CD44 is a cell surface adhesion molecule for several extracellular matrix components. We previously showed that CD44 expressed in cancer cells is proteolytically cleaved at the ectodomain through membrane-anchored metalloproteases and that CD44 cleavage plays a critical role in cancer cell migration. Therefore, cellular signals that promote the migration and metastatic activity of cancer cells may regulate the CD44 ectodomain cleavage. Here, we demonstrate that the expression of the dominant active mutant of Ha-Ras (Ha-RasVal-12) induces redistribution of CD44 to the newly generated membrane ruffling area and CD44 ectodomain cleavage. The migration assay revealed that the CD44 cleavage contributes to the Ha-RasVal-12-induced migration of NIH3T3 cells on hyaluronate substrate. Treatment with LY294002, an inhibitor for phosphoinositide 3-OH kinase, significantly inhibits Ha-RasVal-12-induced CD44 cleavage, whereas that with PD98059, an inhibitor for MEK, does not. The active mutant p110 subunit of PI3K has also been shown to enhance the CD44 cleavage, suggesting that PI3K mediates the Ras-induced CD44 cleavage. Moreover, the expression of dominant negative mutants of Cdc42 and Rac1 inhibits the Ha-RasVal-12-induced CD44 cleavage. These results suggest that Ras > PI3K > Cdc42/Rac1 pathway plays an important role in CD44 cleavage and may provide a novel molecular basis to explain how the activated Ras facilitates cancer cell migration.

CD44 is a transmembrane receptor for the extracellular matrix (ECM) components including hyaluronic acid (HA) and is implicated in a wide variety of adhesion-dependent cellular processes including lymphocyte homing, cell migration, and tumor cell metastasis and invasion. Our previous studies showed that CD44 expressed in tumor cells is proteolytically cleaved at the extracellular domain (ectodomain) through a membrane-associated metalloprotease and that this ectodomain cleavage generates a membrane-bound COOH-terminal cleavage product and a soluble NH2-terminal fragment released into the culture supernatant. The cleavage was found to play a crucial role in an efficient cell detachment from hyaluronate substrate during the cell migration and to promote the CD44-mediated cell migration of cancer cells (8). These results led us to speculate that cellular signals activated in the cancer cells having high motility and migration activity may contribute to the regulation of CD44 cleavage.

Stimulations by various growth factors and ECM proteins are known to activate locomotion of tumor cells, which contributes to invasion and metastasis of the cells (9, 10). Ras small GTPases (Ha-Ras, Ki-Ras, and N-Ras) are indispensable for such cellular signaling. Signals from the growth factor-dependent activation of receptor tyrosine kinase or the integrin-dependent cell adhesion to ECM induce the activation and/or membrane recruitment of guanine nucleotide exchange factors, which convert inactive GDP-bound Ras into active GTP-bound Ras (11). The active form of Ras specifically makes contact with downstream effector proteins including Raf serine/threonine kinase family (consisting of Raf-1, A-Raf, and B-Raf) (12–16), phosphoinositide 3-OH kinase (PI3K) (17, 18), and Rap GDP dissociation stimulator (19–21). Mutation of the Ras protein is associated with not only the development of naturally occurring tumors but also invasive and metastatic behavior of tumor cells in vitro (22, 23). Several lines of evidence suggest the involvement of Ras activation in cell motility and migration. First, Ras-transformed NIH3T3 cells exhibited metastatic behavior in vivo (24, 25). Second, microinjection of cells with a dominant negative mutant of Ras or neutralizing antibody against Ras inhibited the growth factor-induced cell migration (26). Third, microinjection of an active mutant of Ras induced marked alteration in the actin cytoskeleton organization, which is essential for cell movement (27).

Previous studies have shown that Ras-induced cytoskeletal reorganization and transformation are mediated by the Rho family of small G proteins, consisting of the Rho, Rac, and Cdc42 subfamilies (28–34), which modulate different aspects of the actin organization and cell morphology; Rho regulates the formation of stress fibers and focal adhesions (35), Rac regulates lamellipodia and membrane ruffling formation (36), and Cdc42 regulates filopodia formation (37, 38). It has also been reported that the active form of Ras induces PI3K activation (39), which subsequently activates Rac and then Rho, resulting in lamellipodia and stress fiber formation, respectively (36, 40). Cdc42 also was thought to be involved in Ras-induced cytoskeletal reorganization since its dominant negative mutant reverts...
transformed morphology of Ha-RasVal-12-expressing cells (30). We have recently found that the Rho family of small G proteins are involved in the regulation of the subcellular distribution and cleavage of CD44 (41). Therefore, we hypothesized that the downstream signaling events of Ras activation promote the cell migration by regulating CD44 cleavage.

In this study, we have investigated the role of activated Ha-Ras in the regulation of CD44 cleavage and the CD44-dependent cell migration. Our findings suggest that oncogenic Ras promotes the CD44-dependent cell migration on hyaluronic substrate by induction of the CD44 cleavage through PI3K and the Rho family of small G proteins, Rac and Cdc42.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals—**An antibody against the cytoplasmic domain of CD44, anti-CD44cyto polyclonal antibody, was prepared as described previously (8, 41). The monoclonal antibody KM114 (PharMingen) is directed against the ectodomain epitope common to all murine CD44 isoforms. The anti-Ha-Ras polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Akt antibody, anti-phospho-Akt antibody, anti-extracellular signal-regulated kinases 1/2 (ERK1/2) antibody, and anti-phospho-ERK1/2 antibody were purchased from New England Biolabs (Beverly, MA). The anti-Myc monoclonal antibody was prepared from 9E10 cells. Secondary antibodies linked to horseradish peroxidase used for Western blot analysis were obtained from Amersham Pharmacia Biotech. Myc monoclonal antibody was prepared from 9E10 cells. Secondary antibodies linked to fluorescein isothiocyanate and Texas Red were purified from BIOSOURCE (Camarillo, CA) and Amersham Pharmacia Biotech, respectively.

Chemicals were obtained as follows: carbobenzoxy-l-leucyl-l-leucinyl-leucinal (MG132) from Peptide Institute (Osaka, Japan); PD98059 from New England Biolabs, Inc. (Beverly, MA); LY294002 from Sigma; and isopropyl-β-D-thiogalactoside (IPTG) from Takara (Tokyo, Japan).

**Cell Culture and Transfection—**CHO-K1 cells and NIH3T3 cells were obtained from the Japanese Collection of Research Bioreresources (JCRB, Tokyo, Japan). NIH3T3 cells harboring Ha-RasVal-12 gene under the control of an IPTG-inducible promoter (designated as NIH3T3 RasValA1) were established as described previously (42). All cells were grown in Dulbecco’s modified Eagle’s medium with Ham’s F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at 37 °C in an atmosphere containing 5% CO₂.

pBj-CD44s-Myc and pOPRSVI-Ras(V12) plasmids were constructed as described previously (8, 43). pmycBD110, which was designed for expression of an active mutant bovine p110 subunit of PI3K (44), was kindly provided by Dr. Y. Fukui. pEF-BOS-HA-Cdc42N17, -Rac1N17, and RhoAN19 plasmids were kindly provided by Dr. K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan).

For confocal microscopic analysis, parental NIH3T3 cells were sparsely seeded in 35-mm dishes and transfected with 1.0 µg of pOPRSVI-Ras(V12) by FuGENE6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For Western blot analysis shown in Fig. 2, NIH3T3 RasValA1 cells were seeded in 6-well plates and transfected with 2.0 µg of pBj-CD44s-Myc plasmid by the liposome-mediated gene transfer method (45). CHO-K1 cells (2 × 10⁶ cells per well) were seeded in 6-well plates and cotransfected with 0.4 µg of p38 SS plasmid, 0.8 µg of pOPRSVI-Ras(V12) plasmid, and 0.8 µg of pBj-CD44s-Myc plasmid. For ELISA analysis shown in Fig. 6, NIH3T3 RasValA1 cells or CHO-K1 cells (2 × 10⁶ cells per well) were seeded in 6-well plates and transfected with 0.5 µg of pBj-CD44s-Myc plasmid and 1.5 µg of pmycBD110 plasmid. For ELISA analysis shown in Fig. 7, NIH3T3 RasValA1 cells were seeded in 6-well plates and transfected with 0.2 µg of pBj-CD44s-Myc plasmid and 1.8 µg of either pEF-BOS-HA-mock, Cdc42N17, Rac1N17, or RhoAN19.

**Enzyme-linked Immunosorbent Assay (ELISA)—**Supernatants of treated cells were filtered using a 0.22-µm Millipore filter (Bedford, MA) before analysis. Soluble human CD44s in the culture supernatant was quantified using a soluble CD44s ELISA kit (Bender MedSystem, Vienna, Austria) as described previously (8, 41).

**Immunoprecipitation—**Cells were lysed on ice for 30 min with 0.5% Nonidet P-40 lysis buffer consisting of 0.5% Nonidet P-40, 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.2% (w/v) sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 0.1 M dithiothreitol, 2 mM Tris-HCl, pH 8.0, 0.2% bromphenol blue) incubated for 5 min. Samples containing equal amounts of lysates extracted from equal numbers of cells were electrophoresed on a SDS-polyacrylamide gel and transferred to nitrocellulose filters with a constant current of 120 mA for 2 h. The filters were incubated in PubMed containing 10% skim milk for 30 min at room temperature and then incubated with primary antibodies diluted in PBS containing 0.03% Tween 20 for 1 h, and washed three times for 7 min each time with PBS containing 0.03% Tween 20. The filters were then incubated for 40 min with the appropriate secondary antibodies diluted in PBS containing 0.03% Tween 20, and specific proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**Western Blot Analysis—**For the Western blot analysis, the cultured cells were preincubated with 10 µM MG132 for 2 h (41), directly lysed with sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 0.1 M dithiothreitol, 20 mM Tris-HCl, pH 6.8, 0.02% bromphenol blue) and centrifuged at 14,000 × g for 20 min. Aliquots of supernatant were incubated for 1 h at 4 °C with anti-Myc antibody and another 1 h of incubation after adding protein G-Sepharose beads (Amersham Pharmacia Biotech). After being washed, the bound proteins were analyzed Western blot analysis.

**Fluorescence Microscopic Analysis—**Ha-RasVal-12-expressing NIH3T3 cells grown on 35-mm culture dishes were fixed with 4% paraformaldehyde/0.25% glutaraldehyde/PBS for 10 min followed by 0.1% Triton X-100/PBS for 5 min. After being washed with PBS, the cells were incubated in primary antibodies diluted in PBS containing 0.2% bovine serum albumin (BSA) for 60 min at room temperature, washed three times in PBS, and then incubated for 60 min at room temperature with the appropriate secondary antibodies diluted in PBS containing 0.2% BSA. After washing with PBS, samples were mounted in 80% glycerol and visualized with a confocal microscope (Fluoview, Olympus, Tokyo, Japan) equipped with an argon gas laser and appropriate filter sets to allow the simultaneous recording of fluorescein. Fluorescence micrographs were recorded using PL APO 40× objectives and were sampled at a resolution of 1024 × 1024 pixels and 8-bits per color. Throughout this study, we confirmed that no bleed-through occurred between different channels by comparing the results obtained by depleting one primary antibody.
Migration Assay—Cell migration was assayed using 48-well modified Boyden chambers (Neuro Probe Inc., Bethesda) with polycarbonate Nucleopore filters of 8-µm pore size (Nucleopore Corp.). The undersides of filters were precoated with 0.5% BSA in the presence of 5 mM IPTG or not, and the ECM-coated filters were placed into the chamber with the coated membrane side facing the lower compartments. Parental NIH3T3 cells or NIH3T3 RasValA1 cells in a logarithmic phase of growth were detached by brief exposure to trypsin-EDTA and resuspended at 10^6 cells/ml in DMEM/F-12 containing 0.5% BSA in the presence of 5 mM IPTG or not. These cells were added to the upper compartments at 5 × 10^4 cells/compartment. For studies using antibodies, the cells were incubated with 20 µg/ml KM114 or isotype-matched control Rat antibody (PharMingen) diluted in DMEM/F-12 containing 0.5% BSA in the presence of 5 mM IPTG or not 10 min prior to and during the migration assay. For studies using BB2516, the cells were incubated with 100 µM BB2516 diluted in DMEM/F-12 containing 0.5% BSA in the presence of 5 mM IPTG 10 min prior to and during the migration assay. Me2SO was used as a buffer control. Chambers were subsequently incubated at 37 °C in a 5% CO2 atmosphere for 24 h. After removal of filters, cells on the non-coated upper membrane side were gently wiped off. Filters were fixed in methanol, stained with Giemsa solution, and mounted on glass slides. Cells that had migrated to the coated side of the filters were counted in a blinded fashion using light microscopy under high power field (× 400). The number of cells in three defined high power fields was counted, and the average was determined. Each assay was performed in triplicate.

RESULTS

Redistribution of CD44 to Ha-RasVal12-induced Membrane Ruffling Area—Our previous study showed that CD44 is enzymatically cleaved at the ectodomain in accordance with its redistribution to membrane ruffling area, which is induced by the treatment with 12-O-tetradecanoyl phorbol 13-acetate (TPA) or expression of dominant active Rac1 (41). Active mutant of Ras was demonstrated to induce membrane ruffling and lamellipodia formation (27, 36). Therefore, we first investigated the distribution of CD44 during Ras-induced cytoskeletal reorganization using confocal microscopic analysis. When we transiently transfected NIH3T3 cells with pOPRSVI-Ras(V12) plas-
mid which expresses the active mutant of the Harvey-Ras, Ha-RasVal-12, membrane ruffling was induced (Fig. 1, A and B). Concomitantly, CD44 was redistributed to the newly generated ruffling area (Fig. 1C). These data indicate that the active form of Ras evokes CD44 redistribution at the plasma membrane.

Enhancement of CD44 Cleavage by Conditional Expression of Ha-RasVal-12—Next, we analyzed the CD44 cleavage activity in Ras-activated cells. For this purpose, we utilized NIH3T3 RasValA1 cells that were stably transfected with pOPRSVI-Ras(V12) and p3' SS that expresses the repressor of Escherichia coli lactose operon (43). In these cells, Ha-RasVal-12 is expressed under Lac repressor control (42, 43). Western blot analysis showed that the addition of IPTG efficiently induced expression of Ha-RasVal-12 (Fig. 2A). Expression of Ha-RasVal-12 became detectable within 4 h of treatment with IPTG, and thereafter, the level of Ras protein was continuously elevated for the duration of IPTG treatment as described previously (42, 43) (data not shown). These cells were transiently transfected with human full-length CD44s expression plasmid (hCD44s-Myc), which was epitope-tagged with Myc at the COOH terminus of CD44s. In these transfectants, as shown by Western blot analysis, the amount of the membrane-bound CD44 cleavage product was increased by the treatment with IPTG for 8 h when compared with untreated cells (Fig. 2A). In contrast, parental NIH3T3 cells transfected with human CD44s-Myc alone did not induce CD44 cleavage in response to IPTG.

To verify further the effect of activation of Ras on CD44 cleavage, NIH3T3 RasValA1 cells transfected with hCD44s-Myc were subjected to an ELISA to examine whether the NH2-terminal cleavage products were increased by Ha-RasVal-12 induction. Induction of the activated-Ras expression results in an increase in the release of human soluble CD44s (hsCD44s) (Fig. 2B). Furthermore, preincubation of cells with BB2516, the hydroxamic acid-based metalloproteinase inhibitor, blocked the production of both the COOH- and NH2-terminal cleavage products (Fig. 2, A and B). These results suggest that Ha-RasVal-12 induces the metalloproteinase-mediated cleavage of CD44 at the ectodomain. Additionally, we tested the effect of Ha-RasVal-12 on the CD44 cleavage in another cell line, CHO-K1 cell. When CHO-K1 cells were transiently transfected with hCD44s-Myc, pOPRSVI-Ras(V12), and p3' SS, the amount of the membrane-bound CD44 cleavage product was increased by the treatment with IPTG (Fig. 2C). All these results indicate that CD44 cleavage at the ectodomain is promoted by activation of Ras.

Fig. 3. Expression of Ha-RasVal-12 promotes migration of NIH3T3 RasValA1 cells on HA through the metalloproteinase-mediated CD44 cleavage. A, migration of parental NIH3T3 (columns 1 and 2) and NIH3T3 RasValA1 cells (columns 3–6) on HA was assessed by modified Boyden chamber type migration assays. The cells were treated (columns 2, 4, and 6) or untreated (columns 1, 3, and 5) with IPTG in the presence of KM114 antibody (columns 5 and 6) or isotype-matched control antibody (columns 3 and 4). Columns and bars represent the mean and S.D. obtained from three independent experiments. Statistical differences were determined with Student’s t test; *, p < 0.002. B, migration of NIH3T3 RasValA1 cells on fibronectin was assessed by the migration assays. The cells were treated (columns 2 and 4) or untreated (columns 1 and 3) with IPTG in the presence of KM114 antibody (lanes 3 and 4) or isotype-matched control antibody (lanes 1 and 2). C, effect of BB2516 (BB) on migration of NIH3T3 RasValA1 cells on HA was assessed by the migration assays. The cells were treated (columns 2 and 3) or untreated (column 1) with IPTG in the presence of BB2516 (column 3) or Me2SO (columns 1 and 2) as a buffer control.

Activation of Ras Promotes Migration of NIH3T3 Cells on Hyaluronic Acid (HA) through the Metalloproteinase-mediated Cleavage of CD44—We previously showed that CD44 cleavage plays a critical role in cancer cell migration on HA. In order to test whether the induction of Ha-RasVal-12 promotes the CD44-dependent cell migration, we performed modified Boyden chamber type migration assays. As shown in Fig. 3A, Ha-RasVal-12 induction by IPTG treatment significantly enhanced the migration of NIH3T3 RasValA1 cells through HA-coated membrane, whereas IPTG treatment did not affect the migration of parental NIH3T3 cells on HA. Furthermore, anti-
CD44ecto monoclonal antibody significantly inhibited the Ha-RasVal-12-induced migration on HA compared with an isotype-matched control monoclonal antibody. In contrast, anti-CD44ecto monoclonal antibody did not reduce Ha-RasVal-12-induced migration of NIH3T3 RasValA1 cells on another extracellular matrix substratum, fibronectin (Fig. 3B). These results strongly support the idea that the Ha-RasVal-12-induced migration of NIH3T3 RasValA1 cells on HA is CD44-dependent.

Next, we examined the effect of BB2516 on the migration of NIH3T3 RasValA1 cells on HA. The presence of BB2516 markedly reduced Ha-RasVal-12-induced cell migration on HA (Fig. 3C) but no significant reduction on fibronectin (data not shown). These results suggest that metalloproteolytic activity is crucially involved in the cell migration on HA and that CD44 cleavage significantly contributes to the Ras-induced cell migration on HA. Interestingly, the treatment of Ha-RasVal-12-transfected NIH3T3 cells with BB2516, which strongly prevented RasVal-12-induced CD44 cleavage (Fig. 2, A and B), did not affect the Ras-induced CD44 redistribution to the ruffling

**Fig. 4.** Ras-induced CD44 redistribution to ruffling areas is not affected by the treatment with BB2516. NIH3T3 cells were transfected with p0RBSV1-Ras(V12). After 24 h of transfection, the cells were incubated with (D-F) or without (A-C) 100 μM BB2516 for an additional 24 h. The cells were fixed, double-stained with anti-Ha-Ras antibody (B and E) and KM114 (C and F), and analyzed by confocal microscopy. Phase-contrast images (A and D) are shown. The CD44 is found at membrane ruffling areas (white arrows) regardless of BB2516 treatment (A, C, D, and F). Bars, 20 mm.

**Fig. 5.** Ha-RasVal-12-induced CD44 cleavage is mediated by PI3K pathway. A, NIH3T3 RasValA1 cells were transfected with pBj-CD44s-Myc. After 24 h of transfection, cells were incubated for 12 h in the presence of various compounds as follows: vehicle (lanes 1 and 2), 100 μM BB2516 (BB) (lane 3), 50 μM LY294002 (LY) (lane 4), and 50 μM PD98059 (PD) (lane 5). The cells were then treated with (lanes 2–5) or without (lane 1) 5 mM IPTG for 8 h. The hsCD44s in the culture supernatant was detected by ELISA analysis (upper panel). Columns and bars represent the mean and S.D. obtained from three independent experiments. Induction of Ha-RasVal-12 was detected by Western blots using anti-Ha-Ras antibody (lower panel). B, activation of ERK induced by Ha-RasVal-12 was detected by Western analysis using antibody that recognizes dually phosphorylated ERK only (upper panel). Cells were incubated for 12 h in the absence (lanes 1 and 2) or presence of 100 μM BB2516 (lane 3), or 50 μM PD98059 (lane 4). This was followed by induction (lanes 2–4) or not (lane 1) of Ha-RasVal-12 expression by 5 mM IPTG for 8 h. The same samples were blotted with anti-ERK antibody (lower panel) to monitor for equal loading. C, activation of PI3K induced by Ha-RasVal-12 was detected by Western analysis using anti-phospho-Akt antibody (upper panel). Cells were incubated for 12 h in the absence (lanes 1 and 2) or the presence of 100 μM BB2516 (lane 3) or 50 μM LY294002 (lane 4). This was followed by induction (lanes 2–4) or not (lane 1) of Ha-RasVal-12 by 5 mM IPTG for 8 h. The same samples were blotted with anti-Akt antibody (lower panel) to monitor for equal loading.
areas (Fig. 4). This result indicates that the ectodomain cleavage is not essential for CD44 to redistribute to ruffling membrane areas.

Involvement of the PI3K Pathway in Ha-Ras Val-12-induced CD44 Cleavage—It is known that Ras can trigger multiple signaling pathways. The Raf > MEK > ERK pathway is a major signal transduction pathway activated by Ras. Previous reports revealed that serum stimulation induces translocation of ERK not only to nucleus but also to membrane ruffling area (46) and that ERK regulates both the proliferation and motility of cells (47). PI3K is also one of the Ras effector molecules and was found to be involved in Ras-induced cytoskeletal reorganization and cell motility (39, 48). Therefore, in order to determine which pathway contributes to the Ha-Ras Val-12-induced CD44 cleavage, hCD44s-Myc transfected NIH3T3 RasValA1 cells were cotransfected with pBj-CD44s-Myc and empty vector (Vector) or pmy-cBD110 (BD110) expressing the Myc-tagged active mutant p110 subunit of PI3K. After 16 h of transfection, hsCD44s released in the culture supernatant was detected by ELISA analysis. Columns and bars represent the mean and S.D. obtained from three independent experiments. B, expression of BD110 and its activity in the transfected NIH3T3 RasValA1 cells were detected by Western blot analysis using an anti-Myc antibody (upper panels) and anti-phospho-Akt antibody (middle panel), respectively. The same samples were blotted with anti-Akt antibody (lower panel) to monitor for equal loading. C and D, the same experiments shown in A and B were performed by using CHO-K1 cells.

To determine whether activation of PI3K could directly induce CD44 cleavage, NIH3T3 RasValA1 (Fig. 6, A and B) and CHO-K1 cells (Fig. 6, C and D) were transfected with hCD44s-Myc and the Myc-tagged active mutant p110 subunit of PI3K, BD110 (44), and the level of hsCD44s in the culture supernatant was determined by ELISA. Activation of PI3K pathway by expression of BD110 was monitored by Western blot analysis of AKT phosphorylation (Fig. 6, B and D). ELISA using the culture supernatants revealed that BD110 expression significantly enhanced the release of hsCD44 from both NIH3T3 RasValA1 and CHO-K1 cells (Fig. 6, A and C). Taken together, it appears that the Ras-induced CD44 cleavage is mediated by PI3K.

Involvement of the Rho Family of Small G Proteins in Ha-Ras Val-12-induced CD44 Cleavage—Recent works have shown that various cellular events caused by activation of Ras and PI3K are mediated by the Rho family of small G proteins (28–32, 36, 49, 50). We previously reported that the Rho family of small G proteins play a crucial role in the regulation of CD44 distribution and cleavage (41). Therefore, we examined the possibility that Rho family GTPases are downstream effector molecules for the Ras-induced CD44 cleavage by expressing dominant negative mutant of Cdc42, Rac1, or RhoA in NIH3T3 RasValA1 cells. After 48 h of transfection, the cells were treated with IPTG for 8 h in serum-free medium, and the level of hsCD44s in the culture supernatant was determined by ELISA. As shown in Fig. 7, the CD44 cleavage induced by Ha-Ras Val-12 was significantly inhibited by the expression of dominant negative Cdc42 or dominant negative Rac1. Moreover, these mutant small GTPases reduced the levels of
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hsCD44s released in the culture supernatant of NIH3T3 RasValA1 cells without Ha-RasVal-12 induction. In contrast, transfection of dominant negative mutant of RhoA did not inhibit the CD44 cleavage induced by Ha-RasVal-12 expression but rather tended to enhance hsCD44s release regardless of induction of Ha-RasVal-12 (Fig. 7). These results suggest that Ha-RasVal-12-induced CD44 cleavage is mediated by activation of Cdc42 and Rac1 and is negatively regulated by Rho activation.

**DISCUSSION**

Mutations of Ras protein are frequently found in sporadic human carcinomas (22), and the expression of the mutated Ras protein in normal fibroblasts has been reported to contribute to transformation and metastasis in the nude mouse model (24, 25). Therefore, the Ras mutations appear to be involved not only transformation but also invasion and metastasis of cancer cells. In this study, we have shown that oncogenic Ha-Ras induces redistribution of CD44 to newly generated membrane ruffling area and promotes metalloproteinase-dependent CD44 cleavage. Furthermore, we demonstrated that migration of NIH3T3 cells on HA, which is promoted by expression of activated Ras, is CD44-dependent and that treatment with the metalloprotease inhibitor BB2516, which strongly prevents the activated Ras-induced CD44 cleavage, suppresses NIH3T3 cell migration on HA. These observations provide a novel molecular basis to explain how the activated Ras facilitates cancer cell migration.

Since Ras triggers multiple signal transduction pathways, we have asked whether the CD44 cleavage is primarily mediated through activation of a single branch, either Raf > MEK > ERK or PI3K pathway. We have demonstrated that the PI3K inhibitor LY294002 effectively blocks Ras-induced CD44 cleavage, whereas the MEK inhibitor PD98059 does not. Additionally, introduction of active mutant p110 subunit of PI3K to CHO-K1 cells has been shown to induce CD44 cleavage. These results indicate that the PI3K pathway is directly involved in the Ras-induced CD44 cleavage. A recent report also showed that PI3K mediates IL-4-induced down-regulation of tumor necrosis factor receptor through the metalloprotease-dependent ectodomain cleavage (51). These findings suggest that activation of PI3K plays an important role in the regulation of cleavage in some membrane proteins. In contrast, the metalloproteinase-dependent cleavage of TGF-α and HB-EGF was reportedly mediated through the Raf > MEK > ERK pathway (52, 53). Therefore, the induction of ectodomain cleavage of diverse transmembrane proteins may be controlled by at least two distinct signaling pathways.

We previously showed that TPA-induced CD44 redistribution and cleavage are inhibited by activation of RhoA and that overexpression of Rac1 dominant active mutants results in the enhancement of CD44 cleavage (41). Furthermore, a wide variety of Ras/PI3K-induced cellular events, such as cytoskeletal reorganization (39, 40, 54) and transformation (28–30, 36, 39), were found to be mediated by Rho family GTPases. These lines of evidence led us to test the possibility that Rho family GTPases are the downstream effector molecules for Ras-induced CD44 cleavage. We have demonstrated that Ha-RasVal-12-induced CD44 cleavage is effectively inhibited by both Cdc42 and Rac1 dominant negative mutants, indicating that activation of Rac1 or Cdc42 is responsible for Ras-induced CD44 cleavage. Activated forms of Cdc42 and Rac are known to stimulate actin reorganization, resulting in formation of microfilament-rich filopodia and lamellipodia which are key elements in cell migration (48, 55). Therefore, it can be speculated that the activation of Rac and Cdc42 promote cell motility by the concomitant induction of CD44 cleavage with cytoskeletal reorganization which results in extension of the leading edge and the formation of new focal complexes (38).

Recent studies revealed that a hierarchy of activation states leading from Ras to PI3K and then to Rac and Cdc42 (Rac > PI3K > Rac/Cdc42) induces various cellular events such as neurite extension (49) and activation of the serine/threonine kinase p65PAK (50). These observations together with our findings suggest that Ras > PI3K > Rac/Cdc42 signal is involved in important cellular functions that are distinct from those regulated by Ras > Raf > MEK > ERK signal. Notably, the dominant negative mutants of Cdc42 and Rac1 inhibited CD44 cleavage in NIH3T3 RasValA1 cells regardless of the incubation with IPTG. Possible reasons for these findings can be envisaged as follows: (a) leakage expression of Ha-RasVal-12 in the noninduced state, and (b) the existence of a Ras-independent pathway that activates Cdc42 and Rac.

In contrast to Cdc42 and Rac, the expression of the dominant negative RhoA did not inhibit the Ras-induced CD44 cleavage but rather enhanced the cleavage (Fig. 5). This finding suggests that activation of RhoA inhibits the ectodomain cleavage, consistent with our previous demonstration that treatment with lysophosphatidic acid, which in known to activate the Rho-dependent pathway, inhibited TPA-induced CD44 cleavage (41). Recent study revealed that activation of Rac down-regulates Rho activity in fibroblasts and suggested that the cross-talk of these Rho family G proteins may determine cellular morphology and adhesion (56). Thus, the regulation of CD44 ectodomain cleavage by the reciprocal balance between Rac and Rho activity might contribute to migratory behavior of the cells.

We have previously shown that CD44 cleavage is mediated...
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Activated Ras induces CD44 cleavage, a process that is critical for tumor cell migration and invasion. CD44, a cell surface glycoprotein, is a member of the immunoglobulin superfamily and plays a crucial role in cell adhesion, motility, and invasion. Ras, a small GTPase, is activated in a variety of human cancers and promotes cell proliferation, migration, and invasion.

The mechanism by which activated Ras induces CD44 cleavage involves the recruitment of the membrane-anchored metalloprotease to the membrane-anchored metalloprotease cleaving CD44. The localization of the metalloprotease cleaving CD44 is still unknown. One plausible explanation is that shedding of CD44 is a result of activation of Ras, which may promote rapid turnover of CD44.

The dynamics of focal adhesion formation plays a major role in cell migration. Interestingly, several reports have demonstrated that Ras transformation induces CD44 expression at the transcriptional level. Therefore, an activated mutation of Ras may promote rapid turnover of CD44, i.e., shedding by ectodomain cleavage and production of the new full-length protein, promoting the tumor cell migration and invasion in ECM.

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by a membrane-bound metalloprotease expressed in cancer cells. The mechanism that links Rac/Cdc42 with activation of the metalloprotease cleaving CD44 is still unknown. One plausible explanation is that these small GTPases contribute to the recruitment of the membrane-anchored metalloprotease to CD44 through actin reorganization, triggering CD44 cleavage. Identification of the metalloprotease and determination of its distribution will provide insight into how the interaction of these molecules is spatially and temporally regulated to enable the CD44 cleavage.

The dynamics of focal adhesion formation plays a major role in cell migration. Interestingly, several reports have demonstrated that Ras transformation induces CD44 expression at the transcriptional level. Therefore, an activated mutation of Ras may promote rapid turnover of CD44, i.e., shedding by ectodomain cleavage and production of the new full-length protein, promoting the tumor cell migration and invasion in ECM.