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The complexity of yolk proteins and their dynamics in the sea star, *Patiria miniata*

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Abstract

Oviparous animals store yolk proteins within the developing oocyte that are used in gametogenesis and as a nutritional source for embryogenesis. Vitellogenin and the major yolk protein are two of the most important yolk proteins among diverse species of invertebrates and vertebrates. Among the Echinoderms, members of the subphyla Echinozoa (sea urchins and sea cucumbers) express the major yolk protein but not vitellogenin, while an initial report documents that two Asterozoa (sea stars), express a vitellogenin (Prowse and Byrne, 2012). Our results indicate that sea stars contain 2 vitellogenins, Vtg 1 and Vtg 2, and the major yolk protein (MYP). In *Patiria miniata* these genes are differentially expressed in the somatic and germ cells of the ovary: Vtg 1 is enriched in the somatic cells of the ovary but not in the oocytes, whereas Vtg 2 accumulates both in oocytes and somatic cells, and MYP is not robustly present in either. Remarkably, Vtg 2 and MYP mRNA reappear in larvae: Vtg 2 is detected within cells of the ectoderm, whereas MYP accumulates in the coelomic pouches, the intestine, and in the posterior enterocoel (PE), the site of germ line formation in this animal. Additionally, the Vitellogenin 2 protein is present in oocytes, follicle cells and developing embryos, but becomes undetectable following gastrulation. These results help elucidate the mechanisms involved in yolk dynamics, and provide molecular information for better understanding the evolution of these important gene products.

Keywords

Vitellogenin; Major Yolk Protein; sea star; oogenesis; embryogenesis; *Patiria miniata*

Introduction

Most egg-laying animals store yolk proteins within the egg. Yolk proteins are important both for the developing gametes, and as a nutritional source during early embryogenesis until the larvae, or juvenile, is capable of feeding. Typically, large yolk protein precursors are

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synthesized in extragonadal tissues (Opresko and Wiley, 1987, Raikhel and Dhadialla, 1992, Reimer and Crawford, 1995, Voogt *et al.*, 1985, Wallace and Selman, 1981) and in the somatic cells that support the developing gametes (Majewska *et al.*, 2014, Reunov *et al.*, 2010, Unuma *et al.*, 2007, Unuma *et al.*, 2010, Unuma *et al.*, 2003). These precursors are then proteolytically cleaved into smaller yolk proteins while within cells in the gonad (Brooks and Wessel, 2002, Brooks and Wessel, 2003, Brooks and Wessel, 2004, Prowse and Byrne, 2012, Yamamura *et al.*, 1995). For most oviparous invertebrate and vertebrate animals, the primary large yolk protein precursor is Vitellogenin (Vtg; (Yamamura *et al.*, 1995)), a glycopospholipoprotein belonging to the Large Lipid Transfer Protein (LLTP) superfamily (Babin *et al.*, 1999, Smolenaars *et al.*, 2007). Vtg proteins contain a lipid-binding domain near the N-terminus, a Domain of Unknown Function 1943 (DUF1943), a von Willebrand Factor Type D domain (VWD), and conserved RXXR furin/subtilisin-like protease cleavage sites (Prowse and Byrne, 2012, Smolenaars *et al.*, 2007). Additionally, Vtg may contain a polyserine phosphovitin domain as seen in some vertebrates (Finn, 2007, Smolenaars *et al.*, 2007). While Vtg is typically found only in female animals, recent reports indicate that in some invertebrate species Vtg is present in both male and female gonads (Prowse and Byrne, 2012). Because Vtg is the primary yolk protein in invertebrates, it may have been inferred in earlier studies on echinoderms that the major protein of the yolk granules (major yolk protein (MYP)) is a Vtg. However, the sea urchin MYP is not a Vtg, but rather is a transferrin-like protein that can bind metals such as iron and zinc (Brooks and Wessel, 2002, Brooks and Wessel, 2003, Brooks and Wessel, 2004, Unuma *et al.*, 2007). Further, the MYP of sea urchins persists throughout development, a result inconsistent with its presumed role in nutrition for gametogenesis or development. Therefore, both functionally, and by sequence, Vtg and MYP are two distinct yolk proteins.

Much of the research on yolk proteins in echinoderms has focused on the Echinozoa subphylum and less on the Asterozoa subphylum. Most studies on sea star yolk proteins provide no direct evidence that indicate whether Vtg, MYP, or both are the primary yolk precursor proteins in sea stars (Kurata *et al.*, 2012, Reimer and Crawford, 1995, Voogt *et al.*, 1985, Yokota *et al.*, 2000). Importantly, Prowse and Byrne (2012) recently demonstrated that Vtg is present in *Patiriella regularis*, a species with small eggs and planktotrophic development, and in *Parvulastra exigua*, a species with large eggs and lecithotrophic development. Vtg from these sea stars contains characteristic vitellogenin sequences, protein domains, and shared features such as multiple furin/subtilisin cleavage sites for processing. It is, however, expressed in both sexes and lacks a phosphovitin domain.

Currently, the literature suggests that the Echinozoa subphylum of Echinoderms expresses MYP (Brooks and Wessel, 2002, Unuma *et al.*, 2007, Unuma *et al.*, 2003, Yokota *et al.*, 2003) while the Asterozoa subphylum expresses a Vtg protein, Vtg1 (Prowse and Byrne, 2012). We lack an understanding though of whether other vitellogenin paralogs exist in sea stars, of receptors for the vitellogenin(s), and whether sea stars have a transferrin-like MYP. Here we report that *P. miniata* contains, and differentially expresses, *Vtg1*, *Vtg2* and *MYP* mRNA in ovaries, oocytes, embryos, and even new expression in subdomains of larvae.

Materials and Methods

Animals and embryo culture

P. miniata were collected from several sites in southern California (www.scbiomarine.com; phalmay@earthlink.net) and embryos were grown as described (Foltz *et al.*, 2004). Briefly, sperm were collected from a gonad biopsy taken from the body cavity and placed into a microfuge tube on ice. Oocytes were collected from a gonad biopsy and some of them were matured *in vitro* with 2 μ M 1-Methyladenine. Resultant eggs were fertilized with a dilute sperm suspension and embryos were cultured as previously described (Hinman *et al.*, 2003). Samples from different developmental stages (ovary; young oocytes; full grown oocytes; mature oocytes; hatched blastula, 19 hpf; early gastrula, 23 hpf; mid-gastrula, 40 hpf; late gastrula, 48 hpf; early larva, 74 hpf; late larva, 164 hpf) were collected, fixed and stored in 70% ethanol at -20°C as previously described (Arenas-Mena *et al.*, 2000).

Yolk granule isolation

Yolk granules (platelets) were isolated from *P. miniata* basically as described (Wessel and Vacquier, 2004). Briefly, (all at 4°C) fertilized eggs were washed twice with calcium-free seawater, and then twice with KCl solution (0.55 M KCl; 1 mM EDTA, pH 7.0). The cells were then resuspended in 5 volumes of the KCl solution and Dounce homogenized. The lysate was centrifuged for 4 minutes at 400 x g, and the supernatant was then re-centrifuged for 10 minutes at 2400 x g. The resulting pellet was resuspended in KCl solution, and centrifuged. This pellet is highly enriched for yolk granules and their content proteins and was used here for further analysis (Appendix Figure 1).

Mass spectrometry analysis

Enriched fractions of yolk protein were loaded on a 4-12% gradient SDS-PAGE for Coomassie Brilliant Blue staining. The six main high molecular weight bands were excised and processed for *in gel* digestion using the *In gel*/tryptic digestion kit (ThermoFisher, Waltham, MA). Proteins were digested overnight at 37°C in 10 ng/ μ l trypsin, and the resultant peptides were fractionated on a reversed phase column, and introduced directly onto a Thermo-Finnigan LTQ linear ion trap mass spectrometer via electrospray ionization. The ensuing ions were then screened against a *Pm* ovary transcriptome transformed for mass-spec output (Reich *et al.*, 2015) (see Table 1).

RNA analysis

Whole mount *in situ* RNA hybridization was performed using a digoxigenin-labeled RNA probe as previously described (Arenas-Mena *et al.*, 2000). A cDNA from oocyte RNA was used as a template for PCR reactions, using specific primers of each gene of interest and adding a T7 RNA polymerase sequence in the 5' end of reverse primers. Genes were amplified using primers shown in Appendix Figure 1. Amplification products were transcribed with T7 RNA polymerase to yield an antisense RNA probe with the DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, IN, USA). Ovary, oocytes and embryos were fixed, hybridized, and the signals detected essentially as described (Ransick *et al.*, 1993). Negative controls for these experiments included the use of a non-relevant transcript

probe (Neomycin resistance, phosphotransferase gene). The sequences for each transcript can be found within GenBank as follows: Vitellogenin 1 (Vtg1) KU569217; Vitellogenin 2 (Vtg2) KU569218; Major yolk protein (MYP) KU569219; Vitellogenin Receptor (VtgR) KU569220.

Antibody Production

Rabbit polyclonal antibodies were generated against three peptides within Vitellogenin 2 from *P. miniata* (1) INPTEDMLDNILRC; 2) KQTDAETDSIDRIA; and 3) PFVKNMEEDPQVRV; see Appendix Figure 2) and affinity purified, all by GenScript (Piscataway, NJ). The results presented herein were from antibody #1, though each of the antibodies gave similar results.

Protein analysis and immunoblotting

Sea star tissues and embryos were collected and homogenized in modified lysis buffer (Cormier *et al.*, 2001), [40 mM Hepes pH 7.4, 100 mM β -glycero-phosphate, 0.2 mM Na_3VO_4 , 100 mM NaF, 20 mM tetrasodium pyrophosphate (PPi), 100 mM NaCl, 0.4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM DTT and one tablet of mini complete protease inhibitor cocktail (Roche) per 10 ml of lysis buffer] using a Dounce homogenizer.

Proteins of the lysates were separated by SDS-PAGE on an 4-12% polyacrylamide gel and blotted electrophoretically onto 0.22 μm nitrocellulose filters (Towbin *et al.*, 1979). The filters were incubated with anti-*Pm* Vtg2 antibody (1:1000, 2h at room temperature) and secondary anti-rabbit IgG antibody conjugated to peroxidase (1:3000, 1h at room temperature) and the antigen-antibody complex was measured by standard chemiluminescence reaction. All antibody incubations were made in 20 mM Tris-HCl (pH 7.5), 3% non-fat dried milk and 0.1% Tween-20. Controls included samples probed with anti-Vtg2 that has been pre-incubated overnight at 4°C with 40 $\mu\text{g}/\text{ml}$ of Vtg2 peptide to test the specificity of the antibody by blocking its capacity to recognize the Vtg2 peptide present in the sea star samples (Figure 3), pre-immune serum used as primary antibody (data not shown), and similar results from the three different anti-peptide antibodies (see above).

Immunofluorescence microscopy

Immunofluorescent labeling was performed on 10 μm paraffin sections of *P. miniata* ovaries that were fixed and processed as previously described (Laidlaw and Wessel, 1994). Paraffin sections were rehydrated in PBS containing 0.05% Tween-20 (PBST). Ovary sections were incubated for 2 hours with anti-Vtg2 (1:50), washed with PBST to remove unbound antibody and then incubated with secondary anti-rabbit IgG antibody conjugated to Alexa 488 (1:500) for 2 h. A last series of PBST washes included a 10-min wash with Hoescht 33342 (1:1000). Controls included pre-incubating Vtg2 with 40 $\mu\text{g}/\text{ml}$ of Vtg2 peptide to test specificity of the antibody and to identify non-specific signals. Images were captured using a LSM 800 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

Results

Mass spectrometry, transcriptome, and bioinformatic analyses of yolk proteins in *P. miniata*

Mass spectrometry was performed on peptides obtained from enriched yolk granule fractions of the sea star, *P. miniata*, that were subjected to SDS-PAGE. Appendix Figure 1 shows the regions that were cut for mass spectrometry analysis and the corresponding peptide sequences that were identified from each band. These peptides enabled us to identify the cognate mRNA and their close relatives by searching a *P. miniata* ovary transcriptome (Reich *et al.*, 2015). The peptide sequences identified as Vtg by mass spectrometry are each found in the full-length Vtg2 sequence (Appendix Figure 2), and many are conserved in Vtg2 from *P. regularis* and from *Patiria pectinifera* (Figure 1). Using the *P. miniata* transcriptome (Reich *et al.*, 2015), the Vtg2 sequence did enable us to identify a paralog Vtg gene with conserved Pfam domains of vitellogenin. Analysis of sequences submitted to the NCBI protein database also indicated that *P. regularis* actually contains two vitellogenins, Vtg1 (AHK12747.1), Vtg2 (AHK12748.1) and a MYP (AHK12749.1) whereas currently, only Vtg1 (AFH56436.1) has been reported in *P. exigua* within the available datasets and literature. The multiple sequences and domains experimentally identified in *P. miniata* are compared with Pfam domains present in sequences from *P. regularis* and *P. exigua*, and we find strong conservation between these multiple disparate species (Figure 1). A partial transcript coding for MYP was also found in the *P. miniata* transcriptome, its amino acid sequence is 90% identical to *P. regularis* MYP and 44% identical to *S. purpuratus* MYP (NP_999740.1), the founding member of this family in Echinoderms (Brooks and Wessel, 2002). By comparison, the next closest MYP-like sequence in echinoderms is a transferrin domain from melanotransferrin of *S. purpuratus* (XP_786179.3; only 25% identity with *P. miniata* MYP). These data suggest that asteroids contain three distinct yolk protein transcripts: two vitellogenins, *Vtg1*, *Vtg2* and one *MYP*.

Cleavage of the pro-Vtg is a crucial step in Vtg biosynthetic processing. For example, in the mosquito, the cleavage of the pro-Vtg is a rate limiting and regulated process (Dhadialla and Raikhel, 1990; Chen and Raikhel, 1996). The RXRR sequence has been predicted as a consensus sequence in the Vtg protein for the recognition site by furin, a proprotein converting enzyme structurally related to bacterial subtilisin (Hosaka *et al.*, 1991). Vitellogenins usually contain at least one furin/subtilisin-like protease minimal cleavage site, and Prowse and Byrne (Prowse and Byrne, 2012) identified this site in *P. exigua* as RKRRS, starting at residue 786. Analysis of the Vtg1 sequences from *P. miniata*, *P. regularis*, and *P. exigua* shows that each Vtg1 protein contains this cleavage site at a location within three amino acids from the site in *P. exigua* (data not shown) but the cleavage sites within Vtg2 are disparate. Analysis of the Vtg2 ORF from *P. miniata* revealed four potential furin/subtilisin-like protease minimal cleavage sites (Appendix Figure 3). These sites were also present in a similar location in *P. regularis* and *P. pectinifera* Vtg2 sequences (data not shown). Overall these results indicate that *P. miniata* ovaries express *Vtg1*, *Vtg2* and *MYP* transcripts, encoding 3 proteins that are distinct from each other, but that do have conserved orthologs within other sea star species.

Expression of yolk protein genes in the gonad and during early embryogenesis of *P. miniata*

Since mass spectrometry and bioinformatics analysis reveal that *P. miniata* ovaries contain three distinct yolk protein precursor transcripts for Vtg1, Vtg2 and MYP, we next wanted to determine the expression pattern of these transcripts in the *P. miniata* ovary. *In situ* RNA hybridizations were performed on ovaries, young oocytes, full-grown oocytes, and mature oocytes. Only somatic cells of the gonad, including the follicle cells surrounding the oocyte, express *Vtg1* mRNA, while *Vtg2* mRNA is expressed not only in these somatic cells but also in developing oocytes (Figure 2). Interestingly, neither the somatic cells of the gonad nor any stage oocyte seem to express significant levels of *MYP* mRNA (Figure 2). These data indicate that *Vtg1*, *Vtg2* and *MYP* mRNA are differentially expressed in the *P. miniata* ovary.

Vtg1 mRNA is not detectable in embryos (Figure 2), but surprisingly, *Vtg2* and *MYP* are differentially expressed during early embryogenesis and larval development in *P. miniata*. Low levels of *Vtg2* are detected at the anterior end of the embryo during mid- and late-gastrula and *Vtg2* transcripts become dramatically enriched at the anterior end of the embryo during early and late larva (Figure 2). A closer look at *Vtg2* expression at the anterior end of early and late larvae reveal that *Vtg2* mRNA is expressed in the ectoderm. The magnification of the anterior end of an early larva showed strong punctate enrichment of *Vtg2* transcripts in this region of the embryo, as well as in individual cells in other areas of the larva. (Appendix Figure 4). Conversely, low levels of *MYP* transcripts are present in the blastula stage and become strongly enriched in the coelomic pouches (CP), the posterior enterocoel (PE) and the intestine in early and late larva stages (Figure 2; left asterisk corresponds to the PE and right asterisk to the CP; arrowhead corresponds to the intestine).

Expression of Vtg2 protein in the sea star *P. miniata*

Based on our mass spectrometry and transcriptome analyses, we raised custom affinity-purified rabbit polyclonal antibodies against three peptides from the deduced amino acid sequence of Vtg2, which has a predicted size of 215 kDa. We tested all three different antibodies and achieved similar results with each. Here we report the results obtained with antibody #1. Figure 3A illustrates the controls for the Western blots: preabsorption with the peptides used to generate the antibody resulted in no detectable signal.

Additional controls included using the three different anti-peptide antibodies (resulting in similar bands identified, data not shown), and pre-immune sera (resulting in no significant bands, data not shown).

To determine the potential utilization of Vtg2 during oogenesis, we performed Western blot analysis on fractions enriched with oocytes of various stages. Two major bands of around 180 kDa and 140 kDa and a minor band of about 115 kDa are present in young oocytes (Figure 3B). In full-grown oocytes and eggs the major doublet band at ~180 kDa disappears and two distinct less intense bands, one slightly above 180 kDa and one slightly under 180 kDa (~170 kDa), are present and the intense band at ~140 kDa remains (Figure 3B). No band at ~115 kDa is detectable in full-grown oocytes and eggs (Figure 3B). The three higher

molecular weight bands around 180 kDa present in all stages of oocytes are also present in the ovary (data not shown). Proteolytic cleavage of Vtg2 may not yet have occurred in young oocytes and therefore the major doublet band of ~180 kDa has not yet been cleaved. Additionally, since young oocytes sometimes have follicle cells attached to them the major doublet band of ~180 kDa may include an unprocessed Vtg2 present in follicle cells.

Interestingly the high molecular mass bands detected in the oocytes may (Figure 3B) correspond to furin/subtilisin-like protease cleavage products that would be detected by this antibody (Appendix Figure 3, numbers in bold). The band above 180 kDa may result from cleavage once at the RTQRS site located at residue 235, which would produce a 189 kDa product, while the band around 140 kDa would be a product if Vtg2 was cleaved once at the RVPRS site located at residue 1098, which would produce a 122 kDa product. If the protease cleaved once at RVQRS at residue 1368 a product of 154 kDa would be produced. Additionally, cleavage at more than one site could produce products of 177, 129 and 97 kDAs in size, all detectable by this antibody. We acknowledge that the bands seen on immunoblots are relative mobilities and not mass determinations, but overall, the sizes and changes of the Vtg2 bands correspond generally with the predictions made by sequence analysis and the furin consensus sites.

Vtg2 is present in embryos during the late blastula to late gastrula stages but is undetectable in 4-day and 9-day larvae (Figure 3B). The two intense bands of slightly over 180 kDa and about 140 kDa are present in these embryos and protein levels appear to decrease from late blastula to late gastrula (Figure 3B). Additionally, two faint bands of over 200 kDa are present in late blastula, mid gastrula and late gastrula stage embryos and decrease as development progresses, becoming undetectable in 4-day and 9-day larvae (Figure 3B). These >200 kDa bands may represent full length unprocessed Vtg2, similar to those detected in ovary, testis and pyloric stomach in adult animals (data not shown). Immunoblot analysis of Vtg2 in larvae that were either fed or unfed for 20-days did not show any perceptible differences compared to the 9-day larva sample (data not shown), suggesting that food does not affect Vtg2 expression in larvae.

Immunolocalization of Vtg2 in the *P. miniata* ovary

Ovary sections (10µm) were immunolabeled with anti-Vtg2 antibody to determine the site of Vtg2 in the *P. miniata* ovary. Figure 4 indicates that Vtg2 is present in the follicles cells and the cells of the ovarian wall. To test the specificity of the antibody, we pre-incubated Vtg2 antibody with 40 µg/ml of Vtg2 peptide (Figure 4C), which resulted in no detectable signal, as was seen by immunoblotting as well (Figure 3A). Thus, the signal detected (Figure 4A-B) appears specific for Vtg2. However, we did not see Vtg2 in oocytes in these 10µm sections of ovary in contrast to the Western blot results (Figure 3B). It is possible that Vtg2 is not recognized by the antibody in oocytes due to its conformational shape, its post-translational modifications of Vtg2 in the oocytes, and/or the packing of the protein into dense, near crystalline arrays (Reimer and Crawford, 1995) that hide the epitope of the antibodies generated here. Each of the peptides used for antibody generation reside within a region of about 150 amino acids within the vitellogenin domain.

Discussion

Here we report that the sea star, *P. miniata*, contains two forms of Vitellogenin, Vtg1 and Vtg2, in addition to MYP. Prowse and Byrne (2012) reported that the sea stars, *P. exigua* and *P. regularis* contain transcripts for a true Vtg, the first such report, and now seeing a larger vitellogenin family in more sea stars validates the original contentions (Prowse and Byrne, 2012). These vitellogenin sequences also appear well conserved; *P. exigua* and *P. regularis* sequences obtained from the NCBI database confirm our findings that *P. miniata* express both Vtg1 and Vtg2. For example, the N-terminal, Internal-1 and Internal-2 peptide sequences used to identify Vtg from *P. exigua* (Prowse and Byrne, 2012) are almost identical in sequence to Vtg1 from *P. miniata*, *P. pectinifera* and *P. regularis* but are not present in Vtg2 (data not shown). Furthermore, the several peptide sequences identified by mass spectrometry on yolk proteins isolated from *P. miniata* (Table 1) are not present in Vtg1 from these three species but are present in Vtg2 from *P. regularis* as well as *P. pectinifera*, a species within the same genus as *P. miniata*.

The presence of Vtg2 in *P. regularis* and *P. miniata* but not in *P. exigua* may shine light on the evolution of yolk proteins in echinoderm subphylum, Asterozoa. Asterozoa phylogeny based on mtDNA indicates that *P. exigua* likely diverged from the stem lineage first followed by *P. regularis*, with *P. miniata* likely branching most recently (Byrne, 2006, Yasuda *et al.*, 2006). The lack of Vtg2 in *P. exigua* suggests that it appeared subsequent to Vtg1, following this split in species (Figure 1). Furthermore, analysis of the Pfam domains between *P. miniata* and *P. regularis* indicate *P. miniata* has a duplicated VWD domain suggesting may have evolved later than *P. regularis* (Figure 1; Appendix Figure 5). Moreover, both *P. miniata* and *P. regularis* have relatively small oocytes and planktotrophic larval development while *P. exigua* has large oocytes and lecithotrophic larval development. Vtg1 and Vtg2 are present in both *P. miniata* and *P. regularis* (planktotrophic) while only Vtg1 appears to be present in *P. exigua* (lecithotrophic). The evolution of Vtg2 could be a result of a switch between lecithotrophic and planktotrophic development in that the oils and lipids within lecithotrophic eggs harbor significant energy, and may in some way obviate utility for an additional vitellogenin gene.

In situ RNA hybridization results indicate that *Vtg1*, *Vtg2* and *MYP* transcripts are differentially expressed during oocyte development and early embryogenesis. MYP protein in sea urchins is made in the adult gut, transported to the ovary (likely by diffusion) and up taken by somatic cells that transport the protein to the oocyte, which endocytoses it via a dynamin-dependent mechanism (Brooks and Wessel, 2002, Brooks and Wessel, 2003, Brooks and Wessel, 2004). The lack of mRNA expression in sea star oocytes does not exclude the possibility that MYP protein is actually present in the oocytes, since MYP may also be synthesized primarily in extra-gonadal tissue and taken up into sea star oocytes much like seen in the sea urchin.

Herein, we report for the first time the re-expression of mRNA encoding yolk proteins during embryogenesis and larval development in the sea star. *Vtg1* mRNA does not appear to be expressed in embryos up to late larva stage whereas substantial, and differential *Vtg2* and *MYP* mRNA expression was surprising. These results are particularly curious since the

MYP transcript is present in tissues that become the major rudiment tissues for larval development and for the germ line (Inoue *et al.*, 1992, Wessel *et al.*, 2014), and we do not see any protein made from the *Vtg2* mRNA (Figure 3). We also do not see any Vtg2 protein even later in larvae (20 days) so we currently have no explanation for any functional role of the *Vtg2* mRNA. Clearly antibodies to MYP are needed in order to test the same question of the *MYP* mRNA.

Vtg2 protein is present in embryos up to late gastrula stage in the sea star, *P. miniata*, and then is gone, presumably from utilization in development (Figure 3). An extremely intense band of around 140 kDa is present in all of these embryonic stages, and likely represents the cleavage product of the large Vtg2 precursor into the N-terminal lipovitellin I product as observed in chicken Vtg2 (Yamamura *et al.*, 1995). Interestingly, *P. miniata* Vtg2 has a furin/subtilisin-like protease cleavage site at residue 1098, which if cleaved here would produce an expected product of 122 kDa similar to that seen in chicken Vtg2, which is cleaved into a 1098 aa lipovitellin I product (Appendix Figure 3). Furthermore, this product is detectable by the anti-Vtg2 antibody and is a major band detected in oocytes and developing embryos. Another similarity between *Pm* Vtg2 and chicken Vtg2 is the presence of 2 more furin proteolytic cleavage sites in the C-terminus, although *P. miniata* cleavage sites are located at different positions. It is also worth noting that some invertebrate Vtgs lack a phosvitin domain, like Vtgs from *P. exigua* and *P. regularis* (Prowse and Byrne, 2012). *P. miniata* also lacks a phosvitin domain, which may contribute to differences in cleavage between chicken Vtg2 and sea star Vtg2. However, the cleavage at position 1098 may be an important and conserved cleavage mechanism between invertebrate and vertebrate Vtg2 precursors.

The finding that sea stars not only contain vitellogenin (Prowse and Byrne, 2012), but two vitellogenins and an MYP (data shown herein) provides great resources to understand the role of yolk storage proteins in development, how they have changed in evolution, and how they may fit within the reproductive strategy of an organism. Since other main taxa of echinoderms also have lecithotrophic and plautotrophic reproductive strategies, we now have an important task of testing whether lecithotrophism impacts the vitellogenin profile of the animal. Perhaps an oil-based nutrient system is counter to import of lipids by vitellogenin proteins, or that two Vtgs then require multiple and different storage vesicles within the oocyte. It is also important to know when and where MYP originated within the phylum. By virtue of the presence of MYP seen here in the early germ line (the posterior enterocoel) and the fact that it is not utilized early in development in the sea urchins tested (Brooks and Wessel, 2002, Brooks and Wessel, 2003, Brooks and Wessel, 2004), suggests that MYP may be doing something different than vitellogenin, even though it is stored in a similar granule. The diversity of developmental and reproductive strategies within echinoderms makes this phylum an excellent resource for these broad, important questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conserved domain composition in yolk proteins

Figure 1. Comparison of protein domains of Vtg1, Vtg2 and MYP in sea stars

Vtg1, Vtg2 and MYP from *P. miniata* (Pm) were analyzed for conserved Pfam domains and compared to proteins from *P. regularis* (Pr), *P. exigua* (Pe) or from the sea urchin *S. purpuratus* (Sp). Right column indicates the percent identity of *P. miniata* sequence to the other sequences determined by alignment with Clustal W for full length sequences, or with NCBI for partial sequences. The domains identified are Vitellogenin-N (PF01347), Duf1943 (PF09172), von Willibrand type A domain (PF00092), and Transferrin (PF00405).

Figure 2. Expression of yolk protein transcripts during *P. miniata* embryonic development

Line 1: Vitellogenin 1 expression is only detected in gonad tissue and in follicle cells (seen surrounding the young oocyte). Line 2: Vitellogenin 2 transcripts are enriched in the gonad, in the oocytes and then re-accumulates during gastrulation in the anterior region of the embryo. Line 3: Major Yolk Protein transcripts are largely absent in gonads and oocytes, but accumulate beginning in blastula/gastrula stages and become strongly enriched in the coelomic pouches (CP, right asterisk), the posterior enterocoel (PE, left asterisk) and the intestine (arrowhead) in larval stages. Line 4: A sequence for the Neomycin (Neo) resistance gene was used as a negative control for the hybridization procedure. Dorsal views of the larva. Scale bar = 100 μ m. Note that the row of young oocytes is magnified.

Figure 3. Expression of Vtg2 protein in the sea star *P. miniata*

A) Specificity of the anti-Vtg2 antibody. Western blot of proteins from ovary and pyloric stomach probed with anti-Vtg2 that has been pre-incubated with 40μg/ml of Vtg peptide. Corresponding loading controls included transfer membranes stained with Ponceau red (data not shown) and tubulin immunodetections. 5 μg of protein were loaded in each lane. Molecular weight markers are indicated on the left. B) Immunoblot detection of Vtg2 during oogenesis oocyte development (40 cells were loaded per line) and early development (40 embryos were loaded for late blastula and mid gastrula. 35 embryos were loaded for late gastrula. 25 embryos were loaded for 4-day and 9-day larva). Molecular weight markers are indicated on the left.

Figure 4. Vtg2 is enriched in the follicle cells of the ovary

Immunolocalization of Vtg2 (signal in A and B) in 10 μ m ovary sections showed that Vtg2 protein is enriched in follicle cells surrounding the oocytes and in the ovarian wall. Cell nuclei are stained with Hoechst. C) Control was made pre-incubating Vtg2 antibody with 40 μ g/ml of Vtg2 peptide to test specificity of the antibody. Only the nuclear Hoechst label is present. OW, ovary wall; Oo, Oocyte Scale bar = 20 μ m.

The peptides detected by mass spectroscopy (Appendix Figure 1) and identical peptides seen in other species

Table 1

Mass spectrometry analysis of purified yolk proteins from *P. miniata*

Peptide sequence	Sea star species
KLQTEENPAKH [*]	<i>Pm</i> , <i>Pp</i> (ts, ov), <i>Pr</i>
KGPVLNGINADKQ	<i>Pm</i>
KINQVCSEVKK	<i>Pm</i> , <i>Pp</i> (ts)
KINQVCSEVKKQ	<i>Pm</i> , <i>Pp</i> (ts)
KAIGNAGLILKQ	<i>Pm</i>
KQTTDAETDSIDRI	<i>Pm</i>
KSKTVVDVDLRV	<i>Pm</i>
RVQGIYSLRR	<i>Pm</i> , <i>Pr</i>
KNLRDIAEKI	<i>Pm</i> , <i>Pp</i> (ts), <i>Pr</i>
RDLDALDGGQLRT	<i>Pm</i> , <i>Pp</i> (ts)
RTTVIDSDTNALKA	<i>Pm</i>
TTVIDSDTNALKA	<i>Pm</i>
KFFGNELRT	<i>Pm</i> , <i>Pp</i> (ts), <i>Pr</i>
RLTDVLSDKA	<i>Pm</i> , <i>Pp</i> (ts, ov)
KLNVDVAKK	<i>Pm</i> , <i>Pp</i> (ts, ov), <i>Pr</i>
KLNVDVAKKL	<i>Pm</i> , <i>Pp</i> (ts, ov), <i>Pr</i>
RSIFNFDEVRS	Not recognized as Vig

^{*} Note: Indicates that the H is Q in translated ORFs in all species it was detected in; ts, testis transcriptome; ov, ovary transcriptome; *Pm*, *P. miniata*; *Pr*, *P. regularis*; *Pp*, *P. pectinifera*.