An extracellular matrix molecule that is selectively expressed during development is important for gastrulation in the sea urchin embryo

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SUMMARY

The extracellular matrix is important in the regulation of many cellular events of early development including migration, shape change, proliferation and gene expression. In the sea urchin embryo, disruption of the extracellular matrix results in selective defects in each of these events during gastrulation. Here we describe a new molecule of the extracellular matrix in *Lytechinus variegatus*, referred to as ECM 18, that has several important features. First, antibody interference of ECM 18 results in a profound but reversible inhibition of primary mesenchyme cell organization and endoderm morphogenesis during gastrulation. Second, during gastrulation, ECM 18 mRNA accumulates to highest levels in the invaginating endoderm and the ECM 18 protein is deposited in the basal lamina surrounding the archenteron as well as in other areas of the blastocoel wall. Immunolocalization by fluorescence and electron microscopy demonstrates the selective accumulation of ECM 18 in the extracellular matrix. Third, although the mRNA encoding ECM 18 is present throughout development, the protein accumulates only during gastrulation. ECM 18 protein is not detected in eggs or early embryos and analysis of polysome-associated mRNA suggests that at least part of the translational regulation of ECM 18 is at the level of ECM 18 mRNA-polysome formation. Finally, sequence analysis of ECM 18 shows that the protein contains a repeat sequence with a conserved cysteine motif, suggestive of involvement in protein-protein interactions. Thus, ECM 18 appears to be important in mediating select morphogenetic changes during gastrulation and the pattern of its expression in the embryo is unique among the extracellular matrix molecules known in this embryo.

Key words: extracellular matrix, basal lamina, regulated translation, gastrulation, sea urchin

INTRODUCTION

Gastrulation is a period of highly regulated cell and tissue rearrangements. The transition from a ball or a layer of cells to a trilaminar structure requires directional information for coordinated cell shape changes and migration. Correlated with these overt morphological changes are requisite changes in gene expression (Davidson, 1986). Although in some organisms, early periods of embryonic development may be regulated by stored maternal factors, gastrulation and subsequent development is dependent on the activation of zygotic transcription (Davidson, 1986). Signals for transcriptional activation may result from cells interacting directly with other cells, growth factors and other diffusible molecules, or with extracellular matrix molecules. In several well-studied examples, a combination of such stimuli has proved important for the selective cell rearrangements and cell fate determinations that take place during gastrulation (see reviews by Jessel and Melton, 1992; Slack, 1993).

Gastrulation in the sea urchin embryo is a well-studied process in which one may easily observe ingress of the primary mesenchyme cells (the lineage that forms the embryonic skeletal system) as well as invagination of the vegetal plate cells. The vegetal plate is a diverse population of cells that includes precursors of the digestive endodermal epithelium, muscle cells, basal cells, coelomic epithelia and the highly migratory and invasive pigment cells. At gastrulation, cells of each lineage begin a defined series of movements that leads to their differentiation and, for the endodermal epithelium, morphogenesis is dependent on interactions with the extracellular matrix environment (Ettenson and Ingersoll, 1992).

Several lines of evidence demonstrate that the extracellular matrix is important for differentiation of the endoderm. First, drugs that block the biosynthesis or processing of ECM (extracellular matrix) molecules interfere with gastrulation. Inhibitors of the biosynthesis and processing of, for example, collagens (using the lathrytic agent, β-aminopropionitrile, or the proline analog, cis-OH-proline, Wessel and McClay, 1987; Butler et al., 1987), glycosaminoglycans (using β-D-xylisides or sulfate-free media, Benson et al., 1991; Karp and Solursh, 1974), or glycoproteins (using tunicamycin, Heifitz and Lennarz, 1979), each block differentiation and morphogenesis of the vegetal plate. In most cases, the inhibition caused by
these drugs was selective: not all of embryonic development was inhibited and the effect was reversible. However, since the inhibitors affect the biosynthesis of all sensitive molecules, and not just those of the blastocoel, the molecular defect(s) responsible for the phenotypic response is (are) not always certain.

A second line of evidence showing the involvement of the ECM in differentiation of the endoderm is in vitro assay. Presumptive endoderm cells cultured on a Matrigel extracellular matrix expressed significantly more of the endoderm-specific markers LvN1.2 and Endo 1, than did cells cultured on non-ECM substrata (S. W. Chen and G. M. Wessel, unpublished data).

A third line of evidence demonstrating the importance of the ECM in gastrulation comes from the restricted expression of ECM molecules, which is correlated with the time and location of a morphogenetic event. Although presence alone is insufficient to assign function to a particular molecule, several ECM molecules have been identified whose expression is correlated with regions of morphogenetic change. For example, monoclonal antibodies have identified ECM determinants that are localized to the stomodeum region of the late gastrula (Wessel et al., 1984) and polyclonal antibodies to a recombinant ECM molecule identified as ECM 3, which is deposited throughout the basal lamina except at the site of stomodeum formation (Wessel and Berg, 1995). Endo 16 is secreted selectively by endodermal cells during gastrulation and accumulates in the ECM surrounding the elongating archenteron (Soltsyik-Espanola et al., 1994). Yet another molecule of the blastocoel, ECM 1, accumulates selectively in the basal lamina of the vegetal region of the embryo, which is associated with the invaginating archenteron, and then concentrates ventrally later in development, at the site of stomodeum formation (Ingersoll and Ettensohn, 1994). Studies using antibodies as blocking reagents suggest that specific determinants of the ECM that participate in gastrulation can be identified by introduction of these reagents into the blastocoel (Ingersoll and Ettensohn, 1994).

Since many ECM molecules accumulate in selective regions within the blastocoel, they must not freely diffuse. More likely, these ECM molecules are stabilized by specific intermolecular interactions to form an ordered heterogeneous compartment. One can envisage that in the blastocoel some molecules interact with cells directly; other molecules serve as structural organizers that interact with other ECM molecules and lead to a proper or stable configuration of the ECM environment; and a third group of molecules may serve to mediate the function or the localized concentration of soluble growth factors (Ramachandran et al., 1993). Were the ECM important in morphogenesis, disruption of any one of these molecular types could lead to dysfunction of the matrix. Thus, understanding the regulation of morphogenesis during gastrulation will require an explanation of the ontology of diverse ECM constituents.

We undertook an immuno-cDNA screen to identify molecules of the blastocoelic ECM, and generated antibodies to recombinant proteins in order to characterize the cognate proteins and their function in vivo. Here we describe a molecule referred to as ECM 18 that has a unique pattern of ECM protein expression in the sea urchin embryo. The evidence suggests that ECM 18 protein expression early in development is limited both by the recruitment of ECM 18 mRNA into polysomes and by accumulation of its mRNA in the invaginating archenteron. In addition, we show that interference with this protein in the blastocoel during gastrulation results in a severe but selective and reversible inhibition of morphogenesis. Thus, ECM 18 appears to be an important constituent of the ECM for gastrulation, and shows a pattern of expression different from known ECM molecules in this embryo.

**MATERIALS AND METHODS**

**Handling embryos**

Adult *Lytechinus variegatus* were obtained from Scott’s Services and from Sue Decker (both of Miami, FL) and from the Duke University Maine Laboratory (Beaufort, NC). 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 by 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 minutes at 4°C with calcium-free seawater containing 0.1% Triton X-100, and a protease inhibitor cocktail (0.5 mM phenylmethylsulfonyl fluoride; 1 mM EGTA; 0.3 units aprotinin; Sigma, St Louis, MO). After several cycles of washing with extraction buffer and centrifugation in a clinical centrifuge, the extract was resuspended in 0.25 M sodium acetate buffer pH 6.5, 20 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% NP-40, 0.1% SDS, then denatured at 100°C for 3 minutes and treated with endoglycosidase F (Boehringer Mannheim) for 16 hours 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 3 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 and Lane, 1988).

**cDNA library screen and production of antibodies**

Antisera showing the highest titer to the blastocoelic extracellular matrix by in situ immunofluorescence were used to screen a λZAP cDNA library constructed from prism stage polyadenylated RNA (Stratagene, La Jolla, CA; Wessel et al., 1989). BB4 cells harboring the AZAP 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 hours with Blotto buffer (50 mM Tris pH 7.5, 0.9% NaCl, 0.05% Tween-20, and 3% nonfat dry milk) and then incubated with the anti-extracellular matrix antibodies (diluted 1/200) at 21°C for 2 hours. The filters were washed overnight with several changes of Blotto, and then incubated with ¹²⁵I-labeled donkey anti-rabbit antibody (10⁶cpm/ml of 10 μCi/μg; Amersham, Arlington Heights, IL) for 2 hours. 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) and recovered as a Bluescript plasmid (Short et al., 1988).

XL1-Blue cells (Stratagene, La Jolla, CA) bearing Bluescript plasmid-derived β-galactosidase/ECM 18 peptides were induced with...
10 mM IPTG for 2 hours, collected by centrifugation and lysed by repeated freeze/thaw cycles. The lysate was mixed with Freund’s adjuvant and injected subcutaneously into a female 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 and Lane, 1988).

**DNA sequencing**

The DNA sequence was determined by the Sanger chain termination method (Sanger et al., 1977) using 35S-dATP (Dupont, Boston, MA) and Taq DNA polymerase (Promega Biotech, Madison, WI). Sequence data were assembled and analyzed using the University of Wisconsin Genetic Computer Group (UWGCG) sequence analysis package (Devereux et al., 1984).

**RNA analysis**

Total RNA isolated from embryos at several developmental stages was analyzed by hybridization of ECM 18 cDNA to RNA gel blots (Bruskin et al., 1981). Accumulation of ECM 18 mRNA was measured by an RNase protection assay using antisense 32P-riboprobes transcribed in vitro from recombinant Bluescript plasmids, essentially as described (Kreig, 1991). The probe template was the 450 bp of the 5’-most region of ECM 18 (see Fig. 1), which was generated by PCR, analyzed by gel electrophoresis, photographed in the presence of a fluorescent ruler (Clontech Laboratories, Palo Alto, CA) and ligated into Bluescript plasmid (Stratagene, La Jolla, CA). For an antisense probe, the ECM 18 PCR plasmid was linearized with HindIII and then transcribed using T7 RNA polymerase. L. variegatus ubiquitin antisense riboprobe was transcribed with T7 RNA polymerase following template linearization with EcoRI. Riboprobes were gel purified and used in an RNase protection assay with a RPAII kit (Ambion, Inc. Austin, TX), using 4 μg total RNA from each stage.

**Polysome isolation**

Polysomes were prepared as described (Kelso-Winemiller and Winkler, 1991) with slight modifications. Eggs and embryos were collected and washed twice in modified buffer IV (0.25 M KCl, 5 mM EGTA, 10 mM MgCl2 and 10 mM Pipes) at 4°C. Embryos were homogenized in buffer IV with 1 mM DTT and were then centrifuged at 18000 g for 12 minutes. Triton X-100 was added to these postmitochondrial supernatants at a final concentration of 0.1% and 20 OD260 were layered on a 15-40% sucrose gradient made in buffer IV. These gradients were centrifuged for 6 hours at 26000 revs/minute in a Beckman SW28 rotor at 4°C. Fractions were collected by upward displacement with 80% sucrose. The protein fraction was collected by precipitation in 70% ethanol and then extracted with phenol-chloroform. An equal amount of RNA from each stage was subjected to RNase protection assays.

**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 mM DTT and a protease inhibitor cocktail (consisting of a final concentration per ml of aprotinin, 1 TIU; benzamidine, 10 μg; soybean trypsin inhibitor, 10 μg; antipain, 1 μg; leupeptin, 1 μg; bestatin, 0.5 μg; E-64, 1 μg; phosphoramidon, 1 μg; phenylmethylsulfonylfluoride, 10 μg; chymostatin, 1 μg; pepstatin, 1 μg), and denatured for 3 minutes 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 immunolabeling, the blots were washed twice for a total of 1 hour in Blotto and then incubated for 1 hour in Blotto containing anti-ECM 18 sera diluted 5000x. The blots were then washed three times in Blotto over 30 minutes and incubated in Blotto with goat anti-rabbit antibodies conjugated to horseradish peroxidase (Organon Teknika, Durham, North Carolina) diluted 5000x. Blots were washed in Blotto three more times over 30 minutes and finally washed in Blotto without milk. The secondary antibody was detected by chemiluminescence using the ECL western blotting kit (Amersham Corporation, Arlington Heights, IL) and Kodak X-Omat film. Controls used in this experiment included blots incubated with preimmune antiserum and secondary antibody alone. Each of these western blots showed no signal (data not shown).

**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 Sall 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 18 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) was diluted 1/40. Electron microscopic immunolabeling was performed as described (Wessel and McClay, 1985) 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 Phillips 410 electron microscope.

**Fab fragment preparation**

ECM 18 Fab fragments were prepared from protein A-purified antiserum using the Immunopure Fab Preparation Kit (Pierce, Rockford, IL) and were purified by protein-A affinity chromatography. Fab fragments were dialyzed versus 1 mM Tris pH 8, lyophilized, resuspended in distilled water and diluted for injection in artificial seawater (ASW; Instant Ocean; Aquarium Systems, Mentor, OH). The purity of the Fab preparation was assessed by western blots using antibodies to whole IgG and the fidelity was verified by immunolocalization on embryo sections (data not shown).
Microinjection

Embryos were loaded in a microinjection chamber (Kiehart, 1982), injected using a screw-driven oil-filled Narishige 1M-5B microinjection and visualized on a Leitz DMIL microscope. Microinjection needles were pulled from 50 μl Microcaps (OD 1.0 mm; Drummond, Broomall, PA) and tips were broken to approximately 3 μm. Injection solution was stored between layers of oil in a loading capillary and was front-loaded for each injection (Kiehart, 1982). Approximately 60 pl of ECM 18 Fab fragment (either 0.2 mg/ml or 0.02 mg/ml) in ASW or control antibody in ASW (0.2 mg/ml fluorescein-conjugated goat anti-rabbit Fab fragment; Organon Teknika, Durham, NC) was injected into the blastocoel of mesenchyme blastula or early gastrula (primary invagination) embryos. Injected embryos were marked with a droplet of dimethylpolysiloxane oil (Sigma, St Louis, MO) and cultured in the injection chamber in ASW at 20°C. Embryos were examined with a Zeiss Axioplan microscope and photographed using Nomarski optics.

RESULTS

Antiserum was raised to polypeptides of the ECM of the blastocoel that were isolated from plutei of *Lytechinus variegatus* (Wessel et al., 1984) and depleted of N-linked carbohydrates by endoglycosidase treatment. This antiserum selectively immunolabeled molecules within the blastocoel and basal lamina of the developing embryo (data not shown), and was used to screen approximately 340,000 plaques of a cDNA library constructed from prism stage poly(A)+RNA. The cDNA clone encoding ECM 18 is 2.6 kb in length and was used to isolate overlapping cDNA clones that extend toward both the N and C terminus of the protein (Fig. 1). Based on a DNA sequence of over 2.5 kb and the immunostaining pattern, ECM 18 does not appear to be similar to any known ECM proteins in this embryo, including Endo 16 (Nocente-McGrath et al., 1989), ECM 1 (Ingersoll and Ettensohn, 1994) and ECM 3 (Wessel and Berg, 1995). One region of ECM 18 sequence has three repeating motifs, each 67 amino acids in length, containing nine absolutely conserved cysteines (Fig. 1). This segment of cysteine repeats has 42% sequence identity to the domain D repeats of the von Willebrand blood clotting factor (Mancuso et al., 1989), and eight of the nine cysteines of ECM 18 are aligned perfectly with the von Willebrand sequence. The function of the domain D repeats in von Willebrand factor is not understood but is thought to be involved in intrachain and interchain disulfide bonding (Mancuso et al., 1989). It is the only sequence of significant similarity to this region of ECM 18 found within the Genbank database, and except for the three possible N-linked glycosylation sites, the cognate protein encoded by the 2.6 kb partial reading frame contains no common structural motifs.

The ECM 18 mRNA was detected both by an RNase protection assay (Fig. 2A) and by a RNA gel blot (Fig. 2B). As quantitated by phosphorimager analysis, the RNase protection assay shows that ECM 18 mRNA is present in eggs and early embryos and increases in abundance approximately 3-fold at mesenchyme blastula and gastrula followed by a slight decrease at pluteus. RNA loading levels in this assay were evaluated by RNase protection of ubiquitin mRNA in the same reactions. Ubiquitin mRNA is known to be present in equal amounts throughout development (Gong et al., 1991). ECM 18 appears as a transcript of approximately 6.5 kb but, at gastrulation, an additional signal is present of up to approximately 7 kb (Fig. 2B).

In situ RNA hybridizations show that ECM 18 mRNA is evenly dispersed throughout the embryo at early developmental stages and that the mRNA is not sequestered in nuclei (Fig. 3). During gastrulation, however, ECM 18 mRNA accumulates to the highest levels in endoderm cells with approximately half as much signal in the ectoderm (quantified autoradiography shows that endoderm contains 0.083 grains per μm² whereas ectoderm contains 0.045 grains per μm²; background levels were 0.008 grains per μm²). Primary mesenchyme cells have no detectable signal. The accumulation of ECM 18 mRNA in endoderm is uniform along the digestive tract, beginning with primary invagination and continuing through development to the larval stage. No regionalization within the digestive tract is seen for ECM 18 mRNA, in contrast to the mRNA and proteins of the endoderm specific markers, Endo 1 and LvN1.2 (Wessel, 1993) or Endo 16 (Soltysik-Espanola et al., 1994).

Polyclonal antiserum was generated to a β-galactosidase fusion protein encoded by the ECM 18 cDNA clone (Fig. 1) and this monospecific antiserum was used to examine ECM 18 accumulation in situ during development, both by immunofluorescence (Fig. 4) and by electron microscopic immunogold localization (Fig. 5). In larvae, ECM 18 accumulates along the blastocoel wall underlying all epithelial tissues (Fig. 4). Signal is detected underlying both endoderm and ectoderm tissues, but very little signal is associated with the mesenchymal cells. During invagination of the endoderm, ECM 18 accumulates in
Fig. 3. In situ RNA localization of ECM 18 mRNA. 3H-labeled sense and antisense RNA probes were used to identify ECM 18 mRNA by autoradiography (sense strand background levels shown in K,L). Bright-field photographs are adjacent to the corresponding dark-field images. In eggs (A,B) and early blastula (C,D), signal is evenly distributed throughout the embryo. During gastrulation, ECM 18 mRNA accumulates to the highest levels in the invaginating archenteron (E,F early invagination; G,H late gastrulation) and this signal persists to the pluteus stage (I,J). bc, blastocoel; ec, ectoderm; en, endoderm; bar in L, 25 μm.

Fig. 4. Immunolocalization of ECM 18. Paraffin sections of oocytes and embryos were processed for immunolocalization using antibody to ECM 18. Immunofluorescence photographs are adjacent to corresponding brightfield photographs. In eggs (A,B) and early blastula (C,D) no ECM 18 is seen. At mesenchyme blastula (E,F) ECM 18 begins to accumulate along the blastocoel wall, but very little signal is associated with mesenchyme cells throughout the blastocoel. During gastrulation (G-J) signal is detected underlying both the ectoderm and the invaginating endoderm. This accumulation pattern continues through pluteus (K,L). Immunofluorescence using preimmune sera (M,N) shows no signal. bc=blastocoel; pmc=primary mesenchyme cells; ec=ectoderm; en=endoderm; bar in N= 25 μm.
the basal lamina underlying the ectoderm and endoderm cells and no signal is detected in the cytoplasm or in the apical lamina of these cells.

Ultrastructural immunolabeling shows that ECM 18 accumulates within the basal lamina and within nearby fibers of the blastocoel (Fig. 5). No consistent immunolabeling was found within the cytoplasm of the epithelial cells, within the apical lamina of the embryo, or surrounding the cell bodies of the primary mesenchyme cells (PMC). Immunolabeling experiments using preimmune serum resulted in no detectable signal anywhere in the embryo. A clustering of the immunogold signal is characteristic of the blastocoelic immunolabeling of ECM 18. We do not know if this aggregated morphology is indicative of its clustering in vivo or whether it is a fixation artifact, but this pattern is distinct from the immunolabeling pattern of antibodies against other ECM proteins, e.g. ECM 3 (Wessel and Berg, 1995), using the same protocol. Constituents of the blastocoel are known to be difficult to preserve so we have tested different fixation protocols for immunofluorescence (e.g. 100% methanol, 4% formaldehyde, Bouins fixative) and for electron microscopy (combinations of formaldehyde and glutaraldehyde, with or without osmium tetroxide). Each of these experiments has resulted in the same localization pattern of ECM 18 as shown in Figs 4 and 5 (data not shown).

Prior to gastrulation, the ECM 18 protein is not evident anywhere within the embryo (Fig. 4B,D). The first appearance of the protein is at mesenchyme blastula stage, coincident with the migration of PMCs from the vegetal plate along the ectodermal walls of the embryo (Fig. 4E,F). In these embryos, ECM 18 is present along the walls of the blastocoel, but not at the vegetal plate or in association with the PMC. During gastrulation, ECM 18 signal is present underlying the ectodermal cells with only low levels associated with the invading endoderm (Fig. 4H,I). However, the amount of label associated with the gut increases during secondary invagination to levels that are associated with the ectoderm.

The lack of ECM 18 protein in early embryos (Fig. 4) is in contrast to the prevalence of ECM 18 mRNA in these same embryos as seen by RNase protection assays (Fig. 2B), RNA gel blots (Fig. 2A) and in situ RNA hybridization (Fig. 3). Therefore, we examined ECM 18 appearance in development by western blot analysis (Fig. 6) to determine whether the lack of ECM 18 was a result of selective epitope destruction by aldehyde fixation or by tissue processing for embedding. The accumulation profile of ECM 18 seen in western blots corresponds closely to the profile seen by in situ immunolocalization; ECM 18 protein is not detected in eggs or early embryos by either of these techniques but accumulates rapidly during gastrulation. Immunoblotting experiments using preimmune serum resulted in no detectable signal. Since the ECM 18 antibody was made to denatured, recombinant ECM 18, and reacts well in immunoblots both to the recombinant protein and to the ECM 18 from postgastrula embryos, we do not believe that lack of ECM 18 immunolabeling in early embryos is the result of a change in the epitope structure required for antibody binding. Instead, it appears that, even though substantial mRNA is present, ECM 18 is not translated in early development, or is rapidly degraded so that it does not accumulate detectably until gastrulation. We have been unable to immunoprecipitate ECM 18 protein from any stage and so cannot test ECM 18 synthesis metabolically.

Western blot analysis reveals a complex pattern of bands extending from approximately 200×10⁻³ M to 50×10⁻³ M. We have no clear explanation for this multiplicity of bands, but it does not appear to be the result of general protein degradation in the embryo samples; the buffer used for SDS-PAGE contained several different protease inhibitors (see Materials...
Early gastrulation; primary control antibody 33 No effect on development in 85% (28/33) of embryos; remaining.

Early mesenchyme blastula Control antibody 46 No effect on development in 79% (36/46) of embryos; remaining.

Stage of embryo Injection examined Resultant phenotype

- Early mesenchyme blastula
  - Control antibody 46 No effect on development in 85% (28/33) of embryos; remaining.
  - ECM 18 antibody 54 In 81% (33/41) of the embryos mesenchymal rings were lacking or disarrayed, and spicule growth was limited to small crystals; limited invagination of the vegetal plate was evident in most cases; in remaining 29% (16/54) of embryos, development was similar to normal.

- Early gastrulation; primary invagination
  - Control antibody 33 No effect on development in 85% (28/33) of embryos; remaining.
  - ECM 18 antibody 55 In 83% (45/55) of the embryos secondary invagination was inhibited or delayed, and skeletal growth was limited to small, but numerous triradiates; 36% (16/45) of these embryos also had disrupted mesenchymal rings; 17% of the embryos had a variety of abnormalities with no consensus phenotype.
  - Dilute ECM 18 antibody 22 In 81% (18/22) of the embryos archenteron elongation was inhibited or delayed, whereas only 50% (11/22) of the embryos had reduced skeletal growth.

Table 1. Effect of ECM 18 antibody on gastrulation

<table>
<thead>
<tr>
<th>Stage of embryo</th>
<th>Injection</th>
<th>Number of embryos examined</th>
<th>Resultant phenotype</th>
</tr>
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<tbody>
<tr>
<td>Early mesenchyme blastula</td>
<td>Control antibody</td>
<td>46</td>
<td>No effect on development in 79% (36/46) of embryos; remaining 21% (10/46) of embryos had a variety of defects</td>
</tr>
<tr>
<td></td>
<td>ECM 18 antibody</td>
<td>54</td>
<td>In 71% (38/54) of the embryos mesenchymal rings were lacking or disarrayed, and spicule growth was limited to small crystals; limited invagination of the vegetal plate was evident in most cases; in remaining 29% (16/54) of embryos, development was similar to normal.</td>
</tr>
<tr>
<td>Early gastrulation; primary</td>
<td>Control antibody</td>
<td>33</td>
<td>No effect on development in 85% (28/33) of embryos; remaining 15% (5/33) of embryos had a variety of defects.</td>
</tr>
<tr>
<td>invagination</td>
<td>ECM 18 antibody</td>
<td>55</td>
<td>In 83% (45/55) of the embryos secondary invagination was inhibited or delayed, and skeletal growth was limited to small, but numerous triradiates; 36% (16/45) of these embryos also had disrupted mesenchymal rings; 17% of the embryos had a variety of abnormalities with no consensus phenotype.</td>
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<td></td>
<td>Dilute ECM 18 antibody</td>
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<td>In 81% (18/22) of the embryos archenteron elongation was inhibited or delayed, whereas only 50% (11/22) of the embryos had reduced skeletal growth.</td>
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Fig. 7. Polysomal associated RNA was isolated from pooled fractions of a sucrose gradient and was used in a RNase protection assay with an anti-sense ECM 18 RNA probe (phosphorimager scanned gel). No ECM 18 signal can be detected in polysomes from eggs but is first detected at the 4-cell stage. A substantial increase in ECM 18 signal is seen at the mesenchyme (Mes) blastula stage, the time of first detectable protein accumulation, which persists to the gastrula stage. Ubiquitin mRNA was used as an internal mRNA marker to show that the RNA in each lane is not degraded. The total RNA sample was isolated from gastrulae and yeast tRNA was used as a background control.

Approximately 60 pl of control or ECM 18 antibody was injected at 0.2 mg/ml or for dilute ECM 18 injections at 0.02 mg/ml.
ECM 18 appears to be important in the morphogenesis of PMCs and vegetal plate descendants during gastrulation. To perturb ECM 18 function in vivo, Fab fragments were made from the ECM 18 antibody and were injected into the blastocoel of embryos during gastrulation. Two time points were chosen for injection: early mesenchyme blastula, when primary mesenchyme cells are ingressing into the blastocoel, but before they have migrated into a mesenchymal ring; and primary invagination, following the initial inpocketing of the vegetal plate but before secondary elongation when the archenteron extends across the blastocoel. These points of development were chosen because they are coincident with the first appearance of ECM 18 protein in the embryo and the occurrence of pronounced morphogenetic changes. Injection experiments are summarized in Table 1, and representative embryos are shown in Fig. 8. In embryos injected at the early mesenchyme blastula stage with approximately 12 pg of ECM 18 Fab fragments, the PMCs continued to migrate through the blastocoel, but a mesenchymal ring did not form (Fig. 8B). In addition, although most embryos showed at least some invagination of the vegetal plate, invagination was delayed in time and reduced in magnitude when compared with the control embryos. In contrast, when embryos were injected with approximately 12 pg of a control antibody (goat anti-rabbit Fab) almost 80% of the embryos developed normally (Fig. 8A). For the 20% of embryos that were judged abnormal in these experiments, no regular or predictable phenotype resulted, but among the phenotypes were death, exogastrulation, complete developmental inhibition and a filling of the blastocoel with unknown cell types. In these experiments, most of the embryos injected with either a control antibody or the ECM 18 antibody appeared healthy; their cilia continued beating, the morphology of ectoderm cells changed normally from cuboidal to squamous epithelium, and pigment cells differentiated and invaded the ectodermal epithelium. Thus the developmental inhibitions seen with anti-ECM 18 injection did not appear to result from nonspecific embryo damage.

When embryos were injected with approximately 12 pg of ECM 18 Fab fragments during early gastrulation, invagination of the vegetal plate ceased and secondary invagination was not detected (Fig. 8D). Injections at this time also inhibited organization of the PMC; these cells continued to migrate throughout the blastocoel, but they did not form a normal mesenchymal ring or synthesize an elongated skeleton. In many cases, clusters of PMCs were seen to form, but the mesenchyme ring pattern was missing and a triradiate spicule was absent. Often embryos showed the initiation of several spicule rudiments but none of these extended beyond a small, tri-radiate form.
Embryos injected with a reduced amount (1-2 pg) of ECM 18 Fab showed a greater ability to gastrulate (data not shown). Although gut morphogenesis was still delayed in these embryos, PMCs were better organized and skeletal structures were closer to normal. We do not know if all the cells in the blastocoel were PMCs or whether they also included secondary mesenchyme cells (SMC), but a majority of the cells appeared migratory. In addition, the pigment cells differentiated normally; they made pigment, migrated throughout the blastocoel and invaded the ectodermal epithelium.

The inhibition caused by ECM 18 antibody was apparent approximately 4 hours after injection of embryos at primary invagination, but 12-16 hours later, recovery was evident (Fig. 8E). In some cases the recovered embryos were not completely normal, particularly in skeletal patterning and symmetry. But, in the majority of cases, the developmental inhibition caused by ECM 18 Fab was transient and nontoxic. One explanation for this transient effect is that the antibody activity was lost by cellular endocytosis. To explore this possibility, we injected control antibodies conjugated to fluorescein into the blastocoel of gastrulating embryos. Fluorescence signal was detected in the blastocoel for approximately 4-5 hours, but after prolonged culture the signal was lost from the blastocoel and instead was detected within cells of the mesenchyme and epithelium. A second explanation for the transient nature of the antibody effect is that new ECM 18 is synthesized and secreted into the blastocoel, overwhelming the limited antibody that is present. In an attempt to prolong the period of inhibition, embryos were injected with approximately 12 pg of ECM 18 Fab at early gastrula, cultured for 2-4 hours, and then injected again with another 12 pg of the antibody. In such embryos, inhibition of the above processes was prolonged, whereas embryos injected with control antibody were largely unaffected (Fig. 8F,G).

**DISCUSSION**

Spatial and temporal heterogeneity of molecules in the blastocoel is created in part by differential gene activity (Venkatesan et al., 1986; Esposito et al., 1994) and differential release of maternal proteins from cytoplasmic stores in the egg (Wessel et al., 1984; Alliegro et al., 1992; Wessel and Berg, 1995). Heterogeneity in the extracellular matrix (ECM) also results from delayed translational activation during early development and the ECM 18 protein shows this unusual pattern of ECM expression. The protein does not accumulate in oocytes during oogenesis like many ECM proteins, and is not released into the nascent blastocoel during blastulation, a pattern that is also shown by many molecules of the ECM (Wessel et al., 1984; Ingersoll and Ettenson, 1994). Instead, the ECM 18 protein first begins to accumulate coincident with PMC migration and with the differentiation of the endoderm. This pattern is particularly unusual considering that the mRNA is present throughout development.

Analysis of polysome-associated mRNA shows that prior to gastrulation, a majority of the ECM 18 mRNA is not associated with polysomes. Instead, it is either insoluble and removed from gradient fractionation, or it is in a non-polysomal, soluble fraction. Although we did not attempt to identify ECM 18 in the nonsoluble fraction of the embryo homogenate, significant ECM 18 mRNA was not detected in the soluble ‘cytoplasmic’ fraction until postgastrula stages. From these results we conclude that a major mechanism of selective expression of ECM 18 is a lengthy translational delay similar to the delays seen, for example, in certain mRNA species in the polar lobe of *Illyanassa* embryos (Brandhorst and Newrock, 1981). In certain mRNA of *Xenopus* and mouse, an increase in polyadenylation appears to be a mechanism for stimulating translation (Richler, 1991). Although the extent of polyadenylation in this message is unknown, RNA gel blot analysis does detect a second species of higher molecular weight (a broad band up to approximately 7 kb versus 6.5 kb) at gastrulation (Fig. 2B), which could be indicative of ECM 18 polyadenylation coincident with the movement of ECM 18 mRNA in the polysome fraction.

Cells of the ectoderm are first to translate ECM 18 mRNA, although these cells do not appear to be sensitive to ECM 18 disruption during gastrulation. This tissue still shows the morphogenetic transition from a cuboidal to a squamous shape, the cilia beat normally, and the oral ectoderm cells at the animal cap elongate to form the characteristic animal-cap thickening. The ectodermal epithelium appears to develop independently of several other ECM molecules as well, including collagen (Wessel and McClay, 1987), ECM 1 (Ingersoll and Ettenson, 1994) and ECM 3 (Wessel and Berg, 1995). However, morphogenesis of these same cells is disrupted with anti-laminin antibodies (McCarthy and Burger, 1987) and select gene expression can be inhibited in the ectoderm with alterations in the ECM (Wessel et al., 1989). Thus, cells of the ectoderm do interact with the ECM during development and this interaction can be disrupted by antibody treatment, but ECM 18 does not appear to be essential for the morphological features of ectoderm development examined here.

Different molecules of the blastocoel influence different events of gastrulation, as shown by antibody-blocking experiments. When a monoclonal antibody to sea urchin laminin was injected into the blastocoel of embryos, the epithelial cells rounded up and detached from the basal lamina (McCarthy and Burger, 1987). Since the cell shape changes in the epithelia are coincident with the timing of laminin expression in the embryo, it was suggested that laminin is essential for epithelial morphogenesis beginning early in development. Also identified by a monoclonal antibody is a glycoprotein(s) termed ECM 1 (Ingersoll and Ettenson, 1994). The ECM 1 determinant is a carbohydrate moiety shared by several ECM molecules. When monoclonal antibodies recognizing this determinant, or when peptides bearing the ECM 1 determinant, were injected into the blastocoel, cell rearrangements and elongation of the archenteron were blocked. No effect, however, was seen on PMC migration or pattern formation. Blocking ECM 18 also inhibits archenteron morphogenesis but, in a manner distinct from anti-ECM 1 and anti-laminin, it alters PMC patterning as well. These results suggest that different processes of gastrulation rely on distinct molecules of the blastocoel.

The vegetal plate of the embryo appears to be particularly sensitive to disruption of ECM 18 function. Assuming a blastocoel volume of 270 pl (diameter, approx. 80 μm), injection of 12 pg Fab yields a final concentration of approximately 40 ng/ml and results in a nearly complete inhibition of the development in the vegetal plate and in PMCs. In comparison with other antibody reagents used for blocking studies, the concentration of ECM 18 Fab required for significant inhibition is quite low. For comparison, the final concentration needed for inhibi-
tion of hyalin interaction was 10 µg/ml of Fab (Adelson and Humphreys, 1988), 1.2 mg/ml of ECM 1 (Ingersoll and Ettensohn, 1994), 8 µg/ml of fibropellin (Burke et al., 1991) and 2 mg/ml of laminin (McCarthy and Burger, 1987). A possible explanation for the increased sensitivity of the embryo to ECM 18 is that the ECM 18 Fabs were derived from a polyclonal antiserum instead of from monoclonal antibodies, as used in the above examples, resulting in a more thorough blocking of ECM 18 determinants. In addition, the region bound by Fabs could be an active site for its function. The region of ECM 18 used for antibody generation contains a cysteine-rich repeat (13.5%) with an absolute conservation in the number and spacing of the cysteines between these repeats. In many cases, cysteine repeats have been shown to influence the structure of a protein, and to be involved in protein-protein interactions (Artavanis-Tsakonas et al., 1995). Even though no extensive primary sequence similarity is seen in the GenBank, the conserved cysteine residues may impart a secondary structure that could be shared by other molecules with significantly different sequences. Thus the structure, but not the sequence, may be conserved, as in the cysteine knot motif of many peptide growth factors (McDonald et al., 1995). Even though no extensive primary sequence similarity is seen in the GenBank, the conserved cysteine residues may impart a secondary structure that could be shared by other molecules with significantly different sequences. Thus the structure, but not the sequence, may be conserved, as in the cysteine knot motif of many peptide growth factors (McDonald and Hendrickson, 1993). A final explanation for the sensitivity of the embryo to ECM 18 is that this molecule may be a key element of the ECM, either in organization of the intermolecular structures of the ECM, or more directly in a signaling pathway ultimately used by PMCs and descendants of the vegetal plate. We hypothesize that the timing of the onset of ECM 18 protein accumulation in the blastocoele is an important step in the differentiation and morphogenesis of these cell types.

We thank Dr. Laurinda Jaffe for assistance in our microinjection techniques and Yuin-Tsee Jennifer Hsu for contributing to the DNA sequencing aspects of this project. Valuable lessons from Chandler Merritt are also gratefully acknowledged. This work was supported by grants from the National Institutes of Health (HD28152), the March of Dimes.

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Function of extracellular matrix in gastrulation


(Accepted 10 November 1995)