Engagement of CD44 Promotes Rac Activation and CD44 Cleavage during Tumor Cell Migration

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CD44 is a major cell surface adhesion molecule for hyaluronan, a component of the extracellular matrix, and is implicated in tumor metastasis and invasion. We reported previously that hyaluronan oligosaccharides induce CD44 cleavage from tumor cells. Here we show that engagement of CD44 promotes CD44 cleavage and tumor cell migration, both of which are suppressed by a metalloproteinase inhibitor KB-R7785 and tissue inhibitor of metalloproteinases-1 (TIMP-1) but not by TIMP-2. We also present evidence that blockade of metalloproteinase-disintegrin ADAM10 (a disintegrin and metalloproteinase 10) by RNA interference suppresses CD44 cleavage induced by its ligation. Engagement of CD44 concurrently induced activation of the small GTPase Rac1 and led to drastic changes in cell morphology and actin cytoskeleton with redistribution of CD44 to newly generated membrane ruffling areas. A fluorescence resonance energy transfer approach to visualize GTTP-bound Rac1 in living cells revealed the localization of the active Rac1 in the leading edge of the membrane ruffling areas upon ligation of CD44. Taken together, our results indicate that the cleavage of CD44 catalyzed by ADAM10 is augmented by the intracellular signaling elicited by engagement of CD44, through Rac-mediated cytoskeletal rearrangement, and suggest that CD44 cleavage contributes to the migration and invasion of tumor cells.

CD44 is a cell surface receptor for several extracellular matrix components including hyaluronan (HA) and is implicated in a wide variety of biological processes including lymphocyte homing, cell migration, and metastasis. It has been demonstrated that CD44 expressed in tumor cells is proteolytically cleaved at the extracellular domain by metalloproteinases and that CD44 cleavage plays a critical role in CD44-mediated tumor cell migration. CD44 cleavage on the tumor cell surface is enhanced by phorbol 12-myristate 13-acetate (PMA) or ionomycin treatment, indicating that the cleavage is under the regulation of the activation of protein kinase C (PKC) or the extracellular calcium influx. CD44 cleavage is also induced by introducing dominant active mutant of Ha-Ras small GTPase. In addition to these CD44 cleavage inducers acting on intracellular signaling molecules, we have shown recently that HA fragments also enhance CD44 cleavage from tumor cells. HA fragments probably bind and cross-link CD44 and then induce certain intracellular signals to cause CD44 cleavage. However, almost no information is available regarding the intracellular signals transmitted upon stimulation of CD44 to the induction of CD44 cleavage. The relationship between CD44 cleavage and the enhanced cell migration also remains largely unknown.

Tumor cells migrate by regulating adhesion to and detachment from extracellular matrix, dynamically changing their cytoskeleton. The mammalian Rho GTPase family consists of three subfamilies, Rho, Rac, and Cdc42, whose activation leads to form stress fibers, lamellipodia, and filopodia, respectively. Upstream events leading to extracellular matrix transcription factor binding to Rho family small GTPases activate these proteins, which then interact with downstream target molecules to produce different biological responses. CD44 functions as one of the major adhesion molecules of tumor cells and also functions as a signaling molecule to induce cell migration and invasion through actin reorganization, raising the possibility that CD44 signaling may enhance cleavage of CD44 itself by inducing such cellular changes.

In the present study, we show that engagement of CD44 with an anti-CD44 monoclonal antibody (mAb) induces cleavage of CD44 and enhances tumor cell migration through CD44 cleavage. We also observed that engagement of CD44 induces cytoskeletal rearrangement and activation of the small GTPase Rac at the leading edge of the membrane ruffling areas. These raise the possibility that intracellular signals elicited by ligation of CD44 induce CD44 cleavage and enhance tumor cell migration and invasion, through activation of Rac.

EXPERIMENTAL PROCEDURES

Reagents

Anti-CD44 antibodies were obtained as follows: rat B cell hybridoma producing mAb IM7 (IM7.8.1) was purchased from the American Type Culture Collection (Manassas, VA); a polyclonal antibody (pAb) against the cytoplasmic domain of CD44, anti-CD44cyo pAb (5), and Hermes-3 (16) were kindly provided by Drs. Hideyuki Saya (Kumamoto

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The human glioblastoma cell line U251MG, kindly provided by Dr. H. Saya, was grown in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO2. Transfection of U251MG cells with the plasmid pEF-BOS-myc-N17Rac1, kindly provided by Dr. Yoshimi Takai (Osaka University, Suita, Japan), was performed as follows. The cells were seeded in 24-well plates at 2.5 × 104 cells/well and transfected with 0.8 μg of plasmid using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions.

Western Blot Analysis
For detection of CD44 cleavage products in cell lysates, U251MG cells were seeded in a 24-well plate at 5 × 104 cells/well and cultured overnight. After preincubation with 10 μM MG132 for 30 min, the cells were incubated with IM7 mAb for 1 h. To detect CD44 cleavage product, the cells were lysed and subjected to Western blot analysis using anti-CD44 cyto pAb as described under “Experimental Procedures.”

Immunofluorescence Microscopy
U251MG cells were seeded on a 4-well Lab-teck chamber plate (Nalge Nunc, Naperville, IL) at a density of 2 × 103 cells/ml and incubated overnight at 37 °C in an atmosphere containing 5% CO2. The cells were fixed with paraformaldehyde/PBS for 10 min followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and rhodamine-conjugated phalloidin (Molecular Probes). After 10 min, the cells were observed with an Olympus fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Pull-down Assay
U251MG cells were plated at a density of 5 × 105 cells per 10-cm dish. After treatment with 1 μg/ml IM7 mAb for different time periods, the cells were lysed, and the pull-down assay for detection of active form of Rac1 was performed as described previously (17).

Plasmid Construction
The coding regions of Rac1 cDNA (Xbal-XbaI fragment) and Cdc42 cDNA (BamHi-BamHI fragment), kindly provided by Dr. Kao Kibuchi (Nagoya University, Nagoya, Japan) (18), were individually inserted into the plasmid pEYFP-C1 (Clontech, Palo Alto, CA) at the corresponding cloning sites. The resulting plasmids were named pEYFP-Rac1 and pEYFP-Cdc42.

In Situ Rac Activity Assay
Microinjection and Imaging—U251MG cells were seeded on a 25-mm diameter glass-bottom dish at a density of 5 × 105 cells/ml and transfected with the plasmid pEYFP-Rac1 or pEYFP-Cdc42 using FuGene 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. After incubation for 24 h followed by serum starvation for 2 h, the cells were microinjected into the cytoplasm with 1 μl BODIPY TR-GTP (Molecular Probes). After 10 min, the cells were incubated with 1 μg/ml IM7 mAb and then observed with an Olympus IX-70 inverted microscope equipped with a confocal laser scanner unit CSU10 (Yokogawa Electronic, Tokyo, Japan). Images were acquired with an AxioZoom 512BFT back-illuminated charge-coupled device camera (Princeton Scientific Instruments), through 515–550-nm and 630DF300
filters (Omega Optical, Brattleboro, VT) for yellow fluorescent protein (YFP) and fluorescence resonance energy transfer (FRET) channels, respectively, under excitation of YFP through a 480/10-nm filter.

**FRET Measurements**—The background was subtracted from the raw images prior to the calculation of FRET signals. The FRET was calculated using “corrected FRET” method (19, 20). The formula for the calculation of FRET is shown in the equation, [FRET] = \( \frac{Y - D}{Y + D} \), where, in notation of Gordon et al. (19), the first letters in each symbol represent the filter set (F for the FRET filter set, D for the donor filter set, A for the acceptor filter set), and the second

![Fig. 2](http://www.jbc.org/)

**Fig. 2. Effect of metalloproteinase inhibitors on mAb-induced CD44 cleavage.** A, effect of a hydroxamate-type metalloproteinase inhibitor on CD44 cleavage. U251MG cells were incubated for 1 h in the presence (lanes 3 and 5) or absence (lanes 1, 2, and 4) of KB-R7785 (10 \( \mu \)M) and left untreated (lane 1) or further incubated with 1 \( \mu \)g/ml IM7 mAb for 1 h (lanes 2 and 3) or with 100 ng/ml PMA for 30 min (lanes 4 and 5). To detect sCD44, the culture supernatants were subjected to Western blot analysis using rabbit anti-human CD44 pAb (right panel). The intensity of the 70-kDa bands that appeared in the blot (lanes 1-5) was quantified by densitometric analysis and expressed in an arbitrary unit (A.U.) (left, columns 1-5, respectively). B and C, effect of TIMPs on CD44 cleavage. After preincubation of U251MG cells for 1 h in the presence of TIMP-1 (B) or TIMP-2 (C) at the indicated concentration, the cells were incubated in the absence (lanes 1 and 6) or presence of 1 \( \mu \)g/ml IM7 mAb for 1 h (lanes 2-5) or 100 ng/ml PMA for 30 min (lanes 7-10). To detect CD44 cleavage product, the cell lysates were subjected to Western blot analysis using anti-CD44cyto pAb as described under “Experimental Procedures.”
letters indicate the fluorochromes present in a cell (f for both donor and acceptor present in a cell, d for donor only, a for acceptor only). Although BODIPY TR-GTP was slightly excited without FRET, its fluorescence could not be detected through either filters used. Thus Ad and Da are effectively zero.

RESULTS

Engagement of CD44 with mAb Induces Cleavage of CD44 Itself—We reported previously (9) that HA oligosaccharides can enhance CD44 cleavage from tumor cells. We thus determined whether ligation of CD44 induces its cleavage, using a highly invasive human glioma cell line U251MG, which was shown previously (5, 7) to have high CD44 cleavage activity. When U251MG cells were incubated with a mAb to CD44, IM7, membrane-tethered CD44 cleavage products of ~20- to 25-kDa, were clearly detected in the lysate of U251MG cells by Western blotting using a pAb against cytoplasmic domain of CD44,
The U251MG cell suspension was incubated at 37 °C for 2 h in the absence (columns 1 and 2) or presence of 10 μg/ml anti-CD44 non-blocking mAb Hermes-3 (column 3) or blocking mAb BRIC235 (column 4), prior to incubation in the Transwell chambers. B, migration of U251MG cells treated with metalloproteinase inhibitors. Migration assay was performed as described under “Experimental Procedures.” The U251MG cell suspension was incubated at 37 °C for 2 h in the absence (columns 1 and 2) or presence of 10 μg KB-R7785 (column 5), Me2SO (DMSO) as a vehicle control for KB-R7785 (column 3), 10 μg/ml TIMP-1 (column 5), or 10 μg/ml TIMP-2 (column 6), prior to incubation in the Transwell chambers. Data are shown as mean ratio to the control migration. The data represent the mean and the standard deviations of triplicate determinations.

Next we examined the effect of metalloproteinase inhibitors used in the cleavage assay on the cell migration. The hydroxamate-type metalloproteinase inhibitor KB-R7785 effectively inhibited the cell migration enhanced by stimulation with IM7 mAb to a greater extent than anti-CD44cyto pAb (Fig. 1). The induction of CD44 cleavage by IM7 mAb was dose-dependent and reached a plateau at 1 μg/ml. The cleavage was not observed when rat IgG was used as a control antibody to IM7 (data not shown). The bands of CD44 full-length (∼90 kDa) were still detected after IM7 mAb treatment, showing that more than 90% of CD44 remains uncleaved on the cell surface, estimated by densitometric analysis. Basically identical results were obtained using the human pancreatic carcinoma cell line Mia PaCa-2 (data not shown).

Next we tried to detect sCD44 released into the culture supernatants. When the culture supernatants of U251MG cells were immunoblotted with anti-human CD44 pAb, which recognizes the ectodomain of CD44, a band of ∼70 kDa was observed (Fig. 2A, right panel). When the cells were treated with IM7 mAb, the intensity of the band increased to almost the same level when CD44 cleavage was induced by PMA (Fig. 2A, columns 2 and 4). The effects of both IM7 mAb and PMA on CD44 cleavage were markedly abrogated by a hydroxamate-type metalloproteinase inhibitor KB-R7785 (Fig. 2A, columns 3 and 5), indicating that the induction of CD44 cleavage by mAb is mediated by metalloproteinases. KB-R7785 is an inhibitor acting on matrix metalloproteinases (MMPs), including MMP-1 and MMP-9, with a broad spectrum (21). To further investigate the involvement of metalloproteinases in CD44 cleavage, we used TIMP-1 and TIMP-2, the natural inhibitors of MMPs. TIMP-2, which inhibits the activity of most membrane-type MMPs (MT-MMPs) (22–24), did not inhibit IM7 mAb-induced CD44 cleavage (Fig. 2C, left panel), whereas TIMP-1, whose inhibitory effect on MT-MMP activities is much less than TIMP-2, effectively inhibited the mAb-induced CD44 cleavage dose-dependently (Fig. 2B, left panel). These results were similar to those of PMA-induced CD44 cleavage (Fig. 2, B and C, right panels). These findings imply that the enzyme responsible for mAb-induced CD44 cleavage is a metalloproteinase(s) other than MT-MMPs.

mAb-induced CD44 Cleavage Is Independent on PKC and Extracellular Calcium Influx—Hitherto, mAb-induced CD44 cleavage exhibits similar pattern as in the case of PMA, a known activator of PKC. Thus to determine whether mAb-induced CD44 cleavage requires activity of PKC, like PMA-induced cleavage, we tested the effects of a specific PKC inhibitor GF109203X (25) on CD44 cleavage. PMA-induced CD44 cleavage was inhibited dose-dependently by GF109203X as expected, whereas mAb-induced CD44 cleavage was not affected (Fig. 3A). These results imply that the signaling mechanisms involved in mAb-induced cleavage may be different from those that operate in PMA-induced cleavage. In addition to IM7 mAb and PMA, CD44 cleavage is also induced by influx of extracellular calcium ion into the cytosol by the ionophore ionomycin (7). Thus we tested whether extracellular calcium ion is required for mAb-induced CD44 cleavage. Although ionomycin did not induce CD44 cleavage in the absence of extracellular calcium ion (Fig. 3B, lane 8), IM7 mAb yet induced CD44 cleavage in the same condition (Fig. 3B, lane 6). These results indicate that the extracellular calcium ion is not required for CD44 cleavage induced by IM7 mAb. Hitherto basically identical results were obtained using another anti-CD44 mAb 5F12 (26) instead of IM7 mAb (data not shown), indicating that CD44 cleavage induced by ligation of CD44 with mAb is mediated by a distinct pathway from those by PMA or ionomycin.

Engagement of CD44 Promotes Migration of U251MG Cells through Cleavage of CD44—CD44 has been reported to be a receptor for HA (1) and to be associated with cell migration (3). CD44 cleavage was shown previously (5, 6, 8) to play a critical role in tumor cell migration. To determine whether the ligation of CD44 promotes CD44-dependent cell migration through metalloproteinase-mediated CD44 cleavage, we performed modified Boyden chamber-type migration assays. As shown in Fig. 4A, IM7 mAb treatment enhanced the migration of U251MG cells on HA. The enhanced migration was inhibited by pretreatment with an anti-CD44 blocking mAb BRIC235 but not by a non-blocking mAb Hermes-3. These mAbs did not inhibit the basal level cell migration (data not shown). These results indicate that the migration enhanced by IM7 mAb treatment was dependent on the CD44-HA interaction.

Next we examined the effect of metalloproteinase inhibitors used in the cleavage assay on the cell migration. The hydroxamate-type metalloproteinase inhibitor KB-R7785 effectively inhibited the cell migration enhanced by stimulation with IM7 mAb to
the basal level (Fig. 4B). When tissue inhibitors were used, TIMP-1 also inhibited the enhanced migration, whereas TIMP-2 did not. Neither of these agents changed the basal level cell migration ability (data not shown). KB-R7785 and TIMP-1 were the inhibitors that suppressed CD44 cleavage as described above (Fig. 2). These results therefore indicate that a certain metalloproteolytic activity that cleaves CD44 is involved in CD44-mediated migration and suggest that CD44 cleavage induced by mAb contributes to the migration of tumor cells.

**ADAM10 Is Involved in CD44 Cleavage Induced by Engagement of CD44**—One of the metalloproteinases whose activity is known to be inhibited by TIMP-1 and not by TIMP-2 is ADAM10 (27). To verify that ADAM10 is the responsible enzyme for CD44 cleavage, endogenous expression of ADAM genes was silenced by RNAi. Efficient and specific knockdown in U251MG cells was monitored by RT-PCR (Fig. 5A) and Western blot analysis of cell lysates (Fig. 5B) after transfection with siRNA. As shown in Fig. 5C, siRNA to ADAM10 sup-
pressed CD44 cleavage induced by ligation of CD44, whereas siRNA to ADAM17 did not. These results indicate that ADAM10 is involved in CD44 cleavage induced by ligation of CD44.

Engagement of CD44 Induces Membrane Ruffling and Accumulation of CD44 in the Ruffling Area—When the cells were treated with IM7 mAb, the cells changed their morphology extending broad lamellipodial membrane protrusions (Fig. 6A). CD44 was redistributed to the membrane ruffling area where polymerized actin was concentrated. We then examined the relationship between the cytoskeletal rearrangement and CD44 cleavage, both of which were induced by ligation of CD44, by disrupting actin polymerization with cytochalasin D. As shown in Fig. 6B, cytochalasin D inhibited the CD44 cleavage induced by IM7 mAb, indicating that the cytoskeletal rearrangement enhances the CD44 cleavage.

Engagement of CD44 Promotes Rac1 Activation, Which Mediates CD44 Cleavage and Cell Migration—mAb-induced cytoskeletal rearrangements shown in Fig. 6 were highly reminiscent of the lamellipodial outgrowth caused by activation of Rac, one of the Rho family small GTPases (12, 28). We therefore speculate that the ligation of CD44 elicits intracellular signals to activate Rac, which causes the membrane ruffling and the lamellipodial protrusion. To confirm this hypothesis biochemically, we performed the pull-down assay for GTP-bound Rac1 (active Rac1), using a recombinant p21-binding domain of PAK1 (PBD) fused with glutathione S-transferase (GST). As shown in Fig. 7A, virtually no active Rac1 was pulled down from lysates of untreated U251MG cells, whereas the amount of active Rac1 was distinctly increased within 5 min of incubation with IM7 mAb, reached a maximum within 10 min, and persisted for at least 30 min. Activation of Rac1 was not enhanced when the cells were incubated with rat IgG, an isotype-matched control IgG (data not shown). Following Rac1 activation, CD44 cleavage was detected significantly within 30 min (data not shown).

Next we determined the causal link of CD44 cleavage to Rac1 activation by introducing dominant negative mutant of Rac1 (N17Rac1) into U251MG cells. As shown in Fig. 7B, the CD44 cleavage induced by its ligation was abrogated in the cells transfected with N17Rac1. Moreover, cell migration enhanced by ligation of CD44 was also inhibited by expression of N17Rac1. These results suggest that CD44 cleavage induced by ligation of CD44 is mediated by Rac1 activation, which up-regulates cell migration.

GTPases of the Rho family cycle between GDP-bound inactive and GTP-bound active forms. The cycle is regulated by the activator, guanine nucleotide exchange factors, and the inactivator, guanine nucleotide dissociation inhibitors, as well as by GTPase-activating proteins that promote hydrolysis of GTP (13). The effects of Rac might be controlled by regulating the subcellular localization of its activation. Thus we developed a method based on FRET (29, 30) that enables us to observe spatial and temporal regulation of Rac1 activity. We designed a fusion protein comprising Rac1 and YFP as illustrated in Fig. 8A and introduced this fusion protein into U251MG cells as a protein biosensor, together with GTP labeled with the acceptor dye BODIPY TR. Binding of BODIPY TR-GTP to YFP-Rac1 brings BODIPY TR close to YFP and increases FRET from YFP to BODIPY TR (Fig. 8A). When U251MG cells harboring YFP-

Fig. 6. Engagement of CD44 induces membrane ruffling and redistribution of CD44. A, immunocytochemical staining of CD44 and actin filament in U251MG cells. U251MG cells were seeded on Lab-teck chamber plate at a density of 2 × 10^6 cells/ml and incubated overnight. After incubation with (lower panels) or without (upper panels) 1 μg/ml IM7 mAb for 1 h, the cells were stained with rhodamine-phalloidin (left panels) and anti-CD44 cyt c-pAb (middle panels) as described under “Experimental Procedures.” Right panels, the merged views. B, U251MG cells were incubated with (lane 3) or without (lanes 1 and 2) 10 μM cytochalasin D at 37 °C for 2 h and further incubated in the presence (lanes 2 and 3) or absence (lane 1) of 1 μg/ml IM7 mAb at 37 °C for 1 h. The cells were then lysed and subjected to Western blot analysis as described under “Experimental Procedures.”
Rac1 and BODIPY TR-GTP were imaged for YFP-Rac1 (515–550 nm) and FRET (615–645 nm), YFP-Rac1 was detected diffusely within the cells, whereas virtually no FRET signals were detected without any stimulation (Fig. 8B), suggesting that Rac1 is localized in the cytosol in its GDP-bound inactive form. When the cells were stimulated with IM7 mAb, membrane protrusion was initiated, and FRET signal distinctly appeared at regions of the leading edge of the cells within 5 min, reached its maximum at 10 min, and persisted at least 30 min (Fig. 8B, upper panels). This time course corresponds to the biochemically acquired data shown in Fig. 7, indicating that Rac1 activation is visualized spatially and temporally. On the other hand, activation of Cdc42 in response to IM7 mAb was not observed by using YFP-Cdc42 (Fig. 8B, lower panels).

**DISCUSSION**

This study shows that CD44 on tumor cells is cleaved upon ligation of CD44 with a mAb and that the cleavage contributes to CD44-mediated tumor cell migration. We also provide evidence that CD44 cleavage induced by engagement of CD44 is mediated by cytoskeletal rearrangement through Rac activation.

We demonstrate that an anti-CD44 mAb IM7 enhances CD44 cleavage from U251MG glioblastoma cells (Fig. 1). Consistent with our findings, Mikecz et al. (31, 32) reported that administration of IM7 mAb to mice with experimental arthritis abrogated inflammation and concomitantly induced increase in serum sCD44 concentration and decrease in cell-surface CD44 of leukocytes. These findings suggest that substantial CD44 cleavage on leukocytes was induced by the anti-CD44 mAb in vivo. They also reported that cleavage of CD44 was induced in fibroblasts derived from mouse synovial tissues and monocytes by incubation with IM7 mAb (33). In addition to IM7 mAb, we tested various mAbs against CD44 (clones 5F12, BJ18, B-F24, B-G15, B-K12, B-R8, and G44–26) (34) and found that only 5F12 could induce CD44 cleavage from U251MG cells.² Because 5F12 inhibits binding of HA to CD44 most strongly among them (26, 34), the cross-linking effect on the ligand binding epitope of CD44 might be important to CD44 cleavage. A major ligand to CD44 is HA (1). In agreement with our previous report (9), HA oligosaccharides enhanced CD44 cleav-

² T. Murai, unpublished observation.
age from U251MG cells, whereas native high molecular weight HA (from human umbilical cord) did not (data not shown). One possible explanation for the difference is that HA oligosaccharides have a strong cross-linking effect on CD44 as the anti-CD44 mAb. Further investigation is required to solve this issue concerning the function of HA, and we thus used IM7 mAb throughout this study as a cross-linking agent specific to CD44.

In addition to HA, we reported previously (35) that versican, a large chondroitin sulfate proteoglycan, is a ligand to CD44. Because U251MG cells have been reported to express versican (36), we attempted to examine whether versican can act as an autocrine inducer to CD44 cleavage. However, the versican, purified from U251MG cells according to the previously described method (37), did not exhibit CD44 cleavage enhancing activity (data not shown). The size of the chondroitin sulfate side chains may be critical for CD44 cleavage induction, as in the case of HA.

The mAb-induced CD44 cleavage from tumor cells is sensitive to certain inhibitors of metalloproteinases (Fig. 2). Kajita et al. (6) reported that CD44 on MIA PaCa-2 cells is spontaneously cleaved by MT1-MMP. In U251MG cells, we found expression of MT1-MMP and MT3-MMP, analyzing by RT-PCR (data not shown). However, based on the present results that mAb-induced CD44 cleavage from U251MG cells is sensitive to TIMP-1 and insensitive to TIMP-2 (Fig. 2), these MT-MMPs are unlikely to have CD44 cleavage activity in this case, because these MT-MMPs can be more efficiently inhibited by TIMP-2 than TIMP-1 (22–24). Other candidates for the CD44 cleavage metalloproteinase are ADAM family enzymes, because several ADAM family proteinases were reported to be the cleavage enzymes for certain membrane proteins: ADAM9 and ADAM12 for heparin-binding epidermal growth factor-like growth factor-like growth factor (38, 39), ADAM10/Kuzbanian for amyloid precursor protein (40) and L1 adhesion molecule (41), and ADAM17/tumor necrosis factor-α converting enzyme for tumor necrosis factor-α, l-selectin, and transforming growth factor-α (42). The facts that U251MG cells express ADAM10 and that ADAM10 can be inhibited by TIMP-1 (27) lead us to speculate that ADAM10 is one of the responsible enzymes for CD44 cleavage in U251MG cells induced by engagement of CD44. In fact, we identified ADAM10 as the responsible proteinase for the CD44 cleavage by using RNAi (Fig. 5). On the other hand, siRNA to ADAM17 did not exhibit any detectable change in the CD44 cleavage, indicating that ADAM17 is not involved in the cleavage significantly, in agreement with the report by Shi et al. (33).

We demonstrate in the present study that ligation of CD44 enhances tumor cell migration in a CD44-dependent fashion.
signaling molecule (14), it is feasible that CD44 itself can introduce signals into the cells. Because CD44 cleavage was induced by PMA, an activator of PKC, we speculate that PKC might be involved in membrane ruffling and lamellipodia. In this study, we demonstrate the spatial and temporal images of Rac activation in response to ligand of CD44 (Fig. 8). It is now extremely important to understand the upstream events close to CD44.

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