Normal Development of Mice and Unimpaired Cell Adhesion/Cell Motility/Actin-based Cytoskeleton without Compensatory Up-regulation of Ezrin or Radixin in Moesin Gene Knockout*

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Ezrin/radixin/moesin (ERM) proteins are general cross-linkers between the plasma membrane and actin filaments. Because their expression is regulated in a tissue-specific manner, each ERM protein has been proposed to have unique functions. On the other hand, experiments at the cellular level and in vitro have suggested their functional redundancy. To assess the possible unique functions of ERM proteins in vivo, the moesin gene located on the X chromosome was disrupted by gene targeting in embryonic stem cells. Male mice hemizygous for the mutation as well as homozygous females were completely devoid of moesin but developed normally and were fertile, with no obvious histological abnormalities in any of the tissues examined. In the tissues of the mutant mice, moesin completely disappeared without affecting the expression levels or subcellular distribution of ezrin and radixin. Also, in platelets, fibroblasts, and mast cells isolated from moesin-deficient mice, targeted disruption of the moesin gene did not affect their ERM-dependent functions, i.e. platelet aggregation, stress fiber/focal contact formation of fibroblasts, and microvillar formation of mast cells, without compensatory up-regulation of ezrin or radixin. These findings favor the notion that ERM proteins are functionally redundant at the cellular as well as the whole body level.

Three closely related proteins, ezrin, radixin, and moesin constitute a gene family called the ERM family. Ezrin, radixin, and moesin were identified in different directions (1–7), but isolation and sequencing of their cDNAs revealed that they were closely related (amino acid sequence identity of 70–80% in the mouse) (8–12). Independently of the lines of study on ERM proteins, another ERM-like protein was identified as a tumor suppressor or hereditary neurofibromatosis type 2 and named merlin (moesin/ezrin/radixin-like protein) or schwannomin (13, 14).

It is now widely accepted that ERM proteins function as general cross-linkers between plasma membranes and actin filaments (for reviews see Refs. 15–19). The highly conserved NH2-terminal half of ERM proteins directly binds to the cytoplasmic domains of integral membrane proteins such as CD44, CD43, ICAM-1, and ICAM-2 (20–24). On the other hand, ERM proteins directly interact with actin filaments (25–28). The co-existence of plasma membrane- and actin filament-binding domains in individual molecules may allow ERM proteins to function as plasma membrane/actin filament cross-linkers. Furthermore, ERM proteins are also thought to be involved in plasma membrane/actin filament cross-linkage through hetero- and/or homo-dimerization (29–32) and through binding to EBP (ERM-binding phosphoprotein) 50/NHE-RF (regulatory cofactor of Na+/H+ exchanger) (33, 34). There is accumulating evidence that the cross-linking activity of ERM proteins is regulated by the Rho-dependent signaling pathway through binding to Rho-dependent dissociation inhibitor and/or Rho-dependent phosphorylation (23, 35–38).

One of the important questions regarding ERM proteins that have not yet been addressed is the extent to which ezrin, radixin, and moesin are functionally redundant. Targeted disruption of ERM protein genes would be one of the most direct ways to approach this issue. Among the ERM proteins, we expected moesin to be rather functionally unique, partly because this molecule lacks the polyproline stretch found in both ezrin and radixin and partly because only moesin is not tyrosine phosphorylated by the epidermal growth factor receptor (39). Therefore, to address the redundancy problem in ERM proteins we generated mice with a targeted null mutation of the moesin gene located on the X chromosome.

EXPERIMENTAL PROCEDURES

Antibodies—Rat anti-ezrin, radixin, and moesin mAbs (M11, R21, and M22, respectively) (40) and rabbit anti-ezrin pAb (TK90) (41) were specific for respective antigens, whereas rabbit anti-ERM pAbs (TK89 and TK88) (41) recognized COOH- and NH2-terminal halves of all ERM proteins, respectively. Rabbit anti-radixin pAb (11) (6) recognized all ERM proteins on immunoblotting but recognized only radixin on immunofluorescence microscopy. Mouse anti-ERM mAb (CR22) reacted strongly with moesin (11). Mouse anti-vinculin mAb (Sigma) were purchased.

Construction of Targeting Vector—Mouse moesin genomic clones were isolated from a 129/Sv mouse genomic library using mouse moesin cDNA (nucleotides 1–315) (11) as a probe. The targeting vector (see Fig. 1A) was constructed by standard recombinant DNA techniques; the 0.7-kb PstI-KpnI fragment containing the 3’ part of exon 3 was deleted and replaced by a loxP-neo cassette in which loxP sequences (42, 43)
flank phosphoglycerate kinase-neo cassette (44) oriented in the opposite orientation to moesin transcription. In addition, a splicing acceptor (SA) sequence (45, 46) and a polyadenylation signal (PA) [XhoI-PolI fragment of pCAGGS (47)] were ligated 3' of this cassette. The coding region of the diphtheria toxin A gene driven by the MCI promoter (DT-A) was then ligated into the homologous region, loxP phosphoglycerate kinase-neo/loxP/SV40/PA, a 1.5-kb 3'-homologous region, and DT-A.

**Disruption of Moesin Gene in Embryonic Stem Cells and Generation of Moesin-Deficient Mice—** Embryonic stem (ES) cells were electroporated with 20 μg of linearized targeting vector DNA using a Bio-Rad Gene Pulser at 0.25 V and 960 microfarad. Cells were plated on feeder cells in normal growth medium for 36–48 h, followed by selection with 175 μg/ml G418. After 8–10 days, G418-resistant colonies were picked up. The colonies were screened individually by cleaving genomic DNA (1 μg) with BamHI and probing the Southern blots with the 230-bp cDNA sequence in exons 4 and 5 downstream from the 3' homologous region (3' probe; see Fig. 1A). Correct targeting was confirmed by Southern blotting of BstXI-digested genomic DNA with the 50-bp cDNA in exon 2 upstream from the 5' homologous region (5' probe; see Fig. 1A). Targeted clones were also checked for single integration by hybridization with a neo probe. Two correctly targeted ES cell clones (clones 145 and 199) were expanded and injected into the blastocysts from C57BL/6 mice, which were then transferred into the uteri of pseudo-pregnant ICR recipients. Male chimeras with extensive ES cell contributions to their coats were bred with C57BL/6 female mice. Tail DNA from agouti F1 offspring was genotyped by Southern blotting analysis. F1 heterozygous females and F1 wild-type males were interbred and the littersmates were genotyped.

**Immunoblotting—** Protein extracts from ES cells (+/N, −N) and various tissues of wild-type and moesin-deficient mice were separated by SDS-polyacrylamide gel electrophoresis (10%) and then electrophoretically transferred from gels onto nitrocellulose membranes, followed by incubation with antibodies. For antibody detection, a blotting detection system from Amersham Pharma Biotech (USA) was used.

**Immunofluorescence Microscopy—** Immunofluorescence microscopy of frozen tissue sections (10 μm) and embryonic fibroblasts were performed as described previously (49). They were examined using confocal imaging system (Bio-Rad) equipped with a Zeiss Axioskop II photomicroscope (Carl Zeiss, Oberkochen, Germany).

**Platelet Aggregation Assay—** Blood was collected by cardiac puncture on sodium citrate (0.38%), mixed with an equal volume of suspension buffer (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 5 mM MgCl2, 0.38% sodium citrate, pH 7.4) and then centrifuged at 120 × g for 10 min at room temperature. The supernatant was collected as platelet-rich plasma, which was diluted to 3 × 105 platelets/μl with plasma. Platelet aggregation was assayed as described previously (50).

**Fibroblast Culture and Cell Motility Assay—** Embryonic fibroblast cells were obtained from clonal wild-type (+/N) and moesin-deficient (−/N, −/−) embryos and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum on fibronectin-coated dishes (IWAKI, Chiba, Japan) for 7 days. Cell movement was recorded using a time lapse video system (LVR-3000N; SONY, Tokyo, Japan) at 37 °C under a 10× phase contrast objective lens.

**Mast Cell Culture—** Mast cells were isolated according to the technique described previously (51). Briefly, bone marrow was carefully taken out from the femurs of wild-type or moesin-deficient mice and suspended in α-minimum essential medium containing 10% fetal calf serum and 40 units/ml recombinant murine interleukin-3. The cell suspensions were placed on culture dishes for 7 days, and nonadherent cells were transferred into fresh medium in new culture dishes every 4 days. After 6–7 weeks of culture, homogeneous populations (>90%) of bone marrow-derived mast cells were obtained. They were placed on poly-L-lysine coated coverslips and then fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer (pH 7.2), and processed for the scanning electron microscopic observation.

**RESULTS**

**Targeted Disruption of Moesin Gene in Embryonic Stem Cells—** The mouse genomic clone used for construction of the targeting vector is shown in Fig. 1A. Judging from the structure of the human moesin gene consisting of 12 exons (52), this mouse clone was thought to contain exons 2–5. The targeting vector was designed with the expectation that homologous recombination between the vector and the moesin gene would result in deletion of a 3' part of exon 3. Because the numbers of nucleotides in exon 1 (12 bp from ATG), exon 2 (84 bp) and exon 3 (96 bp) are multiples of 3, it was possible that an aberrant transcript would be produced from the targeted allele by skipping the disrupted exon 3. To minimize this possibility, a SA and PA were placed at the 3' end of LoxP-neo cassette.

The linearized targeting vector was introduced into J1 ES cells by electroporation, and cells were selected with G418. To screen for homologous recombination events, DNA from resistant clones were subjected to Southern blotting analysis with a probe corresponding to a sequence 3' of the recombination site (3' probe). The wild-type moesin allele displayed a 9.2-kb band on Southern blotting of BamHI-digested DNA, whereas the disrupted locus showed a 5.0-kb band (Fig. 1B). Correct targeting was confirmed by Southern blotting with a 5' probe, and targeted clones were also checked for single integration by hybridization with a neo probe. For 430 G418-resistant clones examined, two had undergone a single homologous recombination event (clones 145 and 199) (Fig. 1B). As the moesin gene is located on the X chromosome (52, 53) and ES cells were established from male mice, the moesin single knockout cells were expected to be deficient in moesin. Immunoblotting analysis was performed using two different pAbs, TK88 and TK89, which recognized NH2- and COOH-terminal halves of all ERM proteins, respectively (Fig. 1C). In wild-type ES cells, both pAbs recognized three bands around 80 kDa, which corresponded to ezrin, radixin, and moesin from the top, whereas both clones 145 and 199 lacked the moesin band in TK88 as well as TK89 immunoblotting. In these moesin single knockout cells, additional bands did not appear at the smaller molecular mass region in TK88 or TK89 immunoblotting, confirming that the moesin single knockout cells are deficient in moesin. This was further confirmed by the immunoblotting with ezrin-, radixin-, and moesin-specific mAbs (M22 and CR22 recognized distinct epitopes of the COOH-terminal half of moesin) (Fig. 1C). Theoretically, of course, the possibility cannot be completely excluded that only the short fragment of moesin molecule, which cannot be detected with pAbs (TK88 or TK89) or mAbs (M22 or CR22), is expressed in small amounts in the moesin single knockout cells. It is not, however, likely that these small fragments of moesin, if any, can work as functional ERM proteins.

**Generation of Moesin Null Mice—** Clones 145 and 199 were injected into C57BL/6 recipient blastocysts, and male chimeric mice were crossed with C57BL/6 females. F1 female agouti pups, which were expected to be heterozygous for the mutant moesin allele, were then crossed with wild-type males and littersmates were genotyped using the 3' probe (Fig. 2A). This interbreeding yielded offspring at the expected Mendelian segregation ratio, 1:1:1:1 of wild-type male (+/N), wild-type female (+/−), heterozygous female (+/−), and hemizygous male (−/−). Moesin-deficient male mice (−/−) developed and grew normally in the laboratory environment and showed no differences in weight, size, or reproductive ability from wild-type mice at least up to 12 months old. Furthermore, interbreeding between F2 hemizygous males (−/−) and F2 heterozygous females (+/−) yielded F3 homozygous females (+/−), which also showed normal development, growth, and reproductive capacity, at least up to 9 months old (data not shown). Various tissues were examined histologically in hematoxylin-eosin-stained sections of moesin-deficient mice (−/− and −/−), and no significant abnormalities were detected (data not shown). Most of the following analyses were performed using hemizygous male mice (−/−).

**Expression Levels and Subcellular Distributions of Ezrin and...
Radixin in Moesin-deficient Mice—We first examined whether the expression levels of ezrin and radixin were elevated in moesin-deficient mice in a compensatory manner by immunoblotting with anti-ERM pAb TK89 that recognized COOH-terminal halves of all ERM family members (Fig. 2B). As reported previously, in wild-type mice most tissues co-expressed ezrin, radixin, and moesin in various expression ratios, but the liver and intestine lacked ezrin and radixin expression, respectively. Unexpectedly, in moesin-deficient mice, neither ezrin nor radixin expression was affected; no compensatory up-regulation of ezrin or radixin was detected in any of the tissues examined. Immunoblotting with ezrin-, radixin-, and moesin-specific mAbs also confirmed this conclusion, and anti-ERM pAb TK88 that recognized NH2-terminal halves of all ERM proteins gave the same results without additional bands at the lower molecular mass region (data not shown).

We next examined the subcellular distributions of ezrin and radixin in various tissues of moesin-deficient mice. When frozen sections of the kidney from wild-type mice were doubly stained with anti-ezrin pAb (TK90)/anti-moesin mAb (M22) (Fig. 3A) or anti-radixin pAb (I1)/anti-moesin mAb (M22) (data not shown), intense ezrin, radixin, and moesin signals were detected from apical surfaces of proximal tubules and glomeruli. As reported previously, in endothelial cells of blood vessels, moesin was abundantly detected, whereas the expression levels of ezrin and radixin were rather low. In the kidney of moesin-deficient mice, in which the moesin signal was not detected by immunofluorescence microscopy, the subcellular distributions of ezrin (Fig. 3A) and radixin (data not shown) did not appear to be affected. In the liver of wild-type mice, the only ERM proteins expressed were radixin and moesin. In this tissue, moesin expression was mostly restricted to sinusoidal endothelial cells (Fig. 3B, panel b), whereas radixin was detected abundantly in bile canaliculi and in small amounts in sinusoidal endothelial cells (Fig. 3B, panel a). In the liver of moesin-deficient mice, the subcellular distribution of radixin did not appear to be affected (Fig. 3B, panels c and d), and no induction of ezrin was detected (data not shown). Further intensive immunofluorescence microscopic analyses led us to conclude that targeted disruption of the moesin gene did not affect...
either the expression levels or subcellular distribution of ezrin or radixin in any of the tissues examined. Because we detected no abnormalities in moesin-deficient mice at the tissue level, we next examined several types of cells isolated from moesin-deficient mice, in which moesin was reported to be important for physiological functions.

**Aggregation of Moesin-deficient Platelets**—Among the ERM proteins, human platelets were reported to predominantly express moesin, and moesin was suggested to be involved in their aggregation process (54). Therefore, we first examined the aggregation activity of platelets isolated from moesin-deficient mice. Upon immunoblotting of wild-type mouse platelets, in addition to large amounts of moesin, significant amounts of ezrin and radixin were also detected (Fig. 4A). In platelets collected from moesin-deficient mice, the moesin band disappeared completely, leaving relatively small amounts of ezrin and radixin without compensatory up-regulation.

We then compared the aggregation ability of platelets between wild-type and moesin-deficient mice (Fig. 4B). Wild-type and moesin-deficient platelets were collected, and they were subjected to the aggregation assay; aggregation was initiated by addition of various concentrations of thrombin, ADP, or collagen, and the extent of aggregation was measured as the turbidity change with a platelet aggregometer. Unexpectedly, as shown in Fig. 4B, no differences were detected between wild-type and moesin-deficient platelets.

These observations led us to conclude that in platelets targeted disruption of the moesin gene did not affect their aggregation activity without any compensatory up-regulation of ezrin or radixin.

**Stress Fiber/Focal Contact Formation and Cell Motility of Moesin-deficient Fibroblasts**—Moesin was reported to be required for the Rho-dependent formation of actin stress fibers and focal contacts in fibroblasts (36). We then isolated and cultured embryonic fibroblasts from wild-type as well as moesin-deficient mice and stained them with rhodamine-phalloidin.
or anti-vinculin mAb. As shown in Fig. 5 (A–D), even in moesin-deficient fibroblasts, well developed stress fibers with vinculin-positive focal contacts were observed, indicating that targeted disruption of the moesin gene did not affect stress fiber/focal contact formation in fibroblasts. We next compared the migration rate of moesin-deficient fibroblasts on coverslips with that of wild-type fibroblasts using a time lapse video system. Again, however, no statistically significant difference was detected by Student’s t test (p, 0.005) (Fig. 5E). The mean migration speeds of moesin-deficient and wild-type fibroblasts on coverslips were 31.45 ± 1.44 and 29.05 ± 1.52 μm/h, respectively (n = 100).

**Microvillar Formation in Moesin-deficient Mast Cells**—Finally, we examined microvillar formation in moesin-deficient cells, in which ERM proteins were reported to be directly involved through experiments with antisense oligonucleotides (40). To obtain a homogeneous population of mast cells bearing well developed microvilli, we cultured bone marrow isolated from wild-type as well as moesin-deficient mice in medium containing interleukin-3 for 6–7 weeks (51). Immunoblotting with anti-ERM pAb TK89 revealed that wild-type mast cells expressed large amounts of moesin, small amounts of radixin, and only trace amounts of ezrin and that targeted disruption of the moesin gene did not elevate the expression levels of ezrin or radixin (Fig. 6A). However, as shown in Fig. 6B, no significant differences were detected in the length or number of microvilli between the wild-type and moesin-deficient mast cells on scanning electron microscopy.

**DISCUSSION**

ERM (ezrin/radixin/moesin) proteins have been implicated as general cross-linkers between the plasma membrane and actin filaments (for reviews see Refs. 15–19). In this study, we generated male and female mice hemi- and homozygous, respectively, for a null mutation in the moesin gene located on the X chromosome. Surprisingly, the mutant mice exhibited no obvious abnormalities in appearance or fertility, and a systemic histological scan of mutant tissues revealed no abnormalities. Our results clearly demonstrated that moesin is not required for normal mouse development or for survival in the laboratory environment. This is surprising in view of the degree of conservation of the moesin gene, for example, the occurrence of a moesin gene in *Drosophila* (55, 56) and the tissue-specific regulated expression of this gene (57–59).

To date immunofluorescence microscopy and immunoblotting analyses have revealed that the ratio of the levels of ezrin, radixin, and moesin expression in individual cells varies between different tissues in wild-type mice. Furthermore, ezrin, radixin, and moesin are not always colocalized. From these *in situ* observations in wild-type mice, ezrin, radixin, and moesin have been suggested to have specific functions. However, experiments in vitro or at the cellular level have not clearly identified differences in function between these molecules. When the expression of any one or two ERM proteins was selectively suppressed by antisense oligonucleotides, no phenotypic changes were detected, and cell-cell/cell-matrix adhesion and microvillar formation were affected only when expression...
and microvillar formation (4, 31, 40, 60, 61), we concluded that only a small fraction of total ERM proteins in wild-type platelets and mast cells are sufficient for their physiological functions. Similar observations, i.e. no phenotypic changes in knockout mice without up-regulation of other family members, has been reported in various systems. For example, mice devoid of components of intermediate-sized filaments such as vimentin and glial fibrillary acidic protein developed normally without compensatory expression of other intermediate filament components (62, 63).

Another issue that we should discuss here is the function of moesin in intracellular signaling. We proposed that the cross-linking activities of ERM proteins were regulated by the Rho-dependent signaling pathway through direct binding to Rho-GDP dissociation inhibitor and/or through Rho-dependent phosphorylation (23, 35–38). Ezrin and radixin bound to Rho-GDP dissociation inhibitor with similar binding constants to phosphorylation (23, 35–38). Ezrin and radixin were phosphorylated by Rho kinase with similar efficiency to moesin in vitro. Mackay et al. (36) found that moesin was required for the Rho-dependent formation of stress fibers and focal contacts in permeabilized Swiss 3T3 cells. Also in this case, moesin was able to be replaced by ezrin and radixin. From the present results, we concluded that the functions of ERM proteins in the Rho-dependent signaling pathway are redundant also at the whole body level.

In conclusion, the present observations were consistent with the notion that ERM proteins are functionally redundant. The possibility cannot be excluded that a more distantly related protein to moesin in the band 4.1 superfamily functionally compensates for the lack of moesin. Further generation of mutant mice lacking ERM proteins, both singly and in combination, will lead to a better understanding of the physiological relevance of the occurrence of these three closely related proteins.

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