主論文

Regulation Mechanism of ERM Protein/Plasma Membrane Association: Possible Involvement of Phosphatidylinositol Turnover and Rho-dependent Signaling Pathway

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Running Title: Regulation of ERM/membrane Association

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Abstract.

The ERM proteins, ezrin, radixin and moesin, are involved in the actin filament/plasma membrane interaction as crosslinkers. CD44 has been identified as one of the major membrane binding partners for ERM proteins. To examine the CD44/ERM protein interaction *in vitro*, we produced mouse ezrin, radixin, moesin, and the GST/CD44 cytoplasmic domain fusion protein (GST-CD44cyt) by means of recombinant baculovirus infection, and constructed an *in vitro* assay for the binding between ERM proteins and the cytoplasmic domain of CD44. In this system, ERM proteins bound to GST-CD44cyt with high affinity (Kd of moesin was 9.3±1.6nM) at a low ionic strength, but with low affinity at a physiological ionic strength. However, in the presence of phosphoinositides (PI, 4-PIP, and 4,5-PIP_2), ERM proteins bound with a relatively high affinity to GST-CD44cyt even at a physiological ionic strength : 4,5-PIP_2 showed a marked effect (Kd of moesin in the presence of 4,5-PIP_2 was 9.3±4.8nM). Next, to examine the regulation mechanism of CD44/ERM interaction *in vivo*, we re-examined the immunoprecipitated CD44/ERM complex from BHK cells and found that it contains Rho-GDI, a regulator of Rho GTPase. We then evaluated the involvement of Rho in the regulation of the CD44/ERM complex formation. When recombinant ERM proteins were added and incubated with lysates of cultured BHK cells followed by centrifugation, a portion of the recombinant ERM proteins was recovered in the insoluble fraction. This binding was enhanced by GTPγS, and markedly suppressed by C3 toxin, a specific inhibitor of Rho, indicating that the GTP-form of Rho in the lysate is required for this binding. A mAb specific for the cytoplasmic domain of CD44 also markedly suppressed this binding, identifying most of the binding partners for exogenous ERM proteins in the insoluble fraction as CD44. Consistent with this binding analysis, in living BHK cells treated with C3 toxin, most insoluble ERM proteins moved to soluble compartments in the cytoplasm, leaving CD44 free from ERM. These findings indicate that Rho regulates the CD44/ERM complex formation *in vivo* and that the phosphatidylinositol turnover may be involved in this regulation mechanism.

ERM family proteins are thought to function as general crosslinkers between plasma membranes and actin filaments (Bretscher, 1983; Pakkanen et al., 1987; Lankes et al., 1988; Tsukita et al., 1989a; Algrain et al., 1993; Arpin et al., 1994). The ERM family consists of three closely related proteins, ezrin, radixin and moesin (Sato et al., 1992; Tsukita et al., 1992). In cultured epithelial and fibroblastic cells, they are expressed and localized at specialized regions where actin filaments are densely associated with plasma membranes, such as cleavage furrows, microvilli, ruffling membranes, and cell-cell/cell-matrix adhesion sites (especially at primordial forms of cadherin- and integrin-based cell adhesion sites), whereas among organs their expression varies, suggesting some dedifferentiation in terms of ERM expression in cultured cells (Bretscher, 1983; Pakkanen et al., 1987; Lankes et al., 1988; Tsukita et al., 1989a,1992; Sato et al., 1991,1992; Berryman et al., 1993; Franck et al., 1993; Amieva et al., 1994; Takeuchi et al., 1994b; Henry et al., 1995). The suppression of ERM expression with antisense oligonucleotides destroys microvilli, cell-cell, and cell-matrix adhesion sites (Takeuchi et al., 1994b), and the introduction of a dominant-negative construct of ezrin and radixin impairs the cortical actin organization and cytokinesis, respectively (Martin et al., 1995; Henry et al., 1995).

Sequencing has revealed that the three ERM proteins are highly homologous; in the mouse, the identity is 75, 72, and 80% for ezrin/radixin, ezrin/moesin, and radixin/moesin, respectively (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992). The sequences of their amino-terminal halves are highly conserved (~85% identity for any pair). A tumor suppresser molecule responsible for neurofibromatosis type 2 named merlin or schwannomin, was recently identified, which has significant sequence similarity to ERM proteins; ~49% identity overall and, especially, ~85% identity in their amino-terminal halves (Trofatter et al., 1993; Rouleau et al., 1993).

The highly-conserved amino-terminal sequence in ERM proteins is also found in the amino-terminal end of some membrane-associated proteins such as band 4.1 protein, talin, PTPH1 and PTPMEG, indicating a band 4.1 superfamily (Conboy et al., 1986; Rees et al., 1990; Gu et al., 1991; Yang and Tonks, 1991; Takeuchi et al., 1994a; Arpin et al., 1994). Considering that this conserved domain in band 4.1 protein is reportedly responsible for its direct association with glycophorin C, an integral membrane glycoprotein of erythrocyte membranes (Bennett, 1989), the amino-terminal half domain of ERM proteins was thought to directly bind to some integral membrane proteins. Immunoprecipitation experiments using cultured cells subsequently revealed that CD44 is directly associated with ERM proteins (Tsukita et al., 1994). CD44 is a polymorphic cell-surface glycoprotein which is found on a wide variety of cells (Haynes et al., 1989, 1991; Lesley et al., 1993), that is also called Pgp-1 (Zhou et al., 1989), HCAM (Goldstein et al., 1989), Hermes antigen (Jalkanen et al., 1986) and ECMRIII (Wayner et al., 1988). In cultured fibroblasts, CD44 is precisely colocalized with ERM proteins, favoring the notion that CD44 is a membrane binding partner of ERM proteins (Tsukita et al., 1994). However, in the body, the expression of CD44 varies among tissues and its distribution is not necessarily identical to that of ERM proteins (Berryman et al., 1995; Nakamura et al., submitted). Furthermore, in some types of cells, integral membrane proteins such as CD43 and the H^+/K^+ ATPase pump are precisely colocalized with ERM proteins (Hanzel et al., 1991; Yonemura et al., 1993). These findings suggest that ERM proteins bind to a specific group of integral membrane proteins, including CD44. Considering that CD44 is rather highly and ubiquitously expressed, however, it could be a major binding partner of ERM proteins.

The carboxy-terminal half of ERM proteins, especially the carboxy-terminal 34 amino acids, interacts with actin filaments (Turunen et al., 1994; Pestonjamasp et al., 1995). The co-existence of plasma membrane-binding and actin filament-binding domains in individual molecules allows ERM proteins to function as a plasma membrane/actin filament crosslinker. Considering that the plasma membrane/actin filament association is dynamically regulated, the next question is how the membrane-binding of ERM proteins is regulated *in vivo*.

In this study, we first analyzed the manner of binding between ERM proteins and the

cytoplasmic domain of CD44 *in vitro* using recombinant ERM and a GST/CD44 cytoplasmic domain fusion proteins produced by means of recombinant baculovirus infection. We found that the CD44 cytoplasmic domain showed very weak binding activity to any of the ERM proteins at physiological ionic strength, but that in the presence of phosphatidylinositol 4,5-bisphosphate (4,5-PIP_2) it bound with high affinity to ERM proteins [the Kd of moesin was 9.3±4.8nM]. These findings are not only the first to show that ERM proteins can bind to the cytoplasmic domain of CD44 *in vitro*, but also suggests that the formation of CD44/ERM complex is dynamically regulated *in vivo*. Next, we then re-examined the CD44/moesin immune complex from BHK cells and found that Rho-GDI, a regulator of a small GTP-binding protein Rho, was tightly associated with the CD44/moesin complex. Rho reportedly regulates the actin filament-plasma membrane association (Hall, 1990; Ridley et al., 1992a,b; Takai et al., 1995; Zigmond, 1995). The Rho family belongs to the small GTP-binding protein superfamily and consists of the Rho, Rac, and Cdc42 subfamilies. Interconversion between active and inactive forms (GTP-Rho and GDP-Rho, respectively) is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rho-GDI, Smg-GDS, Dbl, and Ost have been identified as GEFs. Therefore, the occurrence of Rho-GDI in the CD44/ERM complex suggests that this complex formation is regulated by Rho. We then established a cell-free system with which to measure the binding of the exogenous recombinant ERM proteins to the insoluble fraction of the lysate of BHK cells, and found that the binding of ERM proteins to the cytoplasmic domain of CD44 was enhanced by GTPγS and remarkedly suppressed by C3 toxin, a specific inhibitor of Rho GTPase (Aktories et al., 1988; Kikuchi et al., 1988; Narumiya et al., 1988; Braun et al., 1989). In good agreement with these binding results, when living BHK cells were incubated with C3 toxin, most insoluble ERM proteins moved to the soluble compartments in the cytoplasm, leaving free CD44. We thus concluded that Rho regulates the formation of CD44/ERM complex *in vivo*, and that the elevation of 4,5-PIP_2 concentration in plasma membranes is possibly involved in this regulation pathway.

Materials and Methods

Cells and Antibodies

Sf9 and High Five cells were purchased from Invitrogen Corporation (San Diego, CA) and cultured in TC-100 medium (GIBCO BRL, Gaithersburg, MD) supplemented with Tryptose Phosphate Broth (GIBCO BRL) and 10% FCS at 27℃. BHK cells were cultured in DME supplemented with 5% newborn calf serum and 10% tryptose phosphate broth. The mAbs, M11, R2-1, and M22, were raised in rats against recombinant mouse ezrin, radixin, and moesin, respectively (Takeuchi et al., 1994b). The pAbs, I1 and p-800, were raised in rabbits against purified rat radixin and recombinant mouse radixin, respectively (Tsukita et al., 1989a; Funayama et al., 1991). The pAb I1 was used to detect all ERM proteins by immunoblotting (Tsukita et al., 1989a; Sato et al., 1992). The mouse mAb CR-22 reacts specifically with moesin in immunoprecipitation (Sato et al., 1992; Tsukita et al., 1994). Anti-mouse rho-GDI mAb has been characterized (Shimizu et al., 1991). The rat mAb30189 is specific for hamster CD44 (Tsukita et al., 1994).

Monoclonal antibodies were produced in the rat using the GST fusion protein with the cytoplasmic domain of mouse CD44 (GST-CD44cyt) as described (Tsukita et al., 1989b). The culture supernatant of each hybridoma was screened to select mAbs that recognize not only mouse CD44 (GST-CD44cyt and in mouse MTD-1A cells) but also hamster CD44 (in BHK cells) by immunoblotting.

Production and Purification of Recombinant Ezrin, Radixin, and Moesin

Mouse ezrin, radixin, or moesin full-length cDNA was integrated into the baculovirus genome, and the recombinant virus containing ezrin, radixin, or moesin cDNA was isolated and condensed using a MAXBAC™ kit (Invitrogen, San Diego, CA). Insect Sf9 cells were infected with recombinant viruses, cultured in 200ml spinner flasks at 27℃ for 72h, then

washed with PBS. After being suspended in PBS ($3x10^6$ cells/ml) containing 2mM EGTA, 2mM PMSF and 4 μg/ml of leupeptin, the cells were homogenized in a tight-fitting Dounce homogenizer and sonicated, followed by centrifugation at 100,000xg for 1h at 4℃.

The supernatant was applied to a heparin column (5ml in 15x25mm; Hi TrapHeparin, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with DG buffer (10mM Hepes, pH 7.5, 1mM EGTA, 0.1mM DTT). After thoroughly washing with DG containing 150mM KCl, proteins were eluted with a linear concentration gradient of KCl (150-750mM). Recombinant ERM proteins were eluted around 350mM KCl. The eluate was dialyzed against DG followed by centrifugation at 100,000xg for 1h. The supernatant was loaded onto a Q-sepharose column (1ml in 8x38mm; Econo-PacQ, Bio-Rad Laboratories, Richmond, CA) equilibrated with DG. The column was washed with DG, then proteins were eluted with a linear concentration gradient of KCl (0-200mM). Purified ERM proteins were eluted around 50mM KCl. The purity of each protein was determined by SDS-PAGE (see Fig.1).

Production and Purification of GST-fusion Protein with the Cytoplasmic Domain of CD44

The cDNA encoding the full-length of the cytoplasmic domain of mouse CD44 (1180-1395 bp; CD44cyt) with BamHI and EcoRI sites at the ends was produced by PCR using mouse lung cDNA library as the template, and subcloned into pBluescript SK(-). The insert was then subcloned into the pGEX2T expression vector, which was designed to express a GST fusion protein in *E.coli*. Using this GST-CD44cyt expression vector as a template, the GST-CD44cyt cDNA with NheI and BglII sites at the ends was produced by PCR, then subcloned into pBluescript SK(-). The insert was integrated into the baculovirus genome, and the recombinant virus containing GST-CD44cyt cDNA was isolated and condensed using a MAXBAC™ kit (Invitrogen, San Diego, CA). The correct sequence of the PCR products was confirmed by sequencing with a Taq terminator cycle sequencing kit (DyeDeoxy™; Applied Biosystems, Inc., Foster City, CA).

Insect Sf9 cells were infected, cultured, washed, homogenized and centrifuged as described above. The supernatant was incubated with Glutathione-Sepharose 4B beads (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), which had been washed with PBS, for 30min at room temperature. The Glutathione-Sepharose beads bound to GST-CD44cyt were washed with W buffer (10mM Hepes, pH7.5, 2mM PMSF, 4μg/ml leupeptin), and used in the *in vitro* binding assay. In some experiments, GST-CD44cyt was purified by elution with H-buffer containing 30mM glutathione, dialyzed against H-buffer, then used for the *in vitro* binding assay. The control experiments included GST expressed in E.coli using pGEX2T and purified using Glutathione-Sepharose 4B beads.

Production and Purification of the Amino- and Carboxy-terminal Halves of Moesin

The cDNAs encoding the amino-terminal (1-930 bp) and carboxy-terminal halves (931-1733 bp) of mouse moesin were produced by PCR using the moesin cDNA clone p22T6 (Sato et al., 1992) as the template, and subcloned into pBluescript SK(-). These inserts were integrated into the baculovirus genome, and the recombinant virus containing the cDNA encoding the amino- or carboxy-terminal half of moesin was isolated and condensed using a MAXBAC™ kit (Invitrogen, San Diego, CA). The sequences of the PCR products were confirmed using a Taq terminator cycle sequencing kit (DyeDeoxy™; Applied Biosystems, Inc., Foster City, CA).

To obtain a large amount of the amino-terminal half of recombinant moesin (N-moesin), we used insect High Five (HF) cells instead of Sf9 cells. HF cells were infected with recombinant virus carrying N-moesin cDNA, cultured on 19cm dishes at 27℃ for 48h, then washed with PBS. After being suspended in low salt buffer (2mM Tris-HCl, pH10, 1mM EGTA, 0.5mM PMSF), the cells were sonicated and centrifuged at 100,000xg for 10min at 4℃. The pellet was resuspended and sonicated in TE (10mM Tris-HCl, pH8.0, 0.1mM EDTA) containing 1% Triton X-100 followed by a 60min incubation at 4℃. The

sonicate was centrifuged at 100,000xg for 1h, and the supernatant was dialyzed against DG. The dialysate was applied onto a heparin column (5ml in15x25mm; Hi TrapHeparin) which had been equilibrated with DG. After thoroughly washing with DG, proteins were eluted with a linear concentration gradient of KCl (0-800mM). Recombinant N-moesin was eluted at around 600mM KCl.

To produce the carboxy-terminal half of moesin (C-moesin), Sf9 cells were infected with recombinant virus containing C-moesin cDNA. C-moesin was purified in the same manner as the full-length moesin.

In Vitro Binding Assay between ERM proteins and CD44 Using GST-CD44cyt Beads

After the Glutathione-Sepharose beads containing GST-CD44cyt fusion protein or GST were pretreated with RIPA buffer (0.1% SDS, 0.5% DOC, 1% Nonidet P-40, 50mM Tris-HCl, pH8.0, 150mM NaCl) for 20min at room temperature, they were washed with 20 volumes of H buffer (10mM Hepes, pH7.5, 1mM $MgCl_2$). After a brief centrifugation, 30 μl of the glutathione-conjugated Sepharose bead slurry was suspended in 200 μl of H buffer containing 40mM or 150mM KCl. In some experiments, 150mM KCl H buffer contained 50 μg/ml phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (4-PIP), or phosphatidylinositol 4,5-bisphosphate (4,5-PIP_2) (Sigma Chemical Co., St.Louis, MO). Each phospholipid was dissolved in distilled water to a final concentration of 1mg/ml and sonicated three times each for 10s.

Bead suspension (230 μl) was incubated with 4 μg of purified ezrin, radixin, moesin, N-moesin, or C-moesin in 10-20 μl of 40mM KCl or 150mM KCl H buffer for 30min at room temperature. The beads were washed three times with H buffer by a brief centrifugation, and bound proteins were eluted from the beads with H buffer containing 30mM glutathione. The amount of GST-CD44cyt or GST in each eluate was determined by SDS-PAGE. An appropriate amount of each eluate was again applied to SDS-PAGE to

contain the same amount of GST-CD44cyt or GST, and the amount of ezrin, radixin, moesin, N-moesin or C-moesin in each eluate was evaluated by immunoblotting with specific mAbs.

Estimation of the Dissociation Constant between Moesin and CD44cyt

To estimate the dissociation constant between CD44cyt and moesin, 30 μl of the Glutathione-Sepharose bead slurry containing 40 μg of GST-CD44cyt were incubated in 240μl of 40 or 150mM KCl H buffer containing 0-100pmol (0-13.3 μg) of purified moesin in the presence or absence of 4,5-PIP_2. After the beads were washed three times with H buffer, bound moesin was eluted with H buffer containing 30mM glutathione. The amount of moesin in each eluate was estimated by comparing the silver staining intensity of moesin band with that of various amounts of authentic purified moesin resolved and silver stained in the same gel. The staining intensity was measured by a densitometer using Adobe-Photoshop TM 3.0J histogram and a Scatchard plot of the data was generated. Experiments were repeated three times for each estimation of Kd.

In Vitro Binding between ERM proteins and CD44 Using Ezrin/Radixin-coupled Beads

Purified ezrin (10μg) was coupled to 30 μl of Affigel-10 (Bio-Rad Laboratories, Richmond, CA). These ezrin-coupled beads were incubated with 1% BSA at 4℃ for 2h followed by 1mg/ml GST in H-buffer containing 150mM KCl at 4℃ overnight, then suspended in 200 μl of H-buffer containing 150mM KCl in the presence or absence of 50 μg/ml PS, PC, PI, 4-PIP or 4,5-PIP_2. They were then incubated with 5 μg purified GST-CD44cyt in 40 μl of 150mM KCl H-buffer for 30min at room temperature. In the control experiment, the GST-fusion protein with the cytoplasmic domain of E-cadherin was used instead of the GST-CD44cyt. After washing three times with the incubation solution, bound proteins were eluted from beads with the sample buffer for SDS-PAGE. Each eluate was resolved by electrophoresis and stained with Coomassie brilliant blue.

In some experiments, 20 μg of ezrin or radixin was coupled to 50 μl of CNBr-activated Sepharose (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). These beads were blocked only by 1% BSA at 4℃ for 2h, and 50 μl of beads were suspended in 200 μl of 150mM KCl H-buffer in the presence or absence of 50 μg/ml 4,5-PIP_2. They were then incubated with GST-CD44cyt, and the amount of bound GST-CD44cyt was determined according to the same procedure as that used with ERM-coupled Affigel beads, except that purified GST was used as a control and that bound GST-CD44cyt was detected by immunoblotting with anti-GST pAb.

Gel Filtration Chromatography

Gel filtration was performed on Superose 12R 10/30 (24ml;1x30cm; equilibrated with DG) at 4℃. Each ERM protein or GST-CD44cyt (6 μg) was mixed with no phospholipid, PS, PC, PI, 4-PIP, or 4,5-PIP_2 (20 μg) in ～200 μl of 10mM Hepes (pH7.5) containing 1mM $MgCl_2$ and 150mM KCl, and then passed through the column. Eluted proteins were detected by ELISA using anti-ERM protein pAb, I1, or anti-GST pAb.

Immunoprecipitation

BHK cells were cultured as monolayers in plastic dishes 100 mm in diameter to 80-90% confluence. After washing once with methionine-free medium supplemented with 2% fetal calf serum, the monolayers were incubated with 3 ml of the same medium containing 0.2 mCi [^{35}S]methionine (Amersham International plc., Bucks., UK) for 3h and washed three times with PBS. The labeled cells on one dish were lysed and incubated in 0.4-0.8 ml of RIPA buffer [0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 150mM NaCl, 50mM Tris (pH8.0), 1 mM *p*-amidinoPMSF, and 10 μg/ml leupeptin] for 5 min. The RIPA lysate was removed from the dish after fully dislodging any remaining cellular debris with a rubber policeman. After centrifugation at 12,000xg for 15 min, the RIPA-soluble supernatant was immunoprecipitated with 15μl of protein G-Sepharose 4B (Zymed Laboratories, Inc., South

San Francisco,CA) conjugated with anti-moesin mAb CR-22 or control mouse IgG. In some experiments, to chase the labeled moesin, 100 μl of 0.05mg/ml purified non-labeled recombinant moesin in 50mM KCl /10mM Hepes (pH 7.5) was added to 300 μl of the RIPA lysate of [^{35}S] methionine-labeled BHK cells. Sepharose 4B-bound immune complexes were washed five times with RIPA buffer. Immune complexes were then eluted by boiling in sample buffer that did not contain 2-mercaptoethanol, and resolved by SDS-PAGE. The [^{35}S] methionine signal was quantified by means of a Fujix Bioimage Analyzer Bas 2000 System (Fuji Film Co.Ltd., Tokyo).

For reimmunoprecipitation, the immune complexes were eluted from 15 μl of Sepharose 4B into 1.5-ml test tubes with 50 μl of a high salt solution consisting of 0.98M KCl, 0.02M NaCl, 1mM $MgCl_2$, 10mM MOPS (pH 7.4). The eluates from five tubes were combined and diluted with 6 vol of distilled water, then reimmunoprecipitated with 20 μl of protein G-Sepharose 4B conjugated with mouse anti rho-GDI mAb or control mouse IgG. After five washes with the high salt solution diluted with 6 vol of distilled water, immune complexes were eluted and analyzed as described above.

In Vitro Translation of Mouse Rho-GDI

Translation *in vitro* proceeded as reported (Pelham and Jackson, 1976). RNA was synthesized from the full-length cDNA of rho-GDI which was subcloned into the KpnI-digested Bluescript SK(-) vector using an mCAP™mRNA Capping Kit (Stratagene). Capped RNA was translated in mRNA-dependent rabbit reticulocyte lysate in the presence of [^{35}S] methionine using an *In Vitro* Express™ Translation kit (Strategene). The [^{35}S] methionine (1300Ci/mmol, Amersham International plc., Bucks., UK) radiochemical concentration was 2.6 mCi/ml in a final reaction volume of 25 μl. The reaction product in the *in vitro* translation reaction was then resolved by SDS-PAGE. The [^{35}S] methionine signal was quantified by means of a Fujix Bioimage Analyzer Bas 2000 System (Fuji Film Co. ,Tokyo).

Binding of Purified Recombinant ERM Proteins to the Insoluble Fraction of BHK Cell Lysate

BHK cell lysate was prepared from a monolayer in a 10cm dish at 60-70% confluence at 4℃. Cells were washed three times with PBS, scraped off using a rubber policeman in lysis buffer [0.06% Nonidet P-40, 0.6% glycerol, 130mM KCl, 20mM NaCl, 1mM sodium orthovanadate, 2mM $MgCl_2$, 0.1mM EGTA, 1mM EDTA, 1mM dithiothreitol (DTT), 10mM Hepes (pH 7.4), 1mM phenylmethyl-sulfonyl fluoride (PMSF), and 10 μg/ml leupeptin], then disrupted by 3 strokes in a loose-fitting teflon-glass homogenizer. The protein concentration of each lysate was adjusted to 12 mg/ml using the lysis buffer. The protein concentration was estimated from the absorbance at 280nm using BSA as the standard, after 5 μl of the cell lysate was completely solubilized by 95 μl of 2N NaOH. The BHK cell lysate (5 μl) was brought to 10 μl with 1/15 PBS (1mM sodium phosphate solution, pH 7.5, and 10mM NaCl). To examine the effect of GTPγS, 2 μl of 10mM GTPγS (pH 7.5) and 3 μl of 1/15 PBS were added to the BHK cell lysate (5 μl), and to examine the effect of C3, 2 μl of 10mM GTPγS (pH 7.5), 2 μl of 0.75mg/ml of C3 toxin, and 1 μl of 10mM NAD+ were added to the cell lysate. Thereafter, 5 μl of purified ezrin, radixin, or moesin [~0.16mg/ml eluate from the monoQ column in ~50mM KCl/10mM Hepes (pH 7.5)] was added, and incubated for 8h at 4℃, followed by centrifugation at 300,000xg for 10min in a 7x20mm tube (Beckman Optima TL Ultracetrifuge). The insoluble fraction recovered as a pellet was washed with the lysis buffer without Nonidet P-40/glycerol, and resolved by SDS-PAGE. The amount of ERM proteins in the insoluble fraction was estimated by comparing the staining intensity of each band in immunoblotting with that of the various amounts of authentic ERM proteins resolved by electrophoresis and immunoblotted on the same nitrocellulose sheet.

Exposing Living BHK Cells with C3 Toxin

BHK cells cultured to 80-90% confluence were incubated with 100 μg/ml C3 toxin in 1 ml of Opti-MEM medium (GIBCO BRL, Gaithersburg, MD) in the presence of 10 μl of lipofectamin (GIBCO Ltd.) for 4h. Control cells were incubated with the same culture medium without C3 toxin for 4h. To compare the amount of soluble and insoluble ERM proteins, cells exposed or not to C3 toxin were homogenized in a physiological solution consisting of 130mM KCl, 20mM NaCl, 1mM $MgCl_2$, 10mM Hepes (pH7.5), 1mM *p*-amidinoPMSF, and 0.01mg/ml of leupeptin. After centrifugation at 300,000xg for 20min, the amount of ERM proteins in the pellet and supernatant was quantified by immunoblotting using anti-ERM pAb I1. The cells exposed or not to C3 toxin in 60mm dishes were immunoprecipitated with anti-CD44 mAb (mAb30189) as described above, and the immunoprecipitates were separated by SDS-PAGE followed by immunoblotting with anti-ERM pAb I1.

SDS-PAGE and Immunoblotting

One-dimensional SDS-PAGE (7.5%-18%) proceeded essentially according to Laemmli (1970), and the gels were stained with Coomassie brilliant blue R-250. For immunoblotting, proteins were electrophoretically transferred from gels to nitrocellulose membranes, which were incubated with the first antibody. Bound antibodies were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and the appropriate substrates as described by the manufacturer (Amersham).

One-dimensional Peptide Mapping

Protein bands were excised from the SDS polyacrylamide gels, immersed in the sample buffer, then subjected to limited proteolysis with Staphylococcus aureus V8 protease in 18% SDS polyacrylamide gels as described by Cleveland, et al. (1977). The [^{35}S] methionine signal was quantified by means of a Fujix Bioimage Analyzer Bas 2000 System (Fuji Film Co.Ltd., Tokyo).

Results

In Vitro Binding Assay between GST-CD44cyt and ERM Proteins

To obtain a large amount of purified native ERM proteins for the *in vitro* binding assay, we overexpressed mouse ezrin, radixin, or moesin in insect Sf9 cells by means of recombinant baculovirus infection. The overexpressed ERM proteins were then purified to homogeneity through two chromatographic steps, and used for the *in vitro* binding assay (Fig.1). A fusion protein between GST and the cytoplasmic domain of mouse CD44 (GST-CD44cyt) was first expressed in *Escherichia coli* and purified. However, this fusion protein was degraded into smaller fragments very rapidly in *E.coli*, making it very difficult to purify the full-length GST-CD44cyt required for the *in vitro* binding assay. We thus overexpressed GST-CD44cyt in Sf9 cells by recombinant baculovirus infection and found that it was stable in Sf9 cells. This product was used for the *in vitro* binding assay (Fig.1).

GST-CD44cyt produced in the Sf9 system was incubated with Glutathione-Sepharose beads. After washing, purified recombinant ezrin, radixin, or moesin was added to the beads. After incubation and washing, the proteins associated with GST-CD44cyt were eluted with a buffer containing glutathione, resolved by SDS-PAGE, then immunoblotted with anti-ezrin, radixin, or moesin mAb. The control was GST produced in *E.coli* instead of GST-CD44cyt. All binding assays were performed at low and at physiological ionic strength (40mM and 150mM KCl, respectively). In some experiments, we used GST fusion proteins with the cytoplasmic domain of mouse E-cadherin as additional controls.

As shown in Fig.2, at low ionic strength, ezrin, radixin, and moesin bound to the cytoplasmic domain of CD44. By contrast, at physiological ionic strength, they bound very weakly to GST-CD44cyt. Control experiments with GST as well as with GST/cadherin showed that the non-specific background binding was negligible.

Using this assay, the interaction of moesin with CD44cyt was quantified by determining

the relationship between the amount of 'bound' moesin on beads and that of moesin in the incubation solution. The ratio of bound to total moesin over a range of 0-100pmol was estimated by subtracting the amount on control GST beads from that on GST-CD44cyt beads. As shown in Fig.3, at low ionic strength, moesin binding to CD44cyt was saturable. Scatchard analysis determined the dissociation constant to be 9.3 ± 1.6 nM (inset of Fig.3b). The CD44cyt/moesin interaction at physiological ionic strength was too weak for quantitation.

Effects of Phospholipids on the CD44cyt/ERM Binding

We investigated the regulation mechanism for the CD44/ERM interaction by means of this binding assay. Considering that many of the cellular events occurring just beneath the plasma membranes are regulated by phospholipids, we studied the effects of various phospholipids on the CD44/ERM interaction *in vitro*. As shown in Fig.4, neither phosphatidylserine (PS) nor phosphatidylcholine (PC) affected CD44/radixin or CD44/moesin interaction, whereas phosphoinositides significantly enhanced it at physiological ionic strength. Phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (4-PIP), and phosphatidyl-inositol 4,5-bisphosphate (4,5-PIP_2) apparently enhanced the binding of radixin or moesin to GST-CD44cyt, although there was a slight background binding to control GST. The effect of this enhancement of these three phosphoinositides was in the order of PI < 4-PIP < 4,5-PIP_2, as shown in Fig.4. The dissociation constant between CD44cyt and moesin at physiological ionic strength in the presence of 4,5-PIP_2 was determined to be 9.3 ± 4.8nM, according to the method described above.

The CD44/ezrin interaction also appeared to be enhanced by phosphoinositides. However, for unknown reason, ezrin showed a relatively high affinity for Sepharose beads in the presence of phosphoinositides, making it difficult to conclusively evaluate the phosphoinositide dependence of the CD44/ezrin interaction. To overcome this technical difficulty, we directly conjugated recombinant ezrin (and also radixin as a control) with

CNBr-activated Sepharose beads, and analyzed the interaction between those and soluble GST-CD44cyt in the absence or presence of 4,5-PIP_2 at physiological ionic strength. Fig.5a shows that in the absence of 4,5-PIP_2, only a small amount of GST-CD44cyt was specifically bound to the ezrin beads, whereas in the presence of 4,5-PIP_2 this binding was remarkedly enhanced, an effect that was also observed between radixin beads and GST-CD44cyt. Using the ezrin-coupled Affigel beads, we further confirmed the similar phospholipid-dependence of CD44/ezrin interaction to that of CD44/radixin and CD44/moesin which was shown using GST-CD44cyt beads (Fig.5b). Taken all together, we conclude that the direct interaction of ezrin, radixin and moesin with the cytoplasmic domain of CD44 is remarkedly enhanced by phosphoinositides, especially 4,5-PIP_2.

Binding Affinity of ERM proteins for Phosphoinositides

The enhancement of the CD44/ERM interaction by phosphoinositides suggested that either ERM proteins or the cytoplasmic domain of CD44, or both, have binding affinity for phosphoinositides. We evaluated the binding affinity of ERM proteins and GST-CD44cyt for various phospholipids using gel filtration chromatography (Fig.6).

Phospholipids were eluted in the void volume from a Superose 12R 10/30 column, due to micelle formation. ERM proteins were eluted at a position corresponding to an apparent molecular weight of ~60kD. As shown in Fig.6a, purified recombinant moesin was incubated with various phospholipids, and passed through the column. An incubation with PS, PC, or PI did not affect the behavior of moesin in the column. In sharp contrast, when moesin was incubated with 4-PIP or 4,5-PIP_2, it co-eluted with 4-PIP or 4,5-PIP_2 in the void volume. The same results were obtained using purified recombinant ezrin and radixin. A similar binding assay by means of gel filtration was performed between various lipids and GST-CD44cyt. None of PS, PC, PI, 4-PIP or 4,5-PIP_2 affected the behavior of GST-CD44cyt in the column (Fig.6b).

Phosphoinositide-independent Binding of the Amino-terminal Half of Moesin to the Cytoplasmic Domain of CD44

We produced amino- and carboxy-terminal halves of mouse moesin by means of recombinant baculovirus infection, purified them to homogeneity, then incubated them with the GST-CD44cyt beads at low or physiological ionic strength in the absence or presence of 4,5-PIP_2 (Fig.7). In sharp contrast to full length moesin, the amino-terminal half bound to GST-CD44cyt not only at low, but also at physiological ionic strength in the absence of 4,5-PIP_2, indicating that the binding between GST-CD44cyt and the amino-terminal half of moesin at physiological ionic strength did not require 4,5-PIP_2. The carboxy-terminal half of moesin did not have any binding affinity for GST-CD44cyt under any of the conditions tested.

Rho-GDI Coimmunoprecipitated with the CD44/moesin Complex

The above *in vitro* findings suggest that the formation of CD44/ERM complex is dynamically regulated *in vivo*. As a first step to examine the *in vivo* regulation mechanism of this complex formation, we immunoprecipitated the CD44/moesin complex from cultured BHK cells (Tsukita et al., 1994), and reexamined its constituents, focusing upon the small molecular mass range. Cultured BHK cells were metabolically labeled with [^{35}S]-methionine, solubilized with RIPA buffer, then immunoprecipitated with anti-moesin mAb, CR-22. The immunoprecipitate was resolved in 12.5% SDS-polyacrylamide gels in the absence of mercaptoethanol to bring the IgG in the sample into the high molecular mass region. These conditions allowed small molecular mass constituents of the immunoprecipitates to be analyzed by autoradiography (Fig.8a).

As compared with the control immunoprecipitate, that of moesin reproducibly contained at least three low molecular mass polypeptides (55, 30, and 25kD) in addition to moesin and CD44 (Tsukita et al., 1994). To exclude the possibility of non-specific binding of these polypeptides to the immunoprecipitate, we added the excess amount of purified unlabeled recombinant moesin into the lysate from [^{35}S]-methionine-labeled BHK cells prior to

immunoprecipitation (Fig.8b). As a result of competition, most [^{35}S]-methionine-labeled bands (three low molecular mass polypeptides [55, 30, and 25kD], moesin and CD44) were removed from the immunoprecipitate, indicating that these low molecular mass polypeptides and CD44 were specifically precipitated with anti-moesin mAb through their association with moesin.

To identify these low molecular mass components, we analyzed the crossreactivity of various antibodies specific to known cytoplasmic proteins. Considering that the Rho signaling pathway plays a key role in the regulation of actin filament/plasma membrane association, we investigated whether or not there were Rho and/or Rho-regulators in the above moesin immunoprecipitate. The molecular mass of Rho is around 21kD, so we concluded that Rho itself was not included in the immunoprecipitate. Among Rho-regulators, the molecular mass of Rho-GDI is reportedly ~30kD (Fukumoto et al., 1990). Then we checked whether or not the 30kD band in the moesin immunoprecipitate is identical to Rho-GDI.

The moesin immunoprecipitate was resolubilized, and reimmunoprecipitated with anti-mouse Rho-GDI mAb. As shown in Fig.9a, most of the 30kD band was specifically reimmunoprecipitated by this mAb, leaving the other bands in the supernatant. Since this mAb was raised against mouse Rho-GDI, we examined its specificity using hamster Rho-GDI. When BHK cells were immunoprecipitated with the anti-mouse Rho-GDI mAb, a 30kD polypeptide was specifically recognized (Fi.g9a). Furthermore, we compared the 30kD band in the moesin immunoprecipitates with the *in vitro* translated mouse Rho-GDI by one dimensional peptide mapping. As shown in Fig.9b, the peptide map of the 30kD band was very similar to that of *in vitro* translated mouse Rho-GDI. We thus concluded that Rho-GDI is tightly associated with the CD44/moesin complex *in vivo*.

Binding of Recombinant ERM proteins to the Insoluble Fraction of Crude Cell Homogenate is Suppressed by C3 Toxin

To initially evaluate the notion that the formation of CD44/ERM complex is regulated by Rho-signaling pathway, we examined whether or not a GTP-binding protein is involved in regulating the binding of ERM proteins to the insoluble components of cells including plasma membranes. For this purpose, we established a cell-free system that allowed the estimation of ERM binding affinity to the insoluble components. Since we identified the CD44/ERM complex in BHK cells by immunoprecipitation, we used the same cells in this experiment.

BHK cells were lysed in lysis buffer at physiological ionic strength containing 0.06% Nonidet P-40, and the protein concentration in the lysate was adjusted to ~12 mg/ml. To estimate the binding affinity of ERM proteins to the insoluble fraction, recombinant ezrin, radixin, or moesin was added and incubated with the lysate. When ~0.8 μg of ezrin, radixin, or moesin was added to the lysate to a final volume of 15 μl, ~0.05 μg of each protein was recovered in the insoluble fraction after centrifugation (Fig.10a). The amount of endogenous ERM proteins recovered in the insoluble fraction was too small to be detected under our experimental conditions. The amount of recovered protein was estimated by comparing the staining intensity of each band in immunoblotting with that of the various amounts of authentic ERM proteins resolved by electrophoresis and immunoblotted on the same nitrocellulose sheet (Fig.10b). When the amount of ERM proteins added was increased, their binding to the insoluble fraction was saturable: at the level of ~0.8 μg added ERM proteins, binding was saturated sufficiently. Upon adding GTPγS to the incubation system, the amount of recovered ezrin, radixin, and moesin was increased 1.9 fold±0.5, 2.1 fold±0.3, and 3.1 fold±1.3, respectively, averaged from four experiments. This suggested that the GTPγS activation of some GTP-binding protein in cell

lysates increases the ERM binding ability to the insoluble fraction.

C3 toxin, a specific Rho inactivator, was added to the incubation mixture with NAD+. As shown in Fig.10a, the binding ability of ERM proteins to the insoluble fraction of the lysate was remarkedly suppressed by C3 toxin at a concentration of 0.1mg/ml. In the presence of $GTP\gamma S$, C3 toxin reduced the binding affinity of ezrin, radixin, and moesin to 21%±10%, 29%±5%, and 50%±35%, respectively, of the value in the absence of C3 toxin and $GTP\gamma S$ (control value). Also in the absence of $GTP\gamma S$, the respective binding affinities were reduced by C3 toxin to 23%±11%, 25%±12%, and 32%±30% of the control value, suggesting that there was some stimulation by endogenous active Rho in the lysate. As a control, we added same amount of BSA or IgG to the BHK cell lysate instead of ERM proteins, no Rho-dependent binding of these proteins was identified. As another control, we added the recombinant ERM proteins to the same amount of buffer instead of the BHK cell lysate, no $GTP\gamma S$/C3 toxin-dependent precipitation of ERM proteins was observed. These results indicated that activated Rho is required for the interaction between the insoluble fraction and recombinant ERM proteins.

Binding of Recombinant ERM Proteins to the Insoluble Fraction of BHK Cell Lysate is Suppressed by an Anti-CD44 Cytoplasmic Domain Monoclonal Antibody

We studied whether the Rho-dependent association of exogenous ERM proteins with the insoluble fraction can be attributed to the CD44/ERM or other unknown interactions. We raised mAbs using the GST fusion protein with the cytoplasmic domain of mouse CD44 as the antigen in rats, then studied whether or not they interfere with the Rho-dependent association of ERM proteins in the presence of $GTP\gamma S$. We obtained one mAb (45-4), that recognized the cytoplasmic domain of hamster CD44 as well as mouse CD44 on immunoblots (Fig.11a). When 0.5 μg of purified mAb 45-4 was added to 15 μl of the incubation

solution, the binding affinity of ERM proteins to the insoluble fraction was suppressed (Fig.11b), to a level that was almost equivalent to that of C3 toxin. In contrast, nonimmune rat IgG did not affect the association. Furthermore, this mAb effectively interfered with the *in vitro* binding of recombinant moesin with GST-CD44cyt at physiological ionic strength in the presence of 4,5-PIP_2, while control nonimmune IgG did not affect this interaction (Fig.11c).

C3 Toxin Interferes with ERM-Plasma Membrane Interaction In Living Cells

We examined the effect of C3 toxin on the ERM-plasma membrane interaction in living BHK cells. This strategy is however, technically difficult. C3 toxin-induced morphological changes (rounding-up of cells) are undetectable for 12h in living BHK cells and the effect is not particularly synchronized among treated cells. To circumvent this difficulty, we introduced C3 toxin into cells using the lipofectamin, which rather clarified its effect.

After a 4h-incubation with the lipofectamin-conjugated C3 toxin, BHK cells were homogenized in PBS, then divided into soluble and insoluble fractions by centrifugation. From the untreated BHK cells, ~30% of the endogenous ERM proteins were recovered in the insoluble fraction, whereas only a trace amount was recovered in the insoluble fraction obtained from C3 toxin-treated cells (Fig.12a). The untreated and C3 toxin-treated BHK cells were lysed with RIPA buffer and immunoprecipitated with anti-CD44 antibody (Fig.12b). From untreated BHK cells, some ERM proteins were recovered and detected by immunoblotting in the CD44 immunoprecipitate, whereas ERM proteins were virtually undetectable in the CD44 immunoprecipitate from the C3 toxin-treated cells.

Discussion

ERM proteins directly bind to the cytoplasmic domain of CD44 as a 4,5-PIP_2-dependent manner in vitro.

We found that ERM proteins coimmunoprecipitate with CD44 (Tsukita et al., 1994). However, so far *in vitro* evidence for a direct association between the cytoplasmic domain of CD44 and ERM proteins has not been obtained. Here using an *in vitro* binding assay we revealed that at low ionic strength (40mM KCl) ERM proteins directly bind to the cytoplasmic domain of CD44 at relatively high affinity (Kd of moesin is 9.3±1.6nM). Although this direct binding became undetectable at physiological ionic strength (150mM KCl), 4,5-PIP_2 facilitated the CD44 binding of ERM proteins (Kd of moesin is 9.3±4.8nM). Thus, we concluded that ERM proteins directly bind to the cytoplasmic domain of CD44 in a regulated manner, and that they are essentially indistinguishable with respect to their binding capacity with the cytoplasmic domain of CD44.

Many actin-modulating proteins, such as gelsolin (Janmey and Stossel, 1987), gCap39 (Yu et al., 1990), profilin (Lassing and Lindberg, 1985), cofilin (Yonezawa et al., 1991), and α-actinin (Fukami et al., 1992) reportedly bind to 4,5-PIP_2. 4,5-PIP_2 induces the dissociation of profilin-actin and gelsolin-actin complexes, and the interaction of cofilin with G- and F-actin is also inhibited by PI, 4-PIP, and 4,5-PIP_2. Through these mechanisms, the structural integrity of actin-based cytoskeletons is thought to be regulated depending on the level of phosphoinositides *in vivo*. The interaction of ERM proteins with the cytoplasmic domain of CD44 is enhanced by 4,5-PIP_2, increasing the number of actin filament-association sites on the cytoplasmic surface of plasma membranes. This may also markedly affect the structural integrity of actin-based cytoskeletons. ERM proteins localize and function just beneath the plasma membrane, so compared with other actin-modulating proteins, they have many topological opportunities to interact with phosphoinositides, which are components of plasma membranes.

The mechanism by which 4,5-PIP_2 enhances the association between the cytoplasmic domain of CD44 and ERM proteins.

This study revealed that ERM proteins bind to 4,5-PIP_2 with high affinity. Considering that the cytoplasmic domain of CD44 does not have any affinity for 4,5-PIP_2, the facilitation of the CD44/ERM interaction by 4,5-PIP_2 is derived not from artifactual crosslinking of CD44 and ERM by 4,5-PIP_2 micelles, but from the 4,5-PIP_2-induced conformational change of ERM proteins. During the course of this study, Nigglie et al. (1995) independently reported using different techniques that 4,5-PIP_2 binds ezrin with high affinity, and that its binding domain of ezrin has been narrowed down to the amino-terminal half.

Several studies have indicated that the carboxy- and amino-terminal halves of ERM proteins have distinct functions. The respective halves bind to actin-based cytoskeletons and plasma membranes (Algrain et al., 1993). Some *in vitro* and *in vivo* data suggested that the amino-terminal half of native ERM proteins interferes with the interaction of their carboxy-terminal half with actin filaments (Turunen et al., 1994; Martin et al., 1995). Actually, the amino-terminal half of ezrin binds to its carboxy-terminal half directly (Gary and Bretscher, 1995).

In this study, at physiological ionic strength in the absence of phosphoinositides, full length moesin very weakly bound the cytoplasmic domain of CD44, whereas the amino-terminal half of moesin bound with high affinity. This indicated not only that the amino-terminal half of moesin is responsible for its association with plasma membranes (CD44), but also that in native moesin, the carboxy-terminal half suppresses the direct interaction of the amino-terminal half with the cytoplasmic domain of CD44. These findings indicate that the amino- and carboxy-terminal halves of ERM proteins suppress the functions of their carboxy- and amino-terminal halves; that is, binding to actin-based cytoskeletons and plasma membranes, respectively. This mutual suppression mechanism would be very important for ERM proteins to function as molecular crosslinkers between actin filaments and plasma membranes. This study showed that 4,5-PIP_2 can function as a key modulator

to release this mutual suppression immediately below the plasma membrane.

Rho-GDI is associated with the CD44/moesin complex in vivo.

To examine the *in vivo* regulation mechanism of the CD44/ERM complex formation, we re-examined the immunoprecipitated CD44/moesin complex from BHK cells. Immunoprecipitation and peptide mapping revealed that this immunoprecipitate contained not only moesin and CD44 but also three smaller molecular mass proteins around 55, 30 and 25kD, and that the 30kD protein is identical to hamster Rho-GDI. So far, the physiological function of Rho-GDI is not clear. Rho-GDI was originally thought to form a tight complex with the GDP-form of Rho to form a cytoplasmic pool (Araki et al., 1990). However, it may recruit cytoplasmic Rho to the plasma membrane to be activated there as it is in Rab-GDI (Novick and Garret et, 1994; Takai et al., 1995; Horiuchi et al., 1995). The Rab molecule undergoes a cycle of localization coupled with that of nucleotide exchange and hydrolysis (Novick and Garrett, 1994): Rab remains soluble until the Rab/Rab-GDI complex interacts with the appropriate donor membrane, promoting dissociation from Rab-GDI, membrane attachment and the exchange of GDP for GTP (Ullrich et al., 1994; Soldati et al., 1994; Horiuchi et al., 1995). If this recycling pathway of Rab is also true of Rho, the occurrence of Rho-GDI in the CD44/moesin complex would indicate that this complex is a site where active Rho regulates for plasma membrane/actin filament interaction. The immunoprecipitation results agree with the findings that in MDCK cells expressing myc-tagged RhoA, activated Rho is reportedly translocated from the cytoplasm to ERM-concentrated regions of plasma membranes (Takaishi et at., 1995).

In our immunoprecipitates, probably due to the severe solubilizing conditions, Rho itself was not detected, leaving Rho-GDI bound to the complex. As far as we examined, none of the available antibodies specific for Rho-related proteins such as Rho-GDS and Rho-GAP recognized the other 55 and 25kD bands in the immunoprecipitate. The identification of these proteins will lead to a better understanding of the physiological functions of

CD44/ERM complex. However, the small amount of these proteins recovered as immunoprecipitates makes this identification technically challenging.

The CD44/ERM complex formation is regulated by Rho in vivo.

To evaluate the involvement of Rho in the CD44/ERM complex formation, we developed a cell-free system with which to examine the binding affinity of exogenous ERM proteins to the insoluble fraction of the BHK cell lysate. Using this system, we found that GTPγS facilitated the binding, and that C3 toxin remarkedly suppressed it in the presence as well as in the absence of GTPγS. This indicated that the GTP-form of Rho included in the cell lysate is required for this binding. Furthermore, we found that one of the anti-CD44 cytoplasmic domain mAbs also effectively suppressed the ERM binding to the insoluble fraction, and that this mAb interfered with the association between ERM proteins and CD44 *in vitro*. We then concluded that most of the membrane binding partners in this BHK cell-free system are CD44 molecules, and that the association between ERM proteins and the cytoplasmic domain of CD44 is regulated by Rho. As described in the Introduction, there are other potential ERM membrane binding partners. It remains elusive whether or not the binding of these possible partners to ERM proteins are also Rho-dependent.

Recently, ezrin was reported to be oligomerized possibly through phosphorylation (Gary and Bretscher, 1995; Berryman et al., 1995). This nature of ezrin is probably shared by radixin and moesin (Andreoli et al., 1994). The effective suppression of ERM binding to the insoluble fraction by an anti-CD44 cytoplasmic domain mAb, however, indicates that our cell-free system detected CD44/ERM interaction rather than ERM oligomerization.

The results obtained from the incubating living BHK cells with C3 toxin also favored the notion that the formation of CD44/ERM complex is dependent on active Rho. C3 toxin in living cells recruited most insoluble ERM proteins to the soluble pool, leaving CD44 free from ERM proteins. Considering that the effect of C3 toxin in living cells is not sharp in terms of its time course and synchronization, the effect observed in living cells is highly

compatible to that observed in our cell-free system.

Ridley et al. (1992a,b) have found using serum-starved Swiss 3T3 cells, that Rho plays a central role in the coordinated assembly of focal adhesions and stress fibers induced by growth factors, and that Rac stimulates actin filament accumulation at the plasma membrane, forming membrane ruffles. Furthermore, Cdc42 controls the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). However, the relationship between these three small GTP-binding proteins and actin-based cellular events does not appear simple. For example, in KB and MDCK cells membrane ruffle formation is reportedly regulated by Rho (Takaishi et al., 1993; Nishiyama et al., 1994;), and the cleavage furrow formation is also Rho-dependent in Xenopus and sea urchin eggs (Kishi et al., 1993; Mabuchi et al., 1993). The Rho-dependent enhancement of the CD44/ERM complex formation would provide an increasing number of actin filament attachment sites on the cytoplasmic surface of plasma membranes at microvilli, cleavage furrows, ruffling membranes, and cell-cell/cell-matrix adhesion sites. This speculation could explain the diversified functions of Rho in the regulation mechanism of actin filament/plasma membrane interaction in general.

The elevation of 4,5-PIP_2 level in plasma membranes is possibly involved in the Rho-dependent regulation of the CD44/ERM complex formation.

The question then arises as to how Rho regulates the formation of the CD44/ERM complex *in vivo*. One explanation is that Rho directly regulates CD44/ERM association. This possibility was not considered likely. An alternative is that through one or several Rho downstream components, this complex formation is Rho-dependent. Several intensive studies have been performed to identify the direct target of Rho. However, the list of activities stimulated by Rho as well as Rac and Cdc42 is long, and includes cascades of kinases that regulate gene transcription and cell growth, none of which have been linked to the actin filament/plasma membrane interactions (Zigmond, 1995; Watanabe et al., 1996; Amano et al., 1996). Rho reportedly regulates phosphatidylinositol (PI) turnover (Chong et al., 1994; Divecha and Irvine, 1995; Zhang et al., 1995). Furthermore, considering that Rac also rearranges the actin filaments also through 4,5-PIP_2 synthesis (Hartwig et al., 1995), Rho might control actin-based cellular events by similar mechanisms. The enzymatic activities of phosphatidylinositol 3-kinase (Zhang et al., 1993), phosphatidylinositol 4-phosphate 5-kinase (Chong et al., 1994), and phospholipase D (Bowman et al., 1993; Malcolm et al., 1994) are reportedly regulated by Rho. Among them, the activation of phosphatidylinositol 4-phosphate 5-kinase elevates the 4,5-PIP_2 concentration in membranes.

At least as one of the signaling pathways that links Rho to the actin-based cytoskeleton, we speculate the following. [1] Activation of Rho directly or indirectly upregulates PIP5-kinase. [2] Activated PIP5-kinase elevates the 4,5-PIP_2 level in plasma membranes. [3] The CD44/ERM complex formation is induced by 4,5-PIP_2. The elevated 4,5-PIP_2 level in the plasma membranes could be derived from mechanisms other than PIP5-kinase activation. Regardless, considering that lovastatin, a general inhibitor of small GTP-binding proteins, suppresses the elevation of 4,5-PIP_2 in the plasma membrane *in vivo* (Chong et al., 1994), Rho is a likely upstream regulator of this elevation.

Rho also reportedly regulate the activity of some serine/threonine kinases (Zigmond, 1995; Watanabe et al., 1996; Amano et al., 1996). ERM proteins are highly serine/threonine phosphorylated (Gould et al., 1986; Urushidani et al., 1989; Nakamura et al., 1995), and their phosphorylation is suggested to be involved in the regulation of the ERM/membrane association (Chen et al., 1995). We should examine the possibility that Rho regulates the ERM/membrane association through the serine/threonine phosphorylation of ERM proteins. The *in vitro* binding system developed in this study would be useful for this purpose. Further molecular studies on how active Rho facilitates the formation of CD44/ERM complex *in vivo* and how actin filament association with plasma membranes is facilitated through the formation of this complex, are now underway in our laboratory.

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Figure 1. Recombinant proteins used in the *in vitro* binding assay. Fusion proteins between GST and the cytoplasmic domain of mouse CD44 (GST-CD44cyt), mouse ezrin, radixin and moesin produced by means of recombinant baculovirus infection, were purified to homogeneity. GST was produced in E.coli. The mobility of the molecular markers is shown on the left (110, 88, 48, 33, and 29kD from the top), and each band was stained with Coomassie brilliant blue.

Figure 2. Association of ezrin, radixin, and moesin with GST-CD44cyt-bound beads. GST-CD44cyt or GST bound to Glutathione-Sepharose beads was incubated with purified recombinant ezrin, radixin, or moesin at low (*40mM KCl*) or physiological ionic strength (*150mM KCl*). After washing, the protein associated with GST-CD44cyt (*F*) or GST (*G*) was eluted from the beads with a buffer containing glutathione. Proteins in the glutathione-eluate were separated by SDS-PAGE followed by immunoblotting with anti-ezrin, anti-radixin, or anti-moesin mAbs. The bands of ezrin, radixin, and moesin are indicated by *E*, *R*, and *M* on the left.

Figure 3. Quantitative analysis of the binding between moesin and GST-CD44cyt at 40mM KCl. (*a*) Estimation of the amount of 'bound' moesin. Recombinant moesin (0.75-96pmol) was added to the GST-CD44cyt or GST beads, and 15% of each glutathione-eluate was applied to each lane of SDS-PAGE. The silver staining intensity of moesin band in glutathione-eluates (*left*) was compared with that of the various amounts of authentic purified moesin resolved and silver stained in the same gel (*right*). (*b*) Binding mode of moesin to GST-CD44cyt beads. After subtraction of the GST-dependent background binding, the amount of bound versus added moesin (0-100pmol) was plotted. Scatchard analysis (*inset*) determined that the Kd was 9.3+1.6nM.

Figure 4. Effects of phospholipids on the association of radixin and moesin with the cytoplasmic domain of CD44. As described in Fig.2, radixin or moesin was incubated with GST-CD44cyt or GST bound to beads at physiological ionic strength in the absence (*C*) or presence of phosphatidylserine (*PS*), phosphatidylcholine (*PC*), phosphatidylinositol (*PI*), phosphatidylinositol 4-phosphate (*PIP*), or phosphatidylinositol 4,5-bisphosphate (PIP_2). After washing, the protein associated with GST-CD44cyt (*F*) or GST (*G*) was eluted from the beads with a buffer containing glutathione. Proteins in the glutathione-eluate were separated by SDS-PAGE, then immunoblotted with anti-radixin or anti-moesin mAbs.

Figure 5. Association of GST-CD44cyt with ezrin- or radixin-coupled beads. (*a*) Ezrin- or radixin-coupled Sepharose beads (*Ezrin* or *Radixin*, respectively) were incubated with purified GST-CD44cyt (*F*) or GST (*G*) at physiological ionic strength (150mM KCl) in the absence ($-PIP_2$) or presence ($+PIP_2$) of phosphatidylinositol 4,5-bisphosphate, and bound proteins were eluted with SDS-PAGE sample buffer. The eluates were separated by SDS-PAGE then immunoblotted with anti-GST pAb. (*b*) Ezrin-coupled Affigel beads were incubated with purified GST-CD44cyt at physiological ionic strength (150mM KCl) in the presence of phosphatidylserine (*PS*), phosphatidylchorine (*PC*), phosphatidylinositol (*PI*), phosphatidylinositol 4-phosphate (*PIP*), or phosphtidylinositol 4,5-bisphosphate (PIP_2), and bound proteins were eluted with SDS-PAGE sample buffer. The eluates were resolved by electrophoresis and stained with Coomassie brilliant blue.

Figure 6. Binding affinity of ERM proteins and the cytoplasmic domain of CD44 with phospholipids. (*a*) Interaction of moesin with phospholipids. Purified recombinant moesin was incubated without (*Moesin*) or with phosphatidylserine (*PS*), phosphatidylcholine (*PC*), phosphatidylinositol (*PI*), phosphatidyl-inositol 4-phosphate (*PIP*), or phosphatidylinositol 4,5-bisphosphate (PIP_2), and passed through a column containing Superose 12R gel. Elution profiles of moesin were obtained by ELISA with anti-moesin mAb. An incubation of moesin with PS, PC, or PI did not affect its behavior in the column. By contrast, when moesin was incubated with 4-PIP or 4,5-PIP_2, it co-eluted with 4-PIP or 4,5-PIP_2 in the void volume. 4-PIP and 4,5-PIP_2 had the same effect upon ezrin and radixin (data not shown). (*b*) Binding affinity of GST-CD44cyt with 4,5-PIP_2. Incubating GST-CD44cyt with 4,5-PIP_2 did not affect its binding behavior.

Figure 7. Interaction of amino- and carboxy-terminal halves of moesin with GST-CD44cyt beads. As described in the legend to Fig.2, the amino- or carboxy-terminal half of moesin (*N-Moesin and C-Moesin, respectively*) was incubated with GST-CD44cyt- or GST-bound beads at low (*40mM*) or at physiological ionic strength (*150mM*) in the absence or presence (*+ PIP_2*) of 4,5-PIP_2. After washing, the protein associated with GST-CD44cyt (*F*) or GST (*G*) was eluted from the beads with a buffer containing glutathione. Proteins in the glutathione-eluate were separated by SDS-PAGE, then immunoblotted with anti-ERM pAbs (p-800 and I1) for the amino- and carboxy-terminal halves, respectively. The bands of C- and N-moesin are indicated by *C* and *N* on the left.

Figure 8. Coimmunoprecipitation of low molecular mass proteins with the CD44/ERM complex. (*a*) Cultured BHK cells were metabolically labeled with [^{35}S]methionine, solubilized with RIPA buffer, then immunoprecipitated with anti-moesin mAb CR-22 or non-immune mouse IgG. The immunoprecipitate (*anti-moesin mAb* or *IgG*, respectively) was separated in a 15% SDS-polyacrylamide gel in the absence of mercaptoethanol to bring the IgG band to the high molecular mass regions, and quantified by image analysis. In addition to CD44 (*CD44*) (see Tsukita et al., 1994), three bands around 55, 30, and 25kD coimmunoprecipitated with moesin (*M*). (*b*) Purified, unlabeled moesin was added to the RIPA lysate of [^{35}S]methionine-labeled BHK cells, and immunoprecipitated with anti-moesin mAb CR-22 or non-immune mouse IgG (*anti-moesin mAb* or *IgG*, respectively). [^{35}S]methionine-labeled bands of 55, 30, and 25kD as well as CD44 were undetectable, although a weakly labeled moesin band was evident.

Figure 9. Identification of the 30kD polypeptide in the moesin immunoprecipitate as a hamster Rho-GDI. (*a*) Specific recognition of the 30kD peptide by anti-mouse Rho-GDI mAb. The moesin immunoprecipitate (see Fig.8) was solubilized from the beads with high salt, then the eluate (*high salt eluate*) was again immunoprecipitated with anti-mouse Rho-GDI mAb (*anti-rhoGDI*) or non-immune mouse IgG (*IgG*). The 30kD band (*arrow*) was specifically immunoprecipitated, leaving the supernatant lacking the 30kD band (*supernatant*). This anti-mouse Rho-GDI mAb specifically immunoprecipitated a polypeptide with a molecular mass of 30kD from the BHK cells metabolically labeled with [^{35}S]methionine (*anti-rhoGDI from BHK cells*). (*b*) One-dimensional peptide mapping. Mouse Rho-GDI, which was translated in a cell-free system then labeled with [^{35}S]methionine using a rabbit reticulocyte lysate, and the 30kD band in the moesin immunoprecipitate from BHK cells (see Fig.8) were analyzed by one-dimensional peptide mapping using Staphylococcus aureus V8 protease. The map of mouse Rho-GDI was very similar to that of the hamster 30kD band.

Figure 10. Rho-dependent association of the recombinant ERM proteins with the insoluble fraction of BHK cell lysate. (*a*) Immunoblot analyses. After ~0.8μg recombinant ezrin, radixin, or moesin was incubated with the BHK cell homogenate followed by centrifugation, the pellet was analyzed by immunoblotting with anti-ERM pAb I1. Under these conditions, the amount of endogenous ERM proteins was too small to be detected as compared to that of added ERM proteins, and ~0.05μg of each ERM protein was recovered in the insoluble fraction (*Control*). When GTPγS was added to the incubation mixture, the amount of recovered ERM proteins in the insoluble fraction was significantly increased (*GTPγS*). C3 toxin markedly suppressed the binding of each ERM protein to the insoluble fraction in the presence (*GTPγS+C3*) as well as in the absence (*C3*) of GTPγS. The bands of ezrin, radixin, and moesin are indicated by *E*, *R*, and *M* respectively, on the left. (*b*) Relative binding ability of ERM proteins to the insoluble fraction. The amount of recovered protein was estimated by comparing the staining intensity of each band in immunoblotting with that of the various amounts of authentic ERM proteins resolved by electrophoresis and immunoblotted on the same nitrocellulose sheet. A relative binding ability of 1 was equivalent to the amount of recovered ezrin, radixin, or moesin in the insoluble fraction for each experiment. Values represent relative binding abilities averaged from four experiments ± SEMs.

Figure 11. The cytoplasmic domain of CD44 as a binding partner in the insoluble fraction of BHK cell lysate for recombinant ERM proteins. (*a*) Specificity of mAb 45-4 for the cytoplasmic domain of mouse CD44. Immunoblotting of the lysate of Sf9 cells expressing the GST fusion protein with the cytoplasmic domain of mouse CD44 (*GST-CD44cyt*) or the lysate of E.coli expressing the GST protein (*GST*) with anti-GST pAb (*anti-GST pAb*) or mAb 45-4 (*mAb 45-4*). Anti-GST pAb recognized both GST-CD44cyt (*arrow*) and GST (*arrowhead*), whereas mAb 45-4 recognized only GST-CD44cyt. (*b*) The same binding experiments were performed as those shown in Fig.10, except that C3 toxin was replaced with the purified mAb 45-4 (*mAb 45-4*). As controls, non-immune rat IgG was added instead of mAbs (*IgG*). The mAb 45-4 markedly suppressed the binding of recombinant ERM proteins. The bands of ezrin, radixin, and moesin are indicated by *E*,*R* and *M* respectively, on the left. (*c*) Effects of the mAb 45-4 on the association of recombinant moesin with the GST-CD44cyt beads at physiological ionic strength in the presence of 4,5-PIP_2. Purified mAb 45-4 (*+mAb45-4*) or control non-immune IgG (*+IgG*) was added to the incubation mixture at a final concentration of 30 μg/ml. Moesin bound to GST-CD44cyt (*F*) or GST (*G*) was eluted and detected by immunoblotting with anti-moesin mAb.

Figure 12. Behavior of ERM proteins in the C3 toxin-treated living BHK cells. (*a*) BHK cells incubated in the presence (*C3*) or absence (-) of C3 toxin were homogenized in physiological solution (see Materials and Methods), then divided into soluble supernatant (*S*) and insoluble pellet (*P*) fractions by centrifugation. Each fraction was resolved by SDS-PAGE, and ERM proteins were detected by immunoblotting with anti-ERM pAb I1. In the insoluble fraction of untreated BHK cells (-), ~30% of the total ERM proteins was recovered, whereas only a trace amount was recovered in the insoluble fraction of C3 toxin-treated cells (*C3*). (*b*) The RIPA lysates of C3 toxin-treated (C3) or untreated (-) BHK cells were immunoprecipitated by mAb 30189 which is specific for the extracellular domain of hamster CD44, and each immunoprecipitate was immunoblotted with anti-ERM pAb I1. ERM proteins were reproducibly detected in the CD44 immunoprecipitate in the absence of C3 toxin as described (Tsukita et al, 1994), whereas they were hardly detectable in the immunoprecipitate in the presence of C3 toxin.

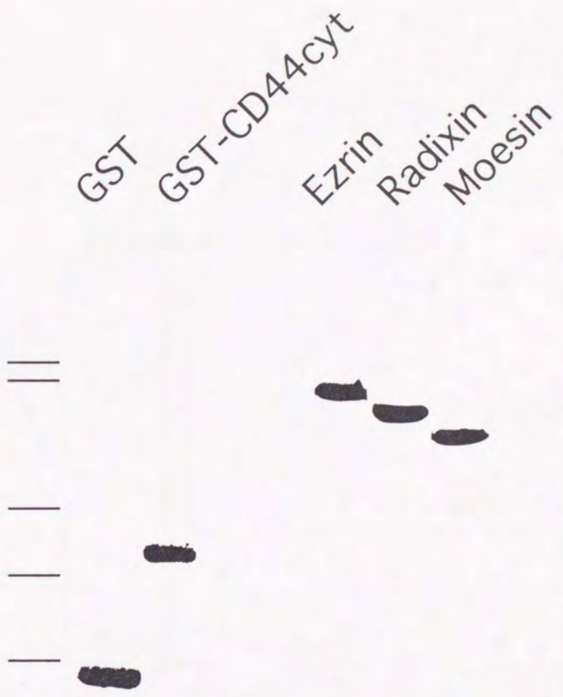


Fig.1 Hirao et al.

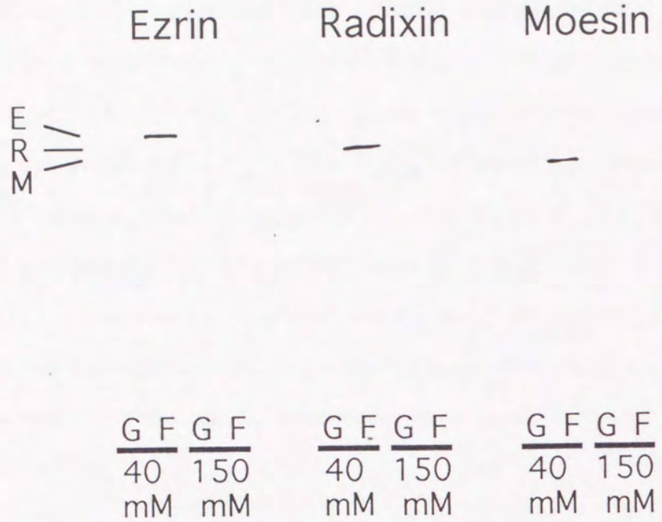
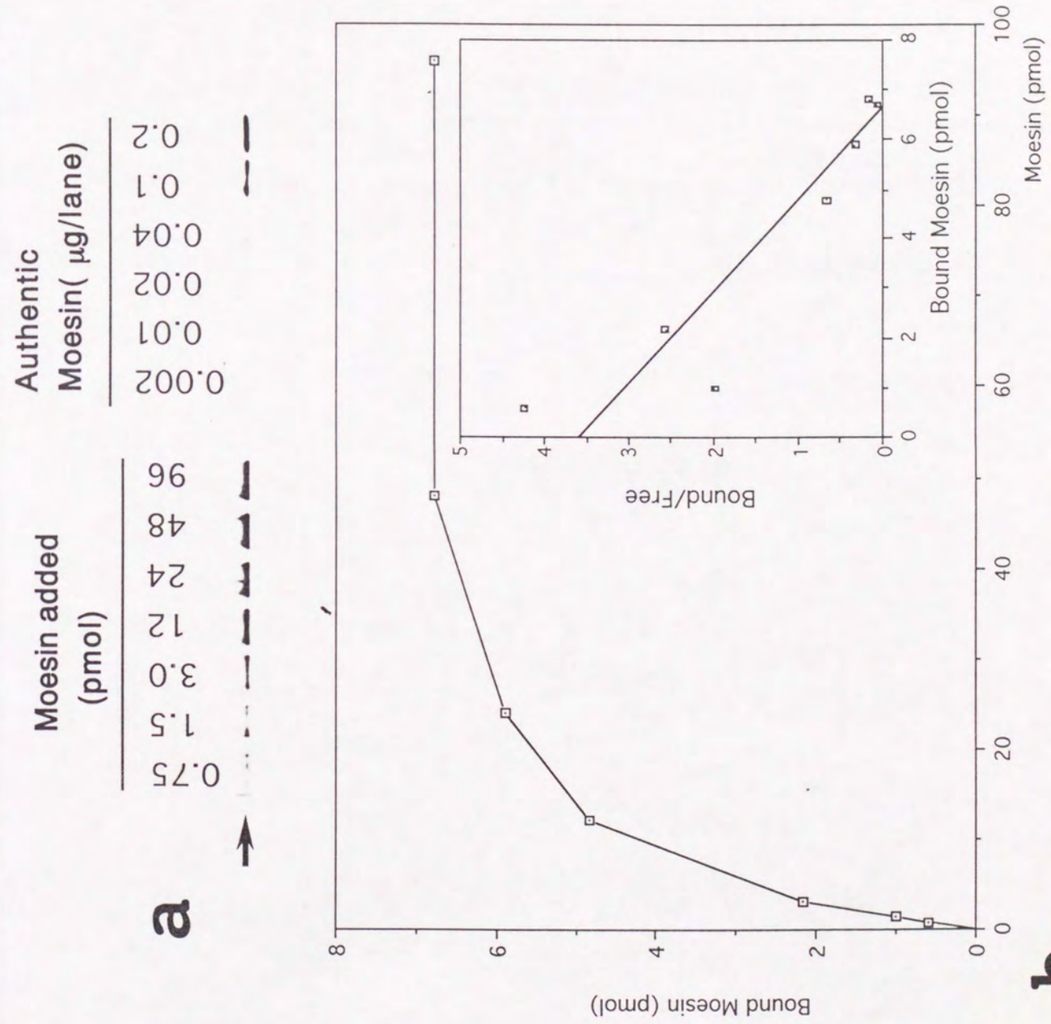


Fig.2 Hirao et al.



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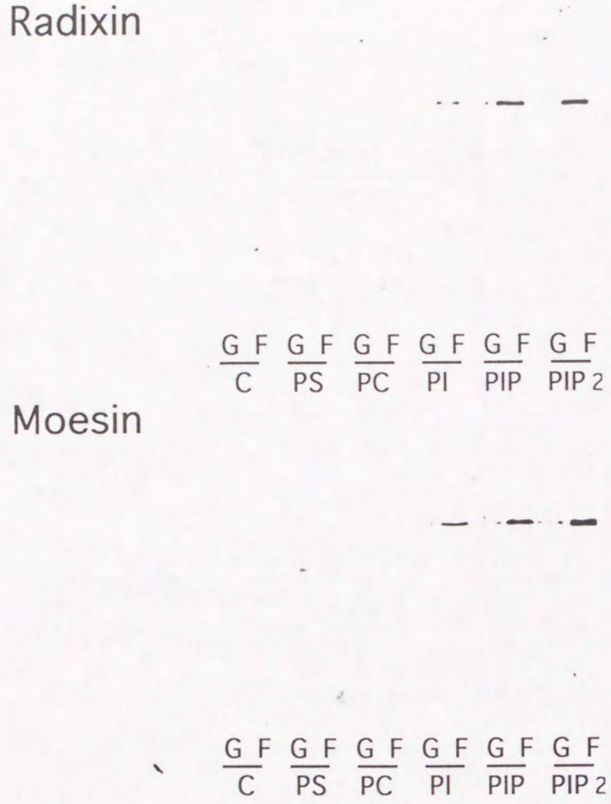
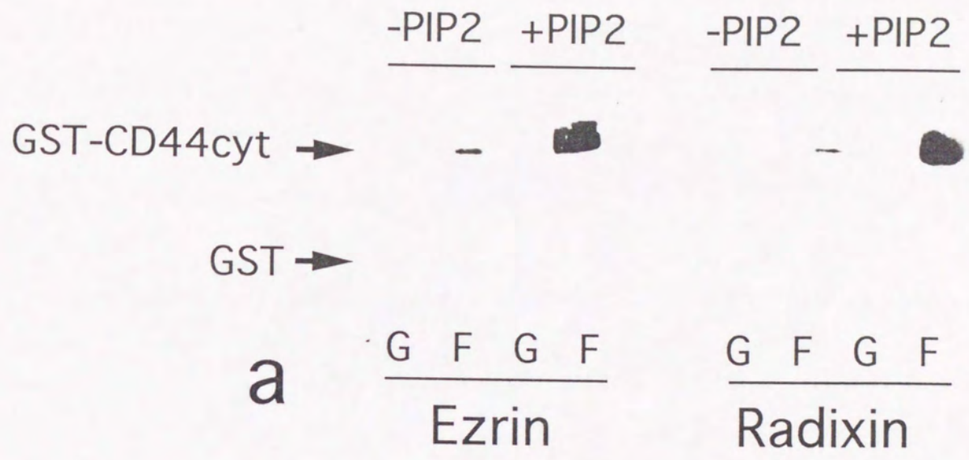


Fig.4 Hirao et al.



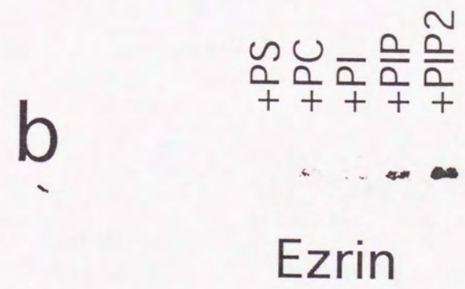
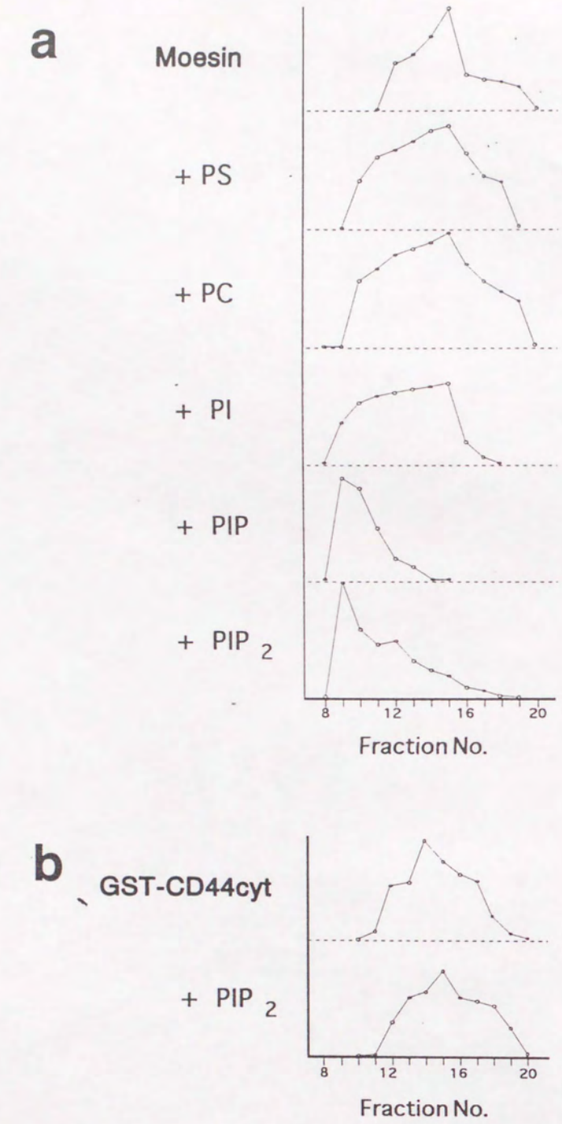


Fig.5 Hirao et al.



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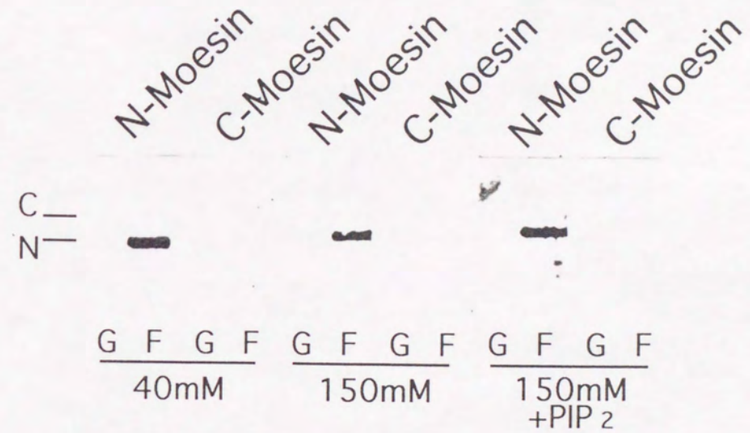

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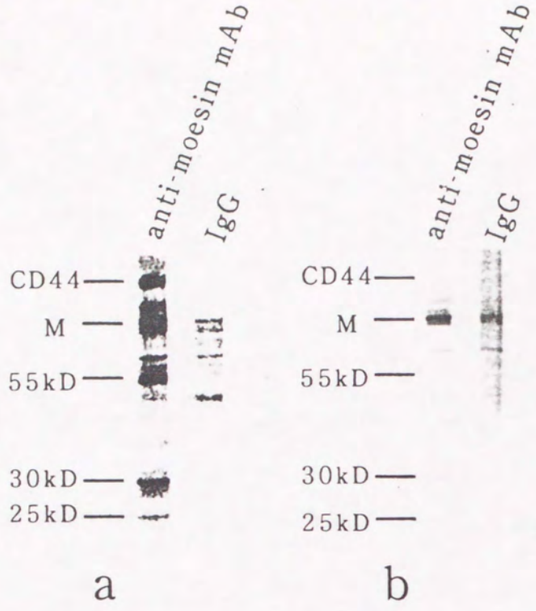

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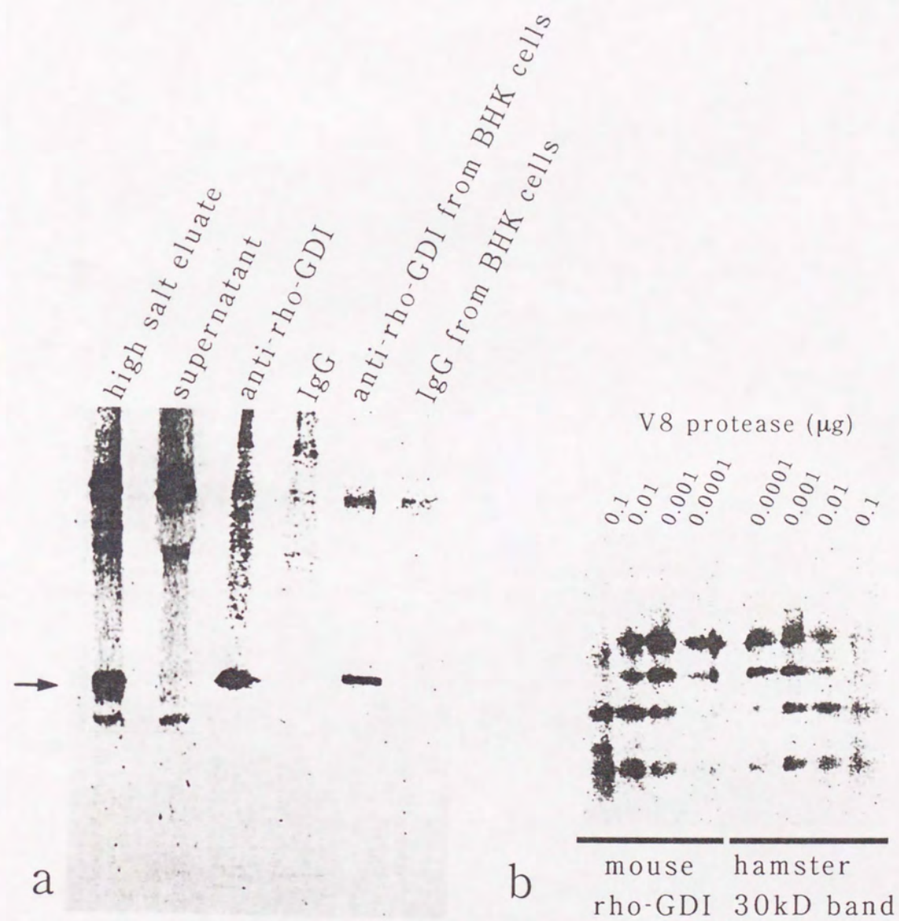

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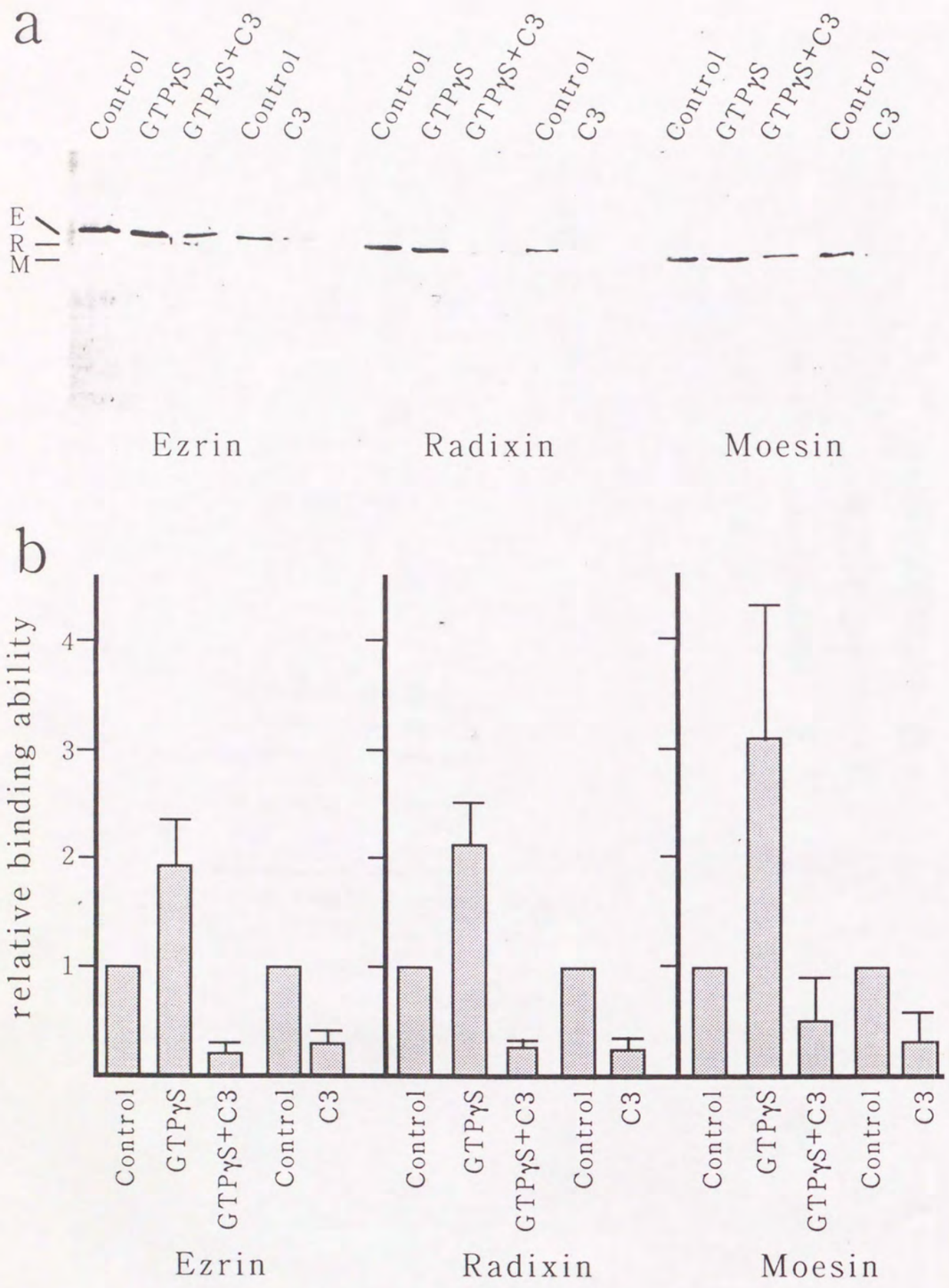
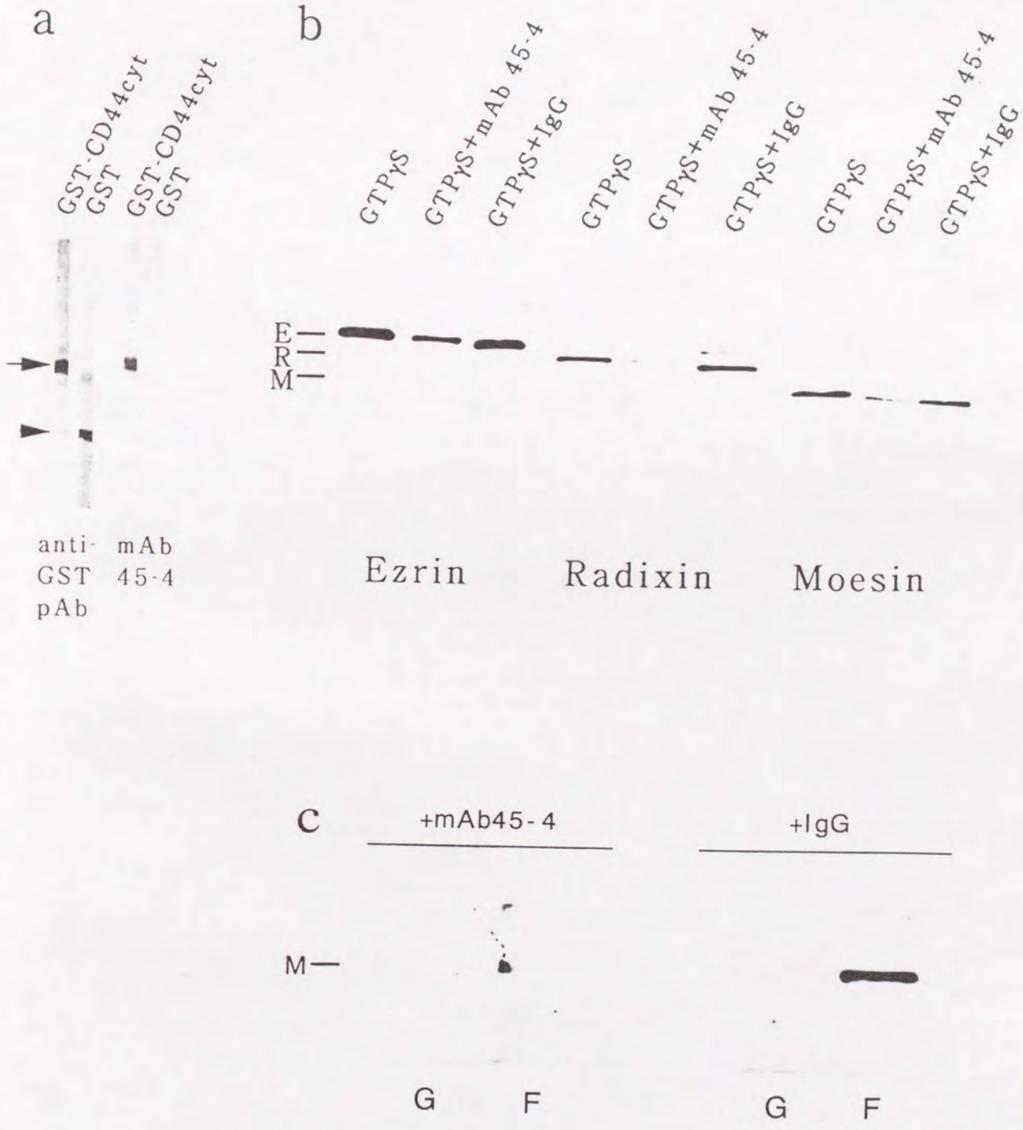


Fig. 10 HIRAO et al.



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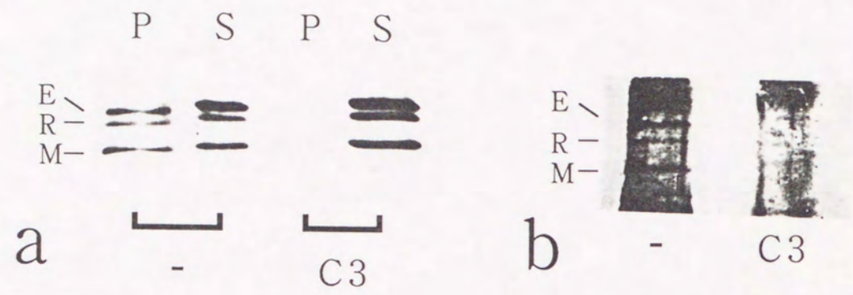

Fig. 12 HIRAO et al.