Sulfated Fucans from the Egg Jellies of the Closely Related Sea Urchins Strongylocentrotus droebachiensis and Strongylocentrotus pallidus Ensure Species-specific Fertilization*

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Sulfated polysaccharides from egg jelly are the molecules responsible for inducing the sperm acrosome reaction in sea urchins. This is an obligatory event for sperm binding to, and fusion with, the egg. The sulfated polysaccharides from sea urchins have simple, well-defined repeating structures, and each species represents a particular pattern of sulfate substitution. Here, we examined the egg jellies of the sea urchin sibling species Strongylocentrotus droebachiensis and Strongylocentrotus pallidus. Surprisingly, females of S. droebachiensis possess eggs containing one of two possible sulfated fucans, which differ in the extent of their 2-O-sulfation. Sulfated fucan I is mostly composed of a regular sequence of four residues ([4-α-L-Fucp-2(OSO₃)₂]-1-[4-α-L-Fucp-2(OSO₃)₂]-1-[4-α-L-Fucp-2(OSO₃)₂]-1-[4-α-L-Fucp-2(OSO₃)₂]-1) whereas sulfated fucan II is a homopolymer of 4-α-L-Fucp-2(OSO₃)₂-1 units. Females of S. pallidus contain a single sulfated fucan with the following repeating structure: [3-α-L-Fucp-2(OSO₃)₂]-1-[3-α-L-Fucp-2(OSO₃)₂]-1-[3-α-L-Fucp-4(OSO₃)₂]-1-[3-α-L-Fucp-4(OSO₃)₂]-1]. The egg jellies of these two species of sea urchins induce the acrosome reaction in homologous (but not heterologous) sperm. Therefore, the fine structure of the sulfated α-fucans from the egg jellies of S. pallidus and S. droebachiensis, which differ in their sulfation patterns and in the position of their glycosidic linkages, ensures species specificity of the sperm acrosome reaction and prevents interspecies crosses.

In addition, our observations allow a clear appreciation of the common structural features among the sulfated polysaccharides from sea urchin egg jelly and help to identify structures that confer finer species specificity of recognition in the acrosome reaction.

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EXPERIMENTAL PROCEDURES

Extraction—Mature females of *S. droebachiensis* and *S. pallidus* were collected near Friday Harbor, WA. Atlantic *S. droebachiensis* females were collected in Bergen, Tromsø, and Svalbard, Norway. Eggs were spawned into filtered sea water after intracelomic injection of 0.55 mm KCl. Egg jelly was isolated by pouring eggs repeatedly through nylon mesh, prepared as a 20,000 × g supernatant, and stored at −20 °C or lyophilized after dialysis against distilled water (8). The acidic polysaccharides were extracted from the jelly coat by papain digestion and partially purified by ethanol precipitation as described previously (11).

Purification—The crude polysaccharides (10 mg) from the egg jelly coats were applied to a Mono Q FPLC (HR5/5; Amersham Biosciences, Inc.) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with 10 ml of the same buffer and then eluted by a linear gradient of 0–4.0 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and fractions of 0.5 ml were collected. Fractions containing the sulfated α-L-fucan and the sialic acid glycoconjugate were pooled, dialyzed against distilled water, and lyophilized. SG indicates sialic acid-rich glycoconjugate.

**FIG. 2.** Purification of the sulfated α-fucans from sea urchin egg jelly by Mono Q FPLC. A mixed sample of sulfated α-fucans from 31 Pacific (A) and 19 Atlantic (B) *S. droebachiensis* females or from 25 Pacific *S. pallidus* females (C) was applied to a Mono Q FPLC column (HR5/5) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was developed by a linear gradient of 0–4.0 M NaCl in the same buffer. Fractions were assayed by metachromasia using 1,9-dimethylmethylene blue (○), the Dubois reaction for fucose (●), and the Ehrlich assay for sialic acid (▲). The NaCl concentration was estimated by conductivity (— —). Fractions containing the sulfated fucans were pooled, dialyzed against distilled water, and lyophilized. SG indicates sialic acid-rich glycoconjugate.
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**RESULTS AND DISCUSSION**

Egg Jelly of the Sea Urchin *S. droebachiensis* (but Not *S. pallidus*) Possesses Two Isoforms of Sulfated Fucans—Agarose gel electrophoresis in 1,3-diaminopropane acetate buffer followed by toluidine blue staining showed that egg jelly isolated from individual females of East Pacific *S. droebachiensis* contained either a slow (sulfated fucan I) or fast (sulfated fucan II) migrating fucan isoform (Fig. 1A). Of 22 individual females, 9 had eggs with sulfated fucan I, and 13 had eggs with sulfated fucan II. Surprisingly, nine individual females of the same species, but collected in the Atlantic Ocean, contained only the slow migrating sulfated fucan (isotype I) (Fig. 1B).

Small differences in the electrophoretic mobility of sulfated fucans I and II (Fig. 1, A and B) could reflect intermediate sulfation degrees, variation in the molecular mass of the polymers (11, 23), or even interaction of the sulfated fucan with other macromolecules (24) because the agarose gel electrophoresis was performed with crude egg jelly. These aspects were further investigated using Mono Q FPLC of mixed samples of egg jelly from a large number of *S. droebachiensis* females. Egg jelly from 31 Pacific females showed two distinct fractions of sulfated fucans (Fig. 2A), whereas egg jelly from 19 Atlantic females contained a single fraction eluted at lower NaCl concentration (Fig. 2B). A peak rich in sialic acid was eluted completely by 0.7 mM NaCl from the two samples and termed “sialic acid-rich glycoconjugate” in analogy with similar compounds described in other species of sea urchins (25).

The absence of intermediate fractions between sulfated fucans I and II suggests that females of *S. droebachiensis* synthesize either type of fucan with a defined sulfation pattern. If the difference between the sulfated fucans from females of *S. droebachiensis* were a consequence of temporal variation in the sulfation or related to the stage of oogenesis, one would expect to see intermediate fractions between sulfated fucans I and II upon agarose gel electrophoresis (Fig. 1, A and B) and anion-exchange chromatography (Fig. 2, A and B).

Seven individual females of the sea urchin *S. pallidus* from the Pacific coast, collected at the same site as the *S. droebachiensis* females used in the experiment in Fig. 1A, contained a single sulfated fucan isoform (Fig. 1C). Mono Q FPLC of a mixed sample of egg jellies from 25 *S. pallidus* females confirmed the occurrence of a single sulfated fucan (Fig. 2C) eluting at high NaCl concentration, like sulfated fucan II from *S. droebachiensis*, in addition to the sialic acid-rich glycoconjugate.

Overall, these results indicate that spawned eggs from individual females of the sea urchin *S. droebachiensis* have one of two possible sulfated fucan isoforms. This polymorphism was observed only in one population. In contrast, all assayed fe-
males of the sea urchin \textit{S. pallidus} contained a single type of sulfated fucan.

\textit{Sulfated \(\alpha\)-Fucans from \textit{S. droebachiensis} Are Linear 4-Linked Polysaccharides, but Differ in the Extent of Their 2-O-Sulfation—}Both sulfated fucans (purified as in Fig. 2, A and B) migrated on agarose gels (Fig. 3) identically as crude egg jelly (shown in Fig. 1, A and B). The slow and fast migrating sulfated fucans were eluted at low and high NaCl concentrations, respectively. Chemical analysis of the purified sulfated fucans (Table I) revealed fucose as the only sugar with a high content of sulfate ester, which increased from sulfated fucan I to sulfated fucan II, as expected from their migration upon agarose gel electrophoresis (Fig. 1A) and elution by anion-exchange chromatography (Fig. 2A).

Methylation of native sulfated fucan I from \textit{S. droebachiensis} yielded equimolar proportions of 2,3-di-O-methylfucose and 3-methylfucose, whereas 2,3-di-O-methylfucose was the predominant methyl ether derivative from desulfated fucan I (Table II). This indicates a polysaccharide composed of 4-linked fucopyranoside residues, partially 2-O-sulfated.\(^2\) This structure was confirmed and further detailed by NMR analysis. The \textsuperscript{1}H one-dimensional and \textsuperscript{1}H/\textsuperscript{13}C HMQC spectra of native and desulfated fucan I from \textit{S. droebachiensis} are shown in Figs. 4 (A and B) and 5 (A and B), respectively. The chemical shifts in Table III are based on the interpretations of TOCSY, COSY, and HMQC spectra.

NMR spectra of desulfated fucan I show a single anomeric signal (Fig. 4B) with a strong downfield shift (\(-11\) ppm) of C-4 (Fig. 5B and Table III), compatible with a linear homopolymer of 4-linked \(\alpha\)-fucopyranoside residues. NMR spectra of native sulfated fucan I contain four anomeric signals in near-equal proportions by integration (Figs. 4A and 5A). TOCSY and COSY spectra confirmed that the four anomeric signals of native sulfated fucan I correspond to four spin systems, each consistent with \(\alpha\)-fucose. The spin systems can be traced, giving the values in Table III. Strong downshifts (approximately \(-0.65\) ppm) of H-2 of residues A and B relative to H-2 of residues C and D indicate that two of the residues are sulfated at C-2. Thus, sulfated \(\alpha\)-fucan I from \textit{S. droebachiensis} is mostly a tetrasaccharide repeat unit consisting of 4-linked residues, two sulfated at the O-2-position and two that are unsulfated.

The order of the four residues can be easily deduced. The only possible array is two consecutive 2-O-sulfated residues followed by two unsulfated residues. If the 2-O-sulfated and unsulfated units alternate, the fucan would contain a disaccharide instead of a tetrasaccharide repeating structure. Our proposition was confirmed by the NOESY spectrum (Fig. 6). As in the NOESY spectra of other fucans from echinoderms (5, 26, 27), NOEs between protons of different units can be seen, and they were used to reveal the sequence (besides, of course, NOEs on other protons in the same residue). In sulfated \(\alpha\)-fucan I

\(^2\) An additional round of methylation did not increase the proportion of 2,3-di-O-methyl fucose. Possibly, the sample still contained small amounts of 2-O-sulfate ester. A different sample of desulfated fucan I was used for NMR analysis.

\begin{table}
\centering
\caption{Methylated derivatives obtained from native and desulfated fucans from the egg jelly of \textit{S. droebachiensis}}
\begin{tabular}{lcccc}
\hline
\textbf{Alditols}\(^a\) & \textbf{\(t_R\)}\(^b\) & \multicolumn{2}{c}{\textbf{Sulfated fucan I}} & \multicolumn{2}{c}{\textbf{Sulfated fucan II}} \\
 & & Native & Desulfated & Native & Desulfated \\
\hline
\textbf{} & \textbf{min} & \% of total peak area & \% of total peak area & \% of total peak area & \% of total peak area \\
2,3-Me\textsubscript{2}Fuc & 25.8 & 49 & 71 & <1 & 100 \\
3-MeFuc & 30.1 & 51 & 29 & 100 & <1 \\
\hline
\end{tabular}
\\(a\) The identity of each peak was established by mass spectrometry. \\
\(b\) Retention time on a DB-1 capillary column.
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{\textsuperscript{1}H one-dimensional NMR spectra at 600 MHz of native (A and C) and desulfated (B and D) \(\alpha\)-fucan I (A and B) and \(\alpha\)-fucan II (C and D) from \textit{S. droebachiensis}. The spectra were recorded at 60 °C for samples in \(\text{D}_2\text{O}\) solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water has been suppressed by presaturation. The \(\alpha\)-anomeric signals assigned by \textsuperscript{1}H/\textsuperscript{13}C HMQC (see Fig. 5A) are labeled A–D in native sulfated \(\alpha\)-fucan I. Expansion of the 4.9–5.5 ppm region of the \textsuperscript{1}H spectrum is shown in the inset in A. The integrals listed under the anomeric signals are normalized to a total number of anomeric protons.}
\end{figure}
The structure of sulfated α-fucan II from *S. droebachiensis* was investigated using the same methodologies. Methylation of native sulfated fucan II yielded 3-methylfucose, whereas 2,3-di-O-methylfucose was obtained from totally desulfated fucan II (Table II). Clearly, this indicates a linear homopolymer composed of 4-linked and 2-O-sulfated fucopyranoside residues, the structure of which was confirmed by NMR analysis (Figs. 4 (C and D) and Fig. 5 (C and D)). The 1H spectrum of sulfated α-fucan II resulting from desulfation processes shows a reduction in intensity of the anomeric residue at 5.30 ppm and a corresponding increase at 5.05 ppm.3 Again, the chemical shifts were based on the interpretations of TOCSY, COSY (data not shown), and HMBC spectra (Fig. 5, C and D). The chemical shifts of the desulfated residues from fucans I and II are similar, indicating that both polysaccharides have the same saccharide backbone. But, in contrast with sulfated fucan I, sulfated fucan II cannot be ruled out. For example, small amounts of three consecutive 2-O-sulfated fucose units followed by three unsulfated residues may occur in the polysaccharide. In this case, the additional structures are either undetectable due to their low proportions or cannot be discriminated by the NMR spectra. Nevertheless, the near-equal proportions by integration of the four anomeric signals (Figs. 4A and 5A) indicate these additional structures cannot account for a substantial proportion of the sulfated fucan structure.

**Note:**

3 Different samples of desulfated fucan II were used for methylation and NMR analyses. Totally desulfated fucan II was employed for methylation analysis (Table II), whereas a partially desulfated preparation was used for NMR analysis (Figs. 4D and 5D).

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**Figure 5.** 1H/13C HMQC spectra of native (A and C) and desulfated (B and D) α-fucan I (A and B) and α-fucan II (C and D) from *S. droebachiensis*. The assignment was based on TOCSY and COSY spectra. The values of chemical shifts in Table III are relative to external trimethylsilylpropionic acid at 0 ppm for 1H and to methanol for 13C. Values in boldface type indicate positions bearing a sulfate ester, and those in italic type indicate glycosylated positions.

**Figure 6.** Expansion from the NOESY spectrum of the sulfated α-fucan I from *S. droebachiensis*. The four fucose residues in the repeating unit are marked A–D as in Fig. 4A. We can observe NOEs from H-1 of each residue to the following ring proton, in particular the sequence-defining NOEs A1–B4, B1–C4, C1–D3, and D1–A2.
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Fig. 7. Structures of sulfated α-l-fucans and sulfated α-l-galactan from sea urchin egg jelly. Shown are eight fully characterized structures of sulfated polysaccharides from the egg jelly of seven species of sea urchins. The specific pattern of sulfation, the position of the glycosidic linkage, and the constituent monosaccharide vary among sulfated polysaccharides from different species (57, 27). See “Results and Discussion” for details.

The Sulfated α-Fucan from S. pallidus Has a 3-Linked Tetrasaccharide Repeating Unit Defined by a Specific Pattern of Sulfation at the 2-O- and 4-O-Positions—The sulfated fucan from S. pallidus that eluted from an anion-exchange chromatography column at high NaCl concentration (Fig. 2C) contains fucose as the only sugar with a high content of sulfate ester (Table I), but has a slower mobility upon agarose gel electrophoresis than the two sulfated fucans from S. droebachiensis (Fig. 3). The electrophoretic mobility of sulfated polysaccharides in 1,3-diaminopropane acetate buffer depends on the structure of the glycan, which forms a complex with the diamino groups (20, 28). Thus, the retarded electrophoretic mobility of the sulfated fucan from S. pallidus is a preliminary indication of its distinctive polysaccharide structure.

As in the case of the polysaccharides from S. droebachiensis, the structure of this new sulfated fucan was determined by NMR analysis. The native sulfated fucan showed four anomeric residues in near-equal proportions by integration (Figs. 8A and 9A), whereas after desulfation, a single anomeric signal was seen (Figs. 8B and 9B), as already observed for sulfated fucan I from S. droebachiensis (Figs. 4 (A and B) and 5 (A and B)). But, in the case of desulfated fucan from S. pallidus, a strong downfield shift (−8 ppm) of C-3 (Table IV, values shown in italic type), and not of C-4, is compatible with a 3-linked polysaccharide. The NMR spectra of the native sulfated fucans from the two species of sea urchins also differ significantly. For S. pallidus, strong downshifts of H-2 of residues A and B (−0.50 ppm) and H-4 of residues C and D (−0.70 ppm) (Fig. 10A and Table IV) indicate that two of the four residues are 2-O-sulfated and that the other two are 4-O-sulfated. Minor structural components, which may occur in this sulfated α-fucan (such as those indicated by arrows in Fig. 8A), do not account for >5% of the total signals in the anomeric region based on integration of the peaks in this region of the 1H spectrum. In addition, the proportions of these minor components (but not those of the A–D spin systems) vary among different preparations of sulfated α-fucan.

The order of the four residues can be easily deduced, as already discussed for sulfated fucan I from S. droebachiensis. The only possible array is two consecutive 2-O-sulfated residues followed by two 4-O-sulfated residues. Again, if the 2-O- and 4-O-sulfated units alternate, the fucan would contain a disaccharide instead of a tetrasaccharide repeating structure. There is no indication of disulfated units in the TOCSY spectrum. Although only one inter-residue NOE could be unambiguously identified in the NOESY spectrum (Fig. 10B), it was enough to confirm the proposed structure. Thus, it was possible to identify NOEs from H-1 of residue A to H-4 of residue D, whereas H-1 of residue B, C, or D does not have any inter-residue NOEs. These NOEs are in agreement with the repeat-
plex, heterogeneous structures. Their regular repeating sequences are not easily deduced; even high-field NMR is at the limit of its resolution, and complete description of their structure is not available at present (9, 27). Recently, we isolated and characterized several sulfated α-L-fucans from echinoderms, mostly from sea urchin egg jelly. In contrast to the algal fucans, these sea urchin polysaccharides have simple, linear structures composed of well defined repeating units of oligosaccharides (5–7).

The specific pattern of sulfation and the position of the glycosidic linkage vary among sulfated α-L-fucans from different species of sea urchins. S. droebachiensis (sulfated α-L-fucan I) and Arbacia lixula (5) have a 4-linked sulfated α-L-fucan with the same tetrasaccharide repeating sequence (Fig. 7A). S. pallidus and Lytechinus variegates (5, 27) have 3-linked sulfated α-L-fucans with tetrasaccharide repeating units that differ in specific patterns of sulfation (Fig. 7, C and F, respectively). S. purpuratus has two structures, found in different individuals: a monosaccharide with variable sulfation at one position (sulfated α-L-fucan I) and a trisaccharide repeating sequence (sulfated α-L-fucan II) (Fig. 7, D and E, respectively) (6). S. droebachiensis (sulfated fucan II), Strongylocentrotus franciscanus (7), and Echinometra lucunter (5) have polysaccharides with a single 2-O-sulfated monosaccharide unit that differ either in the position of their glycosidic linkage or in their constituent monosaccharide (Fig. 7, G, H, and respectively). S. droebachiensis (sulfated fucan II) and S. franciscanus contain 4- and 3-linked α-L-fucopyranose, respectively, whereas E. lucunter has 3-linked α-L-galactopyranose.

**Structural Features in the Sea Urchin Polysaccharides That Confer Finer Specificity of Recognition in the Sperm Acrosome Reaction**—Sulfated polysaccharides from sea urchin egg jelly are responsible for inducing the sperm acrosome reaction, which is an obligatory event for fertilization (5–7). Shortly after fertilization, the sulfated α-fucan disappears (32), which indicates that it has no further role in embryo development. These polysaccharides are species-specific as inducers of the sperm acrosome reaction and may represent one of the barriers that prevent interspecific fertilization.

We have now fully characterized eight sulfated polysaccharides from the egg jellies of seven species of sea urchins (Fig. 7). We can now formulate questions such as follows. What are the common structural features among these polysaccharides? Can we identify the structures that confer finer specificity of recognition in the acrosome reaction?

Clearly, as we examine the eight structures shown in Fig. 7, the common feature shared by these polysaccharides is always the occurrence of 2-O-sulfation at the first unit of the oligosaccharide repeating sequence. In this way, the sea urchin S. franciscanus, which contains a sulfated fucan composed exclusively of the common 2-O-sulfated α-L-fucose unit (Fig. 7G), has a less strict species specificity in sperm recognition of sulfated polysaccharide. The potency of acrosome reaction induction clearly depends on the extent of 2-O-sulfation in the chain of 3-linked α-fucose units (7).

As a distinctive feature for a different polysaccharide backbone, the sea urchin E. lucunter synthesizes sulfated α-L-galactan (Fig. 7H) instead of sulfated α-L-fucan (5). However, the majority of the sea urchin species contain sulfated α-fucans with increased complexity due to variable 2-O- and 4-O-sulfation of their oligosaccharide repeating units as well as 1–3 or 1–4 glycosidic linkage. In the case of a species enriched in 4-O-sulfated units, as exemplified by S. purpuratus (Fig. 7, D and E), a more strict species specificity is observed than in S. franciscanus, and the sperm react only with homologous polysaccharide or, to a lesser extent, with heterologous 3-linked

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**Summary of Variants of Sulfated α-L-Fucans from Sea Urchin Egg Jelly**—A variety of sulfated fucans have been described in marine algae (29–31). These compounds are among the most abundant and widely studied of all sulfated polysaccharides of non-mammalian origin. The algal fucans have...
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The spectra were recorded at 600 MHz in 99.9% D₂O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for 1H and to methanol for 13C. Values in boldface type indicate positions bearing a sulfate ester, and those in italic type indicate glycosylated positions.

| Proton | A        | B        | C        | D        | Nativea | ppm | ppm |
|--------|----------|----------|----------|----------|---------|-----|-----|
| H-1    | 5.522    | 5.430    | 5.200    | 5.120    | 5.230   |     |     |
| H-2    | 4.594    | 4.602    | 4.016    | 4.062    | 4.090   |     |     |
| H-3    | 4.180    | 4.180    | 4.194    | 4.199    | 4.090   |     |     |
| H-4    | 4.120    | 4.120    | 4.853    | 4.894    | 4.133   |     |     |
| H-5    | 4.469–4.560 | 4.469–4.560 | 4.469–4.560 | 4.469–4.560 | 4.430   |     |     |
| H-6    | 1.322–1.430 | 1.322–1.430 | 1.322–1.430 | 1.322–1.430 | 1.380   |     |     |
| Carbon |          |          |          |          |         |     |     |
| C-1    | 94.50    | 97.00    | 97.20    | 95.70    | 94.52   |     |     |
| C-2    | 75.90    | 75.90    | 70.05    | 70.05    | 65.35   |     |     |
| C-3    | 78.55    | 78.55    | 78.55    | 78.55    | 74.01   |     |     |
| C-4    | 71.80    | 71.80    | 81.80    | 81.80    | 67.59   |     |     |
| C-5    | 69.13    | 69.13    | 69.13    | 69.13    | 65.23   |     |     |
| C-6    | 15.00    | 15.00    | 15.00    | 15.00    | 13.00   |     |     |

The sulfated fucan from S. pallidus contains four types of α-fucose residues (see Figs. 8A and 9A).

Overall, the experiments summarized in Table V indicate that the sulfated α-fucans from the egg jellies of S. pallidus and S. droebachiensis induce the acrosome reaction in homologous (but not heterologous) sperm. This was confirmed by recent assays of acrosomal exocytosis using immunofluorescence microscopy and anti-bindin antibody.4 Again, the immunological staining of sperm after incubation with the purified sulfated α-fucans demonstrated that the egg jelly polysaccharides induce the acrosome reaction in homologous (but not heterologous) sperm. This is the major limitation for interspecific fertilization between these two species of sea urchins. It is interesting, and suggestive of adaptation, that these two closely related species, which co-occur over a huge geographic range, show such a strong specificity early in the cascade of gamete recognition events.

Two Sulfated α-Fucan Isotypes in a Single Species of Sea Urchin—We have extended to S. droebachiensis our observation in S. purpuratus (6) that individual females spawn eggs possessing only one of two sulfated α-fucan isotypes (Fig. 1, A and B). As in S. purpuratus, both S. droebachiensis isotypes induce the acrosome reaction with similar potency in homologous sperm, as revealed by the immunofluorescence microscopy assay. It appears that in S. droebachiensis, one of the isotypes does not occur or occurs at lower frequencies in a population from a different ocean. Additional studies with a larger number of...
Fertilization success of plain sperm and sperm pre-reacted with egg jelly for crosses among three Strongylocentrotus species

| Sperm          | Eggs          | Pre-reaction with conspecific egg jelly | Mean % of eggs fertilized | Ratio of fertilization after and before pre-reaction with conspecific egg jelly |
|----------------|---------------|----------------------------------------|---------------------------|--------------------------------------------------------------------------------|
| *S. droebachiensis* | *S. droebachiensis* | –                                      | 80                        | 1.06                                                                            |
| *S. pallidus*    | *S. droebachiensis* | +                                      | 85                        |                                                                                   |
| *S. purpuratus*  | *S. droebachiensis* | –                                      | 9                         |                                                                                   |
| *S. droebachiensis* | *S. pallidus*   | –                                      | 60                        | 6.67                                                                            |
| *S. pallidus*    | *S. purpuratus*  | +                                      | <1                        |                                                                                   |
| *S. pallidus*    | *S. purpuratus*  | +                                      | 24                        | 3.67                                                                            |
| *S. purpuratus*  | *S. pallidus*    | +                                      | 88                        |                                                                                   |
| *S. purpuratus*  | *S. purpuratus*  | –                                      | 78                        | 1.07                                                                            |

* The values are the percentage of eggs fertilized at a sperm concentration of 200 sperm/μl before (−) and after (+) pre-reaction with conspecific egg jelly.

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of females and collected at a variety of geographic sites are necessary to further clarify the role of genetic or environmental factors.

The two sulfated α-fucans of *S. droebachiensis* have well-defined sulfation patterns and are not a consequence of variable degrees of sulfation (Fig. 7, A and B). The inheritance of such sulfation patterns is unknown. We expect that they are produced by site-specific sulfotransferases by analogy with the extensive studies on the biosynthesis of mammalian glycosaminoglycans. Sulfated fucan II requires a single sulfotransferase, but sulfated fucan I requires two sulfotransferases, one that recognizes the first α-fucose residue of the repeating sequence and a second that recognizes the 2-O-sulfated fucose unit and sulfates the second residue. Of course, we cannot exclude unique metabolic pathways, as reported for the biosynthesis of a sulfated α-1-galactan from ascidians (34, 35). For example, an alternative to explain the presence of either sulfated fucan I or II in separate females of *S. droebachiensis* is to postulate that, in both types of females, all fucose residues become 2-O-sulfated, but in females containing sulfated fucan I, specific sulfotransferases remove the sulfate esters from the third and fourth residues.

Another noteworthy observation is that *S. droebachiensis* and *A. lixula*, unrelated sea urchin species from the Arctic and tropical Atlantic Oceans, respectively, synthesize sulfated α-fucans with the same repeating structure (Fig. 7A). Our recent experiments (not shown) with immunological staining of *S. droebachiensis* sperm with anti-bindin antibody after incubation with the purified polysaccharides indicate that *A. lixula* sulfated fucan is indeed equivalent to *S. droebachiensis* sulfated fucan I in its physiological activity in vitro. According to phylogenetic analysis, these two species diverged ~200 million years ago (36). The species *S. droebachiensis*, *S. pallidus*, and *S. purpuratus* diverged 3.5 million years ago (37), but their egg jelly sulfated fucans are markedly different. Therefore, the genes involved in the biosynthesis of the sulfated fucans and their sperm receptors (8) did not evolve in concordance with the evolutionary distance between these echinoderms, but were possibly driven to diverge by natural selection where several species co-occur.

In the case of *S. pallidus*, two additional sulfotransferases may be involved in the biosynthesis of the sulfate fucan: one transferase to recognize the two consecutive 2-O-sulfated fucose units and then to sulfates C-4 of the third residue and another transferase to recognize the sulfation pattern of the first three fucose residues and then to sulfate the fourth unit at C-4 to obtain the repeating sequence shown in Fig. 7C.
Sulfated Fucans from the Egg Jellies of the Closely Related Sea Urchins
*Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* Ensure
Species-specific Fertilization
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