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The nematode *Caenorhabditis elegans* as a model to study the roles of proteoglycans

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The nematode *Caenorhabditis elegans* is a powerful animal model for exploring the genetic basis of metazoan development. Recent genetic and biochemical studies have revealed that the molecular machinery of glycosaminoglycan (GAG) biosynthesis and modification is highly conserved between *C. elegans* and mammals. In addition, genetic studies have implicated GAGs in vulval morphogenesis and zygotic cytokinesis. The extensive knowledge of *C. elegans* biology, including its elucidated cell lineage, together with the completed and well annotated DNA sequence and availability of reverse genetic tools, provide a platform for studying the functions of proteoglycans and their GAG modification.
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The free-living nematode *Caenorhabditis elegans* has been recognized as a valuable model system for the study of the roles of proteoglycans during development of multicellular organisms. This is because of the available knowledge of its development and physiology, as well as its complete genome sequence [1]. In addition, reverse genetic approaches, such as dsRNAi, allow robust inactivation of specific genes [2]. *C. elegans* is easily grown in the laboratory and develops in 3 days from an embryo to a 1 mm long adult. This fact has allowed recent biochemical studies that have revealed the structure of several glycosaminoglycans (GAGs) in *C. elegans*, suggesting that the molecular pathways leading to the biosynthesis of these complex polysaccharides are largely conserved in multicellular eukaryotes although some important differences have also been revealed.

The physiological role of proteoglycans in *C. elegans* development has been established from the study of mutants isolated by classic genetic screens. These were designed to understand specific biological processes, regardless of the nature of the genes involved.

The *sqv* (squashed vulva) genes define a proteoglycan biosynthetic pathway

The development of the *C. elegans* vulva is a powerful model that has been used to study a variety of processes related to morphogenesis and signaling events [3]. Invagination of specific epithelial cells during the third (L3) and fourth (L4) larval stages creates a tube connecting the outer epithelium to the layer of epithelial cells that enclose the uterus. In a screen for mutations that affect vulval invagination, Herman et al. isolated a series of 25 mutants, corresponding to eight genes, in which proper invagination is disrupted. These genes were called *sqv-1* to *sqv-8* (squashed vulva) [4]. In addition to causing a defect in vulval invagination, loss of *sqv* gene function causes a severe reduction in hermaphrodite fertility. Moreover, some of the *sqv* mutants produced eggs that arrested during embryogenesis, suggesting that these genes are required maternally for embryonic development [4].

The molecular identification of the *sqv* genes revealed homology to enzymes involved in glycosylation [5]. Subsequent *in vitro* and *in vivo* biochemical analyses showed them to be specifically involved in a proteoglycan biosynthetic pathway. SQV-7 is a multi-transmembrane protein, similar to members of a family of nucleotide sugar transporters [6]. Heterologous expression followed by *in vitro* biochemical analyses showed that SQV-7 is a novel nucleotide sugar transporter, able to translocate UDP-glucuronic acid (GlcA), UDP-N-acetylgalactosamine (GalNAc) and UDP-galactose (Gal) [7]. SQV-3 and SQV-8 have Gal transferase I and GlcA transferase I activities, respectively, based on their ability to transfer the corresponding sugar to the appropriate substrates *in vitro* and to complement corresponding mammalian cell mutants [8].

Consistent with the demonstrated enzymatic activities, biochemical analyses of *C. elegans sqv-3* and *sqv-8* mutants

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revealed reduced levels of chondroitin and abnormal profiles of chondroitin-modified proteoglycans, while *sqv-7* mutants display defects in both chondroitin and heparan sulfate biosynthesis *in vivo* [8].

The molecular identities of the three *sqv* genes, together with the biochemical studies on *sqv* mutants strongly suggest that the molecular defect responsible for their phenotype is related to GAG biosynthesis. The multiple substrate specificity of SQV-7 also suggests that it may be involved in the biosynthesis other Gal- or N-acetylgalactosamine (GalNAc)-containing glycans. The recent finding in *C. elegans* of nucleotide sugar transporters with specificities that partially overlap with that of SQV-7 opens the possibility that the expression of nucleotide sugar transporters is spatially and temporally regulated (P. Berninsone and C. Hirschberg, unpublished results).

The molecular identity of additional *sqv* genes support the model in which the SQV proteins would act in the biosynthesis of chondroitin and heparan sulfate. The *sqv-1, -2, -4, and -6* genes encode UDP-GlcA decarboxylase [9], Gal transferase II [10], UDP-glucose (Glc) dehydrogenase [11], and xylosyl (Xyl) transferase [10] respectively.

Heparan sulfate-modifying enzymes have important roles in neuronal development

Development of the nervous system requires complex interactions mediated by molecular cues which through many signaling pathways ensure that axons extending over variable distances make connections with their appropriate synaptic partners. Kallman syndrome is a genetic neurological disorder causing various behavioral and anatomical defects. The molecular lesion of the X-linked form of the disease affects a secreted protein expressed in specific areas of the brain that is similar to neural adhesion molecules [12,13]. Bullow et al. [14] found that mis-expression of the *C. elegans* homolog of KAL-1 causes axon branching and misrouting phenotypes. In a suppressor screen, several loci that either suppress or enhance the *kal-1*-induced axonal defects, were isolated. One encodes the only worm homolog of vertebrate heparan 6-O-sulfotransferase (*hst-6*) [14] and a second one encodes a GlcA C5 epimerase (*hse-5*) (O. Hobert, personal communication). These results suggest that heparan sulfate modifications are relevant to neuronal development, and specifically that KAL-1 activity requires a heparan sulfate accessory factor.

Structural analysis of *C. elegans* glycosaminoglycans

Recent studies have demonstrated that GAGs of the chondroitin and heparan sulfate types are present in *C. elegans* with structural features similar to both *Drosophila* and vertebrate GAGs. Analysis of enzymatically released disaccharides, derived from worm GAG fractions, revealed considerable amounts of non-sulfated chondroitin and significantly lower amounts of heparan sulfate [15,16]. Structural analysis identified the major disaccharide released by chondroitinase ABC as ΔHexAα1-3GalNAc; enzymatic analysis indicated that this non-sulfated unsaturated disaccharide is derived from chondroitin, which is composed of $-$4GlcAβ1-3GalNAcβ1- units, rather than from dermatan which contains $-$4IdoAα1-3GalNAcβ1- units [15,16]. No additional evidence of sulfated disaccharides was obtained in these studies. Other studies, however, provide evidence consistent with the existence of a chondroitin sulfate-dermatan sulfate co-polymer in *C. elegans* [17,18]. The reason for these differences is not clear and may be related to the differences in the preparations used.

The disaccharide composition of the less abundant *C. elegans* heparan sulfate fractions contained non-sulfated ΔHexAα1-4GlcNAc (50%), as well as ΔHexAα1-4GlcN(NS) (14–18%), ΔHexA(2S)α1-4GlcN(NS) (18%), ΔHexA(2S)α1-4GlcN(NS, 6S) (11–16%) and ΔHexAα1-4GlcNAc(6S) (6%) [15,16].

Recent studies suggest that GAG chains in *C. elegans* are synthesized on the core proteins via the conventional linkage region tetrasaccharide sequence found in other organisms [19, 20]; therefore the functions of this region in GAG synthesis appear to have been conserved during evolution. *C. elegans* chondroitin is synthesized as proteoglycans and covalently bound probably to a Ser residue on the core protein by a non-sulfated linkage region. The hexasaccharide (-GlcAβ1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-) [19] and pentasaccharide (GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-) [20] were independently isolated and structurally characterized. Although a linkage region was not detected for heparan sulfate, this fact may be due to the limited amount of heparan sulfate in this organism.

Other linkage region structures cannot be ruled out in *C. elegans*. The possibility of short chondroitin-like chains directly linked to the protein backbones through an O-linked GalNAc has been postulated [20]. Substantial amounts of oligosaccharides with structures typical of the chondroitin repeating region (GalNAcβ1-4GlcAβ1-3GalNAc-ol; GlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc-ol and GalNAcβ1-4 GlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc-ol) were isolated using mild alkaline conditions, in which degradation of chondroitin chains is unlikely [20]. The possibility that these oligosaccharides were released by β-elimination directly from the peptide backbone of core glycoproteins suggests that at least some of *C. elegans* chondroitin chains may be linked to the core protein through a GalNAc.

In contrast with linkage regions from other organisms, no phosphorylated Xyl or sulfated Gal were detected in the linkage region of *C. elegans* chondroitin structures [19,20]. This finding suggests that the glycosyltransferases involved in the biosynthesis of chondroitin in this organism have different specificities than those of higher organisms.

How conserved are the biosynthetic mechanisms of chondroitin and heparan sulfate in *C. elegans* compared to those of other organisms?

The current evidence supports conservation of the linkage region biosynthetic pathway among *C. elegans*, *Drosophila* and mammals. However, *C. elegans* possibly has mechanisms for initiation and polymerization of both heparan sulfate and chondroitin as described below, that appear distinct from those of other organisms.

In addition to *sqv-7*, *sqv-3* and *sqv-8*, which were isolated by genetic screens and biochemically characterized *in vitro* and *in vivo*, a number of homologues to the enzymes responsible for the biosynthesis and modifications of the GAG of proteoglycans in other organisms are present in the *C. elegans* genome. These are mostly consistent with the structures of the GAG products described in the previous section and are summarized in Table 1.

Chain initiation

Two vertebrate β4-Xyl transferases (1 and 2) have been reported [21]; however a single homolog exists in *C. elegans and Drosophila* [22]. Attachment of two Gal residues by Gal transferases I and II [23] and GlcA by GlcA ltransferase I [24] by a common pathway is supported by biochemical experiments in *sqv-3* (Gal transferase I) and *sqv-8* (GlcA transfease I) mutants.

Table 1. *C. elegans* genes related to chondroitin/heparan sulfate biosynthesis by homology to vertebrate enzymes

C. elegans *gene*	*Postulated enzyme activity*	*Mutants or loss-of-function phenotype*	*Biochemical activity*
sqv-7	Transport of UDP-GlcA, UDP-GalNAc and UDP-Gal into Golgi	Zygotic loss affects vulval invagination, required maternally for oocyte formation and embryogenesis [4]	Transport of UDP-GlcA, UDP-GalNAc and UDP-Gal into S. cerevisiae vesicles expressing SQV-7, complements UDP-Gal transport-deficient MDCK mutant [7]
sqv-6	Xyl transferase	Zygotic loss affects vulval invagination, required maternally for oocyte formation and embryogenesis [4]	Complements Xyl transferase-defective CHO mutant [10]
sqv-3	Gal transferase I	Zygotic loss affects vulval invagination, required maternally for oocyte formation and embryogenesis [4]	Complements Gal transferase I-defective CHO mutant, SQV-3 expressed in COS-7 cells transfers Gal to β-linked xylosides [8]
sqv-2	Gal transferase II	Zygotic loss affects vulval invagination, required maternally for oocyte formation and embryogenesis [4], 74% embryonic lethality by dsRNAi [35]	Recombinant SQV-2 has Gal transferase II activity [10]
sqv-8	GlcA transferase I	Zygotic loss affects vulval invagination, required maternally for oocyte formation and embryogenesis [4]	SQV-8 expressed in COS-7 cells transfers GlcA to Galβ1,3Galβ [8]
rib-2	α1,4GlcNAc transferase I and II	Essential for embryonic development [36]	Consistent with both GlcNAc transferase I and II activities [30]
rib-1	Unknown	Unknown	
hst-1	N-deacetylase N-sulfotransferase	Unknown	ND
hse-5	5′-uronosyl epimerase		ND
hst-2	Heparan sulfate 2-O-sulfotransferase	Unknown	ND
hst-6	Heparan sulfate glucosamine 6-O-sulfotransferase	Involved in axon outgrowth and navigation. Suppresses KAL-1 overproduction axon branching and mis-routing phenotypes [14].	ND
hst-3	Heparan sulfate glucosamine 3-O-sulfotransferase	Unknown	ND
T24D1.1	Chondroitin synthase and β1,4-N-acetylglactosaminyl transferase I	Unknown	ND

Heparan sulfate and chondroitin/dermatan sulfate polymerization and modification

In vertebrates, the GAGs are built onto this linkage region by the alternate addition of N-acetylhexosamine and GlcA residues. Heparin/heparan sulfate is synthesized once GlcNAc is transferred to the common linkage region, while chondroitin/dermatan sulfate is formed if GalNAc is added (reviewed in [25,26]). The enzymes responsible for this crucial biosynthetic sorting are different from those involved in chain polymerization. Activity of the initiating GlcNAc transferases (EXT-L2 and EXT-L3) commits the intermediate to heparin/heparan sulfate biosynthesis [27,28]. Chain elongation proceeds by stepwise, alternate, addition of GlcA and GlcNAc by one or more members of the EXT gene family. Both EXT-1 and EXT-2 have GlcA and GlcNAc transferase activity *in vitro*, but synergistic effects upon co-expression suggest both may participate in heparan sulfate polymerization [29]. Only two homologs of the EXT family exist in *C. elegans*, *rib-1* and *rib-2*. Biochemical studies indicate that the *rib-2* protein is a novel and unique α1,4-N-GlcNAc transferase, likely involved in the initiation and elongation of heparan sulfate [30]. The biochemical activity of *rib-1* has remained elusive.

The polysaccharide chains of chondroitin sulfate are assembled by a similar mechanism. The human chondroitin synthase is able to transfer both GlcA and GalNAc to the polymer chondroitin [31], and has a single *C. elegans* homolog. A human chondroitin GalNAc transferase which has both initiating GalNAc transferase I activity and elongating GalNAc transferase II activity *in vitro* has recently been characterized, and is postulated to be the chondroitin-initiating enzyme in vertebrates and *Drosophila* [32]. A homolog of the initiating GalNAc transferase I is not present in *C. elegans*; it has been postulated that a single enzyme, the *C. elegans* chondroitin synthase, is responsible for transfer of the first GalNAc, as well as subsequent GlcA and GalNAc residues in this organism [32].

Conclusions and future directions

Defining the proteins modified by GAGs in *C. elegans*, in particular those that are the targets of the genes defined by mutations, such as the *sqv* genes and the recently isolated *hst-6*, would aid in understanding, at the cellular level, the molecular events leading to their phenotypes. Several homologs of well-characterized vertebrate proteoglycan core proteins exist in *C. elegans*. However, it is not known if these proteins are indeed modified by GAGs. UNC-52, a perlecan homolog, was initially identified in genetic screens and is found in the basement membrane between the body-wall muscle and the hypodermis [33]. The *unc-52* pre-mRNA gives rise to a number of distinct proteins isoforms through regulated alternative splicing, with a complex spatial and temporal expression pattern [34]. Single genes encoding homologues to vertebrate syndecan and glypican are found in the *C. elegans* genome. Analyses of their primary sequence reveals that they do not contain the canonical consensus sequence for GAG attachment defined for vertebrate proteoglycans, leading to the question whether or not they are modified by GAGs.

Despite the enormous difference in biological complexity between humans and *C. elegans*, the recent structural and biochemical studies indicate that *C. elegans* is an attractive animal model to study the role of proteoglycans during development and function of metazoans. Two different approaches are currently underway to understand *C. elegans* gene function on a global genomic scale by studying loss-of-function phenotypes. The *C. elegans* Gene Knockout Project is a worldwide consortium whose ultimate goal is to produce null alleles of all known genes in the *C. elegans* genome using a PCR-based knockout technology. In addition, several groups have started to analyze gene function by systematic inactivation of genes using dsRNAi, an experimental technique that can also be employed in a smaller scale to specifically inactivate genes involved in proteoglycan biosynthesis. These approaches, combined with biochemical analyses, offer a bright future in the understanding of the role of proteoglycans in development of multicellular organisms.

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