Occurrence of Heparin in the Invertebrate Styela plicata (Tunicata)
Is Restricted to Cell Layers Facing the Outside Environment

AN ANCIENT ROLE IN DEFENSE?*

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Heparin is an intracellular product of vertebrate mast cell currently used as exogenous anticoagulant. Despite the potent biological activities of exogenous heparin, its physiological function has not been clearly established yet. Here, a heparin with similar structure and anticoagulant properties to the mammalian counterpart was shown to occur as the intracellular product of test cells, a cell monolayer that surrounds egg of the invertebrate Styela plicata (Chordata-Tunicata). As in the case of mammalian mast cells, heparin from the ascidian test cells is removed from the intracellular granules after incubation with compound 48/80. Following fertilization, the test cells surrounding the developing larva still retain heparin as metachromatic granulation. In the adult invertebrate, heparin occurs as intracellular granules at the apical tip of epithelial cells surrounding the lumen of both intestine and pharynx, in close contact with the external environment. This is the first description of the presence of heparin in cytoplasmic granules of epithelial-like cells around the lumen of sites exposed to external agents. This arrangement may reflect the participation of heparin in defense mechanisms in this invertebrate.

Heparin is a highly sulfated glycosaminoglycan composed by disaccharide repeats of hexuronic acid (α-L-iduronic acid or β-D-glucuronic acid) linked 1,4 to α-D-glucosamine. The heparin molecules are made up of a heterogeneous mixture of polymers with a similar backbone, which results from variations of sulfation on the D-glucosamine (N-acetylated, N-sulfated, O-sulfated at C6 and/or C3) and on the uronic acid residue (O-sulfated at C2) (1). So far, heparin has been found exclusively as an intracellular product in the secretory granules of mast cells, and is released only when mast cells degranulate in response to extracellular signals (2–4).

Because of its unique binding to antithrombin, involving a specific pentasaccharide sequence containing a 3-O-sulfated glucosamine, heparin is endowed of a potent anticoagulant activity (1, 5). However, apparently, its physiological function is not to regulate blood coagulation. This function is mostly achieved by endothelial cell-derived heparan sulfate proteoglycan of the syndecan family (6). Recent publications suggest that the physiological roles of heparin are more likely related to the regulation of the activity and release of mast cell proteases (7, 8).

In some species of invertebrates, coagulation of blood fluid has some analogy to blood clotting in vertebrates, but involving totally different proteins (9–11). Coagulation prevents the loss of hemolymph from a wound and also immobilizes microorganisms that invade the body. In tunicates, however, hemolymph coagulation does not occur, and the prevention of body fluid loss and the defense against microorganism invasion involves aggregation of hemocytes (12, 13). The hemagglutinating activity of hemocytes is inhibited by heparin and is mediated by heparin in the 160-kDa membrane glycoprotein that binds heparin and various bacteria (14). However, no physiological role has been reported for heparin in invertebrates, besides the report of the occurrence of a heparin-like glycosaminoglycan (denoted as mactin) in some species of mollusk (15).

A possible clue concerning the physiological roles of heparin has come from an apparently unrelated project. In the course of our extensive studies about sulfated polysaccharides from different species of ascidians (16–21), we found that one of the species possesses high amounts of heparin. In contrast to the mammalian and mollusk counterparts, heparin occurs in the ascidian as the intracellular product of cell monolayer that surrounds egg and the lumen of both intestine and pharynx of the invertebrate Styela plicata (Chordata-Tunicata). This arrangement of heparin-rich cells may reflect the participation of heparin in defense mechanisms in this invertebrate.

EXPERIMENTAL PROCEDURES

Animals and Preparation of the Ascidian Eggs—Adult specimens of S. plicata were collected at Guanabara Bay, Rio de Janeiro, Brazil. Animals were maintained in an aerated aquarium at 20 °C until use. The gonads of several ascidians were carefully separated from other tissues, under magnifying lenses, and the eggs isolated by filtering the gonads several times through a 0.5-mm-diameter net in sea water (pH
The dried eggs (1 g) were suspended in 20 ml of 0.1M sodium acetate buffer (pH 5.5), containing 100 mg of papain, 5 mM EDTA, and 5 mM cysteine and incubated at 60 °C during 24 h. The incubation mixture were then centrifuged (2000 × g for 10 min at room temperature) and another 100 mg of papain in 20 ml of the same buffer, containing 5 mM EDTA and 5 mM cysteine was added to the precipitate and incubated for another 24 h. The clear supernatant from the two extractions were combined and the polysaccharides precipitated with two volumes of 95% ethanol and maintained at 4 °C for 24 h. The precipitates formed by heating and centrifugation were collected by centrifugation (2000 × g for 10 min at room temperature), freeze dried and dissolved in 2 ml of distilled water (“pool 1”). The preponderent sulfated polysaccharides obtained after these procedures have a non-glycosaminoglycans structure (23). In order to solubilize heparin, it is necessary to repeat the extraction procedure with papain two more times (“pool 2”).

**Fractionation of the Invertebrate Heparin**—The glycosaminoglycans (~20 mg) obtained from the third and fourth fractions with papain (pool 2, see above) were applied to a Mono Q-FPLC column, equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The glycosaminoglycans were eluted by a linear gradient of 0–2.0 mM NaCl (10 ml) at a flow rate of 0.45 ml/min. Fractions of 0.5 ml were collected and checked by metachromasia assay using 1.9-dimethyl methylene blue (24). The fractions containing heparin were pooled, dialyzed against distilled water, and lyophilized.

**Enzymatic Treatments with Heparan Sulfate and Heparin Lyases**—Ascidian heparin and standard mammalian heparin (50 μg as dry weight of each) were incubated with 0.005 unit of either heparan sulfate-lyase or heparin-lyase (25) in 100 μl of 100 mM sodium acetate buffer (pH 7.0), containing 10 mM calcium acetate for 17 h at 37 °C. At the end of the incubation period, the mixtures were analyzed by agarose gel electrophoresis (26) in order to determine digestion of the glycosaminoglycan with heparan sulfate- or heparin-lyase.

In order to determine the disaccharide composition, the ascidian and mammalian heparins were incubated simultaneously with both heparin- and heparan sulfate-lyases, as described in the previous paragraph. Disaccharides were recovered by a Superdex peptide column (Amersham Pharmacia Biotech) linked to a HPLC system from Shimadzu (Tokyo, Japan). The column was eluted with distilled water:acetonitrile:triﬂuoroacetic acid (80:20:0.1, v/v) at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and monitored for UV absorbance at 232 nm. Fractions corresponding to disaccharides (>90% of the degraded material) were pooled, freeze-dried, and stored at −20 °C. This disaccharide preparation and standard compounds were subjected to a SAX-HPLC analytical column (250 × 4.6 mm, Sigma-Aldrich), as follows. After equilibration in the mobile phase (distilled water adjusted to pH 3.5 with HCl) at 0.5 ml/min, samples were injected and disaccharides eluted with a linear gradient of NaCl from 0 to 1.0 M over 45 min in the same mobile phase. The eluant was collected in 0.5-ml fractions and monitored for UV absorbance at 232 nm for comparison with lyase-derived disaccharide standards.

**NMR Spectroscopy**—1H and 13C spectra were recorded using a
Heparin in Invertebrate

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Invertebrate and mammalian heparins were dissolved in 0.5 ml of 99.9% D$_2$O (CIL). All spectra were recorded at 60 °C with HOD suppression by presaturation. COSY, TOCSY, and $^1$H/$^13$C heteronuclear correlation (HMQC) spectra were recorded using states-time incremented phase incrementation for quadrature detection in the indirect dimension. TOC SY spectra were run with 4,096 × 400 points with a spin-lock field of about 10 kHz and a mixing time of 80 ms. HMQC were run with 1,024 × 256 points and globally optimized alternating phase rectangular pulses for decoupling. Nuclear Overhauser effect spectroscopy spectra were run with a mixing time of 100 ms. All chemical shifts were relative to external trimethylsilylpropanionic acid and $^{13}$C-labeled methanol.

Anticoagulant Action Measured by Activated Partial Thromboplastin Time (APTT)—Activated partial thromboplastin clotting time assays were carried out as follows: normal human plasma (100 μl) was incubated with 10 μl of a solution of heparin (0.01–1 μg) at 37 °C for 1 min. Then 100 μl of APTT reagent (Celite -Biolab) were added and incubated at 37 °C. After 2 min of incubation, 100 μl of 0.25 mM CaCl$_2$ were added to the mixtures and the clotting time recorded in a coagulometer (Amelung KC4A).

Inhibition of Thrombin by Antithrombin or Heparin Cofactor II in the Presence of Heparin—These effects were evaluated by the assay of antithrombin activity of thrombin using chromogenic substrate, as described (21). Incubations were performed in disposable UV semi-microcuvettes. The final concentrations of reactants included 50 nM antithrombin or 68 nM heparin cofactor II, 15 nM thrombin, and 0–10 μM heparin in 100 μl of 0.05 M Tris/HC1, 0.15 M NaCl, and 1.0 mg/ml polystyrene glycol (pH 7.4) (TS/PEG buffer). Thrombin was added last to initiate the reaction. After a 60-s incubation at room temperature, 500 μl of 100 μM chromogenic substrate S-2238 (Chromogenix AB, Molndal, Sweden) in TS/PEG buffer was added and the absorbance at 405 nm was recorded for 100 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with antithrombin or heparin cofactor II in the absence of heparin. Nor did inhibition occur when thrombin was incubated with heparin alone over the range of concentrations tested.

Degranulation Induced by Compound 48/50—Ascidian eggs surrounded by test cells (1 × 10$^6$ cells/ml) were incubated with 0.2 mM compound 48/50 (27) in filtered sea water (pH 8.0) for 15 min at 37 °C, centrifuged at 500 × g for 10 min and staining with 1,9-dimethylmethylen blue for 5 min at room temperature. After degranulation, the cells were immediately examined under microscope (Olympus, BH-2).

RESULTS AND DISCUSSION

The Eggs from the Ascidian S. plicata Contain a Heparin-like Glycosaminoglycan—The sulfated polysaccharide (pool 2) extracted from the eggs of the ascidian S. plicata with protease digestion eluted from a Mono Q-FPLC column as a symmetric peak at 1.5 mM NaCl, as mammalian heparin (Fig. 1A). The invertebrate glycosaminoglycan is not degraded by heparan sulfate lyase, but completely disappears from the gel after degradation with heparin lyase (Fig. 1B).

Analysis of the disaccharides produced by digestion with heparin lyase on a Spherisorb SAX-HPLC, reveals that the invertebrate glycosaminoglycan yields mainly the disaccharide ΔUA(2SO$_4$)-1→4-β-D-GlcN(SO$_4$)-6(SO$_4$), originated from the disulfated units (Fig. 2 and Table I). The disaccharide ΔUA-1→4-β-D-GlcN(SO$_4$)-6(SO$_4$) was also detected (−30% of the total products). This disaccharide composition is close to those reported for heparin preparation from mammalian tissues and by far more sulfated than heparan sulfate samples (4).

NMR Analysis Confirm the Similarity between Ascidian and Mammalian Heparins—The $^1$H one-dimensional NMR spectra of the invertebrate and mammalian heparins (Fig. 3) and interpretations of $^1$H/$^13$C HMQC (Fig. 4), TOCSY (Fig. 5), and COSY (data not shown) confirm the similarity between these two glycosaminoglycans. Thus, one-dimensional $^1$H NMR spectra show two main anomic resonances at 5.39 and 5.19 ppm, assigned as α-D-glucosamine and α-L-iduronic acid residues, respectively (Fig. 3A). Two additional anomic protons at 5.29 and 4.96 ppm are also present in a 1:1 ratio in the spectrum of the ascidian heparin (Fig. 3B). These anomic signals are

![Table I: Disaccharide composition of the ascidian and mammalian heparins](http://www.jbc.org/)

<table>
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<tr>
<th>Peak number*</th>
<th>Disaccharide</th>
<th>$t_r$*</th>
<th>Proportion of the disaccharides*</th>
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<td></td>
<td></td>
<td>Ascidian heparin</td>
</tr>
<tr>
<td>1</td>
<td>ΔUA-1→4-GlcN(SO$_4$)</td>
<td>21.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>ΔUA-1→4-GlcN(6SO$_4$)</td>
<td>22.8</td>
<td>&lt;1</td>
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<tr>
<td>3</td>
<td>ΔUA-1→4-GlcN(SO$_4$(6SO$_4$</td>
<td>29.3</td>
<td>25.0</td>
</tr>
<tr>
<td>4</td>
<td>ΔUA(2SO$_4$)-1→4-GlcN(SO$_4$(6SO$_4$</td>
<td>36.2</td>
<td>75.0</td>
</tr>
</tbody>
</table>

* Standard peak number in order of elution from a Spherisorb-SAX column (see Fig. 2).

* Retention time of the disaccharides on a Spherisorb-SAX column connected to a HPLC system.

* The areas under the disaccharide peaks eluted from the HPLC column were integrated to obtain the disaccharide proportions.
easily recognized by the $^{1}H/^{13}C$ HMQC spectrum (Fig. 4).

The $^{1}H$ and $^{13}C$ chemical shifts of the four types of residues found in ascidian heparin are presented in Tables II and III, respectively, and are based on interpretations of TOCSY (Fig. 5), COSY (data not shown), and $^{1}H/^{13}C$ HMQC (Fig. 4). All four spin systems could be traced in the TOCSY and COSY spectra, except for H5 and H6 of the $\alpha$-D-glucosamine. Due to overlaps, the connectivity was not seen in the case of these residues. The assignment was done based on comparison with mammalian heparin. The values obtained are in agreement with a sulfated H6, as expected from the analysis of the disaccharides formed by digestion with heparan sulfate $^{1}$heparin lyases (Fig. 2 and Table I).

Based on literature data, we concluded that the two extra spin systems in the ascidian heparin correspond to a disaccharide unit in which $\alpha$-D-glucosamine is linked to a non-sulfated $\alpha$-L-iduronic acid unit (Tables II and III). Glucosamine residues linked to either 2-O-sulfated or non-sulfated $\alpha$-L-iduronic acid units have approximately the same chemical shifts for H2, H3, H5, and H6 (see TOCSY spectrum in Fig. 5), but differ in the chemical shifts of H1 and H4. The $\alpha$-L-iduronic acid residues from the two types of disaccharide units have the same chemical shifts for H4 and H5, but the H2 is 0.76 ppm downfield in the 2-O-sulfated unit, as expected.

The integrals of the four anomeric resonances in the $^{1}H$ spectrum of ascidian heparin (Fig. 3B, inset) suggests a 7:3 ratio of disaccharide units containing 2-O-sulfated and non-sulfated $\alpha$-L-iduronic acid residues. The anomeric resonance of non-sulfated iduronic acid unit can be seen in the $^{1}H$ spectrum of mammalian heparin (Fig. 3A), but the signal intensity is much smaller than in ascidian heparin. The corresponding glucosamine anomeric resonance appears as a shoulder in the predominant unit.

The NMR spectra are in agreement with the analysis of the

![FIG. 3. $^{1}H$ spectra at 600 MHz of mammalian (A) and invertebrate (B) heparins. The glycosaminoglycan samples (~5 mg) were dissolved in approximately 0.7% D$_2$O and the spectra recorded at 60 °C with suppression of HOD signal by presaturation. Expansion of the 4.9–5.3 ppm region of the spectrum of invertebrate heparin is shown in the inset of panel B. Signals designated as a and b refer to those produced by 2-O-sulfated and unsulfated $\alpha$-L-iduronic acid residues of invertebrate heparin. The integrals listed under the region of anomeric protons were normalized to the total Glc-H1 intensity (residues a and b).](http://www.jbc.org/)

![FIG. 4. $^{1}H/^{13}C$ HMQC spectrum of the ascidian heparin. The assignment was based on TOCSY and COSY spectra except for H6, which was based on comparison with mammalian heparin. A and I refer to $\alpha$-D-glucosamine and $\alpha$-L-iduronic acid residues, respectively, from [4-$\alpha$-L-IdoA-2(SO$_4$)$_3$]-4-$\alpha$-D-GlcN(SO$_4$)$_2$-6(SO$_4$)-1] units while A$'$ and I$'$ refer to similar residues but from the disaccharide [4-$\alpha$-L-IdoA-1-4-$\alpha$-D-GlcN(SO$_4$)$_2$-6(SO$_4$)-1]. The values of $^{1}H$ and $^{13}C$ chemical shifts are presented in Tables II and III, respectively, and are relative to external trimethylsilylpropionic acid at 0 ppm for $^{1}H$ and to methanol for $^{13}C$. The spectrum was acquired with 1024 $\times$ 200 points and 512 scans.](http://www.jbc.org/)

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disaccharides produced by heparin lyase digestion, since both confirm the occurrence of 30% of non-sulfated \(a\)-L-iduronic acid units in the invertebrate heparin, which are undetected in the mammalian heparin standard we used.\(^2\)

**Anticoagulant Activity of the Ascidian Heparin**—The invertebrate heparin has an anticoagulant activity of 19 units/mg, as determined by the APTT assay using a parallel standard curve based on Standard Heparin (193 units/mg), 4th International Standard (Fig. 6a). The \(I_{C_{50}}\) for thrombin inhibition in the presence of antithrombin is 0.01 and 0.0005 \(\mu\)g/ml for the invertebrate and mammalian heparins, respectively (Fig. 6b). When antithrombin is replaced by heparin cofactor II, both heparins have approximately the same \(I_{C_{50}}\) for thrombin inhibition (Fig. 6c). These coagulation assays demonstrate that the invertebrate heparin has a lower anticoagulant activity than the mammalian standard. Lower activity may be related to the relative paucity of the high affinity pentasaccharide (28, 29) in the invertebrate heparin. The heparin cofactor II activity, on the other hand, does not show significant variation, as expected, since the activation of heparin cofactor II by heparin is charge-dependent and does not involve a specific structure sequence (30). Overall, these experiments demonstrated the

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**TABLE II**

Proton chemical shifts for residues of \(a\)-L-iduronate and of \(a\)-D-glucosamine in ascidian and mammalian heparins

<table>
<thead>
<tr>
<th>Unit</th>
<th>Proton(^a)</th>
<th>Ascidian heparin</th>
<th>Mammalian heparin</th>
<th>Literature values</th>
</tr>
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<tbody>
<tr>
<td>([4-a-L-IdoA-(2SO_4)-a-D-GlcNSO_4(6SO_4)-1])</td>
<td>I1</td>
<td>5.22</td>
<td>5.19</td>
<td>5.2</td>
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<tr>
<td></td>
<td>I2</td>
<td><strong>4.33</strong></td>
<td>4.34</td>
<td><strong>4.4</strong></td>
</tr>
<tr>
<td></td>
<td>I3</td>
<td>4.20</td>
<td>4.2</td>
<td>4.2</td>
</tr>
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<td>4.11</td>
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<td></td>
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</tr>
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\(^a\) Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicate positions bearing sulfate ester.

\(^b\) Protons designated as "I" refer to those of \(a\)-L-iduronic acid residues, whereas those of \(a\)-D-glucosamine are designated as "A."

\(^c\) See Ref. 36.

\(^d\) See Ref. 37.

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The biosynthesis of mammalian heparin, the 2-O-sulfation occurs first and then the 6-O-sulfotransferase will act on IdooA(2SO_4)-GlcN(SO_4) residues (34, 35). At the end, the major disaccharide unit of heparin is IdooA(2SO_4)-GlcN(SO_4)(6SO_4). However, if some regulatory mechanism led to the 6-O-sulfation to occur first, the 2-O-sulfotransferase cannot act and the end product is IdooA-GlcN(SO_4)(6SO_4). During the biosynthesis of the invertebrate heparin, this later mechanism is more relevant than in mammalian tissues.

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\(^2\) During the biosynthesis of mammalian heparin, the 2-O-sulfation occurs first and then the 6-O-sulfotransferase will act on IdooA(2SO_4)-GlcN(SO_4) residues (34, 35). At the end, the major disaccharide unit of heparin is IdooA(2SO_4)-GlcN(SO_4)(6SO_4). However, if some regulatory mechanism led to the 6-O-sulfation to occur first, the 2-O-sulfotransferase cannot act and the end product is IdooA-GlcN(SO_4)(6SO_4). During the biosynthesis of the invertebrate heparin, this later mechanism is more relevant than in mammalian tissues.
parallel between the anticoagulant action of heparin extracted from the ascidian eggs and standard heparin preparation from mammalian tissue.

Ascidian Heparin Is Located in the Test Cells—We attempted to identify the cell type in the ascidian eggs that contains heparin. In *S. plicata*, the oocytes are surrounded by somatic test cells that reside at the surface of zygotes during development. The function of these cells during embryogenesis is unknown (31). Histochemical analysis of the ascidian eggs, using the cationic dye 1,9-dimethylmethylene blue (24), revealed a strong metachromatic staining associated with intracellular granules in the test cells around the oocyte (Fig. 7), suggesting the occurrence of heparin. Immunofluorescence staining with anti-heparin monoclonal antibody ST-1 confirmed that antibody labeling was restricted to the test cells (Fig. 7, b–e).3 No specific labeling was detected in the oocyte. Following fertilization, the test cells continue to surround the developing larvae, and still retain the metachromasia (data not shown). Thus, the presence of heparin in these cells is not related with the fertilization process. A rather interesting aspect of the test cells, which may reflect their function, is the fact that compound 48/80, a potent stimulator of mast cell degranulation, also promote degranulation of test cells (Fig. 8), as suggested by the loss of metachromasia of the test cells after incubation with 48/80. Preliminary experiments, in which tryptase activity was measured in the supernatants of pure preparations of test cells after degranulation with compound 48/80, indicated that degranulation is associated with protease release (data not shown). However, future experiments are required to further address this issue.

Adult Ascidians Still Retain Heparin—Next, we investigated whether the tissues of the adult ascidian still retain heparin. Sulfated glycosaminoglycans were extracted from the adult invertebrate by protease digestion and purified by anion ex-

Table III

Carbon chemical shifts for residues of α-L-iduronate and of α-D-glucosamine in ascidian and mammalian heparins

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3 Histamine was also detected in the ascidian test cells using anti-histamine monoclonal antibody and the enzyme histamine N-methyltransferase.
change chromatography. Again, we detected heparin eluted from Mono Q-FPLC column at 1.5 M NaCl, but, in contrast to the ascidian eggs, we also detected dermatan sulfate in adult tissues. Heparin from the adult invertebrate produces the same types and proportions of disaccharides after digestion with heparin lyase (data not shown), indicating that both adult and egg heparin have the same structure.

In order to investigate the localization of heparin in the adult invertebrate, we stained sections of intestine and pharynx with 1,9-dimethylmethylene blue and with anti-heparin monoclonal antibody (Fig. 9). A strong metachromatic staining is associated with cytoplasmic granules at the apical tip of epithelial cells, surrounding the lumen of both intestine (Fig. 9a) and pharynx (Fig. 9d), in close contact with the external environment of the animal. The metachromatic staining co-localizes with anti-heparin immune staining in both intestine (Fig. 9b) and pharynx (Fig. 9e). This is a similar arrangement as observed in the test cells, surrounding the oocyte (Fig. 7). The metachromatic staining of the cells resists incubation with chondroitin ABC lyase, excluding the occurrence of high amounts of dermatan and chondroitin sulfate in these cells (data not shown).

Does the Distribution of Heparin in Ascidian Indicate an Ancient Role in Defense?—This is the first report of the occurrence of heparin in cytoplasmic granules of epithelial-like cells around the lumen of sites exposed to the exterior. A glycosaminoglycan similar to heparin was described previously in invertebrates (mostly in species of mollusk) and denoted as "mactin" (15). However, in this case, heparin occurs again as the intracellular product of connective tissue cell similar to mast cells, with a diffuse distribution as in vertebrates (32). We can speculate that the arrangement of heparin-containing cells around the lumen of sites exposed to the exterior may reflect the participation of heparin in defense mechanisms in the invertebrate. In ascidians, the defense against microorganism invasion and the prevention of body fluid loss involves aggregation of hemocytes that migrate from the hemolymph. The hemagglutinating activity is inhibited by heparin and is mediated by hemagglutinin, a 160-kDa membrane glycoprotein that

FIG. 7. Localization of heparin in the ascidian eggs. a, metachromatic staining of cytoplasmic granules in the test cells with 1,9-dimethylmethylene blue. Immunofluorescence labeling of test cells with anti-heparin monoclonal antibody (c and e) and control without the primary anti-heparin antibody (b and d). Bars, 50 μm.

FIG. 8. Degranulation of the test cell-heparin-containing granules with compound 48/80. The test cell-containing ascidian eggs were incubated with compound 48/80, as described under "Experimental Procedures." After incubation, the cells were stained with 1,9-dimethylmethylene blue and immediately examined under microscope. A, control, ascidian eggs without treatment with compound 48/80. B, eggs treated with compound 48/80. Bars, 50 μm.

FIG. 9. Histological sections from the intestine and pharynx of S. plicata stained with 1,9-dimethylmethylene blue and immunostained and with anti-heparin monoclonal antibody ST-1. Sections from intestine (a–c) and pharynx (d–f) were stained with the cationic dye 1,9-dimethylmethylene blue (a and d) or treated with anti-heparin monoclonal antibody ST-1 (b and e), as described under "Experimental Procedures." c and f, show controls without anti-heparin monoclonal antibody ST-1. L, lumen; E, exterior. Bars, 50 μm.
bonds heparin and various bacteria (33). Perhaps, heparin released from the epithelial cells act as a regulator of the hem-agglutinating activity of hemocytes during microorganism invasion.

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REFERENCES
Occurrence of Heparin in the Invertebrate *Styela plicata* (Tunicata) Is Restricted to Cell Layers Facing the Outside Environment: AN ANCIENT ROLE IN DEFENSE?

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