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A protein of the basal lamina of the sea urchin embryo

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The purification, biochemical characterization and functional features of a novel extracellular matrix protein are described. This protein is a component of the basal lamina found in embryos from the sea urchin species *Paracentrotus lividus* and *Hemicentrotus pulcherrimus*. The protein has been named *Pl*-200K or *Hp*-200K, respectively, because of the species from which it was isolated and its apparent molecular weight in SDS-PAGE under reducing conditions. It has been purified from unfertilized eggs where it is found packed within cytoplasmic granules, and has different binding affinities to type I collagen and heparin, as assessed by affinity chromatography columns. By indirect immunofluorescence experiments it was shown that, upon fertilization, the protein becomes extracellular, polarized at the basal surface of ectoderm cells, and on the surface of primary mesenchyme cells at the blastula and gastrula stages. The protein serves as an adhesive substrate, as shown by an *in vitro* binding assay where cells dissociated from blastula embryos were settled on 200K protein-coated substrates. To examine the involvement of the protein in morphogenesis of sea urchin embryo, early blastula embryos were microinjected with anti-200K Fab fragments and further development was followed. When control embryos reached the pluteus stage, microinjected embryos showed severe abnormalities in arms and skeleton elongation and patterning. On the basis of current results, it was proposed that 200K protein is involved in the regulation of sea urchin embryo skeletogenesis.

Key words: basal lamina, extracellular matrix, sea urchin embryo, skeletogenesis.

Introduction

In the sea urchin embryo, blastocoele formation involves secretion of extracellular matrix (ECM) molecules, creating an environment that is biochemically different from the outside of the embryo. The blastocoelic ECM is constituted of the basal lamina and the blastocoel matrix. The basal lamina is a fibrous layer lining the blastocoel wall and the archenteron, and it seems to fill out the blastocoele cavity constituting the so-called blastocoele matrix (Katow & Solursh 1979; Spiegel *et al.* 1989). Many studies have provided information concerning the components of the basal lamina and its structure, including proteoglycans (Solursh & Katow 1982) and glycoproteins already described in vertebrate systems, as collagen (Wessel *et al.* 1984; Benson *et al.* 1990), fibronectin (Iwata & Nakano 1983; De Simone *et al.* 1985) and laminin (Spiegel *et al.* 1983; McCarthy & Burger 1987). Recently, a number of other ECM components of the basal lamina and/or blastocoele matrix have been described, giving new information about the role of each ECM molecule in morphogenesis. These include ECM 1 (Ingersoll & Ettensohn 1994), Endo 16 (Soltysik-Espanola *et al.* 1994), ECM 3 (Wessel & Berg 1995), pamlin (Katow 1995) and ECM 18 (Berg *et al.* 1996). The basal lamina, in fact, has been thought for many years to be involved in some morphogenetic events during sea urchin development, such as primary mesenchyme cell (PMC) migration (Katow *et al.* 1982; Fink & McClay 1985) and gastrulation (Spiegel *et al.* 1983). In order to study the role of ECM molecules during development, numerous experiments involving function-blocking antibodies have been performed. For example, microinjection of monoclonal antibodies to laminin in the blastocoele cavity induces a morphological change in cell shape and a deformation of the embryonic epithelium (McCarthy & Burger 1987). On the other hand, antibodies to ECM 18 inhibit PMC organization and endoderm morphogenesis during gastrulation, when

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microinjected in the blastocoele of early mesenchyme blastula or early gastrula embryos (Berg *et al.* 1996).

We describe here a newly purified ECM protein, designated 200K, obtained from two different sea urchin species living in the Mediterranean and Pacific seawater, and describe its localization and function in the embryo. We found that 200K protein has an affinity binding to cell surfaces, by an *in vitro* adhesion assay, and to type I collagen and heparin by affinity chromatography. The 200K protein is localized to the basal lamina lining the blastocoele wall and on PMC surface at the mesenchyme blastula and gastrula stages. In addition we show that blocking of the protein, by monospecific antibodies, results in a severe inhibition of morphogenesis, with major defects in skeletogenesis.

Materials and Methods

Sea urchins

Paracentrotus lividus was collected from the Sicilian coast near Palermo and *Hemicentrotus pulcherrimus* was collected from the Himaka Island in Aichi Prefecture, Japan on the Pacific coast.

Preparation of 200K

The purification of 200K proteins resulted from the procedure used for the purification of *Pl*-nectin (Matranga *et al.* 1992). All steps were performed on ice and solutions were supplemented with 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and a cocktail of protease inhibitors (2 µg/mL aprotinin, antipain, leupeptin, pepstatin A and 1 mmol/L benzamidine). Fresh unfertilized eggs were pelleted by centrifugation and resuspended with 3 volumes (v/v) of 50 mmol/L Tris buffer, pH 7.5 containing 1% Triton X-100, 1% dimethyl sulfoxide (DMSO), 7 mmol/L ethylene diamine tetraacetic acid (EDTA). The suspension was homogenized in Dounce and sonicated; the homogenate was centrifuged at 16 000 rpm in a Beckman JA-20 rotor at 4°C for 45 min. The supernatant was diluted three times (v/v) with Tris buffer and loaded on a 5 mL gelatin-Sepharose 4B column (Pharmacia, Uppsala, Sweden). The column was washed with 10 volumes of Tris buffer (omitting DMSO and EDTA) and eluted with 15 volumes of 50 mmol/L Tris pH 7.5, 1 mol/L NaCl. The column flow rate was 0.5 mL/min, the absorbance at 280 nm was monitored and 1 mL fractions were collected. The peak obtained with NaCl elution was collected, dialyzed against 10 mmol/L Tris buffer, pH 7.5, lyophilized by speed vacuum centrifugation and stored at − 20°C. Aliquots were analyzed by SDS-PAGE. For cell-substrate adhesion assays, the protein used to coat microtiter plates was in NaCl. We found that sometimes *Hp*-200K protein is obtained, in addition to NaCl elution, also with 8 mol/L urea elution. Protein concentration was determined by optical density at 280 nm.

Binding to heparin

About 150 µg of 200K protein purified by gelatin-Sepharose was dialyzed and loaded on a heparin-Sepharose column (1 mL), previously equilibrated with Tris buffer containing PMSF. The column was washed with 10 volumes of Tris buffer, and eluted with 15 volumes of 1 mol/L NaCl, 50 mmol/L Tris, pH 7.5 and with 10 volumes of 8 mol/L urea, 50 mmol/L Tris, pH 7.5. The NaCl and urea elution peaks were collected, dialyzed against 10 mmol/L Tris, pH 7.5, lyophilized by speed vacuum centrifugation and stored at − 20°C. Aliquots were analyzed by SDS-PAGE.

Preparation of hyaline-apical lamina and basal lamina

To isolate the hyaline-apical lamina (Hy-AL) complex we utilized the procedure originally developed by Hall and Vacquier (1982), with some modifications. Briefly, middle gastrula embryos were allowed to self-dissociate in 1.1 mol/L glycine, 2 mmol/L EGTA, pH 8.0 for 1 min. Hyaline-apical lamina complexes were gently washed several times in the glycine solution containing 1% Triton X-100 and then pelleted. Basal laminar bags were isolated according to McClay and Marchase (1979). Briefly, middle gastrula embryos were lyzed in 10 mmol/L sodium bicarbonate, 0.1% Triton X-100, 10 mmol/L EDTA for 10 min with occasional gentle trituration and centrifuged at 1000 rpm for 2 min. These steps were repeated twice. Pellets were washed three times in 10 mmol/L sodium bicarbonate and centrifuged at 1000 rpm for 10 min. Proteins from both preparations were separated by SDS-PAGE and immunoblotted.

SDS-PAGE and western blot

Lyophilized material was resuspended in denaturation buffer and analyzed by 6% SDS-PAGE under reducing conditions according to Laemmli (1970). Molecular weight markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa) and bovine serum albumin (66 kDa) from BioRad (Hercules, CA, USA). Gels were stained with Coomassie brilliant blue. Immunoblot analysis was carried out according to the procedure reported by Towbin *et al.* (1979). Transfer of protein was performed at 100 V for 1 h in the cold. After washing and blocking free sites, nitrocellulose was incubated overnight at room temperature with specific antibodies. Anti-*Hp*200K polyclonal antibody, raised in rabbit as described in the following, was used at 1:500

dilution. An anti-hyalin polyclonal antibody (Gratwohl *et al.* 1991) was used at 1:200 dilution. Bound antibodies were detected with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG; Cappel, West Chester, PA, USA) diluted 1:2000. Antibodies were diluted into PBS-0.05% Tween 20 and incubated overnight or for 2 h at room temperature, respectively. Immunoreactive bands were visualized using a 0.3% 4-Cl-1-naphthol solution.

Preparation of anti-Hp200K

For preparation of monospecific polyclonal antibodies, a 200 kDa band was cut out from SDS-PAGE, soaked in phosphate-buffered saline (PBS), homogenized with Freund's complete adjuvant (Gibco, Life Technologies, Italy) and injected subcutaneously into female rabbits five times at an interval of 10 days. Ten days after the last injection, the blood was collected for preparation of the sera. Fab fragments were prepared as follows: IgG was purified by affinity chromatography on Protein-A Sepharose, and digested by papain (1/100 of the IgG amount) in 0.1 mol/L PBS, pH 7.0, 10 mmol/L cysteine and 2 mmol/L EDTA at 37°C for 16 h. The digestion was stopped by addition of iodoacetamide at a final concentration of 10 mmol/L. The Fc fragments and IgG not digested were adsorbed with Protein-A Sepharose. The flow through fraction of the papain digest from Protein-A Sepharose was dialyzed against seawater.

Immunofluorescence

Indirect immunofluorescence was carried out according to the procedure reported by Matranga *et al.* (1992). Briefly, eggs and embryos were fixed in Bouin's fixative and embedded in paraffin. Sections of 4 µm were incubated at room temperature for 30 min with 50 µL of anti-*Hp*200K diluted 1:100. After washing, sections were incubated as previously with FITC-conjugated goat anti-rabbit antibodies, diluted 1:200 (Sigma, Milan, Italy).

Cell-substrate adhesion assay

To measure the adhesion of cells to the substrate we utilized an assay originally developed by McClay and Fink (1982) with some modifications as described by Matranga *et al.* (1992, 1995). Briefly, 100 µL of purified protein, at increasing concentrations, were bound to microtiter plate wells for 3 h at room temperature. Blanking of the wells was performed by incubation with 10 mg/mL bovine serum albumin (BSA) overnight at 4°C. *Paracentrotus lividus* embryos were metabolically labeled by culturing them with 0.6 µCi/mL of [^{3}H]lysine (Amersham, Buckinghamshire, UK) until the mesenchyme blastula stage and dissociated into single cells by the technique fully described by Matranga *et al.* (1986). Radiolabeled cells, 1×10^5 per well, were settled onto 200K-coated wells for 1 h at $1 \times g$, then the plates were inverted and spun off for 8 min at $100 \times g$. The percentage of cells bound was calculated relative to the radioactivity associated to 1×10^5 ^{3}H-labelled cells counted separately. Reported values are the mean of four replicates for which the standard error is calculated

Microinjection experiments

Eggs of *Hemicentrotus pulcherrimus* were fertilized in Millipore-filtered sea water (MFSW) containing 3 mmol/L *P*-aminobenzoic acid to prevent hardening of the fertilization membrane. Twenty minutes after fertilization, eggs were passed through 82 µm nylon mesh to remove the fertilization membrane and washed with MFSW by settling and removal of the supernatant. The denuded eggs were allowed to develop with gentle stirring at room temperature. At the early blastula stage, embryos were injected with Fab fragments at a concentration of 0.17 µg/µL in MFSW using a Drummondo microsyringe. The injected amount was about 100 pL. Following microinjection, embryos were cultured in microtiter plate wells at 16°C and the development was observed with an Olympus inverted microscope.

Results

Purification and biochemical properties of 200K protein

We have previously described the isolation and characterization of an ECM molecule, *Pl*-nectin, from *Paracentrotus lividus*, which localizes on the apical surface of blastula cells (Matranga *et al.* 1992). The procedure used for *Pl*-nectin purification, based on affinity chromatography on gelatin-Sepharose of an egg extract, is provided with two affinity separation steps, which follow Tris-Triton elution of unbound material. These are: elution with NaCl first and with urea later. *Pl*-nectin is eluted in the urea peak, which is supposed to contain material that strongly binds to gelatin. In an effort to purify other ECM molecules, on the basis of their affinity for type I collagen, we analyzed the weakly bound material contained in the NaCl elution peak of gelatin-Sepharose columns used for the purification of *Pl*-nectin. On 6% SDS-PAGE under reducing conditions, the NaCl eluate showed one major band with an approximate molecular weight of 200 kDa (Fig. 1A, *Pl*). A faint band with an apparent molecular weight of about 45 kDa was sometimes observed, possibly being a degradation product of 200K protein, or a different

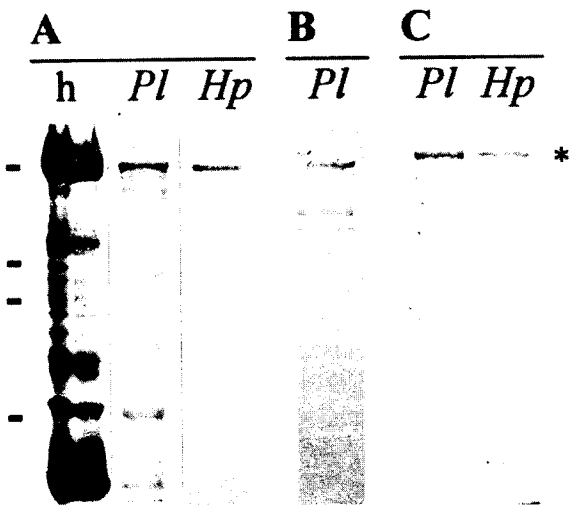

Fig. 1. Electrophoretic analysis on 6% SDS-PAGE under reducing conditions. (A) Gelatin-Sepharose affinity chromatography NaCl elution of *Paracentrotus lividus* (*Pl*) or *Hemicentrotus pulcherrimus* (*Hp*). h, homogenate from *P. lividus* eggs. (B) Western blot analysis of *P. lividus* homogenate with anti-*Hp*200K antiserum. (C) Heparin-Sepharose affinity chromatography urea elution of *Paracentrotus lividus* (*Pl*) or *Hemicentrotus pulcherrimus* (*Hp*). On the left the position reached by molecular weight markers is indicated. On the right, the asterisk indicates the position reached by the 200 kDa band.

gelatin-binding molecular species. The yield obtained was about 150 µg of purified protein from 10 mL of packed eggs, as determined by comparison with a known concentration of BioRad molecular weight markers run on the same gel.

In order to study the native structure of the protein, 200K was analyzed on 4% SDS-PAGE under non-reducing conditions. However, the protein could not enter the lower gel (data not shown), suggesting that the native protein may form large supramolecular structures as reported for fibronectin (Morla *et al.* 1994). However, we do not know if this would reflect the situation occurring *in vivo*.

We were able to purify from the species *Hemicentrotus pulcherrimus*, a protein with the same apparent molecular weight as that purified from the Mediterranean species (Fig. 1A, *Hp*). To verify whether or not the two proteins were the same molecule displayed by different species, we prepared polyclonal antibodies against the *Hp*-200K and the antiserum was used on western blot of *Pl*-200K. As shown in Fig. 1(B) the anti-*Hp*200K antiserum recognizes the *Pl*-200K protein, indicating that the two molecules share common epitopes and suggesting they are homologs found in closely related species.

In addition to the collagen-binding domain, many ECM molecules contain heparin-binding domains that have been shown to be involved in the interaction to proteoglycans or to the cells (for a review see Yamada 1991). As an example, it has been shown that heparin-binding regions of fibronectin modulate cell interactions *in vitro* (Izzard *et al.* 1986). Therefore it was of some interest for the biochemical characterization of the molecule to investigate on binding affinities to heparin. To this purpose the gelatin-purified material from both species was dialyzed against Tris 50 mmol/L, pH 7.5, and applied to the heparin-Sepharose column. Sodium chloride and urea elution peaks were analyzed by 6% SDS-PAGE under reducing conditions. A single band with a molecular weight of 200K was found only in the urea elution peak (Fig. 1C), suggesting a strong affinity of the protein to heparin. This applies to both proteins from Mediterranean (*Pl*) or Japanese (*Hp*) species. As a control, purified 200K protein was loaded on a Sepharose column, showing no binding affinity to Sepharose (data not shown).

Pattern of expression of 200K protein during embryo development

In order to study the temporal and spatial distribution of 200K protein throughout development, indirect immunofluorescence experiments were performed on sections of *Paracentrotus lividus* embryos with anti-*Hp*200K antiserum (Fig. 2). Like many other ECM components found in the sea urchin embryo (Hall & Vacquier 1982; Wessel *et al.* 1984; Matranga *et al.* 1992; Ingersoll & Ettensohn 1994; Wessel & Berg 1995), 200K protein is stored in cytoplasmic granules in the unfertilized egg (Fig. 2A). By the hatching blastula stage, the staining becomes localized at the basal lamina lining the blastocoele wall, although some staining was still observed in the cytoplasm of the cells (Fig. 2B). At the mesenchyme blastula stage, the signal became more evident at the basal lamina and it appeared also associated with PMC ingressing into the blastocoele (Fig. 2C). During early gastrulation, the signal is even more concentrated around the PMC, being still detectable at the basal lamina, including the portion of the lamina that overlies the archenteron (Fig. 2D).

In order to confirm biochemically that 200K protein is a component of the basal lamina, crude preparations of the hyaline-apical lamina complexes and basal laminar bags, isolated from *Paracentrotus lividus* embryos at the middle gastrula stage, were analyzed by immunoblotting with anti-*Hp*200K and anti-hyalin antibodies. As shown in Fig. 3, when comparable amounts of proteins were used, a distinct band with an approximate molecular weight of 200 kDa, was observed in the basal lamina preparation probed with anti-*Hp*200K (see solid arrow). A very faint band was also observed in the hyaline-apical lamina compartment, possibly

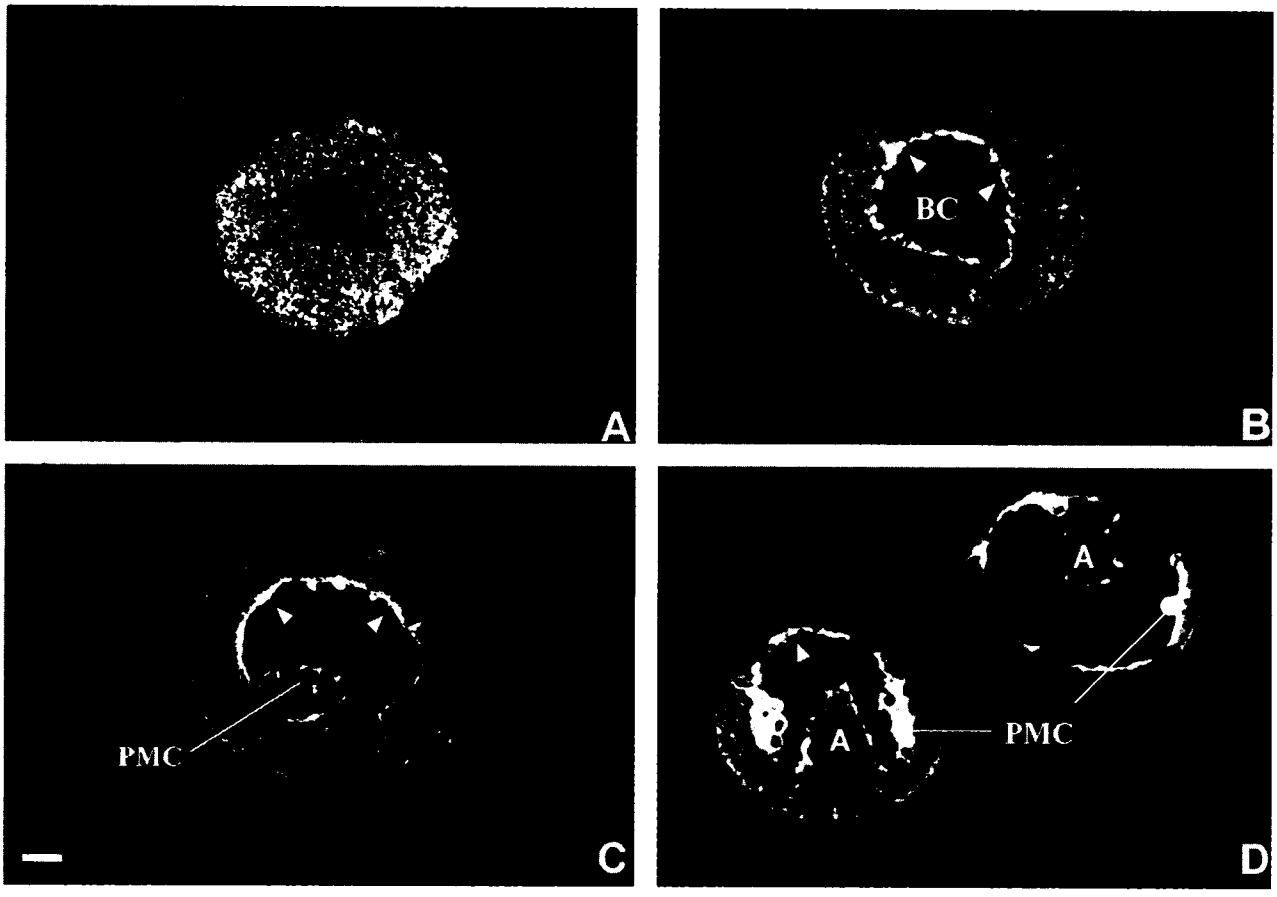

Fig. 2. Indirect immunofluorescence by anti-*Hp*200K monospecific antibodies on sections of *Paracentrotus lividus* eggs and embryos at different developmental stages. (A) unfertilized egg, (B) blastula, (C) mesenchyme blastula, (D) middle gastrula. A, archenteron; BC, blastocoele; PMC, primary mesenchyme cell. Arrowheads indicate basal lamina labeling. Bar, 20 μm.

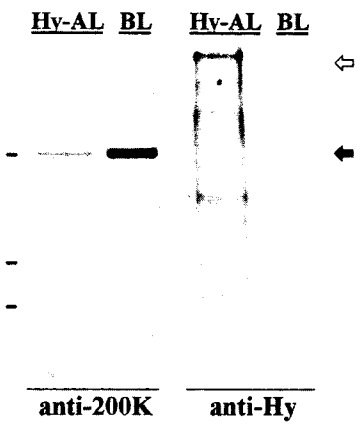

Fig. 3. Immunoblotting analysis of ECM from hyaline-apical lamina and basal lamina of *Paracentrotus lividus*. Hyaline-apical lamina (Hy-AL) complexes and basal laminar bags (BL) from middle gastrulae were run on SDS-PAGE, transferred to nitrocellulose and reacted with anti-*Hp*200K (anti-200K) or anti-hyalin (anti-Hy) antibodies. On the left the position reached by molecular weight markers is indicated. On the right, empty arrow indicates the position reached by hyalin. Solid arrow indicates the position reached by 200K protein.

caused by contamination of the layers during preparation. It should be noted that 1 min in glycine, which is used to extract apical lamina complexes, is sufficient to dissociate embryos and therefore render the basal lamina extractable. As a control, samples of the same preparations were immunoblotted with anti-hyalin antibody. Specific localization of hyalin in the hyaline-apical lamina compartment was observed and no reactivity was associated to the basal ECM compartment (see empty arrow). From these results and previous immunofluorescence labeling, we conclude that 200K protein is compartmentalized to the basal lamina of embryos at the middle gastrula stage.

Cell attachment promoting activity of 200K protein

In the last 20 years, it has been demonstrated that the adhesive interactions of cells with proteins of the ECM influence some important morphogenetic processes (Hay 1981; Trelstad 1984; McClay & Ettensohn 1987; Adams & Watt 1993). A number of assays are available for the analysis of these interactions, ranging from

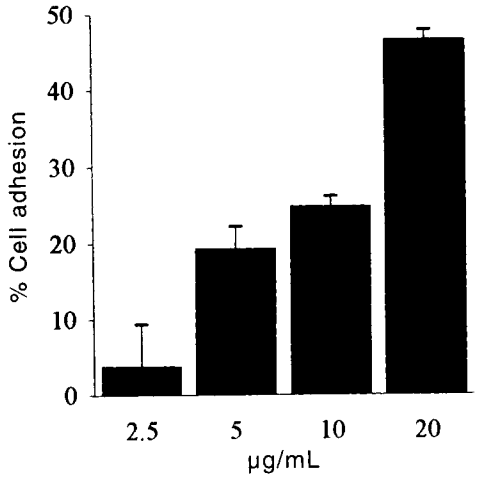

Fig. 4. Cell-substrate adhesion assay of *P. lividus* blastula cells on *Pl*-200K protein. Cells were incubated on plates coated with increasing amounts of purified 200K protein. The percentage of cells bound to the substrate after centrifugal dislodgement was measured. Values are the mean of four replicates for which SE is shown.

in vitro studies of cell attachment to coated substrates, to characterization of morphogenetic alterations in whole embryos induced by specific inhibitors, like drugs and antibodies, or genetic modification.

Evidence for the presence of collagen and heparin binding sites in the 200K protein and its localization on the basal lamina of the embryo, suggested to us that the protein could be important for cell binding to the ECM. In order to confirm this hypothesis, we measured the binding of mesenchyme blastula dissociated cells to 200K-coated substrates, by the method used for functional characterization of *Pl*-nectin and fibronectin-like proteins (Matranga *et al.* 1992, 1995). Figure 4 shows the results of a typical experiment in which dissociated cells bind in a dose-dependent manner to *Pl*-200K-coated substrates. The minimal concentration needed to obtain an increase in cell adhesion was 5 µg/mL, with about 20% of cells adhering to the substrate. The maximum percentage of cell adhesion, corresponding to about 50%, was obtained with 20 µg/mL of coated *Pl*-200K. Values of controls made

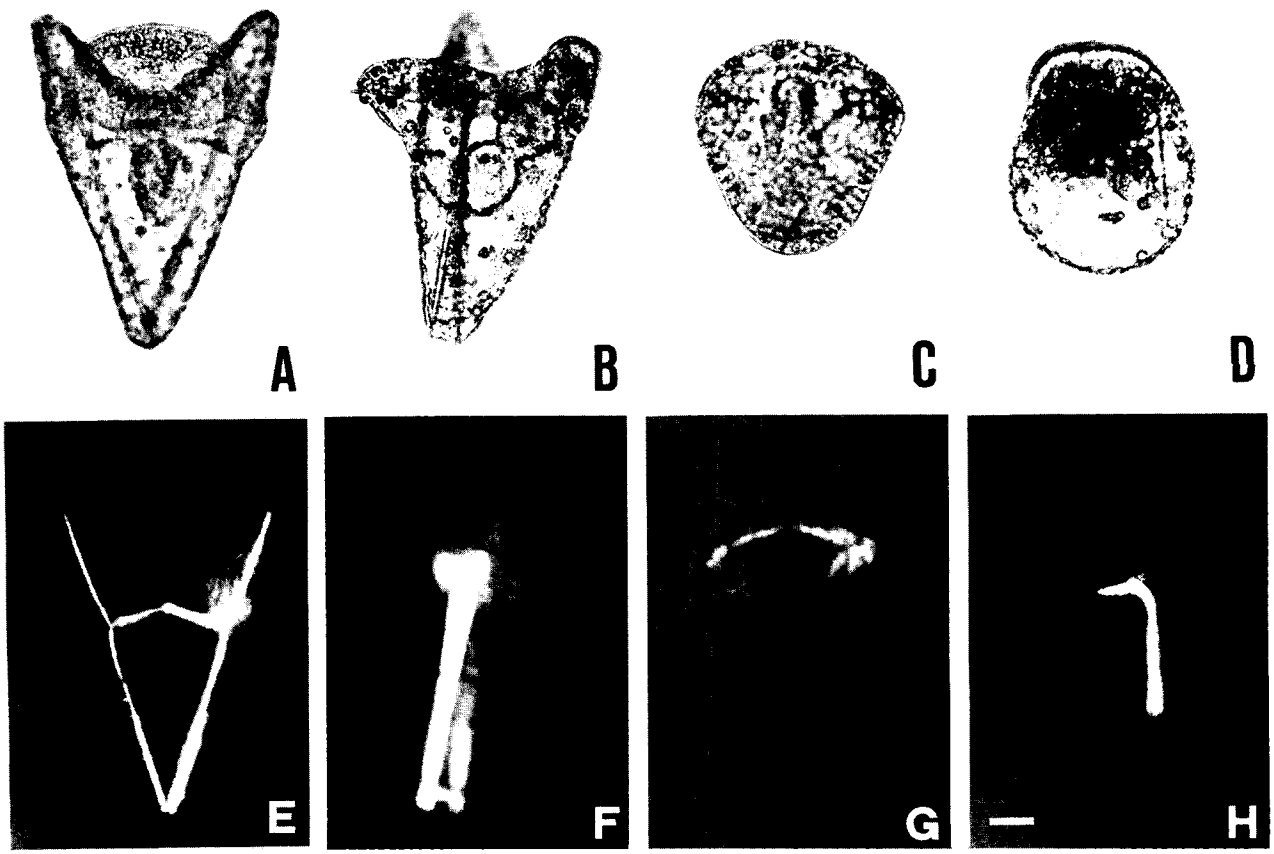

Fig. 5. Effect of anti-*Hp*200K antibodies on the shape and skeleton of larvae. Embryos were injected at the early blastula stage with unrelated Fabs (A,E) or anti-200K Fabs (B–D, F–H). Pictures were taken 54 h after fertilization. (A) control embryos 52 h after fertilization. (B–D) embryos microinjected 10 h after fertilization and observed 54 h after fertilization. Bar, 20 µm

by plating cells onto BSA, giving typically about 10–20% of unspecific binding, were subtracted. These results indicate that 200K protein serves as an adhesive substrate for cells of the mesenchyme blastula embryos.

In vivo *function of 200K protein*

Results obtained on the cell-binding function of 200K protein imply that it could be involved in the morphogenetic cell rearrangements during sea urchin embryo development. Therefore, in an attempt to clarify the presumptive morphogenetic function of 200K, anti-*Hp*200K Fab fragments were injected into the blastocoele of early blastula embryos and the effect of this treatment was scored when control embryos had reached the pluteus stage.

Figure 5 shows representative embryos 44 h after microinjection. The main defects observed in embryos microinjected with anti-*Hp*200K were shortening or lack of arms, and malformation of the skeleton. In many cases the digestive tract developed normally, while the skeleton showed incomplete development. When the anal rods of the skeleton did not stretch (Fig. 5B), the arms were shorter than those of control plutei injected with unrelated Fabs (Fig. 5A). In case skeletal rods were rudimentary, no arms were formed, although the digestive tract developed (Fig. 5C). Figure 5D shows a completely disorganized larva, in which only one body rod developed, no arms were formed and the digestive apparatus did not develop at all. Polarizing microscopy shows more clearly the skeletal abnormalities in injected embryos (Fig. 5E–H).

Table 1 summarizes the results obtained in a series of experiments to give quantification to various developmental defects observed.

Discussion

In the sea urchin embryo, the blastocoele contains a heterogeneous number of molecules, interacting with cells that live in or move into the blastocoele and with a basal surface of ectoderm and endoderm cells. These cell–ECM interactions are known to be involved in cell fate determination and morphogenesis in this embryo (for a review see Ettensohn & Ingersoll 1992).

In the present study, we report the purification of a new ECM protein that is uniformly localized on the basal lamina and on the surface of PMC, at the blastula and gastrula stages. The protein has an apparent molecular weight of 200 kDa, when analyzed on SDS-PAGE under reducing conditions. As we found that 200K protein has binding affinities to cell surfaces, type I collagen and heparin, it is conceivable to suppose that the protein bridges cells to these components present in the basal lamina.

Other ECM proteins have been identified that are uniformly distributed in the basal lamina, including laminin (McCarthy & Burger 1987) and pamlin (Katow 1995). On the contrary, there is evidence for spatially localized components in the basal lamina, such as ECM 1, concentrated in the vegetal and ventral regions of the embryo (Ingersoll & Ettensohn 1994); ECM 18, deposited in the basal lamina surrounding the archenteron (Berg *et al.* 1996) and ECM 3, accumulated only in the basal lamina adjacent to the ectoderm, in all regions except for the animal pole (Wessel & Berg 1995). Of the ECM components already mentioned, none have been observed on the PMC, where the 200K protein is located. These data suggest that the sea urchin basal lamina has different regions with different molecular composition, which may be important in mediating various events during morphogenesis.

In experiments where cell–ECM interactions were disrupted by monospecific polyclonal antibody to 200K protein, we showed that skeleton elongation and patterning of treated embryos is greatly affected. As 200K protein is localized on the surface of PMC, in addition to the basal lamina, it seems that the effects observed are produced by a direct action of antibodies on the PMC. On the contrary, differentiation of ectoderm and endoderm territories, whose cells likewise interact with the basal lamina during development, do not appear to be affected by antibody treatment. One possible hypothesis is that the anti-200K antibody locally disrupts

Table 1. Microinjection of anti-*Hp*200K Fabs into the blastocoele of blastula and block of development

Culture (h)	Fab (μg/μL)	Injected embryos	Surviving embryos	Normal plutei	Short-arm plutei	Trapezoid larvae	Ovoid larvae
44	0.17	32	22	6	11	4	1
44	0.17	23	14	4	5	3	2
44	0.17	30	16	4	6	5	1
42	0.15	26*	13	11	2	0	0
42	0.15	21*	15	14	0	0	1

*Embryos injected with unrelated Fabs.

or blocks the ECM where PMC move and exert their spicule-forming activity. On the other hand, in other systems it has been known for a long time that ECM may serve as a microreservoir of growth factors, which have been shown to bind to ECM molecules (Adams & Watt 1993). Another possibility is that 200K protein has *per se* a growth factor activity, as reported for example in laminin and tenascin (Chiquet-Ehrismann *et al.* 1986; Panayotou *et al.* 1989). It has been demonstrated, in fact, that many ECM proteins contain repeated epidermal growth factor-like sequences (Engel 1989). Recently, we have shown the involvement in skeletogenesis of another ECM protein, named *Pl*-nectin (Zito *et al.* 1998), localized on the apical surface of ectoderm cells. In that case, we proposed that ectoderm fails to address proper signals, maybe growth factors, which regulate the expression of genes involved in skeletogenesis in PMC. Confirmation of this hypothesis comes from recent studies indicating that skeletal rod growth is controlled by short-range signals coming from ectodermal territories (Guss & Ettensohn 1997). Here, we demonstrate that skeleton formation and patterning is also modified by interfering with a different ECM protein, named 200K, found on the basal surface of ectoderm cells and PMC. We propose, on the basis of earlier work and current study, that the establishment of a proper extracellular microenvironment surrounding PMC is crucial to the regulation of skeletal morphogenesis in the sea urchin embryo.

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