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Drosophila syndecan: Conservation of a cell-surface heparan sulfate proteoglycan

(coreceptor/glycosaminoglycans/growth factors/evolution)

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ABSTRACT **In mammals, cell-surface heparan sulfate is required for the action of basic fibroblast growth factor, fibronectin, antithrombin III, as well as other effectors. The syndecans, a gene family of four transmembrane proteoglycans that participates in these interactions, are the major source of this heparan sulfate. Based on the conserved transmembrane and cytoplasmic domains of the mammalian syndecans, a single syndecan-like gene was detected and localized in the *Drosophila* genome. As in mammals, *Drosophila* syndecan is a heparan sulfate proteoglycan expressed at the cell surface that can be shed from cultured cells. The single *Drosophila* syndecan is expressed in embryonic tissues that correspond with those tissues in mammals that express distinct members of the syndecan family predominantly. Conservation of this class of molecules suggests that *Drosophila*, like mammals, uses cell-surface heparan sulfate as a receptor or coreceptor for extracellular effector molecules.**

Analyses of the fruit fly *Drosophila melanogaster* have revealed many developmentally important genes with counterparts in humans—e.g., genes for homeobox proteins, growth factors, or tyrosine kinases (1). Moreover, recent data on polysialic acid in *Drosophila* suggest phylogenetic conservation of potentially important glycans (2). It has become apparent that heparan sulfate, a glycosaminoglycan that is ubiquitous on adherent cells in mammals, is involved in the functions of several classes of effector molecules. For example, to induce proliferation, migration and differentiation, fibroblast growth factor (FGF) family members must bind to heparan sulfate to activate their tyrosine kinase receptors (3–5). The binding specificity of the heparan sulfate may switch developmentally to accommodate different FGFs (6). This coreceptor function of cell-surface heparan sulfate (7) also involves the binding of a variety of extracellular matrix components like fibronectin, cell adhesion molecules like N-CAM, or protease inhibitors like antithrombin III. In mammals, the syndecans are a family of four distinct gene products that contain a cluster of heparan sulfate attachment sites near the N terminus in the extracellular domain and highly conserved transmembrane and cytoplasmic domains (for review, see ref. 7). These proteoglycans are developmentally regulated and are thought to play an important role in signal transduction because they link the cytoskeleton via the cytoplasmic domains to extracellular ligands bound to the heparan sulfate chains (7).

Whether *Drosophila* contains proteins, like the FGFs and fibronectin, that bind to heparan sulfate and function in a comparable manner is not known. These ligands have been elusive in *Drosophila*, although homologues of their receptors—e.g., integrins and FGF receptors, are well studied (8–11). Heparan sulfate has been documented in insects (12–14) and proposed to be involved in pioneer axon guidance in cockroaches (15), but there is no evidence for cell-surface heparan sulfate proteoglycans in invertebrates. We describe here a transmembrane proteoglycan in *Drosophila*.¶ This proteoglycan is related structurally to members of the vertebrate syndecan family. As in mammals, this proteoglycan bears heparan sulfate chains, is expressed at the cell surface, and is shed as an intact extracellular domain. However *Drosophila*, unlike mammals, appears to contain only a single syndecan, apparently derived from a common ancestor with the vertebrate syndecan family, which is expressed in a wide variety of tissues.

MATERIALS AND METHODS

Molecular Cloning. For PCR, two oligonucleotides (o79, o96), corresponding to the most conserved regions of the stop transfer domain, and one reverse primer (o97), matching the nucleotide sequence encoding the C-terminal four amino acids Glu-Phe-Tyr-Ala and the stop codon, were synthesized as a combination of the respective sequences of murine syndecan 1 (16) and human syndecan 2 (17). These contained 15–18 specific nucleotides and, at the 5′ end, restriction enzyme cleavage sites: (o79) CGTCGACGSATGARRAAGAAG, (o96) CGTCGACATGARRAAGAAGGAYGA, and (o97) CTCTAGAYYASGCRTARAAYTC, using the code Y = C or T, S = C or G, and R = A or G. For PCR, 1 μg of genomic DNA was heated at 95°C for 5 min in 100 μl of PCR mixture (10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/200 μM dNTPs/1 μM primers) and cooled to the annealing temperature for 30 sec. Then 2.5 units of Taq polymerase and a drop of mineral oil were added, followed by a 1-min extension at 72°C. After 20 cycles of 1 min at 95°C, 30 sec at 35°C, and 1 min at 72°C with primers o79 and o97, 90 μl of the reaction products was separated on a 3% NuSieve 3:1 agarose gel. The region corresponding to the expected size of syndecan-related products of 90–120 nt was cut out from the gel and transferred to a Spin-X centrifuge filter unit and centrifuged at 14,000 × *g* for 30 min to elute the DNA. Ten microliters of the eluate was reamplified as above with the primers o96 and o97 for 10 cycles at an annealing temperature of 37°C followed by an additional 30 cycles at 55°C. The reaction products were separated on a 5% NuSieve 3:1 agarose gel; visible bands were cut, purified, sequenced, and if they were similar to syndecan sequences, subcloned into the plasmid pBluescript SK−. An aliquot of an 8- to 12-hr *Drosophila* embryo cDNA library in the plasmid pNB40 was screened by PCR using an SP6 primer with two specific

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Abbreviation: FGF, fibroblast growth factor.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U03282).

primers for the candidate *Drosophila* syndecan sequence, (o106) GCGGTTATTCGCATTTT and (o108) GGCGATCTCTTTGGCTC. After 40 cycles with primers SP6 and o106 with 1 min at 95°C, 30 sec at 45°C, and 2 min at 72°C, 1 μl of the 100-μl reaction was reamplified with primers SP6 and o108 in a new reaction under the same conditions. Both reactions were run on a 3% NuSieve 3:1 agarose gel revealing the appearance of a prominent band in the second amplification. This 654-nt PCR fragment, corresponding to a part of the extracellular, the transmembrane, and a part of the cytoplasmic domain, was purified, subcloned into the plasmid pBluescript SK-, sequenced, and used as a probe throughout this work—e.g., RNA blot, screening for full-length cDNA clones from a λgt11 library, and chromosomal localization. For the latter, the fragment was used as a template in a random-primed labeling reaction incorporating biotin-16-dUTP, according to manufacturer's instructions (Boehringer Mannheim).

Sequences were analyzed with the Genetics Computer Group package (18), MacVector 4.0, or at the National Center for Biotechnology Information using the BLAST network service.

Northern Analysis. The syndecan probe was labeled with digoxigenin by PCR and hybridized overnight to 5 μg of poly(A)$^+$ RNA from 7.5- to 15-hr *Drosophila* embryos, separated on a denaturing formaldehyde gel, and blotted on a nylon membrane. Hybridization was done at 42°C in 50% (vol/vol) formamide/5× standard saline/citrate (SSC) (0.75 M NaCl/75 mM sodium citrate, pH 7.0)/0.1% *N*-lauroylsarcosine/0.2% SDS/yeast tRNA at 50 μg/ml/2% Boehringer Mannheim blocking reagent, prepared and autoclaved as a 10% solution in 0.1 M maleic acid buffer, pH 7.5. The membrane was washed in 2× SSC/0.1% SDS at room temperature and at 68°C with 0.1% SSC/0.1% SDS. After equilibration in 150 mM NaCl/100 mM Tris·HCl, pH 7.5, and 3 hr in 2% blocking reagent as above, the blot was incubated in antidigoxigenin alkaline phosphatase conjugate at 150 units/ml in blocking reagent for 30 min, washed with 150 mM NaCl/100 mM Tris·HCl, pH 7.5, and soaked in LumiPhos 530 for 1 min. After overnight equilibration the blot was exposed for 15 min to x-ray film.

Antibody Preparation. Fusion proteins encoding glutathione *S*-transferase in frame with amino acids 165–337 of the extracellular domain of *Drosophila* syndecan were prepared by inserting a part of the 654-nt fragment described above into vector pGEX-2T. After induction of expression with 0.1 mM isopropyl β-D-thiogalactopyranoside for 2 hr, the bacteria were lysed by sonication in phosphate-buffered saline (PBS)/1% Triton X-100. The fusion protein was recovered by binding of the glutathione *S*-transferase domain to glutathione agarose beads. Either the fusion protein was eluted with 5 mM reduced glutathione/50 mM Tris·HCl, pH 8, or the syndecan portion of the fusion protein was released by incubation with thrombin at 4 μg/ml in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/2.5 mM $CaCl_2$. Polyclonal antisera were raised in two rabbits by injection of 50 μg of fusion protein, a first boost with 50 μg of fusion protein, and subsequent boosts with 50 μg of the thrombin-cleaved recombinant *Drosophila* syndecan portion. An aliquot of the fourth bleed was affinity-purified by passing it first over a column containing glutathione *S*-transferase and then a column with the syndecan portion coupled to cyanogen bromide-activated Sepharose. The antibodies retained on the syndecan column were eluted with 0.1 M glycine (pH 2.5)/0.1 M triethylamine (pH 11.5), neutralized, combined, and dialyzed against PBS/0.2% sodium azide. Alternatively, antibodies were adsorbed against an acetone powder (19) made from Schneider L2 cells, which express only a small amount of syndecan.

Immunoanalysis. Conditioned medium was brought to 2 M urea/150 mM NaCl/50 mM sodium acetate, pH 4.5/0.1% Triton X-100 and rocked overnight with DEAE-Sephacel. The beads were washed with 2 M urea/150 mM NaCl/50 mM sodium acetate, pH 4.5/0.1% Triton X-100 and the same buffer without urea. Elution was achieved by raising the salt concentration to 1 M. The samples were precipitated by adding 3 vol of ethanol with 1.3% potassium acetate at −20°C overnight, centrifuged at 14,000 × *g* for 30 min, dried, suspended in 100 mM Tris·HCl, pH 7.2/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride, and incubated with heparitinase at 10 milliunits/ml or chondroitinase ABC at 250 milliunits/ml as indicated for 1 hr at 37°C or for 10 min with an equal volume of freshly prepared nitrous acid at pH 1.5, according to the method of Shively and Conrad (20).

RESULTS AND DISCUSSION

Molecular Cloning of a *Drosophila* Syndecan. To determine whether the syndecans are evolutionarily conserved, we used PCR and degenerate oligonucleotide primers derived from the sequences encoding the conserved cytoplasmic domains of the murine syndecan 1 (16) and human syndecan 2 genes (17). Amplification products from a first set of primers were separated by agarose gel electrophoresis, the gel region with the size expected for a syndecan-like sequence was cut out, and the eluted materials were reamplified by using a new internal primer in combination with one of the original primers. In identical experiments, genomic DNA from *Drosophila* yielded a single product with a syndecan-like sequence, and genomic DNA from the mouse yielded four such products, corresponding in sequence to syndecan 1, 2, 3, and 4. Lowering the initial annealing temperature from 35° to 34° or 33°C resulted in the appearance of additional bands, but no further syndecan-like sequences were discovered in either *Drosophila* or mouse. To confirm this sequence and obtain a longer probe, an 8- to 12-hr *Drosophila* embryo cDNA library in the plasmid pNB40 was screened by PCR using a plasmid-specific SP6 primer with two primers derived from the candidate *Drosophila* syndecan sequence. A 654-nt fragment was obtained that was used for Northern analysis to screen for full-length cDNA clones and for chromosomal localization and preparation of a fusion protein.

Hybridization of the digoxigenin-labeled probe to 7.5- to 15-hr *Drosophila* embryo mRNA revealed a major transcript of 3.9 kb and a minor band at 2.3 kb (Fig. 1*A*). Sequencing of several independent cDNA clones showed that the difference between these transcripts is in the 3′-untranslated region, presumably due to alternative use of two polyadenylylation sites; however, the second polyadenylylation site was not represented in the cDNA clones identified.

Sequencing of the cDNA clones revealed an open reading frame of 395 residues starting at the second ATG that is preceded by stop codons in all three reading frames (Fig. 1*B*). The N-terminal methionine is followed by charged and hydrophobic residues conforming to the pattern of a signal peptide with a predicted cleavage site between Ala-28 and Gln-29 (21). Analysis of the hydrophilicity of the predicted amino acid sequence shows a signal sequence and a second hydrophobic domain near the C terminus with the characteristic length of a membrane-spanning domain distinct from the otherwise hydrophilic sequence (Fig. 1*C*). The predicted mature protein has a molecular mass of 39 kDa and contains a putative extracellular domain of 307 residues with five Ser-Gly dipeptides as potential glycosaminoglycan attachment sites, a putative transmembrane region of 25 hydrophobic amino acids, and a short putative cytoplasmic domain of 35 residues.

The *Drosophila* sequence is significantly similar only to vertebrate syndecan proteins. Because the extracellular do-

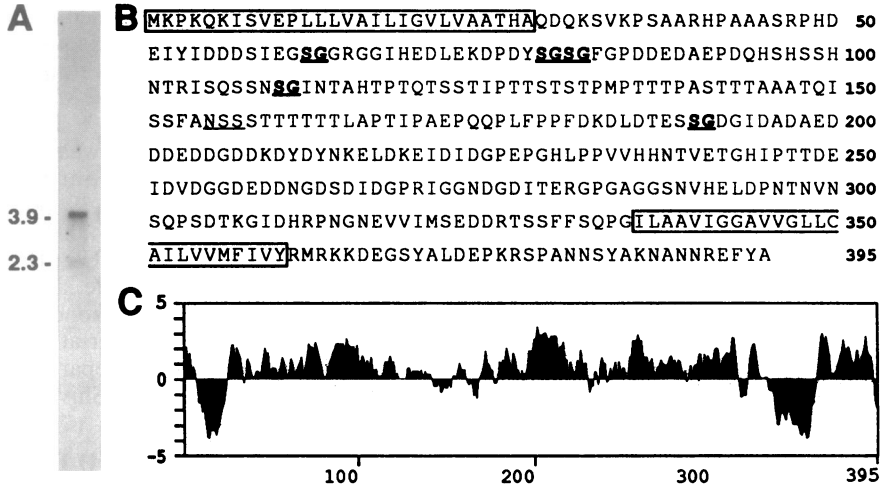

FIG. 1. Molecular analysis of *Drosophila* syndecan. (*A*) RNA blot of 7.5- to 15-hr *Drosophila* embryo mRNA, revealing a major transcript of 3.9 kb and a minor transcript of 2.3 kb. (*B*) Derived amino acid sequence of the coding region of *Drosophila* syndecan. The signal peptide of the derived syndecan sequence, with predicted cleavage site at glutamine Q29, and the transmembrane domain are boxed. All Ser-Gly (SG) sequences, putative attachment sites for glycosaminoglycans, are shown in boldface type and underlined. A single putative N-linked carbohydrate attachment site (NXS/T) is underlined. (*C*) Hydrophilicity plot of the derived amino acid sequence of *Drosophila* syndecan. Values indicated were determined with MacVector using the scale of Kyte and Doolittle with a window size of 7.

main of *Drosophila* syndecan is rich in aspartic acid, threonine, serine, and proline, other matches are found, but the transmembrane and cytoplasmic domains of the *Drosophila* sequence are >50% identical to each vertebrate syndecan protein (Fig. 2*A*). As with the extracellular domains of the vertebrate syndecans, similarities with that of the *Drosophila* syndecan are hardly recognizable, except for a predominance of prolines and hydrophilic residues in regions that separate the conserved Ser-Gly glycosaminoglycan attachment sites from the transmembrane domain.

The dendrogram representation of the sequence similarities (Fig. 2*B*) suggests that the *Drosophila* gene is derived from a common ancestor of all four vertebrate syndecan genes and that syndecan 1 and 3 and syndecan 2 and 4, respectively, form two subfamilies. An intron near the junction between the extracellular and transmembrane domains is precisely conserved in at least the *Drosophila* gene (Fig. 2*A*), murine syndecan 1, and chicken syndecan 3 (22, 23), consistent with common ancestry. The vertebrate syndecan genes apparently arose by two gene duplications after the divergence of the insect and vertebrate lineages some 500 million years ago.

***Drosophila* Syndecan Is a Cell-Surface Heparan Sulfate Proteoglycan.** To confirm whether the *Drosophila* syndecan sequence corresponds to a cell-surface proteoglycan, polyclonal antibodies were raised against a fragment of the extracellular domain of the *Drosophila* protein. The antibodies were used to probe Schneider L2 cells and Kc cells, two *Drosophila* cell lines showing low or high syndecan mRNA levels, respectively (data not shown).

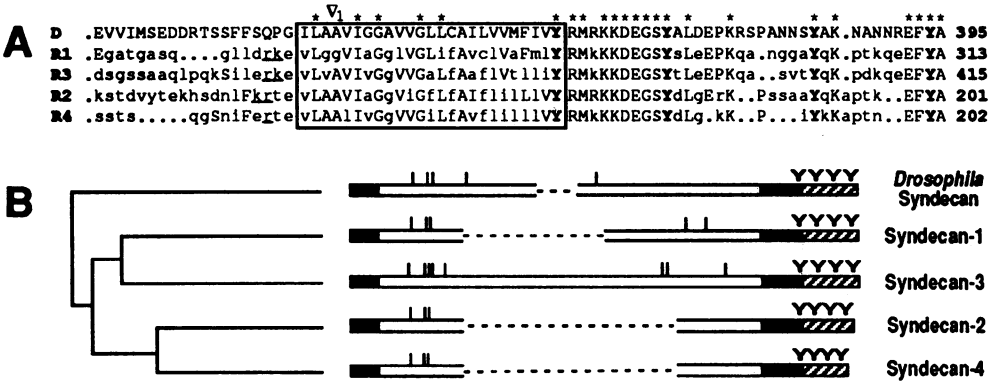

FIG. 2. *Drosophila* and four vertebrate syndecans share a common ancestor. (*A*) Multiple sequence alignment of the C-terminal region of *Drosophila* syndecan (D) with syndecan 1, 3, 2, and 4 from the rat (R1, R3, R2, and R4, respectively). The alignment was calculated with PILEUP (18) and was modified by inspection to conform with the prior alignment of the rat sequences (7). The transmembrane domains are boxed, and the four tyrosines conserved among all syndecans at the end of transmembrane regions and in cytoplasmic domains are shown in boldface type. Amino acids identical to the *Drosophila* sequence are shown in capital letters; asterisks mark positions conserved in all five sequences. The position of the intron conserved in *Drosophila* syndecan, mouse syndecan 1, and chicken syndecan 3 is indicated by $\nabla 1$ to indicate a phase-one intron (22, 23). The basic amino acids just outside the transmembrane region, thought to be protease-cleavage sites in the vertebrate syndecans, are underlined, although none occur at the equivalent position in the *Drosophila* sequence. (*B*) Dendrogram of the sequences compared in *A* with a schematic comparison of the five full-length syndecan sequences. The domain structures of the five syndecans are aligned at the signal peptides and the transmembrane domains (black), leaving gaps with variable sizes in the extracellular domain (white). All Ser-Gly dipeptides that are potential glycosaminoglycan attachment sites are marked by a dash, and the four conserved tyrosines in the cytoplasmic domain (hatched) are indicated. The four vertebrate syndecans are represented by rat sequences because all four syndecan sequences are known in this species, but similar results were obtained with human syndecan 1 and 2, mouse syndecan 1, hamster syndecan 1, and the partial sequence of chicken syndecan 3 (7). The published partial amino acid sequence of rat syndecan 3 (24) lacked the N-terminal 64 residues (D. Carey, personal communication).

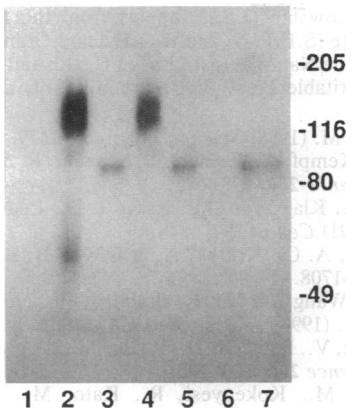

FIG. 3. *Drosophila* syndecan is a heparan sulfate proteoglycan. Material from conditioned medium of *Drosophila* Schneider L2 cells (lane 1) or Kc cells (lanes 2–5 and 7) was purified, and the equivalent of 1.5 ml of conditioned medium was boiled in sample buffer for 10 min, separated on a 7.5% SDS/polyacrylamide gel and blotted on the cationic poly(vinylidene difluoride) membrane Immobilon-N. The filters were incubated with affinity-purified polyclonal antibodies against a recombinant *Drosophila* syndecan fragment followed by a goat anti-rabbit horseradish peroxidase conjugate, incubated for 1 min in the chemiluminescense solution ECL from Amersham, and exposed to an x-ray film for 3 min. Medium from L2 cells (lane 1) showed no signal, whereas medium from Kc cells (lane 2) showed a proteoglycan-like smear of 120–160 kDa. The minor smear around 60 kDa is probably a degradation product because its intensity varies in different preparations. Equal amounts of material as in lane 2 were analyzed after digestion with heparitinase (lane 3), chondroitinase ABC (lane 4), both enzymes (lane 5), or nitrous acid (lane 7). Heparitinase and chondroitinase ABC alone do not react with the antibodies (lane 6). Nitrous acid and heparitinase treatment lead to a core protein of ≈90 kDa. The *Drosophila* core protein shows an apparent molecular mass of two to three times the calculated value, like the vertebrate syndecan core proteins. Apparent molecular masses in kDa for prestained markers are indicated.

As in murine cells, where the extracellular domain of syndecan 1 accumulates in the conditioned medium (7), Kc cells shed a proteoglycan with an apparent molecular mass ranging from 120 to 160 kDa. This broad smear is due to heparan sulfate chains because treatment with heparitinase or nitrous acid, but not with chondroitinase ABC, results in a shift into a sharp band at 90 kDa (Fig. 3). Thus, the proteoglycan bears heparan sulfate chains that behave as those on mammalian syndecans.

Immunostaining of living cells showed substantial punctate stain that circumscribed Kc cells; this staining pattern was similar in type but was much less intense on L2 cells. The stain on Kc cells was markedly enhanced at sites of cell–cell contact and was prominent on filopodia and other cellular extensions near the substratum but was not limited to these sites (Fig. 4). Immunoblots of cell extracts showed a smear migrating at 120–160 kDa, which was converted by heparitinase treatment to a protein band migrating at 100 kDa (data not shown). Thus, this proteoglycan, like the syndecans of mammalian cells, can apparently be shed from the cell surface by cleavage of its extracellular domain, despite the lack of conservation of a potential cleavage site for trypsin-like proteases near the plasma membrane.

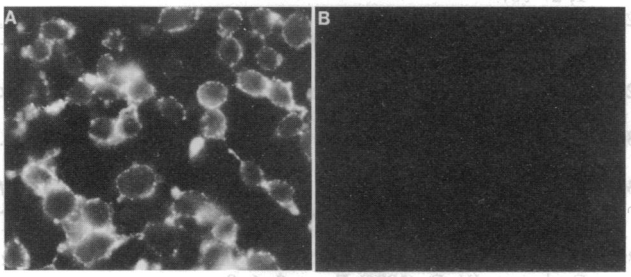

FIG. 4. Syndecan is expressed on the surface of *Drosophila* Kc cells. Live cells on glass coverslips were stained (1 hr at 4°C in PBS) with (*A*) or without (*B*) polyclonal antibodies against the extracellular domain of *Drosophila* syndecan. After incubation with fluorescein-conjugated goat anti-rabbit IgG in 10% normal goat serum, cells were fixed in 4% (wt/vol) paraformaldehyde, rinsed, and mounted in Gelvatol (Monsanto). Stain, seen in *A* but not in *B*, is at the cell periphery and is enhanced at sites of cell–cell contact, which are characteristics of surface staining.

Expression Pattern of Syndecan in the *Drosophila* Embryo. To determine where the syndecan protein is expressed in the embryo, whole embryos were stained with antisyndecan antibody and sectioned (Fig. 5). In embryos 13–16 hr of age, syndecan is expressed in several tissues and is prominent in the lymph glands (hematopoietic organs), in the peripheral and central nervous system, and along the basal surfaces of gut epithelia. The comparable mammalian tissues express predominantly distinct syndecan family members: lymphatic tissues, syndecan 1 (27); neural tissues, syndecan 3 (24); intestine and liver, syndecan 1 (28) and syndecan 2 (29); nearly all tissues, syndecan 4 (30). Hence, the single *Drosophila* syndecan may subserve the functions of the entire complement of mammalian syndecans. Because cell-surface heparan sulfate is required for the action of basic FGF, it is interesting to note that the expression of *Drosophila* syndecan in the central nervous system coincides with the expression of two *Drosophila* FGF-receptor (DFR) homologues in the central nervous system; DFR1 RNA is distributed along the longitudinal connectives while DFR2 RNA is expressed

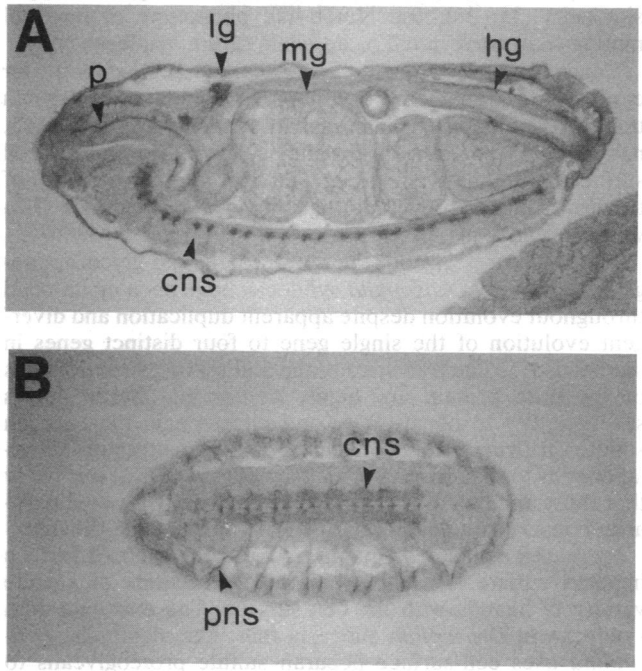

FIG. 5. Expression pattern of syndecan in the *Drosophila* embryo. (*A*) Sagittal section (anterior left, ventral down) of a whole-mount *Drosophila* embryo (stage 16) (25) stained with *Drosophila* syndecan antiserum followed by horseradish peroxidase-conjugated second antibody (26). This section shows staining in the lymph gland (lg), in the central nervous system (cns), and along the basal surface of the pharynx (p), midgut (mg), and hindgut (hg). (*B*) Ventral section (anterior right) of a whole-mount *Drosophila* embryo (stage 17) stained with antisyndecan antibody. This antibody stains both anterior and posterior commissures and the longitudinal connectives of the cns. Note there is staining in cells along the midline, as well as in the peripheral nervous system (pns).

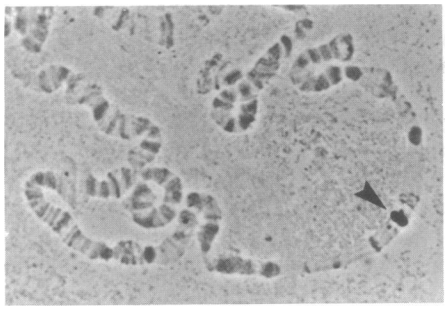

FIG. 6. The syndecan gene is on the second chromosome of *Drosophila*. Chromosome spreads and hybridization with the *Drosophila* syndecan probe were done, essentially as described, and detected with a streptavidin/alkaline phosphatase conjugate (31, 32). Hybridization signal (indicated by arrowhead) shows that the syndecan gene is located at cytological position 57E2 (33).

along the midline and probably functions in the formation of the axon commissures (11).

Localization of the *Drosophila* Syndecan Gene. The results indicate that *Drosophila* contains a single syndecan-like sequence, making subsequent genetic analysis less complex than in the mouse, which contains four syndecan family members. Hybridization with a *Drosophila* syndecan probe to polytene chromosomes from *Drosophila* salivary glands localized the syndecan gene to 57E2 on the second chromosome (Fig. 6). Both viable and lethal mutations have been identified in this region, including the maternal-effect mutant mat(2)N, which has a phenotype similar to that seen in Notch mutants (33, 34). Because the Notch product is a transmembrane protein that interacts with a counterreceptor on opposite cells (35, 36), the Notch-like phenotype of mat(2)N mutant may correspond to an allele of the syndecan gene.

Conclusions. Our results indicate that *D. melanogaster* expresses a transmembrane heparan sulfate proteoglycan that is homologous to mammalian syndecans in sequence, nature of its polysaccharide chains, expression at the cell surface, potential for shedding, and expression in a variety of tissues. Thus, we conclude that this proteoglycan is a *Drosophila* syndecan.

Major features of both the core protein and glycosaminoglycan chains of *Drosophila* syndecan have been maintained throughout evolution despite apparent duplication and divergent evolution of the single gene to four distinct genes in mammals (7). The transmembrane and cytoplasmic domains of the core protein are highly conserved. Based on its susceptibility to heparitinase and nitrous acid the heparan sulfate on *Drosophila* syndecan is similar in structural characteristics to that on mammalian syndecans. Thus, it is likely that these regions of the syndecans interact with evolutionarily conserved elements both within and outside the cell.

Increased evidence in mammals indicates that cell-surface heparan sulfate is involved in the interactions of a wide variety of ligands with the cell surface. The existence of a syndecan in *Drosophila* suggests that, like mammals, *Drosophila* uses cell-surface heparan sulfate proteoglycans to bind these extracellular effector molecules. Thus, it is likely that ligands that bind to heparan sulfate will be found in *Drosophila* and that *Drosophila* homologues of integrins, FGF receptors, and other receptors will be modulated by heparan sulfate proteoglycan coreceptors (7).

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