Myosin-1c Couples Assembling Actin to Membranes to Drive Compensatory Endocytosis

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Summary

Compensatory endocytosis follows regulated exocytosis in cells ranging from eggs to neurons, but the means by which it is accomplished are unclear. In Xenopus eggs, compensatory endocytosis is driven by dynamic coats of assembling actin that surround and compress exocytosing cortical granules (CGs). We have identified Xenopus laevis myosin-1c (XMyo1c) as a myosin that is upregulated by polyadenylation during meiotic maturation, the developmental interval that prepares eggs for fertilization and regulated CG exocytosis. Upon calcium-induced exocytosis, XMyo1c is recruited to exocytosing CG membranes where actin coats then assemble. When XMyo1c function is disrupted, actin coats assemble, but dynamic actin filaments are uncoupled from the exocytosing CG membranes such that coats do not compress, and compensatory endocytosis fails. Remarkably, there is also an increase in polymerized actin at membranes throughout the cell. We conclude that XMyo1c couples polymerizing actin to membranes and so mediates force production during compensatory endocytosis.

Introduction

Dynamic interactions between the actin cytoskeleton and cellular membranes are required for essential processes including endo- and exocytosis, cell locomotion, cell division, and vesicle motility. Force production is inherent to these processes, and intense effort has been expended to identify the means by which the actin cytoskeleton exerts force on membrane compartments. Two general mechanisms have been described: force exerted on membranes by actin assembly, exemplified by actin polymerization at the plasma membrane (PM) of the leading edge of crawling cells (Welch and Mullins, 2002), and force exerted via myosin-based translocation of actin-associated membranes, exemplified by myosin-2-powered PM ingression during cytokinesis (Yumura and Uyeda, 2003).

The unconventional myosins (classes 1 and 3–18) have attracted considerable attention as potential motors for actin-dependent force production on membranes because these motors typically bind membranes in vitro and associate with membrane compartments in vivo (Sokac and Bement, 2000). Furthermore, disrupting the function of specific unconventional myosins can alter the distribution or motility of their associated membrane compartments (e.g., Doberstein et al., 1993).

Myosins-1 are the most abundant and diverse class of unconventional myosins. In vertebrates, they are represented by four distinct subclasses (Coluccio, 1997) and have been implicated in endocytosis (Soldati, 2003) and exocytosis (Bose et al., 2002). But how myosins-1 contribute to these processes in vertebrates is unknown. While the textbook model of unconventional myosins as membrane transport motors predicts that they carry endosomes and secretory compartments along actin filaments (F-actin), recent work in budding yeast suggests that myosins-1 can participate in endocytosis both by promoting actin assembly and by transport of F-actin (Sun et al., 2006).

To understand the roles played by myosins-1 in vertebrate cells, we have analyzed their expression and function during activation of Xenopus laevis eggs. Egg activation in Xenopus and other vertebrate and invertebrate species is triggered at fertilization by an increase in intracellular free calcium ([Ca2+]i) and entails a series of rapid, essential events that depend on F-actin/membrane interactions. These include cortical contraction, required for pronuclear fusion (Elinson, 1977), cortical granule (CG) exocytosis, required for the slow block to polyspermy (Wolf, 1974), assembly of dynamic actin coats around exocytosing CGs, required for compensatory endocytosis (Sokac et al., 2003), and assembly of actin comets (Taunton et al., 2000). The Xenopus system is ideal for analysis of myosin function during egg activation in that the events of fertilization can be mimicked by artificial elevation of [Ca2+]i, exogenous constructs can be expressed by microinjection of mRNA, and four-dimensional (4D) imaging permits detailed analysis of cytoskeletal dynamics with exceptional clarity (Sokac et al., 2003).

Using an expression screen, we identify Xenopus Myosin-1c (XMyo1c; formerly known as myosin-1b or myr 2) as a myosin specifically upregulated by polyadenylation during meiotic maturation, the developmental interval that prepares oocytes for fertilization. We show that XMyo1c is rapidly recruited to exocytosing CGs and that disruption of XMyo1c function perturbs several events of egg activation, including compensatory endocytosis. Surprisingly, the primary phenotype underlying these egg activation defects is misdirected actin assembly whereby dynamic actin is uncoupled from the appropriate membrane compartments. Thus, rather than acting as a transporter, XMyo1c instead links polymerizing actin to membranes such that actin assembly exerts force on the membranes.
Results

Identification and Expression of *Xenopus* Myosins

*Xenopus* oogenesis proceeds through six defined growth stages, followed by progesterone-triggered meiotic maturation to convert the stage 6 oocyte into the fertilizable egg, followed by calcium-dependent egg activation at fertilization. Oogenesis occurs over the course of months, meiotic maturation takes 3–12 hr, and activation commences within seconds of sperm-induced calcium elevation. To identify myosins that might participate in *Xenopus* egg activation, we screened by degenerate polymerase chain reaction (PCR) (Bement et al., 1994) and amplified small fragments of 12 different myosins, representing seven myosin classes (see Experimental Procedures). Six myosins (Myosin-1c, 1e, 2a, 3, 9b, and 10) were selected for amplification of larger fragments by using exact-match primers paired to downstream degenerate primers. The fragments thus obtained, as well as γ-actin, were used to probe poly(A)⁺ RNA blots of stage 1–6 oocytes as well as meiotically mature eggs. Most myosins and γ-actin showed high expression in early oogenesis (stages 1–3), decreasing expression up to stage 6, and a sharp decline during meiotic maturation (Figure 1A).

*XI*Myo1c Is Upregulated by Polyadenylation during Meiotic Maturation

This decrease presumably reflects the de-adenylation of most mRNAs during meiotic maturation (Liu and Smith, 1994). The sole exception to this pattern was *Xenopus* Myosin-1c (*XI*Myo1c): while the general decrease during oogenesis was observed, poly(A)⁺ levels increased during meiotic maturation such that eggs have more *XI*Myo1c poly(A)⁺ RNA than stage 6 oocytes (Figure 1A).

*XI*Myo1c is specifically upregulated by polyadenylation during meiotic maturation

(A) Poly(A)⁺ northern blots of six myosins and γ-actin from the six stages of oogenesis (1–6; “Oogenesis”) and the egg following meiotic maturation (E; “MM”). Levels of *XI*Myo1c Poly(A)⁺ RNA increase during meiotic maturation, while levels for all of the other myosins and γ-actin sharply decrease during the same interval (compare 6 to E). Equal amounts of RNA were loaded in each lane. (B) Poly(A)⁺ and total RNA before (6) and after (E) meiotic maturation for *XI*Myo1c, *XI*Myo2a, and γ-actin. Total RNA levels do not change for any message, while poly(A)⁺ levels drop sharply for *XI*Myo2a and γ-actin. In contrast, poly(A)⁺ levels increase for *XI*Myo1c. Thirty oocyte equivalents of RNA were loaded for each lane. (C) Immunoblot comparison of protein levels before (6) and after (E) meiotic maturation for *XI*Myo1c, *XI*Myo2a, and γ-actin. *XI*Myo1c levels increase during meiotic maturation, while those of *XI*Myo2a and γ-actin do not. (D) The complete coding sequence for *XI*Myo1c. The ATP-binding region (dark underline) and actin-binding region (dashed underline) are indicated, as are the three IQ motifs (dark underline, gray text). (E) Schematic diagram of *XI*Myo1c. (F) Alternative 3’UTRs for *XI*Myo1c. Both 3’UTRs include nuclear polyadenylation signals (PolyA) but only *XI*Myo1cA(A) has the cytoplasmic polyadenylation element (CPE) necessary to direct polyadenylation during meiotic maturation.

**Figure 1.** Poly(A)⁺ northern blots of six myosins and γ-actin from the six stages of oogenesis (1–6; “Oogenesis”) and the egg following meiotic maturation (E; “MM”). Levels of *XI*Myo1c Poly(A)⁺ RNA increase during meiotic maturation, while levels for all of the other myosins and γ-actin sharply decrease during the same interval (compare 6 to E). Equal amounts of RNA were loaded in each lane. (B) Poly(A)⁺ and total RNA before (6) and after (E) meiotic maturation for *XI*Myo1c, *XI*Myo2a, and γ-actin. Total RNA levels do not change for any message, while poly(A)⁺ levels drop sharply for *XI*Myo2a and γ-actin. In contrast, poly(A)⁺ levels increase for *XI*Myo1c. Thirty oocyte equivalents of RNA were loaded for each lane. (C) Immunoblot comparison of protein levels before (6) and after (E) meiotic maturation for *XI*Myo1c, *XI*Myo2a, and γ-actin. *XI*Myo1c levels increase during meiotic maturation, while those of *XI*Myo2a and γ-actin do not. (D) The complete coding sequence for *XI*Myo1c. The ATP-binding region (dark underline) and actin-binding region (dashed underline) are indicated, as are the three IQ motifs (dark underline, gray text). (E) Schematic diagram of *XI*Myo1c. (F) Alternative 3’UTRs for *XI*Myo1c. Both 3’UTRs include nuclear polyadenylation signals (PolyA) but only *XI*Myo1cA(A) has the cytoplasmic polyadenylation element (CPE) necessary to direct polyadenylation during meiotic maturation.
poly(A)^+ RNA levels increased, confirming that the latter is selectively polyadenylated. Immunoblotting showed that XlMyo1c protein levels concomitantly increased during meiotic maturation, but XlMyo2a and γ-actin levels did not (Figure 1C).

The complete XlMyo1c coding sequence was obtained by rapid amplification of cDNA ends (RACE) (Figure 1D). Its deduced primary structure predicts all of the expected features of a myosin-1c: an N-terminal “head” with the actin and ATP-binding motor domain (Reizes et al., 1994), a “neck” comprised of three IQ motifs that bind calmodulin (Zhu et al., 1998; Tang et al., 2002), and a highly basic (pl = 10.4) C-terminal “tail” that directs membrane interaction (Tang et al., 2002) (Figures 1D and 1E). In addition, two alternative 3′untranslated regions (UTRs) were identified, one of which contains a cytoplasmic polyadenylation element (Figure 1F) that could account for the polyadenylation of XlMyo1c during meiotic maturation (Gray and Wicksens, 1998).

**XlMyo1c Localizes to the PM and to Exocytosing CGs upon Egg Activation**

To characterize the subcellular distribution of XlMyo1c, an eGFP-XlMyo1c fusion was generated and expressed in oocytes and eggs. In both oocytes and eggs, confocal analysis of fixed samples showed that eGFP-XlMyo1c localizes to the PM, coincident with cortical F-actin. In regions far from the prick site, where exocytosis had just began upon fixation, CGs with eGFP-XlMyo1c were surrounded by both XlMyo1c and actin (Figure 2A). After calcium ionophore-induced activation, eGFP-XlMyo1c moved to some CGs, as revealed by eGFP-XlMyo1c on spherical compartments of ~1–3 μm diameter immediately beneath the PM (Figures 2A and 2B). These CGs were also encased by coats of F-actin (Sokac et al., 2003). Z views of prick-activated eGFP-activated indicated that XlMyo1c was recruited to exocytosing CGs before actin coats formed (Figure 2B). That is, pricking induces a wave of [Ca^{2+}], increase and, therefore, a wave of CG exocytosis that emanates from the prick site. In regions far from the prick site, where exocytosis had just begun upon fixation, CGs with XlMyo1c, but not actin, were observed. However, in regions close to the prick site, where the exocytosis stimulus had been present longer, CGs were surrounded by both XlMyo1c and actin. In intermediate areas, XlMyo1c surrounded CGs, while actin coats tended to only partially encase these compartments (Figure 2B).

To confirm the localization results obtained from fixed samples expressing exogenous XlMyo1c, two additional approaches were employed. First, the distribution of endogenous XlMyo1c was assessed in activated eggs with an anti-mammalian myosin-1c antibody (Wagner et al., 1992). Consistent with the above results, endogenous XlMyo1c localized to both the PM and exocytosing CGs (Figure 2C). Second, oocytes were fractionated to generate a cortical pellet comprised of the PM and CGs and a supernatant comprised of the cytoplasm and other membrane compartments (Gundersen et al., 2002). Immunoblotting showed that both exogenous and endogenous XlMyo1c associated with the cortical fraction, along with markers for the PM and CGs (Figure 2D).

EGFP-XlMyo1c distribution was next analyzed in living cells activated by photolyis of caged inositol-1,4,5-trisphosphate (IP_3). Briefly, eggs expressing eGFP-XlMyo1c were subjected to IP_3 uncaging in the presence of extracellular Texas red dextran (TR-dextran), which reveals CG exocytosis by filling the compartments created upon CG-PM fusion (Sokac et al., 2003). Time-lapse confocal imaging in a single optical plane revealed that eGFP-XlMyo1c is rapidly recruited to exocytosing CGs (within 0.15 s ± 0.17 s of dextran; mean ± SEM) (Figures 2E and 2F; see Movie S1), whereas actin is recruited later (Sokac et al., 2003; see also below). Exocytosing CGs surrounded by eGFP-XlMyo1c shrank over time, as observed in both en face views (Figure 2E) and z views (Figure 2F). To directly confirm that XlMyo1c is recruited to CGs before actin, localization of both eGFP-XlMyo1c and fluorescent actin were monitored simultaneously. Consistent with the above findings, XlMyo1c was invariably recruited before actin (Figure 2G). We conclude that in resting eggs XlMyo1c localizes to the PM, and upon egg activation XlMyo1c is rapidly recruited to exocytosing CGs before actin coats assemble.

**Contributions of XlMyo1c Domains to Localization**

Unconventional myosins are directed to their sites of localization via their tails (Sokac and Bement, 2000). Thus, isolated tail fragments have been widely used as dominant negatives for a variety of unconventional myosins including myosin-1c (Bose et al., 2002). We therefore generated a series of constructs encoding eGFP fused to either the head and IQ domains (HIQ), the IQ domains alone (IQ), the IQ domains and the tail (IQT), or the tail alone (T) and assayed their distribution in oocytes, eggs, and activated eggs (Figure 3A). In oocytes and eggs, eGFP-HIQ and eGFP-IQ were cytoplasmic, while eGFP-IQT and eGFP-T localized to the PM (data not shown). Following activation, eGFP-HIQ remained cytoplasmic, while eGFP-IQT and eGFP-T rapidly redistributed to some CGs, similar to full-length XlMyo1c (Figure 3B). Interestingly, eGFP-IQ was also recruited to CG compartments after activation, although the high cytoplasmic signal made this recruitment harder to discern than that of eGFP-IQT or eGFP-T (Figure 3B). Thus, the tail is sufficient to direct the localization of XlMyo1c, but the IQ domains also target to exocytosing CGs.

**Disruption of XlMyo1c Perturbs Cortical Contraction, CG Exocytosis, Actin Coat Compression, and Results in Unrestrained Actin Assembly**

Based on the above results, the IQT construct was employed to disrupt XlMyo1c function. Coexpression of IQT with GFP-HIQ displaced the latter from the PM in a concentration-dependent manner (Figures 4A and 4B), as expected if this construct competes with full-length XlMyo1c for membrane binding sites. To assess the effects of IQT on the events of egg activation, oocytes were allowed to express IQT overnight, while undergoing progesterone-induced meiotic maturation. IQT had no apparent effect on meiotic maturation relative to either un.injected or HIQT-injected controls (Figures 4C and 4D), suggesting that IQT did not grossly perturb the cortical F-actin cytoskeleton, which is required for proper meiotic maturation (Gard et al., 1995). IQT did result in specific changes in cortical F-actin, however, in that oocytes expressing IQT consistently had longer

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**Results in Unrestrained Actin Assembly**

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microvilli than either uninjected or HIQT-injected controls (Figure 4E).

IQT expression induced two phenotypes that were not evident until eggs were artificially activated with calcium ionophore. First, cortical contraction was strongly suppressed (Figures 4F and 4G). This effect was concentration dependent and could be rescued by coexpression of HIQT, demonstrating its specificity. Second, fertilization envelope elevation, a marker for CG exocytosis, was slower in eggs expressing IQT (Figure 4H).
Again, this phenotype was both concentration dependent and rescued by HIQT coexpression.

To then examine the effects of IQT on CG exocytosis as well as constitutive exocytosis, a biochemical approach was taken (Gundersen et al., 2002). Two-day expression of IQT completely suppressed CG exocytosis in oocytes and eggs challenged with phorbol 12-myristate 13-acetate (PMA) and eggs challenged with ionophore (Figure 4I). Complete suppression of exocytosis following 2 day expression of IQT was not rescuable by HIQT coexpression, possibly because of the increased efficiency of IQT translation relative to the larger HIQT (data not shown). Nevertheless, this phenotype was specific, in that 2 day expression of IQT had no effect on constitutive exocytosis of either the PM Na\(^+\)-K\(^+\) ATPase or syntaxin-1a (Figure 4J). Since constitutive exocytosis in Xenopus oocytes is actin dependent (e.g., Colman et al., 1981), these results again demonstrate that IQT expression is neither simply toxic nor causing wholesale disruption of the actin cytoskeleton.

In fact, we found that IQT expression actually promoted actin assembly. That is, treatment of Xenopus eggs with PMA results in small endosome propulsion via actin "comets," structures comprised of rapidly polymerizing actin (Taunton et al., 2000). IQT increased the number of comets that formed in response to PMA treatment by more than 10-fold relative to uninjected and HIQT-injected controls (Figure 5A). Since constitutive exocytosis in Xenopus oocytes is actin dependent (e.g., Colman et al., 1981), these results again demonstrate that IQT expression is neither simply toxic nor causing wholesale disruption of the actin cytoskeleton.

To provide independent support for the effects of IQT on actin assembly, cortical contraction, and CG exocytosis, antisense morpholinos were employed (Heasman et al., 2000). A morpholino complementary to the 5’UTR of XlMyo1c resulted in a 59% reduction in XlMyo1c protein relative to a five base mismatch control morpholino (Figure 6A). As with IQT expression, the XlMyo1c morpholino suppressed cortical contraction (Figure 6B), increased the number of actin comets observed following PMA treatment (Figure 6C), and suppressed CG exocytosis (Figure 6D).

The microvilli and comet phenotypes, as well as the increased cortical actin levels, suggest that actin assembly goes unrestrained when XlMyo1c is disrupted. This ectopic actin may perturb some events of egg activation, and if so, then inhibiting actin assembly should rescue the XlMyo1c phenotypes. Obviously, this approach cannot be used for comet formation, cortical contraction, or actin coat assembly since these are all critically dependent on F-actin. However, it can be used to test CG exocytosis, which is actin independent in Xenopus oocytes and eggs (Sokac et al., 2003). Indeed, the effects of IQT on CG exocytosis were rescued by preincubating cells with cytochalasin D (Figure 6E). Thus, unrestrained actin assembly is likely a primary cause of the egg-activation defects observed following XlMyo1c disruption.

XlMyo1c Expression Uncouples Actin Coats from Exocytosing CG Membranes

The consequences of XlMyo1c disruption were next analyzed in more detail by time-lapse confocal microscopy of eggs injected with Alexa488-globular-actin (G-actin) and either IQT or, as a control, HIQT. Further, biochemical analysis confirmed that IQT expression elevated the level of actin associated with the cortical fraction, and this elevation was reduced by coexpression of HIQT (Figure 5C).

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results, exocytosis was slower than in HIQT-expressing cells, based on both the increased time elapsed between uncaging and appearance of TR-dextran (Figure 7A; see Movie S3) and the reduced speed with which dextran signal reached its peak in exocytosing CGs (HIQT 2.2 ± 0.15 s, IQT 4.2 ± 0.39 s; mean ± SEM; p < 0.05). Actin coats assembled around exocytosing CGs in IQT-expressing cells and appeared at
least as robust as those in HIQT-expressing cells (Figure 7A). However, coats in IQT-expressing cells failed to compress over time and eventually dissipated. Furthermore, compression of exocytosing CGs was severely compromised (89.9% ± 2.8% in HIQT cells compressed to at least half their initial diameter within 1 min versus 13.9% ± 5.6% in IQT cells; p < 0.05). Consequently, many exocytosing CGs were not retrieved in IQT-expressing cells (Figure 7A; see Movie S3).

Cells were then analyzed by 4D imaging, which has the advantage of allowing visualization of dynamic processes that occur in the z dimension such as actin coat assembly and compression (Sokac et al., 2003). Apical views showed that in HIQT-expressing cells the dilated fusion pores of exocytosing CGs were rapidly covered over with actin (data not shown, but see Sokac et al., 2003), whereas in IQT-expressing cells, the fusion pores remained open (Figure 7B; see Movie S4). Similarly, basal views showed that actin coats in HIQT-expressing cells spread over the entire cytoplasmic surface of exocytosing CGs (Figure 7C; see Movie S6), while in IQT-expressing cells, most coats failed to fully enclose the exocytosing CGs (Figures 7B and 7C; see Movie S5). Rather, the coats started to form and extend into the cytoplasm but remained separated from exocytosing CGs and did not track closely along their surface (Figure 7C; see Movie S7). Tilt views of the underside of the PM showed actin fingers extending from the PM into the cytoplasm near exocytosing CGs in IQT-expressing cells (Figure 7D; see Movie S8). Such fingers grew and shrank but failed to completely enclose the CGs. The partially enclosed CGs were often squeezed to one side but were not compressed, even after many minutes (Figure 7D).

Z views further supported the notion that XIMy1c tethers assembling actin coats to exocytosing CGs. In HIQT-expressing cells, the coats extended rapidly down either side of exocytosing CGs, enclosed them, and compressed upward and inward (Figure 7E; see Movie S9). In contrast, in IQT-expressing cells, actin coats began to enclose exocytosing CGs and then shrank back, leaving the exocytosed CGs stranded beneath the PM (Figure 7E; see Movie S10).

Z views of dextran alone provided a potential explanation for the slowed exocytosis in IQT-expressing cells. Specifically, the necks of fusion pores in IQT-expressing cells were much narrower than in controls (Figure 7F). These results suggest that IQT expression might somehow limit dilation of fusion pores following CG-PM fusion, perhaps due to unrestrained actin assembly at the pore region either prior to or coincident with CG exocytosis.

Discussion

The most remarkable findings of this study are that a developmentally regulated myosin, XIMy1c, participates in egg activation by coupling dynamic actin to membranes and that when the function of this myosin is disrupted, unrestrained actin assembly results. Egg activation entails the rapid onset of several processes dependent on precisely coordinated actin-membrane interactions (see Introduction). The consequences of XIMy1c disruption, thus, are numerous and severe: suppressed cortical contraction, superfluous actin comet formation, perturbed CG exocytosis, and impaired compression and retrieval of those CGs that do exocytose.

Of the above phenotypes, dissection of the last was the most informative. That is, XIMy1c normally localizes to exocytosing CGs. Following disruption of XIMy1c
Figure 7. XMyo1c Couples Dynamic Actin to Exocytosing CG Membranes

(A) Time-lapse images from a single plane just below the PM of Alexa488-G-actin (green) in eggs expressing either HIQT or IQT. Eggs were activated by IP$_3$ uncaging in the presence of extracellular TR-dextran (red) to reveal exocytosing CGs. In HIQT eggs, actin coats rapidly enclose and compress exocytosing CGs (arrowheads). See Movie S2. In IQT eggs, CG exocytosis is slower, as judged by delay in the appearance of dextran. Also, actin coats assemble around exocytosing CGs but fail to compress and eventually dissipate (arrowheads). See Movie S3.

(B) Low magnification, 4D time-lapse images of eggs expressing IQT. Uncaging triggers exocytosis; however, CGs are not covered over by actin coats on either the exoplasmic surface (Apical) or the cytoplasmic surface (Basal). See Movies S4 (Apical) and S5 (Basal).

(C) High magnification, 4D time-lapse images of eggs expressing either HIQT or IQT. In HIQT eggs, basal views show that the actin coat tracks closely over the cytoplasmic surface of the exocytosing CG, eventually enclosing it. See Movie S6. In IQT eggs, basal views show that the actin coat forms (arrowhead) but is separated from the cytoplasmic surface of the CG (arrows). The coat eventually regresses. See Movie S7.

(D) High magnification, 4D time-lapse images of an egg expressing IQT. This tilted z view shows perturbed actin-coat assembly in the form of actin fingers (arrowheads) that partially track along the CG but then extend into the cytoplasm. The fingers grow and shrink over time, but the CG
function, actin coats still assemble but fail to remain attached to the CG membrane. Thus, XrMyo1c serves to tightly link the actin coat to the CG membrane, and this linkage is essential for CG compression and retrieval. How, exactly, might this work? While it is possible that XrMyo1c functions by myosin-based contraction in the classic bipolar filament sense, it is predicted to be monomeric and to lack a second actin-binding site outside the motor domain and so is not well suited to this role. Further, the F-actin that comprises the coats is highly dynamic and nucleated by the Cdc42-WASP-Arp2/3 pathway (Sokac et al., 2003). Thus, it is presumably comprised of branched networks with the plus ends of the filaments facing the membrane. We therefore suggest that XrMyo1c works by linking dynamic actin to the membrane of CGs and so transduces the force provided by polymerizing filaments to compress the membrane.

Recent work on mammalian myosins-1c supports the idea that this motor could play such a role. First, to efficiently couple actin assembly to membrane compression, XrMyo1c would need to be firmly anchored to the CG membrane; otherwise, its interaction with actively polymerizing actin might rip the myosin from the membrane surface. Consistent with this, the tail domain of mouse myosin-1c binds tightly and specifically to PI(4,5)P2-containing membranes (Hokanson and Ostap, 2006). Exocytosing CGs contain PI(4,5)P2 (Sokac and Bentread, 2006), thus, the means for tight linkage between CG membranes and actin coats by XrMyo1c is available in this system. Second, the ideal myosin transducer would entrain force production by the polymerizing actin filament to its own ATPase cycle. Consistent with this, release of ADP by rat myosin-1c is thought to be strain-dependent (Batters et al., 2004). Thus, myosins linked to an actively growing filament on CGs would preferentially move through the ATPase cycle as the filament pushed on the membrane and then rebind filaments near the membrane, in a ratchet-like manner. Conversely, myosins linked to a nongrowing filament would preferentially hang on to the filament, ensuring some constant tethering between the membrane and assembling actin. Third, the optimal orientation for a motor that directs actin assembly-dependent force production on membrane compartments is parallel to the surface of the membrane compartment. Consistent with this, myosin-1c releases calmodulin from IQ domains upon calcium increase (Zhu et al., 1998), exposing other potential membrane-binding sites (Swanljung-Collins and Collins, 1992; Barylko et al., 2005; but see also Hokanson and Ostap, 2006). Thus, it is thought that under conditions of elevated calcium, the motor domain is oriented parallel to the membrane (Barylko et al., 2005).

How can the above model account for the finding that XrMyo1c disruption results in increased actin assembly, as revealed by biochemical fractionation and increased numbers of actin comets? At least two, nonexclusive hypotheses suggest themselves. First, and most simply, by maintaining the plus ends of actin filaments in close contact with the membrane, XrMyo1c might normally limit intercalation of actin monomer between the actin filament plus end and the membrane. Indeed, that immediate proximity to the membrane acts as a physical restraint on actin-filament growth is a basic assumption of a variety of models of actin-based cell locomotion (Mogilner and Oster, 1996; Alberts and Odell, 2004). Consequently, it is reasonable to imagine that increased distance between the filament plus ends and the membrane would permit greater access of monomer to the plus ends and, thus, faster assembly. Second, XrMyo1c might normally antagonize factors that bind to growing filaments and promote further filament nucleation or growth, such as the Arp2/3 complex or other myosins-1 that interact with CARMIL, a protein that binds to and inhibits capping protein (Yang et al., 2005).

Whether myosins-1 in other contexts can restrain actin assembly in vivo remains to be seen, but previous work is consistent with this possibility. Specifically, disruption of myosins-1 in Dictyostelium results in formation of ectopic pseudopodia (e.g., Titus et al., 1993). Similarly, acute disruption of mammalian myosin-1c in growth cones results in a rapid outgrowth of lamellipodia (Diefenbach et al., 2002).

Interestingly, the disruption of XrMyo1c also impairs cortical contraction. The suppression of cortical contraction is unlikely to reflect a role for XrMyo1c in powering this event, both for the reasons presented above for coat compression and because cortical contraction in eggs is myosin-2 dependent (Christensen et al., 1984). We instead suspect that the cortical contraction deficit either reflects improper anchorage of cortical F-actin to the PM or the inability of myosin-2 to efficiently utilize the ectopic F-actin resulting from XrMyo1c disruption.

The effects of XrMyo1c disruption on CG exocytosis are particularly fascinating given the previous report that expression of mammalian myosin-1c IQT in adipocytes suppresses exocytosis of the GLUT4 glucose transporter by an unknown mechanism (Bose et al., 2002). We can envision several possible explanations for inhibition of CG exocytosis. The first is that excess actin resulting from IQT expression provides a physical block to exocytosis, as observed in other systems (e.g., Muallem et al., 1995) and supported by our finding that cytochalasin treatment rescues the exocytosis defect. The second potential explanation is that IQT itself acts as a physical barrier to CG-PM fusion. However, constitutive exocytosis is unaffected by IQT expression at levels sufficient to completely suppress CG exocytosis, arguing against simple physical inhibition. A third possibility is that XrMyo1c abets exocytosis by limiting actin assembly at the fusion pores and thus promoting their dilation. This idea is consistent with the observation that expression of IQT slows incorporation of

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is never completely surrounded by an actin coat. Compression of the incomplete coat causes the CG to squirt out through a region with no coat. ("Out" and "in" indicate the outside and the inside of the cell, respectively.) See Movie S8.

(E) High magnification, 4D time-lapse images of eggs expressing either HiQT or IQT. Z views show in HiQT eggs, actin coats extend downward from the PM (arrowheads), enclose the exocytosing CG, and compress upward. See Movie S9. In IQT eggs, while coats start to assemble downward from the PM (arrowheads), they subsequently regress upwards. See Movie S10.

(F) Z views from eggs expressing either HiQT or IQT. Only the TR-dextran is shown. In HiQT eggs, exocytosing CGs have broad necks where they fuse to the PM (arrowhead). In IQT eggs, the necks are narrow. Time is in min:sec; bars are 5 μm.
dextran into exocytosing CGs and produces constricted fusion pore necks in these compartments. It is also consistent with the results of the cytochalasin rescue experiments. Of course, the above mechanisms are not mutually exclusive, and it is possible that each may contribute to the deficit in CG exocytosis.

Finally, in addition to demonstrating a key role for XMyo1c in egg activation, this study also provides a striking confirmation of the proposal that F-actin coats are responsible for retrieval of exocytosing CG membranes (Sokac et al., 2003). This point could not be directly tested before because inhibition of coat assembly by latrunculin treatment also results in immediate collapse of exocytosing CGs into the PM (Sokac et al., 2003). In contrast, disruption of XMyo1c does not result in collapse but does prevent coat compression. Consequently, the CG membranes are not retrieved and remain trapped beneath the PM. Because F-actin coats form in other eggs as well as in secretory cell types (reviewed in Sokac and Bement, 2006), it may be that myosin-1 similarly contributes to compartment retrieval following regulated exocytosis in a number of different systems.

Experimental Procedures

Degenerate PCR Screen
Degenerate primers ATP3 (5'-GGGAGRIWSIGGCGIGQNAARAC-3') and EAF-A (5'-GTYYTTCRGTCCRAIGGYTC-3'), corresponding to amino acids GESGAGKT and EAFGNAKT, respectively, were used to amplify 12 different myosins from Xenopus ovary cDNA. Based on the closest vertebrate homolog identified by BLAST searching, these myosin fragments represent XMyo1b (100%, XMyo1b), XMyo1c (92%, RcMyo1c), XMyo1d (100%, HsMyo1d), XMyo1e (89%, HsMyo1e), XMyo1f (100%, HsMyo1f), XMyo2a (92%, HsMyo2A), XMyo3 (87%, GgMyo3), XMyo6 (100%, XMyo6), XMyo9a (100%, HsMyo9a), XMyo9b (89%, HsMyo9b), XMyo10 (70%, TrnMyo10), and XMyo18 (72%, TrnMyo18) where the percentage indicates percent amino acid identity, and initials indicate the following organisms: X, Xenopus tropicalis; R, Rana catesbeiana; Hs, Homo sapiens; Gg, Gallus gallus; Trn, Tetraodon nigroviridis. Larger myosin fragments were then amplified with upstream-specific primers for Myo1c (5'-CAGGTTGGAGACCCCTAAGT-3'), Myo1e (5'-GGGAGGGCTGGCCTAAAGGT-3'), Myo2a (5'-GAACACTCACAAGCTC-3'), Myo6 (5'-TTGGCTGGCTGACCCCTA-3'), and Myo10 (5'-GAAGCCACCACACTGCTG-3'), corresponding to amino acids QVETVK, GGGPKV, ATSHKS, ESTKLL, and ORF respectively of the gene. The fragments were used in downstream degenerate primers LDY (5'-TSCRAACRWRADTRTCR-3') or FEQF (5'-ARTDTRCARAAATYGCTCRAA-3'), corresponding to amino acids LDYF/FGE and FEQFCIN, respectively.

5/0 RACE and Morpholinos
For 5/0 RACE, cDNA was made from egg poly(A)+ RNA with a XMyo1c-specific primer (5'-GAGATAGTTGAATTGACCC-3'), corresponding to amino acids GHLNVL. For subsequent PCR, a nested XMyo1c primer (5'-GGAGGCTAGGAGAGGGCC-3'), corresponding to amino acids RLQSN, was paired with a generic oligo-dT anchor primer (Roche Applied Biosciences, Indianapolis, IN). For 5/0 RACE, two specific primers were designed against the XMyo1c 5'UTR (5'-CTGAAGATGTTAGGATGGTTCGAG-3') and 5'-GTTGAGCTGGCTGAAGCTCAGG-3') and used in sequential rounds of PCR. The morpholino (GeneTools; 5'-TTTCCCATGTTTGGACCGTTCG-3') was designed against the XMyo1c 5'UTR; the control morpholino (5'-TTCACCCTAGTCACACCGGCTTCG-3') incorporated a five base pair mismatch.

RNA Isolation and Northern Blotting
Total RNA was extracted from staged oocytes and further fractionated to yield stage-specific poly(A)+ RNA with the MicroPoly(A) Pure mRNA Isolation Kit (Ambion, Austin, TX). For developmental Northern, poly(A)+ RNA was quantified by spectrophotometry and equal quantities loaded per oogenic stage. For total versus poly(A)+ RNA/stage 6 oocyte versus egg analysis, 30 oocyte/egg equivalents were loaded per lane. RNAs were blotted and processed according to Northern Max Blotting Kit (Ambion) specifications. Antisense RNA probes were transcribed in vitro, purified over G-50 spin columns, and labeled and detected with the Bright-Star Psoralen-Biotin Nonisotopic Labeling and Detection Kit (Ambion).

Protein Extraction and Immunoblotting
For stage 6 oocyte versus egg analysis of endogenous myosin or actin, 90 stage 6 oocytes or eggs were homogenized in 240 μl PHEME Buffer (60 mM K-PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2 [pH 7.0] with KOH, 1% Triton X-100, 5 mM MgATP, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc SC, 10 μg/ml chymostatin, 10 μg/ml pepstatin) with a mechanical homogenizer. Homogenates were spun at 14,000 × g, and the cytoplasmic layer collected and combined with sample buffer and then separated by SDS-PAGE. For cortical fractionations, single oocytes were disrupted in Solution A (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 2 mM MgCl2 [pH 7.4] supplemented immediately before use with 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) by six to seven gentle passages through the orifice of a yellow pipet tip. Sample was spun at 720 × g for 3 min. Low-speed supernatant was collected and combined with sample buffer. Low-speed pellet was re-extracted in Solution B (1% Triton X-100, 0.1 M NaCl, 50 mM Tris, 1 mM EDTA [pH 7.4]) by trituration through a yellow pipet tip. Sample was spun at 16,000 × g for 1 min. Supernatant was collected, combined with sample buffer, and subjected to SDS-PAGE.

For immunoblots primary antibodies anti-Myosin-1c (M3) (Wagner et al., 1992), anti-Myosin-2 (Biomedical Technologies, Stoughton, MA), and anti-actin (Amersham Biosciences) were used at a dilution of 1:500. Secondary antibodies anti-mouse IgG-HRP conjugate and anti-rabbit IgG-HRP conjugate (Promega, Madison, WI) were used at a dilution of 1:5000. Chemiluminescent detection proceeded according to standard ECL protocols.

Secretion Assays
For analysis of constitutive secretion, oocytes were injected with 10–50 ng of capped RNAs encoding GFP-raft syntenin 1A (construct supplied by Dr. T. Coppola; Laussanne) or α-1[III] subunits (1:41 ratio) rat Na+-K+ ATPase (construct supplied by Dr. J.D. Horisberger; Lausanne) and incubated at 18°C for 48 hr. Cells were fractionated as above, and the fractions immunoblotted. Cortical granule secretion from single oocytes was monitored as previously described (Gundersen et al., 2002) following 30 min treatment with 1 μM PMA or 10 μM ionomycin at 20°C–22°C.

Capped RNA Synthesis
For expression of untagged or N-terminally eGFP-tagged XMyo1c constructs, myosin sequences were cloned into pC52 or custom vector pCS2+eGFP, respectively. Transcription of capped RNA proceeded according to the SP6 or T7 Message Machine High Yield Capped RNA Transcription Kit (Ambion) protocol with 1 μg of template. RNA was extracted with an equal volume of phenol/chloroform (1:50). For rescue experiments, cells were injected with 80 ng HiQ7 and 40 ng IQ7.

Cell Fixation and Staining
Egg activation was induced and proceeded for 2 min in 10 mM lonomycin (Calbiochem) in 1/10× OR2. The eGFP-XMyo1c-expressing oocytes, eggs, and activated eggs were fixed for 4 hr in 3.7% paraformaldehyde, 0.1% glutaraldehyde, and 0.4 units/ml Alexa568-phalloidin (Molecular Probes, Eugene, OR) in Myo1c Fix Buffer (10 mM HEPES, 10 mM EGTA, 100 mM KCl, 3 mM MgCl2, 150 mM sucrose [pH 7.6]). Cells were bisected prior to staining with 1 unit/ml Alexa655-phalloidin for 12–14 hr. For immunofluorescence localization of XMyo1c, activated eggs were fixed for 30 min in the presence of Alexa655 phalloidin, washed for 1 hr, bisected, and processed for immunofluorescence with a monoclonal anti-myosin-1c antibody (Wagner et al., 1992).

Microscopy and Image Analysis
Oocytes and eggs were prepared, injected, cultured, and mounted as previously described (Sokac et al., 2003). Imaging was performed
with a Zeiss Axiovert 100 M microscope (Carl Zeiss, Inc., Thornwood, NY) with Bio-Rad 1024 x 1024 Lasersharp Confocal Software (Bio-Rad Laboratories, Hercules, CA) with a numerical aperture 1.4, 63 x objective lens. For quantification of fluorescence intensities, the NIH Object-Image v2.06 software was used. Z series and time-lapse, single optical plane movies and 4D renderings were generated with Velocity v10 software (Improvision, Lexington, MA). All other image processing was done with Adobe Photoshop 7.0 (Adobe Systems).

Supplemental Data
Supplemental Data include ten movies and corresponding movie descriptions and are available at http://www.developmentalcell.com/cgi/content/full/11/5/629/DC1/.

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References

**Accession Numbers**

The myosin sequences obtained in this study have been entered into GenBank with the following accession numbers: XMMyo1b, EF026166; XMMyo1c, EF026164; XMMyo1d, EF026167; XMMyo1e, EF026163; XMMyo1f, EF026168; XMMyo2a, EF026169; XMMyo3, EF026170; XMMyo6, EF026171; XMMyo9a, EF026172; XMMyo9b, EF026173; XMMyo10, EF026174; XMMyo18, EF026165.