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

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Ho-Yon Hwang‡, Sara K. Olson§, Jillian R. Brown§, Jeffrey D. Esko§, and H. Robert Horvitz‡¶

From the ¶Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the §Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California 92093-0687

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 GlcAβ1,3Galβ1,4Galβ1,4Xylβ1,3-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)§ are important in animal development, and defects in GAGs are responsible for certain human disorders. For example, mutations in the Drosophila melanogaster genes tout-tetu (1) and sulfateless (2), which encode homologs of heparan sulfate co-polymerase and heparan sulfate N-deacytelase/N-sulfotransferase, respectively, cause zygotic lethality and defects in segmentation. Mutations in the mouse tout-tetu homolog EXT1 disrupt gastrulation and the extension 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-tetu, 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 (GlcAβ1,3Galβ1,4Galβ1,4Xylβ1,3-O-(Ser) 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 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) AF244927 and AF244928.

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¶ Investigator of the Howard Hughes Medical Institute. To whom correspondence and reprint requests should be addressed: Howard Hughes Medical Inst., Dept. of Biology, Massachusetts Inst. of Technology, Rm. 68-425, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-253-4671; Fax: 617-253-8126; E-mail: horvitz@mit.edu.

1 The abbreviations used are: GAG, glycosaminoglycan; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, N-acetylgalcosamine; Xyl, xylose; EDS, Ehlers-Danlos syndrome; ORF, open reading frame; CHO, Chinese hamster ovary; UTR, untranslated region; CMV, cytomegalovirus.
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 cosmid carrying genomic DNA into sqv-2(n2821) and sqv-6(n2845)/unc-60(e6777)/unc-34(e138) 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. COST 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) pgk-c745 Cells by sqv-6—The galactosyltransferase-deficient CHO pgkA745 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 35SO4 into GAG 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. 1A).

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...
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FIG. 2. SQV-6 is similar to xylosyltransferases. A. genetic and physical maps showing sqv-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.

Y110A2AL.14 (Fig. 1B). The two stronger alleles of sqv-2, n3037 and n3038, cause a maternal-effect lethal phenotype and are anopal 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 a maternal-effect lethal phenotype, cause a maternal-effect lethal phenotype, and are anopal nonsense mutation at arginine 225 and a methionine-to-isoleucine missense mutation of the predicted 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.

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 (see “Experimental Procedures”). Average incorporation of [3H]xylose from UDP-[1-3H]xylose into soluble silk acceptor ± S.D. (n = 3) are shown.

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, [35S]SO4 incorporation into GAGs (see “Experimental Procedures”). Black bars, [35S]heparan sulfate (HS). White bars, [35S]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 [3H]xylose from UDP-[1-3H]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).

This document is a scientific paper discussing the role of SQV-2 and SQV-6 genes in glycosaminoglycan biosynthesis. The authors identified these genes using molecular biology techniques and demonstrated their function in cell-surface glycosaminoglycan synthesis. The SQV-2 gene encodes a protein similar to galactosyltransferase II, while SQV-6 is a xylosyltransferase, and both genes are involved in the biosynthesis of chondroitin and heparan sulfate. The paper includes genetic and physical maps, sequence alignments, and biochemical assays to support these findings.
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. 2A).

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. 2B).

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. 2B). 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 35S into GAGs (16–27% 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. 3A). Stable expression of sqv-6 in pgsA-745 cells enhanced the incorporation of 35S into GAGs to ~50% of wild-type levels, compared with 1% for the untreated mutant or mutant transfected with empty vector (Fig. 3B). The 35S 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. 3C).

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, 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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REFERENCES
1. Bellaiche, Y., The, I., and Perrimon, N. (1996) Nature 384, 85–88
2. Lin, X., and Perrimon, N. (1999) Nature 400, 281–284
3. Lin, X., Wei, G., Shi, Z., Dryer, L., Esco, J. D., Wells, D. E., and Matzuk, M. M. (2000) Dev. Biol. 224, 299–311
4. Zak, B. M., Crawford, B. E., and Esco, J. D. (2002) Biochim. Biophys. Acta 1573, 346–355
5. Quentin, E., Gladen, A., Roden, L., and Kresse, H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1342–1346
6. Almeida, R., Levery, S. B., Mandel, U., Kresse, H., Schwientek, T., Bennett, E. P., and Claussen, H. (1999) J. Biol. Chem. 274, 26165–26171
7. Okajima, T., Fukumoto, S., Furukawa, K., and Urano, T. (1999) J. Biol. Chem. 274, 28841–28844
8. Esco, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
9. Herman, T., Hartweg, E., and Horvitz, H. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 968–973
10. Hwang, H.-Y., and Horvitz, H. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14218–14223
11. Herman, T., and Horvitz, H. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 99, 974–979
12. Balik, D. A., Wei, G., Toyoda, H., Kinoshita-Toyoda, A., Waldrip, W. R., Esco, J. D., Robbins, P. W., and Selleck, S. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10638–10643
13. Hwang, H.-Y., and Horvitz, H. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14224–14229
14. Bernie, P.-P., Hwang, H. Y., Zemplena, I., Horvitz, H. R., and Hirschberg, C. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3738–3743
15. Okajima, T., Yoshida, K., Kondo, T., and Furukawa, K. (1999) J. Biol. Chem. 274, 22915–22918
16. Brenner, S. (1974) Genetics 77, 71–94
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Melio, C. C., Kramer, J. M., Stinchcomb, D., and Ambrose, V. (1991) EMBO J. 10, 3959–3970
19. Stringham, E. G., Dixon, D. K., Jones, D., and Candido, E. P. (1992) Mol. Biol. Cell 3, 221–233
20. Wei, Z., Svedler, S. J., Ishihara, M., Orenda, A., and Hirschberg, C. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3885–3888
21. Bai, X., Zhao, D., Brown, J. R., Crawford, B. E., Hennet, T., and Esco, J. D. (2001) J. Biol. Chem. 276, 48189–48195
22. Esco, J. D., Stewart, T. E., and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3197–3201
23. Bai, X., Wei, G., Sinha, A., and Esco, J. D. (1999) J. Biol. Chem. 274, 13017–13024
24. Bame, K. J., and Esco, J. D. (1989) J. Biol. Chem. 264, 8659–8665
25. Herman, T., and Horvitz, H. R. (1997) Cold Spring Harbor Symp. Quant. Biol. 62, 353–359
26. Krause, M., and Hirsh, D. (1987) Cell 49, 753–761
27. Gensch, C., Kohn, J., Zahn, R., Brinkmann, T., and Klessieck, K. (2000) J. Mol. Biol. 304, 517–528