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Exogenous hyalin and sea urchin gastrulation. Part IV: a direct adhesion assay – progress in identifying hyalin's active sites

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Summary

In *Strongylocentrotus purpuratus* the hyalins are a set of three to four rather large glycoproteins (hereafter referred to as 'hyalin'), which are the major constituents of the hyaline layer, the developing sea urchin embryo's extracellular matrix. Recent research from our laboratories has shown that hyalin is a cell adhesion molecule involved in sea urchin embryo-specific cellular interactions. Other laboratories have shown it to consist of 2–3% carbohydrate and a cloned, sequenced fragment demonstrated repeat domains (HYR) and non-repeat regions. Interest in this molecule has increased because HYR has been identified in organisms as diverse as bacteria, flies, worms, mice and humans, as well as sea urchins. Our laboratories have shown that hyalin appears to mediate a specific cellular interaction that has interested investigators for over a century, archenteron elongation/attachment to the blastocoel roof. We have shown this finding by localizing hyalin on the two components of the cellular interaction and by showing that hyalin and anti-hyalin antibody block the cellular interaction using a quantitative microplate assay. The microplate assay, however, has limitations because it does not directly assess hyalin's effects on the adhesion of the two components of the interaction. Here we have used an elegant direct assay that avoids the limitations, in which we microdissected the two components of the adhesive interaction and tested their re-adhesion to each other, thereby avoiding possible factors in the whole embryos that could confound or confuse results. Using both assays, we found that mild periodate treatment (6 h to 24 h in sodium acetate buffer with 0.2 M sodium periodate at 4 °C in the dark) of hyalin eliminates its ability to block the cellular interaction, suggesting that the carbohydrate component(s) may be involved in hyalin's specific adhesive function. This first step is important in identifying the molecular mechanisms of a well known cellular interaction in the NIH-designated sea urchin embryo model, a system that has led to the discovery of scores of physiological mechanisms, including those involved in human health and disease.

Keywords: Archenteron attachment, Direct adhesion assay, Hyalin, Periodate oxidation, Sea urchin embryos

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Introduction

Hyalin is a sea urchin glycoprotein set (hereafter termed 'hyalin') consisting (in *Strongylocentrotus purpuratus*, (*S. purpuratus*)) of three to four large components (native molecular weights vary from 280–920 kDa; Gray *et al*., 1986; Justice *et al.*, 1988, 1992) that on average (in species studied to date) contains 2–3% carbohydrate (Stephens & Kane, 1970; Citkowitz, 1971), hyalin repeat domains (termed HYR by Callebaut *et al.*, 2000) and non–repeated sequences (Wessel *et al.*, 1988).

The HYR has been identified in organisms as diverse as bacteria, worms, flies, mice and humans, as well as sea urchins (Callebout *et al.*, 2000) and has stimulated increased interest in this glycoprotein set. The sea urchin embryo is a NIH designated model system that has led to the discovery or a better understanding of scores of physiological mechanisms of general importance in organisms and in human health and disease (Davidson & Cameron, 2002; Davidson, 2006). This model is the best organism for studying hyalin function because of the quantity of embryos obtainable and because of the availability and transparency of the sea urchin embryo. Hyalin has been implicated in controlling general adhesive interactions in the sea urchin embryo (Kondo, 1973; Wessel *et al.*, 1998) and our laboratories provided evidence that hyalin is a cell adhesion molecule involved in mediating specific cellular interactions during the process of archenteron elongation and attachment to the blastocoel roof (Razinia *et al.*, 2007; Alvarez *et al.*, 2008; Carroll *et al.*, 2008; Contreras *et al.*, 2008), cellular interactions that have interested investigators for over a century (Herbst, 1900).

In past studies we used a quantitative microplate assay for assessing the effects of exogenous hyalin and anti-hyalin antibody on the specific cellular interactions in whole, living embryos (Razinia *et al.*, 2007; Alvarez *et al.*, 2008; Carroll *et al.*, 2008; Contreras *et al.*, 2008). This assay was indirect because it did not directly measure the effects of hyalin on the specific interactions and did not even assure that hyalin entered the embryo to probe the interactions being studied. Here, in addition to the microplate assay, we used an elegant assay to directly assess the influence of hyalin on the adhesion of the archenteron to the blastocoel roof, a major development for our hyalin studies. This assay utilized microdissected pieces of the cellular interaction, termed the 'pieces' assay (Coyle-Thompson & Oppenheimer, 2005), consequently allowing us to study the effects of hyalin directly on the adhesive interaction of the isolated pieces.

In order to determine if the small amount of carbohydrate present in hyalin is required for its function in our system, preparations were treated with periodate, a reagent that degrades some carbohydrates by selectively oxidizing C–C bond between vicinal diols. Previous work from our laboratories using native polyacrylamide gel electrophoresis indicated that the highest molecular weight components of several hyalin preparations stained positively for carbohydrate with the periodic base–acid Schiff reaction (unpublished). Here to test our direct assay in the hyalin system, we have compared the results with the indirect microplate assay, examining the effects of periodate-treated hyalin on the adhesive interaction, as a first step in identifying the nature of hyalin active sites.

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 using the Marine Biological Laboratory (Woods Hole, MA, USA) formula. Low calcium seawater (LCASW) with a reduced calcium concentration of 1.5 mM and calcium-free seawater (CFASW) were prepared according to Bidwell & Spotte (1985).

Preparation of hyalin

Adult *S. purpuratus* sea urchins were obtained from Marinus Scientific, Garden Grove, CA, USA. Gametes were obtained by intracoelomic injection of 0.55 M KCl. Eggs were collected by inverting the female over a beaker of artificial seawater at 11°C. Sperm were collected 'dry' in 100 × 15 mm plastic Petri plates and held on ice. Eggs were rinsed through 202 μm Nitex mesh and washed three times with large volumes of ASW prior to acid dejellying. The dejellying procedure involved bringing a suspension of 0.5% eggs rapidly to pH 5.5–5.7 with 1 N HCl, letting the suspension incubate for 2 min without stirring and then returning the suspension to pH 8.0 with 2 M Tris base. The dejellied eggs were washed three times with large volumes of ASW and their vitelline envelopes were disrupted with 0.01 M dithiothreitol, 0.1 M Tris base, pH 9.2 for 3 min. Eggs were then washed extensively with 0.01 M Tris base-buffered ASW, pH 8.0. Four volumes of eggs were inseminated with one volume of dilute sperm (1 ml sperm/25 ml 0.01 M Tris base-buffered ASW, pH 8.0). At 45–90 s post insemination, the suspension was diluted into eight volumes of ASW and the hyaline layers were allowed to develop for 45 min while the eggs settled. Hyalin protein was isolated and purified by the method described by Gray *et al.* (1986) with the following variations. The supernatant seawater containing the embryos with fully formed hyaline layers was aspirated leaving a mat of loosely adherent cells. The hyaline layers were dissolved from the embryo surfaces by the addition of 50 ml of 0.475 M NaCl, 0.025 M KCl. Embryos were stirred in this medium for 15 min until the hyaline layers appeared to be substantially reduced. Embryos were allowed to settle down and the supernatant solution containing crude hyalin proteins was collected. The crude hyalin preparation was centrifuged in a Sorvall SA 600 rotor at 15 000 rpm for 15 min at 4°C, to remove residual sperm and contaminants. The supernatant solution containing partially purified hyalin proteins was collected and stored on ice (from Razinia *et al.*, 2007).

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Protein determination

Protein concentration measurements were estimated using a NanoDrop™ 1000 Spectrophotometer Model ND-1000 (Thermo Fischer, Inc.). The protein, 280 nm setting was used on the instrument with a blank of 2.5 µl autoclaved, distilled water. A portion of 2.5 µl CFASW was then used to zero the instrument and 2.5 µl of a series of hyalin dilutions were used to determine absorbance at 280 nm.

Periodate oxidation of hyalin

Mild periodate oxidation was performed on samples with a starting protein concentration of approximately 1.7 mg/ml (Spiro, 1964, 1966a,b; Kondo, 1973). Four reaction mixtures (6 h, 12 h, 18 h and 24 h) were prepared using 600 µl of hyalin protein (at 1.7 mg/ml), 600 µl of 0.1 M sodium-acetate buffer pH 4.5 and 240 µl of 0.2 M sodium-periodate (freshly prepared) in 2.5 ml centrifuge tubes. The reaction mixtures were incubated for the indicated times at 4°C in the dark. The reactions were terminated by adding an excess of ethylene glycol, 150% of total moles of periodate; about 4 µl of 100% ethylene glycol. The untreated, 6 h, 12 h, 18 h and 24 h periodate-treated samples were dialyzed in the same dialysis solution simultaneously using 7 Spectra/Por Dialysis Membrane MWCO: 25000 (Fisher Scientific), first against 2 litres of 0.1 M Tris–HCl pH 8.0 and then against 2 litres of CFASW pH 8.0 at 4°C.

Gel electrophoresis

Gel Electrophoresis was performed with a Mini Protean III slab gel apparatus under native conditions using 4–20% polyacrylamide gradient gels (Bio Rad). The running buffer was 0.0248 M Tris–HCl, 0.192 M glycine pH 8.3 and was prepared from 10× stock. Hyalin samples were diluted into native sample buffer (final concentrations: 0.017 M Tris–HCl, 7% glycerol pH 6.8) (Bio Rad) and 30 µl of sample was loaded on each lane. Gels were run at constant current of 20 mA for 2.5 h. The gels were then fixed using 10% (w/v) glacial acetic acid, 40% (w/v) methanol for at least 1 h before silver staining (Bio Rad). A Sony (Tokyo, Japan) 7.2 megapixel DSC-W80 digital camera was used to photograph the gels. The gel presented in the Results was scanned on a UMAX Astra 2400 S scanner (Techville, Inc.). The colour image was imported into Adobe Photoshop, Version 7.0 (Adobe Systems, Inc.), converted to greyscale and cropped to eliminate extraneous gel. The single adjustment made was to push brightness to +60 so as to reduce the background of the silver stain and better interpret all bands present in the Control lane. Two barely visible bands noted in the control lane located in the portion of the gradient gel which was removed in the cropping process were not observed in any of the periodate-treated lanes and easily disappeared upon pushing the brightness and thus were cropped as part of the extraneous gel portion.

Photography

For the microplate assay work, photographs were at magnifications of ×100 and ×200 using a Zeiss Axiolab microscope mounted with a Sony 7.2 megapixel DSC-W80 digital camera.

For the microdissected pieces assay work, a Zeiss Axiolab compound photomicroscope was used to photograph the whole embryos and the dissected pieces. A Canon A95 digital camera was used for all pieces photographs on a Leica FZLIII stereo fluorescence dissecting microscope and the Zeiss Axiolab compound photomicroscope.

Embryo preparation and culture

Gametes of *Lytechinus pictus* (*L. pictus*) sea urchins were prepared as described above. Hyalin from *S. purpuratus* is biologically active with *L. pictus* (Alvarez *et al.*, 2008) and antibody to hyalin from one species cross reacts with hyalin from other species (Vater & Jackson, 1990). Eggs collected from individual females were washed separately two times with 100 ml of ASW, pH 8.0. Individual collection of eggs (1–2 ml) suspended in 30 ml of ASW were inseminated with 1.5 ml of freshly diluted sperm (1 ml concentrated sperm/10 ml ASW, pH 8.0). The eggs were then microscopically examined to check for fertilization by confirming the presence of fertilization envelopes. Batches of eggs giving less than 95% fertilization were discarded before proceeding. The embryos were then washed three times with 100 ml of ASW, pH 8.0 to remove residual sperm. The embryos from individual females were then transferred to 150 ml beakers respectively containing 30 ml of ASW final volume, pH 8.0 and incubated at 19°C for 24 h.

Microplate assay

For the microplate assay the starting concentrations of hyalin in CFASW for the untreated, 6 h, 12 h, 18 h and 24 h periodate-treated samples were adjusted to 0.34 mg/ml. Each of these samples was then serially diluted (by $^1/_2$ at each dilution) to 2.66×10^{-3} mg/ml thus yielding eight different concentrations of hyalin in CFASW to be examined (0.34 mg/ml, 0.17 mg/ml, 8.50×10^{-2} mg/ml, 4.25×10^{-2} mg/ml, 2.13×10^{-2} mg/ml, 1.06×10^{-2} mg/ml, 5.31×10^{-3} mg/ml and 2.66×10^{-3} mg/ml). Eleven total replicate experiments were done for the untreated hyalin and periodate-treated hyalin samples. In addition, there were 16 replicates for each of the following controls: dialyzed 0.2 M mock periodate with 10 µl of 100% ethylene glycol and no hyalin protein using 7 Spectra/Por

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Dialysis Membrane MWCO: 25000 (Fisher Scientific), 0.1 M Tris–HCl pH 8.0 (from first round of dialysis) and no hyalin protein, CFASW pH 8.0 (from second round of dialysis) and no hyalin protein, ASW pH 8.0 and no hyalin protein, LCASW pH 8.0 and no hyalin protein and CFASW pH 8.0 and no hyalin protein. Each well of the 96-well polystyrene flat-bottom microplate contained 75 µl of media. Wide-mouthed pipette tips were used to transfer 25 µl of 26 h *L. pictus* embryos in ASW at first invagination to each respective well giving a 1:3 (1 to 4 final dilution) ratio of embryos to media. The number of embryos per well varied as the embryos were swimming and an exact sample quantity could not be added to the wells. The embryos were incubated at 19°C for 24 h and at 48 h when the controls had fully attached archenterons the embryos were fixed with 100 µl of 10% formaldehyde solution in ASW giving a final formaldehyde concentration of 5%. The embryos were observed using a Zeiss Axiolab microscope and their morphologies were recorded as complete attached archenterons, incomplete unattached archenterons, no invagination, exogastrulation or embryos that were dead.

Statistical analysis of data

An unpaired one tail two-sample assuming equal variances *t*-test (Quinn & Kough, 2006) was performed, using Microsoft Excel 2007, to compare the means of complete archenterons of embryo samples incubated with untreated hyalin and the means of complete archenterons of embryo samples incubated with periodate treated hyalin at the two highest concentrations of hyalin (0.17 and 0.34 mg/ml, in LCASW). *p*-values of less than 0.05 were considered significant and *p*-values of less than 0.001 were considered highly significant. The standard error of the mean (SEM) was used to evaluate any variation among the means of the complete archenteron and unattached archenteron morphologies at all concentrations of hyalin examined.

Microdissected pieces assay

Fixation of embryos

Embryos at 50–58 h of incubation were collected in 50 ml and 15 ml Falcon™ (BD Biosciences) polypropylene centrifuge tubes and fixed with a final concentration of 3.7% formaldehyde (Ted Pella) in ASW pH 8.0. The centrifuge tubes containing the formaldehyde and embryos were placed on their sides overnight. Fixed embryos were stored in 3.7% formaldehyde (Ted Pella) in ASW pH 8.0 at room temperature in uncrowded 50 ml Falcon polypropylene centrifuge tubes. Previous studies showed that fixed embryos behaved as did live embryos in the pieces assay (Coyle-Thompson & Oppenheimer, 2005).

Slide preparation

Frosted on one side, precleaned Clay Adams (BD Biosciences, San Jose, CA) microscope slides (3 inch × 1 inch) were coated with 0.5 ml of Sigmacote™ (Sigma) and allowed to dry in a fume hood for 24 h. Slides were stored in a lint-free slide box. Just prior to use, the coated slides were wiped with a KimWipe™ (Kimberly Clark).

Washing fixed embryos

A 200 µl drop of ASW pH 8.0 was placed on the slide coated with Sigmacote™ inside the lid of a 15 × 100 mm Petri dish (Becton Dickinson). A total of 100 µl of fixed embryos were pipetted into the drop using a 3 ml sterile plastic Pasteur pipette with the narrow end cut off with a razor blade. The surrounding solution was removed by observing the embryos under the microscope and by pipetting carefully around the embryos to remove any debris. A 25 µl drop of ASW was pipetted forcefully onto the embryos to wash the fixative solution off the embryos. Three more 25 µl drops of ASW were pipetted forcefully onto the embryos to wash the fixative solution off the embryos. Excess solution was removed and the wash steps were repeated three times. A final 200 µl drop of ASW was added to the embryos.

Dissection of whole fixed embryos

Washed fixed embryos were placed in a 200 µl drop of ASW on the slide coated with Sigmacote™ and photographed. Whole fixed embryos were dissected in ASW on Sigma coated slides. Fine Elephant brand (Austria, BioQuip) size 00 or 000 insect pins were used to puncture the side of the blastocoel and catch the tissue of the embryo. A second pin was used to tease away the roof of the blastocoel from the tip of the archenteron. Careful dissection was performed to prevent the tearing of the archenteron by viewing the specimens during dissection with a Leica FZLIII stereo fluorescence dissecting microscope. Dissected pieces were photographed. The dissected pieces were placed together to determine if the pieces would adhere to each other using the criteria described. Thus, pieces that appeared to adhere were dragged through the solution to separate them. ASW was forcefully pipetted against the embryos and the pieces did not come apart. In addition the dissected adhered pieces were shaken several times and the pieces did not come apart. Pieces that adhered to each other did not come apart when dragged or bobbled in the solution by wiggling the slide, or by pipetting solutions at the pieces placed together. Pieces were judged to not adhere if they came apart with this probing.

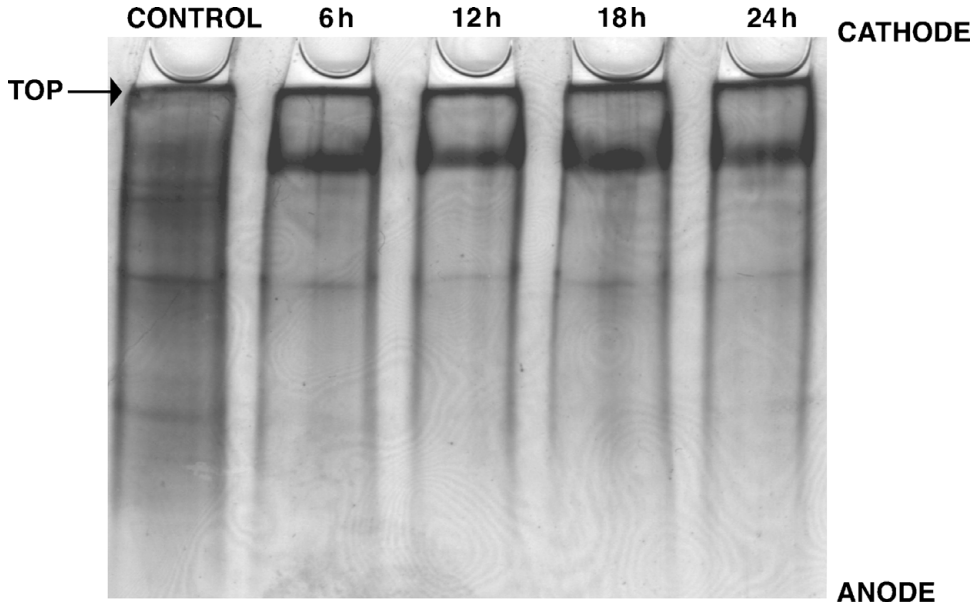

Figure 1 A silver-stained 4–20% native polyacrylamide gradient gel. A hyalin preparation (control) was periodate treated (for 6 h, 12 h, 18 h and 24 h) as described in Materials and methods. The gel has been cropped to remove the bottom portion, which had no stained bands. Migration was from top (cathode) to bottom (anode).

Incubation of dissected pieces in untreated and periodate-treated hyalin

Only archenteron pieces and blastocoel roof pieces shown to adhere were used in the hyalin incubation adhesion assay. A 100 µl solution of untreated hyalin was added to 100 µl of ASW containing the adhered dissected pieces and they still adhered even when the forceful stream of hyalin was pipetted against them. The adhered pieces were separated and then attempted to be put back together in the hyalin ASW solution. Next, additional embryos were dissected in 100 µl of ASW and 25 µl of hyalin was added to the pieces for a 1:5 dilution of untreated hyalin to match the concentration of the periodate-treated hyalin (see below). Untreated hyalin–ASW was pipetted against the adhered embryo pieces and the dish was shaken.

Again only archenteron pieces and blastocoel roof pieces shown to adhere were used in the periodate-treated hyalin assay. A 125 µl drop of periodate-treated hyalin was added to the adhered dissected pieces as above. The adhered pieces were separated and attempted to be put back together in the periodate-treated hyalin ASW solution. Periodate-treated hyalin–ASW was pipetted against them and the dish was shaken to determine if the adhesion was strong.

Results

In order to determine if the small amount of carbohydrate present in hyalin is required for biological function in our system, hyalin was treated with periodate under mild conditions. Native polyacrylamide gel electrophoresis was used to assess the state of the preparation after periodate treatment. As shown in Fig. 1, using a 4–20% silver stained polyacrylamide gradient gel, the untreated hyalin preparation contained several components. Following treatment of the preparation with periodate, the 6–24 h periodate-treated samples showed a single major band and several minor bands. Periodate-treated samples showed focusing of the hyalin band, consistent with removal of varying amounts of sugar mass and sialic acids that could decrease or increase the mobility of the native glycoprotein set on a native gel. Thus, it is possible that the group of components seen in the control lane collapsed to a common core after periodate treatment. As also can be seen, the periodate-treated components appeared to be essentially intact with no indication of major degradation. Thus, treatment of the hyalin preparation for 6–24 h with periodate yielded samples with a constant gel pattern. Similar results were obtained on several replicate gels.

Untreated and periodate-treated hyalin preparations were used in microplate assays for biological activity. As shown in Fig. 2 untreated hyalin caused unattached archenterons while periodate-treated hyalin (Fig. 3) did not. In over 16 000 *L. pictus* embryos assessed, periodate-treated hyalin (0.17–0.34 mg/ml LCASW) did not block the cellular interaction, while untreated hyalin (0.17–0.34 mg/ml LCASW) did. A range of 76.4–77.2% ± 2.3% of the embryos incubated with periodate-treated hyalin possessed complete

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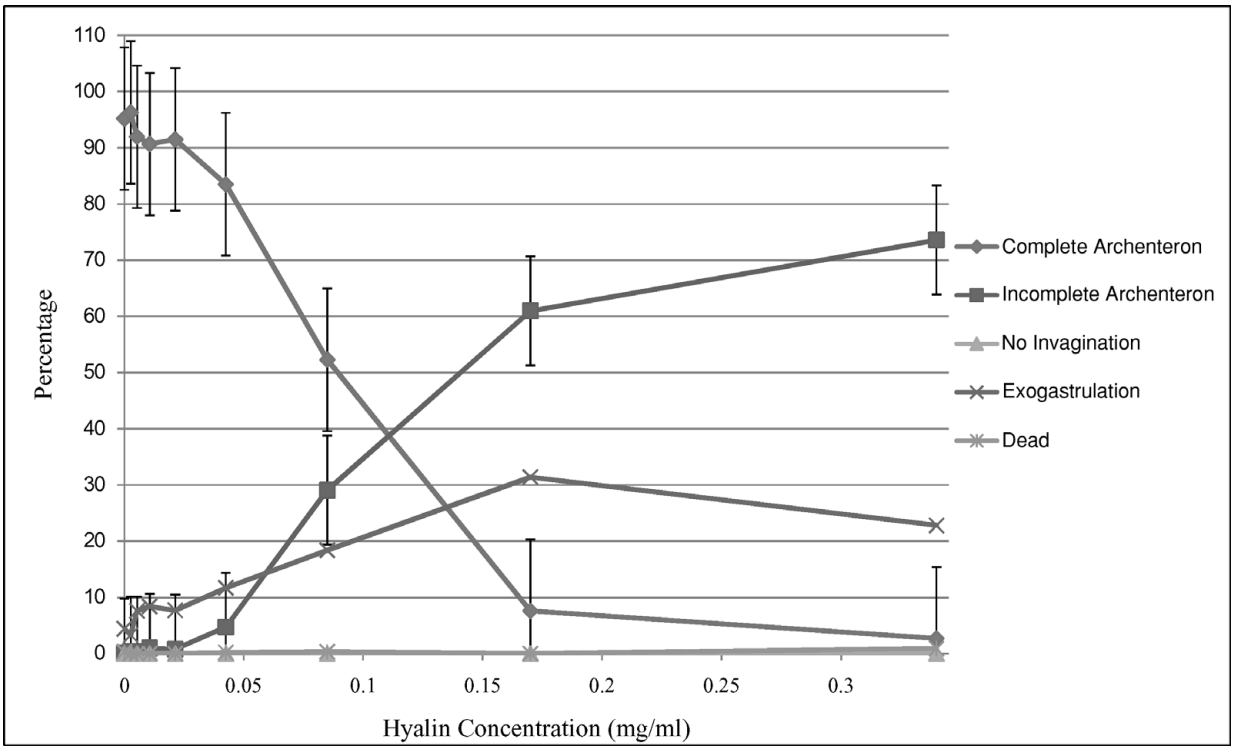

Figure 2 Dose–response curves showing the effects of untreated and dialyzed hyalin on archenteron development in 48 h *L. pictus* embryos. The preparations were serially diluted from 3.4×10^{-1} mg/ml to 2.7×10^{-3} mg/ml with 0 mg/ml being the LCASW control. Data were plotted as mean percentage ± standard error of the mean for the complete archenteron and incomplete archenteron morphology.

attached archenterons, while 2.7–7.6% ± 12% possessed complete attached archenterons in the untreated hyalin samples with a *p*-value of less than 0.001 in an unpaired one tail two-sample assuming equal variances *t*-test (Quinn & Kough, 2006).

Figure 4 shows that various control solutions used in the periodate procedure had no effect on archenteron morphology, except for one that caused embryo death: the Tris–HCl pH 8.0 buffer solution used in the first dialysis, before the final dialysis procedure against CFASW. Figure 5 shows an example of a 48 h embryo with an unattached archenteron following incubation with untreated hyalin and one with an attached archenteron following incubation with periodate-treated hyalin or no additions or other controls.

A total of 38 whole fixed embryos were dissected in ASW. All 38 pieces of archenteron adhered to their respective roofs of the blastocoel when placed together. All 38 pieces continued to adhere when untreated hyalin or when periodate treated hyalin was added to the drop of pieces on the siliconized slide containing dissected adhered embryo pieces. The pieces remained adhered even when the solutions were forcefully pipetted against the tissues and again when the dish was shaken forcefully under the microscope while observing the adhered pieces. The pieces stayed adhered in the solutions suggesting that the adhesion was strong. Once the archenteron/blastocoel roof pieces adhered they remained adhered unless physically pulled apart with insect pins.

The untreated hyalin incubated adhered archenterons and blastocoel roof pieces were all pulled apart using insect pins under the microscope and allowed to incubate in the untreated hyalin solutions for 20 min. The pieces were put back together so they could adhere and none of the 24 sets of pieces in untreated hyalin solutions re-adhered. The dissected pieces would not re-adhere in untreated hyalin solutions even after several attempts to put them together again. The roof of the blastocoel was shown to adhere to the tip of the archenteron in ASW and when left adhered together in the hyalin solutions. However when pulled apart and then attempted to be put back together in the untreated hyalin–ASW the pieces no longer adhered. They came apart when untreated hyalin–ASW was pipetted against them and when the dish was shaken.

When periodate-treated hyalin was added to the adhered-dissected pieces, they still adhered even with the forceful stream of periodate-treated hyalin pipetted against them. The adhered pieces were

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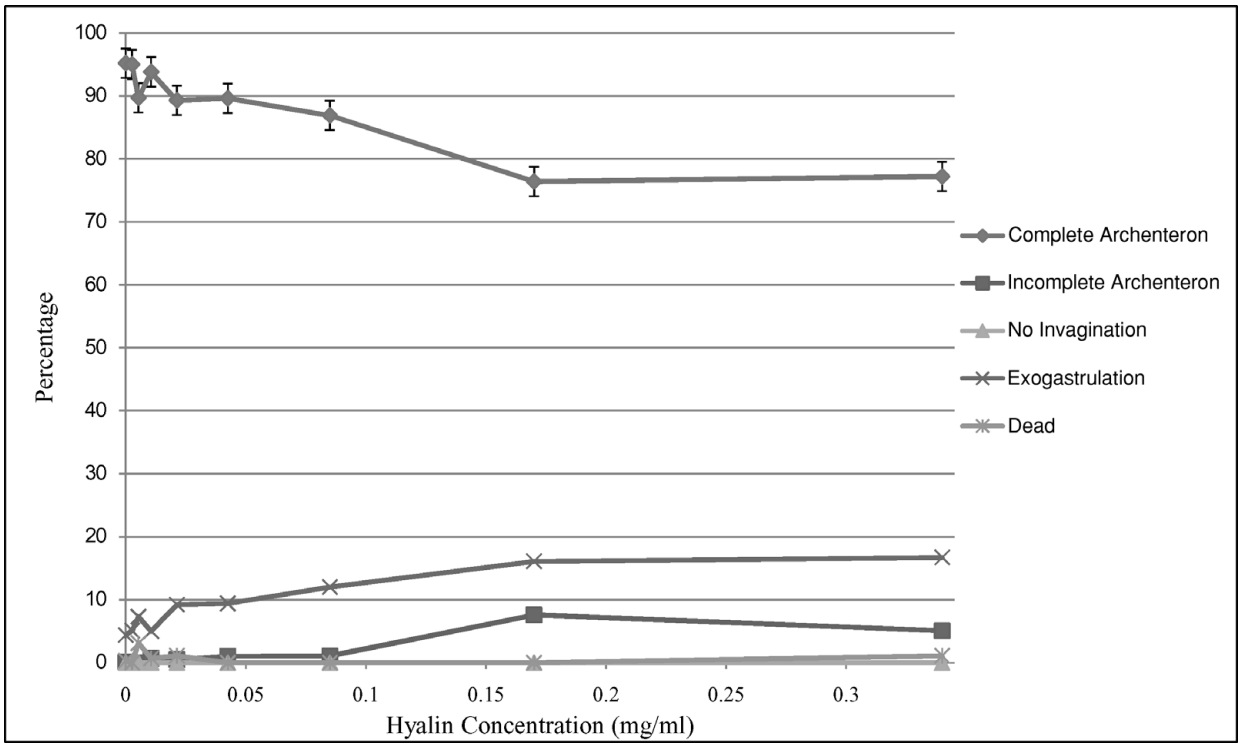

Figure 3 Dose–response curves showing the effects of periodate-treated hyalin on archenteron development in 48 h *L. pictus* embryos. Six hour sodium periodate-treated and dialyzed hyalin was serially diluted from 3.4×10^{-1} mg/ml to 2.7×10^{-3} mg/ml with 0 mg/ml being the LCASW control. The data were plotted as mean percentage ± standard error of the mean for the complete archenteron and incomplete archenteron morphology. The small error bars for the incomplete archenteron morphology are not visible.

separated and when put back together in the periodate-treated hyalin– ASW the dissected archenteron/roof of blastocoel pieces re-adhered. All 14/14 sets remained attached together when periodate-treated hyalin–ASW was pipetted against them and when the dish was shaken.

Figure 6 shows examples of unattached pieces and attached pieces to illustrate what microdissected pieces assay results described above look like.

Discussion

The pieces assay results indicate that untreated hyalin blocks the adhesion of the archenteron and blastocoel roof, that was previously only suggested using the microplate assay (Razinia *et al.*, 2007; Alvarez *et al.*, 2008; Contreras *et al.*, 2008). In addition, using both the pieces assay and the microplate assay, we show that periodate-treated hyalin did not block the adhesive interaction, while untreated hyalin did.

This is a first approach to identify hyalin's active sites. Under the mild periodate treatment used here, it is unlikely that hyalin protein was substantially affected and rather hyalin carbohydrate was primarily oxidized. Several pieces of evidence support this contention. The gels presented do not suggest that hyalin protein bands were substantially degraded. Although hyalin contains only 2–3% carbohydrate (Stephens & Kane, 1970; Citkowitz, 1971), removal of carbohydrates could be expected to show slightly altered protein band mobility on polyacrylamide gels. This was observed in several replicate gels (e.g., Fig. 1). The suite of high molecular weight hyalins would not be expected to be resolved on the 4–20% gradient gels used here to focus the bands for mobility observations. Finally, the classic experiments of Kondo (1973) showed that while trypsin abolished hyalin effects on cell reaggregation, mild periodate treatment, similar to that used here, had no influence on hyalin's effects in the cell reaggregation assay. Kondo's results are important for two reasons. First, they showed that trypsin degradation of hyalin protein wiped out hyalin effects in their reaggregation system. Periodate-treated hyalin had no effect, suggesting that the mild periodate conditions used oxidized carbohydrates and did not substantially degrade the protein because hyalin's protein was active in this cell reaggregation

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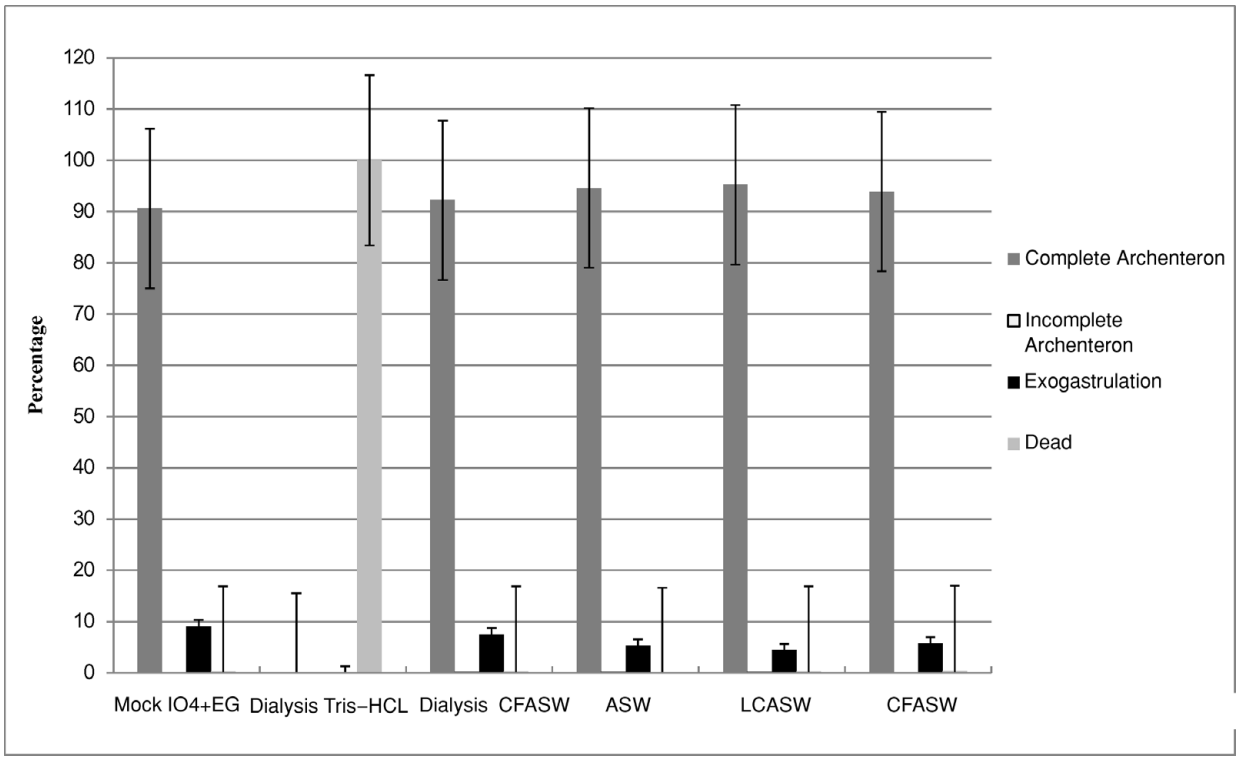

Figure 4 Various controls showing no effect on archenteron morphology, except for the first Tris–HCl dialysis supernatant that caused embryo death. There are no incomplete archenterons. Mock IO4 + EG: 0.2 M periodate and ethylene glycol with no hyalin, dialyzed against CFASW (pH 8.0). Dialysis Tris–HCl: 0.1 M Tris–HCl (pH 8.0) used for first round of dialysis. Dialysis CFASW: calcium-free artificial seawater (pH 8) used for second round of dialysis. ASW: artificial seawater (pH 8.0). LCASW: low calcium artificial seawater (pH 8.0). CFASW: calcium-free artificial seawater (pH 8.0) Note: a small percentage of incomplete archenteron morphology is not visible.

assay. Kondo's results are in agreement with those of Wessel *et al.* (1998), whose cloned sugar-free hyalin was found to be active in their cell reaggregation system.

The evidence suggests that the components of hyalin that are active in the reaggregation system (Kondo, 1973; Wessel *et al.*, 1998) are not the same components that are active in our specific structural adhesion system. As Kondo's results suggest that mild periodate treatment does not greatly alter hyalin protein and instead oxidizes hyalin carbohydrate, also suggested by our gels, we suggest that hyalin glycopeptides are active in our system, while glycosylated peptides are not active in Kondo's cell reaggregation system (Kondo, 1973) or in Wessel's reaggregation system (Wessel, *et al.*, 1998).

Our working hypothesis to explain what is occurring in this system is as follows. We showed that hyalin is localized on both the archenteron and blastocoel roof (Carroll *et al.*, 2008). We showed that hyalin and anti-hyalin antibody block the cellular interaction using the microplate assay (Razinia *et al.*, 2007; Alvarez *et al.*, 2008; Carroll *et al.*, 2008; Contreras *et al.*, 2008) and finally here, using exogenous hyalin, in the direct pieces assay. We propose that exogenously added hyalin binds to the hyalin on the two pieces of the cellular interaction (hyalin is known to selfassociate) and in this way blocks adhesion. Alternatively, hyalin may block a hyalin binding receptor or ligand on one or both components of the cellular interaction. Hyalin carbohydrate may be involved in hyalin's binding activity as suggested by the findings in this report. Independent of these results, we have provided a great deal of evidence suggesting that carbohydrate is involved in the cellular interaction under study (Latham *et al.*, 1999; Khurrum *et al.*, 2004; Coyle-Thompson & Oppenheimer, 2005; Sajadi *et al.*, 2007).

Here, for the first time, we use the direct microdissected pieces assay in the hyalin system. Because this assay directly measures the cellular interaction under study, it is likely that future work, using this assay, will help solve the problem of the molecular basis of a cellular interaction that has intrigued investigators for over a century.

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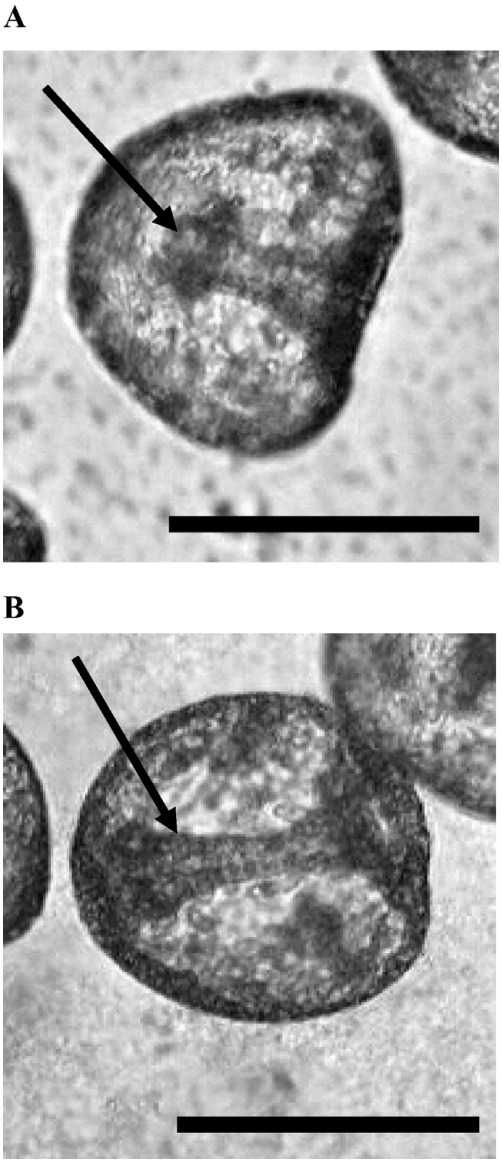

Figure 5 Examples of a 48 h *L. pictus* embryo with unattached archenteron incubated with untreated hyalin (*A*, top) and a 48 h *L. pictus* embryo with an attached archenteron incubated with periodate-treated hyalin (*B*, bottom). Similar results to (*B*) were found in the 'no addition' and other controls. Scale bar = 100 μm. Arrows show archenterons.

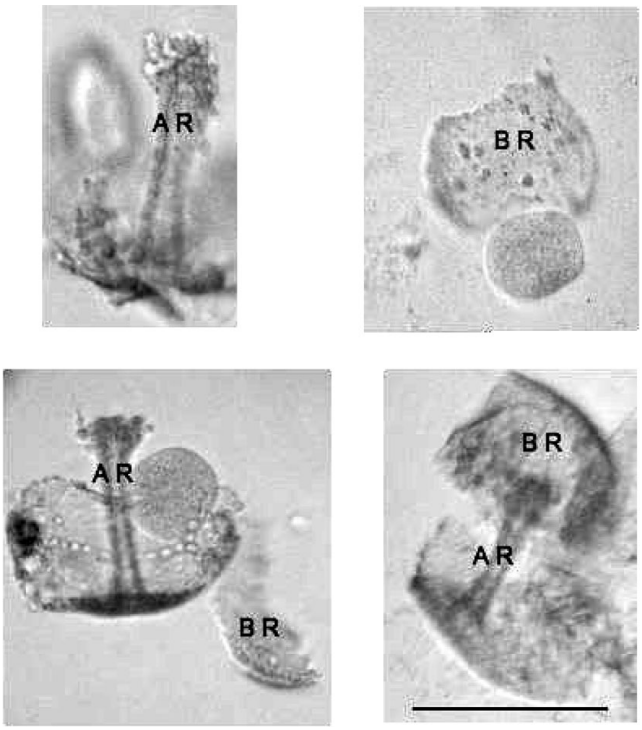

Figure 6 The microdissected pieces assay. Top left, microdissected archenteron (AR). Top right, microdissected blastocoel roof (BR). Bottom left, microdissected archenteron and microdissected blastocoel roof not adhering (found in 24/24 of the untreated hyalin samples) Bottom right, microdissected archenteron adhering to microdissected blastocoel roof (found in 14/14 of the periodate-treated hyalin samples). These images are examples of what the components of the microdissected pieces assay look like. Scale bar = 100 μm.

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