In this study we have explored the interaction between CD44 (the hyaluronic acid (HA)-binding receptor) and Tiam1 (a guanine nucleotide exchange factor) in metastatic breast tumor cells (SP1 cell line). Immuno-precipitation and immunoblot analyses indicate that both the CD44v3 isoform and the Tiam1 protein are expressed in SP1 cells and that these two proteins are physically associated as a complex in vivo. Using an Escherichia coli-derived calmodulin-binding peptide-tagged Tiam1 fragment (i.e. the NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain designated as PHn-CC-Ex, amino acids 393–738 of Tiam1) and an in vitro binding assay, we have detected a specific binding interaction between the Tiam1 PHn-CC-Ex domain and CD44. Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in the PHn-CC-Ex domain of Tiam1 with an apparent dissociation constant (Kd) of 0.2 nm, which is comparable with CD44 binding (Kd = 0.13 nm) to intact Tiam1. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1-binding region for CD44. Most importantly, the binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration. Transfection of SP1 cells with Tiam1 cDNA promotes Tiam1 association with CD44v3 and up-regulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with PHn-CC-Ex cDNA and Tiam1 cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors. Taken together, we believe that the linkage between CD44v3 isoform and the PHn-CC-Ex domain of Tiam1 is required for HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration during breast cancer progression.

The transmembrane glycoprotein CD44 isoforms are all major hyaluronic acid (HA) cell surface receptors that exist on many cell types, including macrophages, lymphocytes, fibroblasts, and epithelial cells (1–6). Because of their widespread occurrence and their role in signal transduction, CD44 isoforms have been implicated in the regulation of cell growth and activation as well as cell-cell and cell-extracellular matrix interactions (1–7). One of the distinct features of CD44 isoforms is the enormous heterogeneity in the molecular masses of these proteins. It is now known that all CD44 isoforms are encoded by a single gene that contains 19 exons (8). Of the 19 exons, 12 exons can be alternatively spliced (8). Most often, the alternative splicing occurs between exons 5 and 15, leading to an insertion in tandem of one or more variant exons (v1–v10 (exon 6-exon 14) in human cells) within the membrane-proximal region of the extracellular domain (8). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations and glycosaminoglycan additions (9–12). In particular, CD44v3-containing isoforms have a heparin sulfate addition at the membrane-proximal extracellular domain of the molecule that confers the ability to bind heparin sulfate-binding growth factors (9, 10). Cell surface expression of CD44v isoforms changes profoundly during tumor metastasis, particularly during the progression of various carcinomas including breast carcinomas (13–17). In fact, CD44v isoform expression has been used as an indicator of metastasis.

It has been shown that interaction between the cytoskeletal protein, ankyrin, and the cytoplasmic domain of CD44 isoforms plays an important role in CD44 isoform-mediated oncoenic signaling (6, 18, 19). Specifically, the ankyrin-binding domain (e.g. NGNGNTVEDRKPSEL between amino acids 306 and 320 in the mouse CD44 (20) and NGSGNGAVEDRKPSSL amino acids 304 and 318 in human CD44 (21)) is required for the recruitment of Src kinase and the onset of tumor cell transformation (21). Furthermore, HA binding to CD44 stimulates a concomitant activation of p185^{HER2} linked tyrosine kinase (linked to CD44s via a disulfide linkage) and results in a direct cross-talk between two different signaling pathways (e.g. proliferation versus motility/invasion) (22). In tumor cells, the transmembrane linkage between CD44 isoform and the cytoskeleton promotes invasive and metastatic-specific tumor phenotypes (e.g. matrix degradation (matrix metalloproteinases) activities (23, 24), “invadopodia” formation (membrane projections), tumor cell invasion, and migration) (23). These findings strongly suggest that the interaction between CD44 isoform and the cytoskeleton plays a pivotal role in the onset of oncogenesis and tumor progression.

The Rho family proteins (e.g. Rho, Rac, and Cdc42) are members of the Ras superfamily of GTP-binding proteins structurally related to but functionally distinct from Ras itself (25, 26). They are associated with changes in the membrane-linked cytoskeleton (26). For example, activation of RhoA, Rac1, and Cdc42 have been shown to produce specific structural changes in the plasma membrane-cytoskeleton reorganization leading
to membrane ruffling, lamellipodia, filopodia, and stress fiber formation (26). The coordinated activation of these GTPases is considered to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (27–29). In particular, Rac1 activation is known to initiate oncogenic signaling pathways that promote cell shape changes (33, 34), influence actin cytoskeleton organization (33, 34), and stimulate gene expression (35–37). The question of whether Rac1 activation is also involved in CD44v3-related cytoskeleton function that results in the metastatic phenotypes (e.g., tumor cell migration) of breast tumor cells remains to be answered.

Tiam1 (T lymphoma invasion and metastasis 1) has been identified as an oncogene because of its ability to activate Rho-like GTPases during malignant transformation (38, 39). Specifically, Tiam1 is capable of activating Rac1 in vitro as a guanine nucleotide exchange factor and inducing membrane cytoskeleton-mediated cell shape changes, cell adhesion, and cell motility (34, 40–42). It also acts as a Rac-specific guanine nucleotide exchange factor in vivo and induces an invasive phenotype in lymphoma cells (40). These findings have prompted several research groups to investigate the mechanisms involved in the regulation of Tiam1. For example, addition of certain serum-derived lipids (e.g., sphingosine-1-phosphate and lysophosphatidic acid) to T lymphoma cells promotes Tiam1-mediated Rac1 and Cdc42 signaling and T lymphoma cell invasion (43). Tiam1 has also been found to be phosphorylated by protein kinase C in Swiss 3T3 fibroblasts stimulated by lysophosphatidic acid (44) and platelet-derived growth factor (45). Most recently, Exton and co-workers (46) demonstrate that phosphorylation of Tiam1 by Ca2+/calmodulin-dependent protein kinase II (but not protein kinase C) regulates Tiam1-catalyzed GDP/GTP exchange activity in vitro. These findings support the notion that posttranslational modifications of Tiam1 by certain serine/threonine kinase(s) during surface receptor-mediated activation may play an important role in Tiam1-Rac1 signaling. Tiam1 transcript has been detected in breast cancer cells (39). However, it is not known at the present time whether there is any structural and functional relationship(s) between Tiam1-Rac1 signaling and CD44v3-mediated invasive and metastatic processes of breast cancer cells.

In this paper, using a variety of biochemical, molecular biological, and immunocytochemical techniques, we have found that the cell adhesion molecule, CD44v3 isoform, which binds directly to HA, is closely associated with Tiam1 (in particular, the NH2-terminal pleckstrin homology (PHn), a putative coiled coil region (CC), and an additional adjacent region (Ex), designated as PHn-CC-Ex domain of Tiam1) in SP1 breast tumor cells. Most importantly, HA binding to CD44v3 isoform stimulates Tiam1-specific GDP/GTP exchange for Rho-like GTPases such as Rac1 and promotes cytoskeleton-mediated tumor cell migration. These findings suggest that a transmembrane interaction between CD44v3 and Tiam1 plays an important role in promoting oncogenic signaling and tumor cell-specific phenotypes required for HA-mediated breast tumor cell migration.

MATERIALS AND METHODS

Cell Culture—Mouse breast tumor cells (e.g., SP1 cell line) (provided by Dr. Bruce Elliott, Department of Pathology, and Biochemistry, Queen’s University, Kingston, ON, Canada) were used in this study. Specifically, SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen’s University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (47). These cells were cultured in RPMI 1640 medium supplemented with 5–7% fetal calf serum, folic acid (290 mg/ml), and sodium pyruvate (100 mg/liter). COS-7 cells were obtained from American Type Culture Collection and grown routinely in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin.

Antibodies and Reagents—for the preparation of polyclonal rabbit anti-Tiam1 antibody or rabbit anti-CD44v3 antibody, specific synthetic peptides (15–17 amino acids unique for the COOH-terminal sequence of Tiam1 or the CD44v3 sequence) were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using conventional solid-phase synthesis or automated synthesizers, respectively. These Tiam1-related or CD44v3-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies, respectively. The anti-Tiam1-specific or anti-CD44v3-specific antibody was collected from each bleed and stored at 4°C containing 0.1% azide. The anti-Tiam1 IgG or anti-CD44v3 IgG fraction was prepared by conventional protein chromatography, respectively. Mouse monoclonal anti-HA (hemagglutinin epitope) antibody (clone 12 CA5) was purchased from Roche Molecular Biochemicals. Mouse monoclonal anti-green fluorescent protein (GFP) was purchased from Pharmingen.

Escherichia coli-derived GST-tagged Rac1 was kindly provided by Dr. Richard A. Cerione (Cornell University, Ithaca, NY).

Cell Surface Labeling Procedures—SP1 cells suspended in PBS were surface labeled using the following biotinylation procedure. Briefly, cells (105 cells/ml) were incubated with sulfo-succinimidyl-4-biotinamido-hexanoate (Pierce) (0.1 mg/ml) in labeling buffer (150 mM NaCl, 0.1% HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44v3-mediated immunoprecipitation as described previously (23). The biotinylated material precipitated by anti-CD44v3 antibody were analyzed by SDS-polyacrylamide gel electrophoresis, transferred to the nitrocellulose filters, and incubated with ExtrAvidin-peroxidase (Sigma). After an addition of peroxidase substrate (Pierce), the blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Immunoprecipitation and Immunoblotting Techniques—SP1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rabbit anti-CD44v3 antibody or rabbit anti-Tiam1 antibody followed by goat anti-rabbit IgG, respectively. The immunoprecipitated material was solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-Tiam1 antibody (5 μg/ml) or rabbit anti-CD44v3 antibody (5 μg/ml), respectively, for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) or goat anti-mouse 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.

In some experiments, SP1 cells or COS cells (e.g., untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1cDNA (FL1591) or HA-tagged NH2-terminally truncated C1199 Tiam1cDNA or GFP-tagged PHn-CC-ExcDNA or C1199Taim1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector only) were immunoblotted with mouse anti-HA (hemagglutinin epitope) antibody (clone 12 CA5) or rabbit anti-CD44v3 antibody (5 μg/ml), respectively, for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-mouse 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.

Cloning, Expression, and Purification of CD44 Cytoplasmic Domain (CD44cyt) from E. coli—The procedure for preparing the fusion protein of the cytoplasmic domain of CD44 was the same as described previously (48). Specifically, the cytoplasmic domain of human CD44 (CD44cyt) was cloned into pFLAG-AST using the PCR-based cloning strategy. Using human CD44 cDNA as template, one PCR primer pair (left, FLAG-EcoRI; right, FLAG-XhoI) was designed to amplify complete CD44 cytoplasmic domain. The amplified DNA fragments were one-step cloned into a pCR2.1 vector and sequenced. Then the DNA fragments were cut out by double digestion with EcoRI and XhoI and subcloned into EcoRI/XhoI double-digested pFLAG-AST (Eastman Kodak Co., Rochester, NY) to generate FLAG-pCD44cyt construct. The nucleotide sequence of FLAG/CD44cyt junction was confirmed by sequencing. The recombinant plasmids were transfected into Cos7/B12I-DE3 to produce FLAG-CD44cyt fusion protein. The FLAG-CD44cyt fusion protein was further purified by anti-FLAG M2 affinity gel column (Eastman Kodak Co.). The nucleotide sequence of primers used in this cloning protocol are: FLAG-EcoRI, 5′-GAGAGGTGCTGATGGGAAAGAGCTGATCTTTAAGGC-3′, and FLAG-XhoI, 5′-AGCTCTAGATTTACCCCAATCCCATC-3′.

Expression Constructs—Both the full-length mouse Tiam1cDNA.
(FL1591) and the NH2-terminally truncated Tiam1cDNA (C1199) were kindly provided by Dr. John G. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Specifically, the full-length Tiam1 (FL1591) cDNA was cloned into the eukaryotic expression vector, pMT2SM. The NH2-terminally truncated C1199 Tiam1 cDNA (carrying a HA epitope tag at the 3′-end fragment of Tiam1) was subcloned into the eukaryotic expression vector, pUTS51 (Eurongectec, Belgium). The Tiam1 fragment, Pbn-CC-Ex domain was cloned into calmodulin-binding peptide (CBP)-tagged vector (pCAL-n) (Stratagen) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, Pbn-CC-Ex domain was amplified by PCR with two specific primers (left, 5′-AAGACCGAGAT- CTTAGCAACACGTGGA-3′; and right, 5′-CATCTGGAACTAGTCTAC-3′) linked with specific enzyme digestion site (XhoI or HindIII). PCR product digested with XhoI and HindIII was purified with QIAquick PCR Purification Kit (Qiagen). The Pbn-CC-Ex domain cDNA fragment was cloned into pCAL-n vector digested with XhoI and HindIII. The inserted Pbn-CC-Ex domain sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed into E. coli strain BL21-DE3 to produce CBP-tagged Pbn-CC-Ex fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma).

The Pbn-CC-Ex domain cDNA fragment was also cloned into pEGFPN1 vector (CLONTECH) digested with XhoI and HindIII to create GFP-tagged Pbn-CC-Ex cDNA. The inserted Pbn-CC-Ex domain sequence was amplified by nucleotide sequencing analyses. The recombinant plasmids were then used for transient expression in SP1 cells as described below. The molecular mass of the GFP-tagged Pbn-CC-Ex is expressed as 68 kDa in SP1 or COS-7 cells by SDS-polyacrylamide gel electrophoresis and immunoblot analyses.

Cell Transfection—To establish a transient expression system, SP1 cells (or COS-7 cells) were transfected with various plasmid DNAs (e.g. HA-tagged C1199 Tiam1cDNA, GFP-tagged Pbn-CC-ExDNA, or HA-tagged C1199Tiam1cDNA plus GFP-tagged Pbn-CC-ExDNA) or transfected with either the full-length Tiam1 cDNA or NH2-terminally truncated Tiam1cDNA plus GFP-tagged Pbn-CC-ExDNA (co-transfection) or vector alone using electroporation methods according to the procedures described previously (74). Briefly, SP1 cells were plated at a density of 2 × 104 cells/100-mm dish and transfected with 25 μg/dish plasmid cDNA using electroporation at 230 V and 960 microfarad with a Gene Pulser (Bio-Rad). Transfected cells were grown in the culture medium at least for 24–48 h. Various transfectedants were then analyzed for their protein expression (e.g. Tiam1-related proteins) by immunoblot, GFP/TPX reaction on Rac1, and tumor cell migration assays as described below.

In Vitro Binding of Tiam1/Tiam1 Fragment to CD44—Aliquots (0.5–1 ng of protein) of purified FLG-CD44cet fusion protein bound to Anti-FLG MoAb 52 as study immunoblot, transfected with either the full-length Tiam1cDNA, GFP-tagged Pbn-CC-ExDNA, or HA-tagged C1199Tiam1cDNA plus GFP-tagged Pbn-CC-ExDNA (co-transfection) or vector alone) were washed extensively in binding buffer, and the bead-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding, which was approximately 20% during the incubation of cells on HA-coated wells, was subtracted.

Double Immunofluorescence Staining—SP1 cells (transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1cDNA, GFP-tagged Pbn-CC-ExDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged Pbn-CC-ExDNA as co-transfection) or vector alone) were first washed with PBS buffer (0.1 M phosphate buffer (pH 7.5) and 150 mM NaCl) and fixed by 2% paraformaldehyde. Subsequently, SP1 transfectedants were stained with rhodamine (Rh)-labeled rabbit anti-CD44v3 antibody. In some cases, Rh-labeled cells were then rendered permeable by ethanol treatment followed by incubating with FITC-conjugated mouse anti-HA IgG. To detect nonspecific antibody binding, RH-CD44v3-labeled cells were incubated with FITC-conjugated normal mouse IgG. No labeling was observed in such control samples. The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProtocol 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Cell Adhesion Assay—SP1 cells were metabolically labeled with TranS32 label (20 μCi/ml) as described above. After labeling, the cells were washed in PBS and incubated in PBS containing 5 μl of 32P at 37 °C to obtain a nonadherent single cell suspension. Labeled cells (∼9.1 × 103 cpm/104 cells) (in the presence or absence of anti-CD44v3 antibody) were plated on the HA-coated plates at 4 °C for 30 min. Subsequently, transfected or nontransfected cells were washed three times in PBS, the adherent cells were solubilized in PBS containing 1% SDS, and the well-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by including 300 μg/ml soluble HA during the incubation of cells on HA-coated wells. The nonspecific binding was 10–15% of the total well-associated radioactivity and has been subtracted.

Cell Migration Assays—Twenty-four transwell units were used for monitoring in vitro cell migration as described previously (23). Specifically, the 5-μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SP1 cells (∼1 × 104 cells/well in PBS) were placed in the upper chamber of the transwell unit. In some cases, cells were transfected with either C1199Tiam1cDNA, Pbn-CC-ExDNA, C1199Tiam1cDNA plus Pbn-CC-ExDNA, or vector alone. The growth medium containing high glucose Dulbecco’s modified Eagle’s medium 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, the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Promega) was added at a final concentration of 0.2 mg/ml to both the upper and the lower chambers and incubated for an additional 4 h at 37 °C. 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 migrating to the lower side of the polycarbonate filter with standard cell counting methods as described previously (23, 49). 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-CD44v3 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 the Student's $t$ test, and statistical significance was set at $p < 0.01$.

RESULTS

Identification of CD44 Variant Isoform(s) as HA Receptor(s) in SP1 Cells—The expression of CD44 variant isoforms such as CD44v3 is known to be closely correlated with metastatic and proliferative behavior of a variety of tumor cells including various carcinomas such as human breast tumor cells (14–19). Immunoblotting with anti-CD44v3 antibody (recognizing the v3-specific sequence located at the membrane-proximal region of the extracellular domain of CD44) indicates that a single CD44v3 protein (molecular mass $= 260$ kDa) is expressed in SP1 cells (Fig. 1, lane 1). Furthermore, we have utilized surface biotinylation techniques and anti-CD44v3-mediated immunoprecipitation to characterize this CD44v3 molecule. Our results show that the 260-kDa CD44v3 molecule can be surface-biotinylated and is located on the surface of SP1 cells (Fig. 1, lane 3). No CD44v3-containing material is observed in control samples when preimmune rabbit serum is used in either immunoblot (Fig. 1, lane 2) or immunoprecipitation experiments (Fig. 1, lane 4). Further analyses using reverse transcriptase-PCR, cloning, and nucleotide sequence techniques indicate that this CD44v3 belongs to the CD44v3,8–10 isoform in SP1 cells (data not shown). This CD44v3,8–10 variant exon structure was previously identified in human breast carcinoma samples (14–19), and its molecular mass (expressed at the protein level) has been shown to $= 260$ kDa (9).

CD44 is the major hyaluronan cell surface receptor (50), and a cellular adhesion molecule in many different cell types (51). Specific HA-binding motifs have been identified and localized in the extracellular domain of all CD44 isoforms (52, 53). To determine whether HA promotes cell adhesion, breast tumor cells (SP1 cell line) were incubated with plastic dishes coated with HA. As shown in Table I, SP1 cells adhere to the HA-coated dishes very well. In addition, because preincubation with anti-CD44v3 antibody blocks the adhesion of SP1 cells to HA-coated dishes, these data clearly indicate that CD44v3 isoform involves a specific binding interaction with the extracellular matrix component such as HA and is a cell surface adhesion molecule in SP1 cells.

Analysis of a Complex Formed between CD44v3 and Tiam1 in SP1 Cells in Vivo—Both CD44v4 isoforms (14–19) and Tiam1 (39) have been detected in a variety of tumor cells. In this study we have addressed the question of whether there is an interaction between CD44v3 isoform and Tiam1 in breast tumor cells (e.g. SP1 cells). First, we have analyzed Tiam1 expression (at the protein level) in breast tumor cells such as SP-1 cell line. Immunoblot analysis, utilizing anti-Tiam1 antibody designed to recognize the specific epitope located at the COOH-terminal sequence of Tiam1 reveals a single polypeptide (molecular mass $= 200$ kDa) (Fig. 2, lane 2). We have demonstrated that Tiam1 detected in SP1 cells revealed by anti-Tiam1-mediated immunoblot is specific because no protein is detected in these cells using preimmune rabbit IgG (Fig. 2, lane 1). Furthermore, we have carried out anti-CD44v3-mediated and anti-Tiam1-mediated precipitation followed by anti-Tiam1 immunoblot (Fig. 2, lane 3) or anti-anti-CD44v3 immunoblot (Fig. 2, lane 4), respectively, using SDS-polyacrylamide gel electrophoresis analyses. Our results clearly indicate that the Tiam1 band is revealed in anti-CD44v3-immunoprecipitated materials (Fig. 2, lane 3). The CD44v3 band can also be detected in the anti-Tiam1-immunoprecipitated materials (Fig. 2, lane 4). These findings clearly establish the fact that CD44v3 and Tiam1 are closely associated with each other in vivo in breast tumor cells.

In Vitro Binding Between Tiam1 (or PHn-CC-Ex Domain) and CD44—Previous studies indicate that Tiam1 membrane localization (through its NH$_2$-terminal PHn domain and an adjacent protein interaction domain (designated as PHn-CC-Ex, a sequence between amino acids 393–738 of Tiam1)) (Fig. 3, A and C) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and c-Jun NH$_2$-terminal kinase activation (37, 54). To test whether CD44 is one of the membrane proteins involved in the direct binding to Tiam1, we have used purified CBP-tagged PHn-CC-Ex fusion protein (Figs. 3C and 4, lane 1) and the FLAG-tagged cytoplasmic domain of CD44 (FLAG-CD44cyt) fusion protein (Fig. 4, lane 2) to identify the CD44 binding site on the Tiam1 molecule. Specifically, we have tested the binding of FLAG-CD44cyt to $^{125}$I-labeled CBP-PHn-CC-Ex (or $^{125}$I-labeled intact Tiam1 under equilibrium binding conditions. Scatchard plot analyses presented in Fig. 5 indicate that PHn-CC-Ex binds to the cytoplasmic domain of CD44 (CD44cyt) at a single site (Fig. 5A) with high affinity (an apparent dissociation constant ($K_d$) of $\approx 0.2$ nM). This interaction between PHn-CC-Ex and CD44 is comparable in affinity with CD44 binding ($K_d = 0.13$ nM) to intact Tiam1 (Fig. 5B). These findings clearly indicate that Tiam1 (in particular, PHn-CC-Ex domain) contains the CD44 binding site.

Further analyses using an in vitro binding assay show that surface biotinylated CD44v3 (isolated from SP1) specifically binds to Tiam1 (including intact Tiam1 (Fig. 6, lane 1), HA-tagged C1199 Tiam1 (Fig. 6, lane 2) or Tiam1 fragment (PHn-CC-Ex) (Fig. 6, lane 3)-coated beads. In the presence of an excess amount ($\approx 100$-fold) of recombinant PHn-CC-Ex Tiam1 fragment, the binding interaction between CD44v3 and these-Tiam1-related proteins is readily abolished (Fig. 6, lanes 4–6). These observations suggest that (i) the breast tumor cell-spe-
specific CD44v3 is also capable of interacting with Tiam1 (e.g. intact Tiam1 (Fig. 6, lane 1), HA-tagged C1199 Tiam1 (Fig. 6, lane 2), or Tiam1 fragment (PHn-CC-Ex) (Fig. 6, lane 3)); and (ii) the Tiam1 fragment such as PHn-CC-Ex acts as a potent competitive inhibitor for Tiam1 binding to CD44v3 in vitro (Fig. 6, lanes 4–6).

**Tiam1-catalyzed Rac1 Activation in SP1 Cells**—Rac1 GTPase becomes activated when bound GDP is exchanged for GTP by a process catalyzed by guanine nucleotide (GDP-GTP) exchange factors or GDP dissociation stimulator proteins (i.e. promoting GTP binding to RhoA by facilitating the release of GDP) (25,
CD44-Tiam1 in Rac1 Signaling and HA-mediated Breast Cancer

Tiam1 is known to function as an exchange factor for the Rho-like GTPases such as Rac1 (34, 40–42). To investigate whether Tiam1 complexed with CD44v3 acts as a GDP/GTP exchange factor (or a GDP dissociation stimulator protein) for E. coli-derived GST-Rac1, we have isolated Tiam1 complexed with CD44v3 from SP1 cells using anti-Tiam1-conjugated Sepharose beads. Our data show that Tiam1 complexed with CD44v3 from SP1 cells causes the exchange of preloaded GDP for [35S]GTP-S on GST-Rac1 in a time-dependent manner (Fig. 7, lines a and b). Most importantly, addition of HA to CD44v3 containing SP1 cells stimulates the total amount of bound [35S]GTP-S to GST-Rac1 (Fig. 7, line b) (at least 1.5-fold increase) as compared with Tiam1 isolated from untreated SP1 cells (Fig. 7, line b) or HA-treated SP1 cells in the presence of anti-CD44v3 antibody (data not shown). No [35S]GTP-S-bound material was detected in these samples containing GST alone under the same GDP/GTP exchange reaction using Tiam1 isolated from SP1 cells (in the presence of Fig. 7, line c) or absence (Fig. 7, line d) of HA treatment. These findings suggest that the HA interaction with CD44v3 isoform-containing SP1 cells promotes Tiam1 activation of Rac1.

CD44v3-Tiam1 Interaction in Rac1 Signaling and Cytoskeleton-mediated Tumor Cell Migration—Previous studies indicate that the invasive phenotype of tumor cells characterized by an invadopodia structure (or membranous projections) (56, 57) and tumor cell migration (28, 29) is closely associated with CD44v3- and Rac1-linked cytoskeleton function (23). In this study we have transiently transfected breast tumor cells (e.g. SP1 cells) with HA-tagged NH2-terminally truncated C1199 Tiam1 cDNA (Fig. 3B). Our results show that the C1199 Tiam1 is expressed as a 150-kDa protein (Fig. 8A, lane 1) detected by anti-HA-mediated immunoblot in CD44v3-positive breast tumor cells (SP1 cells). No protein band was detected in vector-transfected SP1 cells by anti-HA-mediated immunoblotting (Fig. 8A, lane 3). Using anti-CD44v3 immunoprecipitation of SP1 cellular protein followed by immunoblotting with anti-HA antibody, we have found that the 150-kDa C1199 Tiam1 is co-precipitated with CD44v3 (Fig. 8A, lane 2). In control samples, immunoblotting of rabbit preimmune IgG-purified material using anti-HA antibody does not reveal any protein associated with this material (Fig. 8A, lane 4). Double immunofluorescence staining data also confirms the close association between CD44v3 (Fig. 9A) and the C1199 Tiam1 (Fig. 9B) in the plasma membranes and long membrane projections. In contrast, vector-transfected cells expressing CD44v3 on the surface (Fig. 9A, inset a) (with no detectable C1199 Tiam1 by anti-HA label (Fig. 9, inset b) fail to display long membrane projections. Furthermore, we have demonstrated that transfection of SP1 cells with C1199 Tiam1 cDNA stimulates CD44v3-associated Tiam1-catalyzed GDP/GTP exchange on Rac1 (Table II) and induces a significant amount of increase in CD44v3-specific and HA-mediated breast tumor cell migration (Table II) compared with vector-transfected SP1 transfectants (Table II). These results are consistent with previous findings indicating that transfection of NIH3T3 cells with the NH2-terminally truncated C1199 Tiam1 cDNA confers potent oncogenic properties (42). Treatment of SP1 cells (e.g. untransfected cells or transfected cells) with certain agents (e.g. cytochalasin D (a microfilament inhibitor)) causes a remarkable inhibition of CD44v3/HA-specific tumor cell migration (Table II). These observations suggest that CD44v3-associated Tiam1 signaling and cytoskeleton-mediated tumor cell motility are closely coupled.

Moreover, we have found that SP1 cells transfected with GFP-tagged PHn-CC-Ex Tiam1 cDNA express a 68-kDa pro-
**CD44-Tiam1 in Rac1 Signaling and HA-mediated Breast Cancer**

![Fig. 9. Double immunofluorescence staining of CD44v3 and Tiam1 cDNA (e.g. C1199 Tiam1 cDNA or PHn-CC-Ex cDNA)-transfected SP1 cells. SP1 cells (transfected with HA-tagged C1199 Tiam1 cDNA or GFP-tagged PHn-CC-Ex cDNA) were fixed by 2% paraformaldehyde. Subsequently, cells were surface labeled with Rh-labeled rabbit anti-CD44v3 antibody. Some cells were then rendered permeable by ethanol treatment and stained with FITC-labeled mouse anti-HA IgG. A and B, Rh-labeled anti-CD44v3 staining (A) and FITC-anti-HA-labeled C1199 Tiam1 staining (B) in HA-tagged C1199 Tiam1 cDNA transfected SP1 cells. Insets a and b, Rh-labeled anti-CD44v3 staining (a) and FITC-anti-HA staining (b) in vector-transfected SP1 cells. C and D, Rh-labeled anti-CD44v3 staining (C) and GFP-tagged PHn-CC-Ex domain (D) in GFP-tagged PHn-CC-Ex cDNA transfected SP1 cells. Insets c and d, Rh-labeled preimmune IgG staining (c) and GFP-tagged PHn-CC-Ex domain (d) in GFP-tagged PHn-CC-Ex cDNA transfected SP1 cells. E and F, Rh-labeled anti-HA staining of C1199 Tiam1 (E) and GFP-tagged PHn-CC-Ex domain (F) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged PHn-CC-Ex cDNA. Insets e and f, Rh-labeled anti-CD44v3 staining (e) and GFP-tagged PHn-CC-Ex domain (f) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged PHn-CC-Ex cDNA.](Image 130x486 to 474x729)

protein as detected by anti-GFP antibody (Fig. 8B, lane 1). In vector-transfected SP1 cells, we are not able to detect any protein band by anti-GFP-mediated immunoblotting (Fig. 8B, lane 3). Using anti-CD44v3 immunoprecipitation of SP1 cellular protein followed by immunoblotting with anti-GFP antibody, we have found that the 68-kDa PHn-CC-Ex Tiam1 fragment is co-precipitated with CD44v3 (Fig. 8B, lane 2). No protein band was found when immunoblotting of rabbit preimmune IgG-precipitated materials with anti-GFP antibody was used (Fig. 8B, lane 4). It is also noted that both GFP-tagged PHn-CC-Ex domain (Fig. 9D) and CD44v3 are co-localized in the plasma membranes (Fig. 9C). However, no significant stimulation of long membrane projection was observed in these cells (Fig. 9, C, D, and insets c and d). Furthermore, we have demonstrated that CD44v3 staining detected in these SP1 transfectants revealed by anti-CD44v3-mediated immunostaining is specific because no surface label (Fig. 9, inset c) is detected in these GFP-PHn-CC-Ex overexpressed cells (Fig. 9, inset d) using preimmune rabbit IgG (Fig. 9, inset c). Additionally, we have demonstrated that overexpression of GFP-tagged PHn-CC-Ex domain in SP1 transfectants does not cause any significant changes of breast tumor cell properties (e.g. CD44v3-associated Tiam1-Rac1 signaling or HA-mediated tumor cell migration (Table II))

Finally, we have conducted co-transfection of SP1 cells with HA tagged C1199 Tiam1 cDNA and GFP-tagged PHn-CC-Ex cDNA. Our results indicate that C1199 Tiam1 and PHn-CC-Ex Tiam1 fragment are co-expressed as a 150-kDa protein (Fig. 8C, row a, lane 1) and a 68-kDa protein (Fig. 8C, row b, lane 1), respectively, in SP1 cells. No protein band was revealed in vector-transfected SP1 cells by anti-HA (Fig. 8C, row a, lane 3) or anti-GFP-mediated (Fig. 8C, row b, lane 3) immunoblotting. Using anti-C44v3 antibody immunoprecipitation of SP1 cell lysate followed by immunoblotting with anti-GFP antibody and anti-HA, respectively, we have found that the 68-kDa PHn-CC-Ex Tiam1 fragment (Fig. 8C, row b, lane 2) (but not 150-kDa C1199 Tiam1 (Fig. 8C, row a, lane 2)) is co-precipitated with CD44v3. In control samples, immunoblotting of rabbit preimmune IgG-precipitated material using anti-HA antibody (Fig. 8C, row a, lane 4) or anti-GFP antibody (Fig. 8C, row b, lane 4) does not reveal any protein associated with this material. Immunocytochemical staining results confirm that the PHn-CC-Ex Tiam1 fragment (Fig. 9, inset c) is co-localized with CD44v3 (Fig. 9, inset f) in the plasma membranes of SP1 transfectants. In contrast, the C1199 Tiam1 (Fig. 9E) fails to display plasma membrane localization as the PHn-CC-Ex domain does (Fig. 9F). Co-expression of PHn-CC-Ex domain and C1199 Tiam1 also efficiently blocks CD44v3-associated Tiam1-Rac1 activation and CD44v3-dependent and HA-mediated breast tumor cell migration (Table II). These results are consistent with a previous report showing that co-transfection of COS-7 cells with PHn-CC-Ex cDNA and C1199 Tiam1 cDNA results in an inhibition of C1199 Tiam1-induced Rac1 signaling and membrane ruffling (54). These findings suggest that the NH2-terminal PHn domain and an adjacent protein interaction domain (PHn-CC-Ex) play an important role in regulating Tiam1 localization to the plasma membrane proteins such as CD44v3 isosforms and for oncogenic signaling during extracellular matrix component (e.g. hyaluronic acid)-regulated breast tumor cell invasion and migration.

**DISCUSSION**

CD44 denotes a family of glycoproteins (e.g. CD44s (standard form), CD44E (epithelial form), and CD44v (variant isosforms)) that are expressed in a variety of cells and tissues (1–6). Clinical studies indicate that a number of CD44v isoforms have been detected at high levels on the surface of tumor cells during tumorigenesis and metastasis (13–17). As the histologic grade
of each of the tumors progresses, the percentage of lesions expressing an associated CD44v isoform increases. In particular, the CD44v3-containing isoforms are detected preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (14, 17, 57).

It has been speculated that some of these CD44v3 isoforms on epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions (9, 10) and subsequent tumor formation. The CD44-related glycoproteins are also known to mediate cell adhesion to extracellular matrix components (e.g. HA) and to function as the major hyaluronate receptor (50). In this study we have demonstrated that a 260-kDa CD44v3 isoform is expressed on the surface of breast tumor cells (SP1 cell line) (Fig. 1) and that it interacts with extracellular matrix HA as an adhesion receptor (Table I). Furthermore, addition of HA to SP1 cells stimulates tumor cell migration in a CD44v3-specific and cytoskeleton-dependent manner (Fig. 2). The invasive phenotype of CD44v3-mediated breast tumor cell migration is shown to play important roles. Tsukita and co-workers (60) have reported that Rho-like proteins participate in the interaction between the CD44 and the ERM cytoskeletal proteins. Our recent study determined that RhoA is physically linked to CD44v3 isoform (e.g. CD44v3, 8–10) in breast tumor cells (48). Rho-kinase stimulated by activated RhoA (GTP-bound form of RhoA) appears to play a pivotal role in promoting CD44v3, 8–10-ankyrin interaction during membrane-cytoskeleton function and metastatic breast tumor cell migration (48). Signaling to the RacGTPase known to regulate actin assembly associated with membrane ruffling, pseudopod extension, cell motility, and cell transformation (33–37) has been shown to be abnormal in breast tumor cells as compared with normal breast epithelial cells (61). The fact that Rac1 induces stress fiber formation in a Rho-dependent manner suggests that cross-talk occurs between the Rho and Rac1 signaling pathways (33). The question of whether the activation of Rac1 signaling is involved in CD44v3-cytoskeleton-mediated breast tumor-specific events remains to be answered.

Tiam1, which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior in vitro, has been shown to regulate Rac1 activation (38, 39). This molecule is largely hydrophilic and contains several functional domains including a Dhl homology domain (38, 62, 63), a Disc large homology region (38, 64), and two pleckstrin homology (PH) domains (e.g. PHn) of the PDZ domain located at the NH2-terminal region of the molecule; and PHc (the PH domain located at the COOH-terminal region of the molecule) (Fig. 3) (38). In particular, the Dhl homology domain of Tiam1 exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (62, 63) and plays an important role in Rac1 signaling and cellular transformation (33–37). In breast tumor cells (e.g. SP1 cells), Tiam1 is detected as a 200-kDa protein (Fig. 2) that is capable of carrying out GDP/GTP exchange for Rac1 (Fig. 7), similar to Tiam1 described in other cell types (34, 40–42, 65, 66). Other functional domains such as Disc large homology region have been implicated in the binding of membrane protein networks (38, 64). The PD domain may mediate association with the submembrane region of the cell via protein-protein or protein-lipid interactions (67). Based on mutational analyses and immunofluorescence staining, Col- lard and co-workers (37, 54) report that the NH2-terminal PHn domain (but not PHc) and an adjacent protein interaction domain (e.g. PHn-CEx domain) (Fig. 3) are required for Tiam1 targeting to the plasma membrane and Rac1 activation in fibroblasts. At the present time, identification of the membrane protein(s) involved in Tiam1 binding has not been established.

In this study we have presented new evidence that a close interaction occurs between Tiam1 and certain plasma membrane proteins such as CD44v3 isoform. Using two recombinant proteins (CBP-tagged PHn-CEx domain (Fig. 4, lane 1) and FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44Cy) (Fig. 4, lane 2)), we have demonstrated that the PHn-CEx domain of Tiam1 is directly involved in the binding to the cytoplasmic domain of CD44 (Figs. 5 and 6). In fact, the binding affinity of the PHn-CEx domain of Tiam1 to CD44 is comparable with the intact Tiam1 binding to CD44 (Figs. 5 and 6). In the presence of PHn-CEx, the binding between Tiam1 and CD44 (e.g. CD44v3) is greatly reduced (Fig. 6). The ability of PHn-CEx to effectively compete for Tiam1 binding to the plasma membrane proteins such as CD44v3 (Fig. 6) strongly suggests that the PHn-CEx of Tiam1 is responsible for the recognition of CD44 in vitro.

In addition, we have detected that Tiam1 and CD44v3 are physically linked to each other as a complex in vivo (Figs. 2, 8, 1836)
and 9) and that HA binding to CD44v3 promotes Tiam1-catalyzed Rac1 activation (Fig. 7 and Table II) and tumor cell migration (Table II). Our data also indicate that overexpression of Tiam1 (by transfecting SP1 cells with C1199 Tiam1cDNA) (Figs. 8 and 9) not only promotes C1199 Tiam1 association with CD44v3 (Figs. 8 and 9) but also enhances the metastatic capability of tumor cells (e.g., Rac1 activation and tumor cell migration (Table II)). These results suggest that Tiam1 and CD44v3 are not only structurally linked but also functionally coupled. Previously, it has been shown that Tiam1-activated Rac1 initiates oncogenic signaling cascades that involve activation of c-Jun NH2-terminal kinase (37, 54) and a novel family of serine/threonine kinases, Paks (p-21 activated kinases) (68, 69). However, the identification of CD44v3-Tiam1-mediated downstream targets (e.g. c-Jun NH2-terminal kinase and/or Paks activities) during HA-mediated breast tumor progression and metastasis remains to be answered.

Furthermore, we have found that co-transfection of SP1 cells with PHn-CC-Ex cDNA and C1199 Tiam1cDNA (Figs. 8 and 9) effectively blocks tumor cell-specific behaviors (e.g., C1199 Tiam1 association with CD44, integrin, or E-cadherin, etc.) or extracellular matrix components (e.g., HA, collagen, laminin, or fibronectin, etc.), which may result in selective Tiam1-activated Rho-like GTPases and distinct biological outcome. In summary, we believe that Tiam1-CD44v3 interaction plays a pivotal role in regulating oncogenic signaling required for RhoGTPase activation and cytoskeleton function during HA-mediated metastatic breast tumor cell progression. This could be one of the critical steps in CD44 variant isoform-mediated breast tumor spread and metastasis.

Acknowledgments—We gratefully acknowledge Dr. Gerard J. Bourguignon’s assistance in the preparation of this paper. We also thank Dr. Dan Zha for help in reviewing the manuscript.

REFERENCES

1. Lesley, J., Hyman, R., and Kincade, P. W. (1993) Adv. Immunol. 54, 271–335
2. Picker, L. J., Nakache, M., and Butcher, E. C. (1989) J. Cell Biol. 109, 927–937
3. Bourguignon, L. Y. W., Lakeshwar, V. B., He, J., Chen, X., and Bourguignon, G. J. (1992) Mol. Cell. Biol. 12, 4646–4655
4. Zhu, D., and Bourguignon, L. Y. W. (1996) Oncogene 12, 2309–2314
5. Brown, T. A., Boucher, T. J., St. John, T., Wayner E., and Carter, W. G. (1991) J. Cell Biol. 113, 207–221
6. Bourguignon, L. Y. W. (1996) Curr. Topics Membr. 43, 293–312
7. Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1991) EMBO J. 10, 2653–2670
8. Screteton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 12160–12164
9. Bennett, K. L., Jackson, D. G., Simon, J. C., Tanczos, E., Peach, R., Modrell, B., Stamenkovic, I., Fowlman, G., and Aruffo, A. (1995) J. Cell Biol. 129, 687–698
10. Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J., and Whittle, N. (1995) J. Cell Biol. 126, 675–685
11. Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J., and Whittle, N. (1995) J. Cell Biol. 126, 675–685
12. Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J., and Whittle, N. (1995) J. Cell Biol. 126, 675–685
13. Dall, P., Heider, K.-H., Sinn, H. P., Skroch-Angel, P., Adolf, G., Kaufmann, M., Herrlich, P., and Pon, H. (1995) Int. J. Cancer 60, 471–477
14. Ida, N., and Bourguignon, L. Y. W. (1995) J. Biol. Chem. 270, 17983–17989
15. Iida, N., and Bourguignon, L. Y. W. (1995) J. Biol. Chem. 270, 17983–17989
16. Rodriguez, C., Monges, G., Rouanet, P., Dutrillaux, B., Lefrancois, D., and Theillet, C. (1995) Int. J. Cancer 60, 347–354
17. Kalisch, E., Iida, N., Moffat, R. L., and Bourguignon, L. Y. W. (1999) Front. Biosci. 4, 1–8
18. Bourguignon, L. Y. W., Zhu, D., and Zhu, H. B. (1998) Front. Biosci. 3, 637–649
19. Bourguignon, L. Y. W., Ida, N., Welsh, C. F., Zhu, D., Krongrad, A., and Pasquale, D. (1995) J. Neurol- Oncol. 26, 201–208
20. Lobeswar, V. B., Frejneg, N., and Bourguignon, L. Y. W. (1994) J. Cell Biol. 128, 1099–1107
21. Ida, N., and Bourguignon, L. Y. W. (1998) Cell Motil. Cytoskeleton. 39, 209–222
22. Bourguignon, L. Y. W., Zhu, H., Chiu, A., Ida, N., Zhang, L., and Hung, H. C. (1997) J. Biol. Chem. 272, 27913–27918
23. Bourguignon, L. Y. W., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J. T., and Pasquale, D. (1995) J. Neurol Oncol. 26, 201–208
24. Rodriguez, C., Monges, G., Rouanet, P., Dutrillaux, B., Lefrancois, D., and Theillet, C. (1995) Int. J. Cancer 60, 347–354
25. Ridley, A. J., and Hall, A. (1995) Cell 70, 389–399
26. Hall, A. (1998) Science 279, 509–514
27. Dickson, R. B., and Lippman, M. E. (1995) The Molecular Basis of Cancer (Mendelsohn, J., Howlwy, F. W., and Israel, M. A., and Liotta, L. A., eds) pp. 268–289, W. B. Saunders Company, Philadelphia, PA
28. Jiang, W. G., Punet, M. C. A., and Hallett, M. B. (1994) Br. J. Surgery 81, 1576–1590
29. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
30. Deleted in proof
31. Deleted in proof
32. Deleted in proof
33. Deleted in proof
34. Ridley, A. J., Paterson, C. L., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
35. Nobe, C. D., and Hall, A. (1995) Cell 81, 53–62
36. Coia, O. A., Ciarchiello, M., Casini, A., Ferramonti, H., Crespo, P., Xu, N. M., Gki, T., and Oudj, A. (1995) Cell 81, 1137–1147
37. Minden, A., Lin, A. N., Claret, F. X., Abo, A., and Karin, M. (1995) Cell 81, 1147–1157
38. Michelis, F., Stamm, J. C., Bordijk, P. L., van der Kamen, R. A., Ruuls-Van Stalle, L., Feltkamp, C. A., and Collard, J. G. (1997) J. Cell Biol. 137, 387–398
39. Habets, G. G. M., Schultes, E. H. M., Zaydeyveld, D., van der Kamen, R. A., Stum, J. C., and Collard, J. G. (1994) FEBS Lett. 345, 446–447
40. Fleming, I. N., Elliott, C. M., and Exton, J. H. (1998) Biochim. Biophys. Acta 137, 387–398
41. Fleming, I. N., Elliott, C. M., and Exton, J. H. (1998) FEBS Lett. 429, 229–233
46. Fleming, I. N., Elliott, C. M., Bruchanan, F. G., Downes, C. P., and Exton, J. H. (1999) J. Biol. Chem. 274, 12753–12758
47. Elliott, B. E., Maxwell, L., Arnold, M., Wei, W. Z., and Miller, E. R. (1988) Cancer Res. 48, 7237–7245
48. Bourguignon, L. Y. W., Zhu, H. B., Shao, L., Zhu, D., and Chen, Y. W. (1999) Cell Motil. Cytoskelet. 43, 269–287
49. Zhang, Y., Hart, M. J., and Cerione, R. A. (1995) Methods Enzymol. 256, 77–84
50. Underhill, C. B., Green, S. J., Comoglio, P. M., and Tarone, G. (1987) J. Biol. Chem. 262, 13142–13146
51. Lesley, J., Hyman, R., and Kincade, P. (1993) Adv. Immunol. 54, 271–235
52. Peach, R. J., Hollenbaugh, D., Stamenkovic, I., and Aruffo, A. (1993) J. Cell Biol. 122, 257–264
53. Liao, H. X., Lee, D. M., and Haynes, B. F. (1995) J. Immunol. 155, 3938–3945
54. Stam, J. C, Sander, E. E., Michiels, F., van Leeuwen, F. N., Kain, H. E. T., van der Kamen, R. A., and Collard, J. G. (1997) J. Biol. Chem. 272, 28447–28454
55. Deleted in proof
56. Mueller, S. C., and Chen, W. T. (1991) J. Cell Sci. 99, 213–225
57. Sinn, H. P., Heider, K. H., Skroch-Angel, P., von Minckwitz, G., Kaufmann, M., Herrlich, P., and Ponta, H. (1995) Breast Cancer Res. Treat. 36, 307–313
58. Horst, E., Meijer, C. J., Radaszkiewicz, T., Ossekoppele, G. J., Van Krieken, J. H., and Pals, S. T. (1996) Leukemia 4, 595–599
59. Jalknen, S., Joensuu, H., Oderstr, S., Ko, O., and Klemi, P. (1991) J. Clin. Invest. 87, 1835–1840
60. Hirao, M., Sato, N., Kondo, T., Yonemura, S., Mondon, M., Sasaki, T., Takai, Y., Tsukita, S., and Tsukita, S. (1996) J. Cell Biol. 135, 36–51
61. Johnston, C. L., Cox H. C., Gomm, J. J., and Coombes, R. C. (1995) Biochem. J. 306, 609–616
62. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A., and Cerione, R. A. (1991) Nature 354, 311–314
63. Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A., and Zheng, Y. (1994) J. Biol. Chem. 269, 62–65
64. Pontings, C. P., and Phillips, C. (1995) Trends Biochem. Sci. 20, 102–103
65. Van Leeuwen, F. N., Kain, H. E., Kamen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997) J. Cell Biol. 139, 797–807
66. Horndijk, P. L., ten Kloost, J. P., van der Kamen, R. A., Michiels, F., Oomen, L. C., and Collard, J. G. (1997) Science 278, 1464–1466
67. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) Cell 85, 621–624
68. Moler, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) Nature 367, 40–46
69. Knaus, U. G., Morris, S., Deng, H., Chernoff, J., and Bokoch, G. M. (1995) Science 269, 221–223
70. Bourguignon, Lilly Y. W., H. Zhu, L. Shao, and Y. W. Chen, (1999) Proc. Am. Assoc. Cancer Res. 40, 196
71. Nishiyama, T., Sasaki, T., Takaishi, K., Kato, M., Yaku, M., Araki, K., Matsuura, Y., and Takai, Y. (1994) Mol. Biol. Cell. 14, 2447–2456
72. Sander, E. E., ten Kessel, J. P., Reid, T., van der Kamen, R. A., Michiels, F., and Collard, J. G. (1998) J. Cell Biol. 143, 1385–1398
73. Michiels, F., and Collard, J. G. (1999) Biochem. Soc. Symp. 63, 125–126
74. Chu, G., Hayakawa, H., and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326