Regulated CD44 Cleavage under the Control of Protein Kinase C, Calcium Influx, and the Rho Family of Small G Proteins*

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CD44 is a cell surface receptor for several extracellular matrix components and is implicated in tumor cell invasion and metastasis. Our previous studies have shown that CD44 expressed in cancer cells is proteolytically cleaved at the extracellular domain through membrane-associated metalloproteases and that CD44 cleavage plays a critical role in CD44-mediated tumor cell migration (Okamoto, I., Kawano, Y., Tsuiki, H., Sasaki, J., Nakao, M., Matsumoto, M., Suga, M., Ando, M., Nakajima, M., and Saya, H. (1999) Oncogene 18, 1435–1446). In the present study, we first demonstrate rapid degradation of the membrane-tethered CD44 cleavage product through intracellular proteolytic pathways, and it occurs only after CD44 extracellular cleavage. To address the mechanisms regulating CD44 cleavage at the extracellular domain, we show that 12-O-tetradecanoylphorbol 13-acetate (TPA) and the calcium ionophore ionomycin rapidly enhance metalloprotease-mediated CD44 cleavage in U251MG cells via protein kinase C-dependent and -independent pathways, respectively, suggesting the existence of multiple distinct pathways for regulation of CD44 cleavage. Concomitant with TPA-induced CD44 cleavage, TPA treatment induces redistribution of CD44 and ERM proteins (ezrin, radixin, and moesin) to newly generated membrane ruffling areas. Treatment with lysophosphatidic acid, which is known to activate the Rho-dependent pathway, inhibits TPA-induced CD44 redistribution and CD44 cleavage. Furthermore, overexpression of Rac dominant active mutants results in the redistribution of CD44 to the Rac-induced ruffling areas and the enhancement of CD44 cleavage. These results suggest that the Rho family proteins play a role in regulation of CD44 distribution and cleavage.

The CD44 glycoprotein is a cell surface receptor for several extracellular matrix (ECM) components including hyaluronic acid (1, 2). CD44 is involved in a wide variety of biological process, including lymphocyte homing and activation (3, 4), cell adhesion (5), cell migration (6, 7), and metastatic spread of cancer (8). Although a number of experimental observations have shown that CD44 is associated with tumor invasion (9–12) and metastasis (8, 13, 14), the detailed molecular mechanisms remain to be elucidated.

We have recently demonstrated that CD44 expressed in cancer cells is cleaved proteolytically at the extracellular domain through membrane-associated metalloproteases and that CD44 cleavage plays a critical role in CD44-mediated tumor cell migration through the highly dynamic regulation of interaction between CD44 and ECM (15). These observations present CD44 as being dynamically regulated during the migration process. Little is known, however, about the mechanisms that regulate CD44 cleavage. Identification of the molecular components involved in regulated CD44 cleavage is crucial for a better understanding of CD44 dynamics in tumor cell migration and invasion.

Consistent with the implication of CD44 in cell migration, CD44 has been known to associate with actin-cytoskeleton via binding to ERM proteins (16). The ERM proteins consist of three closely related proteins, ezrin, radixin, and moesin, and behave as regulatable scaffold proteins that anchor actin filaments to the plasma membrane (17). CD44-ERM complex formation has been reported to be regulated by the small GTP-binding protein Rho (18), which belongs to the Rho family proteins. The Rho family proteins, consisting of the Rho, Rac, and Cdc42 subfamilies, regulate various cell functions, including cell migration. The best characterized function involves their regulation of the organization of the actin cytoskeleton (19–21). Rho is involved in the assembly of stress fibers and focal adhesions in many types of cells (22), Rac induces lamellipodia and membrane ruffling (23), and Cdc42 regulates the formation of filopodia (24). These dynamic actin structures provide the driving force for cell migration. Considering that cell migration is undoubtedly complex, requiring coordinated activities of the cytoskeletal, membrane, and adhesion systems (25, 26), we hypothesize that the Rho family proteins may not only internally regulate actin-cytoskeleton but also externally regulate the CD44-ECM interaction in CD44-mediated tumor cell migration.

To address mechanisms regulating CD44 cleavage at the extracellular domain, we determined the effect of 12-O-tetradecanoylphorbol 13-acetate (TPA) and the calcium ionophore ionomycin on CD44. We found that each agent rapidly enhanced CD44 cleavage through distinct pathways. Concomitant with the induction of CD44 cleavage during TPA stimulation, we also observed the coordinated regulation of Rac and Rho in TPA-treated U251MG cells. Therefore, we have focused on the role of the Rho family proteins in regulating CD44 cleavage.
Materials and Chemicals—The monoclonal antibody BU52 (Ancell, Bayport, MN) is directed against the ectodomain epitope common to all CD44 isoforms. An antibody against the cytoplasmic domain of CD44, anti-CD44cyto pAb, was raised in rabbits as described previously (15). The M11, R21, and M22 mAbs, which were raised in rats against recombinant mouse ezrin, radixin, and moesin, respectively, were kindly supplied by Dr. Sachiko Tsukita (Kyoto University, Kyoto, Japan) (27). The anti-HA monoclonal antibody was prepared from 12CA5 cells. mAb065, an anti-rat β-tubulin mouse monoclonal antibody that reacts with β-tubulin from all species (plant to human), was purchased from Chemicon (Temecula, CA) and Amersham Pharmacia Biotech, respectively.

Antibodies were obtained as follows: carbobenzoxy-leucinyl-eucinyl-lysophosphatidic acid (LPA) were from Sigma; GF109203X was from Wako Pure Chemical Industries (Osaka, Japan); and calcium ionophore (ionomycin) was from Calbiochem (La Jolla, CA). Hydroxylamine-based metalloprotease inhibitor, BB2516 (marimastat) was kindly provided by Dr. M. Nakajima (Novartis Pharmaceutical, Takarazuka, Japan).

Cell Culture and Transfection—The human glioblastoma cell line U251MG was grown in Dulbecco’s modified Eagle’s medium with Ham’s F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Bio-Whittaker, Walkersville, MD) at 37 °C in an atmosphere containing 5% CO₂.

pEF-BOS-HA-RhoAV12, -Rac1N17, and -Rac1V12 plasmids were constructed as described previously (28, 29). These expression plasmids were transfected into U251MG cells using FuGENE6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Briefly, 5 × 10⁴ cells in 2 ml of medium supplemented with 10% fetal bovine serum were seeded on 35-mm culture dishes or 6-well plates in the absence or presence of MG132 or BB2516 (100 μM). The cells were directly lysed with SDS sample buffer without mechanical scraping. The samples containing equal amounts of cell lysates were electrophoresed on a 10–20% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-CD44cyto pAb against the cytoplasmic domain of CD44.

Induction of CD44 Cleavage at the Extracellular Domain by Mechanical Stimulation and Subsequent Degradation of Membrane-bound CD44 Cleavage Products—We showed previously that CD44 cleavage at the extracellular domain in cancer cells generated a membrane-tethered cleavage product that was detected by Western blot analysis using an antibody (anti-CD44cyto pAb) against the cytoplasmic domain of CD44 (15). The CD44 cleavage product was clearly detected by anti-CD44cyto pAb in the highly invasive human glioma cell line U251MG, when the cells were mechanically scraped in preparing the lysates, as shown in our preceding paper (Fig. 1A, lane 1) (15). In contrast, the CD44 cleavage product was not observed in the presence of the specific metalloprotease inhibitor BB2516 (marimastat) during mechanical scraping of the cells (Fig. 1A, lane 2). These results are in line with our previous demonstration that CD44 expressed in cancer cells is proteolytically cleaved at the extracellular domain by metalloproteases (15).
In the course of further studies, we found that the CD44 cleavage product was hardly detectable when the U251MG cells were directly lysed with SDS sample buffer without scraping (Fig. 1B, lane 1). Interestingly, when the lysates were prepared from the proteasome inhibitor MG132-treated U251MG cells without mechanical scraping, three bands of with apparent molecular masses between 20 and 30 kDa became apparent (Fig. 1B, lanes 2 and 3). The bands were absent in the presence of the metalloprotease inhibitor BB2516 (Fig. 1B, lane 4), indicating that these fragments are generated by metalloprotease-mediated CD44 cleavage at the extracellular domain. The difference in the size of the three fragments suggests existence of plural cleavage sites in the membrane proximal of CD44 ectodomain or modifications of the CD44 cleavage product. Our data indicate that CD44 cleavage product is rapidly degraded through intracellular proteolytic pathways, and thereby, the membrane-bound CD44 cleavage product is not detectable in the lysate prepared without mechanical scraping. On the other hand, mechanical stimulation itself, such as scraping of cells, which may activate diverse intracellular signalings, strongly enhances the CD44 cleavage reaction in cancer cells having high CD44 cleavage activity, and the enhancement enables us to detect the resultant cleavage product.

**Induction of CD44 Cleavage at the Extracellular Domain by PKC Activation in U251MG Cells**—These observations prompted us to investigate what type of intracellular signaling can enhance the metalloprotease-mediated CD44 cleavage at extracellular domain. Because phorbol esters have been shown to facilitate proteolytic cleavage of several cell surface receptors and release the soluble form (30, 31), we examined whether phorbol esters can induce CD44 cleavage in cancer cells. U251MG cells were incubated with TPA, and then the levels of soluble CD44 released into the culture supernatants were determined by an ELISA system. TPA treatment induced a dose- and time-dependent increase in the release of soluble CD44 (Fig. 2A).

To verify that the increase in the soluble CD44 level was caused by enhancement of CD44 cleavage, the cell lysates prepared without mechanical scraping were resolved by 10% (C and F, upper panels) or 15% (C and F, lower panels) SDS-polyacrylamide gel and subjected to immunoblotting with BU52 directed against the extracellular domain of CD44 or anti-CD44cyto pAb against the cytoplasmic domain of CD44. The results shown are representative of three independent experiments.

![Fig. 2. Induction of CD44 cleavage by TPA or ionomycin treatment. U251MG cells were plated (7 × 10⁴ cells/well) in a 6-well culture plate. After overnight incubation at 37 °C, cells were washed and incubated with 1.0 ml of control medium (●), medium containing TPA (100 □ or 500 □ ng/ml) (A) or ionomycin (1 □ or 5 □ µM) (D) for the indicated periods of time. Then soluble CD44 released into the cell-free supernatants was determined by an ELISA system. U251MG cells were treated with TPA (100 ng/ml) (B and C) or ionomycin (5 µM) (E and F) for 30 min in the presence or absence of EGTA (2 mM), GF109203X (2.5 µM), or BB2516 (100 nM). The cell-free supernatants were analyzed for soluble CD44 using an ELISA system (B and E). The cell lysates prepared without mechanical scraping were resolved by 10% (C and F, upper panels) or 15% (C and F, lower panels) SDS-polyacrylamide gel and subjected to immunoblotting with BU52 directed against the extracellular domain of CD44 or anti-CD44cyto pAb against the cytoplasmic domain of CD44. The results shown are representative of three independent experiments.

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cleaved on the cell surface. The effects of TPA were completely blocked in the presence of BB2516 (Fig. 2, B and C, lane 5), indicating that the induction of CD44 cleavage by TPA treatment is mediated by metalloprotease. This observation is consistent with our previous demonstrations that CD44 cleavage at cancer cells surface is evoked by metalloproteases (15).

TPA is a known activator of PKC. To examine whether TPA-induced proteolytic cleavage of CD44 is PKC activation-dependent, U251MG cells were pretreated with the specific PKC inhibitors GF109203X prior to addition of TPA and then analyzed for the effect on CD44 cleavage by ELISA and Western blot using anti-CD44cyto pAb. As demonstrated in Fig. 2 (B and C), pretreatment with GF109203X prevented TPA-induced CD44 cleavage (Fig. 2, B and C, lane 4). These results suggest that the induction of CD44 cleavage in the presence of TPA requires PKC activity.

**Induction of CD44 Cleavage by Extracellular Calcium Influx**—Because the activity of certain PKC isoforms is Ca^{2+}-dependent (32, 33), the elevation of intracellular Ca^{2+} levels may activate PKC and subsequently produce effects similar to those of TPA on CD44 cleavage. We therefore treated U251MG cells with the calcium ionophore ionomycin and then analyzed for the effect on CD44 cleavage as described above. Treatment of cells with ionomycin-induced release of soluble CD44 in a dose- and time-dependent manner and yielded the CD44 cleavage products detected with anti-CD44cyto pAb (Fig. 2, D and F, lower panel, lanes 1 and 2). The fact that the ionomycin effect was abolished in medium containing the Ca^{2+} chelator EGTA indicated that CD44 cleavage can be induced by the influx of Ca^{2+} across the plasma membrane followed by the elevation of intracellular Ca^{2+} levels (Fig. 2, E and F, lane 3). Furthermore, the ionomycin-induced CD44 cleavage was completely blocked in the presence of BB2516, as seen with TPA (Fig. 2, E and F, lane 5). These results indicate that extracellular Ca^{2+} influx enhances CD44 cleavage mediated by metalloproteases.

We subsequently examined whether PKC plays a role in ionomycin-induced proteolytic cleavage of CD44. Interestingly, pretreatment with GF109203X had no significant inhibitory effect on ionomycin-induced CD44 cleavage (Fig. 2, E and F, lane 4), indicating that PKC activity is not required for the cleavage induction mechanism, whereas it was essential for TPA-induced CD44 cleavage. Moreover, in contrast with its effect on ionomycin, chelation of Ca^{2+} in the medium had minimal inhibitory effects on TPA-induced CD44 cleavage (Fig. 2, B and C, lane 3). Thus, TPA- and ionomycin-induced CD44 cleavage were distinct in terms of their sensitivities to GF109203X and chelation of Ca^{2+}, whereas metalloprotease inhibitors blocked CD44 cleavage induced by both TPA and ionomycin. Taken together, these findings show that there are at least two independent pathways leading to the induction of CD44 proteolytic cleavage mediated by metalloproteases.

**Redistribution of CD44 and ERM Proteins to TPA-induced Membrane Ruffling**—To further investigate the role of TPA and ionomycin in the regulation of CD44 cleavage, we examined the effect of these treatments on CD44 localization at the plasma membrane. Confocal microscopic analysis of U251MG cells without any treatment showed that CD44 was mainly located at filopodia and membrane ruffling areas where polymerized actin was concentrated at the inner surface of the plasma membrane (Fig. 3, A–D, M, and O). The ERM proteins (ezrin, radixin, and moesin) also showed a distribution similar to that of CD44 (Fig. 3, E–H), and double immunofluorescence analysis revealed that CD44 was precisely colocalized with the ERM proteins at filopodia and ruffling areas (Fig. 3, I–P). These staining patterns are consistent with the earlier reports that CD44 is associated with the actin cytoskeleton through its interaction with the ERM proteins, which are thought to function as cross-linking proteins between plasma membranes and actin filaments (16).

Notably, treatment of U251MG cells with TPA-induced membrane ruffling over the entire cell surface within 10 min (Fig. 4, A–D) followed by a loss of actin stress fibers at 30 min (Fig. 4, E–H). The newly generated membrane ruffling was always observed during the treatment, and both CD44 and the ERM proteins were redistributed to the TPA-induced ruffling areas where the colocalization of these proteins was observed (Fig. 4, C, G, and I–L). Pretreatment of U251MG cells with BB2516, which strongly prevented TPA-induced CD44 cleavage (Fig. 2, B and C), did not affect the TPA-induced CD44 redistribution (Fig. 4, M–P), indicating that CD44 cleavage is not necessary for TPA-induced CD44 redistribution. On the other hand, ionomycin treatment did not show apparent alterations in the localization of CD44 and the ERM proteins under the condition that enhanced CD44 cleavage (data not shown).

**The Role of Rho Family Proteins in Redistribution of CD44 to TPA-induced Membrane Ruffling Areas**—The Rho small G protein family is reported to be involved in phorbol ester-induced membrane ruffling (34–36). We therefore examined the regulation of the Rho family proteins in CD44 redistribution to TPA-induced membrane ruffling areas. We transiently transfected the plasmid encoding HA epitope-tagged dominant active mutant of RhoA (RhoaAV14) into U251MG cells. Treatment of the RhoaAV14 transfected cells with TPA for 30 min did not reduce the stress fibers (Fig. 5, A and B), consistent with earlier observations that Rho activation enhanced the formation of stress fibers (22). Moreover, in the RhoaAV14-transfected cells, the membrane ruffling and CD44 redistribution were not observed during TPA treatment (Fig. 5, C and D).

Because it is well known that membrane ruffling is induced by activation of Rac (23), we transiently transfected U251MG cells with an HA-tagged Rac1N17 dominant negative mutant. Treatment of the Rac1N17-transfected cells with TPA did not induce the membrane ruffling and CD44 redistribution during the treatment (Fig. 5, E–H). Furthermore, we transfected an HA-tagged Rac1V12 dominant active mutant into U251MG cells. As reported previously (23), the expression of Rac1V12 in U251MG cells induced ruffling at the margin of the plasma membrane, and the expressed active Rac itself was found to be accumulated in the ruffling areas (Fig. 5, I and L). In the Rac1V12-transfected cells, relocation of CD44 and the ERM proteins to the Rac1V12 induced ruffling areas was clearly observed (Fig. 5, I–N). Thus, the redistribution of CD44 and the ERM proteins to membrane ruffles induced by Rac1V12 were similar to observations in TPA-treated U251MG cells. Taken together, the coordinated regulation of the Rho family proteins may be involved in the redistribution of CD44 and the ERM proteins to TPA-induced membrane ruffling.

**Involvement of the Rho Family Proteins in TPA-induced CD44 Cleavage at the Extracellular Domain**—Because the TPA-induced CD44 redistribution and CD44 cleavage at the extracellular domain took place simultaneously, we next examined possible involvement of the Rho family proteins in TPA-induced CD44 cleavage. To address the possibility, we took advantage of LPA, which is known to activate the Rho-dependent pathway (22). U251MG cells were treated with LPA in serum-free medium for 3 h and then stimulated with TPA. In most U251MG cells pretreated with LPA, the TPA-induced reduction of actin stress fibers and CD44 redistribution was impaired (Fig. 6A) consistent with our observations in dominant active Rho-transfected U251MG cells (Fig. 5, A–D), although these effects of LPA appeared to be less than those by introduction of active Rho. The treatment of cells with LPA
clearly inhibited the TPA-induced increase in soluble CD44 and CD44 cleavage product in a concentration-dependent manner (Fig. 6, B and C) but not the ionomycin-induced CD44 release (Fig. 6D), which acts through a different mechanism from TPA (Fig. 2). These results suggest that the finely regulated Rho family proteins are involved in regulation of TPA-induced CD44 cleavage.

To obtain further evidence of the involvement of the Rho family proteins in CD44 cleavage, based on the regulation of Rac activity found in TPA-treated U251MG cells, we tested effects of the expression of dominant active or negative Rac on the induction of CD44 cleavage. U251MG cells were transfected with HA-tagged Rac1V12 dominant active or HA-tagged Rac1N17 dominant negative or control vector plasmids (Fig. 7A). At 8 h post-transfection, cells were washed and incubated for 30 min with fresh medium, and the level of soluble CD44 in the conditioned medium was determined. As shown in Fig. 7B, the dominant active Rac-transfected cells released significantly larger amounts of soluble CD44 than the control vector- or dominant negative Rac-transfected cells (Fig. 7B). These results suggest that activation of Rac plays a role in the promotion of CD44 cleavage at the extracellular domain.
DISCUSSION

In this study, we have firstly demonstrated that the membrane-tethered cleavage products generated following the proteolytic cleavage of CD44 at the extracellular domain is degraded through intracellular proteolytic pathways. Western blot analysis in the lysates prepared without mechanical scraping of the cells shows that the CD44 cleavage product is normally unstable and that the products are stabilized by MG132, which inhibits intracellular proteolysis activities including the proteasome, calpains, and lysosomal cysteine proteases. Our present data give the evidence that CD44 is processed by two sequential proteolytic events at the extra- and intracellular domains.

We have identified two independent pathways that promote proteolytic cleavage of CD44 at the extracellular domain in U251MG cells. The first pathway is activated by extracellular Ca\(^{2+}\) influx and is PKC-independent. Elevations in intracellular Ca\(^{2+}\) concentration are known to activate PKC (32, 33). However, the lack of inhibitory effects of PKC inhibitor GF109203X on ionomycin-induced CD44 cleavage indicates that possible downstream PKC activation is not required for functional activation of the first pathway. The second pathway is activated by the phorbol ester TPA and is PKC-dependent. TPA can often stimulate proteolytic processing of the extracellular domain of several cell surface molecules (30, 31). Although TPA is known as a potent PKC activator, the proteolytic stimulation by TPA has been insufficient to demonstrate the involvement of PKC-dependent regulation. For example, TPA-induced cleavage of angiotensin converting enzyme has been reported to be resistant to several PKC inhibitors, indicating the activation of the PKC-independent pathway by TPA (37).

Fig. 4. Redistribution of CD44 and ERM proteins to TPA-induced membrane ruffling areas. U251MG cells were seeded as described in Fig. 3 and treated with 100 ng/ml TPA in the absence (A–L) or presence of BB2516 (M–P). The cells were fixed at 10 min (A–D) or 30 min (E–F) after TPA treatment, double stained with rhodamine-phalloidin (B, F, and N) and BU52 (C, G, and O) or with anti-ERM mAb (J) and BU52 (K), and then analyzed by confocal microscopy as described under “Experimental Procedures.” Phase-contrast images (A, E, I, and M) and merge images (D, H, L, and P) are shown. The results shown are representative of at least three independent experiments. Bars, 20 μm.
TPA rapidly enhances CD44 cleavage and that PKC activation is necessary for the enhancement. The existence of such several distinct pathways for the regulation of proteolytic cleavage at the extracellular domain have been also shown in other membrane proteins, including transforming growth factor-α, Kit ligand, c-kit receptor, and heparin-binding epidermal growth factor (39–42).

It is notable that the induction of CD44 cleavage through the extracellular Ca²⁺-mediated pathway or the PKC-mediated pathway was blocked by a specific metalloprotease inhibitor, indicating that these distinct pathways share a common component, metalloproteases, in the CD44 cleavage mechanisms. These results are supported by our previous demonstration that membrane-associated metalloproteases cleave CD44 at the extracellular domain in several cancer cell lines, including U251MG cells (15).

In parallel with the induction of CD44 cleavage at the extracellular domain, we have found that TPA treatment results in the redistribution of CD44 and ERM proteins to the newly formed membrane ruffling areas in U251MG cells. ERM proteins have bipartite domains, composed of the N-terminal domain responsible for binding to a transmembrane protein and the C-terminal domain that binds to actin filaments, and serve as cross-linkers between plasma membranes and actin filaments (17). CD44 has been proposed to be an integral membrane target for ERM proteins (16). Evidence has accumulated

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**FIG. 5.** Effect of Rho family proteins on redistribution of CD44 to TPA-induced membrane ruffling areas. U251MG cells were seeded as described in Fig. 3. The cells were transiently transfected with HA-RhoAV14 (A–D) or HA-Rac1N17 (E–H) and treated with 100 ng/ml TPA for 30 min at 24 h after the transfection. The cells were then double stained with 12CA5 anti-HA mAb (A, C, E, and G) and rhodamine-phalloidin (B and F) or anti-CD44cyto pAb (D and H) and analyzed by confocal microscopy. The cells transfected with HA-Rac1V12 (I–N) were fixed at 24 h after the transfection and double stained with 12CA5 (I and L) and anti-CD44cyto pAb (J) or anti-ERM mAb (M) and analyzed by confocal microscopy. K and N show merge images. The results shown are representative of at least three independent experiments. Bars, 20 μm.
Regulation of CD44 Cleavage

**A**

Phase contrast

F-actin

CD44

**B**

Relative soluble CD44 release

- no treat
- TPA
- LPA20+TPA
- LPA50+TPA

**C**

CD44 cleavage product

Blot Anti-CD44cyto pAb

1 2 3 4

Blot Anti-β-Tubulin Ab

**D**

Relative soluble CD44 release

- no treat
- Ionomycin
- LPA20+Ionomycin
- LPA50+Ionomycin

**Fig. 6.**
that the association of ERM proteins with the plasma membrane and actin filaments is regulated by the Rho family proteins, and this regulatable scaffold (membrane-ERM-actin filaments) is thought to be an essential prerequisite for both Rho- and Rac-induced cytoskeletal reorganization (18, 19, 43–45). We found that dominant active Rho and dominant negative Rac impair the TPA-induced membrane ruffling and redistribution of CD44 and ERM proteins, whereas dominant active Rac induces their redistribution to the Rac-induced membrane ruffling areas. Taken together, these observations suggest that TPA-induced redistribution of CD44 and ERM proteins is under the control of coordinated regulation of Rho and Rac.

In the present study, we have shown that treatment of the cells with LPA inhibits both TPA-induced CD44 redistribution and promotion of CD44 cleavage. LPA have been thought to activate Rho based on an ability to induce stress fiber formation in a Rho-dependent manner (22). Our findings that LPA treatment, as well as dominant active Rho, inhibited TPA-induced formation of membrane rufflings followed by reduction of stress fibers likely result from impairment of the fine regulation between Rac and Rho activity, although we cannot completely exclude the possibility that LPA may have other actions. Therefore, the fact that U251MG cells pretreated with LPA no longer respond to TPA-induced CD44 cleavage suggests that regulation of the Rho family proteins is involved in the CD44 cleavage. Furthermore, the promotion of CD44 cleavage in U251MG cells overexpressing dominant active Rac supports this notion and suggests that activation of Rac plays a role in the regulated processing of CD44.

We have demonstrated previously that CD44 cleavage is mediated by a membrane-bound metalloprotease expressed in cancer cells (15). If the metalloprotease and CD44 are localized apart at the membrane, the redistribution followed by clustering of the protease and substrate might be essential for triggering this proteolytic processing. Therefore, we propose that one plausible role for the Rho family in CD44 cleavage may be the spatial organization between the CD44 cleavage metalloprotease and CD44. As in our demonstration that CD44 and ERM proteins are dramatically redistributed and colocalized on the membrane through activation of Rac, the Rho family proteins may finely regulate the recruitment of the CD44 cleavage metalloprotease to CD44. Identification of the metalloprotease and its distribution on the membrane will provide further insight into the mechanisms of regulated CD44 cleavage.

The Rho family proteins have been shown to regulate cadherin-dependent cell-cell adhesion (46–49) and integrin-mediated signaling and cell motility (50–52). Concomitant with their effects on the organization of the actin cytoskeleton, the Rho family proteins thus act as key regulatory molecules in the formation of cell-cell and cell-ECM adhesions through cell surface receptors. In our preceding paper (15), CD44 cleavage at the extracellular domain was demonstrated to play a critical role in CD44-mediated tumor cell migration by providing offsetting changes in adhesive interactions between CD44 and ECM. The results of the present study suggest the involvement of the Rho family proteins in the CD44 regulatory mechanism.

**Fig. 6. Effect of LPA on TPA- or ionomycin-induced CD44 cleavage**. U251MG cells were plated as described in Fig. 3. Cells were pretreated for 3 h with 50 μM LPA in serum-free medium and subsequently incubated for 30 min in serum-free medium containing 100 ng/ml TPA in the presence of 50 μM LPA. The cells were then double stained with rhodamine-phalloidin and BU52 and analyzed by confocal microscopy (A). U251MG cells were plated as described in Fig. 2 and pretreated for 3 h with LPA (20 or 50 μM) or vehicle in serum-free medium. The cells were subsequently incubated for 30 min in serum-free medium or in serum-free medium containing 100 ng/ml TPA (B and C) or 5 μM ionomycin (D) in the presence or absence of LPA (20 or 50 μM). The cell-free supernatants were analyzed for soluble CD44 using an ELISA system (B and D). Mean values and standard deviation of triplicate samples for one experiment are shown and are representative of three independent experiments. The cell lysates prepared without mechanical scraping were resolved by 15% SDS-polyacrylamide gel and subjected to immunoblotting with anti-CD44cyto pAb against the cytoplasmic domain of CD44 (C, upper panel) or anti-β-tubulin mAb (C, lower panel).

**Fig. 7. Activation of Rac enhanced CD44 cleavage**. U251MG cells were plated (5 × 10⁴ cells/well) in a 6-well culture plate 12 h before transfection and transfected with vector only, HA-RacN17, or HA-RacV12, as indicated. At 8 h post-transfection, cells were washed and incubated for 30 min with 1.0 ml of fresh medium. A, cell lysates were analyzed by immunoblotting with 12CA5 antibody (upper panel) and anti-β-tubulin mAb (lower panel). B, the cell-free supernatants were analyzed for soluble CD44 using an ELISA system. The results are presented as mean values and standard deviation from two independent experiments.
