On the Ultrastructure of Hyalin, A Cell Adhesion Protein of the Sea Urchin Embryo Extracellular Matrix

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Abstract. Hyalin is a large (ca. 350 × 10^3 kD by gel electrophoresis) molecule that contributes to the hyalin layer surrounding the sea urchin embryo. In previous work a mAb (McA Tg-HYL), specific for hyalin, was found to inhibit cell–hyalin adhesion and block morphogenesis of whole embryos (Adelson, D. L., and T. D. Humphreys. 1988. *Development.* 104:391–402). In this report, hyalin ultrastructure was examined via rotary shadowing.

Hyalin appeared to be a filamentous molecule ~75-nm long with a globular "head" about 12 nm in diameter that tended to form aggregates by associating head to head. Hyalin molecules tended to associate with a distinct high molecular weight globular particle ("core"). In fractions containing the core particle often more than one hyalin molecule were seen to be associated with the core. The core particle maintained a tenacious association with hyalin throughout purification procedures. The site(s) of McA Tg-HYL binding to the hyalin molecule were visualized by decorating purified hyalin with the antibody and then rotary shadowing the complex. In these experiments, McA Tg-HYL attached to the hyalin filament near the head region in a pattern suggesting that more than one antibody binding site exists on the hyalin filament. From the ultrastructural data and from the cell adhesion data presented earlier we conclude that hyalin is a filamentous molecule that binds to other hyalin molecules and contains multiple cell binding sites.

Attempts were made to demonstrate the existence of lower molecular weight hyalin precursors. Whilst no such precursors could be identified by immunoprecipitation of in vivo labeled embryo lysates, immunoprecipitation of in vitro translation products suggested such precursors (ca 40 × 10^3 kD) might exist.

Sea urchin embryos are surrounded by an extra-embryonic matrix called the hyaline layer (HL)[1] which is required for normal morphogenesis (Herbst, 1908; Citkowitz, 1971, 1972). The major component of the HL is hyalin, an extremely large and acidic protein secreted at fertilization by the cortical granules (Faust et al., 1959; Kane, 1970; Stephens and Kane, 1970; Hylander and Summers, 1982). Hyalin is a heterogenous species as judged by sedimentation (Gray et al., 1986); by its migration as a doublet in electrophoresis gels (Citkowitz, 1972; Gray et al., 1986; Adelson and Humphreys, 1988) whose molecular weight has been estimated variously from 100 to 900 × 10^6 (Stephens and Kane, 1970; Gray et al., 1986). Hyalin precipitates to form a gel in the presence of Ca^{2+} (Kane, 1973), and this requires disulphide bond-dependant conformation (Stephens and Kane, 1970). Although hyalin is synthesized during oogenesis, it is not produced in the embryo until mesenchyme blastula stage (Citkowitz, 1972; McClay and Fink, 1982; Adelson and Humphreys, 1988).

A functional role for the HL in morphogenesis was proposed by Gustafson and Wolpert (1967). They hypothesized that epithelial folding could result from inequalities in cell–cell adhesion relative to cell-substrate adhesion. Indeed, some evidence (Dan, 1960) had suggested that cell–hyalin interactions early in development were stronger than cell–cell adhesion. Dan's observations were based on the behavior of embryos subjected to hypotonic sea water in which the cells remained attached to the HL but lost their attachment to each other. More recently, quantitative measurements of adhesion showed that primary mesenchyme cells lost affinity for hyalin at the mesenchyme blastula stage while presumptive ectoderm and endoderm cells retained an affinity for hyalin during the same time period (McClay and Fink, 1982; Fink and McClay, 1985).

A specific role for cell–hyalin interactions in morphogenesis was further confirmed by two observations. First, low concentrations (5–10 μg/mL) of a mAb specific for hyalin (McA Tg-HYL) inhibited cell–hyalin binding in vitro. Second, morphogenetic events in vivo requiring epithelial folding such as gastrulation and arm rudiment formation were

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1. *Abbreviation used in this paper*: HL, hyaline layer.

blocked reversibly when embryos were incubated at similar concentrations of antibody (Adelson and Humphreys, 1988).

Because hyalin appears to play an important role in early morphogenesis of the sea urchin about which relatively little is known, we have undertaken a critical examination of the molecule, its structure, and the site which serves as a cell attachment domain. In this report we describe hyalin ultrastructure and correlate it with previous data.

Materials and Methods

Glycerol Gradient Ultracentrifugation and Rotary Shadowing

S. purpuratus hyalin was first purified by the method of Kane (1970). 100 μl hyalin (1.6 mg/ml) was loaded onto 25–50% linear glycerol gradients buffered with 0.1 M NH_4HCO_3, pH 8.0. Gradients were centrifuged at 37,000 rpm in a SW-41ti rotor (Beckman Instruments, Inc., Palo Alto, CA) for 24 h at 20°C. Fractions (0.5 ml) were collected by piercing the bottom of the tubes and displacing the gradients from the top. Rotary shadowing and EM of the shadowed molecules were performed according to the method of Erickson and Carroll (1983).

Antibody decoration of hyalin molecules for rotary shadowing was performed by incubating 100 μl of a gradient fraction with 1 μg McA Tg-HYL overnight at 4°C.

FPLC Fractionation

Strongylocentrotus purpuratus hyalin was purified by repeated cycles of Ca^{2+}-induced precipitation (Kane, 1970). 0.5 ml hyalin (1.6 mg/ml) which had been dialyzed against 0.05 M Tris-HCl, pH 8.1, was applied to a Mono-Q column (Pharmacia Fine Chemicals, Piscataway, NJ). A 20 ml linear gradient of NaCl (0–100% buffer B) at a flow rate of 1 ml/min was used to elute the various molecular species, which were collected as 1-ml fractions. Buffer A consisted of 5 M urea, 1% Taurine (wt/vol), 20 mM Hepes, pH 7.3. Buffer B consisted of buffer A plus 1 M NaCl.

In Vitro Translations and Immunoprecipitations

The embryo lysate for in vitro translations was made (Luca and Ruderman, 1989) by lightly homogenizing 4 ml of packed gastrulae (previously washed 2× with calcium-free sea water) with 1 ml buffer containing 300 mM Glycine, 120 mM K-Gluconate, 100 mM Taurine, 40 mM NaCl, 2.5 mM $MgCl_2$, 10 mM EGTA, and 100 mM Hepes, pH 7.2. The lysate was clarified by centrifugation and stored at −80°C. In vitro translations were performed by mixing 50 μl rabbit reticulocyte lysate, 6 μl translation reaction mix (Bethesda Research Labs, Gaithersburg, MD) and 100 μCi [^{35}S]methionine with 50 μl embryo lysate made from *S. purpuratus* gastrulae.

For in vivo labeling, *S. purpuratus* embryos were reared at 15°C in filtered sea water buffered to pH 8.0 with 10 mM Tris-HCl. Embryos at various stages of development were collected, concentrated by centrifugation, washed 2× with filtered sea water and diluted to provide 80,000 embryos in 2-ml buffered sea water. 125 μCi[^{35}S]methionine was added to the cultures which were allowed to incorporate label for 1 h. Embryos were then collected and washed 2× with acid sea water prior to flash freezing in liquid nitrogen. Frozen embryos were stored at −80°C until needed.

Surface iodination of embryos was performed using Iodogen (Pierce Chemical Co., Rockford, IL) according to manufacturers instructions. 50 μCi ^{125}I was used for 45,000 eggs/embryos.

Immunoprecipitations were performed as previously described (Adelson and Humphreys, 1988) except that protein A agarose (Repligen Corp., Boston, MA) was substituted for fixed *Staphylococcus aureus*.

Gel Electrophoresis and Western Blotting

SDS-PAGE was performed by the method of Laemmli (1974). Gels were silver stained by the method of Merrill et al. (1984). Western blots were performed as previously described (Adelson and Humphreys, 1988).

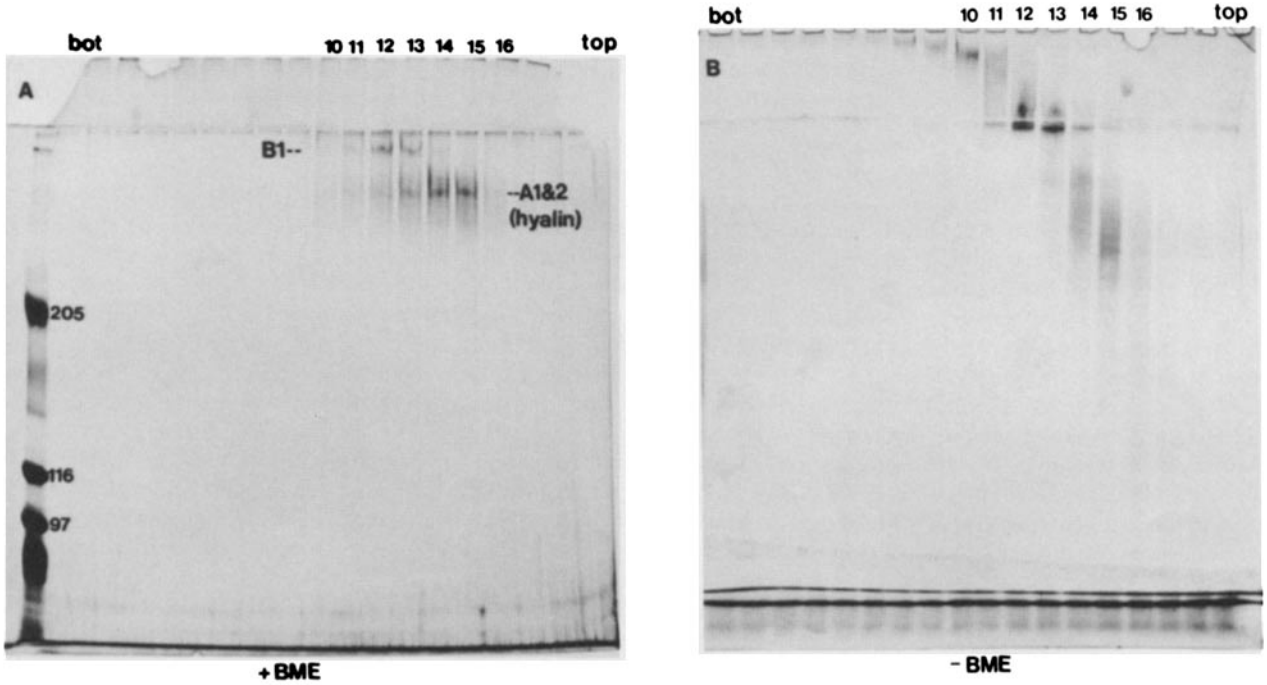

Figure 1. Glycerol gradient fractionation of *S. purpuratus* hyalin. Aliquots for gel electrophoresis were mixed with sample buffer either with (reducing) 5% β-Mercaptoethanol (vol/vol) (BME) or without (nonreducing) BME. Numbering of fractions begins at the bottom of the glycerol gradient. (*A*) A silver stained 5% gel (4% stacking gel) run under reducing conditions (*+BME*). Most of the hyalin was present in fractions 12-15 and ran as poorly defined band within a smear under these conditions. Another molecular species (*Bl*) of higher apparent molecular weight was found in overlapping fractions further towards the bottom of the gradient. (*B*) A silver stained 5% gel (4% stacking gel) run under nonreducing conditions (*−BME*). Hyalin was seen to run as a smear, with the bulk of the material at a much lower apparent molecular weight. Band Bl ran at a much higher apparent molecular weight, barely entering the running gel. Under these conditions, very high molecular weight species were detected further down the gradient and are of unknown composition.

Results

Glycerol Gradient Purification of Hyalin

Initial purification of *S. purpuratus* hyalin was by the method of Kane (1970), which relies on repeated cycles of Ca^{2+} induced precipitation alternated with solubilization in the absence of Ca^{2+}. This semi-pure hyalin was further purified for rotary shadowing by ultracentrifugation through 25–50% (wt/vol) glycerol gradients. Fractions were collected, aliquots electrophoresed on both reducing and non-reducing SDS gels, and silver stained (Fig. 1, *a* and *b*). Hyalin was identified as the poorly resolved doublet of lower apparent molecular weight (nominally estimated as ca. 350 kD) seen on reducing gels across fractions 11 through 16, but primarily in fractions 14 and 15. The distribution of hyalin overlapped partially with a higher apparent molecular weight component referred to henceforth as B1, found primarily in fractions 12 and 13. This high molecular weight band is thought to be the globular "core" particle that will be described below. There was a strong correlation between the presence or absence of band B1 and the presence or absence of the "core" in the macromolecular structures from a given fraction.

Previous work (Adelson and Humphreys, 1988) has shown that McA Tg-HYL is specific for hyalin and has indicated that both bands within the *Tripneustes gratilla* hyalin doublet are recognized by McA Tg-HYL. In *S. purpuratus* other investigators have shown that the presence of multiple components seems to be a function of purification technique (Gray et al., 1986). The results from our glycerol gradients would seem to support the existence of this higher molecular weight species associated with *S. purpuratus* hyalin.

On non-reducing gels, hyalin was found to migrate as a smear ranging from its reduced size to considerably smaller. A poorly defined doublet was present within the smear, at

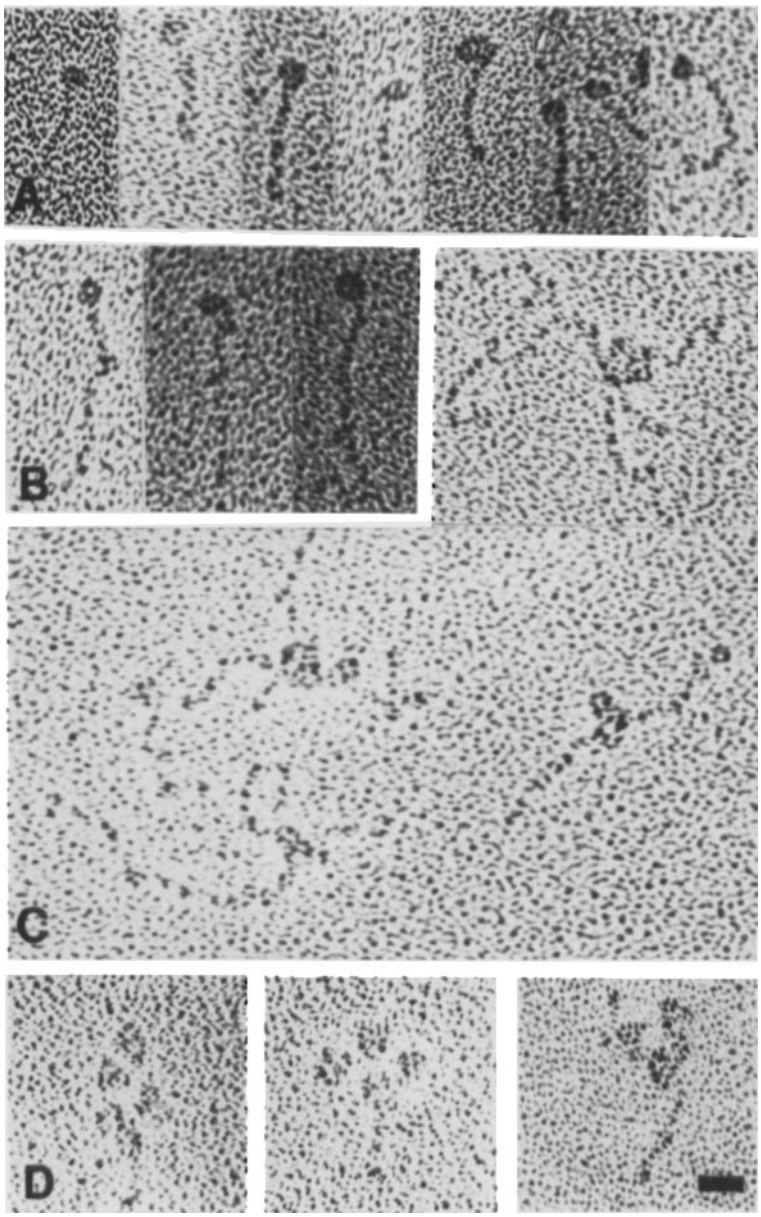

Figure 2. Rotary shadowing of glycerol gradient fractions. (*A*) Material from fraction 15 showing typical 75 nm hyalin filaments terminated by a single 12 nm globular domain. (*B*) Material from fraction 15 showing typical 125 nm hyalin filaments also terminated by a single 12 nm globular domain. (*C*) Material from glycerol gradient fraction 12 showing "spider" aggregates of hyalin filaments clustered with their globular domains around B1 cores. (*D*) McA Tg-HYL decorated material from fraction 15. Note the clustering of antibody molecules around the globular end of the hyalin filament. Bar, 25 nm.

a much lower apparent molecular weight than the doublet seen in reduced material. From this we deduced that reduced hyalin had a much more extended conformation (on SDS gels) than when it is unreduced. In contrast, the mobility of the higher molecular weight band B1 was decreased in non-reducing gels. The hyalin smear was probably caused by partial disulphide bond reduction in our unreduced sample, implicating disulphide bonds in maintaining hyalin's normal conformation. These observations are in agreement with those of previous investigators who have shown that disulphide bonds are abundant in hyalin and are involved in maintaining the conformation required for Ca^{2+} induced polymerization (Stephens and Kane, 1970).

Rotary Shadowing of Glycerol Gradient Fractionated Material

Rotary shadowing (Fig. 2) of material from glycerol gradient fraction 15 (which consisted mainly of hyalin) demonstrated the presence of filamentous, flexible molecules, with a globular domain at one end. The filaments were often folded over one another or crumpled into compact blobs. The filaments are of two lengths, with a predominant isoform of 75-nm and a club-shaped head of 12 nm in diameter (Fig. 2 *a*). A less abundant isoform of 125-nm length was detected as well (Fig. 2 *b*). Aggregates of these filamentous molecules were often seen and resembled tangled pieces of yarn (Fig. 2 *b*). These molecules bore no resemblance to laminin, which has been identified in the sea urchin embryo and implicated in morphogenesis (McCarthy et al., 1987; McCarthy and Burger, 1987).

Glycerol gradient fraction 15 consisted mainly of hyalin, which therefore must form the observed monomeric, tangled filaments. Further down the gradient, as B1 increased in abundance, the filaments were observed to be part of spider-like structures with the globular ball domains clustered around a central core and filaments radiating outwards. In particular, from the shadowed material of fraction 12 (Fig. 2 *c*), many "spiders" were observed. Most of the "arms" were 75 nm in length, but each had one arm of ~125 nm in length.

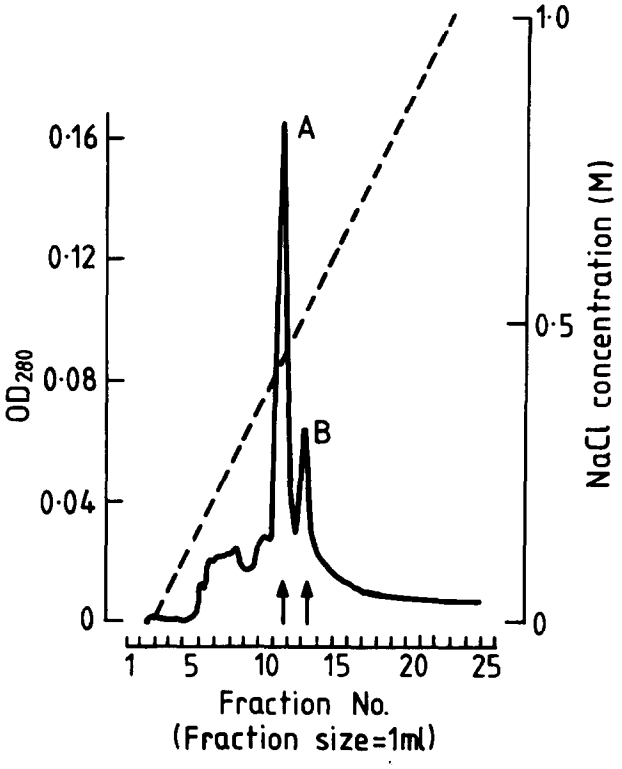

Figure 3. FPLC fractionation of *S. purpuratus* hyalin. Hyalin prepared by the method of Kane (1970) was applied to a Mono-Q column (Pharmacia Fine Chemicals) and eluted with a linear salt gradient in the presence of Urea and Taurine (see Materials and Methods). Most of the material applied to the column was eluted at approximately 0.5 M NaCl as two well resolved symmetrical peaks. Both peaks (*A* and *B*) were collected in fractions 11 and 13, respectively.

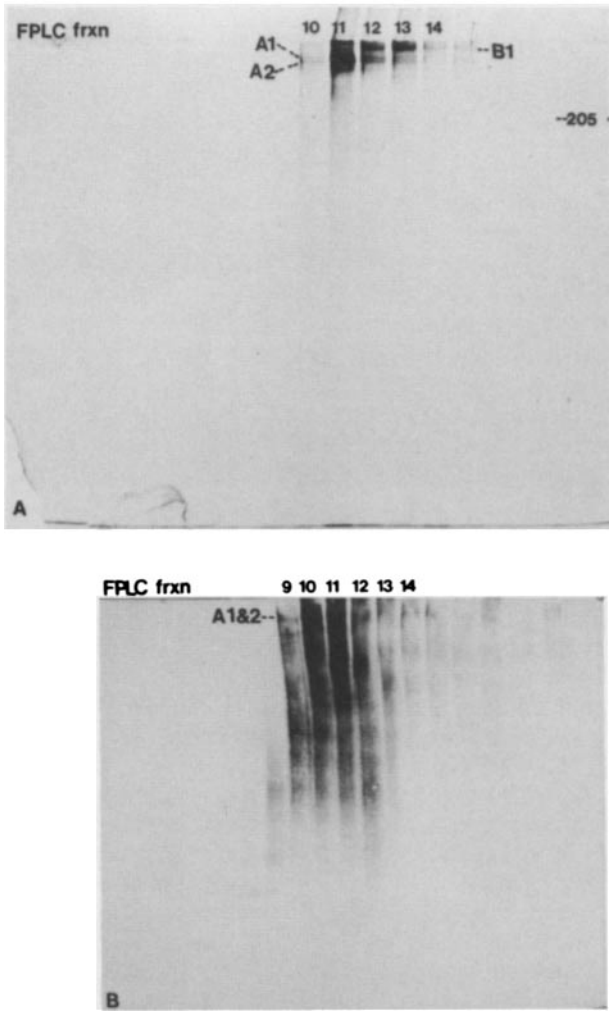

Figure 4. Molecular composition of FPLC peaks. (*A*) SDS-PAGE of the FPLC fractions on a silver stained 8% gel (4% stacking gel). Peak A (maximum at fraction 11) is enriched for hyalin, which ran as a doublet denoted as A1 and A2. Peak B (maximum at fraction 13) was enriched for a higher apparent molecular weight band (*B1*). (*B*) Western blot of an identical gel showed that McA Tg-HYL recognized bands A1 and A2 and not band B1. Note the smear of immunoreactive lower molecular weight material which probably represented degradation products of hyalin.

We concluded that hyalin made up the arms, while B1 went into the cores.

Antibody Decoration of Hyalin with McA Tg-HYL

Having shown the structure of hyalin and the core particle, we wanted to determine where on the molecule the cell binding site was located. Since the mAb specifically blocked cell–hyalin interactions at very low antibody concentrations (Adelson and Humphreys, 1988), it was probable that the antibody recognized an epitope in or near the cell binding domain of the hyalin molecule.

We decorated hyalin by adding McA Tg-HYL to glycerol gradient fractions followed by overnight incubation. Aliquots from these fractions were then rotary shadowed. Fig. 2 *d* shows antibody decorated hyalin molecules. Each of the molecules was decorated near the globular end, often by three to five antibody molecules, indicating possible tandem repeats of the epitope.

Association of B1 and Hyalin

Because hyalin ran as a doublet within a smear, and maintained some association with B1 in the glycerol gradients, we attempted to purify the polypeptides by anion exchange chromatography on a FPLC Mono-Q column (Pharmacia Fine Chemicals). By eluting with a 0–1 M NaCl gradient in 5 M urea, 1% taurine (wt/vol) at pH 8.1 we succeeded in separating two well resolved, symmetrical, 280-nm absorbance peaks (Fig. 3). A large peak (peak A) was eluted at fraction 11 with ~0.45 M NaCl and a smaller peak at fraction 13 (peak B) with ~0.55 M NaCl. Peak A contained approximately five times as much protein as peak B based on the ratio of their integrated 280-nm absorbances (45.0:9.5).

Although both FPLC peaks were sharp and symmetrical, they were composed of multiple polypeptides (Fig. 4 *a*). Silver-stained gels showed that peak A was enriched for the hyalin doublet, while peak B was enriched for band B1 which barely entered the running gel. Western blotting (Fig. 4 *b*) confirmed that McA Tg-HYL bound only to the hyalin doublet and to a lower molecular weight smear of bands which were probably degradation products. McA Tg-HYL did not recognize band B1, indicating that B1 ("core") was not directly involved in the antibody-mediated inhibition of morphogenesis. Remarkably, B1 remained associated with hyalin throughout multiple cycles of Ca^{2+} precipitation and solubilization and to some degree through the FPLC fractionation, as judged by its presence of fraction 11 (Fig. 4 *a*). This tenacious association may have been a reflection of a structural role for B1 in the crosslinking of the hyaline layer.

Evidence for Lower Molecular Weight Precursors

Hyalin's large size suggested to us that the filaments might be produced by covalently linking lower molecular weight subunits. To test for the existence of lower molecular weight precursors, McA Tg-HYL was used to immunoprecipitate hyalin in vivo labeled with [^{35}S]methionine. As the fluorograph in Fig. 5 *a* shows, no lower molecular weight precursors could be unambiguously identified in this experiment. Two developmentally regulated proteins of ~114 × 10^3 and 97 × 10^3 kD were non-specifically precipitated by the immunoprecipitation procedure; they were synthesized until

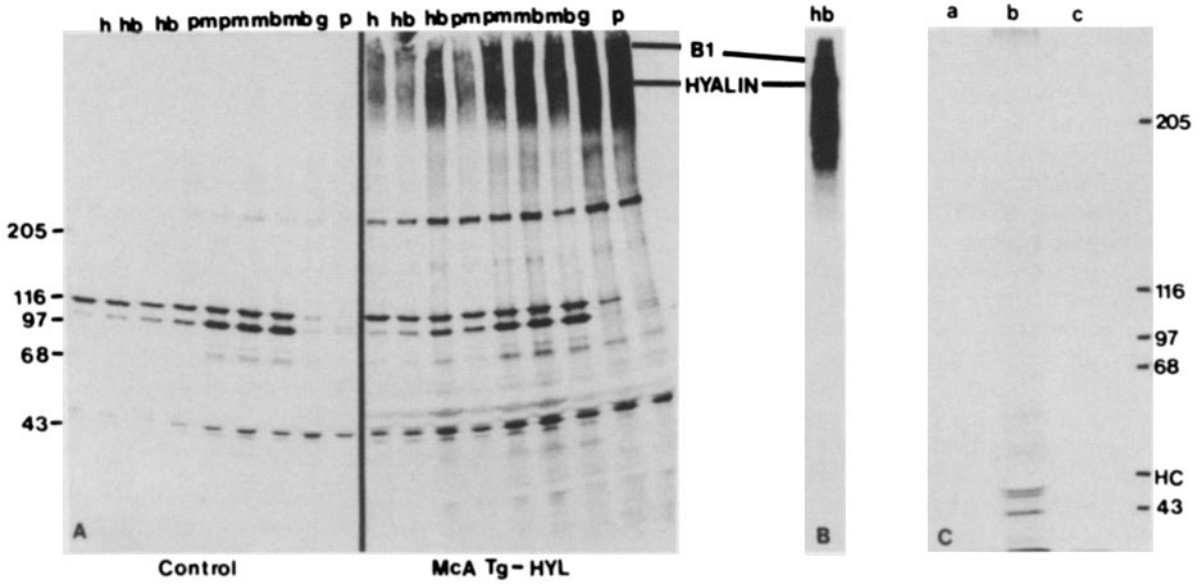

Figure 5. Examination for hyalin precursors. (*A*) McA Tg-HYL immunoprecipitates of in vivo [^{35}S]methionine-labeled *S. purpuratus* embryos at several stages of development. *H*, hatching blastula; *HB*, hatched blastula; *PM*, beginning of primary mesenchyme cell ingression; *MB*, mesenchyme blastula; *G*, mid-gastrula stage; and *P*, pluteus stage. Immunoprecipitates were run out on 2.5–17.5% gels, but no lower molecular weight precursors could be identified due to the poor signal to noise ratio of the experiment. (*B*) McA Tg-HYL immunoprecipitate of ^{125}I surface-labeled embryos demonstrated that most of the background seen in *A* was attributable to intracellular species. (*C*) McA Tg-HYL immunoprecipitate of an in vitro translation of gastrula/rabbit reticulocyte mixed lysate. The three lanes shown are (*A*) protein A–agarose beads only; (*B*) McA-Tg-HYL + rabbit anti-mouse + protein A beads and (*C*) rabbit anti-mouse + protein A beads only. Several low molecular weight bands were specifically immunoprecipitated by McA-Tg-HYL (lane *B*).

between mid-blastula and mid-gastrula when their rate of synthesis decreased substantially. These proteins presumably bound to either the agarose beads, the protein A or the rabbit anti-mouse antibody. Curiously enough, the copious number of background bands observed in the in vivo labeled immunoprecipitates was probably not due to proteins with extracellular domains as they were not evident in Fig. 5 *b*, showing immunoprecipitated material from ^{125}I surface-labeled embryos.

One further attempt was made to demonstrate the likelihood of low molecular weight hyalin precursors by immunoprecipitating in vitro translation products with McA Tg-HYL. In order to maximize mRNA "translatability" we used equal parts rabbit reticulocyte lysate and mid-gastrula embryo lysate, with the mRNA provided by the embryo lysate. Fig. 5 *c* shows the resulting fluorograph of a 4% gel where McA Tg-HYL precipitated not only hyalin but several low molecular weight bands. These bands were unambiguous and the immunoprecipitate was free of the background bands seen in Fig. 5 *a*. Although this evidence was not conclusive, it suggested that the lower molecular weight proteins identified were in fact hyalin precursors.

Discussion

Hyalin Ultrastructure

Previous attempts to characterize hyalin have been hampered by the molecule's intrinsic biophysical properties. Hyalin has been described as an extremely large protein, possibly glycosylated (see Introduction). Unfortunately, estimates of hyalin's molecular weight have been unreliable for a number of reasons. Hyalin's size precludes meaningful molecular weight estimates from gel electrophoresis other than to say that it is "very big." Molecular size or weight estimates based on centrifugation techniques rely on the assumptions that the material is homogeneous and does not form aggregates. As we have demonstrated, hyalin violates both of these assumptions in that it appears to have two size isoforms, it remains associated with Bl (both on glycerol gradients and to some degree in the FPLC fractions), and it can form aggregates (in vitro at least) of variable composition and size. In addition, hyalin's native conformation is dependent on disulfide bonds for stability. Partial or complete reduction of these disulfides bridges causes enormous changes to the electrophoretic migration of hyalin on polyacrylamide gels (Fig. 1).

In this report, hyalin was shown to be a long filamentous molecule, with a primary isoform ~75 nm in length, and a globular domain ~12 nm in diameter at one end. Filaments of the secondary isoform, of much lower prevalence, measured ~125-nm long. Both the filamentous nature and size heterogeneity are in agreement with the hydrodynamic data of Gray et al. (1986). The filaments of both isoforms tended to form aggregates along their length and had a cell-binding domain near the globular end. The existence and location of multiple cell binding sites were inferred from the frequency of multiple antibody molecules found decorating the globular end of single filaments. The filaments appeared to be flexible and examination of a larger number of filaments has revealed no preferential region of bending. Filaments not only associated along their lengths, but radially, with their globular ends inward, surrounding a "core" which we conclude to be a distinct molecule (Bl) of great size. Bl was judged to be distinct from hyalin because the antibody did not bind to it in either Western blots, or in antibody decorated fractions.

Calcium's Role

Ca^{2+} is required to form both precipitable hyalin aggregates and to stabilize hyalin conformation in the presence of high concentrations of NaCl as found in sea water (Robinson, 1988; Robinson et al., 1988). Hyalin aggregates are not inhibited or dissociated by McA Tg-HYL (Adelson and Humphreys, 1988). This was confirmed by observations (not shown), that the antibody, although binding to individual hyalin molecules, did not appear to inhibit their association, as judged by the presence of antibody-labeled aggregates. It is thus possible that the association of hyalin filaments both with each other and with "core" particles are Ca^{2+}-dependant interactions. Cell binding studies suggest that the cell-hyalin interaction is not Ca^{2+} dependent (McClay and Matranga, 1986).

Hyalin Precursors

Immunoprecipitation of in vivo labeled embryo lysates did not identify any possible precursors. This kind of negative result is not meaningful because a short precursor half life, coupled with significant background in the immunoprecipitation, could easily produce such a result.

In vitro translation experiments suggested that hyalin may be the product of covalently linked small subunits. Although this evidence is by no means conclusive, taken with the extremely large size of hyalin, it is not unreasonable to speculate that the lower molecular weight bands immunoprecipitated in the in vitro translation experiment constitute evidence of hyalin precursors. Until DNA sequence information and hence gene structure becomes available we cannot exclude the possibility that hyalin is translated as a single polypeptide chain.

Conclusion

The structure of hyalin appears to be unique and does not resemble any known molecule, including laminin or collagen type IV, both of which have been implicated in sea urchin embryo morphogenesis (McCarthy et al., 1987; McCarthy and Burger, 1987; Wessel and McClay, 1987). Whilst further characterization of hyalin and Bl will have to await both the cloning and sequencing of the genes which encode them and in situ ultrastructural work, we have been able to describe the appearance of hyalin. We can unambiguously state that hyalin is a unique type of extracellular matrix adhesion protein which is required for sea urchin embryo morphogenesis.

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