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The *Caenorhabditis elegans* Genes *sqv-2* and *sqv-6*, Which Are Required for Vulval Morphogenesis, Encode Glycosaminoglycan Galactosyltransferase II and Xylosyltransferase*[S]

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In mutants defective in any of eight *Caenorhabditis elegans sqv* (squashed vulva) genes, the vulval extracellular space fails to expand during vulval morphogenesis. Strong *sqv* mutations result in maternal-effect lethality, caused in part by the failure of the progeny of homozygous mutants to initiate cytokinesis and associated with the failure to form an extracellular space between the egg and the eggshell. Recent studies have implicated glycosaminoglycans in these processes. Here we report the cloning and characterization of *sqv-2* and *sqv-6*. *sqv-6* encodes a protein similar to human xylosyltransferases. Transfection of *sqv-6* restored xylosyltransferase activity to and rescued the glycosaminoglycan biosynthesis defect of a xylosyltransferase mutant hamster cell line. *sqv-2* encodes a protein similar to human galactosyltransferase II. A recombinant SQV-2 fusion protein had galactosyltransferase II activity with substrate specificity similar to that of human galactosyltransferase II. We conclude that *C. elegans* SQV-6 and SQV-2 likely act in concert with other SQV proteins to catalyze the stepwise formation of the proteoglycan core protein linkage tetrasaccharide GlcAβ1,3Galβ1, 3Galβ1,4Xylβ-O-(Ser), which is common to the two major types of glycosaminoglycans in vertebrates, chondroitin and heparan sulfate. Our results strongly support a model in which *C. elegans* vulval morphogenesis and zygotic cytokinesis depend on the expression of glycosaminoglycans.

Glycosaminoglycans (GAGs)[1] are important in animal development, and defects in GAGs are responsible for certain human disorders. For example, mutations in the *Drosophila melanogastger* genes *tout-velu* (1) and *sulfateless* (2), which encode homologs of heparan sulfate co-polymerase and heparan sulfate *N*-deacetylase/*N*-sulfotransferase, respectively, cause zygotic lethality and defects in segmentation. Mutations in the mouse *tout-velu* homolog EXT1 disrupt gastrulation and the generation of mesoderm (3), while mutations in human EXT1 and EXT2 have been associated with hereditary multiple exostoses (reviewed in Ref. 4). Mutations in the human galactosyltransferase I have been associated with a progeroid variant of the connective-tissue disorder Ehlers-Danlos syndrome (EDS) (5–7). EDS is a group of heritable disorders characterized by hyperelasticity of the skin and hypermobile joints. Tout-velu, EXT-1, EXT-2, and Sulfateless affect the biosynthesis of heparan sulfate specifically, while galactosyltransferase I deficiency affects the biosynthesis of both chondroitin and heparan sulfate.

The backbones of chondroitin and heparan sulfate consist of repeating disaccharide units: GlcAβ1,3GalNAcβ1,4 for chondroitin and GlcAβ1,4GlcNAcα1,4 for heparan sulfate (reviewed in Ref. 8). Their polymerization occurs on a tetrasaccharide primer (GlcAβ1,3Galβ1,3Galβ1,4Xylβ-) that is linked to the protein core of a proteoglycan. The addition of these four sugars is catalyzed stepwise in the lumen of the Golgi apparatus and requires three nucleotide sugars, UDP-Xyl, UDP-Gal, and UDP-GlcA, and four glycosyltransferases.

Eight *sqv* (squashed vulva) genes were genetically identified in a screen for *Caenorhabditis elegans* mutants defective in vulval morphogenesis (9). All *sqv* mutants fail to form a large fluid-filled vulval extracellular space and have a reduced separation of the anterior and posterior halves of the vulva from the early to middle phases of L4 larval development. Strong mutant alleles of all eight *sqv* genes also cause maternal-effect lethality. Most progeny of mothers homozygous for a strong *sqv* mutant allele arrest at the one-cell stage (9). The nuclei of the arrested progeny divide normally, but the extrusion of the polar bodies and the initiation of cytokinesis are impaired (10). These mutant eggs fail to form the normal fluid-filled extracellular space between the membrane of the egg and the eggshell. We have postulated that the *sqv* genes control the biosynthesis of GAGs that are secreted and become hydrated to form fluid-filled extracellular spaces (10, 11).

The molecular identification of five *sqv* genes has led to a model implicating the biosynthesis of chondroitin and/or heparan sulfate in *C. elegans* development. *sqv-1*, *-3*, *-4*, *-7*, and *-8* encode UDP-GlcA decarboxylase (10), galactosyltransferase I

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The nucleotide sequence(s) reported in this paper has been submitted to GenBank™/EBI Data Bank with the accession number(s) AY241927 and AY241928.

[S] The on-line version of this article (available at http://www.jbc.org) contains a Supplementary Materials and Methods and Refs. 1–4.

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[1] The abbreviations used are: GAG, glycosaminoglycan; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; Xyl, xylose; EDS, Ehlers-Danlos syndrome; ORF, open reading frame; CHO, Chinese hamster ovary; UTR, untranslated region; CMV, cytomegalovirus.

(12), UDP-glucose dehydrogenase (13), UDP-GlcA/UDP-Gal/UDP-GalNAc transporter (14), and glucuronosyltransferase I (12), respectively. *sqv-3* was used to identify the human galactosyltransferase I, which has been implicated in the progeroid variant of EDS (7, 15). In this paper, we show that *sqv-6* encodes the xylosyltransferase that adds Xyl to the protein core, thus initiating GAG biosynthesis. *sqv-2* encodes a galactosyltransferase that adds the second Gal residue to the linkage tetrasaccharide.

EXPERIMENTAL PROCEDURES

C. elegans Maintenance—Strains were cultured as described (16) and were grown at 20–22 °C unless indicated otherwise.

Molecular Biology—Standard molecular biological techniques were used (17). The sequences of all PCR-amplified DNAs used for cloning were confirmed to exclude unintended mutations. Oligonucleotide sequences used for amplification or mutagenesis of DNA are shown in Supplementary Materials and Methods.

Rescue of C. elegans sqv-2 and sqv-6 Mutants—For germ line rescue, we injected cosmids carrying genomic DNA into *sqv-2(n2821)* and *sqv-6(n2845) unc-60(e677)/unc-34(s138)* animals with the dominant roller marker pRF4, as described by Mello *et al*. (18). Rol lines were established, and Rol animals and Unc-60 Rol animals were examined for rescue of the *sqv-2* and the *sqv-6* mutant phenotype, respectively. We injected *sqv-2(n2821)* hermaphrodites with plasmids containing the *sqv-2* open reading frame (ORF) under the control of the *C. elegans* heat-shock promoters (19) and pRF4 as the coinjection marker. We injected *sqv-6(n2845)/nT1(n754)* hermaphrodites with plasmids containing the *sqv-6* ORF under the control of the *C. elegans* heat-shock promoters (19) and pRF4. Rol lines were established, and Rol (non-Unc) animals were examined for rescue of the *sqv-2* and *sqv-6* mutant phenotype following induction of *sqv-2* and *sqv-6* expression by 30 min of heat-shock treatment at 33 °C.

SQV-2 Galactosyltransferase II Assay—A sequence encoding amino acids 25–330 of SQV-2, thus lacking the presumptive transmembrane domain at the amino terminus, was cloned into pDEST-CMV-protA. This plasmid was designed to express a secreted fusion protein containing protein A and SQV-2 amino acids 25–330. COS7 cells were transiently transfected with pDEST-CMV-protA-*sqv-2* using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After 72 h of incubation, the fusion protein was recovered from the cell culture supernatant by affinity chromatography using IgG-agarose (20). Galactosyltransferase II activity was assayed as described by Bai *et al.* (21).

Rescue of the Xylosyltransferase Defect in Chinese Hamster Ovary (CHO) pgsA-745 Cells by sqv-6—The xylosyltransferase-deficient CHO pgsA-745 cells (22) were transfected with *sqv-6* ORF, which was cloned into pcDNA3.1. Stable transfectants were selected with 400 μg/ml geneticin (Invitrogen). Several drug-resistant colonies were isolated and screened by flow cytometry for *sqv-6* expression based on binding of biotinylated FGF-2 as described (23). Incorporation of $^{35}SO_4$ into GAG chains of wild-type CHO or pgsA-745 cells with or without *sqv-6* was assayed essentially as described by Bame and Esko (24), labeling cells overnight at 30 °C with 50 μCi/ml $^{35}SO_4$ (PerkinElmer Life Sciences).

SQV-6 Xylosyltransferase Assay—Cell extracts of wild-type CHO, pgsA-745, and *sqv-6* or empty vector stable transfectants of pgsA-745 were prepared as described previously (22). Xylosyltransferase activity was assayed essentially as described (22) by incubating 25 μg crude cell extract with 50 μg of soluble silk acceptor and 6×10^5 cpm UDP-[1-^{3}H]xylose (PerkinElmer Life Sciences, 8.9 Ci/mmol) at 26 °C for 5 h. Product formation was dependent on the addition of silk. The concentration of substrate was saturating.

RESULTS AND DISCUSSION

Molecular Identification of sqv-2—sqv-2 was previously mapped to the left of *lin-31* on LGII (25). We further mapped *sqv-2* to an interval between *sup-9* and *lin-31* (see Supplementary Materials and Methods). We assayed 27 cosmids in this interval for the ability to rescue the *sqv-2* mutant phenotype, but none showed rescuing activity (Fig. 1*A*).

We examined the DNA sequence corresponding to the gaps between the cosmids in this interval and found a predicted gene, Y110A2AL.14, that is weakly similar to galactosyltransferases. Because all previously cloned *sqv* genes are implicated in the biosynthesis of chondroitin and/or heparan sulfate, we suspected that *sqv-2* also encodes a protein involved in GAG biosynthesis. Specifically, it seemed plausible that Y110A2AL.14 encodes the galactosyltransferase II involved in the formation of the protein core linkage tetrasaccharide and that had not been identified molecularly in any organism at the time.

We identified three molecular lesions corresponding to three of the four identified alleles of *sqv-2* in the ORF of

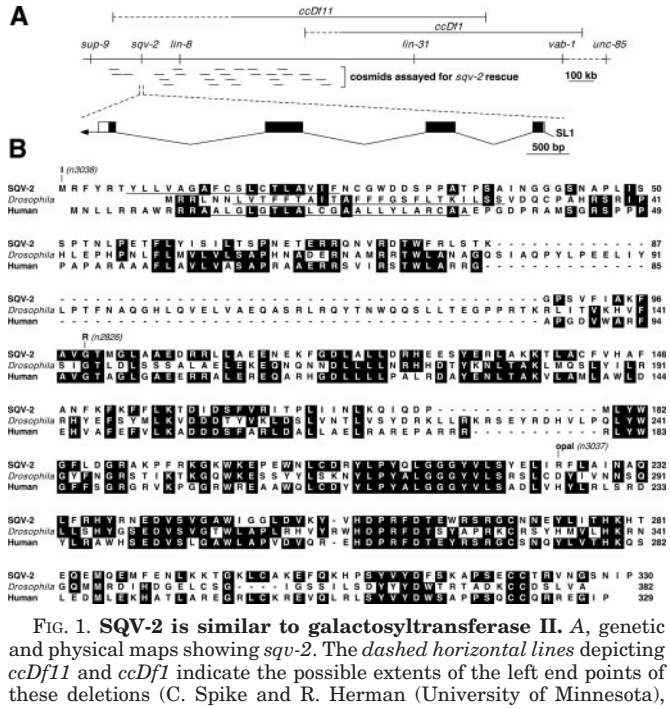

FIG. 1. **SQV-2 is similar to galactosyltransferase II.** *A*, genetic and physical maps showing *sqv-2*. The *dashed horizontal lines* depicting *ccDf11* and *ccDf1* indicate the possible extents of the left end points of these deletions (C. Spike and R. Herman (University of Minnesota), personal communication; E. Davison and H. R. Horvitz, unpublished observations). *Short solid lines* represent cosmid clones that were assayed in germ line transformation experiments. Below is the structure of the *sqv-2* gene as deduced from genomic and cDNA sequences. *Solid boxes* indicate exons, and *open boxes* indicate untranslated sequences. The *trans*-spliced leader SL1 is indicated, and the *arrow* indicates the poly(A) tail. *B*, alignment of SQV-2, the *Drosophila* homolog, and human galactosyltransferase II. Identities between at least two proteins are *shaded* in *black*. The predicted transmembrane domains are *underlined*. The three *sqv-2* mutant alleles are indicated. The *numbers* on the *right* indicate amino acid positions.

TABLE I

The SQV-2 fusion protein has acceptor specificity consistent with its being galactosyltransferase II

Galactosyltransferase activity was assayed *in vitro* using UDP-[^{3}H]galactose together with various acceptor substrates. No substrate controls ranged from 339 to 357 cpm. The range for all substrates shown as "0" activity was 137–619 cpm. The range for the substrate Galβ1,4Xylβ-*O*-Bn was 141,000–142,000 cpm. Bn, benzyl; NM, naphthalenemethanol; C_{10}, *O*-decenyl $(CH_2)_8CH{=}CH_2$

Acceptor substrates	Enzyme activity
	pmol/h/ml medium
Monosaccharides (5 mM)	
Xylβ-*O*-Bn	0
Xylβ-*O*-naphthol	0
Galβ-*O*-NM	0
GalNAcα-*O*-Bn	0
GlcNAcβ-*O*-NM	0
Disaccharides (5 mM)	
Galβ1,4Xylβ-*O*-Bn	2660
Galβ1,3GalNAcα-*O*-NM	1
Galβ1,3Galβ-*O*-NM	3
Galβ1,4GlcNAcβ-*O*-NM	0
Galβ1,3GlcNAcβ-*O*-NM	0
GlcNAcβ1,3Galβ-*O*-NM	6
Manα1,6Manα-*O*-C_{10}	0

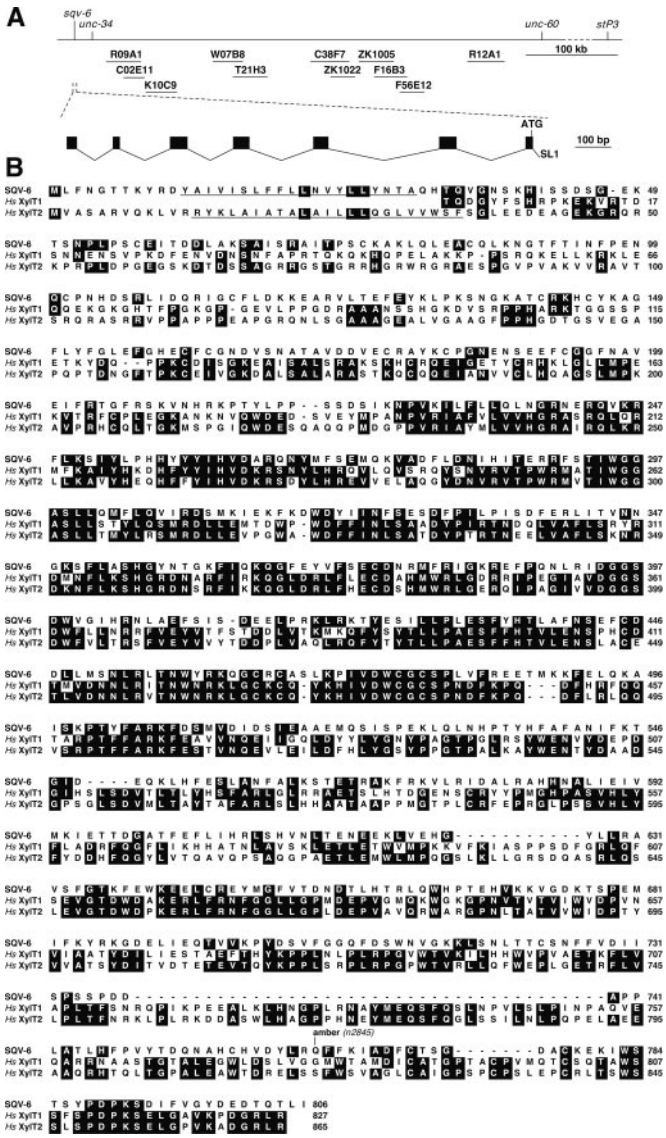

FIG. 2. **SQV-6 is similar to xylosyltransferases.** *A*, genetic and physical maps showing s*qv-6*. *Short solid lines* represent cosmid clones that were assayed in germline transformation experiments. Below is the structure of the *sqv-6* gene as deduced from genomic and cDNA sequences. *Solid boxes* indicate exons. The *trans*-spliced leader SL1 and the start codon (ATG) are indicated. *B*, alignment of SQV-6 and two human xylosyltransferases. Identities between at least two proteins are *shaded* in *black*. The predicted transmembrane domains are underlined. The single *sqv-6* nonsense allele is indicated. The *numbers* on the *right* indicate amino acid positions.

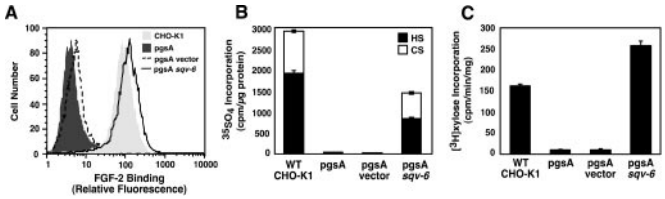

FIG. 3. ***sqv-6* rescues a xylosyltransferase-deficient CHO cell line.** *A*, FGF-2 binding to cell-surface heparan sulfate as assayed by flow cytometry (23). *Light gray shading*, wild-type CHO-K1. *Dark gray shading*, mutant pgsA-745. *Dashed line*, pgsA-745 with empty vector. *Solid line*, pgsA-745 with *sqv-6*. *B*, $^{35}SO_4$ incorporation into GAGs (see "Experimental Procedures"). *Black bars*, [^{35}S]heparan sulfate (*HS*). *White bars*, [^{35}S]chondroitin sulfate (*CS*). The average values ± S.D. (n = 3) are shown. *C*, xylosyltransferase activity in crude cell extracts (see "Experimental Procedures"). Average incorporation of [^{3}H]xylose from UDP-[1-^{3}H]xylose into soluble silk acceptor ± S.D. (n = 3) are shown.

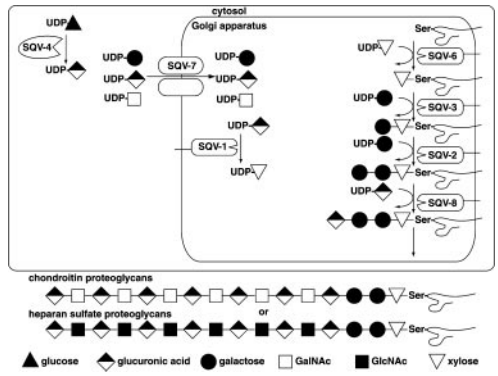

FIG. 4. **Model for the role of seven *sqv* genes in glycosaminoglycan biosynthesis.** SQV-4 converts UDP-glucose to UDP-GlcA (13). SQV-7 transports UDP-GlcA, UDP-Gal, and UDP-GalNAc from the cytoplasm to lumen of the Golgi apparatus (14). SQV-1 converts UDP-GlcA to UDP-Xyl in the lumen of the Golgi apparatus (10). SQV-6 is xylosyltransferase (this study). SQV-3 is galactosyltransferase I (12). SQV-2 is galactosyltransferase II (this study). SQV-8 is glucuronosyltransferase I (12). In other organisms, two additional sets of glycosyltransferases act in later steps of the biosynthesis of chondroitin and heparan sulfate (8).

Y110A2AL.14 (Fig. 1*B*). The two stronger alleles of *sqv-2*, *n3037* and *n3038*, cause a maternal-effect lethal phenotype and are an opal nonsense mutation at arginine 225 and a methionine-to-isoleucine missense mutation of the predicted start codon, respectively. A weak allele, *n2826*, that results in live progeny is a missense mutation causing a glycine-to-arginine substitution at amino acid position 99. The molecular lesion of the weakest allele, *n2840*, has not been identified yet.

We determined the sequences of two cDNA clones, yk94e4 and yk292g2 (see Supplementary Materials and Methods), that correspond to Y110A2AL.14. The yk292g2 clone contains 990 bases of ORF, 17 bases of 5′-untranslated region (UTR), and 121 bases of 3′-UTR. The 5′ end contains three bases that correspond to the sequence of 5′ SL1 *trans*-spliced leader, which is found at the 5′ end of many *C. elegans* transcripts (26). The 3′ end contains a poly(A) sequence. The longest ORF in this cDNA is identical to Y110A2AL.14 and is predicted to encode a protein of 330 amino acids. The yk94e4 clone lacks the 5′ end of Y110A2AL.14. Expression of the longest ORF in yk292g2 under the control of the *C. elegans* heat-shock promoters (19) rescued the defect in *sqv-2* vulval morphogenesis in all five isolated lines.

sqv-2 Encodes a Protein Similar to Galactosyltransferase II—The predicted SQV-2 protein contains a putative transmembrane domain near the amino terminus, suggesting it may be a type II transmembrane protein (Fig. 1*B*). Most known glycosyltransferases that act in the lumen of the ER and the Golgi apparatus are type II transmembrane proteins. Of 330 amino acids of SQV-2, 93 (28%) and 133 (40%) are identical to the *Drosophila* and human homologs, respectively (Fig. 1*B*). Recently, the human homolog of SQV-2 was identified as galactosyltransferase II (21).

SQV-2 Has Galactosyltransferase II Activity—We assayed recombinant protein A-SQV-2 fusion protein expressed in COS7 cells for galactosyltransferase II activity (see "Experimental Procedures"). The SQV-2 fusion protein specifically catalyzed the addition of galactose to a disaccharide acceptor, Galβ1,4Xylβ-O-benzyl that had been used to demonstrate the acceptor substrate specificity of the human galactosyltransferase II (21) (Table I).

Molecular Identification of sqv-6—sqv-6 was previously mapped to the left of the polymorphism *stP3* on LGV (9). We

further mapped *sqv-6* to the left of the cosmid W07B8 and within about 0.2 map units of *unc-34* (see Supplementary Materials and Methods). We assayed 11 cosmids to the right of *unc-34* for the ability to rescue the *sqv-6* mutant phenotype, but none showed rescuing activity (Fig. 2*A*).

We examined the DNA sequences in the gaps in the cosmid coverage near the cosmid W07B8 and *unc-34* and found a gene, Y50D4C.d, that is similar to two human xylosyltransferases (27). Using DNA from the only allele of *sqv-6*, *n2845*, we identified in the ORF of Y50D4C.d an amber nonsense mutation causing the elimination of the last 42 amino acids of the predicted protein product (Fig. 2*B*).

We determined the sequence of PCR-amplified cDNA and 5′-rapid amplification of cDNA ends products corresponding to Y50D4C.d (see Supplementary Materials and Methods). We found that this cDNA contains a 5′ SL1 *trans*-spliced leader, 23 bases of 5′-UTR, and 2418 bases of ORF, including two additional 5′ exons not in Y50D4C.d. The longest ORF in this cDNA including the additional exons is predicted to encode a protein of 806 amino acids. Expression of this ORF under the control of the *C. elegans* heat-shock promoters (19) prior to the start of vulval morphogenesis rescued the *sqv-6* vulval morphogenesis defect in all animals ($n = 13$) and the maternal-effect lethality of the progeny of *sqv-6* homozygotes generated by +/*sqv-6* heterozygous parents for three of 13 *sqv-6* homozygotes studied.

sqv-6 Encodes a Protein Similar to Xylosyltransferases—Of the 806 amino acids of the SQV-6 protein, 182 (23%) and 193 (24%) are identical to human xylosyltransferases I and II, respectively (Fig. 2*B*). Both the predicted SQV-6 protein and the human xylosyltransferase II contain a putative transmembrane domain near the amino terminus and are likely to be type II transmembrane proteins. Neither the start codon nor a presumptive transmembrane domain has been defined for human xylosyltransferase I (27).

sqv-6 Can Correct a Xylosyltransferase Defect in CHO Cells—We tested the ability of *sqv-6* to act as a xylosyltransferase by testing its ability to complement GAG-deficient CHO mutant cells lacking this enzymatic activity (22). Mutant pgsA-745 cells were transiently transfected with a plasmid containing *sqv-6* under the control of a cytomegalovirus (CMV) promoter. These cells showed partial rescue of the defect, as assayed by the restored abilities to incorporate $^{35}SO_4$ into GAGs (16–27% that of the wild type) and to bind biotinylated FGF-2, which binds cell surface heparan sulfate as assayed by flow cytometry (data not shown). From these transiently transfected cells, we obtained a clonal cell line stably expressing *sqv-6*. This cell line showed full restoration of FGF-2 binding to heparan sulfate on the cell surface (Fig. 3*A*). Stable expression of *sqv-6* in pgsA-745 cells enhanced the incorporation of $^{35}SO_4$ into GAGs to ~50% of wild-type levels, compared with 1% for the untreated mutant or mutant transfected with empty vector (Fig. 3*B*). The $^{35}SO_4$ incorporation into GAGs was similar in wild-type CHO cells and pgsA-745 cells transfected with *sqv-6*: 30–40% was released by treatment with chondroitinase ABC and 55–65% by a heparin lyase mixture in both cells, indicating that the composition of chondroitin and heparan sulfate was comparable. Expression of *sqv-6* also resulted in restoration of xylosyltransferase activity, as measured by the transfer of xylose from UDP-xylose to a soluble silk acceptor (22), whereas pgsA-745 cells transfected with empty vector had virtually no activity (Fig. 3*C*).

The sqv-2 and sqv-6 Genes Act in the C. elegans Chondroitin and Heparan Sulfate Biosynthesis Pathway—Our findings indicate that *sqv-2* and *sqv-6* encode galactosyltransferase II and xylosyltransferase, respectively. With the previously identified *sqv-3* galactosyltransferase I and *sqv-8* glucuronosyltransferase I, all four *C. elegans* genes responsible for the biosynthesis of the proteoglycan core protein linkage tetrasaccharide of chondroitin and heparan sulfate have now been defined (Fig. 4). Three previously identified genes, *sqv-4* UDP-glucose dehydrogenase, *sqv-1* UDP-GlcA decarboxylase and *sqv-7* UDP-GlcA/UDP-Gal/UDP-GalNAc transporter, act in earlier steps of GAG biosynthesis. All *sqv* genes identified to date affect the biosynthesis of both chondroitin and heparan sulfate. Based upon these observations, we conclude that in *C. elegans* early embryonic cytokinesis and epithelial invagination during vulval development depend on the expression of GAGs.

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