ERM Family Members as Molecular Linkers between the Cell Surface Glycoprotein CD44 and Actin-based Cytoskeletons

Sachiko Tsukita,*‡ Kumiko Oishi,* Naruki Sato,* Junji Sagara,§ Akihiko Kawai,§ and Shoichiro Tsukita*‖

*Laboratory of Cell Biology, Department of Information Physiology, National Institute for Physiological Sciences, Myodaiji-cho, Okazaki, Aichi 444, Japan; ‡Kyoto University, College of Medical Technology, Sakyo-ku, Kyoto 606, Japan; §Department of Molecular Microbiology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan; and ‖Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

Abstract. The ERM family members, ezrin, radixin, and moesin, localizing just beneath the plasma membranes, are thought to be involved in the actin filament/plasma membrane association. To identify the integral membrane protein directly associated with ERM family members, we performed immunoprecipitation studies using antimoesin mAb and cultured baby hamster kidney (BHK) cells metabolically labeled with [^{35}S]methionine or surface-labeled with biotin. The results indicated that moesin is directly associated with a 140-kD integral membrane protein. Using BHK cells as antigens, we obtained a mAb that recognized the 140-kD membrane protein. We next cloned a cDNA encoding the 140-kD membrane protein and identified it as CD44, a broadly distributed cell surface glycoprotein. Immunoprecipitation with various anti-CD44 mAbs showed that ezrin and radixin, as well as moesin, are associated with CD44, not only in BHK cells, but also in mouse L fibroblasts. Furthermore, immunofluorescence microscopy revealed that in both BHK and L cells, the Triton X-100–insoluble CD44 is precisely colocalized with ERM family members. We concluded that ERM family members work as molecular linkers between the cytoplasmic domain of CD44 and actin-based cytoskeletons.

THE ERM family consists of three closely related proteins; ezrin, radixin, and moesin (Sato et al., 1992; Tsukita et al., 1992). These proteins were identified independently in various tissues and cells: ezrin as a constituent of microvilli (Bretscher, 1983; Pakkanen et al., 1987) and as a good substrate for tyrosine kinases in vivo (Bretscher, 1989; Gould et al., 1986; Hunter and Cooper, 1981, 1983); radixin as a barbed end-capping actin-modulating protein in cell-to-cell adherens junctions (Tsukita et al., 1989) and moesin as a heparin binding protein (Lankes et al., 1988). Sequence analyses of their cDNAs revealed that these three proteins are highly homologous (∼75% identity) (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992). Although the distribution of these proteins inside cells has so far been intensively analyzed using various combinations of antibodies and cells, the results have not been consistent (Bretscher, 1983; Pakkanen et al., 1987; Tsukita et al., 1989; Sato et al., 1991, 1992; Lankes et al., 1988; Berryman et al., 1993; Franck et al., 1993).

We revealed that these closely related proteins are colocalized just beneath the plasma membranes at microvilli, ruffling membranes, cleavage furrows and adherens junctions (Sato et al., 1992), although their presence at adherens junctions was claimed to require reevaluation, especially in tissues in vivo (Berryman et al., 1993; Franck et al., 1993). These sites are specialized regions where actin filaments are densely associated with plasma membranes. Furthermore, Sagara, J., Sa. Tsukita, S. Yonemura, Sh. Tsukita, and A. Kawai (manuscript submitted for publication) have found that during the budding process of enveloped, negative-stranded RNA viruses such as the rabies virus, actin and ERM family members of host cells are selectively incorporated into virions. Therefore, we proposed that ERM family members are directly involved in the molecular mechanism of the actin filament–plasma membrane interaction in general (Sato et al., 1992; Tsukita et al., 1992).

Recent experiments with antisense oligonucleotide complementary to ERM sequences revealed that ERM family members play crucial roles at least in cell–cell and cell–substrate adhesion and microvilli formation probably through the regulation of actin filament–plasma membrane interactions (Takeuchi et al., 1994). Furthermore, the tumor suppressor gene of neurofibromatosis 2 has been identified (Trofatter et al., 1993; Rouleau et al., 1993), and its gene product

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Address all correspondence to Sachiko Tsukita, College of Medical Technology, Kyoto University, Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606, Japan.

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was very similar in amino acid sequence to the ERM family members (~49% identity). This product was named merlin (moesin-ezrin-radixin-like protein). This suggests that like merlin, ERM family members are involved in the regulation mechanism of cell growth. Therefore, ERM family members are now attracting increasing interest among cell biologists studying not only actin filaments, but also cell adhesion and cell growth.

The sequence of the NH_2-terminal half of the ERM family is highly conserved (~85% identity for any pair) (Sato et al., 1992). This sequence was also found in the NH_2-terminal half of the band 4.1 protein (Comboy et al., 1986), one of the major accessory proteins of erythrocyte membranes that is associated with spectrin and actin in membrane skeletons (Bennett, 1989). The NH_2-terminal half of this protein is responsible for its specific binding to glycophorin C (also called glycoconnectin), a major integral membrane protein in erythrocytes (Anderson and Lovrein, 1984; Anderson and Marchesi, 1985; Leto et al., 1986). These findings led to the speculation that ERM family members are also directly associated with a single class of integral membrane protein at their NH_2-terminal half. Therefore, to further understand the physiological functions of ERM family members, this putative integral membrane protein must be identified.

In this study, we immunoprecipitated moesin with an anti moesin mAb from the lysate of cultured baby hamster kidney (BHK)[1] cells, and found that one membrane protein with an apparent molecular mass of 140 kD was coimmunoprecipitated. Taking advantage of rabies virions that had been expected to contain the putative ERM-binding membrane protein (Sagara et al., manuscript submitted for publication), an mAb that recognized the 140 kD membrane protein was obtained. We then cloned the cDNA encoding this membrane protein. Sequence analyses of the cDNA revealed that this protein is a broadly distributed cell surface glycoprotein CD44 (reviewed in Haynes et al., 1989, 1991; Lesley et al., 1993), which 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). We further found with various anti-CD44 mAbs that ezrin and radixin, as well as moesin, were coimmunoprecipitated with CD44, not only in BHK cells, but also in mouse L fibroblasts. Furthermore, both in BHK and L cells, the Triton X-100-insoluble CD44 was precisely colocalized with ERM family members. Therefore, we concluded that ERM family members work as molecular linkers between CD44 and actin-based cytoskeletons. We believe this study will help provide a better understanding of the physiological functions, not only of ERM family members, but also of the cell surface glycoprotein CD44.

Materials and Methods

Cells, Virions, and Antibodies

BHK cells were grown in DME supplemented with 5% newborn calf serum and 10% tryptose phosphate broth. L cells were maintained in DME supplemented with 10% fetal bovine serum.

Rabies virions were purified by a combination of polyethylene glycol precipitation and sucrose density gradient centrifugation, as previously described (Sagara et al., 1992).

The mouse mAb CR-22 (Sato et al., 1991) reacts specifically with moesin in immunoprecipitation and all ERM family members in immunoblotting with a bias to moesin. To detect all ERM family members by immunoblotting, we used a mixture of CR-22 and rabbit anti-ERM pAb II, which detects all ERM family members with a bias to ezrin and radixin (Sato et al., 1992). IM7.8.1. is a rat anti-mouse CD44 mAb (Trowbridge et al., 1982).

Production of mAbs against Surface Antigens of BHK Cells

Cultured BHK cells were washed with PBS, detached from dishes with PBS containing 0.5 mM EDTA, collected by centrifugation, and suspended in PBS. Monoclonal antibodies were raised against the cell surface antigens of these BHK cells in rats. Hybridomas were prepared by fusion between rat lymphocytes and mouse P3 myeloma cells, essentially by the method previously described (Tsukita et al., 1989). The culture supernatant of each hybridoma was assayed for antibody production by immunoblotting using purified rabies virions.

Labeling of Cellular Proteins

To metabolically label BHK cells with $[^{35}S]$methionine at 80-90% confluence, cells were grown as monolayers in plastic dishes 100 mm in diameter. The monolayers were washed once with methionine-free medium supplemented with 2% fetal calf serum, followed by a 3-h incubation in 3 ml of the same medium containing 0.1 mCi $[^{35}S]$methionine (Amersham Corp., Arlington Heights, IL). After three washes with PBS, the cells were processed for immunoprecipitation.

To surface label BHK cells with biotin, cells were grown on plastic dishes 100 or 180 mm in diameter. At ~70% confluence, the cells were washed with 0.1 M Hepes buffer (pH 8.0) containing 50 mM NaCl, and were incubated for 15 min at room temperature with 2 (100-mm dish) or 5 ml (180-mm dish) of 0.1 M Hepes buffer (pH 8.0) containing 1 mg/ml sulfosuccinimidobiotin (sulfo-NHS-biotin) (Pierce Chemical Co., Rockford, IL), 50 mM NaCl, 1 mM *p*-amidinoPMSF, and 10 μg/ml leupeptin. Cells were washed with DME followed by PBS, and were processed for immunoprecipitation.

Immunoprecipitation

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, 150 mM NaCl, 50 mM Tris [pH 8.0], 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 from the plate surface with a rubber policeman. The lysate from one dish was incubated in a 1.5-ml tube on ice for an additional 10 min, and was then clarified by centrifugation at 12,000 *g* for 15 min. The RIPA-soluble supernatant was immunoprecipitated with 20 μl of protein G-Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden, or Zymed Laboratories, Inc., South San Francisco, CA) conjugated with monoclonal antibodies or control mouse/rat IgG. Sepharose 4B-bound immune complexes were washed five times with RIPA buffer. Immune complexes were then eluted by boiling in sample buffer for SDS-PAGE, and resolved by SDS-PAGE. The biotinylated proteins were visualized as described below, and the $[^{35}S]$methionine signal was analyzed (Fujix Bioimage Analyzer Bas 2000 System; Fuji Film Co. Ltd., Tokyo).

For reimmunoprecipitation, the immune complexes were eluted from 20 μl of Sepharose 4B-bound in a 1.5-ml test tube with 50 μl of a high salt solution consisting of 0.98 M KCl, 0.02 M NaCl, 1 mM $MgCl_2$, 10 mM 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 monoclonal antibodies or control rat IgG. After washing five times with the high salt solution diluted with 6 vol of distilled water, immune complexes were eluted and analyzed as described above.

Construction of a λ gt11 cDNA Expression Library and Immunoscreening

The poly(A)$^+$ RNA was isolated from BHK cells as described by Sam-

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[1] *Abbreviations used in this paper*: BHK, baby hamster kidney (cells); RIPA buffer, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 8.0), 1 mM *p*-amidinoPMSF, and 10 μg/ml leupeptin.

brook et al. (1989). The cDNA synthesis was primed with oligo(dT), using a cDNA synthesis kit (TimeSaver; Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After ligation with EcoRI/NotI adaptors, the blunt-ended cDNA was ligated into a λ gt11 vector with dephosphorylated EcoRI overhanging ends.

The clones were immunoscreened using the mAb 30189 as described previously (Funayama et al., 1991). One cDNA clone (B10) was isolated, and its insert was subcloned into pBluescript SK(−) and sequenced with a Taq terminator cycle sequencing kit (DyeDeoxy™; Applied Biosystems, Inc., Foster City, CA).

Production of Fusion Proteins

The 5′-fragment, B10b, was prepared from B10 by digestion with EcoRI (see Fig. 4 *A*). This fragment carried EcoRI sites at both ends, by means of which it was cloned directly into pGEX. The results construct encoded a fusion protein of ∼40 kD.

Two other cDNA fragments, B10a and B10c, were synthesized from B10 by means of PCR (see Fig. 4 *A*). B10a with EcoRI sites at both ends, and B10c with BamHI and EcoRI sites at 5′ and 3′ ends, respectively, were cloned directly into pGEX and the resulting respective constructs encoded fusion proteins of ∼30 and ∼38 kD.

300 μg B10b fusion protein was purified electrophoretically and used as an antigen to produce mAbs in rats.

Gel Electrophoresis, Immunoblotting, and Detection of Biotinylated Proteins

One-dimensional SDS-PAGE (7.5–15%) was based on the method of Laemmli (1970), and the gels were stained with Coomassie brilliant blue R-250.

After electrophoresis, proteins were electrophoretically transferred from gels to nitrocellulose membranes, which were then incubated with the first antibody, which was detected with a blotting detection kit (Amersham Corp.).

To detect biotinylated proteins, the nitrocellulose membranes were soaked for 1 h in TBS containing 5% skim milk, followed by a 60-min incubation with avidin alkaline phosphatase. After washing in TBS, biotinylated proteins were visualized using a blotting detection kit (Amersham Corp.).

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed as described previously (Itoh et al., 1991). Cultured BHK and L cells extracted or not with Triton X-100 were fixed in 3% formalin for 10 min. The second antibody was FITC-conjugated goat anti-rat IgG (Tago Inc., Burlingame, CA), rhodamine-conjugated goat anti-mouse IgG (Chemicon, Inc., Temecula, CA), or rhodamine-conjugated donkey anti-rabbit IgG (Chemicon, Inc.). Samples were examined using a fluorescence microscope (Axiophoto photomicroscope; Carl Zeiss, Inc., Thornwood, NY).

Extraction of CD44 with Various Concentrations of Triton X-100

BHK or surface-biotinylated BHK cells were extracted with 0.1, 0.4, or 1.0% Triton X-100 in the solution containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM Tris (pH 7.5), 1 mM *p*-amidinoPMSF, and 10 μg/ml leupeptin for 10 min at 4°C. The Triton X-100 lysate was removed from the dish after fully dislodging any remaining cellular debris from the dish surface with a rubber policeman. The lysate was separated into soluble and insoluble fractions by centrifugation at 12,000 *g* for 15 min.

Results

Identification of a Membrane Protein Directly Associated with Moesin in BHK Cells

Immunoprecipitation was performed using the mAb CR-22 that preferentially recognizes moesin. In a preliminary study, a variety of cell types and detergent conditions were tested to identify the proteins that coimmunoprecipitate with moesin. A combination of BHK cells and RIPA buffer (see Materials and Methods) was the most appropriate: the same results were obtained from other types of cells, such as mouse fibroblasts (L cells) and epithelial cells (MTD-1A cells), but the yield of the immunoprecipitates was significantly larger from BHK cells. When BHK cells were metabolically labeled with [^{35}S]methionine, lysed, solubilized with RIPA buffer, and immunoprecipitated with mAb CR-22, a broad band around 140 kD was detected in addition to the moesin band by immunoprecipitation (Fig. 1 *A*). When immunoprecipitates were washed in RIPA buffer, the association remained intact, but the addition of 1% SDS to the wash eliminated the 140 kD protein, indicating that it was not recognized by mAb CR-22 and was directly associated with moesin in a noncovalent manner.

To determine whether or not this 140-kD protein was a membrane protein, the cell surface proteins of BHK cells were labeled with biotin, lysed, solubilized, and immunoprecipitated with mAb CR-22 under the same conditions as described above. In immunoprecipitates containing moesin, a biotin-labeled band was detected around 140 kD (Fig. 1 *B*, lanes *3* and *3′*). Less intense bands at 85 and 80 kD were also detected. The intensity of the 85-kD band varied among experiments, whereas that of the 80-kD band increased with time after immunoprecipitation, suggesting that at least the 80-kD band is a degradation product of the 140-kD band. In the presence of 1% SDS, the 140-kD membrane protein was not coimmunoprecipitated with moesin (Fig. 1 *B*, lanes *4* and

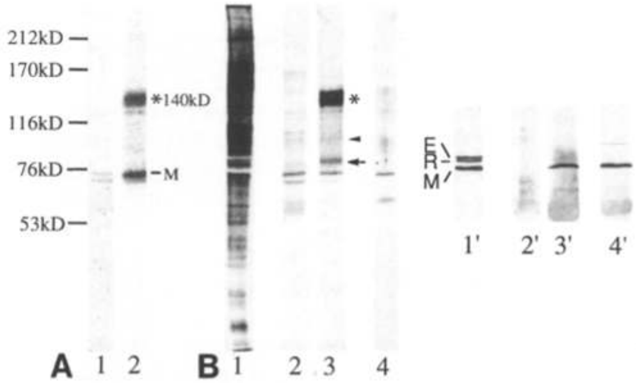


Figure 1. The direct association of moesin with the 140-kD membrane protein. (*A*) Coimmunoprecipitation of a metabolically labeled 140-kD protein (*140-kD*) with moesin (*M*). BHK cells were metabolically labeled with [^{35}S]methionine, lysed, solubilized with RIPA buffer, and immunoprecipitated with normal mouse IgG (lane *1*) or antimoesin mAb, CR-22 (lane *2*). The [^{35}S]methionine signal was detected by a Fujix Bioimage Analyzer. (*B*) Coimmunoprecipitation of a surface-labeled 140-kD protein (*) with moesin. Cell-surface proteins of BHK cells were labeled with biotin, lysed, and solubilized with RIPA buffer (lanes *1* and *1′*). This sample was immunoprecipitated with normal mouse IgG (lanes *2* and *2′*) or with mAb CR-22 (lanes *3* and *3′*). In some experiments, surface-labeled cells were solubilized with the buffer containing 1% SDS, and then immunoprecipitated with mAb CR-22 (lane *4* and *4′*). After samples were separated in SDS-PAGE and transferred to nitrocellulose membranes, the biotinylated proteins were detected by means of the avidin-alkaline phosphatase system (lanes *1–4*), and ezrin (*E*)/radixin (*R*)/moesin (*M*) were detected by immunoblotting with a mixture of pAb II and CR-22 (lanes *1′–4′*). In lane *3*, in addition to the 140-kD biotinylated protein, lower levels of 85 (*arrowhead*) and 80 kD (*arrow*) proteins also coimmunoprecipitated with moesin.

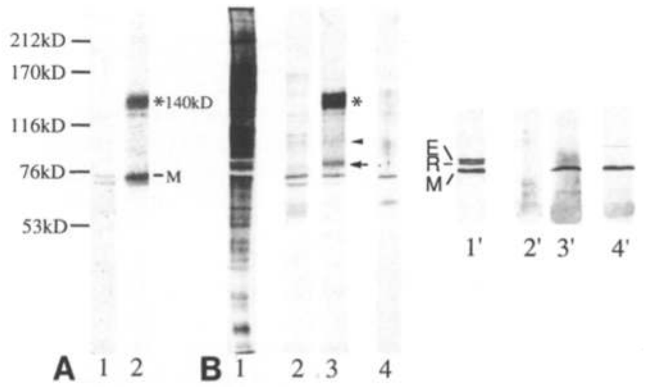


Figure 2. The recognition of the 140-kD moesin-associated membrane protein by a monoclonal antibody (mAb30189). The moesin immunoprecipitates from metabolically labeled BHK cells (lane *1* in *A*; same as lane *2* in Fig. 1 *A*) and those from surface-labeled BHK cells (lane *1* in *B*; same as lane *3* in Fig. 1 *B*) were solubilized using the high salt buffer and immunoprecipitated again using normal rat IgG (lane *2* in *A* and *B*) or with mAb30189 (lane *3* in *A* and *B*). *Arrow,* the 85-kD band (Fig. 1 *B, arrowhead*).

4'). These findings led us to conclude that moesin was directly associated with a 140-kD membrane protein that was specifically trapped by WGA and Con A columns, indicating that it is glycosylated (data not shown).

Production of Monoclonal Antibodies Specific for the 140-kD Membrane Protein

Because of the small amount of immunoprecipitates, they could not be used as antigens to raise mAbs that recognize the 140-kD moesin-associated membrane protein. (Sagara, J., Sa. Tsukita, S. Yonemura, Sh. Tsukita, and A. Kawai, manuscript submitted for publication) have found that during the budding of enveloped, negative-stranded RNA viruses such as the rabies virus, actin and ERM proteins of host cells are selectively incorporated into virions, suggesting that the 140-kD moesin-associated membrane protein is also concentrated in these virions. Actually, SDS-PAGE of rabies virions produced in BHK cells revealed a host cell-derived faint band around 140 kD. Therefore, using living BHK cells as an antigen, we raised many mAbs in rats that recognize the cell-surface antigens, and we selected one (mAb30189) that faintly recognized a band at ~140 kD in rabies virions by immunoblotting.

This mAb recognized the 140-kD membrane protein in the moesin immunoprecipitates both from metabolically and surface-biotinylated BHK cells (Fig. 2, *A* and *B*). This confirmed that the metabolically labeled 140-kD band is identical to the biotinylated 140-kD protein. The 85-kD membrane protein that was also present at low levels in the moesin immunoprecipitates from biotin-labeled cells (Fig. 1 *B*, lane *3*, *arrowhead*) was also recognized by this mAb, suggesting that it is an isoform of the 140-kD protein (Fig. 2 *B*, lane *3*, *arrow*). Furthermore, when BHK cells were lysed, solubilized with RIPA buffer, and immunoprecipitated with this mAb, immunoblotting revealed the presence of not only moesin, but also ezrin and radixin (Fig. 3). Therefore, we concluded that this mAb recognizes the 140-kD membrane protein, and that it is associated with three known members of the ERM family.

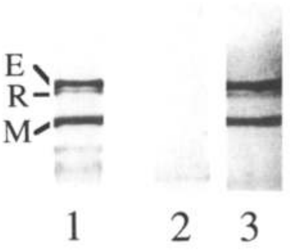


Figure 3. The coimmunoprecipitation of ezrin/radixin/moesin with the 140-kD membrane protein. BHK cells were lysed and solubilized with RIPA buffer (lane *1*). This sample was immunoprecipitated with normal rat IgG (lane *2*) or with mAb30189 (lane *3*). The occurrence of ERM family members in each sample was evaluated by immunoblotting with a mixture of pAb II and mAb CR-22 after SDS-PAGE. *E,* ezrin; *R,* radixin; *M,* moesin.

Isolation and Sequencing of cDNA Encoding the ERM-associated Membrane Protein

Using the mAb30189, we screened ~1 × 10^5 plaques from an oligo(dT)-primed λ gt11 cDNA library made from BHK cells, cloned one positive phage recombinant, B10 (~950

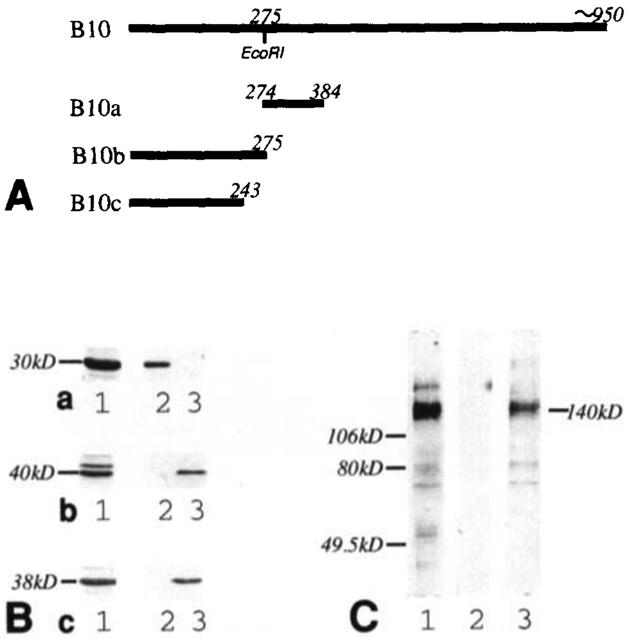
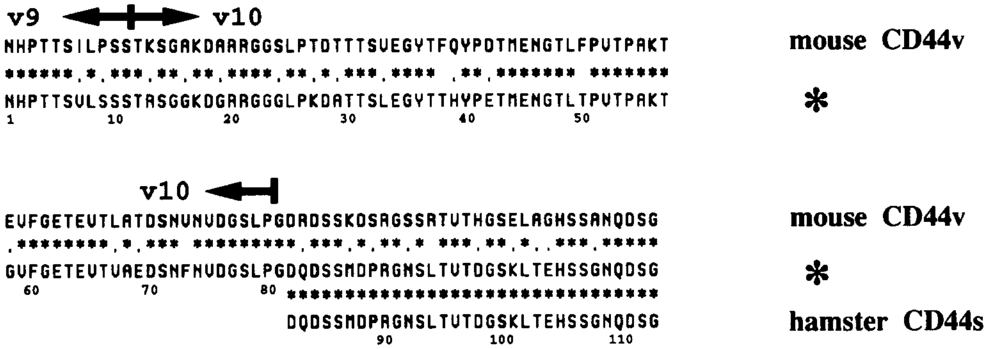


Figure 4. The cloning of a cDNA fragment encoding the 140-kD ERM-associated membrane protein. (*A*) cDNA fragments of the 140-kD protein. Using mAb30189, one positive phage recombinant (*B10*) was cloned from a λgt11 cDNA library made from BHK cells. Using this cDNA fragment, three others (*B10a*, *B10b*, and *B10c*) were constructed to produce fusion proteins in *E. coli*. (*B*) Immunoprecipitation from *E. coli* lysate with which to evaluate the specificity of mAb30189 (lane *2*) and mAbα (lane *3*) to fusion proteins (lane *1*). *a*, B10a fusion protein; *b*, B10b fusion protein; *c*, B10c fusion protein. Fusion proteins (lane *1*) and immunoprecipitates (lanes *2* and *3*) were separated by SDS-PAGE and stained with Coomassie brilliant blue. The mAbα was produced in rats using the B10b fusion protein as an antigen. (*C*) Recognition of the 140-kD ERM-associated membrane protein by mAbα. The moesin immunoprecipitate from surface-biotinylated BHK cells was solubilized using the high salt buffer (lane *1*), and it was immunoprecipitated again with either normal rat IgG (lane *2*) or mAbα (lane *3*).



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NHPTTSILPSSTKSGAKDAARGGSLPTDTTTSVEGYTFQYPDTMENGTLFPVTPAKT
******.*.***.**.**.****.**.*.***.**** .**.******* *******
NHPTTSVLSSSTASGGKDGARGGGLPKDATTSLEGYTTHYPETMENGTLTPVTPAKT
1        10        20        30        40        50

EVFGETEVTLATDSNVNVDGSLPGDRDSSKDSAGSSRTVTHGSELAGHSSANQDSG
.********.*.*** *********.***.*.**.* ***.**.*..***.*****
GVFGETEVTVAEDSNFNVDGSLPGDQDSSMDPRGNSLTVTDGSKLTEHSSGNQDSG
 60        70        80 ******************************
                         DQDSSMDPRGNSLTVTDGSKLTEHSSGNQDSG
                                90        100       110
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Figure 5. Deduced amino acid sequence encoded by the 5′-half (339 bp) of B10 (*large asterisks*) and the corresponding sequences of mouse v9/v10 containing CD44 (*CD44v*) and hamster standard type CD44 (*CD44s*). In the hamster, only the cDNA encoding the standard type has been sequenced so far (Aruffo et al., 1990). Therefore, we also compared the amino acid sequence of mouse v9/v10 containing CD44, which contained exons v9 and v10 (Tölg et al., 1993). Identity and homology are indicated by asterisks and dots, respectively.

bp), then sequenced 339 bp of B10 from its 5′ end. From B10, two cDNA fragments, B10a (111 bp) and B10b (275 bp), were prepared, and fusion proteins were generated in *Escherichia coli* from B10a and B10b (Fig. 4 *A*). Immunoprecipitation revealed that mAb30189 recognized B10a but not B10b fusion proteins (Fig. 4 *B*). We then raised mAbs in the rat using the B10b fusion protein as an antigen, and obtained one mAb (mAb α) that specifically recognized only B10b fusion proteins (Fig. 4 *B*), indicating that the epitope for mAb α is distinct from that for the original mAb30189. As shown in Fig. 4 *C*, this mAbα specifically bound to the 140-kD ERM-associated protein in the moesin immunoprecipitates from the biotin-labeled BHK cells as mAb30189 did. This strongly suggests that the cDNA clone B10 encodes the 140-kD ERM-associated membrane protein.

The amino acid sequence deduced from the 339 bp of B10 from its 5′ end indicated that B10 encodes CD44, a broadly-distributed cell surface glycoprotein (Fig. 5). It is now widely accepted that the great variety of CD44 isoforms are generated by alternative splicing from a single gene (for a review see, Lesley et al., 1993). These isoforms contain various kinds of inserts at the membrane-proximal domain of the extracellular portion as a result of alternative splicing; the isoform without any insertion is called a "standard" type. In the hamster, only the cDNA encoding the standard type has so far been sequenced. As shown in Fig. 5, among 113 sequenced amino acids, the sequence of 82–113a.a. was completely identical to that of a part of the hamster standard type CD44. The remainder (1–81a.a.) was highly homologous in the amino acid sequence to the insert of mouse v9/v10 containing CD44. Therefore, we concluded that B10 encodes a hamster v9/v10 containing CD44.

These data appear to indicate that the 140kD ERM-associated protein is an isoform of CD44 containing at least v9/v10. However, the possibility that it is a standard type CD44 has not been completely excluded because it is still possible that the mAbα epitope resides within amino acid residues 82–91 (see Figs. 4 *A* and 5). Therefore, to check whether or not the mAbα epitope is located within residues 1–81 (exons v9 and v10), we generated a fusion protein corresponding to this domain using B10c (see Fig. 4 *A*). As shown in Fig. 4, *B* and *C*, mAbα bound to this fusion protein, indicating that it recognizes the insert derived from exons v9 or v10. This mAbα recognized the 140-kD ERM-associated protein both by immunoprecipitation (Fig. 4 *C*) and immunoblotting (see Fig. 10 *A*, lane *3*), indicating that the 140-kD ERM-associated membrane protein is an isoform of CD44 that contained inserts at least derived from exons v9 and/or v10.

Colocalization of ERM Family Members and CD44 in Hamster and Mouse Cells

The structure and function of CD44 and the ERM family have been analyzed so far mainly in mice and humans (Lesley et al., 1993; Tsukita et al., 1992). Therefore, using an anti-mouse CD44 mAb, IM7.8.1, which recognizes all types of mouse CD44, mouse L fibroblasts were lysed, solubilized, and immunoprecipitated. As shown in Fig. 6, immunoblotting with a mixture of pAb I1 and mAb CR22 revealed that the CD44 immunoprecipitates contained ezrin, radixin, and moesin, indicating that CD44 is also associated with the ERM family in mouse L cells.

Next, using BHK cells and L fibroblasts, the localization

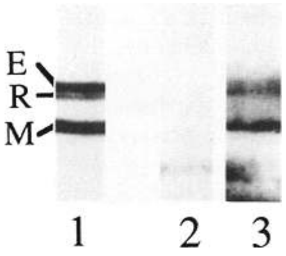

Figure 6. Coimmunoprecipitation of ezrin/radixin/moesin with CD44 in mouse L fibroblasts. L cells were lysed and solubilized with RIPA buffer (lane *1*). This sample was immunoprecipitated with normal rat IgG (lane *2*) or with rat anti-mouse CD44 mAb, IM 7.8.1 (lane *3*). The occurrence of ERM family members in each sample was evaluated by immunoblotting with a mixture of pAb I1 and mAb CR-22 after SDS-PAGE. *E*, ezrin; *R*, radixin; *M*, moesin.

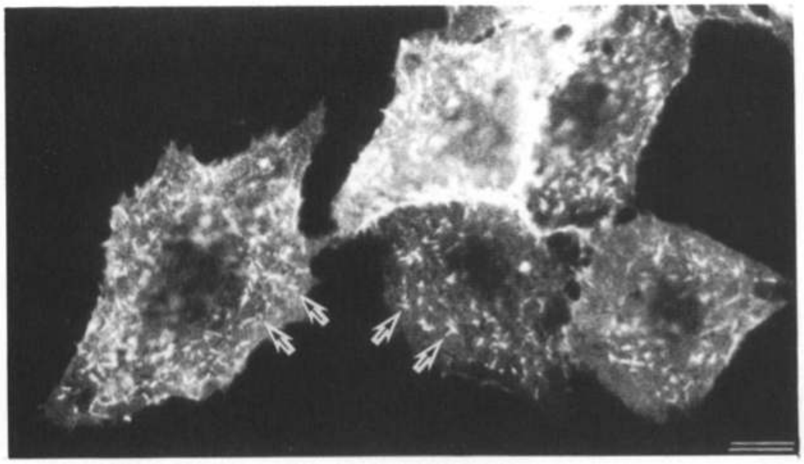

Figure 7. The localization of CD44 in BHK cells without Triton X-100 treatment revealed by immunofluorescence microscopy with mAb30189. In addition to the weak diffuse staining on the cell surface, microvilli (*arrows*) and cell-cell adhesion sites were intensely stained. Bar, 10 μm.

of ERM family members were compared to that of CD44. When these cells were fixed and immunofluorescently stained with anti-CD44 mAb without detergent extraction, in addition to the weak diffuse staining on the cell surface, microvilli and cell-cell adhesion sites were intensely stained (Fig. 7). In sharp contrast, when cells were treated with 0.1-1% Triton X-100 before (data not shown) or after formaldehyde fixation (Figs. 8 and 9), the weak diffuse staining completely disappeared leaving intense staining on microvilli and cell-cell adhesion sites. A close comparison of Triton X-100-treated cells by double immunofluorescence microscopy using anti-CD44 mAb and anti-ERM pAb I1 revealed that the distribution of the Triton X-100-insoluble CD44 completely coincided with that of ERM family members (Fig. 8, *A-D*, Fig. 9, *A* and *B*). In dividing cells, CD44 was highly concentrated at cleavage furrows together with ERM family members (Figs. 8, *E-G* and 9, *C-E*).

The 85- and 140-kD CD44 and their Cytoskeleton Association

When the biotin-labeled BHK cells were lysed, solubilized, and immunoprecipitated with mAb30189, in addition to some minor bands (150-170 kD), two major bands around 140 and 85 kD were detected, and the expression level of the latter was much higher than that of the former (Fig. 10 *A*, lanes *2*). Since only the 140-kD band was recognized by mAbα (Fig. 10 *A*, lane *3*), and since BHK cells were reported to express a large amount of the standard type CD44 (Lesley et al., 1993), it is likely that the 85- and 140-kD CD44 correspond to the standard type and the isoform containing at least v9/v10, respectively.

To examine the association of these isoforms with cytoskeletons, we analyzed the degree of extraction of each from BHK cells with various concentrations of Triton X-100 (Fig. 10 *B*). Immunoblotting and immunoprecipitation revealed that with 0.1% Triton X-100, both the 85- and the 140-kD CD44 were associated with cytoskeletons, that with 0.4-1.0% Triton X-100, most of the 85-kD standard-type CD44 was extracted (Fig. 10 *B*, lane *2*), and that the 140-kD CD44 was hardly extracted, even in the presence of 1.0% Triton X-100 (Fig. 10 *B*, lane *1*). These findings indicate that the 140-kD CD44 binds to cytoskeletons more tightly than the 85-kD CD44.

Discussion

Actin filaments are involved in many kinds of cellular events, and they are found in association with the plasma membrane in a variety of eukaryotic cells (Pollard and Weihing, 1974; Ishikawa, 1979). The ERM family members, ezrin, radixin, and moesin, are thought to play a crucial role just beneath the plasma membrane in the actin filament/plasma membrane association in general (Sato et al., 1992; Tsukita et al., 1992; Berryman et al., 1993). In this study, we searched for an integral membrane protein that is directly associated with the ERM family. We found that immunoprecipitation revealed the direct association of ERM family members with a 140-kD membrane protein. Further analysis of this protein identified it as CD44. Taking into consideration that CD44 was precisely colocalized with ERM family members both in BHK and mouse L cells, we concluded that the ERM family is directly associated with the cytoplasmic domain of CD44.

CD44 is a polymorphic cell-surface glycoprotein that is found on a wide variety of cells (Haynes et al., 1989, 1991; Lesley et al., 1993). Its exact functions have yet to be conclusively defined, although recent reports have implicated CD44 in extracellular matrix binding, cell migration, lymphopoiesis, and lymphocyte homing in normal cells, as well as in metastasis in cancer cells (Günthert et al., 1991.; Arch et al., 1992; Koopman et al., 1993). So far, ERM family members were thought to bind to glycophorin C-like membrane proteins, since their NH_2-terminal half showed a similarity to the glycophorin C-binding domain of the band 4.1 protein (Leto et al., 1986). In this study, we found direct interaction between CD44 and ERM family members. CD44 and glycophorin C are both heavily glycosylated proteins that once span membranes, but they have no significant sequence homology (Colin et al., 1986; Haynes et al., 1989, 1991; Lesley et al., 1993).

CD44 reportedly interacts with components of actin-based cytoskeletons, and this interaction is required for its function (Jacobson et al., 1984; Tarone et al., 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Geppert and Lipsky, 1991; Camp et al., 1991; Neame and Isacke, 1992). Many investigators have attempted to identify the cytoskeletal components that directly bind to CD44. Bourguignon et

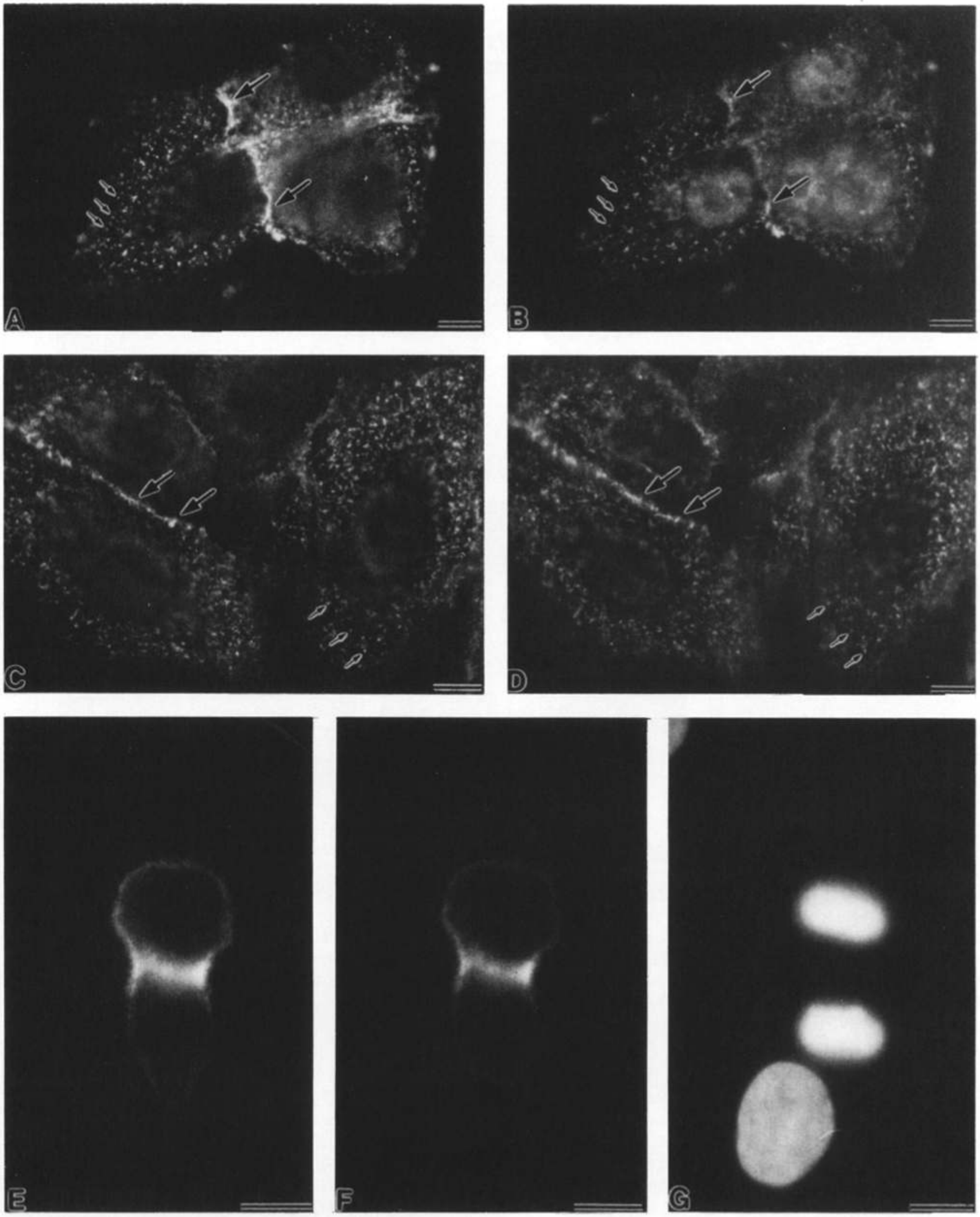

Figure 8. The colocalization of ezrin/radixin/moesin with Triton X-100–insoluble CD44 in BHK cells. BHK cells were fixed with 3% formalin, extracted with 0.2% Triton X-100, and doubly stained with mAb30189 (*A*, *C*, and *E*) and pAb I1 (*B*, and *F*) or mAb CR-22 (*D*). Both ERM and Triton X-100–insoluble CD44 were precisely coconcentrated at cell–cell adhesion sites (*large arrows*) and at microvilli-like structures (*small arrows*). In dividing cells whose nuclei were stained with DAPI in *G*, both are concentrated at the cleavage furrow (*E* and *F*). Bar, 10 μm.

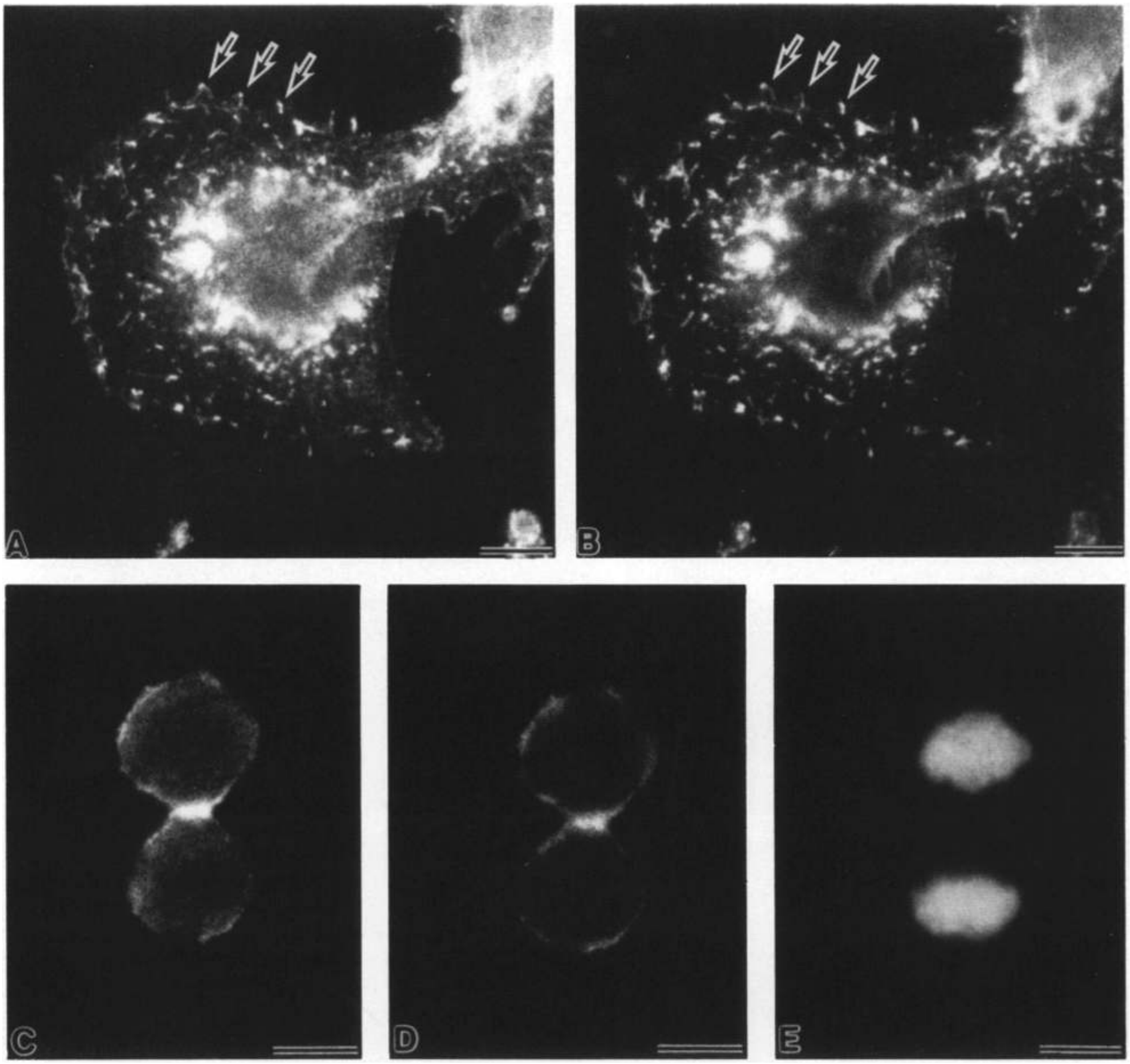

Figure 9. The colocalization of ezrin/radixin/moesin with the Triton X-100–insoluble CD44 in mouse L fibroblasts. L cells were fixed with 3% formalin, treated with 0.2% Triton X-100, and doubly stained with rat anti–mouse CD44 mAb IM7.8.1 (*A* and *C*) and mAb CR-22 (*B* and *D*). CD44 and ERM are colocalized at microvilli-like structures (*arrows*) and at the cleavage furrows (*C* and *D*). (*E*) 4',6-diamidino-2-phenylindole-dihydrochloride staining. Bar, 10 μm.

al. reported that the cytoplasmic domain of CD44 is associated with a 72-kD ankyrin-like protein (Bourguignon et al., 1986, 1992; Kalomiris and Bourguignon, 1988). They found that the 16S complex isolated from lymphoma plasma membranes was mainly composed of CD44 and ankyrin-like 72-kD protein, and they further showed that the cytoplasmic domain of CD44 can bind to the "erythrocyte" ankyrin directly in vivo. In the 16S complex, their molar ratio appeared to be ~1:1. In sharp contrast, the CD44 was coimmunoprecipitated with moesin, but not with the 72-kD polypeptide (see Fig. 1 *B*); at least in the immunoprecipitate, no band around 72 kD was detected by immunoblotting with antiankyrin antibodies (data not shown). This discrepancy may be attributed to the different extraction conditions: the 16S complex was released from plasma membranes with 1% Triton X-100, whereas the CD44-ERM complex was obtained using 0.1% SDS and 1% Nonidet P-40. Given that in the absence of SDS, the CD44 molecule that tightly bound to ERM was totally insoluble in Triton X-100, the lack of ERM in the 16S complex could be explained. Conversely, if the association of the ankyrin-like 72-kD protein with CD44 is not resistant to the SDS treatment, the CD44-ERM complex would lack the 72-kD protein.

At present, it is not clear whether in vivo CD44, ERM, and ankyrin-like 72-kD protein form a single complex (CD44/ERM/72-kD complex) or two distinct types of complex (CD44/ERM and CD44/ankyrin). However, judging from their subcellular distribution, ERM family members may play a central role in connecting CD44 to the underlying cytoskeletons; ERM family members and CD44 are pre-

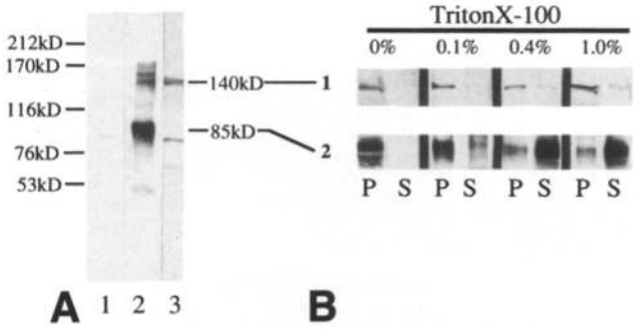

Figure 10. Extractability of the 140- and 85-kD CD44 with various concentrations of Triton X-100 in BHK cells. (*A*) Immunoprecipitates with normal mouse IgG (lane *1*) or mAb30189 (lane *2*) from the surface-labeled BHK cells. In lane 2, in addition to some minor bands (150–170 kD), two biotinylated bands corresponding to the isoform of CD44 containing at least v9/v10 (140-kD) and the standard-type CD44 (85kD) were detected. As shown in lane *3* by immunoblotting, mAbα recognizes the 140- and 80-kD, but not the 85-kD CD44 in BHK cells. The 80-kD band may be a degradation product from the 140-kD CD44. (*B*) In experiment 1 (lane *1*), BHK cells were extracted with various concentrations of Triton X-100, and insoluble (*P*) and soluble (*S*) fractions were obtained as described in Materials and Methods. Each fraction was separated by SDS-PAGE, and the 140-kD CD44 was detected by immunoblotting with mAbα, which recognizes the inserts derived from exon v9 or v10 included in the 140-kD CD44. In experiment 2 (lane *2*), biotinylated BHK cells were extracted with various concentrations of Triton X-100, and insoluble and soluble fractions were obtained. After the insoluble fraction was solubilized with RIPA buffer, the amount of the 85-kD standard-type CD44 in each fraction was evaluated by immunoprecipitation with mAb30189.

cisely colocalized and concentrated at the microvilli and cleavage furrows, while ankyrin is not reportedly concentrated at these structures. The complete colocalization of ERM and CD44 (the Triton-insoluble type) was observed in all types of culture cells we examined (data not shown).

One important question concerning the ERM family is whether or not ezrin, radixin, and moesin are associated with the same integral membrane protein. The present data revealed that they are all associated with the cytoplasmic domain of CD44 molecules. This conclusion is highly consistent with our recent data of the distribution of ERM family members (Takeuchi et al., 1994): close analyses of the localization of each member using antisense oligonucleotide-treated cells revealed that ezrin, radixin, and moesin are all concentrated at specialized regions where actin filaments are densely associated with plasma membranes, and that each protein by itself can concentrate at these regions. The question then naturally arose as to whether or not other band 4.1 superfamily members such as band 4.1 protein (in nonerythroid cells), merlin, protein-tyrosine-phosphatase H1/MEG, and talin can interact with CD44 (Conboy et al., 1986; Rees et al., 1990; Gu et al., 1991; Yang et al., 1991; Trofatter et al., 1993; Rouleau et al., 1993). Especially, considering that the NH_2-terminal half of merlin is highly homologous to that of ERM family members (~60% identity), its interaction with CD44 should be evaluated both in vivo and in vitro.

In humans, ⩾ 18 CD44 transcripts have been described to date (for a review see Lesley et al., 1993). This heterogeneity results from the fact that 12 of 19 exons can undergo alternative splicing (Screaton et al., 1992). As shown in Fig. 10, BHK cells mainly express 85-kD standard-type and the isoform containing at least v9/v10. These isoforms share the same cytoplasmic domain (Screaton et al., 1992). However, the 140-kD isoform was preferably coimmunoprecipitated with ERM family members, and only a lesser amount of the 85-kD isoform was found in immunoprecipitates from biotinylated cells, although the expression level of the former was much lower than that of the latter (Fig. 10). These indicate that two distinct isoforms with the same cytoplasmic domain have different levels of affinity for cytoskeletons, namely, to ERM family members. In fact, Fig. 10 *B* shows that in BHK cells, the 140-kD isoform was much more resistant to Triton X-100 extraction than the 85-kD isoform. This discrepancy can be rationalized as follows.

So far, it was understood that the interaction of CD44 with cytoskeletons is enhanced by clustering CD44 into a multimeric configuration (Geppert and Lipsky, 1991). Therefore, given that the 140-kD isoform has a tendency to form an oligomeric configuration through its insert at the extracellular membrane-proximal portion, the stability of the 140-kD isoform–ERM complex would be much higher than that of the 85-kD isoform–ERM complex. This would result in only the 140-kD CD44-ERM complex being detected by immunoprecipitation in the presence of RIPA buffer. Although no data has been so far reported to directly support this speculation, this speculation is consistent with our preliminary findings that the antibody-induced clustering of CD44 molecules on the cell surface enhanced the interaction of the 85-kD isoform with cytoskeletons. In living cells, because of the following reasons, we speculate that quite a number of ERM family molecules are associated with the 85-kD isoform in an unstable and dynamic fashion, and that the rest of them are tightly and stably bound to the 140-kD isoform: more of the 85-kD than of the 140-kD isoform is expressed (Fig. 10 *A*), and about two thirds of the former are associated with cytoskeletons in the presence of 0.1% Triton X-100 (Fig. 10 *B*); in cells extracted with 0.1% Triton X-100, the insoluble CD44 molecules, most of which may be the 85-kD isoforms, were shown by immunofluorescent means to be precisely colocalized with ERM family members. The general concept that the insertion by alternative splicing at the membrane-proximal portion of CD44 molecules regulates the stability of the CD44/ERM association through CD44 oligomerization is, at present, purely speculative, and it requires experimental elucidation. Of course, the regulation mechanism of the CD44/ERM association may be more complicated. For example, most recently, the manner of interaction of CD44 with cytoskeletons in epithelial cells such as Madin-Darby canine kidney cells has been reported to be completely different from that in fibroblasts (Neame and Isacke, 1993). The phosphorylation of the serine residues and GTP binding in the cytoplasmic domain of CD44 may also be important for regulating the CD44/ERM association (Kalomiris and Bourguignon, 1988; Camp et al. 1991; Lokeshwar and Bourguignon, 1992).

The present study casts a new light on the functions of CD44 molecules: the CD44-ERM-actin filament may work as a fundamental unit in the interaction of actin filaments with plasma membranes in general. We showed here that CD44 is highly concentrated at cleavage furrows in dividing cells, where actin filaments are densely associated with plasma membranes. From a phylogenetic perspective, the most fundamental unit responsible for the actin filament/plasma membrane interactions should be concentrated at the cleavage furrow, because in unicellular organisms, actin filaments are thought to originally emerge for cytokinesis, one of the most fundamental cellular events. In this respect, CD44 (and ERM family members) meets the qualifications as a constituent of the fundamental unit for actin filament/plasma membrane interactions.

Yonemura et al. (1993) demonstrated that the surface protein CD43 was precisely colocalized with ERM family members and concentrated at cleavage furrows in dividing cells. Furthermore, other membrane proteins such as leukocyte adhesion molecule-1 and membrane immunoglobulins are reportedly concentrated at cleavage furrows (de Petris, 1984; Pilarski et al., 1991). Unlike CD44, these membrane proteins are expressed in some restricted types of cells, suggesting that they are not likely constituents of the fundamental unit for actin filament/plasma membrane interactions. Judging from the precise colocalization of CD43 with ERM family members (Yonemura et al., 1993), we speculate that these membrane proteins are laterally associated with the CD44-ERM-actin filament unit to form a large membrane protein complex with CD44 located in the center. Studies to evaluate this hypothesis are now underway in our laboratory.

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