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Cell–matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca^{2+} influx and PKC activation

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CD44 is an adhesion molecule that interacts with hyaluronic acid (HA) and undergoes sequential proteolytic cleavages in its ectodomain and intramembranous domain. The ectodomain cleavage is triggered by extracellular Ca^{2+} influx or the activation of protein kinase C. Here we show that CD44-mediated cell–matrix adhesion is terminated by two independent ADAM family metalloproteinases, ADAM10 and ADAM17, differentially regulated in response to those stimuli. Ca^{2+} influx activates ADAM10 by regulating the association between calmodulin and ADAM10, leading to CD44 ectodomain cleavage. Depletion of ADAM10 strongly inhibits the Ca^{2+} influx-induced cell detachment from matrix. On the other hand, phorbol ester stimulation activates ADAM17 through the activation of PKC and small GTPase Rac, inducing proteolysis of CD44. Furthermore, depletion of ADAM10 or ADAM17 markedly suppressed CD44-dependent cancer cell migration on HA, but not on fibronectin. The spatio-temporal regulation of two independent signaling pathways for CD44 cleavage plays a crucial role in cell–matrix interaction and cell migration.

Introduction

CD44 is a type I transmembrane protein and functions as the major cellular adhesion molecule for hyaluronic acid (HA), a component of the ECM. It is expressed in most human cell types and is implicated in a wide variety of physiological and pathological processes, including lymphocyte homing and activation, wound healing, cell migration, as well as tumor cell growth and metastasis (Aruffo et al., 1990; Gunthert et al., 1991; Lesley and Hyman, 1998; Cichy and Pure, 2003).

We reported previously that CD44 undergoes sequential proteolytic cleavages in its ectodomain and intramembranous domain (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200310024/DC1), which play a critical role in cancer cell migration and metastasis and various physiological events (Okamoto et al., 2001). Cleavage of the ectodomain is mediated by membrane-associated matrix metalloproteinases (MMPs) and is regulated by multiple signal pathways, such as extracellular Ca^{2+} influx, the activation of PKC, the Ras oncoprotein, and Rho family small GTPases (Okamoto et al., 1999a; Kawano et al., 2000). Importantly, we found that the ectodomain cleavage of CD44 induced by PKC activation and extracellular Ca^{2+} influx are regulated by independent mechanisms (Okamoto et al., 1999a; Fig. S1 C).

The release of the soluble ectodomain (soluble CD44) from the membrane-bound COOH-terminal fragment (CD44EXT) is responsible for dynamic regulation of the

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Abbreviations used in this paper: HA, hyaluronic acid; HB-EGF, heparin-binding epidermal growth factor–like growth factor; MEF, mouse embryonic fibroblast; MMP, matrix metalloproteinase; TFP, trifluoperazine.

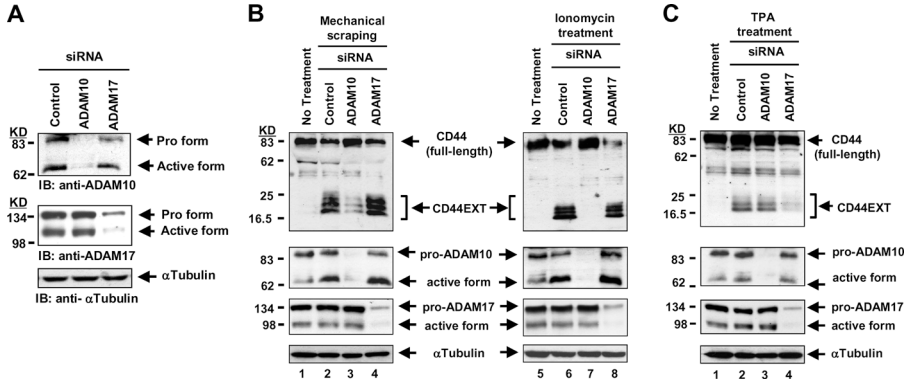

Figure 1. **Distinct regulation of ADAM10 and ADAM17 in the CD44 cleavage induced by different stimulations.** (A) U251MG cells were transfected with control, ADAM10, or ADAM17 siRNAs, as indicated, and cultured for 4 d. Equal amounts of cell lysates were then subjected to immunoblot analysis with antibodies to ADAM10, ADAM17, or α-tubulin. (B) U251MG cells transfected with control, ADAM10, or ADAM17 siRNAs were either subjected to mechanical scraping (left) or treated with 5 μM ionomycin (right) and subsequent incubation for 30 min. Cell lysates were then subjected to immunoblot analysis with antibodies specific for the COOH-terminal region of CD44 (anti-CD44cyto pAb) or with antibodies to ADAM10, to ADAM17, or to α-tubulin (loading control), as indicated. (C) U251MG cells transfected with control, ADAM10, or ADAM17 siRNAs were treated with 100 ng/ml TPA for 60 min (right). Cell lysates were then subjected to immunoblot analysis as in A.

interaction between CD44 and the ECM during cell migration on a HA-containing substratum. The ectodomain cleavage also triggers the intramembrane cleavage of CD44EXT by presenilin-dependent γ-secretase (Murakami et al., 2003) that releases the CD44 intracellular domain (CD44ICD) into the cytoplasm. Furthermore, we found that CD44ICD translocates to the nucleus and activates gene transcription that is mediated through the TPA-responsive element (Okamoto et al., 2002). The ectodomain cleavage event thus initiates the CD44-mediated intracellular signaling pathway.

It has been reported previously that membrane-type 1 MMP forms a complex with CD44 and cleaves the ectodomain at the leading edge of migrating cells (Kajita et al., 2001). However, Ca^{2+} influx inhibits both the conversion of pro–membrane-type 1 MMP to the active form of the protease as well as MMP-2 activation (Yu et al., 1997). Therefore, cleavage of the ectodomain of CD44 triggered by Ca^{2+} influx is likely mediated by other proteases.

CD44 ectodomain cleavage reducing/terminating HA substrate adhesion is markedly enhanced by mechanical stimulation of cells that induces the influx of extracellular Ca^{2+} (Okamoto et al., 1999a). Calcium regulates many of the molecular processes that are required for cell movement (Strohmeier and Bereiter-Hahn, 1984), e.g., in wound healing. Extracellular Ca^{2+} influx was recently shown to promote the shedding not only of CD44 but also of several other membrane proteins including heparin-binding epidermal growth factor–like growth factor (HB-EGF; Dethlefsen et al., 1998) and E-cadherin (Ito et al., 1999). The influx of extracellular Ca^{2+} thus triggers cell proliferation and the disassembly of cell–cell and cell–substratum adhesion complexes during cell motility. However, the precise molecular mechanism by which Ca^{2+} influx induces membrane protein cleavage has remained unknown.

Here, we demonstrate that the Ca^{2+} influx-induced ectodomain cleavage is mediated by ADAM10 metalloproteinase and that ADAM10 activity is regulated by the association with CaM. Contrarily, CD44 ectodomain cleavage induced by phorbol ester stimulation is mediated by ADAM17 through the activation of PKC and small GTPase Rac. Thus, our data indicate that CD44-mediated cell–matrix adhesion is terminated by two independent ADAM family metalloproteinases, ADAM10 and ADAM17, which are differentially regulated in response to Ca^{2+} influx and PKC activation.

Results

ADAM10 activity is involved in CD44 ectodomain cleavage

To characterize the enzymes responsible for cleavage of the CD44 ectodomain, we first tested the effects of various inhibitors of metalloproteinases in a cell-free assay of CD44 cleavage which we developed previously (Ito et al., 1999; Okamoto et al., 1999b). Crude membranes isolated from U251MG human glioma cells, which possess substantial CD44-cleaving activity, were incubated for 120 min at 37°C and subjected to immunoblot analysis with antibodies specific for the COOH-terminal region of CD44 (anti-CD44cyto pAb). Such incubation increased the abundance of a membrane-bound COOH-terminal fragment (CD44EXT; Fig. S2 A, lane 2, available at http://www.jcb.org/cgi/content/full/jcb.200310024/DC1), and the ectodomain cleavage was blocked by the MMP inhibitor BB94 (10 μM; Fig. S2 A, lane 3) and the Ca^{2+} chelator EGTA (10 mM; Fig. S2 B, lane 4), indicating that the reaction is mediated by an MMP and is sensitive to Ca^{2+} concentration in this cell-free system. The cleavage reaction was inhibited by tissue inhibitor of metalloproteinase (TIMP)-1 and by TIMP-3, but it was not affected by TIMP-2 (Fig. S2 A, lanes 4–6). Given that the catalytic activities of all known MMPs, with the exception of ADAM (a disintegrin and metalloprotease domain) family proteins, are inhibited by TIMP-2 (Nath et al., 2001), our results implicated either an unknown MMP or an ADAM proteinase in the Ca^{2+}-dependent cleavage of the CD44 ectodomain.

The activity of ADAM10 is inhibited by TIMP-1 or TIMP-3 (Amour et al., 2000) and ADAM17 (also known as TNF-α–converting enzyme, or TACE) is inhibited by TIMP-3 (Amour et al., 1998; Doedens et al., 2003). Activities of these two ADAMs are not inhibited by TIMP-2. Therefore, to test whether the activity of ADAM10 or ADAM17 mediates CD44 ectodomain cleavage, we attempted to knockdown ADAM10 or ADAM17 by application of RNA interference

in U251MG cells. Immunoblot analysis showed that expression of ADAM17 was not affected by ADAM10 depletion, and vice versa (Fig. 1 A). The CD44 ectodomain cleavage after the incubation at 37°C was markedly inhibited in the membrane fraction prepared from the ADAM10-depleted cells (Fig. S2 B, lane 6) but not in that derived from cells depleted of ADAM17 (Fig. S2 B, lane 8), suggesting that ADAM10 activity mainly mediates this reaction.

ADAM10 and ADAM17 regulate CD44 ectodomain cleavage in response to distinct stimulations

We next examined the roles of ADAM10 and ADAM17 in CD44 ectodomain cleavage in living cells. Depletion of ADAM10, but not that of ADAM17, markedly inhibited the ectodomain cleavage induced by mechanical scraping which leads to extracellular Ca^{2+} influx (Fig. 1 B, left). The ectodomain cleavage induced by exposure to the Ca^{2+} ionophore ionomycin was also inhibited by ADAM10 depletion (Fig. 1 B, right). These results suggested that ADAM10 is responsible for the Ca^{2+} influx-induced CD44 ectodomain cleavage.

As demonstrated in Fig. S1 C, CD44 ectodomain cleavage is triggered not only by Ca^{2+} influx but also by the activation of PKC, and that these two stimuli achieve their effects by distinct signaling pathways. Thus, we examined whether ADAM10 also mediates the PKC-dependent CD44 ectodomain cleavage. Interestingly, depletion of ADAM10 had very little effect on the TPA-induced production of CD44EXT (Fig. 1 C, lane 3). In contrast, depletion of ADAM17 significantly inhibited TPA-induced cleavage of CD44 (Fig. 1 C, lane 4), which is related to the recent observation that phorbol ester PMA (phorbol 12-myristate 13-acetate) induces ADAM17-dependent proteolysis of a neurotrophin receptor TrkA (Diaz-Rodriguez et al., 2002) and TNF-α processing site peptide (Doedens et al., 2003). These results thus suggest that the Ca^{2+} influx-induced cleavage of the CD44 ectodomain is mediated by ADAM10, whereas that induced by PKC activation is mediated by ADAM17.

Ca^{2+} influx-induced CD44 ectodomain cleavage is inhibited in ADAM10-deficient mouse embryonic fibroblasts (MEFs)

To examine further the participation of ADAM10 in the Ca^{2+} influx-induced CD44 cleavage, we used ADAM10-deficient ($ADAM10^{-/-}$) MEFs (Fig. 2 A; Hartmann et al., 2002). Immunoblot analysis showed that the amount of full length CD44 was significantly reduced and CD44 EXT was increased by extracellular Ca^{2+} influx in wild-type MEFs (Fig. 2 B, lane 3). However, CD44 EXT was hardly detected in $ADAM10^{-/-}$ MEFs by mechanical scraping (Fig. 2 B, lane 4). Because the expression levels of endogenous CD44 is very low in MEFs, we confirmed the role of ADAM10 in the CD44 cleavage by transfecting the wild-type and $ADAM10^{-/-}$ MEFs with an expression vector encoding full-length human CD44 (Fig. 2 C). The ectodomain cleavage of the exogenous human CD44 was induced by mechanical scraping in the wild-type and $ADAM10^{+/-}$ MEFs but not in $ADAM10^{-/-}$ MEFs. Moreover, we transfected the mouse ADAM10 expression plasmid (unpublished data) back into

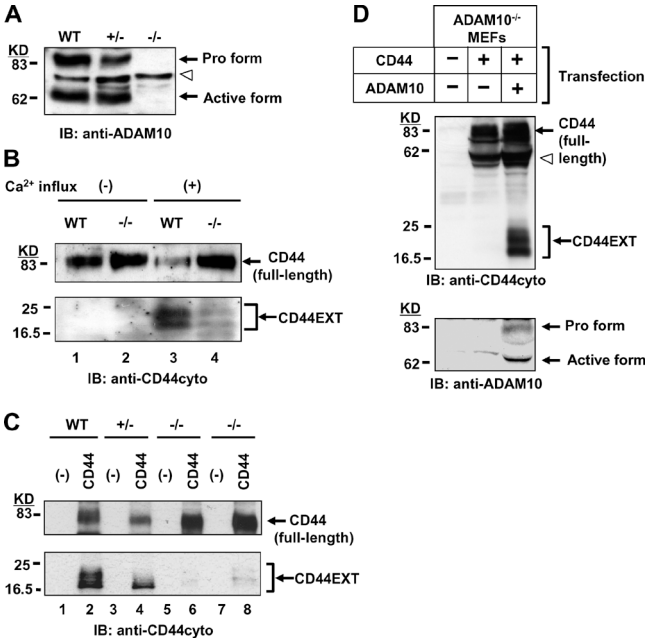

Figure 2. **Ca^{2+} influx-induced CD44 cleavage is inhibited in ADAM10 knockout MEFs ($ADAM10^{-/-}$ MEFs).** (A) Expression of pro form and mature (active) form of ADAM10 in wild-type (WT), $ADAM10^{+/-}$, or $ADAM10^{-/-}$ MEFs. Open arrowhead indicates a nonspecific band. (B) Wild-type (WT) or $ADAM10^{-/-}$ MEFs were induced extracellular Ca^{2+} influx by mechanical scraping. After the scraping in the presence or absence of 10 mM EGTA, membrane fractions of these cells were collected and lysed with Laemmli sample buffer. The lysates were subjected to immunoblot analysis with anti-CD44cyto pAb. (C) Wild-type (WT), $ADAM10^{+/-}$, or $ADAM10^{-/-}$ MEFs were transiently transfected with either an expression plasmid for full-length human CD44 or the empty vector (−). 2 d after transfection, the cells were subjected to mechanical scraping, incubated for 30 min, lysed, and immunoblotted with anti-CD44cyto pAb. Results are shown for two independent transfections of $ADAM10^{-/-}$ MEFs. (D) $ADAM10^{-/-}$ MEFs were cotransfected with an expression vector for full-length human CD44 and an expression plasmid for mouse ADAM10. At 24 h after transfection, the cells were subjected to mechanical scraping, incubated for 30 min, lysed, and immunoblotted with anti-CD44cyto pAb (top) or anti-ADAM10 (bottom). Open arrowhead indicates incompletely glycosylated CD44 protein.

$ADAM10^{-/-}$ MEFs and examine the CD44 cleavage. As shown in Fig. 2 D, the expression of ADAM10 reconstituted the Ca^{2+} influx-induced CD44 ectodomain cleavage in $ADAM10^{-/-}$ MEFs (Fig. 2 D). All these results indicate that ADAM10 is the candidate sheddase for CD44 ectodomain cleavage in response to extracellular Ca^{2+} influx.

Extracellular Ca^{2+} influx and PKC activation differentially activate ADAM10 and ADAM17, respectively

We then examined the effect of Ca^{2+} influx on the activation of ADAM10 by immunoblot analysis with antibodies to this protease. ADAM10 exists as an ~97-kD inactive, pro form that is converted to the 68-kD active, mature form by cleavage at a consensus proprotein convertase cleavage motif (Anders et al., 2001). Prodomain removal is thought to be a prerequisite for ADAM10 activity. Calcium influx, triggered by ionomycin, resulted in a marked increase in the amount of

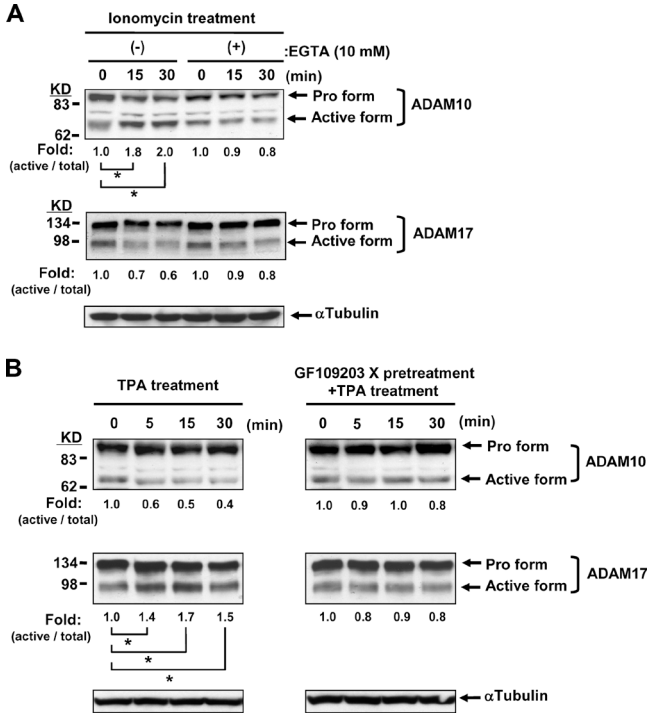

Figure 3. **Activation of ADAM10 and ADAM17 are induced by Ca^{2+} influx and PKC activation, respectively.** (A) U251MG cells were either left untreated or subjected to incubate with 5 μM ionomycin for 15 or 30 min in the absence or presence of 10 mM EGTA. Cells were then lysed and subjected to immunoblot analysis with anti-ADAM10, anti-ADAM17, or α-tubulin. The intensities of the bands were measured with MacBAS2000 (Fuji Film), and the active form/total ADAM10 (or ADAM17) ratios were calculated. The numbers under each lane were expressed as a fold relative to control cells and were presented as mean values from three independent experiments. Statistical differences were determined with *t* test; *, $P < 0.01$. (B) U251MG cells were incubated with 10 μM BB94 containing medium for indicated times in the absence or presence of 100 ng/ml TPA or 2.5 μM GF109203X as indicated. Cell lysates were then subjected to immunoblot analysis with antibodies to ADAM10 (top) or ADAM17 (middle). The active form/total ADAM10 (or ADAM17) ratios were calculated as shown in A. The numbers under each lane were expressed as a fold relative to control cells and were presented as mean values from three independent experiments. Statistical differences were determined with *t* test; *, $P < 0.01$.

the active form of ADAM10 in U251MG cells (Fig. 3 A; Fig. 1 B). Conversion of pro-ADAM10 to the active form induced by ionomycin was inhibited in the presence of 10 mM EGTA, indicating that extracellular Ca^{2+} influx activates ADAM10. Furthermore, in the course of this experiment, we found that TPA treatment did not induce the conversion of pro-ADAM10 to the active form (Fig. 3 B, top left; Fig. 1 C). These results suggest that ADAM10 is activated by extracellular Ca^{2+} influx but not by PKC activation.

Next, we examined the effect of TPA on ADAM17 activation in U251 MG cells. It has been reported recently that PMA increases enzymatic activity of ADAM17 and subsequently active form of ADAM17 undergoes degradation (Doedens et al., 2003). In fact, active form of ADAM17 is degraded in time-dependent manner by TPA treatment in the absence of BB94 (unpublished data). Therefore, to visualize the active form of ADAM17, a metalloproteinase inhibitor needs to be present in the samples prepared for immunoblot analysis. In the presence of BB94, the active form of ADAM17 could be detected by pAbs raised against cytoplasmic domain of ADAM17, and was accumulated in response to TPA within 5–15 min, suggesting that TPA treatment induced ADAM17 activation (Fig. 3 B, middle left). However, accumulation of active ADAM17 was significantly impaired in GF 109203X pretreated U251 MG cells (Fig. 3 B, middle right). In addition, the active form of ADAM17 was not increased but rather reduced in cells treated with ionomycin (Fig. 3 A). Those data thus suggest that Ca^{2+} influx and PKC activation differentially activate ADAM10 and ADAM17, respectively.

CaM inhibitor triggers ADAM10-mediated CD44 cleavage

To elucidate further the mechanism responsible for the Ca^{2+} influx-induced cleavage of CD44, we screened various cell-permeable inhibitors of Ca^{2+} signaling molecules for the ability to affect this process. Neither 50 μM KN93, an inhibitor of Ca^{2+}- and CaM-dependent protein kinase II, nor the calpain inhibitor Zlla1 (5–100 μM) affected CD44 ectodomain cleavage induced by mechanical scraping (unpublished data). In contrast, the CaM inhibitor trifluoperazine (TFP) markedly promoted both the conversion of the pro form of ADAM10 to the active form as well as CD44 ectodomain cleavage in the absence of extracellular Ca^{2+} influx (Fig. 4 A). Moreover, the CD44 ectodomain cleavage induced by TFP was inhibited by depletion of ADAM10, but not by that of ADAM17, in U251MG cells. These findings suggest that inhibition of CaM-mediated signals activates ADAM10 protease, leading to the ectodomain cleavage of CD44. This hypothesis was also supported by the data that TFP treatment induces CD44 cleavage in wild-type MEFs but not in ADAM10-deficient MEFs (Fig. 4 B).

To further investigate the possibility of CaM-mediated inhibition of the CD44 ectodomain cleavage, we examined the effect of CaM on the cell-free assay of CD44 cleavage, which is mediated by ADAM10 activity as shown in Fig. S2 B. Incubation of membrane fractions of U251MG cells with purified CaM significantly impaired CD44 ectodomain cleavage (Fig. 4 C, lanes 4 and 5). However, incubation of membrane fractions with 100 μM TFP enhances CD44 ectodomain cleavage (Fig. 4 C, lane 6) and further CaM-mediated inhibition of CD44 cleavage was significantly impaired in the presence of TFP (Fig. 4 C, lane 7) These observations thus suggested a role for CaM in the regulation of ADAM10-mediated cleavage of the ectodomain of CD44. In fact, immunoblot analysis of CaM-bound proteins with anti-ADAM10 revealed that pro form of ADAM10 specifically associated with CaM in low Ca^{2+} condition (concentration: 10^{-7}M; Fig. 4 D), whereas ADAM17 was not detected in the CaM-binding fraction. These findings suggest that CaM interacts with pro form of ADAM10 to inhibit the activation. Furthermore, this in vitro interaction between ADAM10 and CaM was impaired in the presence of 100 μM TFP (unpublished data). On the basis of these observations, we propose that activation of ADAM10 is regulated through a modification of the CaM–ADAM10 association.

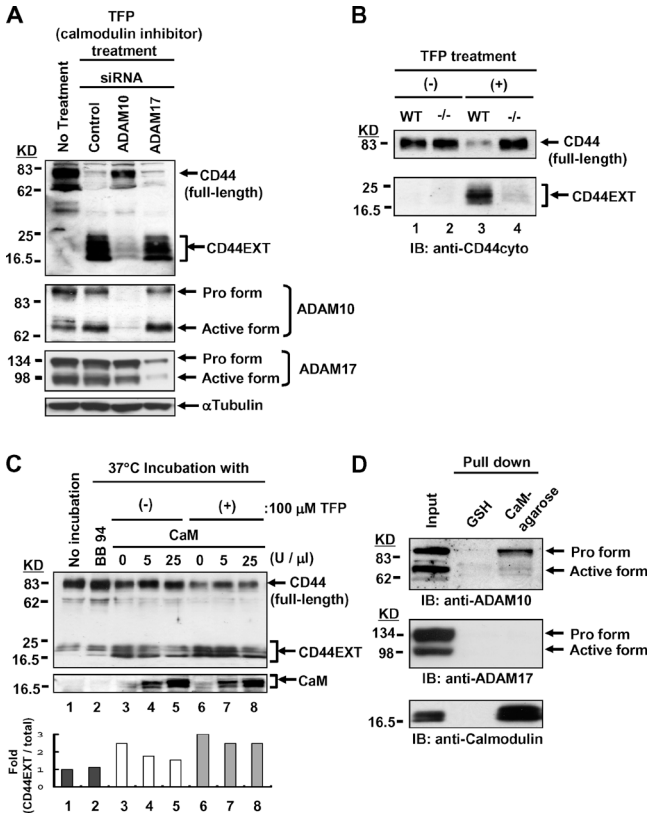

Figure 4. **Interaction between CaM and ADAM10 regulates CD44 ectodomain cleavage.** (A) U251MG cells transfected with control, ADAM10, or ADAM17 siRNAs were incubated with 100 μM TFP, a CaM inhibitor, for 30 min, after which cell lysates were prepared and subjected to immunoblot analysis with anti-CD44cyto pAb or with antibodies to ADAM10, ADAM17, or α-tubulin as indicated. (B) Wild-type (WT) or ADAM10$^{-/-}$ MEFs were incubated with or without 100 μM TFP for 30 min. After the incubation, membrane fractions of those cells were collected with hypotonic buffer containing 10 μM BB94 and 10 mM EGTA. Collected membrane fractions were lysed with Laemmli sample buffer and subjected to immunoblot analysis with anti-CD44cyto pAb. (C) Membrane fractions isolated from U251MG cells were incubated for 2 h at 37°C (lanes 2–8) in the presence of 10 μM BB94 or indicated amounts of bovine brain CaM with or without 100 μM TFP. All samples were then subjected to immunoblot analysis with anti-CD44cyto pAb (top) or anti-CaM antibody (bottom). The intensities of the CD44 bands were measured with MacBAS2000, and the CD44EXT/total CD44 ratios were calculated. The bars under each lane were expressed as a fold relative to control cells. Note that the in vitro CD44 cleavage was inhibited by CaM in a dose-dependent manner (lanes 3–5) but that treatment with TFP significantly blocked the effect of CaM (lanes 6–8). (D) Agarose beads conjugated with either CaM or glutathione (GSH; negative control) were incubated with U251MG cell lysates in the presence of 0.1 μM $CaCl_2$. The input cell lysates and the beads-bound proteins were then subjected to immunoblot analysis with antibodies to ADAM10 (top), ADAM17 (middle), or CaM (bottom).

ADAM10 is responsible metalloproteinase for extracellular Ca^{2+} influx-induced cell detachment from HA matrix

Ca^{2+} influx induces the disassembly of cell–substratum adhesions and is required for detachment of the rear cell margin in locomoting cells (Lee et al., 1999). Therefore, we examined whether the ADAM10 activation induced by Ca^{2+} influx affects cell–substratum adhesion. Ionomycin induced the rapid detachment of U251MG cells from an HA matrix into culture medium and this effect was markedly inhibited by the MMP inhibitor BB94 (Fig. 5, A and D). This effect of ionomycin was observed in U251MG cells transfected with control or ADAM17 siRNAs (Fig. 5, A, C, and D) but not in ADAM10-depleted cells (Fig. 5, B and D). Furthermore, the ionomycin-induced cell detachment was inhibited in ADAM10$^{-/-}$ MEFs in comparison to wild-type and ADAM10$^{+/-}$ MEFs (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200310024/DC1). These data indicate that ADAM10-mediated cleavage of adhesion molecules including CD44 is required for cells to detach from matrix in response to extracellular Ca^{2+} influx.

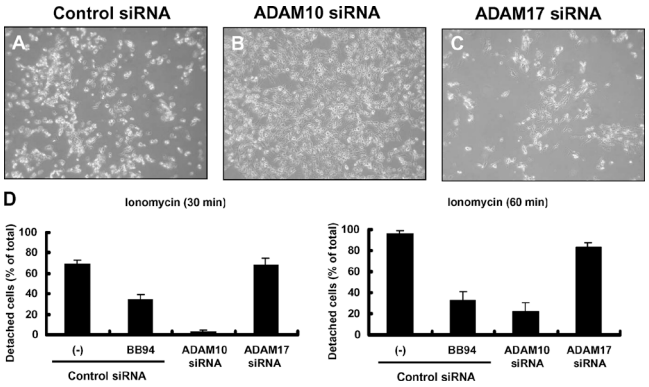

Figure 5. **Inhibition of Ca^{2+} influx-induced cell detachment from an HA matrix by depletion of ADAM10.** (A–C) U251MG cells (2×10^4) were seeded on HA-coated dishes and incubated for 24 h before transfection with (A) control, (B) ADAM10, or (C) ADAM17 siRNAs. 4 d after transfection, the cells were incubated in the presence of 5 μM ionomycin for 30 min and examined by phase-contrast microscopy. (D) Cells transfected with control, ADAM10, or ADAM17 siRNAs as in A–C were incubated with ionomycin for 30 or 60 min in the absence or presence of 10 μM BB94, as indicated, after which floating cells were collected from the culture medium and attached cells were harvested by exposure to trypsin. The numbers of both detached and attached cells were determined with a cell counter, and the number of detached cells was expressed as a percentage of the total cell number. Data are means ± SEM of values from three to five independent experiments.

Small GTPase Rac is involved in ADAM17-mediated CD44 ectodomain cleavage in response to TPA stimulation

TPA treatment induces the redistribution of CD44 to the newly formed membrane ruffling areas, leading to the CD44 ectodomain cleavage in U251 MG cells (Okamoto et al., 1999a). Thus, we investigated the subcellular localization of ADAM17 after TPA treatment. As described previously (Schlondorff and Blobel, 1999), ADAM17 was abundant in the perinuclear region in U251MG cell without any treatment. However, ADAM17 was redistributed to the ruffling areas by TPA treatment and colocalized with CD44 within 10 min (Fig. 6 A).

As reported recently (Burridge and Wennerberg, 2004), TPA treatment induced an increase in the GTP-bound Rac within 5 min, whereas 5 μM ionomycin treatment did not (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/

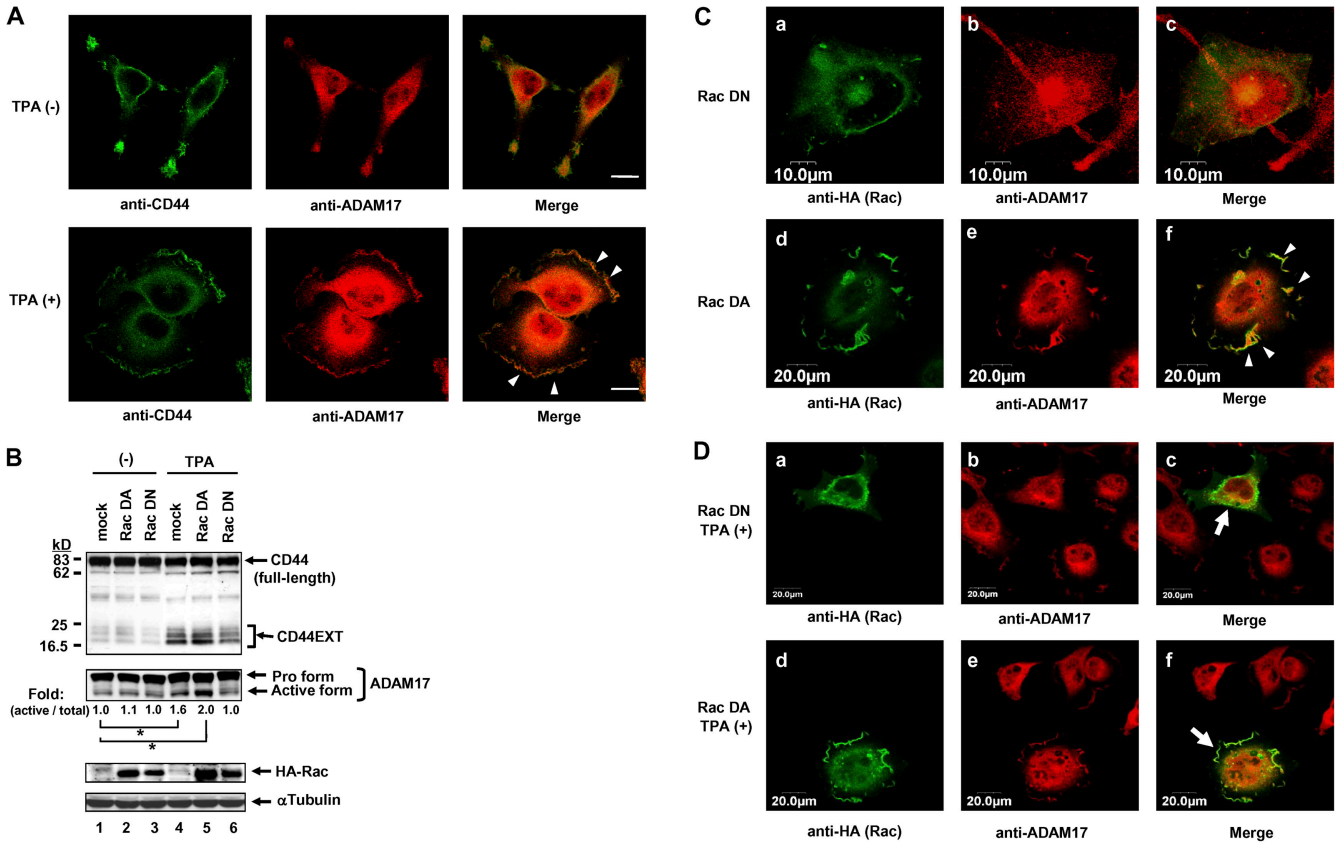

Figure 6. **Small GTPase Rac is involved in TPA-induced ADAM17 activation and CD44 cleavage.** (A) U251 MG cells were incubated with or without 100 ng/ml of TPA for 5 min and cells were double stained with anti-HA or ADAM17 antibody. Bar, 20 μm. Arrowheads indicate redistribution of CD44 and ADAM17 at the TPA-induced ruffling membrane areas. (B) U251 MG cells were transfected with mock vector, HA-Rac1V12 (a dominant active form of Rac1 indicated as RacDA), or HA-Rac1N17 (a dominant negative form of Rac1 indicated as RacDN). At 24 h after transfection, cells were incubated with 100 ng/ml of TPA for 20 min and subjected to immunoblot analysis with anti-CD44cyto, ADAM17, HA (12CA5), and α-tubulin. Note that TPA-induced CD44 cleavage and ADAM17 activation were significantly inhibited by expression of RacN17 (DN) (lane 6). The intensities of the bands were measured with MacBAS2000, and the active form/total ADAM17 ratios were calculated. The numbers under each lane were expressed as a fold relative to control cells and were presented as mean values from three independent experiments. Statistical differences were determined with *t* test; *, $P < 0.01$. (C) The cells transfected with Rac N17 (DN) (a–c) or RacV12 (DA) (d–f) were fixed at 24 h after the transfection, and double stained with anti-HA and anti-ADAM17. Note that expression of RacV12 (DA) induced ADAM17 relocalization on the ruffling membrane (d–f, arrowheads). (D) The cells transfected with Rac N17 (DN) (a–c) or RacV12 (DA) (d–f) were incubated with TPA for 5 min and double stained with anti-HA and anti-ADAM17. Note that TPA-induced membrane localization of ADAM17 was inhibited by expression of Rac N17 (DN) (a–c). Arrows indicate RacV12 (DA) or Rac N17 (DN) expressed cells.

jcb.200310024/DC1). We also found previously that Rac is involved in TPA-induced CD44 cleavage (Okamoto et al., 1999a) and confirmed that expression of dominant negative Rac inhibited the TPA-induced ruffling membrane formation and the CD44 relocalization (Fig. S4 B). These observations prompted us to test the hypothesis that Rac activation is involved in the TPA/PKC-induced, ADAM17-mediated CD44 cleavage. Immunoblot analysis revealed that ectodomain cleavage of CD44 in response to TPA is markedly inhibited in U251MG cells transfected with Rac1N17, a dominant negative form of Rac1 (RacDN; Fig. 6 B, lane 6) but not in those transfected with Rac1V12, a dominant active form of Rac1 (RacDA; lane 5). Furthermore, the conversion of pro ADAM17 to active form after the TPA treatment was significantly reduced in Rac1N17 transfected cells (Fig. 6 B, lane 6) although it was rather enhanced in Rac1V12 transfected cells (lane 5). These results suggest that the activation of Rac is required for TPA-induced ADAM17 activation.

Next, we analyzed the involvement of Rac activation in ADAM17 localization. Expression of dominant active Rac induced ADAM17 relocalization on the ruffling membrane (Fig. 6 C, d–f), and TPA-induced membrane localization of ADAM17 (Fig. 6 D) was clearly inhibited in cells expressing dominant negative Rac (Fig. 6 D, a–c). These data indicate that GTP bound form of Rac facilitates membrane localization of ADAM17 and plays essential role in TPA-induced CD44 cleavage.

ADAM10- and ADAM17-mediated CD44 ectodomain cleavages are both required for cancer cell migration on HA

Interaction of CD44 with HA is involved in response of many cell types to inflammation, wound healing and cancer cell migration (Oksala et al., 1995; Okamoto et al., 1999b; Bourguignon et al., 2000; Tammi et al., 2001). We investigated the role of ADAM10 or ADAM17 in CD44-depen-

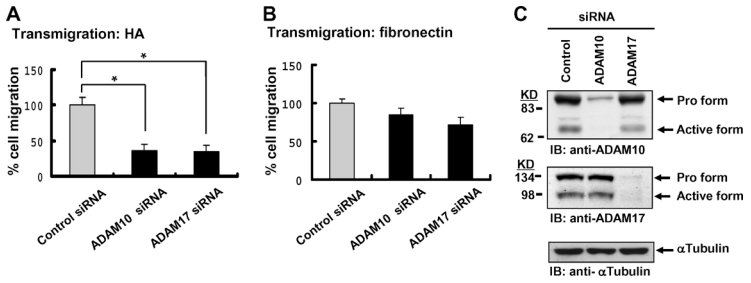

Figure 7. **Effects of ADAM depletion on RERF-LC-OK cells migration on HA.** (A) The effects of ADAM10- or ADAM17-depletion on migration of RERF-LC-OK cells on HA were assessed by modified Boyden chamber-type migration assays. RERF-LC-OK cells were transfected with control, ADAM10, or ADAM17 siRNAs and cultured for 4 d before cells were subjected to migration assays. Columns and error bars represent the mean and SD obtained from three independent experiments. Statistical differences were determined with *t* test; *, $P < 0.01$. (B) Migration of RERF-LC-OK cells on fibronectin was assessed by the migration assays as in A. (C) Inhibition of ADAM10 or ADAM17 expression in RERF-LC-OK cells by RNA interference. Cells were transfected with control, ADAM10, or ADAM17 siRNAs and cultured for 4 d. Equal amounts of cell lysates were then subjected to immunoblot analysis with antibodies to ADAM10 or to ADAM17.

dent cell migration on HA with an in vitro transwell migration assay in cultures of RERF-LC-OK human lung adenocarcinoma cells. In these cells, CD44 cleavage was previously shown to play a critical role in migration on HA (Okamoto et al., 1999a). Treatment with a metalloprotease inhibitor significantly reduced RERF-LC-OK cell migration through HA-coated membrane, demonstrating a requirement for MMP activity in migration on HA. As shown in Fig. 7, depletion of ADAM10 or ADAM17 markedly reduced cell migration through HA-coated membrane although cell migration was not inhibited when fibronectin-coated membrane was used. These results thus suggest that the CD44 ectodomain cleavages mediated by ADAM10 and ADAM17 contribute to cancer cell motility on HA matrix.

Discussion

ADAM10 and ADAM17 are responsible for CD44 cleavage

A large variety of membrane proteins, including collagen XVII (Franzke et al., 2002), β-amyloid precursor protein (Buxbaum et al., 1998), cellular prion protein (Vincent et al., 2001), CX3CL1 (Hundhausen et al., 2003), and HB-EGF (Yan et al., 2002) have been determined to be substrates for ADAM family proteases. However, the relations between the nature of the stimulus and the specific ADAM proteases responsible for the cleavage of these proteins are largely unknown.

It has been reported that ADAM10 and ADAM17 have sequence similarity and share several substrates (Rosendahl et al., 1997; Vincent et al., 2001; Hundhausen et al., 2003). Moreover, ADAM10 and ADAM17 have been reported recently to have critical role in shedding of six different EGFR ligands (TGF-α, amphiregulin, epiregulin, HB-EGF, betacellulin, and EGF) in MEFs (Sahin et al., 2004). Here, we have shown that cleavage of the ectodomain of CD44 is mediated by ADAM10 and ADAM17, and that the activation of these proteases is triggered by distinct stimuli and signaling pathways (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200310024/DC1).

CaM regulates ADAM10-mediated CD44 proteolysis

We have shown that extracellular Ca^{2+} influx induces ADAM10-mediated CD44 cleavage and that CaM inhibitor TFP, which have high affinity to Ca^{2+}/CaM, activates the ADAM10-mediated CD44 cleavage without Ca^{2+} influx. Because we found that pro-ADAM10 interacts with CaM and that the interaction is disrupted by TFP, we hypothesize that Ca^{2+} influx triggers the dissociation of CaM from pro-ADAM10, resulting in the activation of ADAM10. Recently, it was reported that the association between CaM and its binding proteins is defined by the affinities, Ca^{2+} sensitivities and concentrations of all CaM-binding protein in the cell (Tran et al., 2003). Furthermore, it was shown that increasing intracellular Ca^{2+} concentration decreased the amounts of membrane bound CaM in neuroblastoma cells (McGinnis et al., 1998). These observations suggest that CaM may change their localization and substrates by alteration of cytosolic Ca^{2+} concentration. In fact, studies using confocal imaging of CaM labeled with a fluorescent probe showed that the influx of extracellular Ca^{2+} induces redistribution of CaM within the cell (Liao et al., 1999) and that spikes of increasing Ca^{2+} concentration trigger spikes of decreasing cytosolic CaM concentration by translocation of CaM to the secretory granule region or nucleoplasm (Craske et al., 1999; Ashby and Tepikin, 2002). Therefore, the simplest model would be that CaM constitutively associates with pro-ADAM10 in resting cells at low affinity (intracellular Ca^{2+} concentration: 10^{-7}–10^{-6} M) and a rapid decrease in the local cytosolic concentration of CaM by altering their substrates and localization after Ca^{2+} influx (extracellular Ca^{2+} concentration: $\sim 10^{-3}$ M) may promote dissociation of CaM from ADAM10, resulting in activation of ADAM10 and the consequent proteolysis of CD44. The CaM inhibitor TFP has been reported to induce the ectodomain cleavage of several other membrane proteins, such as L-selectin (Kahn et al., 1998), TrkA and proTGFα (Diaz-Rodriguez et al., 2000), raising the possibility that these TFP-sensitive membrane proteins are also cleaved by activated ADAM10.

ADAM10 plays a critical role in Ca^{2+} influx-induced cell detachment from matrix

Ca^{2+} regulates many processes that are required for cell movement, including production of actomyocin-based contractile forces, regulation of the actin cytoskeleton, formation, and disassembly of cell substratum interaction (Strohmeier and Bereiter-Hahn, 1984; Lee et al., 1999). Thus, extracellular Ca^{2+} influx is considered to contribute high cell motility through impairing cell–matrix adhesion. However little is known about the roles of extracellular proteolysis of adhesion molecules triggered by extracellular Ca^{2+} influx in the release of cell–matrix interaction. We have now demon-

strated that extracellular Ca^{2+} influx induces detachment of U251MG cells from HA matrix through ADAM10-dependent proteolysis (Fig. 5). This result suggests that ADAM10 is the responsible enzyme for the termination of links between adhesion molecules and matrix in response to extracellular Ca^{2+} influx.

Small GTPase Rac regulates subcellular localization and activity of ADAM17

We previously showed that the Rho family of small G proteins including Rho and Rac are involved in TPA/PKC-induced redistribution and cleavage of CD44 (Okamoto et al., 1999a). Here, we have shown that Rac activation triggered by TPA treatment induces redistribution of ADAM17 to ruffling areas and that the expression of dominant negative Rac inhibits the TPA-induced ADAM17 redistribution and activation. It was reported that pro-ADAM17 is processed and converted to the active form in the secretory pathway and, thereby, the active form of ADAM17 is detected on the plasma membrane whereas the majority of pro-ADAM17 localizes to a perinuclear compartment (Schlondorff and Blobel, 1999). This observation together with our findings suggests that TPA-mediated Rac activation promotes the redistribution of both pro form of ADAM17 and CD44 to the plasma membrane, leading to ADAM17 activation and clustering of the activated protease and substrate, which might be required for triggering the proteolytic processing.

It should be noted that the expression of a dominant active form of Rac1 (Rac1V12) did not induced CD44 cleavage in the absence of TPA stimulation (Fig. 6 B, lane 2). This result suggests that activation of Rac is required for ADAM17-mediated CD44 cleavage, but Rac activation alone is not sufficient for the induction of cleavage without PKC activation. We speculate that both Rac and PKC activation, which are induced by TPA treatment, are necessary for the activation of ADAM17 to cleave CD44.

Biological significance of CD44 ectodomain cleavage differentially regulated by ADAM10 and ADAM17 in cancer cell migration on HA

HA contributes to cell migration as a structural component of the extracellular space, creating a highly hydrated, elastic matrix that may help cell movement by facilitating detachment and providing space for migration (Tammi et al., 2001). CD44 ectodomain cleavage has shown to require for efficient cancer cell migration on HA matrix (Okamoto et al., 1999b). Our present observation suggests that CD44 ectodomain cleavages mediated by ADAM10 and ADAM17 are equally required for efficient migration of cancer cells on HA (Fig. 7). Although the precise mechanisms by which ADAM10 and ADAM17 are activated to regulate the cell migration on HA matrix remain to be elucidated, we speculate that this process is achieved by two sequential steps: (1) activation of Rac at leading edge of cells induces redistribution and activation of ADAM17, leading to efficient cell crawling on HA through CD44 ectodomain cleavage; and (2) this mechanical stretching of cell is known to induce extracellular Ca^{2+} influx through the stretch activated Ca^{2+} channel (Lee et al., 1999), resulting in rapid activation of ADAM10 followed by CD44 cleavage, facilitating cell detachment from HA at rear end of the migrating cell.

CD44 ectodomain cleavage plays critical roles in not only cancer invasion and metastasis but also various physiological events, such as wound healing, lymphocyte homing, cell migration, and cell proliferation. Therefore, the simple approaches to reduce the cleavage by metalloproteinase inhibition may not be applied to the therapy because diverse physiological processes are potentially impaired. The existence of independent–differential pathways for CD44 cleavage may permit us to develop selective therapeutical approaches to suppress cancer invasion and metastasis.

Materials and methods

Cell culture and reagents

U251MG and RERF-LC-OK cells were grown in DME F12 supplemented with 10% FCS. MEF cell lines from ADAM10$^{-/-}$ mice and respective wild-type mice were generated and characterized as described previously (Hartmann et al., 2002). MEFs were cultured in DME containing 10% FCS. pAb against the cytoplasmic domain of CD44, anti-CD44cyto pAb was generated as described previously (Okamoto et al., 1999a). The mAb directed against the ectodomain epitope of CD44 (BU52) was obtained from Ancell. Anti-ADAM10 antibody directed against cytoplasmic domain was purchased from Calbiochem. Anti-ADAM17 antibody directed against cytoplasmic domain was purchased from CHEMICON International, Inc. and Calbiochem. Anti-ADAM17 (H-300) was obtained from Santa Cruz Biotechnology. Anti-CaM and anti-Rac were purchased from Upstate Biotechnology. The anti-HA mAb was prepared from 12CA5 cells. Materials were obtained as follows: recombinant hTIMP-1, -2, and -3 were provided by K. Iwata (Daiichi Fine Chemical Industries Ltd., Takaoka, Japan). Purified bovine brain CaM were obtained from Sigma-Aldrich; PAK-1 PBD agarose was purchased from Upstate Biotechnology; GF109203X, ionomycin, and TFP were purchased from Calbiochem; and BB94 (batimastat) was provided by W.G. Stetler-Stevenson (National Institutes of Health, Bethesda, MD).

Cell-free assay of CD44 cleavage

Membrane fractions of U251MG cells were obtained as described previously (Okamoto et al., 1999a) with slight modifications. Cultured U251MG cells were incubated for 15 min on ice with hypotonic buffer (20 mM Hepes-KOH, pH 7.5, 10 mM potassium acetate, 1.5 mM magnesium acetate) supplemented with 10 mM EGTA and a protease inhibitor cocktail (Sigma-Aldrich). They were then disrupted by 30 strokes with a Dounce homogenizer. Nuclei were removed from the homogenate by centrifugation at 2,000 *g* for 5 min at 4°C, and the resulting supernatant was then centrifuged at 10,000 *g* for 1 h at 4°C. The new membrane pellet was suspended in an ice-cold solution of 30 mM tris-HCl, pH 7.2, containing 1 mM $CaCl_2$ or 10 mM EGTA, and various test agents and incubated for 2 h at 37°C. The reaction was stopped by the addition of an equal volume of Laemmli sample buffer and heating at 95°C for 5 min.

Immunoblot analysis

Cells were directly lysed in 2× Laemmli buffer or cell lysis buffer containing 0.1 M DTT. For immunoblot analysis of active form of ADAM10 and ADAM17, 10 μM BB94 were added to the culture medium to block degradation. Equal amounts of protein were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with antibodies. All immunoblots were visualized by Chemiluminescence Reagent Plus (Perkin-Elmer).

Plasmids and siRNA transfection

A cDNA encoding full-length CD44 was constructed as described previously (Okamoto et al., 2001). pEF-BOS-HA- Rac1WT (wild-type Rac1), Rac1V12 (a constitutive active form of Rac1), and Rac1N17 (a dominant negative form of Rac1) plasmids were provided by K. Kaibuchi (Nagoya University, Nagoya, Japan; Kuroda et al., 1996). These expression plasmids were transfected into cells using LipofectAMINE and PLUS Reagent (Life Technologies, Inc.) according to the manufacturer's instruction. The sequences of the siRNAs were as follows: human ADAM10, 5′-GACAUUUCAACCUACGAAUTT-3′ and 5′-AUUCGUAGGUUGAAAUGUCTT-

3′; human ADAM17, 5′-CACAUGUAGAAACACUACUTT-3′ and 5′-AGUAGUGUUUCUACAUGUGTT-3′. A double-stranded RNA targeting luciferase (GL-2) was used as a control: 5′-CGUACGCGGAAUACUUC-GATT-3′ and 5′-UCGAAGUAUUCCGCGUACGTT-3′. The 21-nt chimeric RNA-DNA duplexes were obtained from Japan Bioservice. Cells were transfected with annealed siRNAs with the use of Oligofectamine (Life Technologies).

In vitro binding assay

U251MG cells (5×10^6) were washed with ice-cold PBS and incubated for 15 min on ice in 1 ml of a lysis buffer (50 mM tris-HCl, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 10 mM DTT) supplemented with 10 mM NaF, 10 μM BB94, and a protease inhibitor cocktail (Sigma-Aldrich). They were then homogenized by passage through a 25-gauge needle, and the homogenate was centrifuged at 14,000 *g* for 15 min at 4°C. The resulting supernatant (500 μl) was mixed with 20 μl of agarose beads conjugated with either glutathione or CaM (Sigma-Aldrich) and was then rotated for 2 h at 4°C, after which the beads were washed three times with lysis buffer containing 0.1 μM $CaCl_2$ and subjected to immunoblot analysis. For GTP-Rac pull-down assay, control, 100 ng/ml TPA, or 5 μM ionomycin stimulated U251MG cells were lysed with magnesium-containing buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1.0% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA) supplemented with 25 mM NaF, 1 mM sodium orthovanadate, 10 μM BB94, and a protease inhibitor cocktail (Sigma-Aldrich). They were then homogenized by passage through a 25-gauge needle, and the homogenate was centrifuged at 14,000 *g* for 5 min at 4°C. The resulting supernatant was mixed with 5 μg of PAK1 PBD agarose (Upstate Biotechnology) and was then rotated for 1 h at 4°C, after which the beads were washed four times with lysis buffer and subjected to immunoblot analysis with anti-Rac antibody.

HA coating and cell detachment assay

HA coated culture dish was prepared as described previously (Peck and Isacke, 1996). For coating with HA, 35-mm Petri dishes were incubated for 32 h at 4°C with 500 μl of streptococcal hyaluronan (5 mg/ml; Sigma-Aldrich). An equal volume of 2% BSA was added and the plates were incubated for an additional 16 h. They were then washed three times with DME F12 and used immediately. U251MG cells were seeded on the HA-coated dishes 1 d before siRNA transfection and were cultured in DME F12 supplemented with 10% FBS. 4 d after transfection, the cells were incubated with 5 μM ionomycin in serum-free medium for the indicated times.

Immunofluorescence microscopic analysis

U251 cells grown on 35-mm culture dishes were fixed with 4% PFA for 15 min followed by 0.2% Triton X-100 in PBS for 5 min. After being washed with PBS, the cells were incubated in primary antibodies diluted in PBS containing 0.2% BSA for 60 min at RT, washed three times in PBS, and incubated for 60 min at RT with appropriate secondary antibodies linked to fluorescein isothiocyanate and Cy 5 diluted in PBS containing 0.2% BSA. After being washed with PBS, samples were mounted in 80% glycerol and visualized with a confocal microscope (model Fluoview; Olympus).

Migration assay

Migration assay using RERF-LC-OK cells were performed as described previously (Okamoto et al., 1999b). Cell migration was performed using 48-well modified Boyden chambers (Neuro Probe Inc.) with polycarbonate filters of 8-μm pore size (Nucleopore Corp.). The undersides of filters were precoated with 1 mg/ml HA (Sigma-Aldrich).

Online supplemental material

Fig. S1 shows that CD44 cleavage is regulated by different signals. Fig. S2 demonstrates characterization of metalloproteinases responsible for ectodomain cleavage of CD44. Fig. S3 shows that Ca^{2+} influx-induced cell detachment from an HA matrix is suppressed in ADAM10$^{-/-}$ MEFs. Fig. S4 shows that Rac activation is induced by TPA treatment and regulates membrane ruffling in U251MG cells. Fig. S5 illustrates the proposed model for regulation of ADAM10- and ADAM17-mediated CD44 ectodomain cleavages triggered by the distinct signaling pathways. Online supplemental is available at http://www.jcb.org/cgi/content/full/jcb.200310024/DC1.

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