ORIGINAL ARTICLE

Sea urchin arylsulfatase, an extracellular matrix component, is involved in gastrulation during embryogenesis

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Abstract Arylsulfatases (Arses) have been regarded as lysosomal enzymes because of their hydrolytic activities on synthetic aromatic substrates and their lysosomal localization of their enzymatic activities. Using sea urchin embryos, we previously demonstrated that the bulk of *Hemicentrotus* Ars (HpArs) does not exhibit enzyme activity and is located on the apical surface of the epithelial cells co-localizing with sulfated polysaccharides. Here we show that HpArs strongly binds to sulfated polysaccharides and that repression of the synthesis by *HpArs*-morpholino results in retardation of gastrulation in the sea urchin embryo. Accumulation of HpArs protein and sulfated polysaccharides on the apical surface of the epithelial cells in sea urchin larvae is repressed by treatment with β-aminopropionitrile (BAPN), suggesting that deposition of HpArs and sulfated polysaccharides is dependent on the crosslinking of proteins such as collagen-like molecules. We suggest that HpArs functions by binding to components of the extracellular matrix.

Keywords Arylsulfatase · Sea urchin · Extracellular matrix · Gastrulation · Heparin-binding

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Introduction

Sulfatases are an evolutionarily highly conserved gene family. A number of sulfatases are active in vitro against a common set of synthetic small aromatic substrates; hence, some of the enzymes are named as arylsulfatase (Ars) (Parenti et al. 1997). Arses have been reported to be lysosomal enzymes in different animals. Candidates of the natural substrates for the Arses have been reported (Mehl and Jatzkewitz 1968; Yogalingam et al. 1996) and human physiological disorders of Arses are due to the accumulation of nondegradable sulfated compounds in lysosomes. However, we suspected that the specific activity of the mammalian Arses toward natural substrates is too low for Arses to function as a conventional enzyme. It has been reported that V_{max} of human ArsB and feline ArsB toward natural substrate is approximately 1:100 of that toward the synthetic small aromatic substrates (Yogalingam et al. 1996).

Using the sea urchin embryo, we previously demonstrated that the bulk of *Hemicentrotus pulcherrimus* Ars (HpArs) does not exhibit enzyme activity and is located on the apical surface of the epithelial cells (Akasaka et al. 1990; Mitsunaga-Nakatsubo et al. 1998). A signal peptide destined for extracellular space is located in the amino terminal of the HpArs protein. These suggest that the Ars proteins play a role in the extracellular space (Sasaki et al. 1988). During sea urchin development, the expression of *Ars* begins just before the onset of dynamic morphogenetic movement of the epithelial cells (Rapraeger and Epel 1981; Sasaki et al. 1988) and the amount of HpArs protein constitutes more than 0.5% of total protein in the embryo after the gastrula stage (Sasaki et al. 1987). The HpArs protein co-localizes with the sulfated polysaccharide which accumulates mainly on the apical surface of epithelial cells (Mitsunaga-Nakatsubo et al. 1998; Akasaka and Terayama 1983). We also demonstrated that the HpArs proteins

extracted from the sea urchin embryos are insolubilized in the presence of Ca^{2+} (Mitsunaga-Nakatsubo et al. 1998). It has been reported that expansion of the prospective ectoderm cells as a sheet toward the vegetal pole, employing tractoring on components of the apical extracellular matrix, contribute to the primary invagination during gastrulation (Burke et al. 1991, Davidson et al. 1995, 1999; Kominami and Takata 2000, 2004; Takata and Kominami 2001). Components of the extracellular matrix, such as sulfated polysaccharides (Akasaka and Terayama 1983, 1984), hyalin (Adelson and Humphreys 1988; Wessel et al. 1998), fibropellin (Bisgrove and Raff 1993), and echinonectin (Alliegro et al. 1988) located in the apical surface of sea urchin embryos are also involved in cell movements during gastrulation. Here we demonstrate that HpArs binds to sulfated polysaccharides and that HpArs plays an important role as a component of the extracellular matrix to support epithelial cell movement during gastrulation.

Materials and methods

Culture of sea urchin embryos

Gametes of *H. pulcherrimus* were obtained by coelomic injection of 0.55 M KCl. Embryos were cultured in the artificial sea water Jamarin U (Jamarin Lab, Japan) at 16°C in the presence or absence of β-aminopropionitrile (BAPN) (Sigma Chem, USA) at the concentration of 0.39 or 0.78 mM, which specifically inhibits the extracellular collagen crosslinking enzyme, lysyl oxidase (Pinnell and Martin 1968), as reported by Wessel and McClay (1987).

HpArs-antisense morpholino oligonucleotide (MO) and injection into the sea urchin fertilized eggs

HpArs-MO was designed against *HpArs*-mRNA (5'-AUGAAGUCCGCCC CUUUCCUUUUCC-3'; from the translational start codon). Standard control MO commercially obtained from Gene Tools, LLC (Corvallis, OR, USA) was used as a control. Fertilized eggs were injected with different concentrations of the MO as described before (Fuchikami et al. 2002).

Immunohistochemistry of sea urchin embryo

Embryos were fixed in 100% methanol at −20°C for 20 min and stained with affinity purified polyclonal antibodies against HpArs (Mitsunaga-Nakatsubo et al. 1998). The primary antibodies were detected with Oregon green-conjugated goat anti-rabbit secondary antibodies (Molecular

Probes Europe BV, the Netherlands). Images were captured with a digital camera Nikon DXM1200.

Semi-quantitative RT-PCR

Total RNA was isolated from 50 embryos at 28 h after fertilization using ISOGEN (Nippon gene, Japan). The extracted RNAs were used to synthesize cDNA using RNA polymerase chain reaction (PCR) kit (AMV; TaKaRa, Kyoto, Japan). An aliquot of the reverse transcription reaction was then used for PCR containing 0.2 μM concentrations of the gene specific primers as follows; *HpArs*, 5'-CGACGA CATGGGATCTGGC-3' and 5'-CAGCATTTGGTA TAGGCGG-3'; *HpBrachyury*, 5'-CGGCCCCACACCCCAT CAGT-3' and 5'-TGCGGCGGTGGAGGGCCACA-3', and *HpEndo16*, 5'-TACGCCCACGACTTCAACG-3' and 5'-CAGCATTTGGTATAGGCGG-3'. *HpUbiquitin* gene was introduced to use as internal control to quantify RNA amount. *HpUbiquitin* primer sequences; upstream primer: 5'-GAGCTGCGATGTATTTGCCAGATG-3'; downstream primer: 5'-TTTGATGGAATAACAAATAACTGATTGCTT-3'. The reaction mixture was pre-heated at 94°C for 2 min. The PCR amplification conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. All reactions were performed in the linear range of amplification. The products were resolved on 2% agarose gels, transferred to a Nytran membrane (Schleicher & Schuell, Germany) and were detected by Southern hybridization with the appropriate digoxigenin-labeled RNA probes, followed by antibodies to digoxigenin conjugated to alkaline phosphatase (Boehringer Mannheim, Germany). The chemiluminescent signal produced by enzymatic dephosphorylation of CSPD (TROPIX) by hydrolysis with alkaline phosphatase was detected by X-ray film.

Heparin-HpArs binding assay

HpArs protein was extracted and purified from plutei according to the procedure reported previously (Sasaki et al. 1987). Two microgram of purified HpArs protein was mixed with 1 ml of heparin-Sepharose or Sepharose CL-6B (Amersham Pharmacia Biotech AB, Sweden) equilibrated with 10 mM Tris–HCl (pH 7), and then the concentration of Ca^{2+} or Ca^{2+}/Mg^{2+} in the mixture was adjusted to the level found in sea water (9 mM $CaCl_2$, 50 mM $MgCl_2$). The slurry of the Sepharose was poured into the column and washed with ten bed volumes of the buffer. HpArs protein was eluted with a linear gradient of 0–3 M NaCl in the buffer. The aliquot of each fraction was subjected to electrophoresis and the protein was blotted to polyvinylidene fluoride (PVDF) membranes (Immobilon Transfer Membranes; Millipore). The HpArs protein was detected with affinity purified anti-HpArs antibodies (Mitsunaga-Nakatsubo et al. 1998), followed

by anti-rabbit IgG antibodies conjugated to horseradish peroxidase (PIERCE). The chemiluminescence of Super Signal West Dura Extended Duration Substrate (PIERCE) by hydrolysis with horseradish peroxidase was detected by X-ray film.

Immunoblot analysis

Embryos treated with or without BAPN were collected at the prism corresponding stage (42 h after fertilization) and dissolved in sample buffer [final concentration, 100 mM Tris–HCl (pH6.8), 2% SDS, 10% glycerol, 0.004% Bromphenol Blue, 6% 2-mercaptoethanol] and boiled for 5 min. Proteins were analyzed on 12% acrylamide gels by SDS-PAGE and transblotted on to a PVDF membrane (Immobilon Transfer Membranes; Millipore).

In order to verify whether the HpArs proteins are released from the larvae into the sea water, the larvae at the prism corresponding stage cultured with or without BAPN were concentrated by centrifugation ($1,000 \times g$, 1 min) and the 2 ml of larva suspension at a density of 1.3×10^5 was incubated in a 50 ml polypropylene tube at 16°C for 5 h. Then the larva suspension was centrifuged ($8,000 \times g$, 5 min). The pellet was dissolved in 1 ml of the SDS-PAGE sample buffer. The proteins in the supernatant were precipitated with ethanol (final concentration 90%) by centrifugation ($12,000 \times g$, 30 min). The precipitate was washed with 80% ethanol to remove the salt. The pellet was dissolved in 1 ml of the SDS-PAGE sample buffer. Equal aliquots of the samples were analyzed on 12.5% acryl amide gels by SDS-PAGE and transblotted on to a PVDF membrane. The HpArs protein was detected as mentioned above.

Northern blot analysis

Total RNA was extracted from control or BAPN-treated embryos at the gastrula corresponding stage using ISOGEN (Wako, Japan). The total RNA (10 µg) was electrophoresed on each lane of a denaturing formaldehyde–1% agarose gel, transferred to a Nytran membrane (Schleicher & Schuell, Germany), and hybridized to the antisense RNA of *HpArs* labeled with Digoxigenin-11-UTP (Boehringer Mannheim, Germany). The signal was detected as described above.

Biochemical assay for fucose-rich sulfated polysaccharide extracted from sea urchin embryos

Preparation of polysaccharides from sea urchin embryos, chromatography of the polysaccharides on DEAE-cellulose, and biochemical assay for fucose was performed as described before (Akasaka and Terayama 1983).

Results

Repression of HpArs synthesis causes significant retardation of gastrulation of sea urchin embryo

In order to gain insight into the role of Ars during sea urchin development, we designed experiments to perturb the embryo by inhibiting the translation of HpArs by injecting fertilized eggs with *HpArs*-MO. The embryos injected with *HpArs*-MO were morphologically normal until blastula stage, but when the control embryo reached the gastrula stage (Fig. 1a, c), embryos injected with *HpArs*-MO failed to invaginate, though primary mesenchyme cells ingressed into the blastocoel (Fig. 1b, d). The normal decrease in the thickness of the epithelial wall during gastrulation was repressed in the MO embryos. It seemed that the epithelial cells did not spread and remained cylindrical. Immunofluorescence with anti-HpArs antibodies showed that accumulation of the HpArs protein was almost completely repressed in the *HpArs*-MO embryos (Fig. 1d). The extent of repression of the invagination correlated with the amount of *HpArs*-MO injected into the egg (Table 1). The invagination of 85 % of the embryos was retarded by injecting with 7.2×10^8 molecules/egg of *HpArs*-MO. The invagination was rescued in the embryos injected with *HpArs*-MO by co-injection of *HpArs*-mRNA (Fig. 1e). Semi-quantitative reverse transcription (RT)-PCR revealed that the expression of endoderm specific *HpEndo16* (Akasaka et al. 1997) and *HpBrachyury* (Hibino et al. 2004) was not affected by the injection of *HpArs*-MO, while archenteron invagination was blocked. The expression of *HpArs* also was not affected significantly, although the slight reduction of the expression was observed (Fig. 1f).

HpArs binds to heparin

Co-localization of HpArs with sulfated polysaccharides accumulated on the apical cell surface of sea urchin embryos suggests an interaction between these components. In order to analyze the binding activity of HpArs to sulfated polysaccharides, the affinity of HpArs protein for heparin was examined. In the absence of divalent cations, the affinity of HpArs for heparin was very limited. Only a small portion of the proteins bound to heparin and they were almost completely eluted at 1 M NaCl (Fig. 2a). Since HpArs accumulates on the apical surface of the sea urchin embryos, which faces sea water, we postulated that Ca^{2+} is required for the binding activity of HpArs. Unexpectedly, Ca^{2+} inhibited the binding to heparin (data not shown). However, when Ca^{2+} was added to the reaction mixture after mixing of the HpArs with the heparin-Sepharose, a large portion of HpArs bound to heparin (Fig. 2b). The binding activity of HpArs was

Fig. 1 Repression of Ars synthesis causes inhibition of invagination in sea urchin embryo. **a, c** Embryo injected with control MO (7.2×10^8 molecules/egg) fixed at the gastrula stage. **b, d** Embryo injected with *HpArs*-MO (7.2×10^8 molecules/egg) cultured for the same period as control embryo. **e** Embryo co-injected with *HpArs*-MO (7.2×10^8 molecules/egg) and *HpArs*-mRNA (8.4×10^5 molecules/egg). **a, b, e** Bright field. **c, d** Immunofluorescence stained with anti-HpArs antibodies. **f** RT-PCR analysis of different genes in control and *HpAr*s-MO injected embryos. RT-PCR was performed in the linear range of amplification
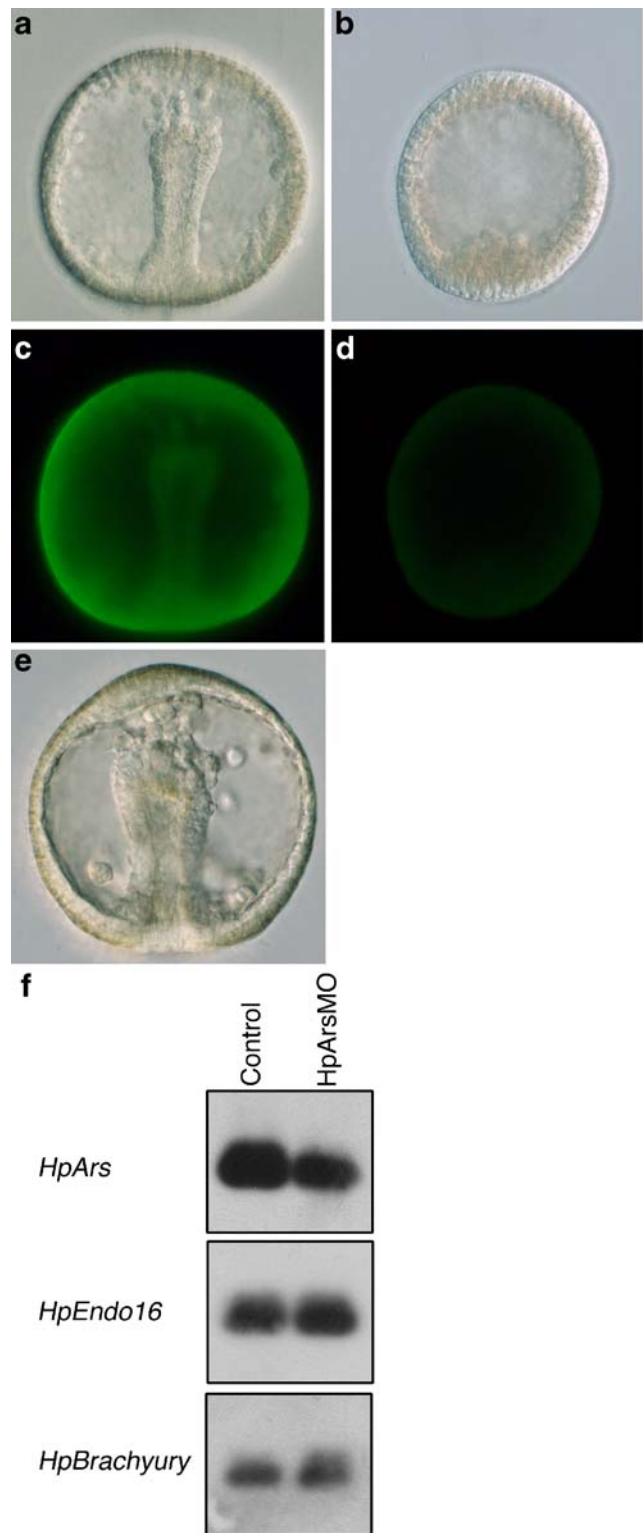
enhanced further by adding Mg^{2+} to the mixture (Fig. 2c). The HpArs protein was eluted with high salt buffer containing more than 1.3 M NaCl. Sepharose was used as a negative control of heparin-Sepharose to examine the binding activity to HpArs. HpArs proteins were mixed with Sepharose CL-6B, and then Ca^{2+} and Mg^{2+} were added to the reaction mixture. The HpArs proteins did not bind to the Sepharose at all and were eluted in flow-through and washing fractions (Fig. 2d). These results suggest that the strong binding activity of HpArs to heparin is Ca^{2+}-dependent via sulfate ion, and that access of HpArs to heparin before exposure of Ca^{2+} may be required for its binding activity.

BAPN induces release of HpArs from sea urchin larvae

Lysyl oxidases are enzymes that stabilize extracellular matrix by crosslinking elastin and collagens (Csiszar 2001; Kagan and Li 2003). BAPN is known to inhibit lysyl oxidases by binding the active site of the catalytic domain (Pinnell and Martin 1968; Tang et al. 1983). It has been reported that gastrulation of sea urchin embryos is arrested in the presence of BAPN (Butler et al. 1987; Wessel and McClay 1987). In order to examine the interaction of HpArs with collagen-like molecules, we examined the effect of BAPN on distribution of HpArs in the larvae. We compared normal prism larvae (Fig. 3a) with BAPN-treated larvae (Fig. 3b) that had developed for the same length of time but had failed to gastrulate as reported before (Wessel and McClay 1987). Accumulation of HpArs protein in the larvae treated with BAPN was almost completely repressed (Fig. 3c). Although the expression of *HpArs* decreased apparently, significant level of *HpArs*-mRNA was observed in the BAPN-treated larvae (Fig. 3d). Deposition of the fucose-rich sulfated polysaccharide which mainly exists on the apical surface of *H. pulcherrimus* embryos (Akasaka and Terayama 1983) was also inhibited by the treatment with BAPN (Fig. 3e). The western blot analysis revealed that release of the HpArs proteins from the larvae into the surrounding sea water was stimulated in the presence of BAPN (Fig. 3f). It is suggested that decrease in the amount of the HpArs protein in the larvae treated with BAPN is due to the acceleration of release of the HpArs protein from the

larvae as well as decrease in the expression of *HpArs*. These results indicate that deposition of Ars and fucose-rich sulfated polysaccharide on the surface of the embryos is dependent on the lysine-derived crosslinks in fibrillar proteins.

Table 1 Inhibition of archenteron invagination by injection of HpArs-MO

HpArs-MO (×10^8 molecules/egg)	Extent of invagination			
	No invagination	<1/2 (Percentage of embryos)	3/4	Full
0.7	0	2.4	1.6	96
1.3	1.7	8.4	3.9	86
2.6	0.8	17	5.9	76
3.6	4.0	36	4.0	56
5.1	10	49	0	41
7.2	18	67	3.6	11

One hundred fifty embryos injected at each of the different levels were scored at the late gastrula stage (28 h after fertilization at 16°C)
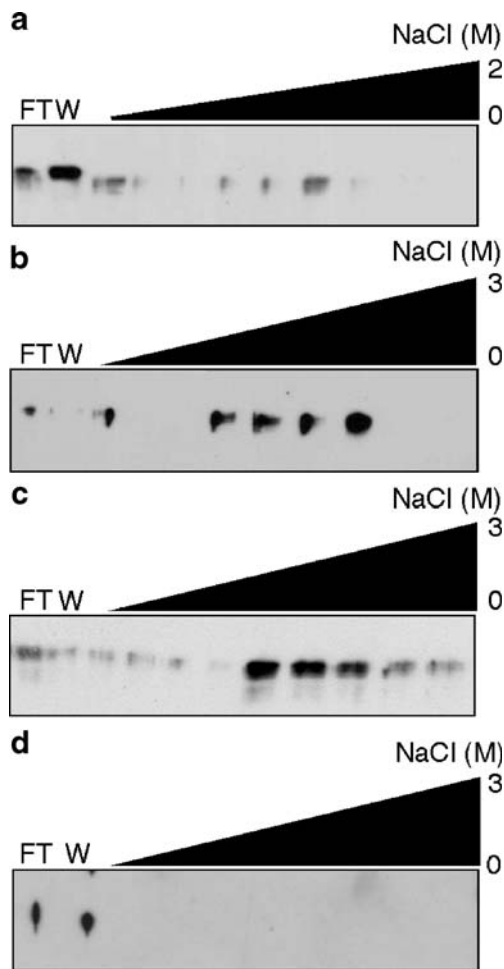

Fig. 2 HpArs binds to heparin-Sepharose. Two micrograms of purified HpArs protein was mixed with 1 ml of the Sepharose equilibrated with 10 mM Tris–HCl (pH 7). The slurry of the Sepharose was poured into the column and was eluted with a linear gradient of 0–3 M NaCl in the buffer. The aliquot of each fraction was analyzed by immunoblot analysis as described in "Materials and methods". All procedures were performed in the absence of divalent cation (**a**); Ca^{2+} was added to the reaction mixture at 9 mM after mixing HpArs with heparin-Sepharose (**b**); Ca^{2+} and Mg^{2+} were added to the reaction mixture at 9 mM and 50 mM, respectively, after mixing HpArs with heparin-Sepharose (**c**), or Sepharose CL-6B (**d**). *FT* flow-through fraction, *W* washing fraction

Discussion

Function of Ars as a component of extracellular matrix in sea urchin development

It has been reported that some of the enzymes have non-enzymatic functions. For example, a mammalian β-1,4-galactosyl transferase located on the surface of the sperm is known to have a binding activity to the egg zona pellucida (Miller et al. 1992). We previously showed that the non-enzymatic HpArs protein exists on the apical surface of epithelial cells of sea urchin (*H. pulcherrimus*) embryos and larvae (Mitsunaga-Nakatsubo et al. 1998). Accumulation of HpArs protein begins just immediately before the onset of dynamic morphogenetic movement (Sasaki et al. 1988; Mitsunaga-Nakatsubo et al. 1998). In this paper, we showed that inhibition of HpArs protein synthesis by injecting embryos with *HpArs*-MO resulted in the suppression of spreading of the epithelial cells and gastrulation. After the prism stage, an abundant accumulation of HpArs protein is prominent on the apical surface of aboral ectoderm, a location where the epithelial cells spread to a great extent (Mitsunaga-Nakatsubo et al. 1998), whereas the expression of HpArs ceases in the oral ectoderm where the epithelial cells remain cylindrical. It has been reported that the gastrulation involves two major kinds of cell movement: the movement of cells as a sheet and the migration of cells as individuals (Ettensohn 1999). During gastrulation, ectodermal cells begin to expand and become thinner as gastrulation proceeds, and the cells near the blastopore are pulled into the base of the archenteron (Wessel and Wikramanayake 1999; Kominami and Takata 2004; McClay et al. 2004). The rearrangement of epithelial cells is required for the invagination of sea urchin embryos (Ettensohn 1985, Burke et al. 1991). In addition, Kominami and Takata suggested that the blastocoele wall: ectoderm plays an important role in progressing the bending of the vegetal plate and exerts the driving force for gastrulation (Kominami and Takata 2000, 2004; Takata and Kominami
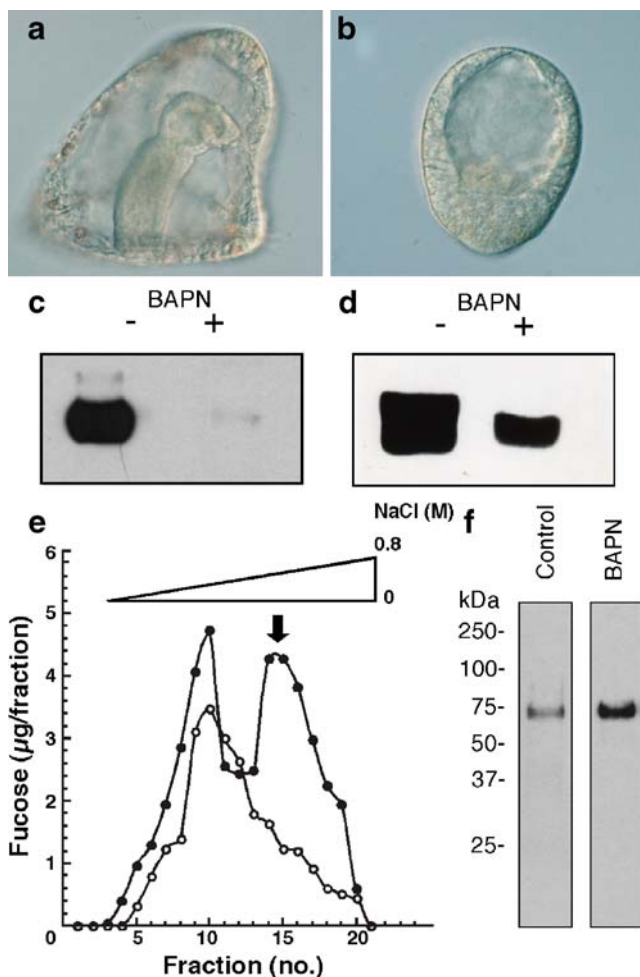
Fig. 3 Effect of BAPN on morphogenesis and the expression of HpArs in sea urchin larvae. **a** Control larva at 42-h post-fertilization. **b** Larva cultured in the presence of 0.39 mM BAPN. **c** Immunoblot analysis of HpArs protein. The aliquot of the protein corresponding to 50 larvae were analyzed by immunoblotting using affinity purified anti-HpArs antibodies as described in "Materials and methods". **d** Northern blot analysis of *HpArs*-mRNA extracted from the control larvae and the larvae cultured with 0.39 mM BAPN. **e** DEAE-cellulose chromatography of polysaccharide fractions. The polysaccharide fractions were prepared from control and the larvae treated with 0.39 mM BAPN at 42-h post-fertilization and the content of fucose was determined by the procedure described in "Materials and methods". *Closed circles* control larva, *open circles* larva treated with BAPN. The *upper inset* indicates the NaCl gradient. The 0.6 M fraction indicated by *filled arrow* mainly accumulates on the apical surface of the sea urchin embryos (*H. pulcherrimus*). **f** HpArs proteins in the sea water fraction released from the larvae. Two milliliters of larvae suspension (1.3×10^5) was incubated in a 50 ml polypropylene tube at 16°C for 5 h in the presence or absence of 0.78 mM BAPN. The aliquot of the released fraction of HpArs (corresponding to 7.5×10^2 larvae) were concentrated by precipitating with 90% ethanol and analyzed by immunoblotting using affinity purified anti-HpArs antibodies as described in "Materials and methods"

2001). We suggest that the HpArs protein accumulated in the apical surface of the epithelial cells is involved in expansion and the migration of the epithelial cells in sea urchin embryos.

Binding activity of HpArs is Ca²⁺-dependent

Ca^{2+} is indispensable for the binding activity of HpArs to heparin; however, if the HpArs is exposed to Ca^{2+} before mixing with heparin, the HpArs loses the binding activity. It has been reported that the Ars are the lysosomal enzymes and that deficiency of the Ars results in the lysosomal storage of sulfated polysaccharides (Evers et al. 1996), indicating co-localization of Ars with sulfated polysaccharides in the vesicles of cytoplasm. We propose that the binding domain of the HpArs interacts with sulfated polysaccharides weakly in the vesicles where the concentration of free Ca^{2+} must be kept at low level. When the mixture of HpArs and sulfated polysaccharides are secreted to the cell surface, high concentration of Ca^{2+} presents in the sea water may change the conformation of the binding domain of HpArs and the HpArs protein binds to sulfated polysaccharides tightly. To date, crystallographic and mutagenesis studies have solved the structures of three kinds of Arses from humans, as well as the Ars from the gram-negative bacterium *Pseudomonas aeruginosa*, revealing that a divalent cation, Ca^{2+} or Mg^{2+} is located at the base of a substrate (sulfate)-binding pocket. Each metal ion is thought to play a role in stabilizing the Ars-sulfate complex in the active site (Hanson et al. 2004; Ghosh 2007). Amino acid sequence homology among the Ars sulfatase family suggests that HpArs also has similar overall folds and divalent metal ion-binding sites. Preceding conformational changes of HpArs induced by Ca^{2+} may disturb the access of the binding domain of the Ars to the polysaccharides, although other possibilities cannot be excluded.

Deposition of HpArs and fucose-rich sulfated polysaccharide on the apical surface of the larvae is dependent on lysine-derived crosslinks in fibrillar proteins

The high affinity binding of HpArs protein to heparin is consistent with the co-localization of HpArs and fucose-rich sulfated polysaccharides which are abundant on the apical surface of sea urchin *H. pulcherrimus* embryos (Mitsunaga-Nakatsubo et al. 1998; Akasaka and Terayama 1983). We showed in this study that accumulation of HpArs and the fucose-rich sulfated polysaccharide on the apical surface of the larvae was inhibited by BAPN, an inhibitor of protein crosslinking. We also demonstrated that the Ars proteins are released from the embryos into the sea water in the presence of BAPN. These results indicate that deposition of Ars and fucose-rich sulfated polysaccharides on the surface of the larvae is dependent on the lysine-derived crosslinks in fibrillar proteins. Presence of collagen-like fibrils on the apical surface of sea urchin embryos was demonstrated by using electron microscopy (Spiegel and

Spiegel 1979). Immunohistochemistry using polyclonal antibodies to vertebrate collagen type I and type IV showed that collagen-like proteins accumulate in the apical region of the epithelium in sea urchin embryos and that the accumulation of collagen-like protein is reduced by treatment of embryos with BAPN (Wessel and McClay 1987). These results suggest that HpArs and fucose-rich sulfated polysaccharide may be deposited on the apical surface of sea urchin larvae via collagen-like molecules in the same manner as mammalian proteoglycans bind to collagen molecules (Scott and Orford 1981; Delehedde et al. 2001), although we have not identified the proteins. Hyalin (Adelson and Humphreys 1988; Wessel et al. 1998), echinonectin (Alliegro et al. 1988), fibropellin (Bisgrove and Raff 1993), and a 350 kDa antigen (Coffman and McClay 1990) have been reported as constituents of the apical extracellular matrix of sea urchin embryos. We suggest that the HpArs, as another component of extracellular matrix, forms insoluble complex with sulfated polysaccharides and collagen-like molecules at the apical surface of the epithelial cells on which the cells spread and migrate. The viewpoint that Ars is a component of the extracellular matrix could be very useful for the analysis of interactions of these proteins in the extracellular matrix, upon which the epithelial cells migrate and spread.

The expression of *HpArs*-mRNA was slightly repressed in the larvae injected with *HpArs*-MO (Fig. 1f). In the larvae treated with BAPN, an apparent repression of expression of the *HpArs* was observed (Fig. 3d). As we have demonstrated in the present study, the accumulation of the HpArs proteins is repressed in the larvae injected with *HpArs*-MO and the deposition of sulfated polysaccharides in addition to HpArs proteins on the apical surface of the larvae is inhibited in the presence of BAPN. It has been reported that the expression of aboral ectoderm specific *LpS1* (Wessel et al. 1989) was repressed in the sea urchin embryos treated with BAPN and that *Spec1* (Benson et al. 1991), which is also expressed in the aboral ectoderm of sea urchin embryos, was repressed when collagen and proteoglycans metabolism is disrupted. The literatures suggest that the extracellular matrix is responsible for their expression in the aboral ectoderm. The expression of *HpArs* may depend on the extracellular matrix such as collagen, sulfated polysaccharides, and HpArs.

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