Characterization of moesin in the sea urchin *Lytechinus variegatus*: redistribution to the plasma membrane following fertilization is inhibited by cytochalasin B

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SUMMARY

We have investigated the distribution and function of an ezrin-radixin-moesin-like (ERM) molecule in the sea urchin. A sea urchin homologue of moesin was cloned that shares 75% amino acid similarity in the conserved N-terminal region to other moesin molecules. A 6.3 kb message is transcribed late in embryogenesis and is present in adult tissues. Polyclonal antibodies were generated to proteins expressed by a bacterial expression vector, and affinity purified. These antibodies recognize a single 75 kDa protein that is present throughout development in approximately equal abundance, and specifically they immunoprecipitate a single protein. We show by immunolocalization that SUmoesin has two predominant patterns during development. First, SUmoesin is rapidly redistributed after fertilization from a location throughout the egg cytoplasm to a location in the egg cortex. Later in embryogenesis, SUmoesin is localized to the apical ends of cells in the regions of cell-cell junctions. We show that SUmoesin is present in actin-rich regions of the embryo. Finally, we show that the location of SUmoesin requires an intact actin-based cytoskeleton. SUmoesin fails to localize to the plasma membrane after fertilization in the presence of cytochalasin B. Furthermore, SUmoesin loses its apical position in the region of cell-cell junctions in the presence of cytochalasin B in later stages of embryogenesis. This effect is reversible, and the microtubule inhibitor colchicine has no effect. These results show that SUmoesin becomes associated with apical plasma membrane structures early in development, and that SUmoesin is both coincident with actin and requires the assembly of actin filaments to maintain its localization.

Key words: moesin, sea urchin, *Lytechinus variegatus*, cytochalasin B, plasma membrane

INTRODUCTION

The organization of the cortical cytoskeleton into microfilaments and associated plasma membrane structures is thought to be influenced by proteins that are encoded by a family of genes known as ezrin, radixin and moesin (now called ERM; reviewed by Bretscher, 1991; Tsukita et al., 1993). These proteins all share extensive similarity at their N terminus to the erythrocyte protein 4.1, which is thought to link the plasma membrane to the cytoskeleton by binding glycophorin to the actin/spectrin network in erythrocytes (Conboy et al., 1986). Because of their structural similarity to 4.1 and talin, ERM proteins are presumed to play a role in linking the actin-based cytoskeleton to the plasma membrane (Rees et al., 1990; Lankes and Furthmayr, 1991). This role is supported by the localization of ERM proteins in actin-rich surface structures such as microvilli, membrane ruffles and adherens junctions (Sato et al., 1992). A recent report also demonstrated that ERM proteins may be functionally redundant, because they may all be necessary for microvillus assembly and cell-cell adhesion (Takeuchi et al., 1994). These data taken together suggest that the roles of individual ERM proteins may be difficult to determine in mammalian cell lines. The push to describe the functions of ERM proteins has been bolstered by a recent report on another ERM protein called merlin, which is the gene associated with human neurofibromatosis 2, may be involved in regulating normal cell growth and may act as a tumor suppressor (Trofatter et al., 1993).

Other biochemical evidence supports a role for ERM proteins as cytoskeleton-plasma membrane linkers. Analysis of ERM proteins has been difficult, however, because antibodies generated to individual ERM proteins have cross-reacted with each other, and the tissue distribution of ERM members overlaps (Berryman et al., 1993). Consistent with what is known about protein 4.1, the localization of ezrin to the plasma membrane and cytoskeleton in cell lines is mediated by the N and C terminus, respectively (Algrain et al., 1993). To date, ERM family members have been shown to interact with actin and the transmembrane molecule CD44 (Tsukita et al., 1989, 1994; Sato et al., 1991). There is little information about the distinctive function of individual ERM family members, and even less is known about these proteins during development.

Model systems have offered important insights into the mechanisms of cytoskeletal-plasma membrane interactions.
For example, protein 4.1 is encoded at the locus responsible for the coracle mutation in *Drosophila* (Fehon et al., 1994). This mutation causes a failure of dorsal closure during embryogenesis, and mutants in the coracle gene interact with a hypermorphic EGFR-Ellipse allele, suggesting the possibility that their protein products interact. We felt that understanding the function of ERM proteins would be advanced by studying these molecules in another model developmental system, the sea urchin. Sea urchin embryogenesis involves a dynamic sequence of cytoskeletal-plasma membrane interactions that leads to the formation of microvilli, polarized epithelia and cell-cell junctions. Accordingly, we have cloned moesin in the sea urchin, and characterized moesin protein during development.

The actin-based cytoskeleton of the sea urchin changes dramatically. After fertilization, a massive reorganization of the cytoskeleton occurs when a large G-actin pool is rapidly polymerized, forming microvilli and a robust, filamentous cytoskeletal cortex in the zygote (Spudich and Spudich, 1979; Spudich et al., 1982; Spudich, 1992). A partially defined series of exocytotic events are stimulated by the fertilization reaction, and these too involve cytoskeletal proteins (Otto et al., 1980; Bonder et al., 1989). Proteins such as fascin, α-actinin and spectrin bundle actin filaments together, but none have been described that link actin filaments or the cytoskeleton to the plasma membrane during sea urchin development (Mabuchi et al., 1985; Bryan, 1986; Fishkind et al., 1987). Later in development, cells become polarized and some proteins require the actin cytoskeleton for maintenance of this polarity (Nelson, 1988). At least five isoforms of actin are associated with well-defined events such as mitosis, cell-cell junction formation and maintenance of cell polarity (McCay et al., 1983; Spiegel and Howard, 1983; Davidson, 1986; Andreuccetti et al., 1987).

All of these cytoskeletal events can be experimentally manipulated in the sea urchin to better understand the role of ERM proteins during embryogenesis.

Changes in the plasma membrane also occur during sea urchin development, and some of these may involve ERM proteins, which are included in a group of proteins that are believed to influence the association of the cytoskeleton with the plasma membrane (Luna and Hitt, 1992). One such influence may be mediated by an interaction of ERM proteins with the integral membrane protein CD44 (Tsukita et al., 1994). Reorganization and recycling of membranes occurs after fertilization during vesicle exocytosis and microvillus formation. The restructuring of the early embryo as it divides and cells become polarized is accompanied by an 100× increase in the surface area to volume ratio by the blastula stage (Wolpert and Mercer, 1963). Against this backdrop and the developmental potential of sea urchin embryos, we have cloned and characterized moesin protein during development.

### MATERIALS AND METHODS

#### Materials

Sea urchins (*Lytechinus variegatus*) were obtained from Susan Decker (University of Miami) and Gail Cannon (Duke University Marine Lab.). Gametes were obtained by intracoelomic injection of 0.5 M KCl, and eggs were dejellied by passing through cheese cloth and washed 3x with artificial sea water (ASW). Later stage embryos were maintained at room temperature with stirring.

#### Cloning moesin in the sea urchin

Poly(A)⁺ RNA was prepared at various stages from 0.2 ml packed embryos processed with the Micropure Kit (Pharmacia). One microgram of RNA from both the midgastrula (14 hours) and pluteus stages (24 hours) was used to generate first-strand cDNA using M-MuLV reverse transcriptase (New England Biolabs). Degenerate oligonucleotide primers to the ERM family (B. McCartney and R. Fehon, unpublished data) were used in a PCR reaction. Conditions were: 94°C, 40 seconds; 45°C, 40 seconds; 72°C, 40 seconds, for 40 cycles. A 340 bp band was isolated and a second round of PCR was carried out under identical conditions using an internal primer. The resultant 300 bp band was ligated into the TA vector (Invitrogen) and sequenced. Screening of cDNA libraries was performed using 32P-labeled probes made to the PCR product and cDNA clones (Stratagene). Filters were washed for 15 minutes 3x at high stringency (0.2x SSC, 0.1% SDS at 60°C). All clones were obtained from Lambda Zap libraries made to poly(A)⁺ RNA isolated from *L. variegatus* embryos at 2 stages; mid-gastrula (constructed by Stratagene with RNA produced in this lab.) and prism (kindly provided by Gary Wessel).

#### Northern analysis

A 1.2% agarose/formaldehyde gel (Sambrook et al., 1989) was loaded with poly(A)⁺ RNA (5 µg/lane) from the indicated stages, blotted onto a nylon membrane and hybridized using the 300 bp PCR product as a probe. The blot was washed at high stringency (as above), stripped and reprobed with a ubiquitin fragment from *L. pictus*.

#### Generation of fusion proteins and antibodies

Clones used in the generation of fusion proteins were produced using the PCR with specific primers made to the 5’ end of moesin. These primers contained 5’ BamHI sites followed by a sequence specific for moesin. Amplification by the PCR was performed using these 5’ primers, the M13 reverse sequencing primer and 20 ng of template DNA from a 1700 bp moesin cDNA clone at 60°C. A near full-length clone (526 amino acids) and a shorter clone (450 amino acids) were ligated into the TA vector (Invitrogen) and sequencing. Cloning moesin in the sea urchin

#### Immunofluorescence and western analysis

Early embryos (prehatching) were fixed for 30-45 minutes in 3.7% paraformaldehyde/ASW at RT, while later (posthatching) stages were fixed for 10 minutes at −20°C in methanol. Embryos were washed 3x in ASW and once in block (10% goat serum/0.1% Triton X-100/ASW), to block nonspecific binding and permeabilize. Multiple washes in ASW and once in block was performed in between the primary and secondary antibody incubations. Primary antibodies to *Sm*moesin were prepared as above, and secondary reagents were all commercially available. Affinity-purified rabbit polyclonal antibody was used at 1:1000 overnight, and secondary Indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (Jackson Immunno Research) was incubated at 1:500 for 2 hours at RT. Embryos were viewed using confocal microscopy. A mouse anti-actin antibody made to chicken actin (gift from NIH hybridoma bank) was used in double-labeling experiments. DAPI ( Molecular Probes) and rhodamine/phalloidin (Sigma) were used for labeling nuclei and filamentous actin, respectively. DAPI (1
mg/ml) was added with secondary antibody at 1:1000 for 2 hours. Rhodamine-phalloidin was added 1:1 (v/v) from a 4 mg/ml stock in methanol to live embryos for 20 minutes on ice, washed 3× in ASW, and immunostaining was carried out as above with anti-moesin.

Protein was prepared for western analysis by homogenizing embryos in 0.1% Triton X-100/150 mM NaCl/1 mM PMSF. Approximately 25 µg/ lane was loaded as measured by the Bradford assay and confirmed by Ponceau staining following SDS-PAGE (Laemmli, 1970). Western blotting was performed using the moesin primary antibody at 1:1000 dilution and secondary phosphatase-conjugated goat anti-rabbit antibody at 1:5000 dilution (Cappel). Leupeptin (1 µg/ml) was added to some homogenization cocktails to prevent proteolysis.

**Immunoprecipitation**

Homogenates of sea urchin embryos were prepared by homogenizing approximately 100 mg wet eggs or embryos in 1 ml of 1.0% Triton X-100 buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM PMSF, 1 mM leupeptin and processed with 20 strokes of Dounce homogenization followed by 10 minutes of centrifugation at 10,000 rpm (7,000 g/ p) in a Beckman microfuge. The supernatant (1 ml) was precleared for 2 hours at 4°C with 40 µl Protein A beads followed by centrifugation to remove the beads. The cleared supernatant was incubated overnight with 1 µg of affinity-purified rabbit polyclonal antibody, followed by addition of 40 µl Protein-P-Sepharose beads for 2 hours followed by 5 washes at 14,000 rpm (6,000 RCF) with 1 ml washes in 150 mM NaCl, 0.1% Triton X-100. The washed and boiled beads were resuspended in SDS sample buffer/BME and analyzed by SDS-PAGE western analysis as above.

**In vivo labeling**

One ml cultures of embryos (10%, v/v) at 5 stages were incubated with 100 µCi of [35S]Met-Cys trans label (ICN) for 1 hour, briefly centrifuged and washed 3× in 1.5 ml sea water, homogenized and centrifuged as above. The supernatant (1 ml) was counted by scintillation, standardized to 7000 cpm/ml, precleared 2× with 40 µl Protein A beads before adding 1 µg of affinity-purified antibody as described. Washing and analysis by SDS-PAGE was performed as above, but proteins were detected by autoradiography after impregnating the gel in PPO (Wessel, 1986).

**Cytochalasin B/colchicine experiments**

Two ml cultures containing approximately 5%, v/v, of dejellied eggs were fertilized by dilute sperm in the presence of 10 µM PABA (p-aminobenzoic acid). After 15 seconds, cytochalasin B (5 mg/ml in DMSO) or colchicine (20 mg/ml in water) were added to final concentrations of 10 and 100 µM, respectively. Control cultures contained 0.1% DMSO. Fertilization was observed to be 100% in all of these experiments (n=4). Fertilization envelopes were stripped by passing fertilized eggs through 73 µm Nitex. After 10-15 minutes, fertilized eggs were fixed in 3% paraformaldehyde/ASW for 30 minutes and treated as above. In washout experiments, cultures were washed 3× in ASW over 5 minutes and fixed at the indicated time points.

Later stage embryos were grown in 2 ml samples of 5% of 5%, v/v, in Costar culture dishes with gentle shaking. Cytochalasin, colchicine and DMSO control were present for 1 hour at the same concentration as above. Recovery from treatment was facilitated by gently spinning cultures down after 1 hour of treatment, washing replicate cultures 3× in ASW and letting cultures recover for 30 minutes in ASW.

**RESULTS**

**Cloning of an ezrin/radixin/moesin-like homologue in the sea urchin**

A 300 bp PCR product was amplified from cDNA derived from mid-gastrula and pluteus stage sea urchin larvae (Fig. 1). This fragment was isolated and ligated into the TA cloning vector and 10 inserts were sequenced using the dye deoxy method. All 10 inserts were identical in nucleotide sequence, and were highly similar to ezrin/radixin/moesin by sequence comparison (Altschul et al., 1990). On the basis of deduced amino acid sequence comparisons (see below), we refer to this as sea urchin moesin, or SUmoesin. The 10 clones were also identical to 10 clones generated from a pluteus cDNA pool. This SUmoesin clone corresponds to amino acids 200-310 of human radixin, and was used for further screening and expression studies. We conclude that the PCR generated a single gene product at both stages of development.

**Isolation of cDNA clones**

A near full-length clone containing the start codon and 138 bp of upstream sequence was obtained from a mid-gastrula cDNA library, and overlapping clones covering the remaining 3′ end were obtained from a prism library (Fig. 1). These clones were sequenced on both strands using the dye deoxy chain termination method (USB). The predicted open reading frame of 573 amino acids (Mg 68,000; pl 5.31) contains an N-terminal region that is highly similar to ezrin/radixin/moesin from many species, while the divergent C terminus does not contain a polyproline stretch and is most similar to moesin (Fig. 2). The overall identity of SUmoesin to human radixin and human moesin is 54%. We are therefore using the name SUmoesin with the understanding that the sequence differences are not clearly distinctive (Lankes and Furthmayr, 1991). There are 4 potential consensus tyrosine phosphorylation start sites at amino acids 161, 192 (261, 262), and 329 according to computer analysis (Geneworks), and these tyrosines have superscripts (P). The 5′ start contains the consensus Kozak sequence and the stop codon is followed by multiple stop codons. In addition, the predicted secondary structure from the carboxy end of the molecule, spanning amino acids 300-500, is alpha-helical (MacVector). The sequence is identical to other ERM family members at the highlighted amino acids.

**Cloning of moesin in the sea urchin**

Northern analysis using 5 µg of poly(A)^+ RNA per lane was performed and the blot was washed under high stringency using the 300 bp PCR product as a probe (Fig. 3). A 6.3 kb message is present in the ovary, presumably in oocytes, and is abundant in adult gut tissue. The mature egg has little or no
moesin RNA; expression during embryogenesis begins at the late blastula stage and peaks at the pluteus larva stage. A ubiquitin control shows that abundant RNA has been loaded in each lane. This developmental profile therefore is classified as an ‘embryonic late gene’ (Davidson, 1986).

Moesin antibody recognizes a single protein throughout embryogenesis

Homogenates from all stages of development were prepared, and 25 µg of each was run on 7.5% SDS-PAGE, blotted and visualized by western analysis using affinity-purified rabbit polyclonal antibody (Fig. 4A). A 75 kDa protein is recognized at all stages of development from eggs through the 24 hour pluteus larva stage, and abundance is similar at each stage. Antibodies prepared independently to both fusion proteins (85 and 95 kDa) in mice and rabbit gave the same staining pattern by western analysis and by whole-mount embryo immunofluorescence. Repeated analysis using the three antibody sources consistently showed a single protein even when blots were overdeveloped or resolved during long runs on 7.5% SDS-PAGE. This antibody is therefore specific for SUmoeisin, and does not cross-react with other potential ERM family members in the sea urchin. The presence of moesin protein in eggs and during early embryogenesis indicates that moesin protein is provided maternally, because moesin RNA is absent from those early stages.

Affinity-purified antibody recognizes a single 75 kDa protein

Immunoprecipitation experiments were performed: (i) to confirm the western analysis; (ii) to investigate possible bio...
Cloning of moesin in the sea urchin

chemical interactions between moesin and other proteins; and (iii) to learn when SUmoesin is translated (Fig. 5). Embryos were incubated in [35S]Met-Cys at five stages of development and SUmoesin was analyzed by immunoprecipitation using 1 µg of affinity-purified antibody, followed by SDS-PAGE and autoradiography. A band of 75 kDa is first detected at the hatching blastula stage. Faint bands at 205, 116 and 50 kDa coprecipitated in both the beads only and antibody/beads fractions (Fig. 5A). At least one of these (116 kDa) may correspond to echinonectin, which is a sea urchin protein that has a high affinity for Sepharose beads (Alliegro et al., 1990). Labeled moesin protein is not detected in early embryonic stages (Fig. 5B), a result that can be explained by the previous observation that moesin mRNA is not present in early embryonic stages (Fig 3). We conclude that zygotic SUmoesin is not translated until the blastula stage, after which it continues to be synthesized through the feeding pluteus larval stage. The pattern of coprecipitated proteins is similar at all stages. The band that migrated at 75 kDa in the metabolically labeled material is identical in size to a protein that is detected by western analysis after immunoprecipitation using nonradioactive cell extracts (Fig. 5C).

Moesin is localized to the plasma membrane after fertilization

To investigate the cellular distribution of moesin, whole-mount indirect immunofluorescence imaging using confocal microscopy was performed on eggs and early fertilized embryos. In eggs, moesin is distributed in a punctate pattern throughout the cytoplasm (Fig. 6A). Within 10 minutes after fertilization, moesin becomes concentrated just beneath the plasma membrane in the egg cortex; over the same interval the punctate cytoplasmic distribution becomes less brightly fluorescent (Fig. 6B). Embryos from 2-cell and early blastula developmental stages are shown in surface and cross-sectional views by confocal microscopy (Fig. 6C-F). SUmoesin in these embryos is seen in the cell cortex in a predominately punctate distribution and continues to be seen in the cytoplasm at lower levels. The patchy staining on the surface of early embryos may correspond to the location of microvilli. In the morula stage, moesin is localized at the cell cortex in the region of cell-cell contacts and on the apical sides of cells (Fig. 6E). There continues to be staining within the cytoplasm in a punctate pattern as well (Fig. 6F). Controls with preimmune serum, preimmune serum with secondary antibody and secondary antibody alone were blank.

Moesin is associated with the region of cell-cell contacts in later embryogenesis

Embryos at hatched blastula, gastrula and feeding pluteus stages are shown in Fig. 7. Surface (A,C) and cross-sectional (B,D) views of two stages are seen. Moesin is concentrated in intercellular borders (Fig. 7A,C,E), and appears in the apical

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**Fig. 4.** Moesin protein is present throughout embryogenesis. A developmental western blot is shown using 25 µg of total protein per stage. The protein was run on 7.5% SDS-PAGE, blotted onto nitrocellulose and incubated with affinity-purified rabbit anti-SUmoesin antibody followed by alkaline phosphatase-conjugated secondary antibody. A 75 kDa protein is present in approximately equal amounts at the 5 stages indicated.

**Fig. 5.** Immunoprecipitation of SUmoesin. (A) Extracts of [35S]Met-Cys-labeled embryos were homogenized, pelleted, precleared twice (beads 1 and 2 lanes) and incubated with 1-2 µg of anti-moesin antibody are shown from left to right in progression (A). A predominant band at 75 kDa is seen in the immunoprecipitate, with other bands faintly seen at 210, 116 and 55 kDa. Adding twice the amount of antibody gave a small increase in protein, including coprecipitating bands. (B) The same immunoprecipitated lanes from different stages are seen, and 75 kDa SUmoesin is not translated 1 hour after fertilization (1 hour lane), but is labeled during the mesenchyme blastula stage. (C) Western blot analysis of immunoprecipitated material from nonradioactive cell extracts, using affinity-purified antibody to SUmoesin, recognizes a single band at 75 kDa and the expected 55 kDa rabbit IgG antibody that brought down the immunoprecipitate. Nonspecific binding to beads used in preclearing was not detectable by western blot (BEADS).
region where adherens junctions are known to be (Fig. 7B,D,F), and in the apical regions of cells in the ectoderm and endoderm. Moesin is not present in mesenchyme tissues. In the pluteus larva, moesin is concentrated in the apical region of cell-cell contacts in the ectoderm, oral hood and mouth (7 E,e). Moesin staining is noticeably reduced in aboral and perianal ectoderm cells. In endoderm, moesin is concentrated between cells at the apical surface of endoderm cells of the fore, mid- and hindgut (anus) regions (Fig. 7E).

**Moesin is concentrated in actin-rich regions of the embryo**

On the basis of the location of moesin protein: (i) in the cortex of fertilized eggs, and (ii) in the region of cell-cell contacts above, we asked whether moesin was localized in regions that contain high concentrations of actin at these two stages of embryogenesis. We analyzed the distribution of moesin and actin by double labeling embryos with rabbit anti-moesin (FITC-conjugated secondary antibody) and either mouse anti-actin antibody or rhodamine/phalloidin (filamentous actin) probes. Moesin in fertilized zygotes is concentrated in the cortex near the plasma membrane, where actin is abundant as judged by anti-actin antibody (Fig. 8A,B). Both moesin and actin are abundant in the cortex of fertilized eggs, but the merged image shows that moesin retains a cytoplasmic, punctate distribution as well (Fig. 8C). Surface staining of moesin and actin in these fertilized eggs is seen as a pattern of small particulate structures that may be microvilli (Fig. 8a,b). Merging the red and green channels shows the overlapping distribution of moesin (green, FITC) and actin (red, Cy3) images in yellow on the surface (Fig. 8c). Gastrula stage embryos were fixed in 50% methanol, 4 mg/ml rhodamine/phalloidin for 30 minutes and analyzed using the same procedure as described above for late stage embryos. A surface view of moesin, filamentous actin and their overlapping distribution is shown (Fig. 8D,E,F).

**Moesin is not recruited to the plasma membrane in the presence of cytochalasin B**

The rapid redistribution of moesin after fertilization, from
Cloning of moesin in the sea urchin throughout the cytoplasm to the plasma membrane, led us to ask what cytoskeletal proteins might be involved. In particular, we asked whether moesin requires microtubules or microfilaments to be associated with the plasma membrane after fertilization. Microfilament and microtubule assembly are disrupted in the presence of cytochalasin B and colchicine, respectively (Banzhaf et al., 1980). Cytochalasin B enters and exits cells within seconds. Accordingly, we added inhibitors of microtubule and microfilament formation 15 seconds after fertilizing eggs with dilute sperm. After 10 minutes, embryos were fixed and stained with anti-SUmoesin antibody as described above. Control embryos in 0.1% DMSO looked like those shown in Fig. 6B. Colchicine-treated zygotes showed staining concentrated at the plasma membrane that appears like a halo around the cell by confocal microscopy (Fig. 9A). Adding cytochalasin B, however, prevented localization of moesin to the cell cortex (Fig. 9B). The effect of cytochalasin B is reversible. Moesin staining near the plasma membrane in cytochalasin-treated cells is restored by rinsing 3× in ASW for 5 minutes before fixation (Fig. 9C).

**Moesin distribution is disrupted by blocking actin polymerization**

We next asked whether the polarized, cortical distribution of moesin seen at later stages of embryogenesis requires an intact cytoskeleton. We again used colchicine and cytochalasin B to inhibit the de novo assembly and maintenance of microtubules and microfilaments, respectively. Embryos at the morula (4

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**Fig. 7.** Moesin localization in post-hatched embryos. Surface and cross-sectional confocal microscopic views of moesin are shown for blastula and gastrula stages. SUmoesin is localized to the region of cell-cell contacts (A), and is found in an apical location in the region of cell-cell junctions (B), in hatched blastula stage embryos. SUmoesin is in the region of cell-cell contacts in an apical location in gastrula stage embryos (C,D). In pluteus larvae, moesin is concentrated between cells mostly in the oral hood and endoderm regions of the pluteus larva (E). A magnified view of the oral hood and mouth is shown (e). Bar, 20 µm.
hours) and hatched blastula (10 hours) stages were incubated as above in 0.1% DMSO, or in DMSO plus colchicine or cytochalasin for either: (i) 1 hour; or (ii) 1 hour followed by washing 3× in ASW and 30 minutes of recovery in ASW before fixation. Embryos were fixed in methanol, stained with antibody and analyzed by indirect confocal microscopy. In both colchicine- and cytochalasin-B-treated embryos, there are binucleate cells that have not completed cytokinesis. This is expected if microtubules or microfilaments are unable to form and take part in karyokinesis or cytokinesis (Cande et al., 1977). Moesin remains at the plasma membrane in an apical location in the presence of colchicine and DMSO (Fig. 9D). In the presence of cytochalasin B, however, the staining pattern at the plasma membrane is disrupted and appears patchy, vesicular and subapical (Fig. 9E). This disruption of cortical localization of moesin is reversible. When embryos are incubated for 1 hour in cytochalasin B, washed thoroughly and incubated in ASW alone for 30 minutes, staining returns near the plasma membrane in a confluent, apical pattern. At the blastula stage (10 hours), colchicine again has no effect on moesin distribution (Fig. 9G). Cytochalasin B, however, disrupts the continuous staining of moesin at the plasma membrane and the organization is again subapical and patchy (Fig. 9H). While moesin remains towards the apical end of cytochalasin-treated cells, staining becomes punctate and patchy, and staining is no longer restricted to the regions of cell-cell junctions. Association of SUmoesin with the plasma membrane is partly reversed when embryos are incubated for 1 hour and allowed to recover for 30 minutes (Fig. 9I). The confluent appearance of moesin at the apical end of blas-
DISCUSSION

While ERM proteins have been examined in cell lines and isolated differentiated tissues, there have been no reports of these proteins as they change during the development of an organism. In addition, the analysis in this report of what appears to be a single ERM protein simplifies an approach to understanding ERM proteins by looking at what is perhaps an ancestral protein to the family. What role does moesin have during reorganization of the cytoskeleton, establishment of cell polarity and formation of cell-cell junctions in the sea urchin embryo? We asked these questions as they pertain to the sea urchin embryo by cloning and characterizing moesin, and then studying its distribution changes during development.

The ERM genes are so highly similar at the nucleotide and amino acid level that identifying a sea urchin protein as a particular ERM homologue is equivocal. We call this ERM member SUmoesin, because of its similarity to other moesin members within the C-terminal, divergent half of these molecules. The 75 kDa size of the mature SUmoesin protein also resembles most closely the ERM family, which is also consistent with this nomenclature. SUmoesin lacks a polyproline stretch of amino acids that is found in ezrin and radixin.

Amino acids predicted from the nucleotide sequence reveals four consensus tyrosine phosphorylation sites, two of which are shared by other ERM family members. Phosphorylation of ezrin has been described in EGF-stimulated carcinoma cells, gastric parietal cells and tumor transplantation antigens (Gould et al., 1986, 1989; Hanzel et al., 1991; Yang and Tonks, 1991; Egerton et al., 1992; Krieg and Hunter, 1992; Fazioli et al., 1993). At present, no evidence exists for direct regulation of ezrin by phosphorylation. It will be interesting to find out whether SUmoesin is phosphorylated in any of its diverse developmental patterns.

The original description of ERM proteins in mammals showed that they can overlap in distribution, and may be functionally redundant. Cross-reactivity of antibodies among ERM family members originally made interpretation of ERM data difficult. For that reason, we attempted to be thorough in establishing that we were investigating a single ERM protein in the sea urchin. SUmoesin is a single ERM family member, based on the following evidence: (i) the antibodies used in this study were affinity purified; (ii) the SUmoesin protein that we describe is always a single species as shown by SDS-PAGE and western blot analysis, and that is true for three independent antibodies; (iii) western blot analysis described above shows only a single band even when low percentage gels are run for extended times; (iv) in vivo labeling experiments show that the rabbit polyclonal antibody described here specifically immunoprecipitates SUmoesin. If ezrin or other ERM members exist in the sea urchin, then these do not seem to associate heterotypically with moesin: an interaction that

![Fig. 9. Moesin distribution is disrupted in the presence of microfilament inhibitors. Three stages of sea urchin embryogenesis were analyzed by indirect immunofluorescence and confocal microscopy after incubation in colchicine (100 µm, A,D,G) or cytochalasin (10 µm, B,E,H) inhibitors. The stages correspond to 10 minutes after fertilization (A,B,C), morula stage (D,E,F) and hatched blastula (G,H,I). Fertilized eggs were incubated for 10 minutes in cytochalasin B or colchicine and recovered for 5 minutes (C). Other embryos were incubated in ASW for 30 minutes after being washed from the cytochalasin B after 1 hour of treatment (F,I).]
occurs in human cell lines (Gary and Bretscher, 1993). The faint band seen at 78 kDa in the in vivo translation experiment could represent a heterotypic association with another ERM member, but this protein is not recognized by the SUmoein antibody. We conclude that our description of SUmoein during sea urchin embryogenesis follows the distribution of a single protein in the ERM family.

The relocation of SUmoein, from punctate structures in the cytoplasm to discrete regions of localization at the plasma membrane later in development, is reminiscent of the changes in ezrin distribution during chicken erythrocyte differentiation (Birgbauer and Solomon, 1989). This movement may represent an association of the protein with vesicles that are trafficked to the plasma membrane. Ezrin, for example, is known to be recruited from tubulovesicles to the apical plasma membrane in gastric parietal cells, and this recruitment is concurrent with a transient phosphorylation of ezrin (Hanzel et al., 1991). SUmoein could be free in the egg, or associated with a class of vesicles that is recruited to the cell surface after fertilization (McCly et al., 1990). The location of SUmoein in the egg is distinct from the pool of G-actin, and seems to correspond to a region internal to the egg cortex described as Z3 (Bonder et al., 1989).

Later in development, SUmoein is located apically in cells in the region of cell-cell junctions. We asked how that apical polarity was maintained. Embryos that were incubated in colchicine and cytochalasin B remained viable, but after one hour formed binucleate cells because of incomplete cytokinesis. Cytochalasin B clearly disrupts the apical, plasma membrane-associated appearance of moesin, whereas microtubule inhibitors have no effect. These experiments were all reversible, indicating that moesin distribution recovers when actin polymerization can proceed. The kinetics of cytochalasin B and D are similar at 10 µM that was used in this study (Cooper, 1987). This reversibility argues that cytochalasin is not toxic to the cell(s), although an effect on pre-established actin filaments must also be considered. Finally, it is possible that the plasma membrane may be disrupted and internalized reversibly in response to cytochalasin B. Our observations are consistent with a role for polymerized actin in the establishment of polarity and the maintenance of SUmoein at the apical ends of cells. These data do not reveal whether moesin interacts directly with actin or is influenced indirectly by the actin cytoskeleton to assume its apical location in the cortex of cells.

At least three different patterns of SUmoein distribution were observed during development: (i) the actin-rich cell cortex of fertilized eggs; (ii) the apical, plasma membrane-associated distribution as soon as cells become polarized; and (iii) in the apical regions of cell-cell junctions in later development. These diverse patterns, and their reversible disruption by cytochalasin, suggest that SUmoein may be involved dynamically in the organization of the these structures. This role for SUmoein would be distinct from those of actin bundling or actin organizing proteins, and would place SUmoein in a position more proximate to the plasma membrane. Whether another molecule, perhaps associated with membranes, associates with SUmoein and the cytoskeleton after fertilization remains to be shown in the sea urchin. A recent study established a link between moesin and CD44 (Tsukita et al., 1994).

These data that show a potential link between the formation of the actin-based cytoskeleton and SUmoein suggest that SUmoein may be constitutively involved in interactions between the plasma membrane and the cytoskeleton. This subcellular structure remains to be identified. The function(s) of SUmoein in the during embryogenesis may be regulated by SUmoein itself or by the group of proteins that SUmoein can interact with. We are investigating the possibility that SUmoein is regulated by phosphorylation during development.

Whether there exist(s) one or several ERM family members in the sea urchin will be of interest with regard to the evolution of the ERM family that now includes the tumor suppressor, Merlin. At least one gene duplication event may have occurred during evolution, according to one report on human radixin (Wilgenbus et al., 1993). If the sea urchin retains a smaller complement of ERM genes than there are in mammals, then the analysis of ERM function(s) will be simplified. We are investigating the possibility that other ERM genes exist in the sea urchin. If they do exist, they are unlike some of their vertebrate counterparts because they do not cross-react immunologically. It is also possible that SUmoein may fulfill the role of two proteins in mammals. The distribution of moesin in the sea urchin during development is similar to that of two different ERM members. Mammalian ezrin and moesin are found in microvilli, and membrane ruffles, and colocalize with actin to the cell cortex (Franck et al., 1993). SUmoein is confined in later development to the apical surface and in regions of cell-cell junctions, which is reminiscent of radixin (Tsukita et al., 1989). These regions of the embryo are also rich in actin filaments.

We are currently testing the hypothesis that moesin is associated with membrane and/or cytoskeletal proteins. The availability of an embryonic system with a predictable temporal pattern of moesin localization should provide an excellent system for this analysis.

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REFERENCES


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