa, lanes 3 and 4) to CD44 (Fig. 7Ad, lanes 3 and 4). These findings suggest that the LARG containing the PDZ domain acts as a potent competitive inhibitor for endogenous, intact LARG binding to CD44 in HSC-3 cells, and it also functions as a strong dominant-negative mutant for blocking EGFR accumulation into HA-induced CD44-LRG signaling complexes.

Furthermore, we have examined signaling events (e.g. LARG-catalyzed GDP/GTP exchange reaction on RhoA (Table 1C), PLCε-mediated IP₃ production (Table 5), Ca²⁺ mobilization (Table 5), CaMKII-mediated filamin phosphorylation (Table 5) and filamin-F-actin binding (Table 5), EGFR tyrosine kinase (Fig. 7Ba), and Ras activation (Fig. 7Bb) as well as Raf-1/ERK phosphorylation (Fig. 7B, c and d)) in vector-transfected or LARG-PDZcDNA-transfected cells treated with HA (Fig. 7B, lanes 2 and 4) or no HA (Fig. 7B, lanes 1 and 3). Our results indicate that LARG-RhoA signaling (Table 1C) together with EGFR kinase (Fig. 7Ba, lanes 1 and 2) and Ras activation (Fig. 7Bb, lanes 1 and 2) occurs after HA addition to vector-transfected cells (Fig. 7B). HA/CD44-mediated Ras activation also stimulates a downstream kinase cascade, the Raf-1/ERK phosphorylation (Fig. 7B, c and d, lanes 1 and 2), leading to cell growth (Table 4C) in the vector-transfected HSC-3 cells. Overexpression of the LARG-PDZ domain by transfecting HSC-3 cells with LARG PDZcDNA (i) impairs the association of CD44 (Fig. 7Ad, lanes 3 and 4) with endogenous LARG (Fig. 7Aa, lanes 3 and 4) and EGFR (Fig. 7Ab, lanes 3 and 4), (ii) inhibits LARG-RhoA signaling (Table 1C) and PLCε-mediated IP₃ production (Table 5) and Ca²⁺ mobilization (Table 5), (iii) abrogates CaMKII-mediated filamin phosphorylation (Table 5) and changes its binding to F-actin (Table 5), and (iv) induces a reversal of the transformed phenotypes (e.g. cell growth and migration) caused by EGFR kinase/Ras/PLCε activation (Tables 3C and Table 4C). All results confirm that LARG is responsible for the HA-mediated CD44 interaction with EGFR and downstream oncogenic signaling required for the concomitant stimulation of HNSCC cell migration and growth.

In addition, we have observed that transfection of HSC-3 cells with LARG PDZcDNA (in the absence of HA) causes a decrease in intracellular Ca²⁺ (Table 5). These results suggest that LARG-PDZ domain overexpression can influence the basal level of Ca²⁺ homeostasis. In the control samples (without HA treatment), there is a low level of LARG.

**TABLE 5**

**Effect of LARG-PDZ overexpression on HA-dependent HSC-3 cell signaling**

The procedures for measuring PLCε-mediated IP₃ production, intracellular Ca²⁺ mobilization, filamin phosphorylation by CaMKII, and filamin-F-actin binding using HSC-3 cells transfected with LARG PDZcDNA or vector alone are described under “Materials and Methods.” The values expressed in this table represent an average of triplicate determinations of 3–5 experiments with a S.D. less than ±5%.

| Cells                          | PLCε-mediated IP₃ production | Ca²⁺ mobilization | Filamin-F-actin binding |
|-------------------------------|-----------------------------|-------------------|--------------------------|
|                               | −HA | +HA | −HA | +HA | −HA | +HA | −HA | +HA |
| Vector-transfected cells (control) | 100 | 257 | 100 | 243 | 100 | 252 | 100 | 57  |
| LARG-PDZcDNA-transfected cells | 55  | 59  | 57  | 53  | 52  | 56  | 108 | 109 |
association with CD44 and EGFR (Fig. 5A, a, b, c, and d, lane 1). Although HA production in HSC-3 cells is low, we cannot preclude the possibility that a small amount of secreted endogenous HA is involved in the formation of CD44-LARG-EGFR complexes. Our recent findings indicate that HSC-3 cells express hyaluronan synthases (HAS1, HAS2, and HAS3), which are the major contributors to endogenous HA production. Therefore, we believe that the interpretation for these pre-existing LARG-CD44-EGFR complexes induced by a low level of endogenous HA is highly plausible.

DISCUSSION

CD44 denotes a family of glycoproteins that are often overexpressed in a variety of human solid neoplasms including head and neck cancers (10–14). HA, the major glycosaminoglycan found in the extracellular matrix of mammalian tissues, is now considered to be a physiologically relevant ligand for CD44 (also known as a hyaluronan receptor) in many cell types including head and neck tumor cells (7–9). Most of malignant solid tumors display high levels of HA (8, 79). HA has been suggested to play an important role in tumor angiogenesis and invasion. Specifically, the large HA polymers are thought to provide a hydrated micro-niche that facilitates the invasion of tumor cells into the extracellular matrix materials (8, 79), whereas the smaller HA fragments (large HA degraded by hyaluronidases) promote angiogenesis and tumor neovascularization (80–82). The invasive potential can be further enhanced by HA-CD44 interaction during tumor progression (10, 83, 84). Both HA and CD44 appear to be overexpressed at the sites of tumor attachment and are known to be involved in tumor cell-specific properties (e.g. tumor cell growth, migration, and invasion) (7, 8, 10–14). In this study we initially determined that CD44 is expressed in HNSCC cells (HSC-3 cells (Fig. 1)). We then address the question of which oncogenic pathways are directly involved in regulating HA-activated and CD44-specific HSC-3 cell behaviors.

The invasive phenotype of tumor cells, characterized by HA/CD44-mediated cell growth and migration, has been linked to cytoskeletal function, which involves the small GTP-binding proteins such as RhoA (35). The activities of members of the RhoGTPases (including RhoA) are often regulated by GEFs that contain a DH domain (42). In recent years a RhoA-specific GEF (LARG) molecule containing several functional domains (e.g. an N-terminal PDZ domain, RGS domain, a DH domain, and a pleckstrin homology (PH) domain) have been identified (42). In particular, the DH domain of LARG by itself exhibits GDP/GTP exchange activity for RhoA and plays an important role in RhoGTPase signaling (41–43). LARG can transduce signals from receptors coupled to the heterotrimeric G proteins, Go12 and Go13 to Rho (85, 86). LARG and its close homologs, p115-RhoGEF and PDZ-RhoGEF, all exhibit an RGS-like domain that is involved in a structural linkage with Go12/13 and may function as a GTPase-activating protein for Go subunits (87). LARG, unlike the related p115-RhoGEF and PDZ-RhoGEF, also serves as an effector for the Go-coupled receptor and mediates RhoA signaling (88). Thus, LARG appears to be selectively involved in certain signaling pathways from G-protein coupled receptors that are linked to different Go subunits. Furthermore, overexpression of LARG in acute myeloid leukemia has been shown to exhibit growth-promoting activity (41). Recent studies show that LARG and its transforming potential can be activated by FAK phosphorylation (89) and/or hetero-oligomerization through the LARG inhibitory C-terminal region (90). Apparently, LARG-RhoGEF activity can be regulated by many different mechanisms in addition to its previously described role in Go subunit signaling by G-protein-coupled receptors. In this study we have focused on the role of LARG (in particular, the PDZ domain) in regulating HA/CD44- and EGFR-mediated RhoA signaling and tumor cell behaviors.

Our results show that LARG is the major RhoGEF detected in the HSC-3 cells (Fig. 1). However, we cannot preclude the possibility that a small amount of other RhoGEFs may be present in these cells. We have also presented evidence for a close, physical interaction between LARG and the transmembrane glycoprotein, CD44, in HSC-3 cells (Fig. 1). Most importantly, the binding of HA to CD44 promotes LARG-catalyzed RhoA activation (Table 1). Therefore, it is likely that HA-CD44 interaction serves as an upstream activator of LARG function.

Furthermore, our results demonstrate that CD44-LARG interacts with EGFR (Fig. 5). HA treatment of HSC-3 cells causes a significant increase in the amount of EGFR recruited into the CD44-LARG signaling complex and stimulates EGFR tyrosine kinase activity (Fig. 5), leading to Ras/Raf-1/ERK2 activation (Fig. 5). HSC-3 cell growth, and migration (Tables 3 and 4). These observations strongly suggest that HA interaction with CD44-LARG complex causes the assembly of other signaling molecules (e.g. EGFR) into a large complex resulting in the activation of multiple signaling pathways involving both RhoA and Ras pathways during HNSCC cell functions.

Unique to LARG compared with other GEFs for small GTPases, is the presence of a PDZ domain that is known to interact with membrane proteins (43). In this study we have demonstrated that the LARG PDZ domain is responsible for the recognition of CD44 and EGFR (Fig. 6). To further assess the role of the LARG-PDZ domain in regulating CD44 and EGFR-mediated oncogenic signaling in human HNSCC cells (HSC-3 cells), we have transfected HSC-3 cells with LARG-PDZcDNA (Fig. 7) or vector alone. Our results indicate that transfection of HSC-3 cells with LARG-PDZcDNA (Fig. 7) effectively blocks endogenous LARG association with CD44 and EGFR (Fig. 7). These findings further support our conclusion that the LARG-PDZ domain acts as a potent competitive inhibitor that is capable of interfering with endogenous LARG interaction with CD44 and EGFR in HSC-3 cells. Moreover, we have observed that the LARG-mediated RhoA activation and EGFR kinase-mediated Ras/Raf/ERK2 signaling becomes significantly up-regulated in vector-transfected cells treated with HA as compared with no HA treatment (Fig. 7). In contrast, the failure of endogenous LARG to become associated with CD44 and EGFR in LARG-PDZcDNA-transfected cells (with or without HA treatment) greatly abolishes LARG-RhoA signaling and EGFR-associated activation events (e.g. EGFR kinase activity and Ras/Raf/ERK2 activation) (Fig. 7). Thus, these results provide strong evidence that LARG not only serves as a RhoA-specific GEF (via the DH domain) but also functions as a linker molecule (via the PDZ domain) to connect CD44 to EGFR leading to HA-mediated RhoA/Ras co-activation in HNSCC (HSC-3 cells). Preliminary data indicate that other members of the family of RhoGEFs (e.g. Lbc, Lfc, Net1, and Xpln) do not effectively substitute LARG-RhoA signaling during HA-mediated CD44-EGFR interaction and that the recombinant fragment of the LARG-RSG domain does not inhibit HA-mediated CD44 signaling or interfere with CD44-EGFR interaction. These observations strongly indicate the importance of LARG-PDZ in HA-mediated CD44-EGFR signaling in HSC-3 cells. These findings are consistent with a previous report showing that HA-mediated CD44 association with p185HER2 signaling complexes is also mediated by molecular scaffolds and adaptors such as Vav2 (a Rac-specific GEF) and Grb-2 (19). Specifically, endogenous Vav2 and Grb2 are associated with CD44 and p185HER2 in a signaling complex and that HA treatment induces recruitment of both Vav2 and Grb2 into CD44v3-p185HER2-containing mul-

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timolecular complexes leading to the co-activation of Rac1 and Ras signaling and ovarian tumor cell growth and migration (19). The binding of HA to CD44 increases intracellular Ca\(^{2+}\) levels in many cell types including HNSCC (HSC-3 cells) (Fig. 3). The mechanism by which HSC-3 cells respond to HA/CD44-induced Ca\(^{2+}\) signaling is not fully understood. Phosphoinositide-specific phospholipase Cs are known to generate 2-s messengers by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form diacylglycerol and IP\(_3\) (47). Diacylglycerol is an activator of certain protein kinase C isoforms, and IP\(_3\) interacts with intracellular membrane receptors (e.g. IP\(_3\) receptors) leading to an increased release of stored Ca\(^{2+}\) ions (47). During growth factor signaling, one of the PLCs, PLC\(_{cyl}\), binds to the tyrosine autophosphorylation sites of the receptor-linked tyrosine kinases, leading to tyrosine phosphorylation and stimulation of PLC\(_{cyl}\) activity (91). Because PLC\(_{cyl}\) tyrosine phosphorylation and enzymatic activity (measured by PLC\(_{cyl}\)-mediated IP\(_3\) production) do not correlate in certain cases (48), we decided to explore additional mechanisms of PLC activation during HA/CD44-mediated Ca\(^{2+}\) signaling. Small GTPases, including RhoA, are interesting candidates for oncogenesis because of their role in both Ca\(^{2+}\) signaling and cytoskeletal arrangement (16, 17, 45). A previous study has shown that CD44 interaction with RhoA-activated Rho kinase plays a pivotal role in IP\(_3\) receptor-mediated Ca\(^{2+}\) signaling and cytoskeletal function during HA-mediated endothelial cell migration (45). Most recently we have found that HA/CD44 association with Rac1-dependent protein kinase N\(_{\gamma}\) plays a pivotal role in PLC\(_{cyl}\)-regulated Ca\(^{2+}\) signaling and contactin-cytoskeleton function required for keratinocyte cell-cell adhesion and differentiation (21). Thus, RhoGTPase signaling appears to be closely associated with HA-activated intracellular Ca\(^{2+}\) mobilization, cytoskeleton activation, and a variety of other biological functions (16, 17, 21, 45).

A new member of PLC family, PLC\(_{c}\), contains several Ras interaction domains including one CDC25 domain and two RA-associated domains (92–94). The CDC25 at the C-terminal region of PLC\(_{c}\) functions as a GEF for H-Ras and/or Rap1, whereas two RA domains at the C-terminal region interact with H-Ras and several Rap isoforms (92–94). Co-expression of both constitutively active H-Ras and PLC\(_{c}\) results in an increase of IP\(_3\) production (95). Heterotrimeric G\(_{\alpha}\) subunits (e.g. G\(_{\alpha}12/13\) but not G\(_{\alpha}q\)) (93, 96) and G\(_{\beta}\)\(_{\gamma}\) subunits have also been shown to stimulate PLC\(_{c}\)-mediated IP\(_3\) production (96). PLC\(_{c}\) has recently been found to directly interact with RhoA (59, 60).

In an effort to identify possible new cellular targets for LARG-activated RhoA, we have focused on PLC\(_{c}\)-associated signaling event. Our results indicate that PLC\(_{c}\) is closely associated with the GTP-bound form of RhoA (but not the GDP-bound form of RhoA). Most importantly, GTP-bound RhoA (but not GDP-bound RhoA) up-regulates the PLC\(_{c}\) activity (measured by PLC\(_{c}\)-mediated IP\(_3\) production) (Fig. 2) required for Ca\(^{2+}\) mobilization. These observations are consistent with previous findings suggesting that small GTPases (e.g. RhoA) are important for PLC\(_{c}\) activation (59, 60). Furthermore, RhoA signaling (Table 1), PLC\(_{c}\)-mediated IP\(_3\) production (Fig. 2), and intracellular Ca\(^{2+}\) mobilization (Fig. 3) are all stimulated by HA and blocked by cells pretreated with anti-CD44 antibody during HA stimulation and C3 toxin treatment (Fig. 3). Transfection of HSC-3 cells with LARG-PDZ-cDNA effectively competes for endogenous LARG interaction with CD44 (Fig. 7), inhibits the ability of LARG to activate RhoA (Table 1), and blocks HA/CD44-mediated PLC\(_{c}\) activity (Table 5) and intracellular Ca\(^{2+}\) signaling (Table 5). These results indicate that LARG-PDZ domain is a potent inhibitor of RhoA/PLC\(_{c}\)-mediated IP\(_3\) production and Ca\(^{2+}\) signaling required for HA/CD44-mediated HNSCC function and suggests that LARG-mediated RhoA signaling and PLC\(_{c}\) activation are closely coupled with HA/CD44-mediated Ca\(^{2+}\) mobilization in HSC-3 cells. It is also noted that overexpression of LARG-PDZ domain interferes with the formation of these multimolecular signaling complexes (possibly induced by endogenous HA), resulting in an inhibition of Ca\(^{2+}\) mobilization. In addition, we have observed that both drug (U-73122/2-APB/KN93) and C-3 toxin treatments and transfection with the LARG-PDZcDNA in cells not treated with HA reduce cell migration/growth capability by 40–50% (Table 3 and 4). These observations suggest that these LARG-CD44/EGFR complexes (possibly induced by endogenous HA) and their downstream signaling events (e.g. RhoA/Ras activation, PLC/IP\(_3\) receptor-regulated Ca\(^{2+}\) mobilization, CaMKII-cytoskeleton interaction, and LARG-PDZ domain) must also be required for the basal level of HNSC-3 cell growth and migration.

Emerging evidence regarding the close connection between Ca\(^{2+}\) signaling and cytoskeletal activation in multiple cell systems leads us to investigate the possible cross-talk between these two signaling pathways during HA-mediated CD44 activation. In HSC-3 cells HA/CD44-mediated Ca\(^{2+}\) signals are mediated by CaMKII, which is capable of phosphorylating a variety of cytoskeletal proteins including filamin (61–64). Filamin is known to be an actin binding protein (63, 64) and also serves...
as a cellular substrate for CaMKII in HSC-3 cells (Fig. 4). Serine/threonine phosphorylation of filamin by CaMKII is known to alter its ability to interact with filamentous actin (Fig. 4). In this study, we have shown that filamin phosphorylation by the CaMKII (isolated from cells treated with HA) significantly down-regulates its ability to cross-link filamentous actin (Fig. 4).

In contrast, the reduction of filamin phosphorylation (in HSC-3 cells treated with various drugs (e.g. a RhoA inhibitor, C-3 toxin, or a PLC inhibitor, U73122, or a CaMK II inhibitor, KN93) or transfected with LARG-PDZcDNA with or without HA treatment) is sufficient to promote cross-linking of actin filaments into bundles (Fig. 4 and Table 5). These results agree with previous findings, suggesting that filamin plays an important role in regulating F-actin-based cytoskeleton function.

HSC-3 cells treated with various inhibitors (e.g. a RhoA inhibitor, C-3 toxin, or a PLC inhibitor, U73122, or a CaMKII inhibitor, KN93, or EGFR inhibitor, AG1478, or MAPK (ERK1/2) inhibitor, U0126) or transfected with LARG-PDZcDNA displayed marked inhibition of HA/CD44-induced HNSCC cell migration (Table 3) and growth (Table 4). These data also strongly suggest that CD44-LARG-mediated RhoA/Ras co-activation is required not only for PLC-regulated Ca²⁺ signaling and CaMKII-regulated cytoskeleton function but also for the Raf-1 and ERK1/ERK2 signaling required for HA-dependent cell migration and growth in HSC-3 cells.

Table 1A indicates that CD44 represents at least 50–52% of total HA binding sites on the HSC-3 cell surface based on the 125I-labeled FITC-HA binding of cells treated with a CD44-specific blocking antibody. However, CD44 appears to play a major role in HA-mediated signaling and function as evidenced by the significant inhibition of HA-mediated activation of HSC-3 cells treated with anti-CD44 antibody (Figs. 1, 3, 4, and 5; Tables 1B, 3A, and 4A). The expression of MMPs by malignant and stromal cells has been studied in squamous cell carcinomas (97). CD44 often associates with MMPs and promotes MMP activity and tumor invasion (98, 99). Preliminary data indicate that MMP-2 is a RhoA inhibitor, C-3 toxin, or a PLC inhibitor, U73122, or a CaMKII inhibitor, KN93) or transfected with LARG-PDZcDNA with or without HA treatment) is sufficient to promote cross-linking of actin filaments into bundles (Fig. 4 and Table 5).

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