A spatially restricted molecule of the extracellular matrix is contributed both maternally and zygotically in the sea urchin embryo

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The extracellular matrix of the early sea urchin embryo is known to have an important functional role in morphogenesis and in the regulation of cell type specific gene expression. We have undertaken an immuno-cDNA screen to identify the constituents of the embryonic blastocoeic-extracellular matrix. Here we describe a newly identified member of the extracellular matrix that we have designated ECM 3. The transcript encoding ECM 3 is approximately 9.5 kb in length and partial DNA sequence contains no significant similarity to other sequences in the Genbank. This transcript is present in eggs and early embryos, and early in gastrulation the transcript accumulation increases approximately 25 fold. In situ RNA hybridization shows that the mRNA is present uniformly throughout eggs and early embryos, but beginning at mesenchyme blastula stage, RNA accumulation is selective to cells of the ectoderm except at the animal pole, where ECM 3 mRNA is greatly reduced. In this species, Lytechinus variegatus, the animal pole ectoderm is the site of fusion with the invaginating endoderm during formation of the mouth. In situ analysis of protein expression using a monospecific polyclonal antisera made against recombinant ECM 3 polypeptides shows that during gastrulation the ECM 3 protein accumulates selectively in the basal lamina and blastocoelar regions adjacent to the ectoderm in all regions except for the ectoderm at the animal pole. The ECM 3 protein is not detected in other regions of the blastocoel e.g. adjacent to the endoderm. ECM 3 is also contributed maternally; the ECM 3 protein is synthesized during oogenesis and stored in oocytes within membrane-bound vesicles in the vicinity of Golgi complexes. Following fertilization ECM 3 is selectively secreted basally into the nascent blastocoel. No accumulation is detected in apical regions of the blastomeres or in the hyaline layer/apical lamina. This newly described molecule of the extracellular matrix thus demonstrates expression regulated both by secretion and by cell type specific gene expression, and shows a correlation between a microenvironment of the extracellular matrix and a morphogenetic event.

Key words: basal lamina, extracellular matrix, gastrulation, regulated secretion.

Introduction

Formation of the blastocoel in sea urchin embryos begins after several cell divisions as molecules are secreted directionally into a nascent basal compartment. Continued expansion of the blastocoel resulting from secreted extracellular matrix molecules forces fundamental changes in the way cells interact. First, as the blastocoel is formed, cells that were adjacent to each other become separated as they form a single cell layer. Cells separated by a blastocoel can only communicate by propagation of signal along a cell layer or by secreted factors that permeate the blastocoel. Second, blastocoel morphogenesis creates an environment that is biochemically distinct from the outside of the embryo; this biochemical distinction may be influential in establishing cell polarity. Lastly, the diversity of ECM molecules, the timing of their secretion, and their localization in the embryo create extracellular microenvironments with the potential for selective influences on different cells.

Molecules of the extracellular matrix in the early sea urchin are known to influence cell type specific gene expression and morphogenesis of the embryo. Gastrulation of the embryo appears to be particularly sensitive to the biogenesis of the extracellular matrix whereas the earlier developmental stages of cleavage and blastulation are less affected. Selective inhibition of the biosynthesis or deposition of certain molecules of the extracellular matrix, for example, sulfated glycosaminoglycans, collagen or ECM 1, results in the inhibition of endoderm and mesoderm differentiation (Karp & Solorsh...
1974; Wessel & McClay 1987; Wessel et al. 1991; Benson et al. 1991; Ingersoll & Ettensohn 1994). This phenotype is not the result of general toxicity since the inhibition is reversible and has no effect on the expression of several proteins and mRNA studied (Wessel et al. 1989a). Instead, certain cell-ECM interactions appear to be important both for cell migration and for signaling pathways that result in specific gene expression.

The extracellular matrix of the sea urchin embryo is a heterogeneous population of proteins many of which are similar to extracellular matrix molecules described in vertebrates. For example, both fibrillar and non-fibrillar collagens have been identified by cDNA cloning (Venkatesan et al. 1986; D’Alessio et al. 1990) and cross-reactivity of vertebrate antibodies have been used to identify laminin (Spiegel et al. 1983; Wessel et al. 1984; McCarthy & Spiegel 1987), fibronectin (Spiegel et al. 1983; Wessel et al. 1984), heparan sulfate proteoglycan (Wessel et al. 1984), and several glycosaminoglycans (Solorsh & Karp 1982). In addition, monoclonal antibodies have identified several other extracellular matrix molecules with unique expression patterns (Wessel et al. 1984; Burke & Tambelino 1990; Ingersoll & Ettensohn 1994). Some ECM molecules, like Meso 1 (Wessel et al. 1984) and Endo 16 (Nocente-McGrath et al. 1989) are not present until gastrulation, whereas others, like ECM 1 (Ingersoll & Ettensohn 1994), laminin (McCarthy & Burger 1987; Wessel et al. 1984), and 1B10 (Wessel et al. 1984) are present throughout development. The oocyte appears to store this latter class of ECM molecules and to provide the embryo with presynthesized ECM components during its period of rapid growth. The maternally contributed, presynthesized ECM molecules presumably make the early embryo insensitive to treatments that disrupt the new synthesis of ECM molecules as occurs later in development. Although the sites of storage in the egg of presynthesized ECM molecules have not been identified, many ECM constituents in this embryo appear to have a maternal contribution.

A goal of our research has been to understand how the extracellular matrix environment is created and what molecules influence cell differentiation at gastrulation. Previously we have identified ECM constituents with monoclonal antibodies generated to the embryonic ECM (Wessel et al. 1984). These reagents were useful in showing the complexity of spatial and temporal heterogeneity of ECM constituents. The ability to characterize different ECM constituents with these MoAb was limited however, because each of the determinants recognized by these monoclonal antibodies included carbohydrates that were shared by several glycoprotein species in the embryo. This precluded both the purification of single gene products and screening of cDNA libraries for further characterization. Biochemical approaches to isolate individual molecules either by immunoprecipitation or by fractionation were also limited because of the heterogeneous size and insolubility of the target molecules. The use of heterologous cDNA screens for extracellular matrix sequences under relaxed stringency has been useful and has, for example, resulted in the identification of several collagen genes in the sea urchin embryo (Venkatesan et al. 1986; Esposito et al. 1994). In an attempt to identify new extracellular matrix molecules in the blastocoel of the embryo, we have used an immuno-cDNA screen with antibodies generated against the peptide backbone of the isolated embryonic extracellular matrix.

We show here a newly identified member of the extracellular matrix, designated ECM 3, and describe its biogenesis both from maternal stores in the eggs and from specific cell types of the early embryo. We find that ECM 3 is present in eggs, where it is stored within membrane bound vesicles. Following fertilization, these molecules are secreted into the nascent blastocoel. During gastrulation, ECM 3 is expressed selectively by both oral and aboral ectodermal cells except by those cells at the animal pole. In the species studied here, Lytechinus variegatus, the animal pole is the target site of archenteron elongation and the point of fusion of the archenteron with the ectoderm during formation of the mouth. Thus the localization of ECM 3 delineates the target site of the archenteron during gastrulation and shows that heterogeneous expression of an ECM protein is correlated with a specific morphogenetic event.

Methods

Handling embryos

Adult Lytechinus variegatus were obtained from Scott’s Services and from Sue Decker (both of Miami, FL, USA) and from the Duke University Maine Laboratory (Beaufort, NC, USA). Gametes were obtained, fertilized and cultured as described (McClay 1986).

Preparation of extracellular matrix and generation of antisera

Polyclonal antisera were generated to blastocoel extracts of early plutei. These extracts were prepared by dissociating embryos with hyaline extraction media (HEM; McClay 1986) to remove ectoderm and endoderm cells. The extracellular matrix ‘bags’ were washed several times by gentle centrifugation with HEM and then extracted by light homogenization with a Teflon pestle for 10 min at 4°C with calcium-free seawater containing 0.1% Triton X-100, and a protease inhibitor
cocktail (0.5 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L EGTA; 0.3 units aprotinin; Sigma, St Louis, MO, USA). After several cycles of washing with extraction buffer and centrifugation in a clinical centrifuge, the extract was resuspended in 0.25 mol/L sodium acetate buffer pH 6.5, 20 mmol/L EDTA, 10 mmol/L 2-mercaptoethanol, 0.5% NP-40, 0.1% SDS, then denatured at 100°C for 3 min, and treated with endo-glycosidase F (Boehringer Mannheim, Indianapolis, IN, USA) for 16 h at 37°C. The extract was frozen at −80°C until needed. Approximately 75 µg of denatured and deglycosylated extract was injected subcutaneously into New Zealand White rabbits every three weeks for 3 months. One week following the last boost, plasma was collected from ear veins and antibodies were purified from the resulting sera by protein A affinity chromatography (Harlow & Lane 1988).

cDNA library screen
Antiserum showing the highest titer to the blastocoelear extracellular matrix by in situ immunofluorescence was used to screen a λZAP cDNA library constructed from prism stage polyadenylated RNA (Stratagene, La Jolla, CA, USA; Wessel et al. 1989b). BB4 cells harboring the λZAP bacteriophage were plated onto NZYM agar plates and cultured at 42°C until plaque formation was visible. Nitrocellulose filters were laid on the bacterial lawn and incubated overnight at 37°C. The nitrocellulose filters were removed from the plates and washed several times for a total of 4 h with Blotto buffer (50 mmol/L Tris pH 7.5, 0.9% NaCl, 0.05% Tween-20, and 3% non-fat dry milk) and then incubated with the antixtracellular matrix antibodies (diluted 1/200) at 21°C for 2 h. The filters were washed overnight with several changes of Blotto, and then incubated with 125I-labeled donkey anti-rabbit antibody (105 cpm/mL of 10 µCi/µg; Amersham, Arlington Heights, IL, USA) for 2 h. After washing overnight with several changes of Blotto, immunolabel signals were detected by autoradiography on Kodak X-Omat film. Plaques reactive to the antibody were purified to homogeneity by repeated plating and immunolabeling, and the recombinant cDNA of each plaque isolate was excised with helper phage R408 (Stratagene, La Jolla, CA, USA) and recovered as a Bluescript plasmid (Short et al. 1988).

Production of fusion proteins and monospecific antibodies
The immuno-positive cDNA clones were subcloned into pWR590 (Guo et al. 1984) and the resulting β-galactosidase-fusion proteins produced in XL1-Blue cells (Stratagene) were isolated by preparative SDS-PAGE and injected subcutaneously into a New Zealand white rabbit. The rabbit was boosted twice at 3-week intervals, and immunoglobulins were purified from the resulting antisera by protein A chromatography (Harlow & Lane 1988).

DNA sequencing
The DNA sequence was determined by the Sanger chain termination method (Sanger et al. 1977) using [32P]dATP and a modified T7 DNA polymerase (Tabor & Richardson 1987). Sequence data were assembled and analyzed using the University of Wisconsin Genetic Computer Group (UWGCGr) sequence analysis package (Devereux et al. 1984).

RNA analysis
Total RNA isolated from embryos at several developmental stages was analyzed by hybridization to RNA gel blots essentially as described (Bruskin et al. 1981). Probes were made from ECM 3 cDNA clones after excision from pBluescript with Eco RI by radiolabeling with [32P]dCTP by random oligonucleotide priming (Feinberg & Vogelstein 1983).

Electrophoresis and western blot analysis
Eggs and embryos were subjected to SDS-PAGE and western blot analysis essentially as described (Towbin et al. 1979). For each stage of analysis, an equal number of embryos were pelleted from the culture, resuspended in SDS-PAGE sample buffer containing 10 mmol/L DTT, and denatured for 3 min at 100°C. The proteins were resolved on a 7.5% acrylamide gel and either stained with Coomassie Blue or transferred to nitrocellulose for immunolabeling as described (Towbin et al. 1979). For immunolabeled blots, the procedure was as described above for plaque lift screening.

In situ RNA hybridization
L. variegatus embryos were fixed in 2% glutaraldehyde and were prepared for in situ RNA hybridization as described (Angerer et al. 1987). 3H-labeled antisense transcripts were synthesized by first linearizing the plasmids with BamHI and then transcribing the probe using T7 RNA polymerase. A sense probe was synthesized by linearizing the plasmid with SalI and then transcribing the probe using T3 RNA polymerase. Sections of embryos were hybridized with the labeled probes, washed at Tm-5°C, and prepared for autoradiography as described (Angerer et al. 1987).

Immunolocalization
Immunofluorescence localization of ECM proteins was performed on sections of embryos that were fixed and
processed as previously described (Wessel et al. 1984). Primary antibodies were diluted between 1/50 and 1/200 and the secondary antibody (fluorescein conjugated affinity purified goat anti-rabbit IgG, Organon Teknika, Research Triangle Park, NC, USA) was diluted 1/40. Electron microscopic immunolabeling was performed as described (Wessel 1989) using embryos fixed with 0.5% glutaraldehyde and 5% formaldehyde and embedded in Spurr’s resin (Spurr 1969). Primary antibodies were diluted 1/500–1/2000 and the secondary antibody [gold-conjugated (15 nm) affinity purified goat anti-rabbit IgG; Janssen, Beerse, Belgium] was diluted 1/30. Specimens were observed at 80 KeV in a Philips 410 electron microscope.

Results

The blastocoelic extracellular matrix (ECM) was isolated as previously described (Wessel et al. 1984) and N-linked carbohydrates were cleaved from protein chains by endoglycosidase treatment. Antisera generated to this ECM preparation selectively labeled molecules within the blastocoel and basal lamina of the developing embryo (data not shown). With this sera we screened approximately 340,000 plaques of a cDNA library constructed from prism stage polyA + RNA. The screen resulted in 26 immunopositive clones (approximately 0.5% of the plaques containing cDNA that randomly would be in the correct orientation and in frame with the β-galactosidase leader peptide of the λ-ZAP vector) which ranged in length from 1.2 to 4.5 kb. These inserts were characterized by restriction mapping and DNA sequence to identify redundancies, false positives and non-ECM sequences. The cDNA representing candidate ECM proteins were then cloned into pWR590 (Guo et al. 1984) to generate β-galactosidase fusion proteins that were used to make monospecific polyclonal antisera in rabbits. These monospecific antisera were then used to test the authenticity of the ECM constituents in situ by immunofluorescence to verify the extracellular nature of the gene product. Of the 26 positive cDNA identified in the library immunoscreen, only two different ECM gene products were found. Six of the cDNA represented the same ECM gene product, designated ECM 3, and one cDNA encoded an independent ECM gene product, designated ECM 18. Several of the isolates from this screen encoded non-ECM proteins, presumably resulting from contaminants in the blastocoel matrix preparation used to raise the antibodies. This report describes our initial characterization of the extracellular matrix protein encoded by ECM 3.

The initial cDNA encoding ECM 3 was 1456 bp in length and was used to isolate overlapping cDNA clones that extend toward both the N- and C-terminus of the protein (Fig. 1). DNA sequence data of over 4 kb of ECM 3 cDNA show no significant similarity to any sequence in Genbank. The mRNA of ECM 3 was detected by RNA gel blots through embryogenesis as a single transcript of approximately 9.5 kb (Fig. 2). ECM 3 mRNA increases in abundance gradually through development until gastrulation, when a greater than 25-fold increase occurs within several hours in development just prior to the mesenchyme blastula stage. Message levels then decrease several fold through gastrulation.

![RNA gel blot of ECM 3. ECM 3 cDNA were oligo-labeled with [32P]-dCTP and hybridized to a gel blot of total RNA (10 μg each) from eggs and different stages of Lytechinus variegatus embryos. A single transcript size of 9 kb was seen with maximal accumulation in mesenchyme blastulae. Shown is an autoradiograph using ECM 3 cDNA, though similar results were obtained using ECM 3A and 3B. Ubiquitin mRNA levels were used to ensure that equivalent RNA amounts were hybridizable in each lane (data not shown). E, egg; EB, early blastula; LB, late blastula; MB, mesenchyme blastula; G, gastrula; P, pluteus.](image-url)
to the larval stage, though signal intensity in larvae is still approximately ten fold greater than in eggs. Approximately equal loading of RNA was ensured by subsequent hybridization of ubiquitin mRNA (data not shown), known to be present at equal levels throughout development (Gong et al. 1991).

The immunolocalization of ECM 3 protein during sea urchin development is shown in Fig. 3. ECM 3 is present in developing oocytes and eggs as a punctate label throughout the cytoplasm. Developing oocytes contain significant ECM 3 signal as does the double basal laminar structure of the ovarian capsule, though accessory cells of the ovary contain only minor label (the majority of the fluorescence in the accessory cells of Fig. 3A is a result of autofluorescence). The subcellular location of ECM 3 in eggs is shown in Fig. 4. The ECM 3 protein is found within membrane bound vesicles that are on average 0.75–1.00 μm in diameter and which have irregularly stained contents. Often these vesicles are close to Golgi organelles, but no label is ever seen to concentrate within the Golgi complex or within other organelles of the egg. Many other membrane bound vesicles of similar size and morphology are present in oocytes in the vicinity of Golgi, yet only a small percentage of these vesicles appear to contain ECM 3. It appears as though ECM is found within a subset of the Golgi associated vesicles and is not uniformly packaged. The localization of ECM 3 in this membrane bound compartment of the egg is consistent with these vesicles being a part of the secretory pathway expected of an extracellular matrix protein.

Following fertilization and early cleavage, ECM 3 is polarized to the basal aspect of the early blastomeres and is secreted into the nascent basal lamina (Fig. 3). No secretion from the apical cell surface is apparent and cytoplasmic vesicles are no longer detected. During formation of the blastocoel, the ECM 3 signal within the blastocoel is concentrated uniformly along the wall of the blastocoel. Since the three dimensional structure of the early blastocoel in this and many other embryos is difficult to preserve upon fixation, the uniform labeling along the blastocoel wall may be the result of molecules collapsing onto the wall of the blastocoel, although this pattern of accumulation was consistently seen with a variety of fixation and embedding protocols that included formaldehyde, glutaraldehyde/formaldehyde combinations, and whole mount preparations fixed in methanol (data not shown).

During late blastula and mesenchyme blastula stages, ECM 3 is localized to the basal lamina of the ectoderm.

**Fig. 3.** Immunolocalization of ECM 3. Oocytes and embryos were processed for immunolocalization using antibody to fusion peptide pWR 3 on paraffin sections. Results are similar using antibody to fusion peptide pWR 3B (data not shown). Immunofluorescence photographs are adjacent to corresponding brightfield photographs. (A, B) In ovaries, ECM 3 is present in developing oocytes and in eggs in a punctate pattern throughout the cytoplasm but not in the nucleus. ECM 3 is also present in the double basal amin of the capsule of the ovary. Fluorescence in the accessory cells is autofluorescence and not a result of selective ECM 3 immunostaining. (C, D) During early development the intracellular ECM 3 signal is deposited into the nascent blastocoel and cytoplasmic label is lost. (E, F) In mesenchyme blastula ECM 3 accumulates selectively at the blastocoel wall underlying the equatorial ectoderm. No accumulation is detectable near the vegetal plate nor the animal pole. (G, H) During gastrulation neither the mesenchyme cells nor the invaginating endoderm have detectable ECM 3 signal. This pattern persists through development to the plateus (I, J, K, L) with limited accumulation underlying the endoderm. Note that the developing arms contain signal about 2/3 to the distal tip. e, egg; oo, oocyte; oc, ovarian capsule; n, nucleus; ac, accessory cells; bc, blastocoel; vp, vegetal plate; ec, ectoderm; en, endoderm; asterisk indicates the animal pole; bar in L = 20 microns.
but is reduced or excluded from the basal lamina at the animal cap (Fig. 3E,G). The animal cap region of the embryo is of interest since this site is targeted by the invaginating endoderm for eventual tissue fusion to form the mouth. The ECM at the animal pole has significantly reduced levels of ECM 3, and through gastrulation its signal remained low relative to other regions of ectoderm and was comparable to signal levels associated with the endoderm during gastrulation and formation of plateus. In larvae ECM 3 continues to accumulate in the ectodermal basal lamina including the developing arm buds. Note that ECM 3 signal is detected in the proximal two-thirds of the arms but not at the very tip of the arms where skeletal elongation occurs.

Utrastructural immunolocalization confirms that ECM 3 is deposited into the blastocoel (Fig. 5). Thin sections of late gastrulae were immunolabeled with ECM 3 antibody and colloidal gold conjugated secondary antibody, and signal was detected only within the blastocoel of the embryo, both along the basal lamina and in nearby extracellular fibers within the blastocoel. The immunogold label was consistently seen along these
Fig. 6. *In situ* RNA localization of ECM 3 mRNA. [3H]-labeled sense and antisense RNA probes were used to identify ECM 3 mRNA by autoradiography (sense strand background levels are not shown). In eggs (A) and early embryos (B) detectable signal is evenly distributed throughout the cytoplasm within all cells. In mesenchyme blastula (C) ECM 3 mRNA accumulates dramatically in all regions of the ectoderm except at the animal pole. Mesenchyme cells (both primary and secondary), cells of the vegetal plate and cells of the invaginating endoderm (D, gastrula stage) do not accumulate significant levels of RNA. This pattern persists through the pluteus (E) although in later stages cells of the endodermal foregut and hindgut show signal above background. Asterisk, animal pole; vp, vegetal plate; en, endoderm; ec, ectoderm; bar in E = 25 microns.

Fig. 7. Western blot characterization of ECM 3. Whole eggs and embryos were prepared for SDS-PAGE followed by western blot immunolabeling using antibody to ECM 3 (anti-pWR 3; data for immunolabeling with antibodies to pWR 3B gave similar results and are not shown). In eggs the major immunolabeled species migrates at 50 kDa which persists as a doublet until mesenchyme blastula. (Note: the apparent increased mobility of the 50 kDa bands is a result of gel ‘smiling’ and not a real change in molecular mobility). In later development, the prevalent immunolabeled species is a doublet at 240/180 kDa. These high molecular weight bands are faint but detectable in eggs and early embryos, they peak at mesenchyme blastula and then decline at pluteus, a pattern very similar to the RNA gel blot. A minor species at 160 kDa is also detected in early stages. Use of preimmune antisera resulted in no immunolabeling. E, eggs; EB, early blastula; LB, late blastula; MB, mesenchyme blastula; G, gastrula; P, pluteus.

extracellular structures and no label accumulated within intracellular compartments.

The spatial localization of ECM 3 protein in the blastocoele is a result of differential mRNA accumulation in the embryo as seen by *in situ* RNA hybridization (Fig. 6). Beginning at gastrulation, ectodermal cells accumulate significant ECM 3 mRNA except for those cells in the animal cap. The oral ectoderm cells of the target site (asterisk in Fig. 6C,D) of the embryo have signal above background, but it is several fold less than other ectodermal regions. This pattern persists through gastrulation and closely resembles the pattern of ECM 3 protein accumulation in the blastocoele. Thus ECM 3 does not appear to diffuse significantly within the blastocoele and is instead deposited adjacent to the cells that synthesize the protein. In larvae, the foregut and hindgut regions of the endoderm also accumulate ECM 3 mRNA, but midgut and mesenchyme show no significant signal.

Although the ECM 3 mRNA signal and *in situ* protein localization suggest a simple expression profile, Western blot data suggest a more complex regulation of protein processing (Fig. 7). Examining whole embryo lysates from egg to pluteus, we detected several forms of the ECM 3 protein. In eggs, the predominant band is a 50 kDa species which persists in embryos to the mesenchyme blastula stage. The 50 kDa species in eggs appears as a single band, but by cleavage stage and later into blastula stage, this species is resolved as a doublet, the smaller of the bands persisting until mesenchyme blastula stage. Beginning at late blastula, as accumulation of ECM 3 in the blastocoele increases...
(Fig. 3), the predominant bands seen by western blots are a doublet of 240/180 kDa. This doublet persists unchanged in size through development to the pluteus. A minor band is also detected at approximately 160 kDa throughout development. The sizes and intensities of bands are identical using two different antibodies made to two different regions of the ECM 3 encoded protein (Fig. 1; data not shown for ECM 3B antibody). Immunoprecipitations of native ECM 3 protein would be informative in revealing protein processing, but we have been unable to detect any metabolically labeled ECM 3 using a variety of immunoprecipitation protocols (e.g., Wessel & McClay 1985). However, we do know that the cDNA encoding ECM 3 represent an open reading frame in excess of the 50 kDa species identified by western blots since fusion proteins constructed in E. coli extend approximately 90 kDa.

In an attempt to elucidate the function of ECM 3 in development, purified antibody solution was injected into the blastocoele of mesenchyme blastulae and the effect of this treatment was analyzed during gastrulation. We studied gastrulation because ECM 3 accumulates sharply and selectively at this time. Injection of approximately 20–50 pg of protein A-affinity purified antibody was performed basically as described (Ingersoll & Ettensohn 1994). Twenty-six embryos injected with antibody to ECM 3 were compared to embryos injected with control antibody (non-relevant, purified IgG) and to un.injected control embryos. No significant differences were observed among these embryo populations during gastrulation (data not shown). The developmental features analyzed included PMC migration, ring formation, and skeletal growth and pattern; pigment cell differentiation and invasion into the ectodermal epithelium; ectodermal epithelium morphology; and primary invagination, archenteron elongation and directionality. Although we were unable to examine the timing and dynamics of filopodial interactions of secondary mesenchyme cells with the blastocoele wall in a manner used by Hardin and McClay (1990), no obvious alteration in secondary mesenchyme cell morphogenesis was detected. We conclude that either the domains of the ECM 3 bound by antibody are not involved in an interaction assayed here, or that the embryo has compensatory gene function for ECM 3. It is also possible that the antibody is inaccessible to the ECM 3 molecule in vivo, but we consider this explanation unsatisfactory since we can readily detect ECM 3 immunolabeling in situ by whole mount preparations. Lastly, the functional portion of ECM 3 may not be the peptide chain used to generate the antibodies. Perhaps, like ZP3 of the zona pellucida in mammals, carbohydrate chains on the protein are the functional domains of the molecule for cell interactions (Wasserman 1993).

Discussion

The blastocoele of the early sea urchin embryo is a heterogeneous compartment of molecules that interacts with cells during development of the embryo. Many cell populations inhabit or transit the blastocoele and rely on cell interactions within this environment for fate and position signals (see Ettensohn & Ingersoll 1992). Both direct cell–cell contact and cell–ECM interactions are known to be involved in cell fate determination and morphogenesis during early development in this embryo (reviewed in Ettensohn & Ingersoll 1992). Here we show a newly identified molecule of the extracellular matrix which shows restricted expression. ECM 3 is deposited into the blastocoele selectively by all ectoderm except for the patch of ectodermal cells at the animal pole. This region of the embryo is of particular morphogenetic interest since it is the site where the invaginating archenteron fuses with the ectoderm to form the mouth. Filopodial extensions from secondary mesenchyme cells (SMC) interact selectively with the animal pole, and following filopodial contractions, are thought to participate in the final extension and directionality of the invaginating archenteron (in Lytechinus variegatus) the stomadeum forms at the animal pole, other species exhibit different sites of stomadeum formation, see Hardin & McClay 1990 for summary). Thus, it has been hypothesized that the 'target site' for invagination at the animal pole is molecularly different from other regions of the blastocoeal wall (Hardin & McClay 1990). The difference may result from ectodermal penetrations through the basal lamina or a difference in the extracellular matrix in that region, or a combination of both.

Results presented here show that the ectodermal cells at the animal pole are different from other ectodermal cells, at least in production of one extracellular matrix molecule, and thus support the hypothesis that the target site for invagination is molecularly distinct from other areas of the blastocoeal wall. The difference in the ECM at the target site may be important in the selective interaction of filopodia emanating from secondary mesenchyme cells with the target site ectoderm. Since both the invaginating archenteron with its associated secondary mesenchyme cells and the target site ectoderm lack ECM 3 expression, perhaps selective cell interactions are favored between these tissue types. This view predicts that ECM 3 might function to block SMCs from contacting the overlying epithelial cells or to repulse the secondary mesenchyme filopodia. Cell repulsion is an important mechanism in nerve growth cone guidance (Edelman & Crossin 1991).

Regulated synthesis of ECM proteins in oocytes and subsequent secretion by early embryos appears to be a general phenomenon in these animals since many ECM proteins exhibit a similar pathway to the nascent
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blastocoel (Wessel et al. 1984, Ingersoll & Ettensohn 1994; but see also Ettensohn & Ingersoll 1992 for examples of zygotic ECM expression). The egg uses at least three distinct vesicle types for the secretion of ECM molecules. First, the cortical granule contents are secreted at fertilization and participate in the block to polyspermy. Their contents contribute to the fertilization envelope, for example proteoliasin and ovoperoxidase (Somers et al. 1989), SFE 9 (Wessel 1995), and to the hyaline layer, the major constituent of which is the glycoprotein hyaline (Hylander & Summers 1982). The second type of extracellular matrix secretion is through apical vesicles. Following fertilization, these secretory vesicles move toward the periphery of the zygote and are released several minutes later. The contents of these vesicles contribute to the apical lamina, containing the fibropelins (Bisgrove & Raff 1993), a glycoprotein 8D11 (Alliegro & McClay 1988), and HLC32 (Brennan & Robinson 1994). The identity of the vesicle harboring 8D11 was determined ultrastructurally and shown to be distinct from the cortical granules and from yolk platelets (Alliegro & McClay 1988).

The third vesicular pathway for secretion used by the egg and early embryo contains extracellular matrix proteins destined for the basal lamina and blastocoel. This storage compartment was first identified with monoclonal antibodies to ECM components of the blastocoel and was shown to be distinct from the cortical granules and from the vesicles of the apical lamina based on mobility in a centrifugal field (Wessel et al. 1984; Alliegro & McClay 1988; Bisgrove & Raff 1993). Shown in this study is the ultrastructural identity of at least one of the vesicle types which secrete blastocoelic ECM. At present we do not know if other ECM molecules destined for the blastocoel are packaged within this same vesicle. Previously we showed that all ECM vesicles have very similar relative densities (Wessel et al. 1984) and thus we would predict that the membrane bound vesicles shown here contain other ECM molecules destined for the blastocoel. However, this hypothesis is inconsistent with the observation that all ECM vesicles destined for the blastocoel are not secreted at the same time. Instead, entry into the blastocoel is varied depending on the protein species examined, and some of the proteins, at least early in development, are secreted both basally and apically (Wessel et al. 1984).

ECM 3 vesicles in oocytes appear to be associated with a Gcgli complex, though without serial sectioning and three-dimensional reconstruction, we are not able to quantify this possible association. We also do not know whether the ECM 3 protein stored in oocytes is in a pre- or a post-Golgi vesicle. In Xenopus oocytes it was shown by metabolic labeling that during meiotic maturation, the secretory pathway is stalled following transit through the Golgi, though earlier steps in the secretory pathway are still functional (Leaf et al. 1990). We were able to efficiently immunoprecipitate ECM 3 from developing oocytes we might be able to identify the step(s) within the biosynthetic pathway where ECM 3 and other ECM molecules are stored. Note that we have used the term storage of ECM 3 and storage vesicle for the membrane-bound vesicles harboring the ECM proteins. This terminology is based on the accumulation of the vesicles within the oocyte during oogenesis. It is not to imply that the vesicle is anything more than a membrane bound compartment stalled in its normal transition through the secretory pathway.

We believe that the protein species detected at 240/180 kDa represents the form of the ECM 3 protein secreted into the blastocoel. These species are enriched in preparations of the blastocoel, and are first detected by western blots in late cleavage stages, coincident with the in situ detection of ECM 3 in the blastocoel. The doublet present at 240 and 180 kDa may represent some post-translational processing differences in the protein or an alteration in the protein upon secretion into the blastocoel. However, were one band to represent the pre-secreted form and the other a post-secreted form, we would expect the secreted form to accumulate to levels in excess of the pre-secreted form. Yet in all Western blots probed with the two ECM 3 antisera, we do not detect significant differences in the relative intensities of these two bands through development. Differential splicing of the ECM 3 mRNA to generate two distinct protein products is also unlikely since we have never seen different transcript sizes even when using probes from several different regions of the ECM 3 cDNA.

The identification of ECM 3 in eggs as a 50 kDa species requires a more complex interpretation of the stored form. Though the ECM 3 mRNA size and the available cDNA sequence encodes a protein much larger than 50 kDa, we do not believe that the 50 kDa form is a cross-reacting protein. Two different antibodies representing two different, non-overlapping regions of the ECM 3 protein to ECM 3 were generated. The sequences of these regions show no similarity to each other, nor to other sequences in the Genbank. Yet both antibodies give the same Western blot profile (Fig. 6), including immunolabeling of the 50 kDa band. Because recombinant proteins produced in E. coli were used to generate the antisera it is unlikely that crossreactivity to shared carbohydrates could explain the 50 kDa band. In addition, no cross hybridization was seen in RNA gel blots, even under relaxed stringency, to support the concept that the 50 kDa band represents a closely related, but distinct gene product.
The 50kDa band could be an incompletely synthesized form of the protein. This explanation presupposes that the storage vesicles identified by ultrastructural immunolabeling are pre-Golgi vesicles, which following fertilization, will re-enter the secretory pathway and complete synthesis of the mature form of ECM 3. The 240/180kDa bands accumulate during cleavage stages, coincident with the secretion of the protein into the nascent blastocoel. This model thus postulates that secretion is not the limiting step leading to ECM 3 protein accumulation in the egg. Instead, completion of protein biosynthesis and transition through the secretory pathway may be blocked at a step earlier than seen in the secretory pathway of Xenopus oocytes (Leaf et al. 1990).

References


