Exogenous hyalin and sea urchin gastrulation. Part III: Biological activity of hyalin isolated from *Lytechinus pictus* embryos

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Summary

Hyalin is a large glycoprotein, consisting of the hyalin repeat domain and non-repeated regions, and is the major component of the hyaline layer in the early sea urchin embryo of *Strongylocentrotus purpuratus*. The hyalin repeat domain has been identified in proteins from organisms as diverse as bacteria, sea urchins, worms, flies, mice and humans. While the specific function of hyalin and the hyalin repeat domain is incompletely understood, many studies suggest that it has a functional role in adhesive interactions. In part I of this series, we showed that hyalin isolated from the sea urchin *S. purpuratus* blocked archenteron elongation and attachment to the blastocoel roof occurring during gastrulation in *S. purpuratus* embryos, (Razinia *et al.*, 2007). The cellular interactions that occur in the sea urchin, recognized by the U.S. National Institutes of Health as a model system, may provide insights into adhesive interactions that occur in human health and disease. In part II of this series, we showed that *S. purpuratus* hyalin heterospecifically blocked archenteron–ectoderm interaction in *Lytechinus pictus* embryos (Alvarez *et al.*, 2007). In the current study, we have isolated hyalin from the sea urchin *L. pictus* and demonstrated that *L. pictus* hyalin homospecifically blocks archenteron–ectoderm interaction, suggesting a general role for this glycoprotein in mediating a specific set of adhesive interactions. We also found one major difference in hyalin activity in the two sea urchin species involving hyalin influence on gastrulation invagination.

Keywords: Archenteron attachment, Hyalin, *Lytechinus pictus* sea urchin embryo

Introduction

At least 25 discoveries of physiological mechanisms have been made using sea urchin embryos that have been shown to be of importance in higher organisms including humans (Davidson & Cameron, 2002;

Davidson, 2006). As a result, the U.S. National Institutes of Health designated the sea urchin embryo as a model system that provides insights into mechanisms important in human health and disease (Davidson & Cameron, 2002; Davidson, 2006). The sea urchin egg and embryo extracellular matrix have long been the subjects of study due, in part, to the spectacular structural and biochemical changes that occur in fertilization and early development. With the advent and maturation of molecular technique and the NIH designation of the sea urchin system as a medically relevant model, we have reinvestigated the possible function(s) of the hyaline layer and its constituent proteins/glycoproteins in embryogenesis.

Hyalin, a large glycoprotein of the sea urchin hyaline layer, and its hyalin repeat domain, a sequence of about 84 amino acids that is associated with the immunoglobulin-like fold, is found in organisms as

diverse as bacteria, sea urchins, worms, flies, mice and humans (Wessel *et al.*, 1998; Callebaut *et al.*, 2000). In addition, p62 and p56, two proteins found in hamster egg cortical granules and in the zygote cortical granule envelope share a common immunological epitope with IL2, a polyclonal rabbit antibody prepared against 11.6 S hyalin (Justice & Carroll, 1989) isolated from *Strongylocentrotus purpuratus* embryos (Gray *et al.*, 1986).

The protein hyalin (actually a complex of three separate, related molecules, see below) is contained within the cortical granules of the unfertilized sea urchin egg (Hylander & Summers, 1982; Vater & Jackson, 1989), and forms the hyaline layer extra-cellular matrix in the fertilized egg within 20 min postinsemination (see the reviews by Schuel, 1978; Shapiro *et al.*, 1981). The protein has been characterized and operationally defined by its solubility in divalent cation free media and its Ca^{2+} and Mg^{2+} precipitability. Extensive work from the laboratories of Carroll & Nagel (Gray *et al.*, 1986; Justice *et al.*, 1988, 1992) has shown, in *S. purpuratus*, that hyalin consists of three large asymmetric molecules of 11.6S, 9.6S, and 6.4S. Poly-clonal rabbit antibody work has shown that the three molecules exist in two immunological families (Justice, 1989; Justice & Carroll, 1989). Although hyalin has been suggested to function in adhesive interactions, some direct evidence for this function has been obtained in sea urchin development, (Herbst, 1900; Fink & McClay 1985; Adelson & Humphreys, 1988; Wessel *et al.*, 1998) and in hamster cleavage (Hoodbhoy *et al.*, 2000).

Microassays have been used to investigate the function of hyalin and other putative adhesive proteins in sea urchin embryos, often using disaggregated single cells (Fink & McClay, 1985; McClay, 1986; Wessel *et al.*, 1998). Recently, we reported the use of a microplate assay that allowed for the quantification of developmental effects of exogenously added hyalin and other molecules in whole, living sea urchin embryos (Alvarez *et al.*, 2007; Razinia *et al.*, 2007; Sajadi *et al.*, 2007) and showed in part I of this series that hyalin isolated from *S. purpuratus* embryos blocked archenteron elongation/attachment to the blastocoel roof in *S. purpuratus* embryos (Razinia *et al.*, 2007). In part II of this series, we showed that *S. purpuratus* hyalin blocked this adhesive interaction in *Lytechinus pictus* embryos. Here we take these studies one important step further to learn if hyalin isolated from *L. pictus* has the same biological activity as *S. purpuratus* hyalin. We isolated hyalin, for the first time, from *L. pictus* embryos and tested its effects on early *L. pictus* development using the microplate assay. We found inhibition of archenteron elongation and attachment to the blastocoel roof and therefore provide evidence for a specific adhesive function of hyalin in addition to its role as an extracellular matrix molecular set.

Materials and methods

Solutions

Artificial seawater (ASW; 423 mM NaCl, 9 mM KCl, 9.3 mM $CaCl_2$, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 8.0) was prepared by following the Marine Biological Laboratory (Woods Hole, MA) formula. Low-calcium seawater (LCSW) was prepared by reducing the calcium concentration to 1.5 mM (Bidwell & Spotte, 1985). All chemicals and reagents were prepared using materials obtained from Sigma Chemical Co, St Louis, MO, USA.

Preparation of hyalin

L. pictus sea urchins were obtained from Marinus Scientific, Garden Grove, CA. Spawning was induced by injecting 0.55 M KCl into the coelomic cavity. Eggs were collected by inverting the female and letting it shed its eggs over a beaker filled with artificial seawater at 11 °C. Sea urchin sperm was collected 'dry' in a petri dish and kept on ice until use. Eggs were filtered through 180 µm Nitex mesh (Tetco Inc.) and washed three times with large amounts of seawater. Subsequent dejellying of the eggs was achieved by bringing a suspension of 0.5% eggs in ASW immediately to pH 5.5–5.7 with 1 N HCl. The eggs were allowed to settle without being disturbed for 2 min before returning the suspension to pH 8.0 with 2 M Tris-base. The dejellied eggs were then washed three times with 500 ml of artificial seawater and their vitelline envelopes were disrupted with 0.01 M dithiothreitol (DTT), 0.1 M Tris-base in ASW, pH 9.2 for 3 min. The eggs were washed several times with 0.01 M Tris-ASW, pH 8.0. Four volumes of eggs were inseminated with 1 volume of dilute sperm (1 ml sperm/25 ml 0.01 M Tris-ASW, pH 8.0). During the 45–90 s post-insemination, the suspension was diluted in 8 volumes of ASW and the hyaline layers were allowed to form for 30–45 min while the eggs settled in the incubator at 15 °C. Hyalin protein was isolated and purified by the method described by Gray *et al.*, 1986 and Razinia *et al.*, 2007 with the following variations. After the fertilized eggs settled, the supernatant was aspirated leaving adherent cells, and the hyaline layers were dissociated from the egg surfaces by the addition of 50 ml of 0.475 M NaCl–0.025 M KCl. Embryos were stirred in this solution for 15 min until the hyaline layers appeared substantially reduced. After the embryos settled, the supernatant that contained the hyalin was collected. Hyalin protein was centrifuged at 20 000 *g* for 15 min at 4 °C to eliminate residual sperm and insoluble contaminants. The supernatant solution containing hyalin protein was collected and frozen at −20 °C.

Protein concentration

Protein concentration was determined at 260 nm and 280 nm (Warburg and Christian, 1941).

Gel electrophoresis

In order to assess hyalin purity, electrophoresis was done with 4% (w/v) polyacrylamide gradient gels on vertical submarine slabs. Proteins were visualized by silver staining using the method of Merrill *et al.* (1984), or using Coomassie Brilliant Blue G250. Prior to silver staining, gels were fixed with 40% methanol, and 10% acetic acid for 30 min.

Embryo extraction/culture

L. pictus gametes were obtained as previously detailed. Eggs were extensively washed three times at 15 min intervals with 500 ml of ASW, pH 8.0. The diluted sperm (1.2 ml concentrated sperm/5 ml ASW, pH 8.0) was combined with 6 ml of eggs suspended in 500 ml of ASW, pH 8.0. Early embryos were washed two times at 15 min intervals with 500 ml of ASW, pH 8.0 to eliminate excess sperm. The embryos were then transferred to a pyrex dish and were maintained in an incubator at 15 °C for a period of 24 h.

Microassay experiments

Using wide-mouthed pipette tips, 25 µl of 24-hour-old *L. pictus* embryos were transferred to 40 wells of a 96-well polystyrene flat-bottom microplate. There were about 10–15 live embryos per well. As the embryos were swimming, a precise sample quantity (number of embryos/well) could not be added to the wells. The embryos in each well were incubated at 15 °C for 24 h with 25 µl of various hyalin concentrations (0.00 mg/ml, 0.018 mg/ml, 0.036 mg/ml, 0.075 mg/ml, 0.100 mg/ml and 0.150 mg/ml) diluted with low calcium seawater. The same process was repeated 10 times for each concentration. Medium containing ASW, 0.475 M NaCl–0.025 M KCl, and LCSW were used as controls for the hyalin protein experimental samples. At 48 h, the embryos were fixed with 2.5 µl of 2.5% formaldehyde solution. Embryos in each well were observed using a Zeiss Axiolab (Oberkochen) photomicroscope and the archenteron morphologies were scored (complete, unattached, exogastrula or no invagination). For each treatment, the total sample size was obtained by combining the number of embryos in each well for the 10 replicates. The particular archenteron morphologies for the 10 replicates were recorded as mean percentages ± standard error of the mean (SEM). An unpooled two-sample *t*-test was used to analyse the significance of the observed differences in numbers of complete archenterons in the combined controls versus the high hyalin-treated samples. The differences were highly significant ($p < 0.001$).

Results

Hyalin was isolated in *L. pictus* as described by Gray *et al.* (1986) for *S. purpuratus* and modified by Razinia *et al.* (2007). The purity of hyalin preparations (0.2 mg protein/ml) was assessed on 4% polyacrylamide gels (Fig. 1). A deliberately overloaded gel is shown in Fig. 1, which indicates the presence of one major band of 11.6S hyalin. In this overloaded condition the small amount of a few minor species that are typically present is shown. These minor species are normally observed in hyalin preparations and probably represent smaller hyalin species as observed and described by Gray *et al.* (1986) in *S. purpuratus*.

In order to determine the morphological effects of exogenous hyalin on gastrulation, 24 h *L. pictus* embryos were incubated for 24 h with a variety of hyalin concentrations (using the preparation described above) and control media. The total sample numbers of embryos for 10 replicates treated with the various hyalin concentrations of 0.00, 0.18, 0.036, 0.075, 0.10 and 0.15 mg/ml in LCSW were: 267, 274, 212, 212, 227, and 262 and the controls were 319, 294, 312 and 208. As shown in Fig. 2*a*, each of the control media embryo samples showed normal archenteron elongation and attachment to the blastocoel roof, while embryos in the
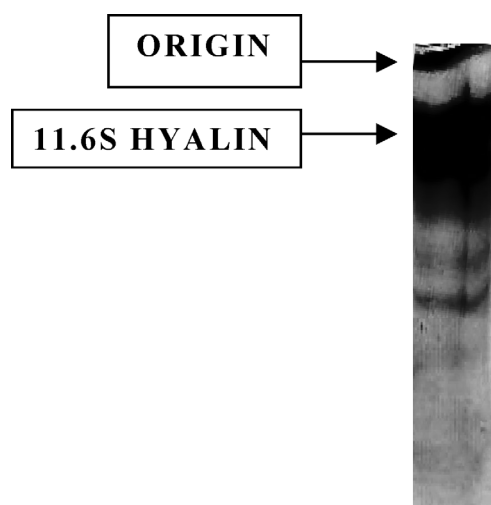
Figure 1 4% (w/v) polyacrylamide gradient gel electrophoresis of *L. pictus* hyalin preparations. The massive 11.6 S hyalin band is indicated with an arrow. The direction of migration, toward the positive pole, is from top to bottom. The other minor species are present in purified hyalin preparations and are believed to represent smaller hyalin subunits (Gray *et al.*, 1985) or minor contaminants.
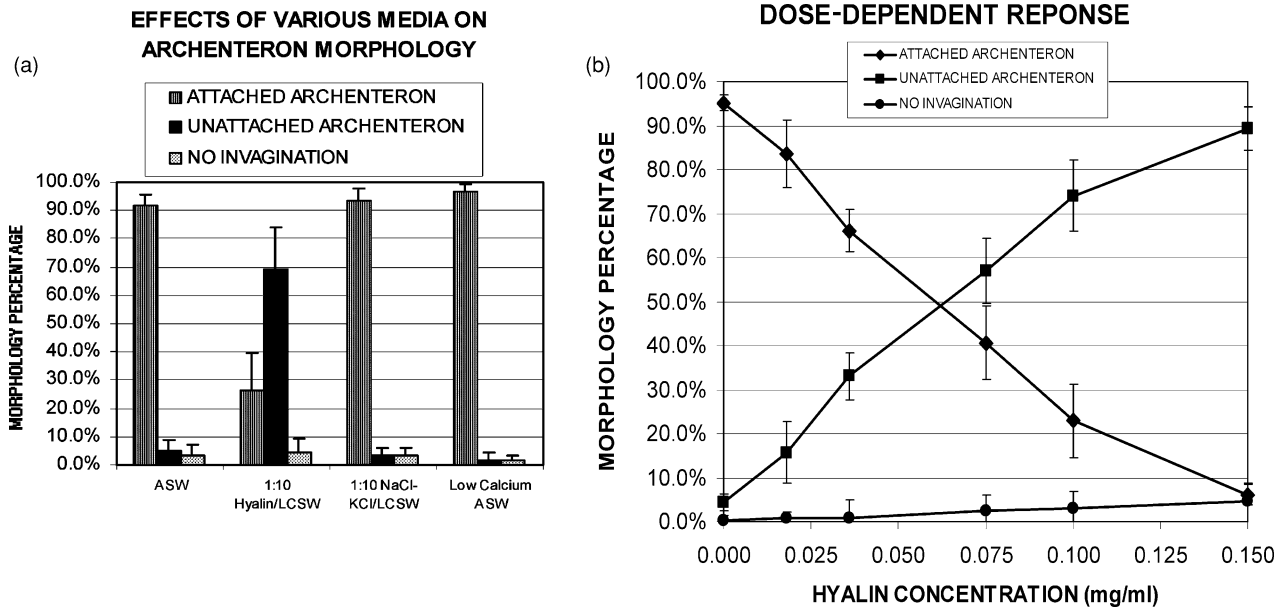
Figure 2 (*a*) The effects of various media on archenteron morphology. *L. pictus* hyalin is at a concentration of 0.2 mg/ml LCSW. Error bars are ± SEM. (*b*) A dose-dependent response curve for various archenteron morphologies. This graph demonstrates the percentage of 48 h embryos that developed the following morphologies: complete archenterons, unattached archenterons and no invaginations when incubated with increasing hyalin concentrations. The data were plotted as mean percentages ± SEM of 10 experiments.

high hyalin concentration tested showed a failure of the archenteron attachment to the blastocoel roof.

Figure 2*b* represents a hyalin dose–response curve for the microassay trials. The mean percentages ± SEM for each morphology (complete archenteron, unattached archenteron and no invagination) were plotted. In the absence of hyalin, 95.1 ± 1.80% of embryos developed full archenteron attachment as seen in Fig. 2*b*. At lower hyalin concentrations, (0.018 and 0.036 mg/ml), there was a reduction in archenteron attachment to 83.6 ± 7.6% and 66.0 ± 4.8% of the embryos respectively. At higher hyalin concentrations (0.10, 0.15 mg/ml), the percentage of embryos with attached archenterons decreased even more notably to 22.9 ± 8.3% and 6.1 ± 2.3% respectively. The differences in archenteron morphology between no hyalin controls and highest hyalin concentration were statistically highly significant ($p < 0.001$). Exogastrulation and inhibition of invagination were almost never observed in any samples. Prior to fixation, embryos were determined to be viable as they were moving. Figure 3*a–c* provides photographs of embryos incubated in the experimental and control media showing attached and unattached archenterons. Figure 3*a* shows control embryos with normal development and complete archenteron attachment. Figure 3*b* shows an unattached archenteron when embryos were exposed to low hyalin concentration (0.018 mg/ml). Figure 3*c* shows the high incidence of unattached archenterons noted at the high hyalin concentration (0.150 mg/ml).

Discussion

In the sea urchin *S. purpuratus*, in part I of this series, hyalin blocked invagination in gastrula stage *S. purpuratus* embryos in a dose-dependent manner from low concentrations (e.g. 0.05 mg/ml) to high hyalin concentrations (0.15 mg/ml and higher), where most embryos exhibited totally blocked invagination at the highest concentrations tested (Razinia *et al.*, 2007). At low hyalin concentrations (e.g. 0.05 mg/ml) most of the embryos invaginated and exhibited unattached archenterons (Razinia *et al.*, 2007). In the present study, high concentration of *L. pictus* hyalin (0.15 mg/ml) had no effect on archenteron invagination at the stages and times tested, while they inhibited archenteron elongation/attachment to the blastocoel roof in *L. pictus* embryos. The dose–response curve for *L. pictus* hyalin showed a nearly straight line of increasing hyalin activity in inhibiting archenteron elongation/attachment to the blastocoel roof, with increasing hyalin concentrations, with about 90% of the embryos exhibiting blocked archenteron attachment at 0.15 mg/ml low calcium seawater. The *L. pictus* results shown here indicate that hyalin blocks archenteron elongation/attachment in viable, swimming embryos, possibly by blocking hyalin/hyalin receptor or ligand interaction within the embryos, with no effect on invagination. It has been previously shown that large molecules can enter the sea urchin blastocoel in normal artificial seawater (Latham *et al.*, 1998) and with
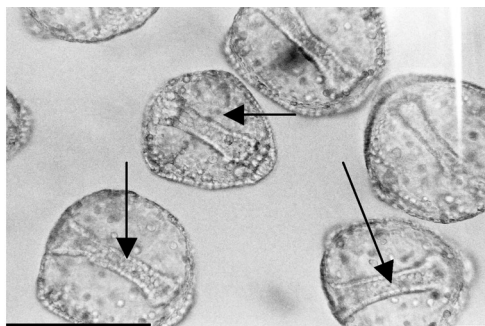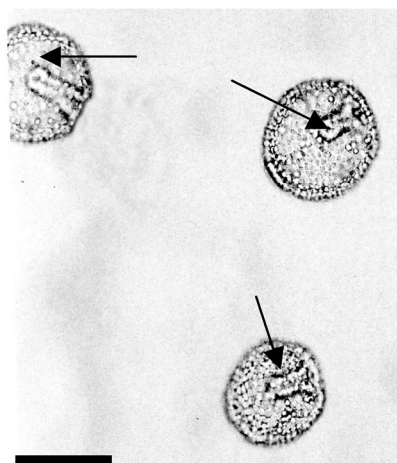
A

B

C

increasing speed in low calcium sea water (Latham *et al.*, 1998; Itza & Mozingo, 2005). We suggest that exogenous hyalin may exert the effects described herein following entry into the blastocoel.

The microplate approach is one of the assay systems that we use in analysing the effects of molecules on sea urchin embryo cellular interactions. We also use a novel assay in which we dissect the archenteron and blastocoel roof from the embryo proper and study the interactions of these isolated components in a pristine environment away from possible confounding factors present in intact embryos (Coyle-Thompson & Oppenheimer, 2005). The third assay system that we are beginning to use involves microinjection of molecules, such as hyalin into the sea urchin blastocoel. In collaboration with Dr Kathleen Foltz at the University of California, Santa Barbara, we have manually microinjected (total volume injected = 35 pl) of varying concentrations of *L. pictus* hyalin, from the same hyalin preparation as used here, into 24 h *L. pictus* embryo blastocoels using mercury-filled micropipettes (Jaffe & Terasaki, 2004). The results of these initial experiments indicated that microinjected hyalin inhibited archenteron attachment in living embryos as compared with control injections that had no effect. Complete microinjection studies will be the subject of another paper. We are unaware of any other studies that approach functional analysis of molecules in the sea urchin model that utilize all three of these assay systems.

For many decades, studies have indicated that hyalin is important in maintaining proper adhesive interactions in developing sea urchin embryos (Herbst, 1900; Fink & McClay, 1985; McClay, 1986; Wessel *et al.*, 1998). We are not aware of any studies that have focused on the role of hyalin in the model specific adhesive interaction described here. Sea urchin hyalin consists of units of the hyalin repeat domain that also have been identified in proteins in organisms as diverse as bacteria, flies, worms, mice and humans (Wessel *et al.*, 1998; Callebaut *et al.*, 2000) and non-repeated regions (Wessel *et al.*, 1998). We are not aware of any studies that have focused on the specific function of the hyalin repeat domain, although the hyalin repeat is similar to the immunoglobulin-like fold that has been associated with the function of various

Figure 3 (*a*) 48 h *L. pictus* embryos incubated in low calcium seawater, pH 8.0, at 24 h in absence of *L. pictus* hyalin. Arrow show complete archenterons attached to the blastocoel roof (scale bar = 80 μm). (*b*) 48 h *L. pictus* sea urchin embryos treated with low *L. pictus* hyalin concentrations (0.018 mg/ml) incubated in low calcium seawater at 24 h demonstrating unattached and attached archenterons. Arrows show unattached archenterons (scale bar = 80 μm). (*c*) 48 h *L. pictus* embryos treated with high *L. pictus* hyalin concentration (0.150 mg/ml) incubated in low calcium seawater at 24 h. Note the unattached archenterons (scale bar = 80 μm).

cell adhesion molecules (Edelman, 1987; Whittaker *et al.*, 2006). Further utilization of the three assay systems just described and the use of anti-hyalin antibody will help elucidate the function of hyalin, which we have termed a cell adhesion molecule that appears to be required for archenteron attachment to the blastocoel roof. In addition, isolation of hyalin binding receptors or ligands, so far under-investigated, should be feasible using hyalin derivatized beads and the three assay systems for assessing activity of isolated hyalin binding ligands or receptors.

The *S. purpuratus* sea urchin genome includes about 400 gene predictions of molecules that include immunoglobulin-like domains as well as many gene predictions of a variety of so-called cell adhesion molecules found across the animal phyla (Whittaker, *et al.*, 2006). These gene predictions have been recently identified with the sequencing of the *S. purpuratus* genome (Whittaker *et al.*, 2006). Little work, however, has been done in identifying specific functions for most of theses molecules during sea urchin development (Whittaker *et al.*, 2006).

The use of the three assay systems could help reveal some of these specific functions. We have used two of the assays in identifying a putative role for glucose/mannose groups in the specific adhesive interaction studied here (Latham *et al.*, 1999; Khurrum *et al.*, 2004; Coyle-Thompson & Oppenheimer, 2005; Sajadi *et al.*, 2007) and are currently examining the relationship between these past findings with respect to sugar involvement, and the hyalin results reported here, in Razinia *et al.* (2007), and in Alvarez *et al.* (2007).

Acknowledgements

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