A Molecular Analysis of Hyalin—A Substrate for Cell Adhesion in the Hyaline Layer of the Sea Urchin Embryo

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The hyaline layer of echinoderm embryos is a classical extraembryonic matrix that functions as a substrate for cell adhesion through early development. The major component of the hyaline layer is the protein hyalin, a fibrillar glycoprotein of approximately 330 kDa that multimerizes in the presence of calcium. Here we provide a molecular characterization of hyalin and identify a region of the protein that is important for its function in cell adhesion. Partial hyalin cDNAs were identified from two sea urchin species, Strongylocentrotus purpuratus and Lytechinus variegatus, by screening expression libraries with monoclonal antibodies to hyalin. The cDNAs each encode a tandemly arranged series of conserved repeats averaging 84 amino acids. These hyalin repeats are as similar between the two species as they are to repeats within each species, suggesting a strong functional conservation. Analysis of this repeat shows that it is a unique sequence within the GenBank database with only weak similarity to mucoid protein sequences. The hyalin mRNA is approximately 12 kb in length and is present in developing oocytes coincident with the appearance of cortical granules, the vesicle in which the hyalin protein is specifically packaged. The mRNA is present throughout oogenesis but is rapidly lost at oocyte maturation so that eggs and early embryos have no detectable hyalin mRNA. The hyalin protein, however, remains at relatively constant levels throughout development. Thus, all the hyalin protein present during early development, when no RNA is detectable, is maternally derived and exocytosed from cortical granules at fertilization. Hyalin mRNA reaccumulates in embryos beginning at the mesenchyme blastula stage; a RNA gel blot and in situ hybridization analysis of gastrulae and larvae shows a progressive confinement of hyalin mRNA to the aboral ectoderm. Recombinant hyalin containing the tandem repeat region of the protein was expressed in bacteria and is shown to serve as an adhesive substrate, almost equal to that of native hyalin, in cell adhesion assays. This adhesive activity is partially blocked by dilute hyalin monoclonal antibody Tg-HYL to the same extent as that for native hyalin. Thus, this hyalin repeat region appears to contain the ligand for the hyalin cell surface receptor. These data help explain some of the classic functions ascribed to the hyalin protein in early development and now enable investigators to focus on the mechanisms of cell interactions with the hyaline layer.

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Key Words: hyalin; hyaline layer; cortical granules; fertilization; cell adhesion.

INTRODUCTION

The hyaline layer of the sea urchin embryo is a classically defined structure now known to be important for development. This structure was first reported by Hertwig (1876), was described by Hammar (1896) as a clear, colorless homogeneous layer, and was suggested by Herbst (1900) to function by binding blastomeres together. More recent experiments show that the hyaline layer serves directly as a substrate for cell adhesion (McClay and Fink, 1982; Fink and McClay, 1985) that is necessary for morphogenesis (Adelson and Humphreys, 1988).

The major component of the hyaline layer is the protein hyalin which is exocytosed at fertilization. Although many...
other proteins also are exocytosed from other vesicles at or shortly following fertilization (Matese et al., 1997), hyalin is secreted from cortical granules, within which the hyalin is selectively packaged into the electron-lucent, homogeneous region (Hylander and Summers, 1982). At fertilization, the hyaline layer forms rapidly and swells to approximately 2–3 μm thick within 10–15 min postinsemination (Harvey, 1956).

The hyaline matrix was shown to be sensitive to calcium ions by Herbst (1900), and this property was later used by investigators to devise isolation schemes, alternating calcium-free solubilization with calcium-induced precipitation (Faust et al., 1959; Vacquier, 1969; Kane, 1970; Citkowitz, 1971). Analysis of isolated hyalin protein has shown it to be about 330 kDa in size, containing approximately 25% acidic residues, only 3.5% basic residues, and 2–3% carbohydrate (Stephens and Kane, 1970; Citkowitz, 1971). In addition, it has distinct heterogeneity. On SDS–PAGE gels hyalin appears as a smear, suggesting that the molecule is either differentially glycosylated and/or is of a heterogeneous length. Physical studies of purified hyalin have also suggested that the protein is heterogeneous with different sedimentation properties (Gray et al., 1986; Justice et al., 1992). Recently, the ultrastructure of the protein was identified by rotary shadowing as a filamentous molecule about 75 nm long with a globular “head” about 12 nm in diameter (Adelson et al., 1992). Though most of the filaments of the protein are 75 nm in length, other filament regions are 25–50 nm longer than the majority, supporting the heterogeneous nature of the protein. In the presence of calcium, the hyalin monomers aggregate with their globular regions to a high-molecular-weight core particle that remains associated with hyalin throughout purification. Antibodies to hyalin bind in several places along the filamentous region of hyalin. These antibodies functionally block cell–hyalin associations and as a consequence disrupt morphogenesis. It is thus believed that hyalin contains multiple cell binding sites on the filamentous region of the molecule (Adelson et al., 1992).

Functionally, hyalin has been shown to act as a substrate for cell adhesion during early embryogenesis (McCay and Fink, 1982; Fink and McCay, 1985; Adelson and Humphreys, 1988). At the beginning of gastrulation primary mesenchyme cells lose contact with the hyaline layer and ingress into the blastocoel. At that time, the primary mesenchyme cells lose an affinity for hyalin and increase their adhesion toward other substrates (McCay and Fink, 1982; Fink and McCay, 1985). Later, the vegetal plate invaginates to form the endoderm. Concurrent with this morphogenesis, the ectoderm and endoderm change in their relative affinities for hyalin; ectoderm cells retain an affinity while endoderm cells lose their affinity (Bursdal et al., 1991). Consistent with its proposed role in the invagination process (Gustafson and Wolpert, 1967), treatment of embryos with a monoclonal antibody to hyalin, known to block cell–hyalin adhesion in vitro, causes the entire embryo to retract from the hyaline layer and inhibits invagination of the archenteron (Adelson and Humphreys, 1988).

Given these functional properties it is of interest to learn more of the molecular nature of the hyalin protein and its regulated expression during development. Accordingly, we identified hyalin cDNAs by screening expression cDNA libraries from two different species and at two different stages with several different anti-hyalin monoclonal antibodies, including an adhesion-blocking antibody. These studies identified an encoded protein domain important for cell adhesion that is unique to the GenBank database. These molecular tools will help us understand this classically described protein.

**MATERIALS AND METHODS**

**Animals and reagents.** Lytechinus variegatus were obtained from the Duke Marine Laboratory (Beaufort, NC) and from Scott’s Services and Sue Decker (both of Miami, FL). Stronglylocentrotus purpuratus were obtained from Marinus (Long Beach, CA). Gametes were obtained by intracoelomic injection of KCl (0.5 M) and eggs were washed with Instant Ocean artificial sea water (ASW; Mentor, OH). Eggs were fertilized and cultured as described (McCay, 1986). S. purpuratus and L. variegatus embryos were cultured at 15 and 20–22°C, respectively. In cases where the fertilization envelope was removed, the eggs were fertilized in 10 mM p-amino benzoic acid and the envelopes were removed mechanically with Nits mesh as described (McCay, 1986). Hyalin was isolated as previously described (McCay and Fink, 1982), using a protocol that was modified from the original hyalin isolation protocols (Vacquier, 1969; Kane, 1970).

To obtain cDNA clones encoding hyalin from L. variegatus, a monoclonal antibody, TgHYL, originally made to hyalin from Tripneustes gratilla (Adelson and Humphreys, 1988), was used to screen two different cDNA expression libraries. This monoclonal antibody has been shown to cross-react with hyalin from a number of echinoid species and strongly stains hyalin specifically from both S. purpuratus and L. variegatus (data not shown). Each of the libraries used were made in λZAP (Stratagene, La Jolla, CA); one was prepared from poly(A)- mRNA isolated from the mid-gastrula stage of L. variegatus, and the others from poly(A)- mRNA isolated from ovaries of either S. purpuratus or L. variegatus containing mixed stages of oocytes. Approximately 3 × 105 recombinants were screened to obtain more than 20 independent clones.

To obtain cDNA clones encoding hyalin from S. purpuratus, monoclonal antibodies generated to the cell surface complex isolated as described previously (Decker and Kinsey, 1983) were screened for anti-hyalin specificity. These hyalin antibodies were then used to probe a cDNA ova library from S. purpuratus as described (Laidlaw and Wessel, 1994). Approximately 10 5 recombinants were screened, resulting in eight different cDNA clones.

**cDNA screening procedure.** The cDNA libraries were screened as previously described (Laidlaw and Wessel, 1994). Briefly, B84 cells harboring the λZAP bacteriophage were plated onto NZCYM 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 in blot buffer (50 mM Tris, pH 7.5, 0.9% NaCl, 0.05% Tween-20, and
scripts were synthesized by first linearizing the plasmids with BglII and then transcribing the template using T7 RNA polymerase. A sense probe was synthesized by linearizing the plasmid with HindIII and then transcribing the template using T3 RNA polymerase (enzymes from Life Technologies,Gaithersburg, MD).

**Immunolocalization in situ.** Immunofluorescence localization of hyalin was performed in whole mounts and on sections of embryos that were fixed and processed as previously described (Laidlaw and Wessell, 1994). Primary antibodies were diluted between 1/50 and 1/200 and the secondary antibody (Cy3-conjugated affinity-purified goat anti-mouse IgG; Kirkegaard and Perry Labs, Gaithersburg, MD) was diluted 1/100.

**Cell adhesion assay.** Embryos from *L. variegatus* were dissociated as previously described (McCay, 1986). An adhesion assay modified from that of McCay et al. (1981) was used to examine cell–substrate adhesive affinity. Briefly, substrates were applied to 96-well microtiter plates at 10 μg per well. After 1 h of incubation with the antibodies used in this study are specific for hyalin.

**RESULTS**

**Hyalin Contains a Region of Conserved Amino Acid Repeats**

Partial hyalin cDNAs were identified independently in two different species using monoclonal antibodies to screen cDNA libraries. Tg-HYL is a monoclonal antibody that cross-reacts with hyalin from several echinoid species, and it blocks the function of hyalin (Smith and Johnson, 1988). Equimolar amounts of native and recombinant hyalin were applied to each well and the wells were then treated with bovine serum albumin (2 mg/ml in ASW), to block nonspecific binding sites. One hundred percent binding controls used cells with no applied substrate, and 0% binding controls used wells that were blocked with bovine serum albumin (BSA). Cells were added to the wells (1 × 10⁵ cells/well), and the wells were filled and then sealed with clear plastic packaging tape. The plates were spun in a low-speed centrifuge at 65 g for 5 min to pellet all the cells as a subconfluent monolayer onto the substrates. The plates were removed from the centrifuge, inverted, and incubated on ice for 30 min. Alternatively, the inverted plates were spun at 65 g with the force acting to pull nondhering or very weakly adhering cells away from the substrate. The well bottoms were then placed, still in an inverted position, on a microscope stage and the number of bound cells present within a standard area was counted. Results are given as the number of cells bound per unit area of substrate. In these experiments, background binding was less than 2% and was subtracted from each of the binding values shown.
diverse genera of echinoderms. Each of these antibodies was used to screen cDNA libraries in several independent screens using libraries from ovaries of L. variegatus and S. purpuratus and from gastrula of L. variegatus. Over 20 different expression clones were identified in these screens.

Sequences of partial hyalin cDNAs from both L. variegatus and S. purpuratus overlapping clones are compared in Fig. 1. Sequence from both species contains a large region of an extended tandem repeat averaging 84 amino acids. Predicted amino acid sequence is strongly conserved among the repeats both within each species (ranging from 38 to 97% identical) and between species (64% identical). The sequence of these cDNA clones encodes a protein with a predicted amino acid composition very similar to that obtained by amino acid analysis of purified hyalin protein (Stephens and Kane, 1970), further supporting the fact that these regions encode hyalin. We do not know if the epitopes recognized by each antibody are shared between repeats, but the presence of a redundant epitope may be one reason for the high frequency of monoclonal antibodies generated to this protein and of identifying cDNA clones to this region of hyalin by expression immunoscreening. This repeat sequence has no strong identity to anything in GenBank. The most similar sequences (less than 25% identity) are mucins, yet hyalin is biochemically unlike mucins in that only 2–3% of hyalin’s mass is carbohydrate (Stephens and Kane, 1970). The binding site of the Tg-HYL antibody was previously mapped to the filamentous region of the molecule using rotary shadowing of immunolabeled native proteins (Adelson et al., 1992). Thus, the tandemly repeated sequence resides within the long filamentous portion of the molecule and not in the globular head region.

**Hyalin Protein in Early Development Is Maternally Derived**

By immunoblot analysis, each of the hyalin antibodies detects a 330-kDa band in hyalin isolated from freshly fertilized eggs or from embryos (data not shown), and a smear in total preparations of eggs, embryos, and larvae (Fig. 2B). This protein heterogeneity has been seen by SDS–PAGE, by FPLC, and by rotary shadowing techniques by several investigators (e.g., Adelson et al., 1992). From the apparent size of the hyalin protein, the mRNA was predicted to be quite large. This prediction is born out by RNA gel blot analysis (Fig. 2A) where the mRNA is estimated to be more than 12 kb in length in both species studied (data not shown for L. variegatus). By RNA gel blot analysis (Fig. 2A) we find abundant mRNA in the oocyte, but no or only very little hyalin mRNA in mature eggs. Hyalin mRNA remains very low or undetectable in embryos until the mesenchyme blastula stage. During gastrulation, hyalin mRNA reaccumulates at the same size as that found in oocytes, and this pattern of mRNA accumulation is identical for both species (data not shown for L. variegatus). Based on genomic DNA blots (data not shown) in which only one hyalin gene is detected, we conclude that the zygotic transcript is derived from the same gene as the maternal transcripts.

The hyalin mRNA detected in ovaries is present specifically in developing oocytes (Figs. 3A and 3B). No signal is found in mature, accessory cells or other somatic cells of the ovarian capsule, eliminating a potential heterosynthetic source of RNA or protein, as is found for some other macromolecules of the egg (like yolk, and glycogen (Anderson, 1974; Shyu et al., 1986). This RNA signal is found in oocytes of all stages and is very abundant. Based on frequency of plaque hybridizations we estimate hyalin to account for 1–2% of the mRNA in ovaries. Although the colorimetric hybridization labeling in these in situ hybridizations is difficult to quantify, it appears that oocytes of all sizes contain an equal density of label. We conclude from this that oocytes accumulate hyalin mRNA throughout oogenesis, which is consistent with the kinetics of mRNA accumulation of other cortical granule proteins (Laidlaw and Wessel, 1994).

Coincident with the appearance of hyalin mRNA is the appearance of cortical granules within the oocytes. These organelles accumulate throughout the cytoplasm during oogenesis (Fig. 3E) and eventually increase to about 15,000 per oocyte (Laidlaw and Wessel, 1994). Following germinal vesicle breakdown, the cortical granules completely translocate to the cortex of the egg where they dock at the plasma membrane in a monolayer (Fig. 3G; Berg and Wessel, 1997; Matese et al., 1997). Because of the temporal coincidence of mRNA and protein appearance, the mRNA appears to be translated immediately. During meiotic maturation of oocytes, which takes approximately 9 h in L. variegatus (Berg and Wessel, 1997), the oocytes degrade hyalin mRNA so that in mature eggs, none is detected (Fig. 3B). This result indicates that the hyalin protein present throughout early development is strictly of maternal origin. The lack of hyalin mRNA during early development (Fig. 3C) explains a previous observation that metabolically radiolabeled hyalin cannot be identified in embryogenesis at least until gastrulation begins (McClay and Fink, 1982).

**Embryonic Hyalin mRNA Expression Is Progressively Restricted to Ectodermal Regions**

Hyalin mRNA reaccumulates in embryos beginning at the gastrula stage. The new hyalin mRNA appears only in the ectoderm in a pattern that is consistent initially with an enriched distribution in aboral ectoderm relative to oral ectoderm (Fig. 4A). A sharp boundary of hyalin message is present in a ring around the vegetal plate, the diameter of which decreases as gastrulation progresses. Based on other lineage markers, these data suggest that hyalin is excluded from endoderm at the ectodermal/endoendermal boundary (Ruffins and Ettenson, 1996; Logan and McClay, 1997). As gastrulation proceeds the RNA becomes further enriched in the aboral ectoderm, and in early plutei the hyalin mRNA appears most exclusively in the aboral ectoderm (Figs. 4C and 4D). This mRNA pattern is distinct from protein immunofluorescence data which shows hyalin in the oral ectoderm and...
Molecular Analysis of Hyalin

**Strongylocentrotus purpuratus**

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**Lytechinus variegatus**

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**FIG. 1.** (a) Sequence and conservation of hyalin repeats. Thirteen full and one partial repeat for *S. purpuratus* and eight full and two partial repeats for *L. variegatus* are aligned to show the high level of amino acid identity both within each species and between repeats of the two species. In both cases the repeats are in tandem. (b) Map of overlapping hyalin cDNA clones containing the repeat region. K, KpnI; S, SstI; B, BamHI; C, Clal.

in the lumen of the endoderm (Fig. 5B). This is probably explained by a long perdurance of the maternal protein, some of which is drawn into the archenteron during gastrulation. In support of this hypothesis, the hyaline layer at the blastopore appears distorted (Fig. 5A); an autoradiographic profile of isolated hyaline layers shows new hyalin deposition in the ectoderm but not in the endodermal hyalin (Citkowitz, 1971), and maternal hyalin is known to persist well into gastrulation (McCoy and Fink, 1982). Further, the previous autoradiographic pattern (Citkowitz, 1971) is now explained by the in situ RNA hybridization pattern seen here.

The hyalin mRNA pattern in larvae is unusual (Fig. 4).
the arms, where no label is detectable, and at the apex of the oral hood. This pattern of hyalin mRNA and protein accumulation is very similar between *L. variegatus* and *S. purpuratus*. The only significant differences are that the clear band around the ciliary band in *S. purpuratus* is not well defined and the apex of the larva is not stained even late in gastrulation (data not shown).

The intracellular distribution of the hyalin message is consistent with its encoding hyalin since it is confined to the region of the rough endoplasmic reticulum around the nucleus as expected for a protein targeted to the secretory pathway (Fig. 4G). The flattened squamous cells of the aboral ectoderm enable this visualization. A comparison with the in situ RNA hybridization of the LvS1 RNA, encoding a cytoplasmic protein, is distinct in that the LvS1 signal is distributed throughout the cytoplasm (Fig. 4F).

When the embryo reaches the feeding stage the endoderm retains hyalin within the lumen, but only in the foregut region. The lumen of the stomach and intestine no longer have hyalin protein, as they did earlier in development (Fig. 5E).

**The Hyalin Repeat Region Contains a Binding Site for Cell Adhesion**

Hyalin is a known adhesive substrate (McClay and Fink, 1982) and cell-hyalin interactions have been proposed to be involved in several morphogenetic movements (Citkowitz, 1971; Fink and McClay, 1985; Adelson and Humphreys, 1988) even though the mechanism of this interaction is unknown. It was of interest, therefore, to determine whether the repeat region of hyalin is the portion of the molecule to which cells bind. One of the monoclonal antibodies used to screen the cDNA expression libraries, Tg-HYL, was previously shown to block gastrulation and it appeared to do so by causing cells to detach from the hyaline layer (Adelson and Humphreys, 1988). Thus, it was reasonable to predict that the repeat region identified by that antibody would be an adhesive substrate. To test this prediction we used a quantitative adhesion assay to ask whether the bacterially expressed peptide fragment of hyalin repeats serves as a substrate for cell binding. Figure 6A shows the cell affinity to native hyalin relative to substrates of mammalian fibronectin, tenascin, and sea urchin ECM bags. BSA is used as a background control. We then tested the ability of cells to bind to the hyalin repeat region identified here. Hyalin-glutathione-S transferase fusion protein was synthesized in bacteria and used in the adhesion assay. Figure 6B shows that this fusion protein serves as a good substrate, at about 80% the level of the native hyalin at the same substrate concentration. In addition, the adhesion-blocking antibody Tg-HYL (McAb 183) used originally to study hyalin function in vivo blocks cell binding to native hyalin by 22%, as well as by 32% to recombinant hyalin repeats (at the same antibody dilution). We thus conclude that the repeat region of hyalin contains at least one type of cell-binding site. Whether only one or more than one repeat has a binding site is not known, but this level of inhibition by

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**FIG. 2.** (A) RNA gel blot analysis of hyalin mRNA. Total RNA of *S. purpuratus* (10 μg), isolated from the cells and embryos indicated, was resolved on agarose gels, blotted to nylon, and hybridized with a 32P-labeled probe of hyalin cDNA. An mRNA of 12 kb is present in ovaries but not eggs or early development. The same sized mRNA reappears at gastrulation and accumulates in larvae. (B) Immunoblot analysis of hyalin protein in eggs and embryos of *S. purpuratus*. The smear of immunolabeling characteristics of hyalin in whole eggs and embryos is present at similar levels throughout development. When aligned to the RNA gel blot (A), it is apparent that the protein present from fertilization until gastrulation is of maternal origin.
FIG. 3. In situ analysis of hyalin protein and mRNA. (A–C). In situ RNA hybridization: (A) Lobe of an ovary showing mature eggs in the background in a honeycomb pattern, and the oocytes (smaller and densely labeled) at the ovary periphery. (B) A fully grown oocyte (top) contains maximal levels of hyalin mRNA, but following maturation (bottom), the hyalin mRNA is lost and does not reappear in early development. (C) Mesenchyme blastula. (D–I) In situ immunolabeling (D, Brightfield; E, Immunofluorescence). Histological section of an ovary showing developing oocytes of various stages surrounded by somatic accessory cells. Oocytes of all stages show cortical granules throughout their cytoplasm, whereas the mature egg at the bottom right has translocated cortical granules. (F) Fully grown oocyte showing cortical granules dispersed throughout the cytoplasm, but not in the germinal vesicle. (G) Mature egg with the cortical granules at the cortex, the source of all hyalin protein for early development to the mesenchyme blastula stage (H, Brightfield; I, Immunofluorescence). Note that hyalin is present throughout the ectoderm and the in-pocketing of hyalin at the blastopore (bottom of embryo). (A, D, E) S. purpuratus; bar, 100 μm. (B, C, F–I) L. variegatus; bar, 50 μm.

the Tg-HYL antibody is consistent with 35% inhibition in adhesion originally described for native hyalin (Adelson and Humphreys, 1988) even though the present experiments use only 1/1000 the concentration of antibody.

DISCUSSION

Hyalin provides the functional backbone of the hyaline extracellular matrix for development of sea urchin embryos.
and here we provide insight into the molecular nature of the functional hyalin protein. Several criteria support the conclusion that the cDNAs identified here encode the hyalin protein. First, monoclonal antibodies to hyalin from two different species were used to screen expression cDNA libraries from two different stages. These independent experiments resulted in several overlapping cDNA clones that each contain the hyalin repeats. Second, the transcripts identified by these cDNAs and the proteins identified by the monoclonal antibodies are each present in the right places and at the right times to be consistent with the classical definitions of hyalin. This includes accumulation of the protein into cortical granules during oogenesis at the same time as mRNA accumulation, the apical accumulation of the protein in the embryo, and the ectodermal accumulation of the mRNA during gastrulation. Third, the size of the mRNA is appropriately large for a protein that is over 300 kDa in size. Finally, the amino acid analysis previously performed on isolated hyalin (Stephens and Kane, 1970) is very similar to the sequence interpreted from cDNA sequence. Differences apparent at this level are likely due to the partial sequence of the hyalin cDNA presented here. Thus, we are confident that the cDNA we have identified is encoded by the hyalin gene.

Cell Adhesion to Hyalin

The tandem hyalin repeats appear to be a portion of the hyalin protein that serves as a cell-adhesive substrate. The Tg-HYL antibody to hyalin earlier showed an ability to block cell-hyalin adhesion (Adelson and Humphreys, 1988) and to bind to the long shaft of the hyalin monomer (Adelson et al., 1992). This same antibody was then used in the expression library screen and identified the tandem repeats both in S. purpuratus and in L. variegatus. We show that expressed peptides of hyalin repeats serve as an adhesion substrate and that adhesion is at least partially blocked by low levels of hyalin antibody. Thus, we conclude that the filamentous portion of the hyalin molecule contains the tandemly arrayed repeats and is the part of the molecule recognized as an adhesive substrate in vivo. The sequence of the hyalin repeats appears to represent approximately two-thirds of the long shaft of the hyalin molecule. This value is estimated from mapping another filamentous extracellular matrix protein, fibronectin. Each of the fibronectin type III repeats contain a similar number of amino acids (90) as does a hyalin repeat (84) and each of the FNIII repeats is estimated to be 3.5 nm in length (Leahy et al., 1996). Were each of the hyalin repeats to be similar to the FN repeats in length, the 13 repeats shown here would extend approximately 45 nm of the 75-nm hyalin shaft. By this reasoning we would predict that the hyalin shaft could contain as many 21 hyalin repeats and account for 230 kDa of the mass of the hyalin molecule.

When blocked by high concentrations of Tg-HYL (10 μg/ml), as was used in Adelson and Humphreys (1988), cells in vivo retract from the hyalin layer and gastrulation is blocked. Using low concentrations of the antibody here (10 ng/ml) we observe inhibition of cell adhesion to recombi-
FIG. 5. In situ hyalin immunolocalization in gastrulae (A–C) and larvae (D, E) of *L. variegatus*. (A) Surface view of gastrula showing the blastopore (arrow). Note that hyalin surrounds the aboral and oral ectoderm. (B) Histological section of gastrula showing hyalin around the entire embryo and in the lumen of the entire gut. (C) Compressed embryos show the lacy nature of the hyalin layer. (D) Surface view and (E) histological section of larvae showing the oral and aboral surfaces covered by hyalin and the mouth (arrow), with hyalin evident within the esophagus only. Bar, 50 µm.

nant hyalin repeats in vitro. These findings do not remove all uncertainties of how hyalin interacts with cells. For example, we have not tested other nonrepeat regions of hyalin for substrate binding activity, so there could be more cell-binding sites. We also do not know whether only one or more than one of the repeats contain the binding activity. We do know that the Tg-HYL antibody can bind multiple places on the shaft of the hyalin molecule with the potential of blocking multiple binding sites shared by the hyalin repeats. Both species also have nonrepeat sequence upstream of the repeats that is distinct in the two species studied here and may represent the globular region of the hyalin molecule. It also has no sequence similarity to anything in GenBank. Since the total size of the native protein is approximately 330 kDa, of which only 2–3% of its mass is due to carbohydrate (Stephens and Kane, 1970), we predict that the sequence shown here represents about 1/3 of the protein.

Regulation of Hyalin Expression

The timing of expression of hyalin at gastrulation is unusual relative to other aboral ectoderm genes that have been studied. The Spec family of genes, CyIIIa, and the arylsulfa-
tase gene are each transcribed much earlier in development than hyalin (Brandhorst and Klein, 1992). Then, when the hyalin message does appear, it is not uniformly expressed throughout the aboral ectoderm. Instead, mRNA accumulates fastest around the ectoderm–endoderm boundary at the blastopore and then in other areas of the aboral ectoderm, with a low level of expression in the oral ectoderm. Later in development, the mRNA signal is also lost in an irregular pattern, beginning at the vertex of the larvae and continuing throughout the ectoderm, except for the strip just oral of the ciliary band. Another protein of the hyaline layer, called Ecto V, is initially expressed all over the surface of the ectoderm as is hyalin, but as development continues it becomes confined specifically to the oral surface. Thus, two proteins in the extraembryonic matrix are both expressed coincidentally all over the embryo and then continued expression is restricted to reciprocal compartments, hyalin in the aboral and Ecto V in the oral. Similar patterns of progressive restriction have been noted for other proteins, e.g., Endo 16 (Ransick et al., 1993).
where it appears thicker than the hyalin over the ectoderm. This endodermal-associated hyalin appears to be maternally derived, from the original cortical granule exocytosis, since cells of the early embryo and the endodermal lineage of the postgastrula embryo do not accumulate detectable hyalin mRNA. Autoradiography of isolated hyalin bags in which the luminal hyaline layer was not detectably labeled with metabolic precursors (Citkowitz, 1971) supports this conclusion. Thus, we believe that the luminal hyalin is drawn into the archenteron during invagination and results from a strong adhesion of the endodermal precursor cells to the hyaline layer. Following formation of the mouth and feeding by the larvae, the luminal hyalin is removed in the midgut and hindgut regions. It is not clear yet whether hyalin is lost by sloughing off as a result of a selective loss of adhesion by cells in that region or whether it is endocytosed by these highly absorptive tissues.

The ciliary band has been shown to be an area where presumptive oral and presumptive aboral clones of cells interact and establish a boundary (Cameron et al., 1990). In the pluteus larva, though, hyalin message appears on both sides of the ciliary band as well as the entire aboral field but is excluded from cells within the band. Such a pattern provides evidence suggesting that patterns of hyalin gene expression are not strictly delimited by lineage but may instead reflect a zone of overlapping and counteracting signals used in establishing the oral–aboral boundary. This means that during transcriptional regulation, which has been shown to be controlled spatially in a modular fashion (Yuh et al., 1994), the hyalin gene regulatory elements must contain a regulatory capacity that is more detailed than that provided in genes transcribed in all cells of a germ layer, for example.

FIG. 6. Adhesion of cells to hyalin. A quantitative adhesion assay was employed using centrifugal force to dislodge cells from attachment to hyalin on the substrate. (A) Hyalin is the preferred substrate of cells when compared to bovine serum albumin (BSA, used to minimize nonspecific binding to the assay wells), fibronectin or tenascin from vertebrates, or whole basal lamina/blastocoel bags of extracellular matrix from sea urchin larvae. (B) Under these same conditions a GST fusion protein of the hyalin repeat region supported binding of cells at 50%, close to the value obtained for native hyalin in this experiment (60%), and several times the background value (BSA, 12.6%). When parallel wells were further treated with the monoclonal antibody Tg-HYL, at dilute concentrations, this hyalin-specific antibody reduced binding to hyalin by 22% and to the fusion protein by 32%. Cell binding to native hyalin is significantly different from binding in the presence of Tg-HYL at P = 0.00409 (t tests) and the difference between binding to recombinant hyalin and binding to recombinant hyalin in the presence of Tg-HYL is significant at P = 0.0240.

The hyalin protein is abundant in the lumen of the invaginating gut and is visible in live embryos, in isolated hyalin bags (Citkowitz, 1971), and in fixed sections of embryos where it appears thicker than the hyalin over the ectoderm. This endodermal-associated hyalin appears to be maternally derived, from the original cortical granule exocytosis, since cells of the early embryo and the endodermal lineage of the postgastrula embryo do not accumulate detectable hyalin mRNA. Autoradiography of isolated hyalin bags in which the luminal hyaline layer was not detectably labeled with metabolic precursors (Citkowitz, 1971) supports this conclusion. Thus, we believe that the luminal hyalin is drawn into the archenteron during invagination and results from a strong adhesion of the endodermal precursor cells to the hyaline layer. Following formation of the mouth and feeding by the larvae, the luminal hyalin is removed in the midgut and hindgut regions. It is not clear yet whether hyalin is lost by sloughing off as a result of a selective loss of adhesion by cells in that region or whether it is endocytosed by these highly absorptive tissues.

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Such a class of vesicles was identified by a monoclonal antibody and appears to carry proteins specifically toward the apical surface of the ectoderm (Alliegro and McClay, 1988; Matrese et al., 1997), but it is not clear whether hyalin is trafficked in that vesicle class or separately in a different vesicle toward the apical surface. Part of the difficulty in reaching conclusions about the timing of secretion later in development is that the maternal hyalin protein continues to be present at the time zygotic hyalin is first made. Thus, it is not possible with the antibodies available to distinguish newly secreted hyalin from maternal hyalin by immunofluorescence. Finally, since by genomic DNA blot analysis only one hyalin gene is detectable in the animal, the same hyalin product is made, packaged, and secreted by two different pathways. We cannot exclude the possibility, however, that alternatively spliced transcripts provide an embryonic molecule with different trafficking properties than the maternal protein, although the adhesive properties of the early and late forms of the molecule appear to be the same (Fink and McClay, 1985).

CONCLUSIONS

Many embryos have an extraembryonic matrix. Little is known, however, about how these matrices are used by the embryos. For most it is thought that the extraembryonic matrix is there primarily to offer protection. This is likely to be a function for the hyalin layer, as well, but the present study with hyalin also indicates that this extraembryonic matrix molecule surrounding the sea urchin embryo is an important and dynamic component of morphogenesis. Hyalin is sequestered in abundance in the egg in a highly specific packaging sequence, released in a highly regulated fashion, and later is expressed in a highly specific pattern at gastrulation as the cells shift in their affinities toward this matrix component. Perhaps similar molecules have such dynamic roles in other species.

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REFERENCES


Hylander, B. L., and Summers, R. G. (1982). An ultrastructural im-


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