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Hyalin is a Cell Adhesion Molecule Involved in Mediating Archenteron - Blastocoel Roof Attachment

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Summary

The U. S. National Institutes of Health has designated the sea urchin embryo as a model organism because about twenty-five discoveries in this system have led to insights into the physiology of higher organisms, including humans. Hyalin is a large glycoprotein in the hyaline layer of sea urchin embryos that functions to maintain general adhesive relationships in the developing embryo. It consists of the hyalin repeat domain that has been identified in organisms as diverse as bacteria, worms, flies, mice, sea urchins and humans. Here we show, using a polyclonal antibody raised against the 11.6 S species of hyalin, that it localizes at the tip of the archenteron and on the roof of the blastocoel exactly where these two structures bond in an adhesive interaction that has been of interest for over a century. In addition, the antibody blocks the interaction between the archenteron tip and blastocoel roof. These results, in addition to other recent findings from this laboratory that will be discussed, suggest that hyalin is involved in mediating this cellular interaction. This is the first demonstration that suggests that hyalin is a specific cell adhesion molecule that may function as such in many organisms, including humans.

Keywords

Hyalin; Sea Urchin Embryo; Blastocoel Roof-Archenteron Tip Interaction; Specific Cell Adhesion Molecule

Introduction

The U. S. National Institutes of Health has designated the sea urchin embryo as a model system for studying physiological mechanisms important in human health and disease. This is because about twenty-five physiological mechanisms have been developed in sea urchins that were found to be important in higher organisms, including humans (Davidson and Cameron, 2002; Davidson, 2006). The structure, function and diseases of the extracellular matrix are important

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in human medicine and the sea urchin system may provide an important tool in contributing to our understanding of them.

A long-studied example of the extracellular matrix in the sea urchin is the fibrillar glycoprotein hyalin, which is a component of the sea urchin embryo hyaline layer. It serves as an adhesive substrate during early development (Herbst, 1900; McClay and Fink, 1982; Wessel *et al.*, 1998) and consists of repeated regions (called hyalin repeats) averaging 84 amino acids (Wessel *et al.*, 1998; Callebaut *et al.*, 2000) and non-repeated regions (Wessel *et al.*, 1998). The Gen Bank database suggests that the hyalin repeat is a unique sequence that shows slight similarity to mucoid protein sequences (Callebaut *et al.*, 2000) and appears to be related to the immunoglobulin-like fold (Callebaut *et al.*, 2000). Since hyalin consists of only 2–3% carbohydrate (Citkowitz, 1971), it is not very similar to mucins that contain more carbohydrate (Wessel *et al.*, 1998). The hyalin repeat has been identified in bacteria, mice, *Caenorhabditis elegans*, and *Drosophila melanogaster* proteins, as well as in a human protein (Callebaut *et al.*, 2000). In recognition of the widespread occurrence of the hyalin repeat sequence, its function is garnering a great deal of interest and hyalin research may yield new information about functions which apply to a wide variety of organisms (Alvarez *et al.*, 2007).

Hyalin and the hyalin repeat appear to function in adhesive interactions, but very little work has been done to explore its specific function. Edelman. (1987) has shown that chick neural cell adhesion molecule is related to the immunoglobulin superfamily. Given the relationship of the hyalin repeat to immunoglobulins, we hypothesize that hyalin may have heretofore been under appreciated and is important specific functions in sea urchin cell adhesion. In the present report, we provide evidence that the sea urchin glycoprotein hyalin is a cell adhesion molecule that is involved in mediating a specific cellular interaction in the model sea urchin embryo system, attachment of the tip of the developing gut (archenteron) to the roof of the blastocoel. This is a classic cellular interaction that has interested investigators for a century, yet its molecular basis has not been elucidated (Herbst, 1900; Gustafson, 1963; Hardin and McClay, 1990; Latham *et al.*, 1999; Khurram *et al.*, 2004; Oppenheimer and Carroll, 2004).

Generally accepted criteria for identifying a molecule as being involved in mediating a specific cellular interaction include: the molecule is present on the interacting cells, exogenously added molecule in solution blocks the cellular interaction and antibody against the molecule also blocks the cellular interaction. Our earlier work has focused on the inhibition of attachment of the archenteron to the roof of the blastocoel by exogenously added hyalin in low calcium artificial seawater (Alvarez, *et al.*, 2007; Contreras, *et al.*, 2007; Razinia, *et al.*, 2007). In the present study, we use anti-hyalin antibodies to further investigate the function of hyalin as a putative, specific cell adhesion molecule.

Material and methods

Embryo collection and culture

Gametes of adult *Lytechinus pictus* and *Strongylocentrotus purpuratus* sea urchins (from Marinus, Inc., Garden Grove, CA) were extracted by intracoelomic injection of 0.55M KCl. Sperm were collected “dry” and held at 15°C. Eggs were filtered through 183 µm Nitex mesh (Tetco, Inc., Briarcliff Manor, NY) to remove debris, rinsed twice in 15°C, pH 8.0 artificial seawater (ASW), and fertilized with dilute sperm suspensions. After settling, embryos were washed three times in ASW to remove excess sperm. *L. pictus* embryos were distributed into Pyrex bowls, and *S. purpuratus* embryos were distributed into Pyrex trays.

Rate of development of *L. pictus* embryos in the incubator was closely monitored and controlled by maintaining separate culture bowls at 15–20°C. *S. purpuratus* embryos were maintained at 15°C.

Antiserum Production

The production of antisera was undertaken in the laboratory of Dr. Edward J. Carroll, Jr. using an Animal Care and Use Protocol approved by the Institutional Animal Care and Use Committee (Chancellor's Laboratory Animal Committee). New Zealand White rabbits were used for antiserum production after pre-immune sera had been collected. The animals were immunized using subcutaneous injection of either 11.6 S or 6.4 S hyalin proteins prepared using the methods of Gray *et al.*, (1986) and Justice *et al.* (1988), respectively. Each antigen was dialyzed using phosphate buffered saline (8.0 gm NaCl, 0.2 gm KH₂PO₄, 2.7 gm KH₂PO₄·7H₂O, 0.2 gm KCl per liter) then mixed with an equal volume of Freund's complete adjuvant. A total volume of 1 ml containing approximately 1 mg of each antigen per animal was injected at six sites on the backs of each of two rabbits for each antigen preparation. At three weeks post initial immunization, booster injections were given, using approximately 0.3 mg antigen per animal in Freund's complete adjuvant. At six weeks post initial immunization, blood was collected from the medial ear vein. All immunization procedures were done with the animals anesthetized using an intramuscular injection of Innovar-Vet. The collected blood was allowed to clot for 2 hr at room temperature and 12 hr at 4° C. The serum was decanted and centrifuged at 2,500g for 15 min and 4° C to remove residual cells. The sera were stored at -15° C in 1 ml aliquots. Each of the sera was monospecific (based on immunoblots, Justice, 1989) with respect to the species of hyalin protein recognized, i.e., anti-11.6 S hyalin serum recognized only 11.6 S hyalin and likewise for the anti-6.4 S serum recognizing only 6.4 S hyalin. Comparative immunoblots of calcium precipitable egg proteins (that can be defined as hyalin) indicated crossreactivity of the antibodies with the hyalin-like proteins in *L. variegatus*, *Arbacia punctulata*, *Eucidaris tribuloides*, and *Ophiothrix spiculata*. This crossreactivity is consistent with the results of Vater and Jackson (1989).

Live *L. pictus* embryo immunoassays

Antisera against 11.6 S and 6.4 S proteins isolated from purified *S. purpuratus* hyalin were distributed at dilutions of 1/250 – 1/64,000 in 48-well microplates. At mesenchyme blastula (when primary mesenchyme cells are ingressing into the blastocoel and delaminating from the hyaline layer) and during the first third of gastrulation (embryo has invaginated and archenteron has elongated 1/3 the distance across the embryo), swimming embryos were concentrated and collected in 63 µm Nitex mesh collection filters partially submerged in glass bowls. Embryos were distributed into wells at an average concentration of 142 embryos per well. Wells containing identical dilutions of pre-immune sera and ASW, and wells containing only ASW with the same distribution of embryos were included as controls along with the embryos incubated with anti-11.6 S and anti-6.4 S sera. Open microplates were incubated at 18°C for 24 hr in humid chambers. Microplate cultures were fixed in a final concentration of 3.3% formaldehyde (Ted Pella Inc., Redding, CA), closed and maintained at room temperature. Fixed cultures were examined and scored using a Zeiss Invertoskop inverted microscope (Zeiss, Inc., Oberkochen, Germany) and micrographs were taken with a Canon Digital IXUS 800 IS camera (Canon, Inc., Tokyo, Japan).

L. pictus immunofluorescence labeling

L. pictus embryos at stages of development from mesenchyme blastula through late prism were concentrated into 63 µm Nitex mesh collection filters partially submerged in glass culture dishes. 2.0 ml of swimming embryos were collected into 2.0 ml microcentrifuge tubes and gently pelleted by allowing the benchtop centrifuge to come to full speed for 2.0 seconds and then to decelerate. ASW was manually aspirated. Embryos were resuspended, fixed and permeabilized in -20°C methanol for 30 min, washed 3 times in phosphate buffered saline (PBS, pH 7.2) and maintained at 4°C in PBS until ready to use (maximum 4 days).

Embryos were blocked with PBS containing 5.0% normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (PBS+N) for 30 min at room temperature with gentle rocking. The anti-11.6 S and 6.4 S primary antibodies were diluted 1/1000 in PBS +N and distributed in 250 µl aliquots into 12 × 75 mm disposable borosilicate glass culture tubes. 4 sets of tubes were prepared, 2 of which contained the anti-11.6 S primary antibody and the other 2 contained the anti-6.4 S primary antibody. 25 µl of packed embryos were added to each tube. Embryos were incubated at room temperature (25°C) for 6 hr while rocking gently. They were then washed 3 times for 5 min in PBS. Following the third wash, FITC and Texas Red™-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories), each diluted 1/300 in PBS+N, were added to each of the 4 sets of embryos. This was to insure each full set of 11.6 S and 6.4 S tagged embryos would be labeled with each fluorochrome. Embryos were incubated overnight at 18°C. Controls used in this assay included embryos incubated with pre-immune sera diluted 1/1000 in PBS+N with and without secondary antibody, embryos incubated in PBS+N with and without secondary antibody, and one sample of embryos in PBS alone to determine autofluorescence.

For the co-localization study, 2 tubes of 25 µl of late gastrula stage embryos, were blocked with PBS+N for 30 min at room temperature with gentle rocking. Then, tube #1 was incubated with the anti-11.6 S primary antibody, and tube #2 with the anti-6.4 S primary antibody (both diluted 1/1000 in PBS+N), at room temperature, for 6 hours, again with gentle rocking. Embryos were then washed 3 times for 5 min in PBS. After the third wash, FITC-conjugated goat anti-rabbit IgG secondary antibody, diluted 1/300, was added to both the anti-11.6 S and anti-6.4 S tubes. Following overnight incubation, all embryos were washed 3 times for 5 min each, then blocked again in PBS+N for 30 min at room temperature. At that time, the anti-6.4 S antibody (diluted 1/1000 in PBS+N) was added to tube #1 which contained the 11.6 S tagged embryos. At the same time, the anti-11.6 S antibody (diluted 1/1000 in PBS+N) was added to tube #2, which contained the 6.4 S tagged embryos. All were incubated at room temperature for 6 hours while gently rocking. Embryos were then washed 3 times for 5 min in PBS. Following the third wash, Texas Red™-conjugated goat anti-rabbit IgG secondary antibody, diluted 1/300 in PBS+N, was added to both tubes and again incubated overnight at 18°C. Duplicates were made of both conditions, and anti-sera controls were prepared in the same manner.

Following overnight incubation, all samples were washed 3 times for 5 min in PBS. A fourth PBS wash included 0.5% 1,4-Diazabicyclo [2.2.2] octane (DABCO, Sigma-Aldrich, St. Louis, MO) anti-fade reagent. Particular care was taken to maintain optimum morphology of the embryos. Thus, mounting medium was not used.

For microscopy, “chambers” were prepared by applying double-thick pieces of Scotch tape to poly-lysine coated slides, over which coverslips were placed with 12 µl of embryos in each chamber. The chambers were sufficiently deep so as to prevent crushing of the embryos.

Samples were examined using an Olympus BH2-BHTU fluorescence microscope (Olympus, Inc., Tokyo, Japan) equipped with a separate Mercury-100 light source (Chiu Technical Corporation, Kings Park, NY) and micrographs were captured with a Canon Digital IXUS 800 IS camera (Canon, Inc., Tokyo, Japan).

S. purpuratus dissection and immunofluorescence labeling

Whole formaldehyde-fixed *S. purpuratus* embryos and the dissected components of the specific cellular interaction under study were used here. The dissection procedure was performed to help determine if some unknown confounding factor in whole embryos might influence labeling results. The dissected pieces, because of their unique characteristics, were treated differently than the whole embryos as follows.

Whole *S. purpuratus* gastrulae (55 hr old, maintained at 14°C) were fixed and preserved in 50 ml centrifuge tubes with 3.7% (final concentration) formaldehyde (Ted Pella Inc., Redding, CA) in ASW at room temperature. The fixative solution was removed and the embryos were transferred to 1.5 ml ClickSeal™ microcentrifuge tubes (National Scientific Supply, Claremont, CA) and washed by adding fresh ASW to the tubes and inverting them several times. The washing step was repeated three times.

The washed, fixed whole embryos were incubated for 30 min in Tris buffered saline, pH 7.6 (TBS) containing 0.4% bovine serum albumin. This was removed, and the embryos were permeabilized by incubation for 30 min in TBS containing 1% Triton-X (TBT). The TBT was removed and incubation in TBT was repeated twice.

Whole fixed embryos were blocked in TBT containing 5.0% normal goat serum (GIBCO, Invitrogen Inc., Carlsbad, CA)(TBT+N) for 30 min in 1.5 ml microcentrifuge tubes. The tubes were inverted several times during the incubation period. The TBT+N was removed and the anti-11.6 S hyalin primary antibody was added to the embryos in a final dilution of 1/1000 in TBT+N. The embryos were incubated in the anti-11.6 S hyalin primary antibody in TBT+N for 2 hr at room temperature and then overnight at 4°C. The control embryos were incubated in pre-immune sera in TBT+N as described above.

Whole embryos were placed in 0.1 ml of the anti-11.6 S hyalin primary antibody in TBT+N solution on a siliconized (Sigma-Aldrich, St. Louis, MO) glass slide, then dissected as previously described (Coyle-Thompson and Oppenheimer, 2005). Whole fixed embryos and the fixed dissected pieces were incubated on the slide in the antibody and TBT+N solution for an additional 2 hr. The primary antibody solution was removed from the slide and the embryos and pieces were incubated in containing 0.2% bovine serum albumin and 0.1% Triton-X and 5.0% normal goat serum (PBT+N) for 30 minutes. Embryos and dissected pieces were then incubated on the slide in FITC-conjugated goat anti-rabbit secondary antibody (Cappel Antibodies, Rockland Immunochemicals, Gilbertsville, PA) diluted 1:200 in PBT+N for 2 hr at room temperature or overnight at 4°C.

Embryos and dissected pieces were observed using a Leica FZLIII stereo fluorescence dissecting microscope (Wetzlar, Germany) and a Zeiss-7082 microscope equipped with an epi-fluorescence condenser (IV FI) (Zeiss, Inc., Oberkochen, Germany). Images were captured using a Canon PowerShot A95 digital camera (Canon, Inc., Tokyo, Japan).

Digital image processing

Micrographs taken on all microscopes used in this body of work were digitally mastered and assembled using Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA).

Statistics

For experiments testing the effects of sera on development, usually four replicate wells were done at each antiserum concentration for both the anti-11.6 S and anti-6.4 S antisera within each experiment. Usually four replicate wells with pre-immune sera were also tested at several concentrations within the range used for the immune sera. Seawater controls were also performed in replicates of four wells and the whole matrix was repeated a minimum of five times. For each experiment, a range of 1000–3000 embryos was scored. The percentage of each effect on development was calculated within each experiment and all were averaged together and the mean and standard error of the means were calculated. A two-tailed t-test analysis was used to determine the significance of key observed differences.

Results

Effects of Anti-11.6 S Hyalin and Anti-6.4 S Hyalin Sera on *L. pictus* Development

As shown in Table 1, the pre-immune serum had essentially no effect on archenteron elongation and attachment when employed at dilutions from 1/350 to 1/2000 and the seawater controls also showed normal development. As can also be seen in the table, the principal effect of the 11.6S antiserum was a dose-dependent inhibition of archenteron elongation/attachment to the roof of the ectoderm. As shown in Fig. 1, low concentrations of anti-11.6 S hyalin serum inhibit archenteron attachment to the blastocoel roof whereas the pre-immune serum had no effect. The antiserum concentration required for 50% inhibition is approximately 1/8000. A two-tailed t-test analysis of the data for a 1/1000 dilution of the anti-11.6 S serum and the pre-immune serum showed that the difference between the pre-immune and immune sera (98.6% archenteron attachment vs. 2.7% archenteron attachment, respectively) was significant at a $p < 0.001$. Exogastrulation, where the developing archenteron everts out of the embryo, was also induced (31%) at a serum dilution of 1/1400 and a dose-dependent inhibition of archenteron elongation was also observed. Micrographs of these developmental effects are shown in Fig. 2. These results suggest that the 11.6 S hyalin molecule is a specific cell adhesion molecule involved in archenteron-blastocoel roof attachment.

The protocol used for the anti-11.6 S hyalin serum was followed to examine the effects of anti-6.4 S hyalin serum on development. As shown in Table 2, archenteron elongation/blastocoel roof attachment was partially inhibited at relatively high antiserum concentrations when compared to the results obtained with the anti-11.6 S hyalin serum. Thus the concentration of antiserum required for 50% inhibition was approximately 1/400. A two-tailed t-test analysis of the data for a 1/500 dilution of the anti-6.4 S serum and the pre-immune serum showed that the difference between the pre-immune and immune sera (99.4% archenteron attachment vs. 58.4% archenteron attachment, respectively) was significant at a $p < 0.001$. Since the antibody titers for the anti-11.6 S and anti-6.4 S hyalin antisera were similar (Justice, 1989, Justice and Carroll, 1989), these results suggest that the 6.4 S hyalin molecule has a quantitatively less important role as a specific cell adhesion molecule involved in archenteron elongation/blastocoel roof attachment. A total of approximately 40,000 embryos were examined in all the microplate experiments.

Localization of 11.6 S and 6.4 S Hyalin During Archenteron Development Period

To determine the cellular localization of the 11.6S and 6.4S hyalins during archenteron development, embryos were cultured and labeled for binding of pre-immune serum (control) and anti-11.6S and anti-6.4S hyaline antibodies. Co-localization was observed throughout development. Control embryos of both species revealed no significant background fluorophore signal. Results are illustrated in Figure 3. A typical example is depicted in Figures 3A and 3B, a *S. purpuratus* embryo. As the archenteron began the process of attachment to the blastocoel roof, labeling intensity of both antibodies in *L. pictus* decreased throughout the blastocoel and increased gradually in the area of attachment. Figures 3C and 3D show the localization of each antibody, and Figure 3E displays the co-localization of both. The labeling intensified significantly as *L. pictus* developed into the prism stage and localized further to the stomodeum (mouth). Once perforation had occurred and the stomodeum formed, labeling intensity in that area was quite dramatic (Fig. 3F). Although there was a low grade of internal fluorescence labeling, it was highly localized to the mouth. The ectodermal cells were all observed to display a lower grade of labeling as well as continued labeling of the extracellular matrix hyaline layer. About 200 embryos were examined in the immunohistochemistry studies showing similar results to those described above.

Immunolocalization of 11.6 S Hyalin in Dissected *S. purpuratus* Embryos

As shown in Fig. 3, the dissected roof of a *S. purpuratus* gastrula labeled brightly with anti-11.6 S hyalin antibody and FITC-conjugated secondary antibody. The brightly labeled lumen of the archenteron was observed as well, the remnant of extracellular matrix hyaline layer which was observed to label evenly and brightly in both species. Labeling of the tip of the archenteron was less intense than the roof of the blastocoel. Cells surrounding the archenteron were also observed to label. Similar results were observed with the dissected pieces in the 65 embryo dissections examined.

Discussion

Previous studies have shown that monoclonal antibodies against *S. purpuratus* hyalin cross react with *L. pictus* hyalin (Vater and Jackson, 1989). *S. purpuratus* hyalin blocks the archenteron/blastocoel roof interaction in *S. purpuratus* and *L. pictus* embryos (Razinia *et al.*, 2007; Alvarez *et al.*, 2007) and *L. pictus* hyalin also blocks this cellular interaction (Contreras *et al.*, 2007). It remained to be seen if antibody against hyalin also blocked the interactions and if it localized to hyalin at the points of the interaction.

For many studies, monoclonal antibodies are too specific to pick out the antigen of interest due to the single determinant against which the antibodies are raised. Therefore, we wanted to have a broad suite of antibodies against the single homogeneous hyalin proteins, each of which was separately used to raise antibodies. These antisera are not available commercially and no other laboratory has prepared antisera against individually purified hyalin proteins. In addition, polyclonal antibodies have been reagents of choice in many studies using echinoderms (*e.g.*, Hertzler and McClay, 1999; Wessel *et al.*, 2000; Hoodbhoy *et al.*, 2001; Katow and Sofuku, 2001). If monoclonal antibodies were used in the present study, many components of the large hyalin molecules would not have been recognized.

Here we show that polyclonal antibodies made against 11.6 S and 6.4 S *S. purpuratus* hyalin labeled the tip of the archenteron and the roof of the blastocoel in *L. pictus* embryos. The anti-11.6 S hyalin antibody also blocked the attachment interaction in *L. pictus* embryos, as *S. purpuratus* embryos were unavailable at the time of the microplate studies. Previous results, however, showed that exogenously added *S. purpuratus* and *L. pictus* hyalin were interchangeable in their activity on both species (Razinia *et al.*, 2007; Alvarez *et al.*, 2007; Contreras *et al.*, 2007) and in antibody cross reactivity (Vater and Jackson, 1989).

In order to more closely examine the internal nature of the interactions, we used immunofluorescence techniques using whole embryos and dissected pieces. Sea urchin embryo dissection has been a successful technique used to study specific interactions (Ettensohn, 1984; McClay and Logan, 1996; Hardin and Armstrong, 1997; Coyle-Thompson and Oppenheimer, 2005). Here we used it in a novel way to compare results obtained by standard immunotechniques.

In a single cell assay, using cells dissociated from *L. variegatus* sea urchin embryos, it was found that cells bound to both native hyalin or a bacterially expressed recombinant hyalin repeat, which does not contain carbohydrate (Wessel *et al.*, 1998). An anti-hyalin monoclonal antibody (Tg-HYL (McAb183) blocked cell binding to both native and recombinant hyalin (Wessel *et al.*, 1998). This same antibody caused cells *in vivo* to retract from the hyaline layer, blocking gastrulation (Adelson and Humphreys, 1988). These studies however, did not examine the role of hyalin and the hyalin repeat domain in the specific cellular interaction studied here. Wessel *et al.*, (1998) state that "We have not tested other nonrepeat regions of hyalin for substrate binding activity, so there could be more cell-binding sites."

Our results show that 11.6 S and 6.4 S hyalin are localized on both partners of the cellular interaction, the tip of the archenteron and the blastocoel roof in *L. pictus*, and that the 11.6 S hyalin has the same localization in *S. purpuratus*. The status of 6.4 S hyalin was not examined in *S. purpuratus*. It is therefore possible that hyalin mediates this cellular interaction by a homophilic hyalin-hyalin interaction without the requirement of a separate hyalin receptor or hyalin binding ligand. It is also possible that the interaction mechanism is heterophilic with both hyalin and a hyalin receptor present in both partners.

Hyalin contains carbohydrate that may include mannose and /or glucose residues (Citkowitz, 1971). This is also suggested by our unpublished observations showing that hyalin adheres to mannose /glucose binding lectins in dot blots. Mannose/glucose groups have been implicated in the interaction of the archenteron tip and blastocoel roof as *Lens culinaris* agglutinin (a mannose/glucose binding lectin) blocked the interaction and was localized, as shown for hyalin, on the blastocoel roof and archenteron surface (Latham *et al.*, 1998; Latham *et al.*, 1999). Also, enzymes that cleave mannose/ glucose residues from glycoproteins, glycolipids and polysaccharides blocked the interaction (Khurram *et al.*, 2004) and of 22 sugars tested, only a six glucose residue cyclic polysaccharide alpha-cyclodextrin, blocked the interaction (Sajadi, *et al.*, 2007). Other studies in echinoderms have shown that hyaline layers label with mannose/ glucose binding lectins (Cerra, 1999). It is possible, therefore, that the carbohydrate of hyalin may be directly involved in the interaction being studied, or mannose/glucose residues may be part of a hyalin-binding receptor or hyalin-binding ligand. We are currently examining these possibilities using carbohydrate-free hyalin and carbohydrate microarrays to see to what hyalin can bind.

In summary, we have shown that 11.6 S hyalin is localized on the structures involved in the cellular interaction and that very low concentrations of antibody against 11.6 S hyalin blocks the interaction of the archenteron and blastocoel roof. This study, coupled with other recent work (Razinia *et al.*, 2007; Alvarez *et al.*, 2007; and Contreras *et al.*, 2007) that showed that exogenously added hyalin or microinjected hyalin blocks this interaction, suggests that hyalin is a cell adhesion molecule that mediates a specific cellular interaction in the NIH designated model sea urchin system. Interest in hyalin (and more recently in the hyalin repeat domain) has existed for a century. We have, for the first time, provided evidence that hyalin plays a specific role, not just a general role (Wessel *et al.*, 1998), as a cell adhesion molecule that may be of importance in organisms as diverse as sea urchins, bacteria, worms, mice and humans (Callebaut *et al.*, 2000; Wessel *et al.*, 1998).

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Figure 1.

The effect of pre-immune and anti-11.6S hyalin antiserum on archenteron attachment to the roof of the blastocoel. The mean values and the standard error of the means are presented for the immune serum dilutions (filled diamonds) and are within the symbols for the pre-immune serum dilutions (filled squares).

Figure 2.

Effects of anti-11.6 S hyalin antiserum on archenteron elongation and attachment to the roof of the blastocoel. (A) A field of control embryos at the completion of archenteron attachment to the roof of the blastocoel; the inset shows a higher magnification view of a single embryo. (B)(C)(D) Beginning stages where the 11.6 S antibody was added. (B) Mesenchyme blastula, where primary mesenchyme cells begin to detach from the extracellular hyaline matrix in the epithelium to mesenchyme transition. (C) Early primary invagination, where the vegetal plate has buckled and the primary mesenchyme cells are migrating into the blastocoel. (D) The developing archenteron has extended 1/3 the distance across the blastocoel. (E) Anti-11.6 S hyalin-induced exogastrulation. (F)(G)(H)(I) Anti-11.6 S hyalin-induced arrest of archenteron

elongation at less than 1/3 to 1/3 (F); greater than 1/3 to 1/2. At 1/2 the secondary mesenchyme cells begin to migrate from the archenteron tip (G); greater than 1/2 to 2/3 (H); greater than 2/3 to archenteron attaching (I). Abbreviations used are AP (animal pole in (A)); VP (vegetal pole in (A)); A (archenteron in (A)); at (archenteron tip in (D)(G)(H)(I)); br (blastocoel roof in (B)); e (exogastrula in (E)); pmc (primary mesenchyme cells in (B)(C)); smc (secondary mesenchyme cells in (G);). All scale bars = 35 μ m.

Figure 3.

Internal localization of the anti-11.6 S and anti-6.4 S hyalin antibodies by immunofluorescence in *S. purpuratus* and *L. pictus* sea urchin gastrulae. (A) *S. purpuratus* control gastrula with $>2/3$ archenteron extension. (B) Same control *S. purpuratus* embryo incubated in pre-immune sera and FITC-conjugated goat anti rabbit secondary antibody. All controls used in this study in both species exhibited similar negative fluorescence signal. (C) *L. pictus* late gastrula stage embryo showing the anti-11.6 S antibody with FITC-conjugated secondary localizing heavily to the area of archenteron attachment to the blastocoel roof. This is the area, which will perforate, and the mouth will form. (D) The same *L. pictus* embryo showing the anti-6.4 S antibody with Texas Red™-conjugated secondary localizing as in (C). (E) The same *L.*

pictus embryo showing co-localization of the two antibodies. (F) An *L. pictus* prism stage embryo showing the anti-11.6 S antibody with FITC-conjugated secondary very heavily localized to the mouth, which has formed following attachment of the archenteron to the blastocoel roof. (G) Dissected roof of a late *S. purpuratus* gastrula showing the internal localization of the anti-11.6 S primary antibody with FITC-conjugated goat anti rabbit secondary antibody. (H) Dissected archenteron and roof piece of a late *S. purpuratus* gastrula showing labeling of the archenteron tip and blastocoel roof with the 11.6 S primary antibody and FITC-conjugated secondary antibody. The lumen also brightly labels as a remnant of the hyaline matrix surrounding the embryo throughout development. Actual micrographs are displayed in insets. Abbreviations used are AP (animal pole in (A)); VP (vegetal pole in (A and F)); br (blastocoel roof in (A and G)), at (archenteron tip in (A and H)); at/br (archenteron tip/blastocoel roof in (C)); M (mouth in (F)); and A (archenteron in (A) (E) (H)). All scale bars = 20 μ m.

Table 1

Effect of anti 11.6 S hyalin serum on *L. pictus* exogastrulation, archenteron elongation and attachment. These data are from five separate experiments and the numbers presented in the table are the means (+/- the standard error of the mean) for each condition. A total of 26,000 embryos was used in a total of five separate experiments.

Anti-11.6 S Serum (Dilution Factor)	Percent Exogastrulation	Percent Archenteron Less than 1/3	Percent Archenteron 1/3 to 1/2	Percent Archenteron 1/2 to 2/3	Percent Attachment of Archenteron	Pre-Immune Serum (Percent Attachment of Archenteron)	Seawater (Percent Attachment of Archenteron)
350	9.9 (6.0)	82 (5.3)	18.0 (10.7)	1.9 (0.8)	2.2 (1.7)	98.5	99.4 (n=6111)
400	7.2 (5.2)	66.7 (10.2)	7.5 (1.3)	22.1 (8.8)	2.0 (0.5)	100	
500	6.5 (2.8)	69.3 (7.5)	12.8 (3.8)	9.9 (6.)	3.2 (1.2)	98.9	
700	16.9 (10.2)	72.6 (9.9)	6.3 (1.5)	3.9 (1.5)	0.3 (0.1)		
800	14.2 (9.3)	42.4 (13.2)	17.4 (6.1)	22.4 (8.6)	3.4 (1.6)		
1000	18.8 (9.3)	43.5 (10.3)	12.7 (2.7)	21.3 (6.9)	2.7 (1.1)	98.2	
1400	31.0 (14.0)	43.0 (18)	15.0 (5.5)	10.9 (3.5)	1.8 (0.8)		
2000	19.6 (7.5)	25.5 (10.3)	31.2 (3.3)	31.6 (63)	7.9 (1.2)	99	
4000	16.3 (4.6)	9.7 (4.0)	18.8 (5.9)	31.4 (5.5)	23.7 (8.0)		
8000	14.8 (6.0)	4.3 (1.8)	31.2 (4.5)	25.0 (6.4)	46.0 (13)		
16000	6.9 (3.1)	0.4 (0.3)	4.8 (4.4)	17.4 (7.9)	70.7 (12.9)		
32000	0.5 (0.3)	0.2 (0.2)	0.6 (0.5)	2.9 (1.4)	95.7 (2.0)		

Table 2

Effect of anti-6.4 S hyalin serum on *L. pictus* exogastrulation, archenteron elongation and attachment. These data are from five separate experiments and the numbers presented in the table are the means (+/- the standard error of the mean) for each condition. A total of 14,000 embryos was used in a total of five separate experiments.

Anti-6.4 S Serum (Dilution Factor)	Percent Exogastrulation	Percent Archenteron Less than 1/3	Percent Archenteron 1/3 to 1/2	Percent Archenteron 1/2 to 2/3	Percent Attachment of Archenteron	Pre-Immune Serum (Percent Attachment of Archenteron)	Seawater (Percent Attachment of Archenteron)
350	3.3 (2.5)	3.0 (1.6)	12.9 (6.1)	17.3 (7.4)	59.0 (14)		99.5 n=(6197)
400	16.0 (9.8)	2.9 (1.6)	12.9 (6.1)	17.3 (7.4)	49.8 (18)		
500	12.8 (6.7)	2.2 (1.0)	5.0(2.0)	22.0 (5.3)	58.0 (12.6)	99.4	
700				16.3 (12)	83.0 (12)		
800							
1000				8.8 (2.7)	78.4 (9.3)	98.6	
1600				1.6 (.6)	96.5 (0.8)		
2000						99.5	