Moesin contributes an essential structural role in Drosophila photoreceptor morphogenesis

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Summary

Ezrin-Radixin-Moesin (ERM) family proteins organize heterogeneous sub-plasma membrane protein scaffolds that shape membranes and their physiology. In Drosophila oocytes and imaginal discs, epithelial organization, fundamental to development and physiology, is devastated by the loss of Moesin. Here, we show that Moesin is crucial for Drosophila photoreceptor morphogenesis. Beyond its requirement for retinal epithelium integrity, Moesin is essential for the proper assembly of the apical membrane skeleton that builds the photosensitive membrane, the rhabdomere. Moesin localizes to the rhabdomere base, a dynamic locus of cytoskeletal reorganization and membrane traffic. Downregulation of Moesin through RNAi or genetic loss of function profoundly disrupts the membrane cytoskeleton and apical membrane organization. We find normal levels and distribution of Moesin in photoreceptors of a Moesin mutant previously regarded as protein null, suggesting alternative interpretations for studies using this allele. Our results show an essential structural role for Moesin in photoreceptor morphology.

Supplemental data available online

Key words: Moesin, ERM, Morphogenesis, Photoreceptor, Cytoskeleton, F-actin, Drosophila melanogaster

Introduction

ERM proteins, dynamic, regulated membrane-cytoskeleton linkers essential to apical membrane traffic and regulation (Bretscher et al., 2002; Reczek and Bretscher, 2001), are attractive candidates to coordinate pathways of cellular morphogenesis. Protein family members contain a conserved N-terminal FERM (4.1-Ezrin-Radixin-Moesin) domain that binds cytoplasmic domains of transmembrane proteins directly or indirectly through coupling proteins and a C-terminal F-actin binding domain. ERM proteins are present in cytoplasm as dormant, non-phosphorylated monomers, and are recruited to plasma membranes and activated there by phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)]-binding and phosphorylation of a C-terminal threonine; upon phosphorylation, binding sites masked by intramolecular association are exposed (Bretscher et al., 1997; Pearson et al., 2000).

Initially identified as a component of the intestinal brush border membrane cytoskeleton, ERM proteins are broadly associated with actin-rich membrane projections, including microvilli and lamellipodia (Berryman et al., 1993). A role for ERM proteins in actin-based morphogenesis is supported by in vitro observations: downregulation of ERM proteins in cultured cells disrupts membrane protrusions (Amieva et al., 1999), and expression of a constitutively active ERM protein produces a profusion of microvilli (Oshiro et al., 1998). In addition to demonstrated structural roles, ERM proteins localize important signalling regulators, including EBP50/NHERF, a regulatory subunit of the sodium-proton pump RhoGDI and others (Hamada et al., 2001; Takahashi et al., 1997; Short et al., 1998).

The single Drosophila ERM gene, Moesin (McCartney and Fechon, 1996), facilitates genetic studies, and Moesin loss in flies results in developmental defects including an inability to correctly localize maternal determinants during oocyte development (Jankovics et al., 2002; Polesello et al., 2002), cytoskeletal abnormalities (Polesello et al., 2002) and a breakdown of epithelial integrity (Speck et al., 2003). Genetic interactions, notably increased survival of Moe mutant flies in a Rho-reduced background, have suggested Moesin facilitates epithelial morphology by antagonizing Rho activity rather than contributing a structural role (Speck et al., 2003). The role of ERM proteins in the orchestration of complex terminal differentiation is largely unexplored.

Photoreceptor sensory organelles, the outer segments of vertebrate rod and cone photoreceptors and the rhabdomeres of arthropods, are extravagant products of a common program of epithelial differentiation: apical membrane expansion and specialization. During photoreceptor terminal differentiation, directed membrane traffic enormously amplifies a central domain of the apical membrane of the cell, and the cytoskeleton folds it into a compact stack rich in Rhodopsin and associated proteins of the phototransduction cascade. Moesin knockout mice reveal fewer defects (Doi et al., 1999); however, strong interpretation of this result is complicated by redundancy with remaining family members Ezrin and Radixin. In Drosophila, loss of epithelial integrity in Moesin mutants confounds investigation of the role of the protein in the epithelium-derived retina; mechanical integrity of the retinal epithelium is a...
Materials and methods

Fly strains and genetics

w^{118} was used as wild type. Flies were raised on a standard cornmeal diet at 20°C. Pupae were developmentally staged by the collection of white prepupae (0% pupal development). From white prepupae to the desired time in development, and then divided this number of hours by 172 to give the % pd. To generate somatic prepupae stage to the desired time in development, and then dividing by 172 to give the % pd. To generate somatic Moe allele, UAS Moesin GFP was first recombined with Act5C>Gal4 and maintained over the balancer. Male over the SM1 examined. Hemizygous and homozygous Moe G0323 recombination was used by crossing Moesin GFP was first recombined with y, w, Moe X5 , FRT101/FM0 P{ActGFP}; Rho1 72R /CyO, P{ActGFP} females (F. Payre, Centre de Biologie du Developement, France) to ovo D1 , FRT101; hs-FLP (Brand and Perrimon, 1993). Generated with P-element-mediated germline transformation. All Independent lines, on the second or third chromosome, were separated by 4 hours. To rescue lethality of the Moe allele, UAS Moesin GFP was first recombined with Act5C>Gal4 and maintained over the SM1 balancer. Male Act5C>Gal4, UAS Moesin GFP/SM1 flies were then crossed to virgin y, w, MoePL54; FRT101/FM0 flies, and y, w, MoePL54; Act5C>Gal4, UAS Moesin GFP/+; FRT101/FM0 females were examined. Hemizygous and homozygous MoeG0323 adults in a reduced Rho background were generated by crossing MoeG0323/FM7i, P[ActGFP]; RhoI-929/CyO, P[ActGFP] females to MoeG0323/ Dp(1;Y)619, B males (R. Fehon, Duke University).

Molecular biology

Two different constructs encoding Moesin inverted repeats (IR-Moesin) were generated by amplifying the Moesin coding sequence (GenBank Accession Number L38909) from positions 327-775 bp and 818-1236 bp by polymerase chain reaction (PCR), using primers that introduced unique restriction sites at the product ends. Each of the DNA fragments were subcloned, in opposite orientations, into AvrII and Nhel sites of the pWIZ plasmid (a gift from R. Carthew, Northwestern University) to produce perfect hairpin RNA loops following splicing under the UAS/Gal4 promoter. A puLV-derided vectors UAS IR-Moe [327-775] and UAS IR-Moe [818-1236] were introduced into the germline by P-element-mediated transformation. Independent transformant lines were established and mapped. Several different UAS IR-Moe [327-775] and UAS IR-Moe [818-1236] strains were tested for double-stranded RNA interference (RNAi) activity by crossing to Gal4 drivers and immunostaining with anti-Moesin antibody.

The full-length UAS Moesin Myc, UAS Moesin GFP and phosphomimetic UAS T559D Moesin Myc transgenic lines were generated by first amplifying the full-length Moesin cDNA by PCR to introduce unique restriction sites at the product ends and then ligating it into pBlueScript (Stratagene, La Jolla, CA). A synthetic mutation primer, CGTGAACAGTACAAAGATCTCCGCAGAGTCCCAGGAGG, was used to mutate Thr559 to Asp and silently introduce a BgIII site. The Myc epitope tag or green fluorescent protein (GFP) was introduced in-frame at the C terminus of Moesin by ligation of a 6-Myc repeat from pCS2+MT (Rupp et al., 1994) or GFP from pBD1010 (B. Dickson, Research Institute of Molecular Pathology). Each sequence was confirmed and subcloned into pUAST. Independent lines, on the second or third chromosome, were generated with P-element-mediated germline transformation. All transgenic lines were expressed with Gal4/UAS-targeted expression (Brand and Perrimon, 1993).

Temperature shifts

Pupae were maintained at 20°C during non-hea shock conditions. A thermocycler was used to conduct a series of heat shocks at 37°C for 45 minutes each. Between the temperature shifts to 37°C, the flies rested at 20°C for 3 hours and 15 minutes. UAS Moesin GFP/+; hsGal4/+; UAS Moesin Myc/+; hsGal4/+ and UAS IR-Moesin/+; hsGal4/+ pupae received two heat shocks. UAS T559D Moesin Myc/+; hsGal4/+ pupae received five heat shocks. After the final heat shock, flies recovered at 20°C for 6-8 hours before dissection.

Immunolocalization and western analysis

Immunostaining and F-actin localization were performed with whole-mount preparations as described (Fan and Ready, 1997), with the following exceptions noted. After dissection, the retina was immediately immersed in PLP fixative (10 mM periodate, 75 mM lysine, 2% paraformaldehyde in 1x PBS) with 0.05% saponin for 20 minutes. Eyes were washed in 10 minutes, three times with 50 mM NH4Cl in PBS. Moesin was visualized using anti-Moesin antisera at 1:5000 dilution (D. Keihart, Duke University). For activated Moesin (p-Moesin) visualization, a rabbit antibody against the Thr phosphorylated Moesin peptide (C-R553-D-K-F-phosphotyrosine) was generated (Bethyl, Montgomery, TX) and used at 1:500. To immunolocalize dominant-active Moesin, Myc antibody 9E10 was used at 1:50. Retinas were incubated overnight in 1:300 diluted primary for Crumbs (U. Temass, University of Toronto) visualization. All whole mounts were examined with a Bio-Rad MRC-1024 confocal microscope using a 60x objective lens. Images were processed using Adobe Photoshop 7.0. Western analysis of 1-day-old adult whole head extract was performed as previously described (Satoh et al., 1997), with the following modifications: anti-Moesin antisera was used at 1:20,000 dilution. Transmission electron microscopy was carried out as described (Fan and Ready, 1997).

Results

Crumbs and Moesin define photoreceptor apical membranes

We used confocal immunofluorescence to observe the membrane cytoskeleton during photoreceptor differentiation (Fig. 1). Adult Drosophila photoreceptor apical plasma membranes are organized into complementary domains: the photosensory rhabdomere and an adjacent supporting membrane, the stalk (Fig. 1A). Rhabdomeres are columns of closely packed, photosensitive microvilli; a collar of stiffened membrane, the stalk, flanks rhabdomeres and supports them on the optical axis of the eye. Prominent coated pits and vesicles in the stalk pocket (Fig. 1A, inset) suggest a special role for the stalk in endocytosis. Molecular specialization of the apical membrane cytoskeleton supports rhabdomeres: microfilaments thread each microvillus (Arikawa et al., 1990), and a terminal web of F-actin extends from the rhabdomere base into the cytoplasm (Chang and Ready, 2000). Activated, phosphorylated Moesin (p-Moesin) localizes to the rhabdomere base and the polarity determinant transmembrane protein Crumbs localizes to the stalk (Fig. 1B) (Izaddoost et al., 2002; Pellikka et al., 2002).

Definitive rhabdomere and stalk primordia are established within the photoreceptor apical membrane at approximately 50% of pupal development (% pd) (Fig. 1C). Future rhabdomeres are covered with short, finger-like microvilli. Smooth membrane defines developing stalks of R2, R4 and R7, and the asymmetrical stalk of R5: R1, R3 and R6 lack stalks at this stage. Concomitant with morphological differentiation,
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Moesin and Crumbs resolve to their respective adult domains (Fig. 1D). The complementary distribution of these determinants of membrane/cytoskeleton interaction suggests that photoreceptor Moesin does not partner with Crumbs, as reported for embryonic epithelia (Medina et al., 2002). Before this stage, Moesin (see Fig. S1 at http://dev.biologists.org/supplemental/) and Crumbs extend fully across the apical surface. The absence of stalks and the lack of Crumbs staining in photoreceptors R1, R3 and R6 suggests Moesin does not require Crumbs to establish the rhabdomere base.

In order to visualize both active phosphorylated and dormant non-phosphorylated Moesin, we used Gal4/UAS-targeted expression of full-length wild-type Myc- and GFP-tagged Moesin. In fixed (Fig. 2A) and live cells (Fig. 2B), both tagged proteins distribute throughout the cytoplasm (Fig. 2A; asterisks) and concentrate at the apical membrane. Cytoplasmic staining probably represents the soluble, dormant Moesin.

To test whether the GFP-tagged Moesin is functionally active, we introduced the transgene into Moe PL54 (Polesello et al., 2002), a homozygous lethal Moe- background. Constitutive expression of Moesin-GFP using the Act5C>Gal4 promoter rescues Moe PL54 viability. Ommatidia examined with confocal microscopy (Fig. 2C) and electron microscopy (Fig. 2D) frequently exhibit rhabdomere size and organization defects, including microvillar gaps. Moesin dosage may play a crucial role in the proper organization of the apical surface.

Moesin RNAi disorganizes photoreceptor apical membrane/cytoskeleton

As epithelial organization, which defines the context of photoreceptor differentiation, fails in flies lacking Moesin (Speck et al., 2003), we expressed IR-Moesin, a transgene carrying an inverted repeat sequence encoding a portion of Moesin to induce RNAi and downregulate Moesin late in eye development. Epithelial integrity is preserved in these eyes, permitting dissection of the role of Moesin in photoreceptor differentiation. The efficiency of UAS IR-Moesin downregulation of Moesin expression was assayed using immunolocalization. Using the heat shock>Gal4 driver
to drive the expression of UAS IR-Moesin in staged pupae, we found photoreceptor morphogenesis to be most sensitive to Moesin loss at the time of rhabdomere/stalk resolution, approximately 50% pd. At this stage, hs>IR-Moesin expression completely abolished Moesin immunodetection in many photoreceptors (Fig. 3A; arrowheads). The membrane cytoskeleton, assayed by F-actin staining, is disorganized in photoreceptors lacking Moesin. Rhabdomeres of these eyes typically have reduced, irregular microvillar fields (Fig. 3B).

Expression of UAS IR-Moesin using the GMR>Gal4 driver, which becomes active soon after photoreceptors are fated in the retinal epithelium, severely disrupts actin cytoskeleton organization and apical differentiation (Fig. 3C,D). Distinct microvillar and stalk membranes are apparent, but are discontinuous and jumbled. It remains to be determined whether higher levels of IR-Moesin, or its expression during an especially sensitive period, account for the stronger phenotype of GMR>IR-Moesin eyes. Downregulation of Moesin during photoreceptor differentiation focuses defects on the rhabdomere; cells appear otherwise healthy and possess apparently normal adherens junctions (Fig. 3B,D; arrowheads), probably accounting for the preservation of epithelial integrity. Once established, adherens junctions may not require Moesin to maintain epithelial integrity; alternatively, severely reduced levels may be sufficient.

Genetic loss of Moesin likewise disrupts rhabdomere morphogenesis

As an alternate strategy to remove zygotic Moesin later in development, we generated eye clones homozygous for either of two Moesin alleles, MoePL54 and MoeX5, shown to be null or strong hypomorphs (Polesello et al., 2002). In pupal MoePL54 mosaic eyes, containing a mixture of Moesin-positive cells and cells with severely reduced Moesin, rhabdomeres of photoreceptors with immuno-undetectable Moesin show severely disrupted apical membranes (Fig. 4A; arrowheads). Assayed with rhodamine-phalloidin, the rhabdomere microvillar array in Moe− cells is irregular and the RTW is replaced with abnormal F-actin accumulations. In MoeX5 mosaic eyes, cells lacking Moesin are rarely observed, but ommatidia containing reduced numbers of photoreceptors are common, suggesting that the missing cells died or were otherwise lost from the developing retinal epithelium (Fig. 4B; asterisks).

Notably, a third hypomorphic allele, MoeG0323, in a Rho-reduced background, which was previously shown to lack Moesin during larval development (Speck et al., 2003), contains normal levels of Moesin at the rhabdomere base (Fig. 4C,D; arrowheads). Both hemizygous MoeG0323/Y; Rho172R/CyO and homozygous MoeG0323/MoeG0323; Rho172R/CyO adult retinas show normal photoreceptors and Moesin localization to the rhabdomere base. Western analysis
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of whole head extracts of these flies shows that Moesin is present, but at a reduced level (see Fig. S2 at http://dev.biologists.org/supplemental/).

Dominant-active Moesin inhibits apical membrane differentiation

To examine the role of Moesin activation in photoreceptor morphogenesis, we generated a transgenic line expressing a constitutively active phosphomimetic mutation, UAS T559D Moesin Myc (Oshiro et al., 1998), under Gal4/UAS control. When hs>T559D Moesin Myc is expressed during apical morphogenesis, a profusion of irregular microvilli dominates the apical surface (Fig. 5B). This result parallels observations in cell culture (Gautreau et al., 2000; Oshiro et al., 1998), where expression of T559D Moesin results in hyper-formation of irregular microvilli. Other cellular structures, including the adherens junctions (Fig. 5B; arrows), appear to be intact. Using confocal immunofluorescence, T559D Moesin Myc localizes to the entire photoreceptor plasma membrane and concentrates at the apical membrane (Fig. 5D). The failure of basolateral Moesin to generate microvilli suggests that additional, apically-limited proteins are needed to initiate rhabdomere microvilli. T559D Moesin severely disrupts the actin cytoskeleton (Fig. 5E), and Crumbs, which should resolve to the stalk, remains distributed across the entire apical surface (Fig. 5C). Failure of Crumbs to re-localize is a harbinger of the loss of stalk/rhabdomere distinction in photoreceptors expressing dominant-active Moesin. We speculate that dynamic turnover and regulation of the protein is important for morphogenesis, and the unregulated binding of dominant-active Moesin ‘freezes’ the apical membrane (Coscoy et al., 2002), compromising its reorganization.

Discussion

Epithelial cell apical plasma membranes host elaborate membrane and sub-membrane protein assemblies that support sensory and effector mechanisms and are often amplified and highly structured by the actin membrane cytoskeleton. Substantial in vitro and in vivo evidence implicates Moesin in

Fig. 3. Moesin RNAi disorganizes F-actin and apical microvilli. (A, C) Confocal micrographs show F-actin staining (red) and Moesin immunolocalization (green). (A) In a 57% pd hs>IR-Moesin retina, photoreceptors lacking Moesin (arrowheads) are reduced in size and exhibit abnormal F-actin distribution. The asterisked ommatidium (enlarged, right) shows lack of Moesin in R1, R3 and R7. (B) An electron micrograph of 57% pd hs>IR-Moesin ommatidium shows loosely organized rhabdomere microvilli (compare with Fig. 5A); the rhabdomere of R5 is reduced. (C) GMR>IR-Moesin photoreceptor apical membrane organization is devastated by Moesin loss. A single photoreceptor (arrowhead) retains strong Moesin signal and an intact rhabdomere. (D) Electron micrograph of 70% pd GMR>IR-Moesin ommatidium shows severe rhabdomere defects. The asterisked photoreceptor lacks a rhabdomere. Adherens junctions appear normal (B,D; arrows). Scale bars: 10 μm in A,C; 1 μm in B,D.
the genesis and regulation of this membrane cytoskeleton, and our results extend this connection to a role for Moesin in the morphogenesis of a complex, compartmental apical specialization, the *Drosophila* rhabdomere. The dynamic distribution of Moesin during photoreceptor development and the impact of Moesin gain-and loss-of-function during morphogenesis suggest an essential role for the protein in organizing the membrane skeleton that supports the microvillar array of the rhabdomere.

The developmental cues that restrict Moesin to the rhabdomere primordium at the onset of overt morphogenesis are not known. At this stage, photoreceptor apices contact each other in a stereotyped pattern of apical cell-cell contacts within a trapped apical pocket that will later open to form the IRS, suggesting that apical contacts may localize future rhabdomeres. In other systems, ERM proteins are recruited to plasma membranes and activated there by PtdIns(4,5)P₂-binding and phosphorylation of a C-terminal threonine (Hirao et al., 1996; Matsui et al., 1998). Rhabdomeres are rich in PtdIns(4,5)P₂ and Rhodopsin-activated cleavage of PtdIns(4,5)P₂ by the phospholipase C NorpA is the substrate for *Drosophila* phototransduction (Hardie et al., 2001). A Moesin-organized nexus of phosphoinositide and actin organizing proteins at the rhabdomere base may represent an economical dual use of the system for structure and physiology.

The restriction of Moesin to the rhabdomere base and its loss-of-function phenotypes suggests that Moesin links microvillar cytoplasmic ends to the underlying actin cytoskeleton, the rhabdomere terminal web (RTW). The full molecular makeup of the Moesin-organized photoreceptor cytoskeleton and the nature of the forces it organizes during morphogenesis remain to be determined, but the catenary-like deformation of the rhabdomere base as developing microvilli elongate suggests the membrane cytoskeleton contributes a sub-apical constraint, a tensile sheet that contains the expanding photosensitive membrane in an orderly microvillar stack. In other systems, Moesin-organized protein scaffolds recruit potent regulators of membrane architecture and function, such as the small GTPase regulators RhoGDI (Hamada et al., 2001), RabGAPs and RhoGAPs (Reczek and Bretscher, 2001). It is plausible that the ability of Moesin to bind F-actin and its regulators may contribute to the establishment of the RTW. Rhabdomere membrane is in dynamic exchange across the rhabdomere base with endosomes of the photoreceptor cytoplasm (Bahner et al., 2002), and it is notable that ERM proteins bind to regulators of membrane recycling (Rochdi and Parent, 2003). Moesin may participate in the membrane exchange that supports a dynamic sensory membrane.

Recent observation that the lethality of flies homozygous for...
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MoeG0323, considered a protein null, could be rescued in Rho-reduced flies was interpreted as showing that Moesin facilitates epithelial morphology not by providing an essential structural function, but rather by antagonizing activity of the small GTPase Rho (Speck et al., 2003). This conclusion rests on MoeG0323 being a protein null. Our observations that Rho-reduced MoeG0323 produces Moesin detectable by western analysis and shows photoreceptor Moesin immunostaining indistinguishable from wild type suggest alternate interpretations. One possibility is that the P-element insertion in the 5' untranslated region of MoeG0323 downregulates embryonic and larval Moesin, but can be over-ridden later in development in Rho-reduced animals. Such interpretation, while not ruling out a role for Moesin in Rho regulation, is compatible with present and previous evidence for a structural role for Moesin.

Given the fundamental parallels between Drosophila and vertebrate photoreceptors, it is interesting to consider whether the ERM-organized membrane cytoskeleton will play a similar role in human photoreceptor development and disease.

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Note added in proof

Observations using photoreceptors from the leopard frog Rana implicate Moesin in rod outer segment biogenesis (Deretic et al., 2004).

References


Fig. 5. Dominant-active T559D Moesin expression prevents photoreceptor apical regionalization. (A,B) Electron micrographs of 65% pd wild type (A) and hs>T559D Moesin Myc (B) ommatidium. The apical membrane, spanning between adherens junctions (A,B; arrows), is severely disorganized by T559D Moesin expression (B). In this ommatidium (B), R5 is less affected, showing clear rhabdomere and stalk domains, presumably due to lower levels of T559D Moesin expression. (C,D) The apical actin cytoskeleton is severely disrupted in dominant-active Moesin-expressing cells, and Crumbs (C) does not properly re-localize to the stalk. (D) The field in C, showing Crumbs staining (blue) merged with staining for F-actin (red) and Myc (green). Red rhabdomeres of 'escaper' cells, not expressing T559D Moesin Myc, appear normal. (E) The ommatidium marked with an asterisk in D is shown at a higher magnification. T559D Moesin was not expressed equally in all cells in this ommatidium, as reflected by the lack of Myc staining in the asterisked R7. F-actin in the rhabdomere of R7 is orderly, and Crumbs was properly cleared from the rhabdomere membrane and localizes to the stalk. Compare with neighboring photoreceptors in D. Scale bars: 1 μm in A,B; 10 μm in D.


