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Carbohydrate-based species recognition in sea urchin fertilization: another avenue for speciation?

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SUMMARY Spawning marine invertebrates are excellent models for studying fertilization and reproductive isolating mechanisms. To identify variation in the major steps in sea urchin gamete recognition, we studied sperm activation in three closely related sympatric *Strongylocentrotus* species. Sperm undergo acrosomal exocytosis upon contact with sulfated polysaccharides in the egg-jelly coat. This acrosome reaction exposes the protein bindin and is therefore a precondition for sperm binding to the egg. We found that sulfated carbohydrates from egg jelly induce the acrosome reaction species specifically in *S. droebachiensis* and *S. pallidus*. There appear to be no other significant barriers to interspecific fertilization between these two species. Other species pairs in the same genus acrosome react nonspecifically to egg jelly but exhibit species-specific sperm binding. We thus show that different cell–cell communication systems mediate mate recognition among very closely related species. By comparing sperm reactions to egg-jelly compounds from different species and genera, we identify the major structural feature of the polysaccharides required for the specific recognition by sperm: the position of the glycosidic bond of the sulfated α-L-fucans. We present here one of the few examples of highly specific pure-carbohydrate signal transduction. In this system, a structural change in a polysaccharide has far-reaching ecological and evolutionary consequences.

INTRODUCTION

Free-spawning marine invertebrates have provided key examples of the evolution of species-specific fertilization (Minor et al. 1991; Vacquier et al. 1997; Palumbi 1999). The discovery of rapidly diverging gamete recognition proteins in sea urchins and snails (e.g., Metz and Palumbi 1996; Hellberg et al. 2000; Galindo et al. 2003) has stimulated investigations in other taxa and led to the revolutionary finding that reproductive proteins are the fastest evolving proteins among mammals as well (Swanson et al. 2001; Swanson and Vacquier 2002a). Here, we return to the sea urchin model system and extend the description of rapidly evolving gamete recognition molecules from proteins to carbohydrates.

Previous researchers have postulated that in sea urchins, gametic mate "choice" is controlled primarily, if not exclusively, by interaction of the sperm protein bindin and its egg receptor (Minor et al. 1991; Vacquier et al. 1995; Cameron et al. 1996; Glaser et al. 1999; Kamei and Glabe 2003). Here we examine a distinct carbohydrate-mediated mechanism for cell–cell recognition that coexists with the bindin-protein paradigm. This signaling event precedes the bindin(g) step in the fertilization cascade (Fig. 1). When sperm approach the sea urchin egg, sulfated polysaccharides in the egg jelly induce the sperm acrosome reaction (AR), which exposes the protein bindin at the tip of the sperm head (Alves et al. 1997; Vacquier and Moy 1997; Vilela-Silva et al. 2002). Only then can sperm attach to the egg and their plasma membranes fuse (Foltz and Lennarz 1993; Glaser et al. 1999). The AR has been well-studied mechanistically (Neill and Vacquier 2004). In this report, we connect the structure of highly specific ligands to their role in reproductive isolation.

The carbohydrates responsible for the induction of the AR have been isolated from the egg jellies of several sea urchin species and structurally characterized (Vacquier and Moy 1997; Alves et al. 1998; Vilela-Silva et al. 1999, 2002; Hirohashi

and Vacquier 2002b). They are large linear fucose polymers (α-L-fucans) with a regular pattern of sulfation. The glycosidic linkage between the monosaccharide units and the sulfation pattern have both been shown to affect the AR induction in sea urchin sperm (Hirohashi et al. 2002). The receptor protein for egg-jelly fucans (REJ) has been localized on the sperm membrane in *Strongylocentrotus purpuratus* (Moy et al. 1996; Vacquier and Moy 1997).

In this study we show that the initiation of the sperm AR is species specific between the two sea urchin species *Strongylocentrotus droebachiensis* and *S. pallidus*, whereas bindin-protein mediated recognition is crucial among some other combinations of species (e.g., for eggs of *S. purpuratus*). We examine the specificity of the AR on multiple levels: (a) fertilization success of sperms on eggs of different species, (b) reactivity of sperm with natural (crude) egg jellies, (c) reactivity of sperm with purified active compounds, (d) structural features of the active compounds, and (e) phylogenetic comparisons of the chemical structures. We demonstrate that the chemical differences responsible for this reproductive barrier are not correlated with phylogenetic distance and therefore may have been under selection.

MATERIALS AND METHODS

Fertilization assays

Strongylocentrotus droebachiensis (O. F. Müller) and *S. pallidus* (G. O. Sars) from Washington State (Northeast Pacific, USA), Newfoundland, and Norway and *S. purpuratus* (Stimpson) from Washington State were induced to spawn by intracoelomic injection of 0.55 M KCl. Sperm suspensions (1:4 at each of five steps, starting with a 1:10,000 dilution of "dry" sperm) were added to aliquots of gently washed eggs (approximately 1 egg/μl filtered seawater). Fertilization success was assessed by counting the proportion of eggs (out of 200–300 per well) having an elevated fertilization envelope or cleaving. Absolute sperm concentrations were determined by 10 counts of fixed sperm suspensions in a hemocytometer. To obtain jelly water to acrosome-react sperm, a 5–10% suspension of eggs in seawater was poured through nitex mesh several times. This stripped the eggs of their soluble jelly; after the dejellied eggs had settled, the supernatant was used for the final sperm dilution in the jelly treatments.

Fertilization curves (the plots of percent of eggs fertilized against the logarithm of sperm concentration) are generally nonlinear. Fitting a kinetics model to these curves (Levitan 2002) requires values for numerous parameters. Another compatibility measure, the sperm concentration needed to achieve 50% fertilization (McCartney and Lessios 2002), does not apply in cases where fertilization success is very low. We used logistic regression (Sokal and Rohlf 1995) to fit a log-linear model to each curve by maximum likelihood (in SAS; SAS Institute, Inc., Cary, NC, USA). The slope and intercept describe fertilization success as a linear equation for each male–female combination and was thus calculated for a given sperm concentration.

Extraction of sulfated α-L-fucans

For *S. droebachiensis* and *S. pallidus*, the jelly coat was stripped off the eggs by pouring an egg suspension repeatedly through nylon mesh with pores the size of the egg diameter. Eggs of *S. purpuratus*, *S. franciscanus*, and *Arbacia lixula* were dejellied by stirring and briefly lowering the pH of the egg suspension to 5 (per Vacquier and Moy 1997). Neither of these dejellying methods has an effect on the sulfated fucans, which were highly purified before use. The jelly water was centrifuged at $>25{,}000\,g$ for >20 min and the supernatant dialyzed into distilled water and lyophilized. The sulfated α-L-fucans were purified and characterized as previously described (Albano and Mourão 1986; Alves et al. 1997; Vilela-Silva et al. 1999, 2002).

AR assays

Sperm were diluted 1:10 in HEPES-buffered (10 mM, pH 7.9, 9°C) filtered seawater (FSW; filtered through 0.2 μm pore size filters Millipore, Billerica, MA, USA). Fifty microliters of the sperm suspension was gently mixed into 100 μl of the test solution (egg-jelly water, purified sulfated fucan in FSW, or FSW as control). The hexose content of the test solutions was quantified by the phenol-sulfuric acid method (Dubois et al. 1956). After 5 min at 9°C, sperm were fixed by the addition of 750 μl of ice-cold hyperosmotic 3% paraformaldehyde in FSW. All subsequent washing and staining steps were done at room temperature by gently pelleting the sperm in a centrifuge and resuspending by pipetting with wide-bore tips or vortexing. After at least 30 min in the fixative, sperm were washed with 500 μl phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) and stained with agitation for at least 30 min with 1 unit Alexa488-labeled phalloidin (Molecular Probes, Eugene, OR, USA) in 100 μl PBS blocker (0.1 M glycine, 1 mg/ml bovine serum albumin, 0.02% sodium azide in PBS, pH 7.4). Then, 20 μl of rabbit bindin-antibody (kindly supplied by V. Vacquier) was added to the blocker-phalloidin solution. After 2 h, sperm were washed twice with 500 μl PBS and resuspended in 100 μl PBS, 10 mg/ml bovine serum albumin, pH 7.4, containing secondary antibody (Texas Red–labeled goat anti-rabbit, diluted 1:400, Jackson Immunoresearch, West Grove, PA, USA).

After agitating the suspension (3 h to overnight), we washed the cells twice in 500 μl PBS, resuspended them in 20–50 μl PBS, mounted them in a thin layer, and sealed the coverslip with a gel pen. Sperm were blindly scored with a Radiance 2000 confocal microscope (BioRad, Hercules, CA, USA) using a 60 × PlanApo 1.4 NA lens (Nikon, Melville, NY, USA). We simultaneously collected green (phalloidin), red (anti-bindin), and transmitted-light channels. The diagnostic validity of the fluorescent stains was confirmed with transmission electron microscopy (not shown).

RESULTS

Effect on fertilization success of treating sperm with conspecific egg jelly

We measured fertilization success among three closely related co-occurring species, *S. droebachiensis*, *S. pallidus*, and *S. purpuratus*. For conspecific gametes, we observed a high percentage of successful fertilization, whereas heterospecific

sperm fertilized only a small percentage of eggs (Fig. 2a). After prereaction of the sperm with conspecific egg jelly, interspecific fertilization between *S. droebachiensis* and *S. pallidus* increased significantly in both directions (Fig. 2b; *t*-tests:

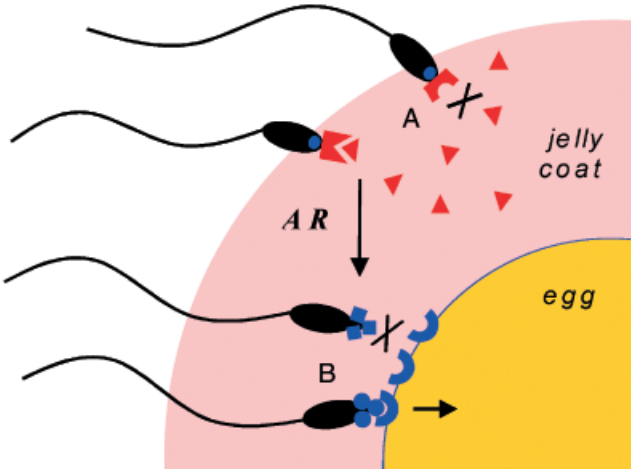

Fig. 1. Schematic depiction of the two hierarchical steps in sea urchin gamete recognition. AR = acrosome reaction. (A) Carbohydrate-based species recognition: the sperm AR is induced when a sperm with the correct receptor type contacts specific sulfated fucans in the egg-jelly coat (red triangles). This reaction exposes the protein bindin (shown in blue). (B) The protein paradigm: the protein bindin, coating the outside of the sperm tip, reacts with a matching egg membrane receptor.

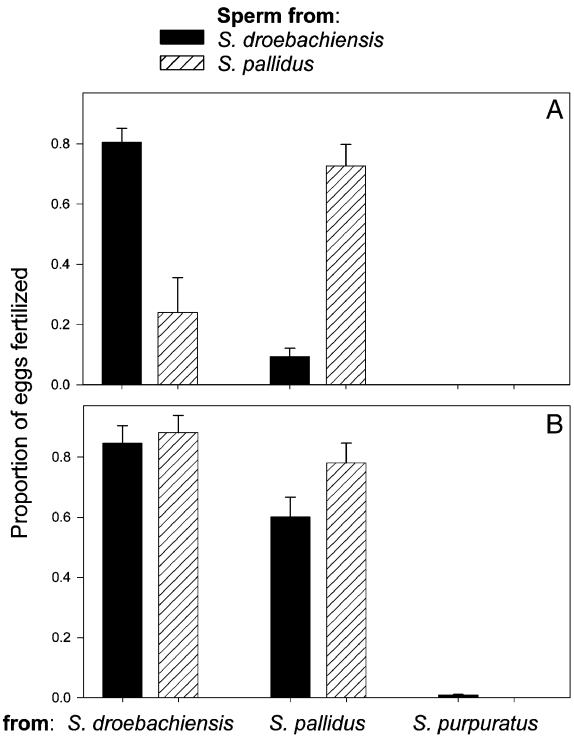

Fig. 2. Fertilization success among three *Strongylocentrotus* species. (A) Sperm diluted in seawater; (B) Sperm prereacted with conspecific egg jelly. The height of the bars show the proportions of eggs fertilized at 200 sperm/µl (plus standard errors).

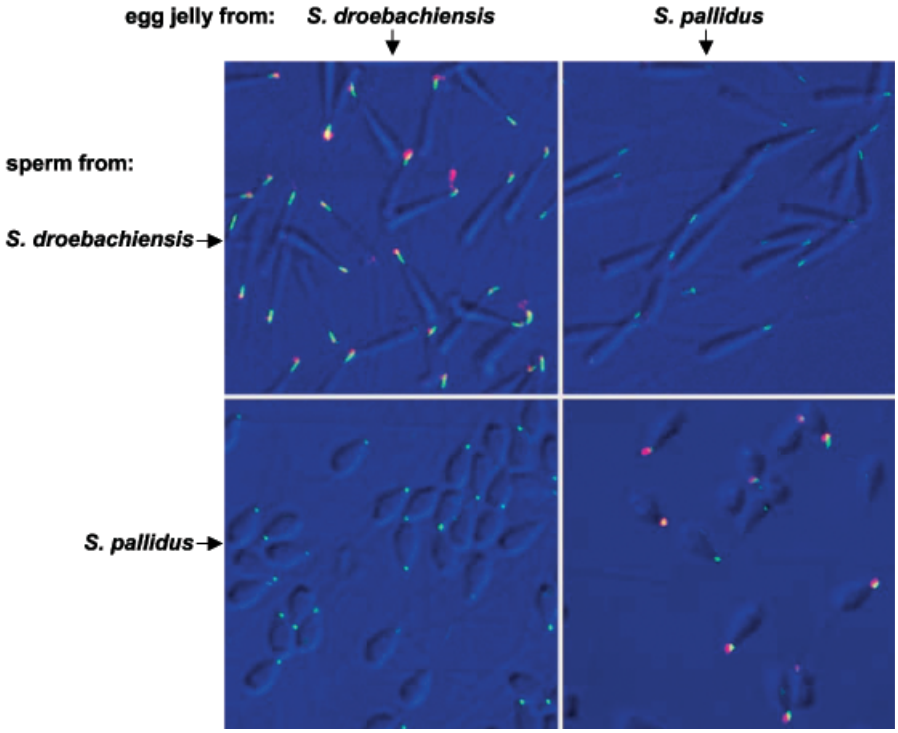

Fig. 3. Confocal images of double-stained *Strongylocentrotus droebachiensis* sperm and *S. pallidus* sperm after incubation with egg jelly (100 µg hexose/ml). When a sperm cell has undergone the acrosome reaction, the acrosomal filamentous actin stains more brightly with phalloidin (green) and the exocytosed attachment protein bindin stains with bindin antibody (red). Not all focal planes were included in these flattened projections.

$P = 0.001$ for *S. droebachiensis* sperm on *S. pallidus* eggs and $P = 0.032$ for the reciprocal cross). In contrast, the eggs of *S. purpuratus* could not be fertilized even after prereaction of the heterologous sperm with conspecific egg jelly (Fig. 2).

Species-specific induction of the AR by egg jelly

To investigate whether a failure of the sperm AR is in fact the major limitation for interspecific fertilization between *S. droebachiensis* and *S. pallidus*, we directly determined the proportion of sperm that undergo the AR in response to egg jelly. Because light microscopy did not provide sufficient resolution of the tips of the sperm heads in these species, we assayed acrosomal exocytosis with fluorescence microscopy using an F-actin stain and anti-bindin antibody (Fig. 3). An initial experiment with the species *S. droebachiensis* and *S. pallidus* indicated that sperm respond selectively to conspecific crude egg jelly (Fig. 3). As we compared the three *Strongylocentrotus* species, we observed that the sperm of *S. droebachiensis* were very selective and reacted only to conspecific egg jelly, whereas sperm of *S. pallidus* and *S. purpuratus* reacted to conspecific and to each other's egg jellies (Table 1). The negative controls for each male's sperm (mixed with FSW only) contained a negligible number of reacted acrosomes.

Sulfated α-L-fucans from the egg jellies as the active molecules inducing the AR

The AR experiments reported in Table 1 were performed with crude egg jellies. We were able to test the biological effects of the active compounds directly, having previously isolated, purified, and characterized the fine structure of the sulfated α-L-fucans from the egg jellies of various sea urchins of the genus *Strongylocentrotus* (Alves et al. 1998; Vilela-Silva et al. 1999, 2002). Fluorescent staining of sperm after incubating them with the purified individual sulfated α-L-fucans confirmed that it is these polysaccharides that induce the AR in conspecific sperm (Fig. 4). As with crude egg jellies, *S. droebachiensis* sperm responded exclusively to the conspecific compound, whereas *S. pallidus* and *S. purpuratus* cross-reacted. For the nine jelly–sperm combinations among these three species, the percent AR in response to natural egg jellies and to purified compounds was significantly correlated (Spearman's rank correlation coefficient = 0.783, $P < 0.05$). The sperms of *S. pallidus* and *S. purpuratus*, but not of *S. droebachiensis*, also reacted to the sulfated fucan from *S. franciscanus* (Fig. 4; compound described in Vilela-Silva et al. 1999).

Functionally insignificant intraspecific polymorphism

Individual females from a population of *S. droebachiensis* collected in the Pacific Ocean spawn eggs possessing one of two 4-linked fucan isotypes, which differ in the extent of their 2-sulfation (Vilela-Silva et al. 2002). One is entirely 2-sulfated (Fig. 4), whereas another one contains two consecutive 2-sulfated residues followed by two unsulfated units (Fig. 5). These two sulfated fucan isotypes were equivalent in their potency to induce the AR (Table 2). The fully sulfated isotype does not appear to occur in a population of *S. droebachiensis* from the Atlantic Ocean (Vilela-Silva et al. 2002). Sperm from *S. droebachiensis* populations collected at the two geographic sites responded similarly to different kinds of sulfated α-L-fucans (Fig. 5; Table 2).

Identical sulfated fucan isotypes in evolutionarily distant sea urchins

Surprisingly, *S. droebachiensis*, a cold-water species, and *A. lixula*, an unrelated sea urchin species from the tropical Atlantic Ocean, synthesize identical sulfated α-L-fucans with the tetrasaccharide repeating structure shown in Fig. 5. The sulfated polysaccharides from these two species were equally able to induce the AR in sperm from *S. droebachiensis* (Fig. 5; live sperm of *A. lixula* for the reciprocal test were not available).

Intergenus comparisons

We further examined the AR-inducing potency of the sulfated fucan from *Lytechinus variegatus*, a species from a different family than the Strongylocentrotidae but in the same superorder (Camarodonta). Its egg-jelly sulfated fucan (Alves et al. 1997) was very similar to the ones from some of the strongylocentrotids and also was functionally equivalent (Fig. 6).

Table 1. Percent of sperm (± standard error) that acrosome reacted in response to natural egg-jelly water (hexose concentration 32 μg/ml)

	Egg Jelly from		
Sperm from	*Strongylocentrotus droebachiensis*	*Strongylocentrotus pallidus*	*Strongylocentrotus purpuratus*
S. droebachiensis	51.2 ± 9.0	13.3 ± 1.7	1.0 ± 0.6
S. pallidus	3.7 ± 3.2	43.8 ± 10.8	49.8 ± 10.1
S. purpuratus	3.5 ± 1.5	74.3 ± 6.9	86.7 ± 9.4

All gametes were from northeastern Pacific sea urchins in the genus *Strongylocentrotus*. Standard errors were calculated with individual males as replicates; each male's response was the average from tests with egg jellies from multiple females.

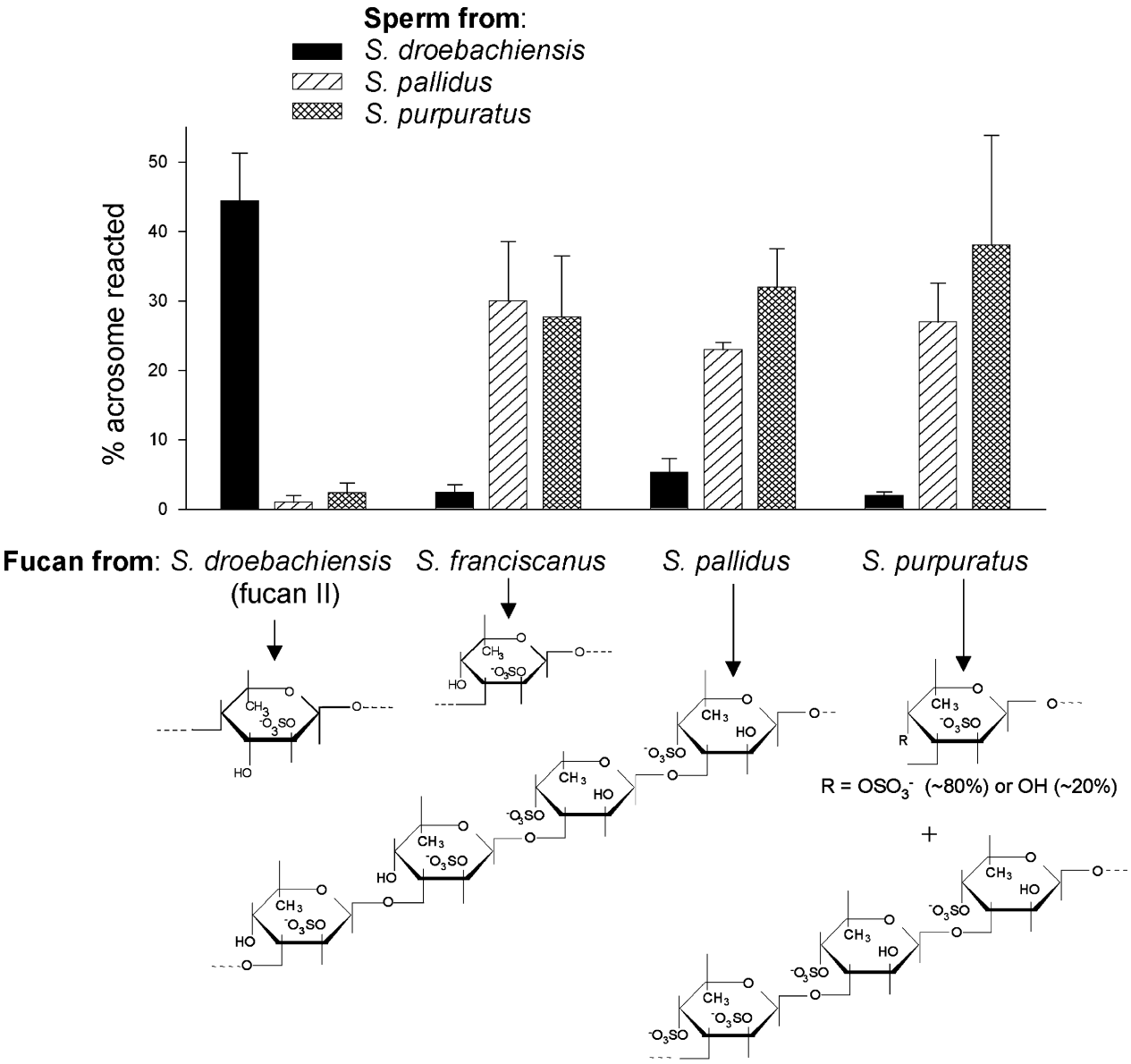

Fig. 4. Structures of sulfated α-L-fucans isolated from the egg jellies of sea urchins and their effects as inducers of the acrosome reaction of three *Strongylocentrotus* species' sperm. The bars show the means and standard errors at a fucan concentration of 100 μg hexose/ml. The structures of the sulfated α-L-fucans were determined by Alves et al. (1998) and Vilela-Silva et al. (1999, 2002).

The species *A. lixula*, whose eggs have a sulfated fucan identical to *S. droebachiensis*, belongs to the superorder Stirodonta, which separated from the Camarodonta over 150 million years ago (Smith et al. 1995). The properties of the egg-jelly fucans we tested are summarized in Fig. 6.

DISCUSSION

We determined that the molecular structure of a sulfated carbohydrate directs a cell physiological event—the induction of the sperm AR in sea urchins. Small chemical changes modulate a system of cell–cell recognition and species-specific fertilization, one that works independently of the sperm–egg binding system. By comparing sperm reactivity among related species, we established that the AR specificity is due to the glycosidic linkages in egg-jelly polysaccharides and that it is not correlated with phylogenetic distance.

Strongylocentrotid sea urchins are a model system for the study of gametic reproductive isolation (Hagström and Lønning 1967; Strathmann 1981; Minor et al. 1991; Biermann and Marks 2000; Levitan 2002). The species we examined

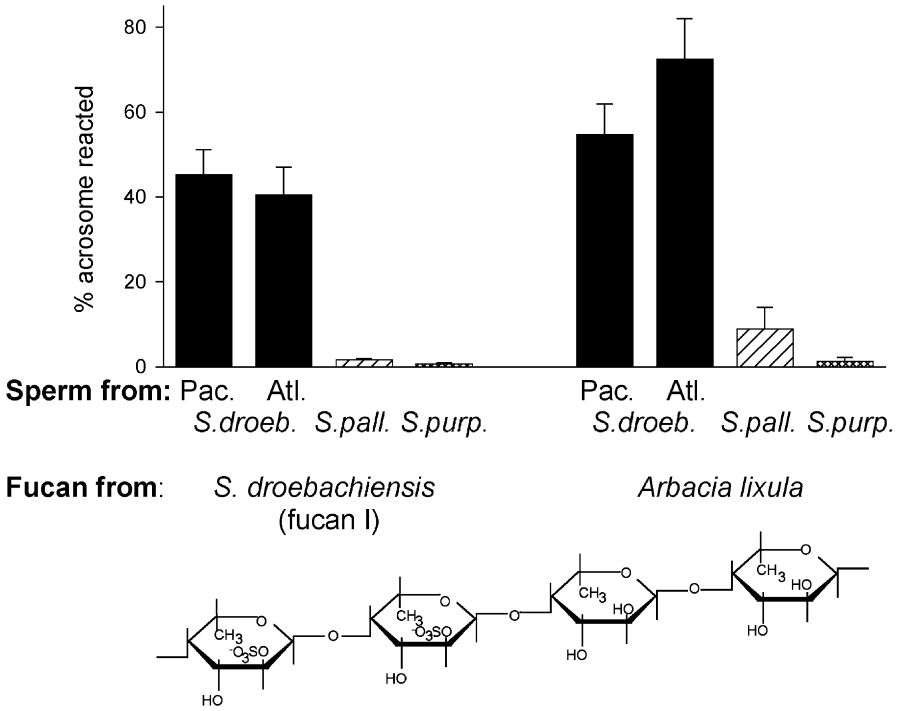

Fig. 5. Effect of sulfated α-L-fucans (100 μg hexose/ml) from the egg jellies of *Arbacia lixula* and *Strongylocentrotus droebachiensis* as inducers of the acrosome reaction in sperm of three *Strongylocentrotus* species. The egg jellies of some *S. droebachiensis* and of the tropical *A. lixula* contain identical sulfated α-L-fucans of the structure shown (Vilela-Silva et al. 2002). Pac., *S. droebachiensis* from the eastern Pacific Ocean; Atl., *S. droebachiensis* from Norway; S. pall., *S. pallidus*; S. purp., *S. purpuratus*.

overlap broadly in their geographic ranges and spawning seasons; they occur together in the North Pacific, with two of the species, *S. droebachiensis* and *S. pallidus*, having a circumarctic distribution (Biermann et al. 2003). The green sea urchin *S. droebachiensis* overlaps not only geographically but also ecologically with its close relatives *S. purpuratus* (which extends upward into the intertidal) and *S. pallidus* (which extends into deeper water and further north; Jensen 1974). The egg-jelly coat of *S. droebachiensis* contains a sulfated fucan with a structure unique among the other strongylocentrotid species examined. This sulfated fucan specifically triggered the AR only in sperm from its own species. Correspondingly, the structural requirements of the sulfated fucan needed to induce the AR in sperm of *S. droebachiensis* were very stringent. Even the similar sulfated α-L-fucan from *S. franciscanus*, which is also 2-sulfated but composed of 1 → 3-linked instead of 1 → 4-linked fucose units, did not induce the AR in *S. droebachiensis* sperm. Sperm from *S. pallidus* and *S. purpuratus* responded to conspecific and heterospecific 1 → 3-linked but not to 1 → 4-linked sulfated α-L-fucans, independent of their sulfation patterns at *O*-2 and *O*-4 positions (Fig. 6).

The results from these experiments clearly indicate that the reactivity of *Strongylocentrotus* sperm does not correlate with the charge density of the sulfated α-L-fucan or with the pattern of 2- or 4-sulfation but with the position of the glycosidic bond. Sperm of *S. droebachiensis* require 1 → 4-linked sulfated fucans to trigger the AR, whereas *S. pallidus* and

Table 2. Comparison of acrosome reaction–inducing activities of the two isotypes of sulfated fucans (I and II) from *Strongylocentrotus droebachiensis* egg jelly

Sperm from	% AR ± SE[1] Sulfated Fucan I[2]	% AR ± SE[1] Sulfated Fucan II[2]	Paired *t*-Test for Comparison of Means	*P*
Pacific				
S. droebachiensis	45.3 ± 5.8	38.3 ± 9.2	$t = 0.712$	0.503
Atlantic				
S. droebachiensis	40.6 ± 6.5	44.2 ± 10.1	$t = -0.577$	0.580
S. pallidus	1.7 ± 0.3	1.0 ± 1.0	$t = 1.177$	0.305
S. purpuratus	0.67 ± 0.3	2.3 ± 1.5	$t = -1.387$	0.300

[1]% AR = percent of sperm having undergone the acrosome reaction (AR) in response to 100 μg hexose/ml of purified sulfated fucan, ± standard error (SE). The sperms' responses to the two isotypes did not differ significantly.
[2]The structures of sulfated fucan I and sulfated fucan II are shown in Fig. 5 and Fig. 4, respectively.

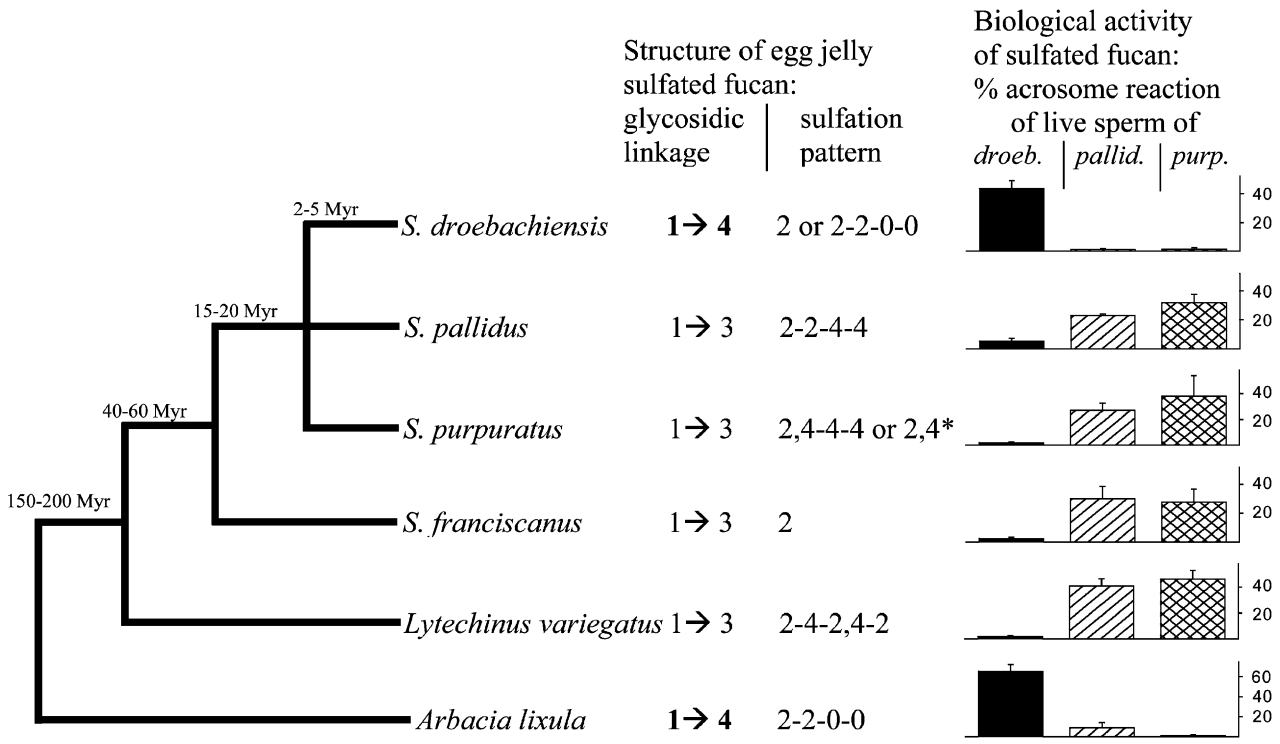

Fig. 6. Phylogenetic relationships and divergence times (Smith et al. 1995; Lee 2003) of sea urchin species in this study and a summary of the structural and functional properties of their egg-jelly sulfated fucans. *S.*, genus *Strongylocentrotus*; *droeb.*, *S. droebachiensis*; *pallid.*, *S. pallidus*; *purp.*, *S. purpuratus*. *In the second type of *S. purpuratus* fucan, about 80% of fucose-units are 4-sulfated.

S. purpuratus need 1 → 3-linked fucans. This constitutes an unusually clear-cut example of a biological event regulated by sulfated polysaccharides, specifically by the position of their glycosidic linkage. The specificity is particularly remarkable in the light of an increasing appreciation of the importance of carbohydrate ligands (Rosen 2004).

It is noteworthy that the egg envelope in mammals, the zona pellucida, contains both fucose and sulfate groups (Berteau and Mulloy 2003) and that the glycoprotein ZP3 is responsible for inducing the sperm AR in mice and humans (Wassarman 1990; van Duin et al. 1992). Given the limitations of studying mammalian fertilization in situ, the sea urchin fertilization system presented here may provide a model for studying the role of egg envelope carbohydrates in triggering the AR and lead to new insights in fertility and contraception.

In broadcast-spawning species with overlapping reproductive seasons, species diversity may be generated and maintained through gametic barriers to reproduction (Palumbi 1992; Vacquier 1998; Swanson and Vacquier 2002a,b; Geyer and Palumbi 2003). We demonstrated that even in seemingly simple free spawners, two independent physiologically different barriers can act to ensure species-specific gamete unions. Evidence from both fertilization experiments and sperm acrosome scoring reveals substantial species selectivity in the compatibility of egg jelly and sperm between *S. droebachiensis* and *S. pallidus*. This was unexpected because the AR occurs nonspecifically between other sea urchin species, including *Echinometra* spp. (Metz et al. 1994) and *S. purpuratus* and *S. franciscanus* (Minor et al. 1991; Cameron et al. 1996). It had therefore been assumed that the sperm binding step rather than the AR would generally be species specific, which is clearly not the case between *S. droebachiensis* and *S. pallidus*; these species cross-fertilize easily when their sperm are prereacted (Fig. 2b).

Induction of the sperm AR is not a limiting step in preventing hybrid fertilization of *S. purpuratus* eggs. *Strongylocentrotus pallidus* sperm are compatible with *S. purpuratus* egg-jelly sulfated fucans but do not fertilize their eggs. Neither do artificially prereacted *S. droebachiensis* sperm fertilize *S. purpuratus* eggs (Fig. 2). Another change in the gamete interaction process must be involved in this blockage, most likely divergent evolution of the acrosomal protein bindin (Minor et al. 1991) and its receptor (Kamei and Glabe 2003). Intriguingly, when divergent selection on the gene for bindin was examined, no evidence for positive selection between *S. droebachiensis* and *S. pallidus* was detected, but the protein had diverged significantly between *S. purpuratus* and *S. droebachiensis* and between *S. purpuratus* and *S. pallidus* (Table 1 in Biermann 1998).

The chance occurrence of the same sulfated α-L-fucan isotype in the egg jelly of two evolutionary distant sea urchins (Figs. 5 and 6) is not relevant for their cross-fertilization, because the populations of *A. lixula* and *S. droebachiensis* do not overlap geographically. However, it allows us to speculate about the evolution of these nonprotein gamete recognition molecules. *Arbacia lixula* and *S. droebachiensis* diverged 150–200 million years ago (Smith et al. 1995), whereas the three *Strongylocentrotus* species examined here (with their markedly different egg-jelly fucans) separated less than 5 million years ago (Biermann 1998; Biermann et al. 2003; Lee 2003). Therefore, the genes involved in the biosynthesis of the sulfated fucans and their sperm receptors (Vacquier and Moy 1997) did not evolve in concordance with evolutionary distance but underwent a dramatic change near the tip of the strongylocentrotid tree—along the branch leading to *S. droebachiensis*. This apomorphy in the egg-jelly recognition system causes reproductive isolation between *S. droebachiensis* and *S. pallidus*, closely related species that overlap ecologically and over a vast geographic range.

Hybrids between *S. droebachiensis* and *S. purpuratus* are less viable than homospecific offspring (Levitan 2002). Hybrids between *S. droebachiensis* and *S. pallidus* can be raised through sexual maturity (Strathmann 1981), but their fitness relative to nonhybrids is unknown. We have not observed obvious *S. droebachiensis*/*S. pallidus* hybrids in the field or by genotyping; hence, their reproductive isolation seems to be fairly complete. The AR specificity could have played a direct role in the separation of these species, or it could have resulted from reinforcement selection (Geyer and Palumbi 2003) if postzygotic factors lower hybrid fitness. It is not possible to demonstrate positive selection on carbohydrates statistically (as it is for DNA sequences). However, the rapid evolutionary change in the egg-jelly fucan in the lineage leading to *S. droebachiensis* (and its strong effect on reproductive isolation) suggests that it was subject to selection.

There is some evidence that *S. droebachiensis* and *S. pallidus* may have separated from *S. purpuratus* before their divergence from each other (Lee 2003). The bindin protein may have functioned as an isolating mechanism during the earlier separation of their joint lineage from *S. purpuratus*, and later the egg jelly–sperm incompatibility between *S. droebachiensis* and *S. pallidus* may have been correlated with a renewed speciation event.

Our findings underscore the importance of looking beyond one model organism and one process. Previous work has concentrated on *S. purpuratus* and on the function of sperm bindin as a determinant of fertilization specificity (Minor et al. 1991; Vacquier et al. 1995; Cameron et al. 1996; Glaser et al. 1999). We discovered that the sibling species of *S. purpuratus* use entirely different molecular and cellular mechanisms for the same purpose.

The compatibility and speciation scenario in North Pacific strongylocentrotids is extremely complicated. For example, *S. purpuratus* sperm fertilize a high proportion of *S. droebachiensis* eggs even though few of the sperm react to *S. droebachiensis* egg jelly, and eggs of *S. droebachiensis* are highly fertilizable in general (Levitan 2002; unpublished observations). There are additional compounds in the egg-jelly coat that aid sperm activation; both the peptide speract and a sialoglycan enhance the AR induction by sulfated fucans (Hirohashi and Vacquier 2002a). This explains the higher AR response to natural egg jelly over purified sulfated fucans even at lower fucose concentrations (Table 1 vs. Table 2 and Figs. 4–6).

Two additional strongylocentrotid species originated at around the same time when the *S. droebachiensis*, *S. pallidus*, and *S. purpuratus* lineages separated: *S. polyacanthus* and *Allocentrotus fragilis* (Biermann 1998; Biermann et al. 2003). Although all five differ in their current distribution and ecological preferences, the order and causes of the speciation events remain unresolved. Examining the two different fertilization barriers among this quintuplet of sibling species—as well as among more distantly related taxa—will reveal much about the importance of the egg-jelly and sperm-bindin recognition systems, respectively. This research, in combination with molecular studies of the egg-jelly molecules and their receptors and sperm bindin and its receptor, will further elucidate the evolutionary forces that affect gamete recognition molecules during and between cladogenetic events.

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