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 of 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 he-magglutinin, a 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 mautcin) 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...
6.0). The eggs collected in the filtrate, free of contaminating tissues, were washed with 500 ml of filtered sea water (pH 6.0) and 500 ml of sulfate-free artificial sea water (pH 8.0). These procedures are important to remove contaminating tissues rich in sulfated polysaccharides, which may be present in preparations from ascidian eggs.

For histochemical preparations, the eggs were fixed in 5% formamide in sea water for 2 h at room temperature. After fixation, the eggs were washed with water, dehydrated in graded ethanol, cleared in xylol and embedded in Paraplast (melting point, 55.6 °C). Approximately 7-μm sections from the egg were cut longitudinally on a Spencer Microtome. Sections were stained with 0.05 M 1,9-dimethyl-methylene blue in acetic acid/ethanol/water (0.1:5.5, v/v).

**Extraction of the Sulfated Polysaccharides from the Ascidian Eggs**—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 mammalian heparins were incubated simultaneously with both heparan sulfate- and heparin-lyases, as described in the previous paragraph. Disaccharides were recovered by a Superose peptide column (Amer sham 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

Strong anion-exchange HPLC analysis of the disaccharides formed by heparin + heparan sulfate lyases. A mixture of disaccharide standards (A) and the disaccharides formed by exhaustive action of heparin + heparan sulfate lyases on the mammalian (B) and ascidian (C) heparins were applied to a 25 cm × 4.6-mm Spherisorb-SAX column, linked to a HPLC system. The column was eluted with a gradient of NaCl as described under "Experimental Procedures." The eluant was monitored for UV absorbance at 232 nm. The numbered peaks correspond to the elution positions of known disaccharide standards as follows. Peak 1, ΔUA-1→4-GlcN(SO₄)(6SO₄); peak 2, ΔUA-1→4-GlcNAc(6SO₄); peak 3, ΔUA-1→4-GlcN(SO₄)₂(6SO₄); peak 4, ΔUA/2SO₄)₂-1→4-GlcN(SO₄)₂(6SO₄); X, unidentified peak.

**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 asidian S. plicata with protease digestion eluted from a Mono Q-FPLC column as a symmetric peak at 1.5 m 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/2SO₄)₂-1→4-β-D-GlcN(SO₄)₂(6SO₄) originated from the disulfated units (Fig. 2 and Table I). The disaccharide ΔUA-1→4-β-D-GlcN(SO₄)₂(6SO₄) was also detected (<30% of the total products). This disaccharide composition is close to that 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 ¹H one-dimensional NMR spectra of the invertebrate and mammalian heparins (Fig. 3) and interpretations of ¹H/¹³C HMQC (Fig. 4), TOCSY (Fig. 5), and COSY (data not shown) confirm the similarity between these two glycosaminoglycans. Thus, one-dimensional ¹H NMR spectra show two main anomic resonances at 5.39 and 5.19 ppm, assigned as α-1-glucosamine and α-1-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**

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Disaccharide</th>
<th>t&lt;sub&gt;0&lt;/sub&gt;</th>
<th>Proportion of the disaccharides&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>minutes</td>
<td>Ascidian heparin</td>
</tr>
<tr>
<td>1</td>
<td>ΔUA-1→4-GlcN(SO₄)</td>
<td>21.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>ΔUA-1→4-GlcNAc(6SO₄)</td>
<td>22.8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3</td>
<td>ΔUA-1→4-GlcN(SO₄)₂(6SO₄)</td>
<td>29.3</td>
<td>25.0</td>
</tr>
<tr>
<td>4</td>
<td>ΔUA/2SO₄)₂-1→4-GlcN(SO₄)₂(6SO₄)</td>
<td>36.2</td>
<td>75.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard peak number in order of elution from a Spherisorb-SAX column (see Fig. 2).  
<sup>b</sup> Retention time of the disaccharide on a Spherisorb-SAX column connected to a HPLC system.  
<sup>c</sup> 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 corresponds 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 NMR spectra are in agreement with the analysis of the
disaccharides produced by heparin lyase digestion, since both confirm the occurrence of 30% of non-sulfated \( \alpha \)-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 \( I_{50} \) 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 IC\(_{50}\) 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

\(^2\) During the biosynthesis of mammalian heparin, the 2-O-sulfation occurs first and then the 6-O-sulfotransferase will act on Idoa(2SO\(_4\))-GlcN(SO\(_4\)) residues (34, 35). At the end, the major disaccharide unit of heparin is Idoa(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 Idoa-GlcN(SO\(_4\))(6SO\(_4\)). During the biosynthesis of the invertebrate heparin, this latter 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. 7a), 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-

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**TABLE III**

<table>
<thead>
<tr>
<th>Unit</th>
<th>Chemical shifts (ppm)</th>
<th>Literature values</th>
<th>Jaseja et al.</th>
<th>Mulloy et al.</th>
</tr>
</thead>
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<tr>
<td>[4-α-L-IdA-(2SO4)-α-D-GlcNSO4-(6SO4)-1]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I1</td>
<td>101.3</td>
<td>101.9</td>
<td>101.4</td>
<td>102.2</td>
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<tr>
<td>I2</td>
<td>77.7</td>
<td>78.7</td>
<td><strong>78.3</strong></td>
<td><strong>79.0</strong></td>
</tr>
<tr>
<td>I3</td>
<td>70.7</td>
<td>71.8</td>
<td>71.4</td>
<td>72.2</td>
</tr>
<tr>
<td>I4</td>
<td>78.0</td>
<td>78.5</td>
<td>78.2</td>
<td>79.1</td>
</tr>
<tr>
<td>I5</td>
<td>71.1</td>
<td>72.2</td>
<td>71.7</td>
<td>72.0</td>
</tr>
<tr>
<td>A1</td>
<td>98.9</td>
<td>98.9</td>
<td>98.5</td>
<td>99.6</td>
</tr>
<tr>
<td>A2</td>
<td>60.2</td>
<td>60.3</td>
<td>60.0</td>
<td>60.8</td>
</tr>
<tr>
<td>A3</td>
<td>72.0</td>
<td>72.0</td>
<td>71.9</td>
<td>72.9</td>
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<tr>
<td>A4</td>
<td>78.5</td>
<td>78.5</td>
<td>79.6</td>
<td>79.1</td>
</tr>
<tr>
<td>A5</td>
<td>72.8</td>
<td>71.6</td>
<td>71.3</td>
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</tr>
<tr>
<td>A6</td>
<td><strong>68.4</strong></td>
<td><strong>68.7</strong></td>
<td><strong>68.6</strong></td>
<td><strong>69.3</strong></td>
</tr>
</tbody>
</table>

---

*Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicate positions bearing sulfate ester. Protons designated as “I” refer to those of α-L-iduronic acid residues, whereas those of α-D-glucosamine are designated as “A.” See Ref. 36. See Ref. 37.*

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\[4-\alpha-L-IdA-\alpha-D-GlcNSO_4-(6SO_4)-1\]

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**FIG. 6.** Anticoagulant properties of mammalian and invertebrate heparin. a, the activated partial thromboplastin time values were determined in citrate-anticoagulated human plasma in the presence of increasing concentrations heparin. b and c, inhibition of thrombin activity by antithrombin or heparin cofactor II in the presence of heparin. Anti-thrombin (b) or heparin cofactor II (c) was incubated with thrombin (20 nm) in the presence of various concentrations of heparin. After 60 s, the remaining thrombin activity was determined with a chromogenic substrate (ΔA405/min). ○, mammalian heparin; ●, ascidian heparin. 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. 7a), 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 metachromatic granulation (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-
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 external agents. 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 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 hemagglutinating activity of hemocytes during microorganism invasion.

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