Molecular survey of myosins in zebrafish oocytes

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Abstract

Actin and myosin are cytoskeletal proteins that are known to mediate several cellular activities, including the movement of cells, secretion, retrieval of membrane by exocytosis and muscle contraction. Based on studies with somatic cells, there is support for the notion that myosins may play a role in early events of vertebrate gametogenesis and fertilization. Two strategies were employed to survey myosin expression in zebrafish ovary tissue and activated eggs: one dimensional electrophoresis and Western blotting of homogenates; and synthesis of cDNA clones using reverse-transcriptase and Polymerase Chain Reaction methods with degenerate primers coding for sequences in the myosin head domain.

Gels of ovary and egg homogenates displayed a single band whose molecular weight was estimated to be 205 kDa (egg) and 210-215 kDa (ovary). Anti-human platelet myosin heavy chain antibody recognized this band in both samples as well as the rabbit myosin protein on Western blots. Standard methods were used to synthesize single and double-stranded cDNA from ovary total RNA. Degenerate primers prepared against the myosin head domain were used. PCR product was successfully ligated into a P-GEM vector.

Introduction

Eukaryotic cells perform a number of activities that require reorganization of the cytoskeleton, including intracellular vesicle transport, cell locomotion, secretion, cell division, and retrieval of membrane by endocytosis. The cytoskeleton is a complex cytoplasmic system of fibers of varying diameters. These include microfilaments (actin filaments; 7-9 nm in diameter), intermediate filaments (10 nm in diameter), and microtubules (24 nm in diameter). In addition to driving cell motility events, the cytoskeleton provides structural support to the cell.

Most non-muscle cells, including egg cells, contain actin and myosin. Actin exists as a globular monomer known as G-actin and as a filamentous polymer termed F-actin (microfilaments or actin filaments). Actin filaments have been localized by fluorescent probes to the cortex of the animal egg where they have been implicated in the movement of the sperm into the egg, the exocytosis of cortical granules, and the formation of the polar bodies. Calcium-sensitive actin regulatory proteins appear to

modulate the rearrangement of the microfilaments during these dynamic changes in cortical structure.

Myosins are a large superfamily of proteins consisting of some fifteen classes that have been identified by genomic analysis. The classes are generally considered to fall into one of two groups: conventional or heavy chain myosins (myosin-II) and unconventional myosins. All myosins are composed of one or two heavy chains and several light chains. The heavy chains are further organized into a globular head domain (with actin- and ATP-binding sites), a neck region, and a tail domain. Non-muscle myosin-II heavy chains exist as two isoforms encoded by at least two genes; these heavy chains have been designated A and B (Phillips, 1995). The cDNAs encoding each of these chains have been cloned and sequenced. Based on studies with *Xenopus*, it would appear that both conventional and unconventional myosins are present in egg cells (Sokac and Bement, 1996). Myosin-II has been identified in frog, starfish (Mabuchi, 1976), zebrafish (Becker and Hart, 1996), and mouse (Simerly et al., 1998) eggs using different methods. In the mouse, both isozymes of myosin-II are distributed over the metaphase-arrested second meiotic spindle.

Although the literature on microfilament distribution and function in eggs and embryos is rather well-established , little is known about myosin localization and function in animal gametes. Most of what is known is based on studies with either eggs or spermatozoa of mammals (Simerly et al., 1998). The objective of this research was to initiate a survey of myosins using the zebrafish as a model system for study. Two strategies were used: 1) one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting of ovary tissue and activated egg samples, and 2) synthesis of cDNA with reverse transcriptase (RT) from total ovary RNA followed by the polymerase chain reaction (PCR).

Materials and methods

Procurement of eggs and ovary

Ovary tissue was carefully dissected from a gravid female immobilized on ice and processed for either SDS-PAGE or RT-PCR. For activation, eggs were collected from a gravid female placed into a medium-sized fingerbowl containing conditioned tank water.

Sample preparation and SDS-PAGE

Ovary and egg (150-200 eggs) samples were homogenized in 400 µl of cold homogenization buffer using a Vibra Cell (Sonics & Materials, Inc) instrument. Homogenates were centrifuged with a refrigerated Eppendorf model 5415C centrifuge according to the protocol shown in Appendix I. Supernatants were then combined with Sample Buffer (0.75 g SDS, 3.12 ml 4X Upper Tris Buffer, pH 6.8, 1.25 ml mercaptoethanol, 0.25 g sucrose and 0.06 g bromophenol blue per 5 ml total volume) and heated for 2 min. Pellets were resuspended in 200-300 µl homogenization buffer and then treated as described above. Samples were either used immediately or stored

at -70°C. Ten and 8% mini-gels were prepared and samples electrophoresed using a Hoefer model 250 electrophoretic apparatus at room temperature. The running conditions were: 50V through the stacking gel and 150V through the separating gel (total time approximately 85 min). Companion lanes included molecular mass standards: high molecular weight mixture (SDS-6H; Sigma) and rabbit myosin standard (#1636, 4.1 mg/ml, Sigma; 205 kDa). Gels were either stained immediately with Coomassise Brilliant Blue R or used for transfer to nitrocellulose membrane (0.45 μm; Biorad) using a Genie Electrophoretic Blotter (Idea Scientific).

Western blotting

The protocol employed for Western blotting is shown in Appendix II. The transfer was run using Towbin's Transfer Buffer (25 mM Tris, 192 mM Glycine, 0.5% SDS, 20% v/v Methanol). Total transfer time was 5.5 hr at 12-13V and 0.9-0.95 amps. The transfer membrane was initially probed with human anti-platelet myosin antibody (Biomedical Technology, Inc.) at a dilution of 1:1000 followed by goat anti-rabbit secondary antibody coupled to phosphatase at a dilution of 1:2000. The staining reaction mixture was: 9.9 ml alkaline phosphatase buffer (pH 9.5), 33 μl 5-bromo-chloro-indolyl phosphate, and 66 μl nitro blue tetrazolium.

Sample preparation for RT-PCR

Ovary was dissected from a gravid female as described above and total RNA isolated. The tissue was homogenized on ice with Trireagent (1 ml/100 mg of sample) and allowed to stand at room temperature for 5 min. Chloroform (200 μl) was then added and, after 2 min, the mixture was centrifuged for 15 min (12,000 *g*). The top aqueous layer was removed from the centrifuge tube and combined with 200 μl of 2-propanol (isopropanol). Following centrifugation (cold, 7500 *g* for 5 min), the alcohol was removed and the pellet allowed to air dry. The pellet was then dissolved in 1 ml of DEPC water. Spectrophotometric assay revealed 5.52 g/l RNA in the ovary sample.

Reverse transcriptase protocol

Samples of zebrafish RNA were stored at -80°C. and were kept on ice when removed from the freezer. M-MLV Reverse Transcriptase 5X buffer was used with M-MLV RT H(-) Point enzyme. The RT was diluted to 2 μl/250 units and the random primers were diluted to 1 μg/μl. RNA concentration was estimated to be 1 μg/μl. Samples of RNA were added to the mixture (11 μl nuclease free water, 2 μl diluted RNA, 2 μl diluted random primers) and heated for five min at 70°C followed by brief centrifugation. The samples were then each added to secondary mixture (5 μl reaction buffer, 1.25 μl 10mM dNTP, 2 μl M-MLV RT, 1.75 μl nuclease free water) and placed in a 37°C water bath with shaker for one hour. A simultaneous control was also run, using 1 μl Kanamycin positive control instead of RNA sample and only 1 μl of random primers.

Synthesis of second strand cDNA then followed using the protocol adapted from *Molecular Cloning, Volume 2.* One half of the results of the RT protocol were treated in this manner, with the other half undergoing both second strand synthesis and amplification during the PCR. The second strand synthesis outside of PCR was as follows: 10 μl of SS DNA from the RT protocol, 5.6 μl 25 mM $MgCl_2$, 2 μl Tris Cl (pH 7.4), 1 μl dNTP, 0.3 μl 1M $(NH_4)_2SO_4$, and 5 μl of 1 unit/μl Klenow Fragments. The mixture was then incubated at 16°C for 4 hours.

The PCR protocol is outlined in the Appendix III. Five samples were run at different dilutions but under the same running conditions. GibcoBRL PCR Supermix was used (Cat #10572-014), which contains Mg^{2+}, deoxyribonucleotide triphosphates, and recombinant Taq DNA. The PCR was run overnight.

Plasmid work-up/transformation

The plasmid used was pGEM 3zF (see Fig. 7). SMA I was used as the restriction enzyme to cleave the plasmid. 3 μl of SMA I, 2 μl of buffer, 5 μl of the pGEM, and 10 μl of nuclease free water was combined to open the circular structure and leave blunt ends.

The products of the PCR were blunt-end ligated into the plasmid. 60 μl of DNA whose second strand was synthesized before PCR and 60 μl of DNA whose second strand synthesis took place during the PCR, were each added to 40 μl of ddH_20 (dd=double distilled) and 250 μl of EtOH, and placed in the -80°C freezer for 20 min. The samples were then washed with 70% EtOH and centrifuged. The pellets were air dried and resuspended in 5 μl of ddH_20, and then placed in a water bath of 65°C for 4 min. 4 μl of this each sample was then added to 3 μl of pGEM, 1 μl of buffer, and 4 μl of DNA ligase.

Each of the above samples were then added to 120 μl of competent *E. coli* cells and placed on ice for 30 min. Heat shock approach was then used for 2 min in a water bath at 42°C to encourage uptake by the competent cells. Samples were then returned to ice for 1 min.

Plating

Each of the samples was then added to 2 ml of LB broth and put on a shaker for 1 hr at 37°C, followed by centrifugation for 1 min. Supernatant was discarded and the pellet was resuspended. Blue/White color screening was utilized, as well as selection for ampicillin resistance. Plated JM109 strains of *E. coli* carry lacZΔM15 and $lacI^Q$ on the F^I episome. Transformed cells of this strain were plated on LB agar plates with 50 ug/ml ampicillin, 0.5 mM IPTG, and 40 ug/ul X-GAL. These plates were then incubated overnight at 37°C. Recombinant colonies were white and larger than the blue colonies. (Colonies with β-galactosidase activity grow poorly relative to cells

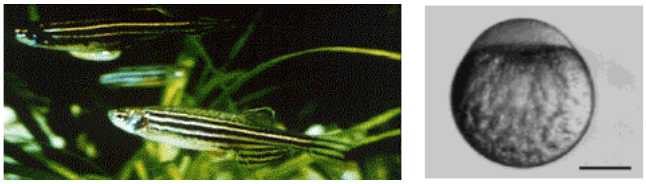
lacking this activity, hence the blue colonies will be small and the white recombinant colonies will be larger, approximately 1 mm.)

Controls during the plating portion of the experiment were as follows: non-recombinant pGEM transformed into competent *E. coli* cells and plated to check efficiency of the transformation; *E. coli* alone was plated onto ampicillin positive plates to ensure efficiency of the ampicillin and the fact that resistance was acquired through transformation alone; an ampicillin negative plate was also run with non-recombinant *E. coli* cells to ensure that the cells were viable.

The selected colonies were isolated and white, and cultured at 37ºC overnight to ensure growth. Mini-prep was then done to prepare the samples for agarose gel to test for plasmid uptake, and for possible later splicing of the recombinant DNA. The mini-prep procedure is outlined in the Appendix IV.

Results

Figure 1: The source of ovary tissue and activated eggs was the female zebrafish (left panel). A 10 min activated egg is shown in the right panel. The magnification bar = 250 μm.

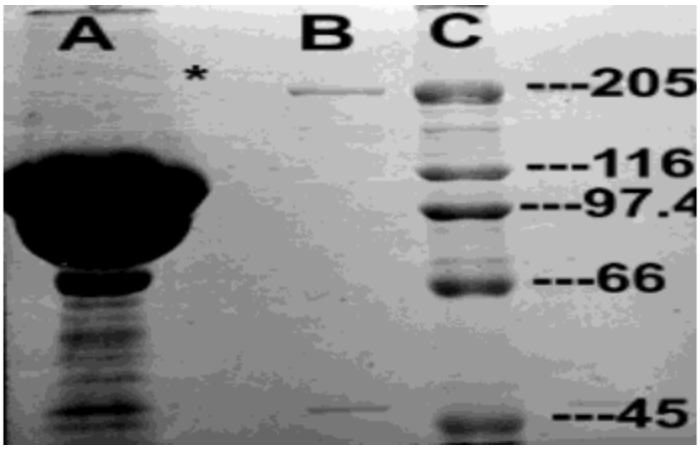


SDS-PAGE and Western blotting

The identification of myosin-II in this study required the development of a protocol that would allow its clear separation - in sufficient amount - from other oocyte proteins. This proved to be a demanding task in part because most of the egg protein was in the form of yolk, visible on gels as two prominent bands with estimated molecular weights of about 115 and 95 kDa (Fig. 2). A number of centrifugation protocols were attempted to drive any putative myosin into the pellet fraction and the yolk proteins into the supernatant fraction. However, irrespective of the centrifugation method, myosin and the yolk proteins partitioned to both supernatant and pellet fractions. The protocol outlined under Materials and Methods is the one upon which the results below are reported. It was also determined that mechanical rather than manual homogenization of ovary and egg samples provided a better crude myosin yield in the extract.

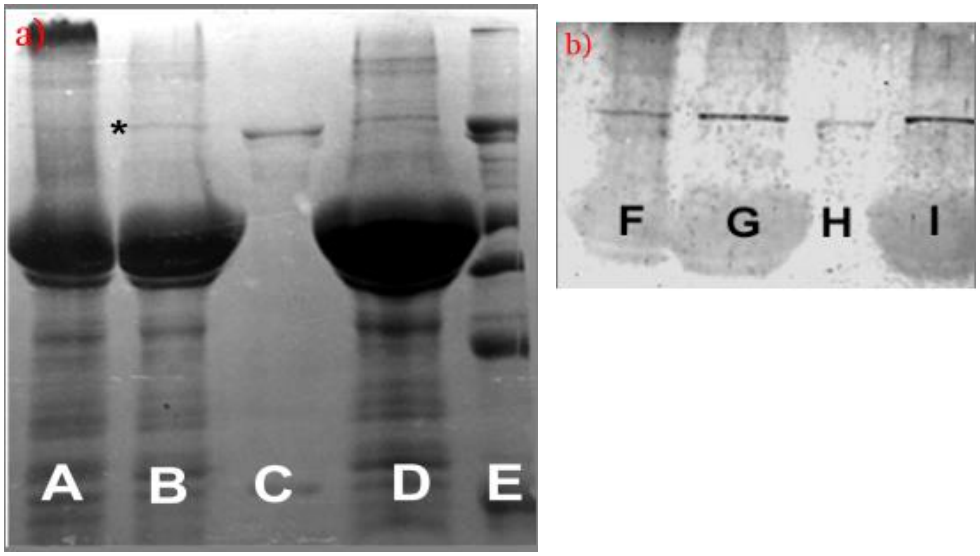
Figure 2: Molecular mass standards (Lanes B,C) were run in gels with samples in companion lanes (A). Lane A, ovary S_1 fraction; Lane B, rabbit muscle myosin, Lane C, molecular mass standard mixture (SDS-6H , Sigma): myosin, rabbit muscle (205

kDa), beta-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa).The asterisk (*) in Lane A designates ovary myosin.



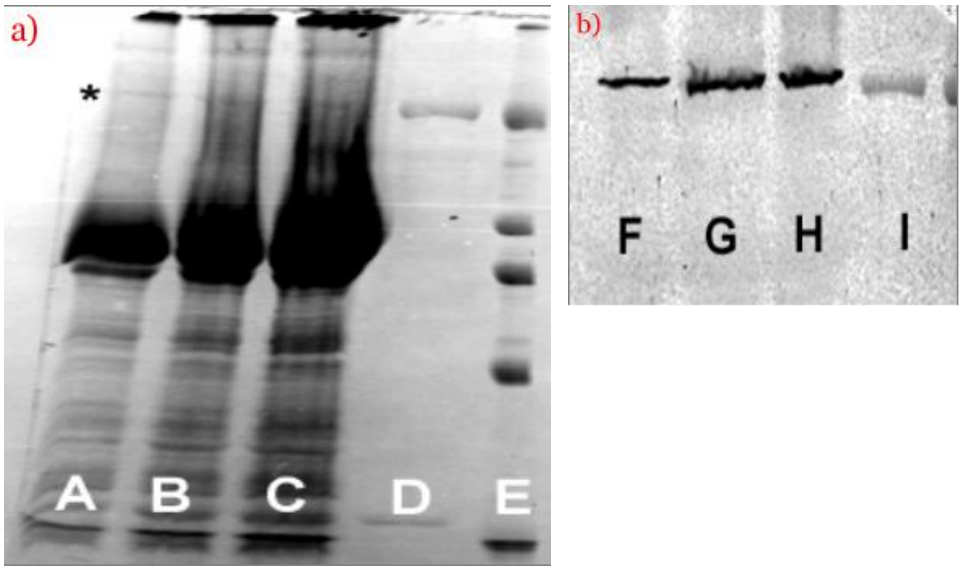
Homogenates of ovary tissue were initially electrophoresed using the S_1 fraction (Fig. 2). A single band with a relatively slower mobility than the rabbit myosin standard was consistently detected. It was tentatively identified as heavy chain myosin. S_2 and P_2 fractions were then analyzed to minimize lane distortion caused by the yolk proteins (Fig. 2). Both fractions displayed a single band with similar mobilities to that in the S_1 fraction (Fig. 3). Based on staining properties, it appeared that more of the myosin partitioned to the P_2 rather than the S_2 fraction (lanes A and B received an equal volume of sample). The estimated molecular mass of this band was 210-215 kDa or slightly higher than the 205 kDa of the rabbit myosin standard. The same band demonstrated a strong reactivity following incubation with the anti-platelet myosin antibody (Fig. 3, lanes F-I).

Figure 3: a) Human platelet antimyosin detects myosin in S_2 and P_2 fractions of zebrafish ovary homogenates. Lane A, S_2; Lane B, P_2; Lane C, rabbit muscle myosin; Lane D, P_2; Lane E, high molecular mass standards; **b)** Lanes F, G, H and I are companion blots to Lanes A, B, C and D. The myosin protein (asterik) has an estimated molecular mass of 210-215 kDa**.**



Since myosin has been implicated in motility events at fertilization, zebrafish eggs were collected and activated for 10 min in conditioned tank water. Work done in our laboratory has shown that the second polar body is separated from the egg at this time. Homogenates were prepared and the S_2 fraction screened for myosin-II by electrophoresis and blotting. A single band was identified that co-migrated with the rabbit myosin standard (Fig. 4, lanes A-C). This band was recognized by the myosin antibody (Fig. 4, Lanes F-H).

Figure 4: a) Human platelet antimyosin detects myosin in 10 min water-activated eggs. Lanes A, B and C, S_2 fractions of egg homogenate; Lane D, rabbit muscle myosin; Lane E, high molecular mass standards. **b)** Lanes F, G, H and I are companion blots to Lanes A, B, C and D. The myosin protein (asterik) has an estimated molecular mass of 205 kDa.

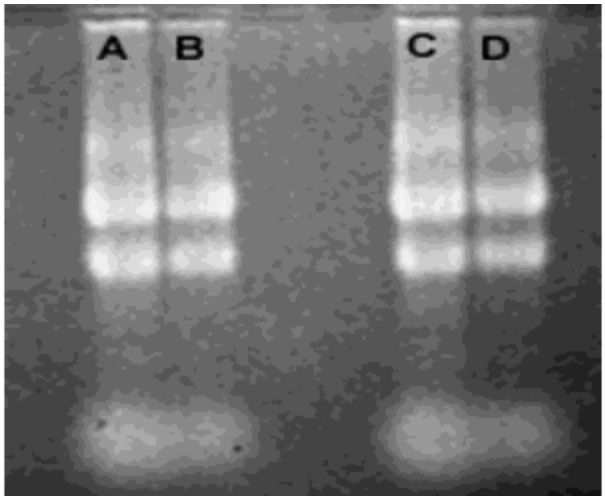


Reverse transcriptase and polymerase chain reaction

An RT-PCR strategy has been employed to identify different myosins in *Xenopus* oocytes (Sokac and Bement, 1996). A similar approach was adopted in this study with the same degenerate myosin primers used by Sokac and Bement.

Total ovary RNA was the source of the template for making cDNA (Fig. 5). At the time of conducting my experiments, spectrophotometric assay showed a total RNA concentration of 4.952 ug/ul.

Figure 5. Agarose /formaldehyde gel showing presence of RNA in ovary tissue. **A, C**: 9 µl RNA + cocktail; **B, D**: 4.5 µl RNA + 4.5 µl water.



Two approaches were employed to synthesize second strand cDNA following the first strand cDNA reaction. In one, the second strand was synthesized before amplification by PCR; in the other, the second strand was synthesized during PCR amplification. Figure 6 shows the PCR products amplified using primers specific to the coding region of the myosin head domain. Amplification was observed using either method.

Figure 6: Agarose (1%) gel of PCR products.
A-D: 10 μl of PCR product (2nd strand synthesis before PCR);
E, F: 10 μl of PCR product (2nd stand synthesis during PCR).

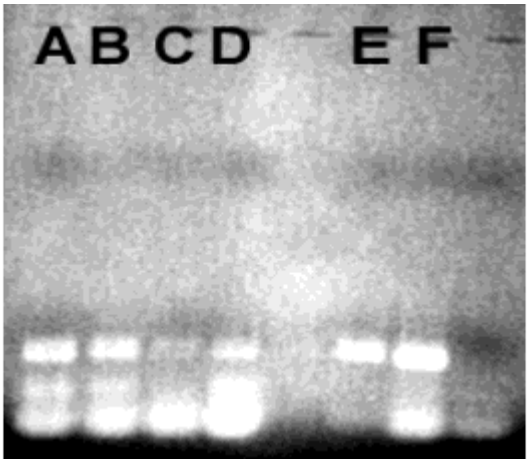


Figure 7: Diagram of the pGEM vector. *Sma I* was used to cut the plasmid before ligation.

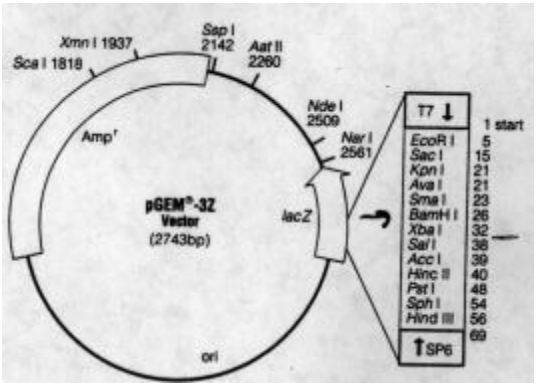
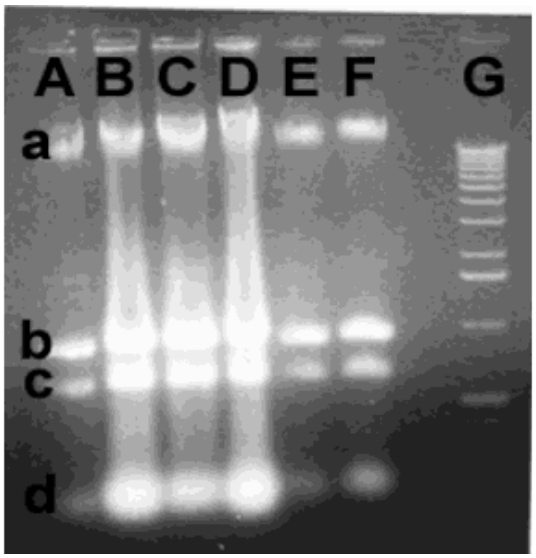


Figure 8: Agarose (1%) gel showing plasmid in isolated cultures after mini-prep.
A-D 10 μl sample + 4 μl dye
E,F 10 μl sample + 4 μl dye
G 1 kDa ladder

a = genomic DNA
b = plasmid

c= supercoiled plasmid
d = RNA



Discussion

The zebrafish egg is an excellent model system for exploring the structure and function of the cytoskeletal system. The egg is large and transparent, permitting in vivo imaging and detailed observations of such events as secretion, ooplasmsic segregation, meiosis, and cell division. Furthermore, the egg is readily activated by conditioned tank water, making it possible to evaluate the role of the egg alone in early events of fertilization.

This study demonstrates: 1) the crossreactivity of an antibody against human blood platelet cells with zebrafish tissues, and 2) the specific presence of myosin-II in ovary and 10 min activated eggs. Both sample extracts display a single band that either co-migrates (egg) or migrates slightly slower than (ovary) the 205 kDa rabbit myosin standard. These results agree with other studies showing myosin-II heavy chain in oocytes of zebrafish (Becker and Hart, 1996) and mouse (Simerly et al., 1998). It would now be useful to determine whether the myosin identified here exists as one or two isoforms. Antibodies against myosin-IIA and myosin-IIB are available. Analysis of other cell types suggests that each nonmuscle isoform is expressed in many tissue and cell lines. If both are present in either ovary or egg, the possibility is raised that each non-muscle isoform may perform different intraoocyte tasks.

The zebrafish egg is arrested in metaphase-II of meiosis at the time of fertilization. Upon fertilization, the egg undergoes a highly regulated program of development The earliest recognizable event is the binding of the sperm to the sperm entry site (within 5 sec of preparing an egg-sperm suspension). Between 30 sec and 3-4 min, membrane-limited vesicles (cortical granules) migrate to the egg surface, fuse with the plasma membrane, rupture, and discharge their contents onto the egg surface. Re-entry into

the cell cycle and formation of the second polar body is initiated by 2 min; at 10 min the egg completes meiosis with the formation of the second polar body. Microfilaments appear to modulate sperm incorporation, sperm incorporation cone formation, and secretion. The apparent presence of myosin-II in unfertilized (Becker and Hart, 1996), recently fertilized (Becker and Hart, 1999) and 10 min activated (this study) eggs suggests a possible role for this protein in these events. Experiments employing specific inhibitors of myosin or myosin antibodies microinjected into the egg should provide additional insight into this issue of egg myosin function.

The zebrafish ovary consists of both somatic (follicle cells, smooth muscle cells) and germ (oocytes) cells. In the absence of localization studies, it cannot be stated with certainty whether the myosin identified in this study is present in one or both cellular compartments. The observation that myosin-II is detected in unfertilized eggs would strongly indicate that the same protein is present in developing oocytes. Possible involvement of myosin-II during oocyte development includes: migration of cortical granules into the cortical cytoplasm, movement and positioning of the first meiotic spindle, maintenance of oocyte shape, and stabilization of the sperm entry site.

An RT-PCR strategy was employed in efforts to replicate and amplify possible unconventional myosin head domain sequences from the total RNA of the zebrafish ovary. This strategy has previously been utilized in the study of myosin expression in *Xenopus* oocytes (Sokac and Bement, 1996). The Sokac and Bement study yielded the identification of fourteen myosins using a *Xenopus* cDNA library and degenerate primers designed, as in our study, from myosin head domains. From these fourteen identified myosins, it was possible to further classify the results into five phylogenetic classes of unconventional myosins. Although our results reported here are quite preliminary, it is a reasonable expectation that further research will yield a high number of unconventional myosins present in the oocytes of the zebrafish.

Although the PCR protocol used with degenerate myosin primers yielded product, it is impossible to definitively conclude that the products were indeed amplified myosin sequences. The competent E. coli cells were successfully transformed and all controls yielded positive results. Upon screening of the presumptive positive cells (those which were isolated, smaller in size, and white in color), the mini-prep procedure was utilized to screen the cells for uptake of the plasmid. Sequences were not excised from the agarose gel and therefore no further blotting analysis was done to conclude that the recombinant plasmid contained myosin sequences native to the zebrafish total RNA. However, this is the likelihood based on the use of myosin primers during the PCR analysis. There has been very little previous work done with the PCR protocol for zebrafish myosin, and therefore more research must be done to establish optimal guidelines for both annealing and denaturing temperatures during amplification, as well as the number of cycles to yield product.

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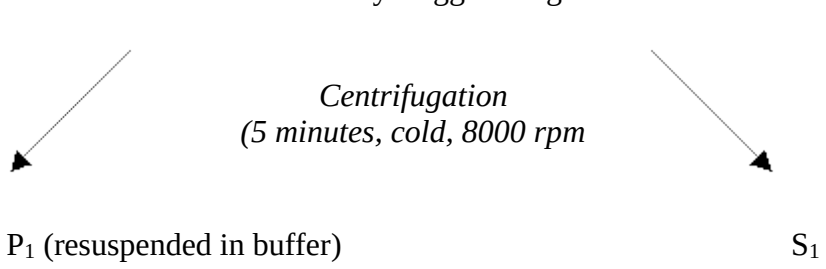
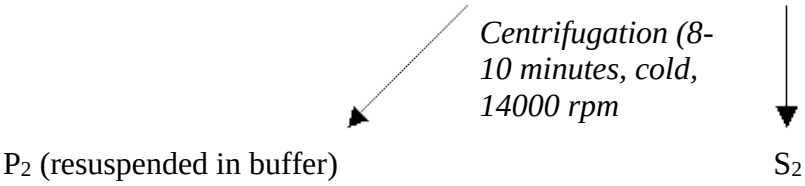
Appendix 1. Gel electrophoresis (sample preparation)





P_1, P_2, S_1, and S_2 homogenous fractions were all run on 8%, 0.75 mm thick polyacrylamide gel.
50 minutes through the separating gel followed by an additional 20 min.

Appendix 2. Western blotting using nitrocellulose paper

Transfer (Towbin's buffer; cold; 5.5 hrs; 12-13V; 0.9-0.95amps)
↓
Blocking (PBST with 5% Carnation evaporated milk)
↓
Rinsing (PBST 4 x 5 min; PBS 2 x 5 min; room temp)
↓
1º Antibody (Anti-human platelet myosin 1:1000 in PBST; overnight in cold room)
↓
Rinsing (Same as first rinsing)
↓
2º Antibody (Goat anti-rabbit IgG 1:2000 coupled to phosphatase)
↓
Staining (BCIP and NBT in alkaline phosphatase buffer)

Appendix 3. Reverse transcriptase (RT) and polymerase chain reaction

Isolate RNA from zebrafish ovary
↓
RT to synthesize 1st strand cDNA
↓
Method 1: DNA polymerase and ligase to synthesize 2nd strand cDNA; followed by PCR for amplification with degenerate myosin primers
OR
Method 2: 2nd strand synthesis/amplification in PCR machine using degenerate myosin primers
↓
Blunt end ligation at Sma I site of vector pGEM 3Z
↓

Transformation of E. coli competent cells
Growth overnight on LB/amp+/xGAL+/IPTG+ plates
↓
Screening successful transformants using blue/white method and growth on amp+ plates
↓
Culture of isolated white colonies overnight at 37°C
↓
Mini-prep analysis of cultures for presence of recombinant plasmid

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Appendix 4. Mini-prep procedure

1. 150 µl of cultured cells centrifuged for 3 minutes; discard supernatant. Add 150 µl more of the cultured cells, centrifuge for 3 additional minutes; discard supernatant.
2. Resuspend pellets by vortex for at least 30 seconds, or until pellet is in solution.
3. Add 300 µl TENS buffer, vortex for at least 15 seconds.
4. Add 150 µl Na acetate (pH 5.2)
5. Centrifuge for 6 minutes.
6. Add 500 µl PCA, vortex for 2 seconds.
7. Transfer the supernatant to a new tube, being careful not to transfer the material at the interface of the two layers.
8. Add 1 ml 100% cold EtOH; place in -80°C freezer for 20 minutes.
9. Centrifuge for 10 minutes, discard supernatant; dry pellets under vacuum for 5 minutes.
10. Resuspend in 10 µl TE buffer.
11. Vortex samples quickly until pellet is in solution, place in H_2O bath at 65°C for 5 minutes.
12. Run samples for desired length of time in an agarose gel treated with ethidium bromide.

Appendix 5. PCR Protocol

Mix: 1 µl of EAFA (upstream) primer, 1 µl of ATP3 (downstream) primer, 2 µl of sterile H_2O, 1 µl of cDNA, and 45 µl of Supermix. The supermix is from GIBCO and contains buffer, taq polymerase, and magnesium chloride.

94°C for 30 seconds
50 °C for 00 seconds (setting on machine allowing cooldown)

50°C for 30 seconds
72°C for 00 seconds (setting on the machine allowing warmup)
72°C for 60 seconds

Run for 30 cycles; followed by 4°C set for infinity.