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Structural and functional insights into sulfated galactans: a systematic review

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Abstract Sulfated galactans (SGs) are highly anionic marine galactose-composed homopolysaccharides. Although their structures vary among species, their main features are conserved among phyla. Green algal SGs are quite heterogeneous, although preponderantly composed of 3-β-D-Gal*p* units. The red algal SGs (like agar and carrageen) are composed of repeating disaccharide units with different sulfation patterns which vary among species. The SGs from invertebrates such as sea urchins and ascidians (tunicates), and from the unique description of a sea-grass, are composed of well-defined repetitive units. Chains of 3-linked β-galactoses are highly conserved in some marine taxonomic groups, with a strong tendency toward 4-sulfation in algae and marine angiosperm, and 2-sulfation in invertebrates. These carbohydrates are extracellular components of the cell wall in plants, of the body wall in tunicates, and of the jelly coat in sea urchin eggs. In sea urchins, the SGs are also responsible to induce the acrosome reaction. However, the wide range of potential pharmacological uses, especially as anticoagulants and antithrombotics, is the main reason for the increasing interest in these sugars. Both natural and clinical actions of SGs have a direct relation to their structural features, since the intermolecular complexes between SG and target proteins are much more stereospecific than only electric charge-dependent. This review will present an overview about the principle structural and functional information of SGs. Other important aspects concerning occurrence, biology, phylogeny, and future directions, will also be reported.

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Abbreviations

aPPT	activated partial thromboplastin time
AR	acrosome reaction
AT	antithrombin
CAM	chorioallantoic membrane
FGF-2	fibroblast growth factor 2
HCII	heparin cofactor II
IC_{50}	inhibitory concentration 50%
SG	sulfated galactan
MW	molecular weight
5-Fu	Fluorouracil

The principle features concerning sulfated galactans: their occurrence and biology

One of the most studied marine sulfated homopolysaccharides is the sulfated galactans (SGs). Together with sulfated fucans, the SGs are, after glycosaminoglycans, the most widely studied sulfated polysaccharides worldwide. In general, the SGs are polymers of α-L- and/or α-D- or β-D-galactopyranosyl units [1]. The structures of these strongly anionic macromolecules vary among species, although their main structural features are conserved among phyla. They usually show high molecular weights (MWs) (≥100 kDa). These glycans posses highly electronegative charge density from their sulfated esters which allows them electrostatic interactions with specific proteins, triggering in consequence their biological effects. However, these intermolecular interactions have been usually stereospecific-dependent rather then a mere or simple consequence of charge interactions [2].

The occurrence of SGs is considerably restricted. They are commonly isolated from the cell wall of seaweeds, such as green (Clorophyta) or red (Rhodophyta) algae. Occasionally, they can be extracted from the egg jelly coats of certain sea urchin species (Echinodermata, Echinoidea), and from the outer tunics of ascidians (Urochordata, Ascidiacea). In addition, there is a single description of a SG isolated from a sea grass, a marine angiosperm (Angiospermae, Spermatophyta). Nevertheless, the SGs are exclusively synthesized by marine organisms [1].

In all of the cases above, SGs occur in high concentrations in the extracellular matrices, which resemble the amount of glycosaminoglycans in proteoglycans found in the extracellular matrices of mammalian connective tissues (especially cartilages).

However, marine SGs have their own structural particularities. Firstly, they are usually more sulfated (and consequently possess higher electronegative charge density) than vertebrate glycosaminoglycans such as chondroitin sulfate and dermatan sulfate, which contain usually one sulfate group per disaccharide unit. Perhaps, interactions between components of the extracellular matrix in marine organisms occur at higher salt concentrations than in vertebrates, and therefore require polysaccharides with higher charge density. Secondly, glycosaminoglycans from mammalian extracellular matrices have molecular masses only between ~15 and ~60 kDa. The covalent complex of these mammalian chains with the core protein results in a high molecular mass complex (>100 kDa). In contrast, SGs from algae and invertebrates are themselves high MW molecules. The attachment to a protein core still needs to be demonstrated and it is apparently irrelevant for the biological activities of this class of polysaccharide [1]. In sea urchin egg jellies, the SGs have masses >100 thousand Da. In addition, the SGs are exclusively from sea organisms, whereas the glycosaminoglycans can be found in marine organisms as well as highly isolated from terrestrial mammalian.

In addition to the SGs found in the extracellular matrices of algae, marine angiosperms, ascidians, the SGs from sea urchins are also localized in the hydrated, usually transparent, jelly layer surrounding the eggs. The sea urchin egg jelly is a complex extracellular matrix containing SG noncovalently linked to other sugars and many unknown proteins of both high and low molecular mass. As described below, the egg SGs are intimately involved in gamete recognition during sea urchin fertilization [3-6].

Unrelated to their natural biological roles as components of the biological wall and/or on the fertilization, the SGs show important and potent pharmacological actions in mammalian systems. These include antiviral [7-14], antitumoral [15-17], immunomodulation [15-17], antiangiogenic [18], anti-inflammatory [18], anticoagulant [13, 19-24], and antithrombotic [23-25] properties. Their beneficial effects on the cardiovascular system are the most studied and exploited clinical actions, especially due to the pressing need for new antithrombotic drugs as a consequence of the continuously increasing incidence of thromboembolic diseases [1]. Generally, the scientific and pharmaceutical interests on these particular sugars, especially due to these clinical applications, have increased significantly over the last 10 years.

Therefore, an overview about the structural and functional data of SGs is presented. Although innumerous articles describing the structure-function relationship of these marine polysaccharides are available, a general and specific review concerning only SGs is not found yet. We will discuss the structures of SGs related to their biological functions (natural roles as well as alternative pharmacological uses), to their phyla of occurrence, including a phylogenetic prospective discussion. Moreover, we will provide in this compilation, some future directions of the research on these particular glycans inside the general glycobiology field.

The preponderant 3-β-D-Gal*p*-1 unit in green algal polymers

Recently, SGs from green algae have been structurally analyzed, particularly those from the genus *Codium.* The *Codium* species have shown large amounts of galactose-composed polysaccharides in their cell wall, together with other heteropolysaccharides. In the past 2 years, the structures of SG isolated from the species *C. isthmocladum* [26] and *C. yezoense* [27] were reported. Both molecules were studied by a combination of chemical reaction analysis plus NMR spectroscopy, as commonly employed for structural determination of many SGs. Coincidently, the green algal SG from these two species exhibited similar backbones, composed preponderantly of 3-β-D-Gal*p*-1 units mainly 4-sulfated (as here exemplified for the polysaccharide of *C. isthmocladum*, Fig. 1a), plus minor amounts of other structures (Fig. 1b–d). These green algal sulfated polysaccharides are also highly pyruvylated at the non-reducing terminal residues forming cyclic ketals like 3,4-*O*-(1′carboxi)-ethylidene-β-D-Gal*p*-1 units (Fig. 1d). The green algal SGs seem to be more complex than those from red algae. Partial characterization of SGs from other green algal species is also available in the literature. SG from *C. fragile* and from *C. cylindricum* revealed heterogeneous polymers. In addition to galactose residues, *C. fragile* is also composed of arabinose residues (sulfated arabinogalactan) [28], and *C. cylindricum* of additional glucose residues [29]. However, the 3-linked 4-sulfated β-D-galactopyranosyl units have seemed to be the most

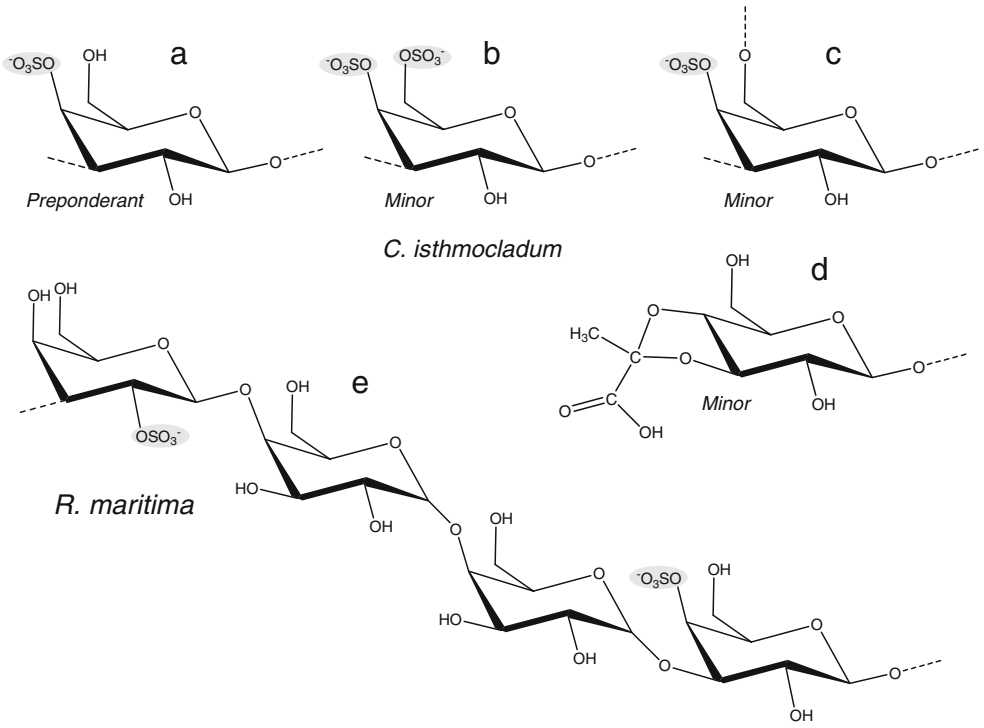

Fig. 1 Chemical structures of the SGs from green algae (**a**–**d**) and marine angiosperm (**e**). The components found in the SG from the green alga *Codium isthmocladum* are: **a** the preponderant unit 3-β-D-Gal*p*-4(OSO_3^-)-1, and minor amounts of **b** 3-β-D-Gal*p*-4,6di(OSO_3^-)-1, **c** 6-β-D-Gal*p*-4(OSO_3^-)-1, and **d** 3,4-*O*-(1-carboxy)-ethylidene-β-D-Gal*p*-1 from the non-reducing terminals [26]. The SG from the marine angiosperm *Rupia maritima* has the tetrasaccharide repeating structure, as following: [→3-β-D-Gal*p*-2(OSO_3^-)-1→4-α-D-Gal*p*-1→4-α-D-Gal*p*-1→3-β-D-Gal*p*-4(OSO_3^-)-1→]$_n$ [35]. All the sulfate groups are highlighted with the grey ellipse

dominant component in the quite heterogeneous backbone of SGs from green algae.

Red algal repeating disaccharide units with usual heterogeneous sulfation patterns: agar and carrageen

Marine SGs are widely abundant in red algae. Carrageenans and agarans are the most common SGs from this type of macroalgae. The origin of the name carrageenan derived from a small village, Carragheen, on the Irish coast, where the carrageenan-bearing seaweed *Chondrus crispus* or "Irish moss" grows [30]. The word agaran, name proposed by Kuntsen and coworkers [31], see also [32] originally derived from the word "agar", which means jelly in the Malay language (agar-agar). Both of these red algal polysaccharides usually have a linear backbone made of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues (Fig. 2a), showing a 'masked repeat' unit of disaccharides similar to the animal glycosaminoglycans. The β-galactoses are always D-enantiomers, whereas the α-galactose residues may be present in the D- or L-configuration [33]. A substantial portion may also exist in the form of 3,6-anhydro derivatives (Fig. 2b, e and g). Considerable structural variation in the red algal SGs occur among different species and in samples collected at different environments, or in different seasons of the year [22]. Furthermore, various hydroxyl groups may be substituted by a sulfate ester, a methyl group or pyruvic acid [33]. The major structural variation in these polysaccharides is the sulfation pattern. The sulfate distribution along the galactose-backbone is quite heterogeneous as in animal glycosaminoglycans, and the sulfate contents are markedly different between different species as depicted in the comparison between the SGs from the red algal species *Botryocladia occidentalis* and *Gelidium crinale* (Fig. 2a) [22].

Although the majority of red algal species express SGs with some heterogeneities, a minor number synthesizes homogeneous galactans, classically named as carrageenans and agarans. Carrageenans are traditionally classified by a Greek prefix according to their sulfation pattern (Fig. 2b–e) and the presence of 3,6-anhydro bridge (carrageenose) on the 4-linked α-D-galactose [34] (Fig. 2b and e). We will not discuss variations in the structures of this class of polysaccharide since this topic has been extensively covered in several other reviews [32-34]. The carrageenans and agarans are extensively exploited due to their industrial applications. The wide uses of these sulfated polysaccharides are based on their unique properties to form strong aqueous gels. These molecules are the major hydrocolloids used as texturing agents for food. A small change from α-D-galactopyranoses in carrageenans to α-L-galactopyranoses in agaran is enough to promote great changes in the physical-properties of these molecules [32]. Other modifications in the backbone of the SG can greatly change their physicochemical properties and, consequently, in industrial applications and biological activities. For example, high levels of 3,6-anhydro-α-L-galactopyranosyl units in agar group polysaccharides (also known as agarose, Fig. 2g) and low sulfate contents are the major structural requirements for gelling [32]. Several types of

these gels are widely exploited by industries in their attempt to obtain the best and specific gel formation under different conditions (regulated by temperature and the combination of ingredients) [32].

The unique description of a SG in a superior plant

A recent article reported at the first time a novel SG isolated from the sea grass *Rupia maritima* [35]. The sea grass is a group of vascular flowering plants (angiosperms) which grow in highly saline marine environments. The structure of the sulfated D-galactan from *R. maritima* is composed of a regular tetrasaccharide repeating unit (Fig. 1e). Like red algae, the marine angiosperm polysaccharide contains both α- and β-D-galactosyl isomers; however, these units are not distributed in an alternating sequence, and the angiosperm molecule has much clearer and well-defined structure. The SG in this marine plant unequivocally contributes for the structural arrangement of the cell wall, but it also seems to be physiologically involved in the osmotic regulation of the epidermal cells, once this plant is ecologically allocated in habitats with high salinity variations. The authors speculate about the influence of the sulfated esters of this polymer on the recruitment of salt ions from the sea water, thus contributing as a regulator of the osmosis in the plant [35].

The characteristic well-defined chemical structures in marine invertebrates

SGs can also be expressed in certain sea urchin species with a well-defined repetitive structure. For example, the species

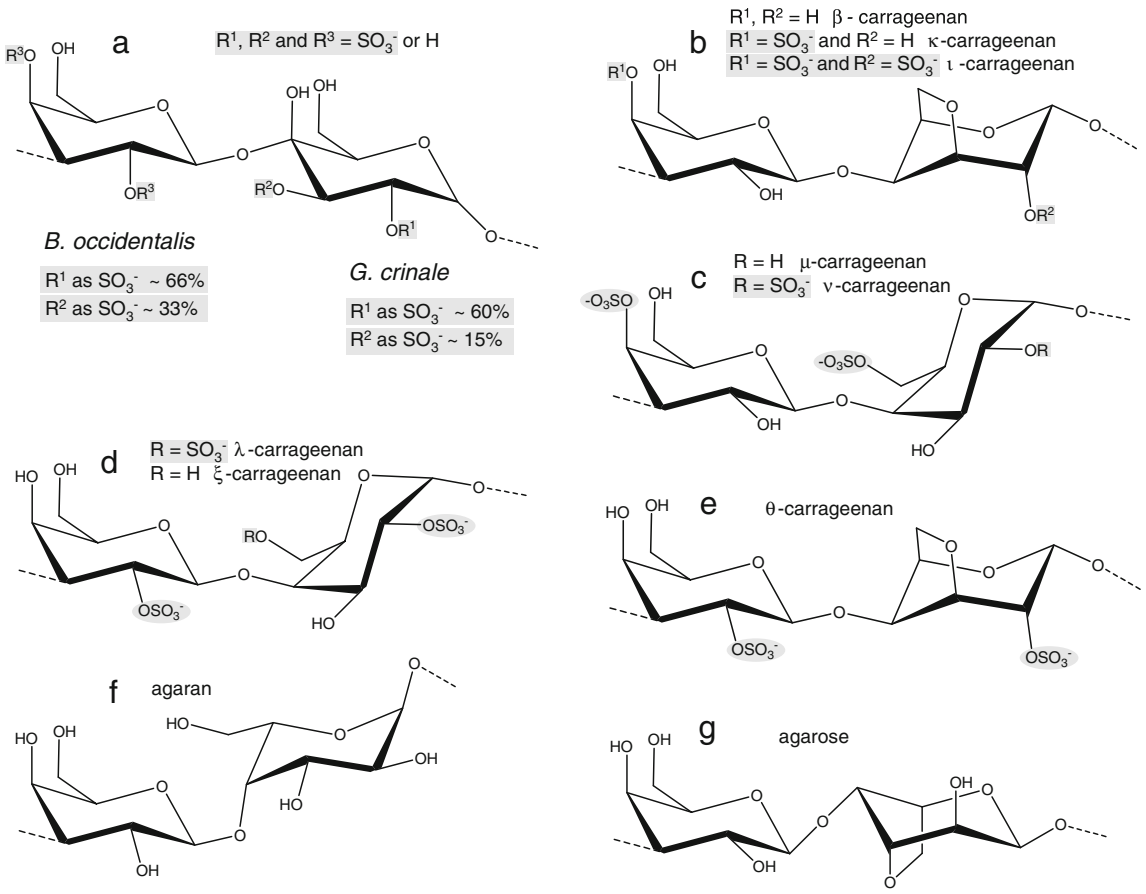

Fig. 2 Chemical structures of the repeating disaccharide units of the SGs from red algae. These units are composed mainly of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues. The species *Botryocladia. occidentalis* and *Gelidium crinale* (**a**) contain SGs which differ exclusively in sulfate contents [19, 22, 25]. The most common SGs from red algae are the carrageenans which differ in the sulfation patterns (**b**–**e**) and also in the replacement of the α-D-galactoses by 3,6-anhydro-α-D-galactose units (**b** and **e**). The structural variety of carrageenans are classified by Greek letters, such as (**b**) β- (beta), ι- (iota) and κ- (kappa), (**c**) μ- (mu) and ν- (nu), (**d**) λ- (lambda) and ξ- (xi) and (**e**) θ- (theta) carrageenan. For more details and carrageenan structures, see [32-34]. The polysaccharides composed of α-L-galactoses (**f**) or 3,6-anhydro-α-L-galactose units (**g**) are named agaran and agarose, respectively [32]. These polymers can also show different sulfation patterns. All the sulfate groups are highlighted in light grey

Echinometra lucunter express a 2-sulfated 3-linked α-L-galactan (Fig. 3a). The species *Glyptocidaris crenularis* exhibit a disaccharide repeating structure of alternating 2- and non-sulfated 3-linked β-D-galactan (Fig. 3b) [4]. This is the first report of a sulfated β-galactan in sea-urchin.

Another source of invertebrate sulfated α-galactans is the ascidians (commonly known as sea squirts or tunicates). The species *Herdmania monus* contains a sulfated polysaccharide composed of 3-sulfated, 4-linked α-L-galactopyranose residues (Fig. 3c) [36], while the SG from *Styela plicata* has a similar glycidic backbone structure with non-sulfated L-galactoses as branched units linked to the position *O*-2 of the central core (Fig. 3d) [37]. This was the first report of the L-enantiomeric form of galactose [37].

Similar to the SGs from algae and marine angiosperm, the invertebrate molecules are components of the extracellular matrix of these marine invertebrates, like the sulfated glycosaminoglycans in vertebrates [37]. The SGs in ascidians compose part of the outer tunic of these marine organisms [36-38]. In the case of the species-specific SGs from sea urchins, they are directly involved in the gamete recognition, triggering the acrosome reaction (AR), an important event for the fertilization, which will be described below [3-6].

Phylogenetic implications: how has the 3-linked β-galactose unit occurred in marine organisms throughout the course of evolution?

A comparison among SGs from different organisms indicates that the galactans with the glycosidic linkage β(1 → 3) are strongly conserved in some taxonomic groups of eukaryotes (rhodophytes, chlorophytes, angiosperms, echinoderms, and mollusks). The SGs found among these phyla differ mainly in sulfation sites, however with a strong tendency toward 4-sulfation in algae and marine angiosperms, and 2-sulfation in invertebrates. The 6-sulfation is dispersed in minor amounts throughout phylogeny. These observations provide grounds for speculation about the evolutionary history of SGs.

The occurrence of the 3-β-D-Gal*p*-1 unit in the SG from the sea urchin *G. crenularis* (Fig. 3b), and its presence in the major units of the SGs from green algae (Fig. 1a) [26, 27], and in sea grass [35] (Fig. 1e) stimulated us to review the distribution of this unit in polymers of the animal and plant kingdoms [39] in order to propose a phylogenetic relationship of this particular unit (Fig. 4). Although this comparison is based only on structural components of the SGs, which are products of action of several genes and biosynthetic enzymes, this taxonomic comparison might allow us to speculate whether there is a relationship among the marine organisms that express sulfated 3-β-D-Gal*p*-1.

Thus, the hypothetical cladogram (Fig. 4) shows that the sulfated 3-β-D-Gal*p*-1 units are preserved among species of specific phyla that inhabit the marine environment, including green algae, red seaweeds, marine sea grass (Angiospermae, Spermatophyta), invertebrates (sea urchins, clams, and tunicates) and vertebrates such as fishes that express keratan sulfate [43].

Although the 3-β-D-Gal*p*-1 unit has been preserved in the major phyla during evolution, the preferential sulfation site on this particular structure varies in a clear tendency toward 2-sulfation for animals, 4-sulfation for algae and marine angiosperms, and a highly dispersive distribution of 6-sulfation.

These observations raise the hypothesis that the galactosyltransferases responsible for the incorporation of 3-β-D-Gal*p*-1 units in the biosynthesis of SG have been maintained during evolution in specific phyla of marine

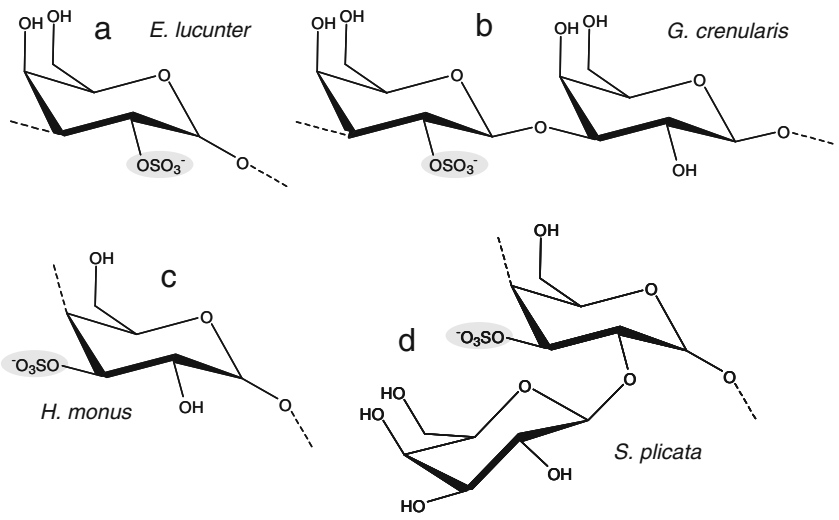

Fig. 3 Chemical structures of the repeating units of the SGs from the egg jelly coat of sea urchins (**a** and **b**), from the outer tunic of ascidians (**c** and **d**), and from red algae (**e**). The structures are the following: (A) *Echinometra lucunter* [→3-α-L-Gal*p*-2 (OSO_3^-)-1→]$_n$ [3]; (B) *Glyptocidaris crenularis* [→3-α-L-Gal*p*-2 (OSO_3^-)-1→3-α-L-Gal*p*-1→]$_n$ [4]; (C) *Herdmania monus* [→4)-α-L-Gal*p*-3(SO_3^-)-(1→]$_n$ [36] and (D) *Styela plicata* {→4)-α-L-Gal*p*-2[→1)- α-L-Gal*p*]-3 (OSO_3^-)-(1→}$_n$ [37]. All the sulfate groups are highlighted with in light grey

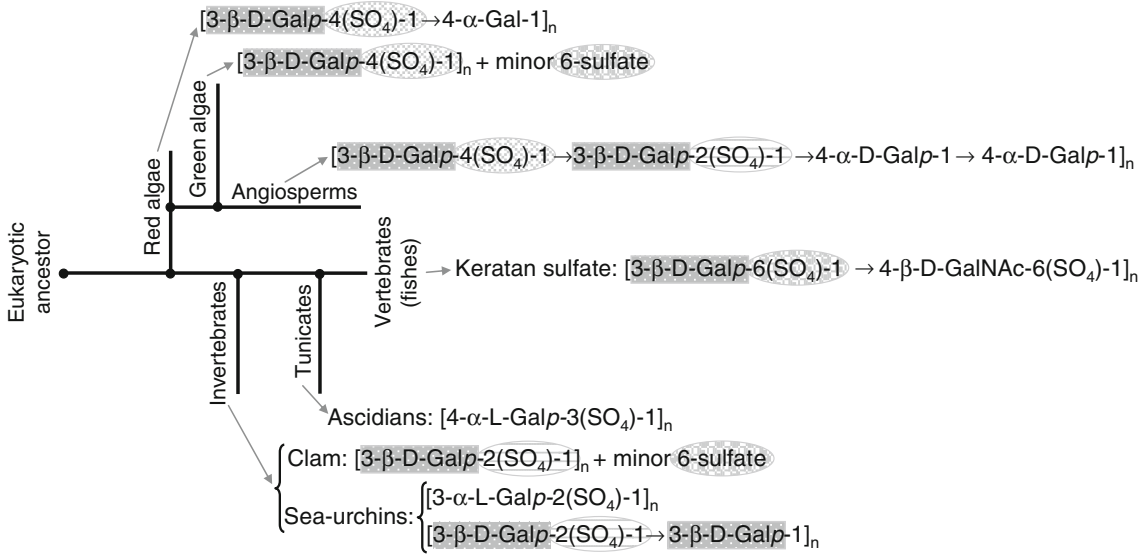

Fig. 4 Schematic phylogenetic tree showing the proposed relationship among SGs from marine organisms of different phyla. The structure 3-β-D-Gal*p*-1 is identified by dark gray boxes. The 2-, 4- and 6-sulfations are indicated with light gray stripped, solid and dotted ellipses, respectively. Red algae (Rhodophyta) exhibit SGs composed mainly of the sequence [3-β-D-Gal*p*-1→4-α-D-Gal*p*-1]$_n$ [19, 22]. Most of them are composed of 4-α-D-3,6-AnGal*p*-1 (3,6-anidrogalactose residues) and 3-β-D-Gal*p*-4(SO_4)-1, as found in carrageenans, the most common sulfated polysaccharides from red algae [32]. The preponderant residue of the SGs from green algae (Clorophyta) is 3-β-D-Gal*p*-4(OSO_3^-)-1 [26, 27]. The marine angiosperms (Angiospermae, Spermatophyta) exhibit the repeating sequence: [3-β-D-Gal*p*-4(OSO_3^-)-1→3-β-D-Gal*p*-2(OSO_3^-)-1→4-α-D-Gal*p*-1→4-α-D-Gal*p*-1]$_n$, comprising structural features of algal and invertebrate sulfated polysaccharides [35]. In invertebrates, the SGs from two species of sea urchins (Equinodermata, Echinoidea) *E. lucunter* [3] and *G. crenularis* [4] exhibit repeating sequences of [3-α-L-Gal*p*-2(OSO_3^-)-1]$_n$ and [3-β-D-Gal*p*-2(OSO_3^-)-1→3-β-D-Gal*p*-1→]$_n$, respectively. The clam *Meretrix petechialis* (Mollusca, Bivalvia) has a polysaccharide composed of the backbone 3-β-D-Gal*p*-1, mainly 2-sulfated and to some extent 6-sulfated [40]. Some species of ascidians (Urochordata, Ascidiacea) *H.* monus, *S.* plicata and Ascidia *nigra*, *Clavelina oblonga* [36, 38, 41, 42 respectively] exhibit the unit [4-α-L-Gal*p*-3(OSO_3^-)-1]$_n$. The glycosaminoglycan keratan sulfate can be found in minor amounts as [3-β-D-Gal*p*-6(OSO_3^-)-1→4-β-D-GalNAc-6(OSO_3^-)-1]$_n$ such as fishes (Teleostei, Chordata) [43]

organisms, but were allowed to vary in the distribution of sulfotransferases types. In favor of this hypothesis is the evidence that the basic backbones are the same, but with a variable position of sulfation that differs from species to species. To some extent, these results are analogous to the biosynthesis of the glycosaminoglycans from vertebrates, where the glycosidic chains vary relatively little among polymers constructed in different tissues, organs, and species. Modifications on the glycosidic core occur mostly after chain elongation, when the principal modification is the sulfation at different sites. Unfortunately, the biosynthesis of the SGs from marine organisms is virtually unknown, and therefore, it is not yet possible to compare the expression of these molecules. The alternative, and just as likely hypothesis, is that the presence of these SGs in such distantly related organisms is an example of independent, convergent evolution of biosynthetic pathways. Although this hypothesis is not based on the gene sequence, not even on the sequence of proteins, and still requires future work to propose a firm and definitive theory, it really offers a plylogenetic perspective in order to predict some tendencies about structural particularities of SGs related to some phyla of marine organisms.

Induction of the AR in sea urchin sperm: a physiological role

A necessary event for the sea urchin fertilization is the sperm AR. The sea urchin AR involves the calcium-triggered exocytosis of the acrosome vesicle and the pH-induced polymerization of actin to form the ~1 μm long, finger-like, acrosomal process which protrudes from the anterior of the sperm head [44]. When sperm approaches the sea urchin egg, the SG binds to sperm receptors, which are homologs of human polycystin, the protein mutated in autosomal dominant polycystic kidney disease [45]. At least two pharmacologically distinct calcium channels open to allow calcium influx from the seawater [46, 47]. The internal pH of the sperm also rises about 0.25 pH units due to sodium/proton exchange [48]. Both the calcium influx and pH rise are required for AR induction. The AR exposes the protein bindin, which coats the acrosome process at the anterior tip of the sperm. The bindin attaches the sperm to the EBR1 receptor on the egg suface. Sperm bindin mediates both the species-specific attachment of sperm to egg and also the fusion of the plasma membranes of the two gametes [49-52]. The sequences of bindins are species-

specific and have been shown to be subjected to positive selection [53].

The purified sulfated polysaccharides of sea urchin egg jellies (especially SGs), devoid of any detectable protein, will by themselves induce the sperm AR [3-6]. Induction by the sulfated polysaccharide is potentiated by a polysialic acid containing-"sialoglycan" also isolated from egg jelly [54].

One reason it is important to investigate the molecular details of AR induction by the egg jelly sulfated polysaccharides is because it is very rare for a pure carbohydrate to induce a signal transduction event in animal cells. The demonstration that sulfated polysaccharides induce the sperm AR in a species-specific manner was extensively discussed in other specific reports: [1, 2, 5, 6].

A broad range of pharmacological application

The marine SGs exhibit potential pharmacological effects in mammalian systems such as antiviral, antitumoral, imunnomodulation, antiangiogenic, antiinflamatory, anticoagulant and antithrombotic activities. This is the most important reason for the continuous rise of interest in both academic and pharmaceutical fields on the research of these particular glycans. Although the invertebrate SGs have much more regular and well-defined chemical structures (Fig. 3), and they allow the best structure-function relationship studies, mainly the algal SGs (Figs. 1 and 2) have been chosen for the general clinical tests. However, invertebrate SGs are those that have showed the most interesting data about anticoagulant and antithrombotic properties, the most studied and desirable pharmacological uses for SGs.

The structure-antiviral effect relationship of red algal SGs against Herpes viruses have been reported in several papers. The herpetic activities of red algal SGs are the most studied antiviral activity of marine galactose-composed homopolysaccharides [7-13]. Mainly, these compounds were shown to interfere with the initial adsorption of viruses to the host cells. Matsuhiro and coworkers have performed a structural analysis and an antiherpetic activity for the SG from the red seaweed *Schizymenia binderi* (Gigartinales, Rhodophyta). They showed that this SG is composed by sulfated groups mainly at positions *O*-2 of 3-linked β-D-galactopyranosyl residues and at position *O*-3 of 4-linked α-galactopyranosyl units, where the latter residues are partially glycosylated at position *O*-2. The SG from *S. binderi* exhibited highly selective antiviral against Herpes simplex virus types 1 and 2, with selectivity indices (ratio cytotoxicity/antiviral property) >1,000 for all assayed virus strains [7].

Similarly, Talarico and coworkers have demonstrated that the SGs from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata* lacked cytotoxic effects in Vero cells and showed a broad spectrum of antiviral activity against HSV-1 and HSV-2 with inhibitory concentration 50% (IC_{50}) values in a good range of 0.5–5.6 μg/mL. Most important from this work is that a significant protection against a murine vaginal infection with HSV-2 was afforded by topical treatment with these algal SGs [8].

The chemical investigation with antiherpetic correlation was also carried out for the high MW SG from the red alga *Gracilaria corticata* (Gracilariaceae, Rhodophyta). Most of the sulfate groups of this SG is located at C-4 of (1-3)-linked D-galactosyl units and C-6 of the (1-4)-linked L-galactose residues. This alga also exhibited in its cell wall an agar polymer with methyl groups at C-6 of its (1-3)-linked D-galactosyl units and at C-2 of the (1-4)-linked L-galactose residues. Bioassays showed that the high MW-SG exhibited selective antiherpetic activity against virus types 1 and 2 [9].

Although Duarte and coworkers showed that the SGs from the red alga *Bostrychia montagnei* have anti-herpetic properties correlated to the MW and sulfate content of the polysaccharides [10], another work from the same author [11] showed that the anti-herpetic activity of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) has a restrict activity correlated to the types of units of the backbone. The 3-linked β-D-galactopyranoses are highly substituted with sulfate groups on C-2 (28–30%), sulfates on C-2 and 4,6-*O*-(1′-carboxyethylidene) groups (9–15%), and only the C-2 sulfate groups (5–8%) with small amounts of C-6 sulfate, 6-*O*-methyl, and non-substituted residues, while the 4-linked α-galactoses are formed mainly by 3,6-anhydro-α-l-galactose (15–16%) and its precursor, α-l-galactose 6-sulfate (10–17%), together with lesser amounts of 3,6-anhydro-α-l-galactose 2-sulfate, α-l-galactose 2,6-disulfate, α-l-galactose 2,3,6-tri-sulfate, α-l-galactose 2,6-disulfate 3-xylose, 2-*O*-methyl-α-l-galactose, and unsubstituted α-l-galactose.

Chattopadhyay and coworkers have also studied the structure-function relationship of the the SG from *Gracilaria corticata*. They reported that the sulfate groups located at C-4 of (1-3)-linked galactopyranosyl residues of the SG appear to be very important for the anti-herpetic activity of the polysaccharide [12]. Some carrageenans have also promising anti-herpetic activities as reported for the work with the tetrasporic *Stenogramme interrupta* (Phyllophoraceae). This red alga synthesizes zeta- and lambda-carrageenans [13].

The antiviral actions of the sulfated carrageenans (kappa, iota, nu) from the red seaweeds *Gymnogongrus griffithsiae* and *Crytonemia crenulata* against four serotypes of dengue virus (DENV) were also described in another work of Talarico [14]. Both seaweed derivatives were selective inhibitors of DENV-2 multiplication in

Vero cells with IC_{50} values around 1 microg/ml and selectivity indices >1,000. The compounds had a lower antiviral effect against DENV-3 (IC_{50} values in the range 13.9–14.2 microg/ml), an even lower effect against DENV-4 (IC_{50} values in the range 29.3 to >50 microg/ml) and were totally inactive against DENV-1 [14].

Zhoug and coworkers have demonstrated in some articles the *in vivo* antitumor and immunomodulation activities of lambda-carrageenan from the red seaweed *Chondrus ocellatus*, an important economic alga in China [15-17]. These authors showed that the native lambda-carrageenan, its low-MW derivatives and co-administration with 5-Fu (a pyrimidine analog, which is largely used as a drug in the treatment of cancers) has great beneficial effects on S180 and H-22 tumors. The anti-cancer effects of these compounds were determined by the weight of immune organ, proliferation ratio of lymphocyte, concentration of Tumor Necrosis Factor-alpha (TNF-α) as well as hystopathology of tumors from transplanted S180 or H-22 tumor mices. The results indicated that the degraded lambda-carrageen could enhance the antitumor activities of 5-Fu and improve the immunocompetence damaged by 5-Fu.

A highly sulfated branched β(1→3)linked galactan was prepared from the arabino-galactan from Larix decidua Miller by partial hydrolysis and subsequent chemical sulfonation, in the work of Bürgermeister [18]. This compound named LaPSvS1 exhibited high *in vivo* anti-angiogenic and antiinflamatory effects in two different modifications of the known CAM-assay. The LaPSvS1 interacts with the fibroblast growth factor 2 (FGF-2) system, and this interaction is reported to be correlated with the potent inhibitor effect of LaPSvS1 on FGF-2 induced angiogenesis and inflammation, since inflammation and angiogenesis are codependent in this mechanism [55].

Indeed, the anticoagulant and antithrombotic properties of SGs are the most studied and desirable pharmacological uses of SGs due to the pressing need for new drugs as a consequence of the increasing incidence of thromboembolic diseases—cardiovascular diseases are the leading cause of death (30% of total) in the world [1, 2]. In addition, heparin preparations have several limitations due to collateral effects and limited source of material [23], once this glycosaminoglycan is widely used for the treatment and prevention of arterial and venous thrombosis [56]. The situation was even more complex recently, because of the alarming notification that heparin preparations have been contaminated with oversulfated chondroitin sulfate [57]. This contaminant induces hypotension associated with kallikrein release when administered by intravenous injection [58].

Red algal SGs [22] and green algae [29] have been known for some time to act as modulators of coagulation. Most of their activities are mediated by both antithrombin (AT) and heparin cofactor II (HCII), although there is a particular case of a SG from a specific green alga that exhibits a serpin-independent anticoagulant effect, possibly due to the inhibition of fibrin polymerization [29]. However, relatively few studies have interpreted the biological activity of SGs in terms of a molecular structure.

A test of red algal SGs from *B. occidentalis* and from *G. crinale* (Fig. 2a) on animal models of venous thrombosis revealed that these polysaccharides have a serpin-dependent anticoagulant activity due to inactivation of thrombin and factor Xa (Table 1). But these polysaccharides have also a pro-coagulant effect due to activation of factor XII. As a consequence of their anti- and pro-coagulant actions, the algal SGs differ in their venous antithrombotic activities in a sulfation pattern-dependent way, as will be described in details below. It was noteworthy that the algal SGs have no hemorrhagic effect even when tested at high doses [25].

The attempts to identify in the algal polysaccharide structural features necessary for their anticoagulant activity have been limited by the fact that algal SGs are heterogeneous mostly on their sulfation patterns. Only in the cases for the SGs of the two red algal species above (Fig. 2a) have shown that the occurrence of 2,3-di-sulfated α-galactose units is a critical structural motif in promoting the interaction of the polysaccharide with the plasma

Table 1 Anticoagulant activities of marine invertebrate and algal SGs measured by APTT[a] and by IC_{50} for thrombin (IIa) and factor Xa inhibition in the presence of antithrombin (AT) or Heparin Cofactor II (HCII) [2]

Polysaccharide	Source	Structure (Fig.)	APTT (IU/mg)	IC_{50} (μg/mL)		
				IIa/AT	IIa/HCII	Xa/AT
Sulfated α-L-galactans	*E. lucunter*	3A	20	3	6	20
	H. monus	3C	~2	>500	>500	>500
	S. plicata	3D	<1	>500	>500	>500
Algal sulfated galactans	*B. occidentalis*	2A	93	0.02	1.1	2.5
	G. crinali		65	0.02	25	1.5

[a] The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg)

protease and the serpins [22]. Obviously, the identification of specific structural requirements in the algal polysaccharides necessary for interaction with coagulation cofactors is an essential step for more rational development of anticoagulant drugs. However, the availability of SGs with well-defined structures as shown for the invertebrate pattern of molecules (Fig. 3), and the possibility to compare their effects in a variety of *in vivo* models of experimental thrombosis can open new perspectives for the development of SGs as therapeutic reagents. This is the particular case of the active anticoagulant SG from the sea urchin *E. lucunter* (Fig. 3a, Table 1).

Special remarks concerning the structure-function relationship of SG

The biological activities of the SGs are a function not so much of charge density, but of specific fine structural features, including the nature of the polysaccharide backbone and the specific patterns of sulfation. This is clearly the case of the molecular interactions of the sea urchin sperm SG-receptor and the SG from the sea urchin egg jelly coat, which will trigger the AR in a very extremely species-specific manner, see reviews [1, 2, 5, 6]. In the same way, the pharmacological effects of SGs have also shown to be highly stereospecific. The intermolecular interactions between SGs and target proteins are directly related to the structural features of the glycans (anomeric configuration, sulfation pattern, position of the glycosidic bond, conformational binding preference). For an overview about the structure-function relationship of SGs (and sulfated fucans), see [2]. Below, we will illustrate two cases describing how the structure-function relationship of SGs can be accomplished. The first example is a sulfation pattern-dependent effect on blood coagulation-inhibition, and the other one, is an anomeric configuration-dependent case of species-specific AR.

For example, through a systematic comparison of the different structures of SGs, we can notice that the SGs from the two red algal *B. occidentalis* and *G. crinale* exhibit identical backbone, same chain size but slight differences in their sulfation patterns (Fig. 2a). As a consequence of these differences these two SG differ in their anticoagulant and venous antithrombotic activities as described by Fonseca and coworkers [25]. The SG from *G.crinale* exhibits pro-coagulant and pro-thrombotic effects in low doses (up to 1.0 mg/kg body weight), but in high doses (>1.0 mg/kg) this polysaccharide inhibits both venous and arterial thrombosis in rats. In contrast, the SG from *B. occidentalis* is a very potent anticoagulant and antithrombotic compound in low doses (up to 0.5 mg/kg body weight), inhibiting venous experimental thrombosis, but these effects are reverted in high doses. Conversely, arterial thrombosis is only inhibited at high doses (>1.0 mg/kg) of the polysaccharide from *B. occidentalis*. These results indicate that slight differences in the proportions and/or distribution of sulfated residues along the galactan chain may be critical for the interaction between proteases, inhibitors and activators of the coagulation system, resulting in a distinct pattern in anti- and pro-coagulant activities and in the antithrombotic action. As summarized in Table 1, these structural differences account for 30% of difference at the anticoagulant activity (aPTT) of these algal macromolecules, especially on the catalytic effect of the sulfated polysaccharide over the HCII.

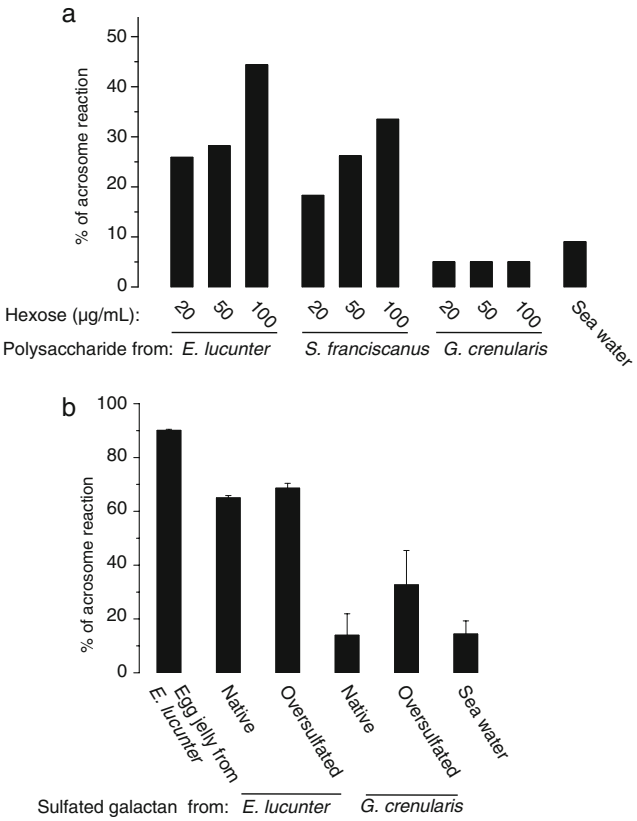

Fig. 5 Effects of native (**a**) or oversulfated (**b**) 2-sulfated α-L-galactan, α-L-fucan and β-D-galactan from the egg jellies of *E. lucunter*, *S. franciscanus* and *G. crenularis*, respectively, as inducers of acrosome reaction (**a** and **b**) of *E. lucunter* sperm. These polysaccharides are identically 3-linked and 2-sulfated but differ in their monosaccharides (galactose or fucose) and/or anomeric configuration (α- or β-form). The SG from *G. crenularis* (Fig. 3b) has an extra non-sulfated residue more than the SG from *E. lucunter* (Fig. 3a). Induction of the acrosome reaction: sulfated polysaccharides and lyophilized egg jelly were dissolved in sea water, incubated with sperm from *E. lucunter* and the acrosome reaction was detected using fluorescence phalloidin. Negative control was done with artificial sea water. Approximately 100-150 sperm were scored per data point. The concentrations of polysaccharides were normalized by hexose content. In Panel B a fixed concentration of sulfated polysaccharides (100 µg/mL) was used in the assays [4]

This same systematic comparison between biological actions of SGs with different structures can also be done to check the species-specific or crossed fertilization of sea urchins, as here exemplified for the sperm AR assay of the species *E. lucunter* (Fig. 5) [4]. This species expresses in the egg jelly coat of its female gametes, a unique 2-sulfated 3-linked α-galactan (Fig. 3a). Sperm from *E. lucunter* were equally sensitive to homologous 2-sulfated 3-linked α-galactan and intriguingly to the heterologous 2-sulfated 3-linked α-fucan from *Strongylocentrotus franciscanus*, but not to the 2-sulfated 3-linked β-galactan from *G. crenularis* (Fig. 3b), even when the polysaccharide was tested at high concentrations (Fig. 5a). This indicates that the sperm receptor for the egg jelly SG of *E. lucunter* does not differentiate between the CH_2OH of L-galactose and CH_3 of L-fucose at position-6. These two polysaccharides present the same sulfation pattern and position of glycosylation, but differ in the sugar moieties. In a similar way, sperm from *S. franciscanus* were sensitive to the homologous sulfated α-fucan and to the heterologous *E. lucunter* sulfated α-galactan [4]. In spite of that, *E. lucunter* sperms markedly distinct between sulfated α- and β-galactans. Another plausible reason for the absence of effect of the sulfated β-galactan on *E. lucunter* sperm is its reduced charge density compared with the homologous polysaccharide (0.5 *vs* 1.0 sulfate/monosaccharide). This doubt was removed through the investigative aspect of the AR of chemically oversulfated galactans. In the 2-sulfated, 3-linked α-galactan from *E. lucunter*, the 4- and 6-positons are the only ones available for further sulfation. Oversulfation of this polysaccharide did not change its responsiveness to homologous sperm (Fig. 5b), indicating that increased sulfates do not increase or inhibit the biological activity. In contrast, oversulfated β-galactan from *G. crenularis* induced acrosome reaction in *E. lucunter* sperm but at a significantly lower potency compared with the homologous α- galactan [4]. Therefore, the anomeric configuration of the glycosidic linkage is really the preferential structural requirement for the effect of these SG on the sea urchin AR. The α- or β-forms of the galactose rings are more effectible for this particular case than the dominant influence of electric charge density of the backbone.

Major conclusions and perspectives

SGs are widespread homopolysaccharides in marine organisms. These carbohydrates vary from species to species, but their major structural features tend to be conserved in each phylum. They have structural functions as components of the extracellular matrix of algal and invertebrate tissues. They are also responsible for the induction of the AR in the sea urchin sperm in a species-specific manner. This biological event triggers the fertilization process of these marine organisms. The algal and invertebrate SGs can also be assayed as alternative pharmacological agents and represent a potential source of phyto-active compounds for clinical purposes. The anticoagulant and antithrombotic effects are indeed the most studied pharmacological actions. Although the SGs offer promising results on this particular field of the medicine, these molecules still require accurate *in vitro*, *in vivo*, and clinical evaluations prior to their uses as drugs. However, the data herein presented revealed that in general, the biological actions of SGs do not simply depend on their negative charge density, but are also directly influenced by their specific structural features.

Further characterization of the action of these polysaccharides involves several challenges. A possible route to follow is the characterization of their binding to target proteins. With respect to their pharmacological actions, the study of the interaction between SGs with invertebrate well-defined structures and human purified proteins is especially attractive. The conformation of the polysaccharide by computational modeling such as molecular dynamics, and/or advanced Nuclear Magnetic Resonance techniques such as STD (saturation transfer difference), RDC (residual dipolar coupling), and trNOE may help to clarify the molecular interactions between SGs and target proteins. Certainly, studies of the specific interaction between the sea urchin egg SGs and the sperm receptor will define the regulation of sea urchin fertilization on a more refined molecular basis.

Possibly, the greatest challenge at this time is the identification of the metabolic pathways involved in the biosynthesis of the invertebrate SGs, especially those from sea urchins. This is not only a fascinating challenge in the glycobiology field, but may help to define the genetic basis for the SGs mechanism of species recognition in sea urchin fertilization. This metabolic accomplishment will also help to enforce the phylogenetic relationship presented here.

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