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Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*

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SUMMARY

In vitro experiments suggest that glycosaminoglycans (GAGs) and the proteins to which they are attached (proteoglycans) are important for modulating growth factor signaling. However, in vivo evidence to support this view has been lacking, in part because mutations that disrupt the production of GAG polymers and the core proteins have not been available. Here we describe the identification and characterization of *Drosophila* mutants in the *suppenkasper* (*ska*) gene. The *ska* gene encodes UDP-glucose dehydrogenase which produces glucuronic acid, an essential component for the synthesis of heparan and chondroitin sulfate. *ska* mutants fail to put heparan side chains on proteoglycans such as Syndecan. Surprisingly, mutant embryos produced by germ-line clones of this general metabolic gene exhibit embryonic cuticle phenotypes strikingly similar to those

that result from loss-of-function mutations in genes of the Wingless (Wg) signaling pathway. Zygotic loss of *ska* leads to reduced growth of imaginal discs and pattern defects similar to *wg* mutants. In addition, genetic interactions of *ska* with *wg* and *dishevelled* mutants are observed. These data demonstrate the importance of proteoglycans and GAGs in Wg signaling in vivo and suggest that Wnt-like growth factors may be particularly sensitive to perturbations of GAG biosynthesis.

Key words: *Drosophila*, glycosamino glycans, GAGs, proteoglycans, syndecan, glucuronic acid, UDP-glucose dehydrogenase, UDPGDH, *suppenkasper* gene, *ska*, *wingless* gene, *wingless* signaling, pattern formation

INTRODUCTION

Glucuronic acid is an essential building block of several classes of glycosaminoglycans (GAGs) that are found in both vertebrates (reviewed in Bernfield et al., 1992; Jackson et al., 1991; Kjellen and Lindahl, 1991) and invertebrates (Cassaró and Dietrich, 1977; Cambiasso and Inestrosa, 1990). GAGs are linear carbohydrate chains composed of a repeating disaccharide that is attached to serine residues of a core protein to produce a proteoglycan. Seven classes of GAG chains are recognized depending on the type of disaccharide repeat that they contain and the manner in which the chains are modified by sulfation and sugar epimerization (Kornfeld and Kornfeld, 1980). These glycans include heparin, heparan sulfate, hyaluronic acid, chondroitin sulfate, dermatan sulfate and keratan sulfate.

The biological functions ascribed to GAGs are quite varied and include biomechanical, structural and regulatory roles (David, 1993; Jackson et al., 1991; Kjellen and Lindahl, 1991; Yanagishita and Hascall, 1992). Of particular interest, GAG chains can concentrate and assemble various proteins on the cell surface including proteases, antiproteases, lipolytic enzymes (Griffith, 1986; Shimada et al., 1981) and, most importantly, growth factors (David, 1993; Jackson et al., 1991). Many growth factors will bind heparin (e.g. heparin-binding-EGF (HB-EGF), PDGF, BMP-2, members of the Wnt and Hedgehog (Hh) families, fibroblast growth factor (FGF) and transforming growth factor β (TGF- β), and several mechanisms have been proposed for the regulation of signal transduction by proteoglycans (Bradley and Brown, 1995; Kelly et al., 1993; Lee et

al., 1994; Reichsman et al., 1996; Ruppert et al., 1996). These include a coreceptor function involving low affinity binding of ligand (Klagsbrun and Baird, 1991; Schlessinger et al., 1995), and possibly sequestering of growth factors in the matrix for later release (Yanagishita et al., 1992).

Despite the abundance of information on GAG chains in vertebrate tissues, little is known about the roles that these polymers play during development. In this report, we describe the phenotypic effects of mutations in the *Drosophila suppenkasper* (*ska*) gene. We show that *ska* codes for the enzyme UDP-glucose dehydrogenase, which makes UDP-glucuronate from UDP-glucose. In the absence of a known salvage or bypass pathway, *ska* mutants are expected to lack all glucuronate-containing GAG carbohydrate chains. *ska* mutants thus provide the first opportunity to investigate the function of the GAGs in vivo. Surprisingly, *ska* mutants do not exhibit highly pleiotropic phenotypes. Developmental defects appear to be primarily the result of reduced Wg signaling. These results are discussed in relation to some of the proposed roles of GAG polymers.

MATERIALS AND METHODS

Drosophila stocks and handling

In this study, the *ska* alleles SG9 (Shearn and Garen, 1974), A31, N71 (Anderson et al., 1995), *l(3)08310* and *l(3)10503* (Bloomington stock center) and the deficiencies Df(3L)W5.4 and Df(3L)XAS96 (Anderson et al., 1995) were used. *ska*^{SG9} and *ska*^{P8310} were recombined with FRT sites at 79 D/E and balanced over TM3, *Ubx-lacZ* (TM3 *P{w⁺mc}*, *Ubx-lacZ*). The *P{w⁺mc}*, *Hs-wg* and *P{w⁺mc}*, *Hs-hh*

transgenes, abbreviated *hs-wg* and *hs-hh* (supplied by A. Bejsovec) and the *P{w^{mc}, Kr-lacZ}* *Kr-lacZ* transgenes were crossed into the *ska^{P8310}* line and homozygous stocks were established. The 2.3 kb cDNA was cloned into the *ubiquitin-hs70-Casper* (*P{w^{mc}, ubi-ska}*) (Brummel et al., 1994) and UAST vectors (Brand and Perrimon, 1993) and injected into *w* embryos. The *P{w^{mc}, ubi-ska}* transformants were used to rescue *ska^{SG9}* and *ska^{P8310}* mutants. The CyO-*wg*lacZ chromosome (CyO, *P{lacZ^{enA}, ry⁺17.2=en1/wg^{en11}}*) (Kassis et al., 1992) was used to make lines heterozygous for *wg*.

To obtain germ-line clones, *hs-FLP/+*, *ska^{P8310}-FRT79/ovo^D-FRT79* females were heat shocked for 2 hours in a 37°C warm room. After recovering from heat shock for several hours, they were crossed to the *ska^{P8310} / TM3,Ubx-lacZ* stock or to the *hs-wg* and *hs-hh* versions of that stock. For ubiquitous *wg* and *hh* expression by heat shock, embryos were collected for 2–5 hours after egg laying and heat shocked for 20–30 minutes in a water bath at 37°C. They were transferred into a 25°C incubator and fixed 1.5–2 hours later with 7% formaldehyde in PBS for 20 minutes.

To produce mitotic clones in adult tissues, females of the following genotype, *y w hsFLP1; mwh³ ska^{P8310} P{ry⁺; hs-neo; FRT180B/P{ry⁺; y⁺66E, P{ry⁺; hs-neo; FRT180B}*, were heat shocked during first and second instar larva stages at 37°C for 1 hour (Xu and Rubin, 1993). The resulting *ska^{P8310}* clones were marked with *mwh* and *y*. Adult structures were dissected in 70% EtOH, dehydrated briefly in isopropanol, placed under a coverslip in Gary's magic mountant (Reichsman et al., 1996) and photographed using a Nikon Optiphot.

In situ hybridizations

wg and *dpp* expression were monitored by whole-mount in situ hybridization using digoxigenin-labeled antisense RNA probes. Plasmids (all in Bluescript) used as templates for probes were F3b (*ska* 2.3 kb cDNA); *wg651* (a kind gift of B. Cohen), a 3 kb *wg* cDNA; *dppE55*, a 4 kb *dpp* cDNA (Padgett et al., 1987); *hhC11*, a 2.3 kb cDNA (Lee et al., 1992). The probes were prepared according to the manufacturer's directions (Boehringer Mannheim 1277 073). Unincorporated nucleotides were removed by LiCl precipitation and the RNA from 1 µg of template was resuspended in 100 µl DEPC water; 20 µl of each probe were hydrolyzed LiCl precipitated, resuspended in 100 µl hybridization solution and diluted 1/50 µl for the hybridization reaction. The prehybridization procedure and hybridization conditions used are based on the protocol of (Tautz and Pfeifle, 1989) with previously described modifications (Mason et al., 1994).

Immunohistochemistry and immunofluorescence microscopy

Antibody stainings were carried out following the protocol published by Frasci et al. (1987), using the Vectastain reagents (anti-mouse-biotin-streptavidin-HRP), AP-anti-rabbit antibodies from Promega, or FITC- and Cy3-conjugated anti-rabbit and anti-rat from Jackson laboratories. Antiserum against En (a kind gift from N. Patel) was used at a 1:2 dilution. Antiserum against β-galactosidase was obtained from Sigma (rabbit) and from Promega (mouse) and used at dilution of 1:5000 and 1:1000, respectively. Embryos were stained in 0.5 mg/ml 3,3-DAB in 0.1 M Tris-Cl, pH 7.5 (HRP) or with NBT/BCIP (AP). Antiserum against Arm (rat), a kind gift from M. Peifer, was used at 1:250. Embryos were mounted in either Canada balsam or 75% glycerol, 50 mg/ml n-propyl gallate (immunofluorescence) and observed with a Bio-Rad MRC 1024 scanning confocal microscope. Antiserum against *Drosophila* Syndecan (Spring et al., 1994), a gift from Stephenie Paine-Saunders, was used at 1:200. Antiserum against Mad (guinea pig) was a kind gift from Stuart Newfeld and used at 1:1000 (Newfeld et al., 1996). Protein bands that are recognized by the anti-syndecan (rabbit) and anti-Mad antisera were visualized using the ECL reagents from Amersham. Proteins that contain heparan sulfate side chains were detected by AP-staining using a monoclonal antiserum obtained from Seikagaku, America, Inc. at a dilution of 1: 2000.

Immunoblotting

Approximately 900 *ska^{P8310}* germ-line mutant embryos were selected by their phenotype. Wild-type embryos were collected as a control. Both batches of embryos were homogenized in 2 M urea/150 mM NaCl/50 mM sodium acetate pH 4.5/0.1% Triton X-100, and protein concentrations were measured by Bradford assay. 42 mg of wild-type and mutant protein extracts were run on a 7% denaturing acrylamide gel and transferred to a nitrocellulose membrane in a wet transfer chamber (Harlow and Lane, 1988).

Cloning of *ska*

Genomic DNA was obtained from *l(3)08310* and *l(3)10503* flies by plasmid rescue. Briefly, the DNA was digested with *Xba*I or *Xba*I and *Spe*I, and then ligated and electroporated into DH5α-bacterial cells. After outgrowth for 1 hour at 37°C, the cells were plated on kanamycin-containing medium and resistant colonies picked for analysis. The P-element insertion sites were identified by sequencing using P-end and 5' specific primers. A 4 kb *Hind*III fragment was used to screen genomic (λ-fix Stratagene) and cDNA λ-zap, a kind gift from C. Thummel) libraries. Several positive cDNA clones were obtained. The longest, a 2.3 kb cDNA (F3b), was isolated and both strands were sequenced.

RESULTS

Identification of the *ska* gene

We have devised several sensitized genetic screens to identify genes that modulate either Wg or Dpp signaling. In one screen, an constitutive active Dpp type I receptor encoded by the *thick veins* (*tkv*) gene is ectopically expressed in wing discs resulting in a highly blistered wing phenotype (Hoodless et al., 1996). Dominant suppressors of this phenotype are recognized by their ability to reduce blistering. In a second screen, modulators of Wg signaling are identified by their ability to enhance a weak *dishevelled* (*dsh^w*) adult phenotype (Theisen et al., 1994). *Dsh* is a downstream component of the Wg signaling pathway (Klingensmith et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995). Since both *dpp* and *wg* are required for proper growth and patterning of imaginal discs, we used our sensitized backgrounds to screen a collection of small disc mutants (Shearn and Garen, 1974) to determine whether loss of one allele of any of these genes would either enhance the weak *dsh* phenotype or suppress the activated *tkv* wing phenotype. One mutation of the collection, SG9, which defines the locus that we now refer to as *ska*, significantly enhances the weak *dsh* phenotype (Fig. 1A) when heterozygous. On average, 5% of the weak *dsh* mutant animals show adult defects consisting primarily of deleted or duplicated antennae and/or deleted wings with a concurrent duplication of notal structures (Fig. 1B,C). When heterozygous for *ska^{SG9}*, the frequency of adult defects increases to 19%. The *ska^{SG9}* allele also shows a mild suppression of the activated *tkv* phenotype (data not shown). Homozygous and hemizygous *ska^{SG9}* animals die at the larval and pupal stage and contain small imaginal discs. Some homozygotes produce pharate adults with reduced and partially differentiated adult tissues that are derived from the imaginal discs while the size of tissues derived from histoblasts is normal (Fig. 2B).

To further characterize this locus, we sought additional alleles of *ska*. The original SG9 mutation was mapped by recombination to the left arm of the third chromosome (Shearn and Garen, 1974), corresponding roughly to the 65 cytological interval. Several deficiencies for this region fail to complement *ska^{SG9}*. Complementation tests for lethal mutations reveal that the EMS

mutations AE31 and N71 are allelic to *ska*^{SG9} (Anderson et al., 1995). In addition, two P element inserts obtained from the *Drosophila* genome project, *l(3)08310* and *l(3)10503*, also fail to complement *ska*^{SG9}. All these alleles appear to be stronger than SG9 since, when heterozygous to a deficiency for the region, they die between the first and second instar larval stages while SG9 dies as a pharate adult. The *ska*^{P8310} allele shows a higher frequency of adult defects (41%) in a weak *dsh* background than SG9 (Fig. 1A). The enhancement of the *dsh*^w phenotype by the strong *ska* alleles is similar to that by strong *wg* alleles suggesting that *ska* may be involved in mediating Wg signaling.

The loss of *ska* primarily affects *wg* signaling

To determine whether *ska* is maternally contributed, we produced germ-line clones of both the *ska*^{SG9} hypomorphic allele as well as the stronger *ska*^{P8310} allele. Cuticles prepared from mutant animals derived from germ-line clones (henceforth referred to simply as *ska* mutant embryos) show a loss of the naked cuticle and a mirror-image duplication of denticle belts (Fig. 2A). This phenotype is strikingly similar to that produced by loss-of-function mutations in genes of the Wg signaling pathway (Bejsovec and Martinez Arias, 1991). In contrast, mutant animals from germ-line clones that inherit a wild-type copy of *ska* from the father (identified in all our experiments by marked balancer chromosomes) are fully rescued and show no defects.

We employed several experiments to address the role of *ska* in Wg signaling. In the first set of experiments, we examined the expression of *engrailed* (*en*) and *wg* in *ska* mutant animals. Transcription of *en* and *wg* is initiated at the blastoderm stage through the action of pair-rule genes in stripes of neighboring cells that form the parasegment compartment boundary (Lawrence and Johnston, 1989; Lawrence and Struhl, 1996). At germ-band extension, *wg* and *en* mutually support each other's expression as the result of a feedback loop involving *hedgehog* (*hh*) (reviewed in Martinez Arias, 1993). In the absence of *wg* signaling, expression of both *wg* and *en* fade during late germ-band extension (DiNardo et al., 1988; Ingham, 1993; Martinez Arias et al., 1988). We observed similar effects in *ska* germ-line mutant embryos. *En* and *wg* stripes are present at early germ-band extension (Fig. 2C,E), but start to fade at late germ-band extension and during germ-band retraction (Fig. 2D,F). During the time that *wg* expression is fading, partial *En* stripes are seen in many embryos even after germ-band retraction (Fig. 2D). Since similar results have been observed in temperature-sensitive mutants of *wg* (*wg*-ts), our results suggests that Wg signaling is reduced but not eliminated in strong *ska* mutants. In wild-type embryos and *wg*-ts mutants, *En* is expressed in stripes that are two cells wide. In contrast, in some of the *ska* mutant embryos, both early and late *En* stripes are up to four cells wide. One possible explanation for this observation is that Wg protein may migrate further from source cells in *ska* mutants than in wild type, but the overall local concentration that a cell perceives is lower and thus signaling is ultimately attenuated.

Since there is a feedback loop that requires Hh to maintain *wg* expression, these effects could also be interpreted as the result of loss of Hh rather than Wg signaling. Since accumulation of Arm in early germ-band-extended embryos is the most direct assay that we know for Wg signaling, we examined the distribution of Arm protein in *ska* embryos. Arm protein accumulates in the cytoplasm only in cells that receive the Wg signal

(Riggleman et al., 1990). In paternally rescued *ska* germ-band-extended mutant embryos, the Arm protein is enhanced in stripes of cells that receive the Wg signal (Fig. 2G). However, in *ska* mutant embryos, the intensity of the Arm stripes are significantly reduced (Fig. 2H). This result indicates that Wg signaling is reduced but not completely eliminated in *ska* mutants at a time when *wg* expression is still independent of Hh.

As a further test for the function of *ska* in Wg signaling, we ectopically expressed *wg* in *ska* germ-line mutants using a heat-shock promoter. In wild-type embryos, *hs* driving *wg* leads to an expansion of the *En* stripes from roughly two cells to four cells (Noordermeer et al., 1992). In contrast, loss of *ska* both maternally and zygotically blocks the ability of *hs-wg* to expand the *En* stripes and leads instead to reduced rather than expanded *En* stripes (Fig. 2J). Paternal rescue of *ska* embryos restores the ability of *hs-wg* to cause an expansion of the *En* stripes after heat shock (Fig. 2I). These results suggest that the Wg signal is not properly received or transmitted in *ska* mutants.

A similar experiment using *hs-hh* (Ingham, 1993) leads to expansion of the *wg* stripes in both wild-type or paternally rescued *ska* embryos (Fig. 2K). However, in *ska* mutant embryos, the *wg* stripes fade (Fig. 2L) suggesting that Hh is not able to induce *wg* expression in *ska* mutant background. However, since Wg autocrine signaling is required for *wg* expression (Hooper, 1994) and Wg signaling is reduced in *ska* mutants, it is difficult to determine whether the observed effect is primarily the result of reduced Wg signaling or the effect of a combined reduction of Wg and Hh signaling.

Since the *ska* mutations did show weak suppression of an constitutive active Tkv phenotype in wings, we examined whether the *ska* germ-line mutants exhibited any evidence of dorsal-ventral patterning defects by asking whether the ventral denticle belts are expanded in *ska* mutant embryos as they are in embryos mutant for *dpp* or its effectors. We find no evidence for expansion of denticle belt width as is typically produced by mutations in genes of the Dpp signaling pathway (Arora and Nüsslein-Volhard, 1992). Further, formation of the amnioserosa, the dorsal-most tissue that is the most sensitive indicator of loss of Dpp signaling (Wharton et al., 1993) appears normal in *ska* mutant embryos when monitored by expression of the *Krüppel* (*Kr*) gene as a marker for amnioserosa (Fig. 3E). Finally, *ska* embryos gastrulate properly. These results suggest that Dpp signaling is not substantially affected in the early *ska* mutant embryos.

ska requirements in imaginal discs

To explore the role of *ska* in adult patterning, we generated clones of the strong *ska*^{P8310} mutation in imaginal discs using FRT-mediated recombination (Xu et al., 1993). Induction of clones during the first or second instar produced large clones in adult tissues but with no patterning defects even when the clones involved regions in which Wg signaling is required as wing margin or ventral legs (Fig. 3A,B).

We also analyzed the zygotic phenotypes of several different *ska* mutants. As described, *ska*^{SG9} is the only allele that survives to the third instar larval stage. *ska*^{SG9} homozygous larvae have smaller but normal-looking imaginal discs (data not shown). In *ska*^{SG9}/*ska*^{A31} transheterozygotes, the eye disc is proportionally much smaller compared to the other discs. When stained with probes against *wg*, *hh* and *dpp*, normal *wg* and *hh* expression is observed but very low *dpp* expression was detected in the morphogenetic furrow compared to wild type (Fig. 3F-H). The

Fig. 1. Interactions of *wg* and *ska* with a weak *dsh* mutant background. (A) Table of the interactions. 5% of the flies rescued by the weak *dsh* transgene show defects in adult structures. About 62% of mutants that are heterozygous for *wg* in this weak *dsh* background have defects similar to those shown in B and C. Flies heterozygous for the weak *ska*^{SG9} and for the strong *ska*^{P8310} mutants exhibit defects with a frequency of 19% and 41% in this background. (B,C) Typical adult defects seen in mutations that interact with the *dsh* mutants rescued by a weak transgene. (B) Heterozygous *wg* mutant that exhibit a defective antenna (arrowhead) and a deletion of the wing (arrow). (C) Leg of a fly that is heterozygous for the strong *ska* mutation in the weak *dsh* background. Note the presence of four claws at the distal end of the leg as well as the reduced sex combs (arrow) indicating a loss of ventral tissue in the leg. Similar dorsal pattern duplications are also seen in *wg* mutants (Theisen et al., 1994).

expression of *dpp* in the antennal portion of this disc appears normal. Since *dpp* expression in the furrow is dependent on *hh* signaling, this result raises the possibility that *hh* signaling may be affected to some degree by the *ska* mutation.

ska encodes UDP-glucose dehydrogenase

To clone the *ska* locus, we used plasmid rescue to isolate genomic DNA flanking the P8310 and P10503 insertion sites. Hybridization probes prepared from the genomic sequences

were then used to screen *Drosophila* cDNA libraries. The largest clone identified, a 2.3 kb cDNA was sequenced (Fig. 4A). The cDNA contains an open reading frame beginning 223 bp from the 5' end and extending to bp 1532, 752 bp from the 3' end. The open reading frame encodes a predicted protein of 53×10³ Mr. Comparison of this clone to the GenBank database revealed that the *Drosophila* sequence is approximately 66% identical at the amino acid level to the *C. elegans*, bovine and soy bean (*Glycine max*) UDP-glucose dehydrogenase enzymes

Fig. 2. The *ska* mutation affects Wg signaling. (A) Cuticle preparations of *ska*^{P8310} germ-line mutant embryos show a segment polarity phenotype similar to *wg*. (B) In *ska*^{SG9} *Df(3L)W5.4* pharate adults, all structures that are derived from imaginal discs are small and not fully developed. (C) En immunostaining in stage 11 *ska*^{P8310} germ-line mutant embryos is detected in stripes that are about 2-4 cells wide. (D) The En stripes fade during stage 12 but are still present in patches that are up to 4 cells wide in many embryos at stage 13 and later. (E) *wg* expression is normal at embryonic stage 9 and fades at stage 10 (F). (G) Arm protein (immunofluorescence) is accumulating in stripes of cells that receive the Wg signal in a paternally rescued embryo (*ska*^{P8310}/TM3, *Ubx-lacZ*). (H) There is very little accumulation of Arm in *ska*^{P8310} germ-line mutant embryos suggesting that Wg signaling is significantly reduced. (I) The domain of En expression (brown) expands anteriorly after ubiquitous expression of *wg* by heat shock in paternally rescued embryos of *ska*^{P8310} germ-line clones (*Ubx-lacZ* staining is faint blue). (J) In *ska*^{P8310} germ-line mutant embryos, En fades even with ubiquitous *wg* expression. (K) *wg* expression expands anteriorly after ubiquitous expression of *hh* by *hs-hh* in paternally rescued embryos (identified by the presence of *Ubx-lacZ* expression). (L) *wg* expression fades in *ska*^{P8310} germ-line mutant embryos despite ubiquitous *hh* expression.

Fig. 3. Effects of *ska* mutations on larval and adult tissues.

(A,B) Somatic clones of *ska*^{P8310} induced in second instar discs show no mutant phenotype in any region of the wing (marked with *mwh* and outlined in red) or other adult tissues. (C) A partially rescuing *ubi-ska* transgene shows severe margin and venation defects in *ska*^{SG9} homozygous animals. (D) Wings of *ska*^{P8310} homozygous animals rescued by a *ubi-ska* transgene are missing the distal portions of veins L2, L4 and L5. (E) *Kr-lacZ* immunostainings of the amnioserosa in *ska*^{P8310} germ-line mutant embryos indicate that Dpp signaling is not significantly affected. (F) *dpp* expression in wild-type third instar eye-antennal disc shows elevated expression in the morphogenetic furrow. (G) *dpp* expression in *ska*^{SG9/ska}^{A31} third instar eye-antennal disc at the same magnification as F. The eye portion of the disc is smaller and *dpp* expression in the morphogenetic furrow is missing. (H) *hh* expression in *ska*^{SG9/ska}^{A31} eye-antennal discs appears normal.

(Fig. 4B). The clone showed much lower similarity to enzymes of different bacteria as well as to other related dehydrogenases (not shown).

To confirm that this sequence corresponds to the *ska* locus, we determined the precise insertion point for the two P elements. These two transposons were located 38 bp from each other in the 5' untranslated region (Fig. 4A). To show conclusively that the transcript represents the *ska* locus, we expressed the 2.3 kb cDNA in transgenic animals using a *ubiquitin* transformation vector *P*{*w*⁺^{mC}, *ubi-ska*} or *ubi-ska* (Brummel et al., 1994). We found that animals homozygous for *ska*^{SG9} as well as *ska*^{P8310} were completely rescued in the presence of most transgenes. These results indicate that the *ska* phenotype is caused by the lack of UDP-glucose dehydrogenase activity.

Interestingly, some transgenic lines show only partial rescue, possibly due to position effects on the *ubiquitin* promoter. One of these transgenes gives partial rescue and produces a bat-shaped wing (Fig. 3C) with margin defects characteristic of loss of Wg signaling (Couso et al., 1994). Other transgenes produce distal deletions of veins (Fig. 3D) that are characteristic of mutants in the EGF- and Dpp-signaling pathways (Spencer et al., 1982; Sturtevant and Bier, 1995; Sutherland et al., 1996).

To further investigate the role of *ska* in Wg signaling at later stages of development, we crossed a *wg* mutant allele into the background of a *ska*^{P8310} mutant that was completely rescued by a single copy of the *ubi-ska* transgene. Animals heterozygous for a *wg* mutation died as second instar larvae whereas animals with two wild-type copies of *wg* were completely rescued. This result shows that a 50% reduction in the Wg signal is able to reverse the rescuing effect of the transgene suggesting that Wg signaling is compromised by *ska* mutations not only in embryos but also in larvae.

ska is differentially expressed during development

To determine the pattern of *ska* expression during development, we hybridized digoxigenin-labeled RNA probes to whole-mount embryos and imaginal discs (Fig. 5). As expected from the germ-line clonal analysis, significant maternally deposited

ska message is found in precellularized embryos (Fig. 5A). At the germ-band-extended stage, expression is enriched in the mesoderm (Fig. 5B) followed by strong staining in the midgut after germ-band retraction (Fig. 5D). After dorsal closure, expression is enriched in the hindgut and pharynx (Fig. 5D,E). In third instar larvae, ubiquitous expression is observed in most imaginal discs (Fig. 5F-H). However, low levels of expression are seen in the eye disc with enriched levels in the morphogenetic furrow (Fig. 5F).

ska mutations lack GAG chains on proteoglycans

UDP-glucose dehydrogenase is required for the synthesis of UDP-glucuronate (GlcUA), a precursor for the production of GAG chains. Since both *ska* P-elements are inserted into the 5' leader region of *ska*, we expected that these strong alleles should produce very little of this enzyme and hence should have little or no GAG modification on proteoglycans. To obtain physical evidence that GAG chain biosynthesis is indeed blocked in *ska* mutants, we monitored GAG-containing proteoglycans on immunoblots of extracts from wild-type and *ska* germ-line mutant embryos using a monoclonal antiserum against heparan sulfate. Heparan sulfate consists of a repetitive unit of GlcUA and GlcNAc that is attached via two galactose and a xylose molecule to Ser/Thr residues of proteoglycans. Since the UDP-xylose that is attached first to the Ser/Thr residue is also synthesized from UDP-GlcUA, we expect that no GAG side chains should be formed in the absence of GlcUA. As shown in Fig. 6A, the wild-type lane shows multiple bands that strongly interact with the antiserum against heparan sulfate. These bands do not appear in the *ska* mutant lane. The same blot was also monitored for the migration properties of *Drosophila* Syndecan (Kan et al., 1993; Spring et al., 1994). Syndecan is a heparan-sulfate-containing transmembrane proteoglycan present in both vertebrate and invertebrates (Bernfield et al., 1992; Couchman and Woods, 1996). On western blots, Syndecan from wild-type embryos migrates as a smear of two or three major bands above 200×10³ M_r (Fig. 6B). In extracts from *ska* germ-line clones, the high molecular mass smear is missing while a novel band

Fig. 4. *ska* encodes UDP-glucose dehydrogenase. (A) Sequence of the *ska* cDNA. The 2.3 kb cDNA contains an open reading frame of 476 amino acids. The sequence surrounding the first ATG is a good match to the *Drosophila* consensus for translational initiation (Cavener, 1987) and is likely to be the true start codon. The integration sites (bold type) of the larval lethal P element mutations *l(3)10503* and *l(3)8310* are located 38 bp from each other in the 5' untranslated region. The GenBank accession number for *ska* DNA is AF 007870. (B) Sequence alignment of UDP-glucose dehydrogenases of *Drosophila melanogaster* (*Dm*), *C. elegans* (*Ce*), *Bos taurus* (*Bt*) and *Glycine max* (*Gm*). The fly, worm, bovine and the soy bean proteins show similar identities of about 66%.

migrating around $125 \times 10^3 M_r$ appears. Control staining using an antiserum against the *mothers against dpp* gene product (Mad) show that equal amount of protein extract were loaded, and that no protein degradation occurred (Fig. 6C). These results suggest that GAG chain biosynthesis is inhibited in *ska* mutants in *Drosophila*.

DISCUSSION

Fates of glucuronic acid

In this report, we show that the *Drosophila ska* locus codes for UDP-glucose dehydrogenase. This enzyme is required for the conversion of UDP glucose to UDP-glucuronic acid. Our results suggest that the most crucial role for this compound during *Drosophila* development is to permit the synthesis of GAGs that help mediate Wg and possibly other growth factor signaling.

In vertebrates, UDP-glucuronic acid serves several functions. In the liver, UDP-glucuronate helps detoxify nonpolar molecules by converting them into more easily secreted polar derivatives (Tephyl and Burchell, 1990). In another pathway, UDP-glucuronate is converted into L-ascorbic acid (vitamin C; Lehninger et al., 1993). Several vertebrate species including humans lack the last enzyme in the vitamin C biosynthetic pathway and therefore require vitamin C in the diet. It seems unlikely that these two pathways could account for the *ska* mutant phenotype in *Drosophila*. First, problems caused by the failure to detoxify ingested compounds are not likely to occur in embryonic epidermal cells, a tissue whose patterning is dramatically affected by *ska* mutants. Second, it can be assumed that the lack of vitamin C synthesis, at least in larva, can be compensated by uptake of this compound from the media during feeding and therefore the larval defects that we observe in partial loss-of-function *ska* mutants are not likely to be caused by a vitamin C deficiency.

The most likely explanation for the *ska* mutant phenotype is a block in the synthesis of the glycosaminoglycan side chains of proteoglycans. As we have shown in Fig. 6A, extracts from *ska* germ-line mutant embryos lack the robust bands of several proteoglycans that are recognized by a monoclonal antiserum against heparan sulfate. In addition, the high molecular bands of the heparan sulfate-containing protein Syndecan are missing in extracts from mutant embryos whereas a novel band of about $125 \times 10^3 M_r$ is recognized (Fig. 6B). Control staining against the Mad protein shows that there is no protein degradation (Fig. 6C) in our samples leading us to conclude that GAG chain synthesis is severely effected in *ska* mutants and that there is no bypass or salvage pathway that permits GAG chain synthesis in the absence of *ska*.

GAGs in Wg signaling

Consistent with our finding that GAG chain removal severely reduces Wg signaling is the recent observation that Wg signaling in *Drosophila* tissue culture cells can be inhibited by the removal of GAGs from the surface of cells receiving the Wg signal and restored by the addition of exogenous heparin (Reichsman et al., 1996). In addition, heparan sulfates are implicated in Wnt -11 autocrine signaling in the ureter epithelium of the mouse (Kispert et al., 1996).

Several mechanisms have been proposed whereby GAGs might influence cytokine signaling. One proposal is that cell surface proteoglycans act as low affinity coreceptors for growth factors such as FGF and TGF- β (Schlessinger et al., 1995). By

this model, proteoglycans would reduce ligand diffusion from three to two dimensions (Bernfield et al., 1992). Binding to the cell surface via interaction with a low affinity proteoglycan would increase the local concentration of ligand, thus enhancing the probability of a productive interaction with a high affinity receptor. The heparan sulfate side chains of cell surface Syndecan may perform this function in mediating FGF signaling (Bernfield and Hooper, 1991, 1993; Steinfeld et al., 1996). For TGF- β betaglycan represents the low affinity type III receptor (Lopez-Casillas et al., 1993). In this case however, TGF- β appears to bind directly to the core protein itself rather than the carbohydrate side chains (Lopez-Casillas et al., 1994).

A second model of GAG action is to oligomerize ligands thus inducing receptor clustering. In the case of FGF, as well as several other heparan sulfate binding growth factors, the high affinity receptors have intracellular tyrosine kinase domains which are activated by transphosphorylation as a result of ligand-induced receptor dimerization. Since FGF binds to the extracellular domain of its high affinity receptor as a monomer (Spivak-Kroizman et al., 1994) that is not capable of inducing receptor dimerization on its own, it has been proposed that a multimeric heparan sulfate FGF complex is required in order to produce a biologically active signal (Mason, 1994; Ornitz et al., 1992).

The phenotypic effects of *ska* mutations are consistent with a role for GAGs in mediating Wg signaling. There are two major effects on Wg signaling seen in *ska* mutants. First, there are initially more cells that express the En protein suggesting that Wg is diffusing further than in wild-type embryos. Second, the level of Arm that accumulates in the Wg-receiving cells is much lower in *ska* mutant background and eventually the En staining also fades. These observations suggest that GAG chains in the extracellular matrix may help to concentrate Wg perhaps by restricting its diffusion. GAG chains may also modulate reception of the Wg signal. The recently identified candidate Wg receptor (DFrizzled 2) is a member of the serpentine class of seven transmembrane G-protein-coupled receptors (Bhanot et al., 1996). There is no known requirement for receptors of this class to oligomerize in order to signal. Thus, the primary role of GAGs in Wg signaling may be to increase surface concentration of Wg on receiving cells as opposed to mediating receptor oligomerization. Alternative models such as stabilization of the ligand from potential proteolytic degradation could also contribute to an increased local concentration of Wg and these can not be ruled out at the present time. It will also be of interest to determine if there is one particular proteoglycan that acts as a Wg co-receptor or whether several different molecules are involved. In this regard, we note that *syndecan* mutants enhance the weak *dsh* phenotype similar to *ska* mutants while *dally* mutants (a *Drosophila* glypican homolog) do not (T. Heslip and J. L. Marsh unpublished). Thus, individual classes of proteoglycans may each have distinct roles in mediating signaling by different types of growth factors. Further study will be required to investigate which proteins are involved in the molecular mechanism of this effect.

Are other growth factor signaling pathways affected in *ska* mutants?

Many vertebrate growth factors bind heparan sulfates and *Drosophila* expresses homologs of several of these including FGF, EGF, BMPs and Hh (Arora et al., 1994; Lee et al., 1992; Neuman-Silberberg and Schupbach, 1993; Padgett et al., 1987; Rijsewijk et al., 1987; Rutledge et al., 1992; Sutherland et al.,

Fig. 5. Expression pattern of *ska* during development. (A) Wild-type stage 3 embryo showing uniform maternal expression. (B) Stage 10 germ-band-extended embryos have enriched expression in the mesoderm. (C) Stage 13 germ-band-retracted embryos show robust staining in the midgut. At stage 16, expression appears in the proventriculus and hindgut (D), and later at stage 17 in the pharynx (E). Expression in eye-antennal (F), leg (G) and wing discs (H) is relatively uniform. Note that expression of *ska* is enriched in the morphogenetic furrow of the eye disc (F).

1996). It is therefore surprising that we do not see a more pleiotropic phenotype in *ska* mutants. The Wg pathway may be more sensitive to perturbations in GAG synthesis than other signaling pathways in *Drosophila*. An alternative explanation may be that Wg in the embryo is the earliest pathway to be affected and therefore involvement of GAGs in mediating later signaling events by other factors might be masked. Certainly this could be the case for *Drosophila* FGF signaling which is required for tracheal development (Sutherland et al., 1996). Tracheal branching and outgrowth in response to FGF signaling begins relatively late during the extended germ-band stage and continues through germ-band retraction and dorsal closure. By this stage, defects resulting from a lack of Wg signaling are quite pronounced and it is difficult to assess whether FGF is impaired or not.

We were also surprised by the absence of *dpp*-like phenotypes in *ska* mutants (e.g. no dorsal-ventral cuticle defects and normal amnioserosa). Likewise, expression of the Dpp responsive transcription factor *spalt* (de Celis et al., 1996) is normal in *ska*^{SG9/ska}^{A31} mutant wing imaginal discs (data not shown) suggesting that Dpp signaling either does not require *ska* function or is much less sensitive to its loss. The recent demonstration that *dally* mutants interfere with Dpp signaling in the wing (Selleck, personal communication) suggests that the effect of *dally* on Dpp signaling may be mediated through the core protein rather than the GAG side chains as has been found to be the case for the effects of betaglycan on TGF- β signaling (Lopez-Casillas et al., 1994).

Even for cytokine genes expressed early in embryogenesis, the requirement for *ska* and GAGs may differ. For example, EGF receptor mutants lack ventral ectoderm (Raz and Shilo, 1992, 1993) but strong *ska* mutants make ventral ectoderm suggesting that EGF signaling in the embryo may not be sensitive to loss of *ska*. A possible requirement for GAGs in later EGF signaling events in the imaginal discs involving the two EGF-like homologs, *vein* and *spitz* (Mason et al., 1994; Schweitzer et al., 1995) has not been directly examined. However, we do find that certain *ubiquitin-ska* transgenic lines only partially rescue *ska* mutants and also show wing phenotypes similar to those produced by mutations in the EGF signaling pathways (Fig. 2E).

The reason why *ska* mutations act as moderate suppressors of the activated Tkv phenotype is not clear. However, heterozygosity for EGF-receptor mutations can suppress the

activated Tkv phenotype similar to the suppression seen with *ska* mutations (unpublished observations). Thus, one explanation for the suppression of the activated Tkv phenotype could be that EGF signaling is reduced in *ska* mutants sufficiently to suppress the activated *thick veins* phenotype but not enough to cause an EGF-like phenotype. Another possibility is that GAGs could directly influence Dpp receptor function. Consistent with this view, BMP-2 signaling is enhanced in a limb bud assay by addition of exogenous heparan sulfates even when the BMP-2 ligand is deleted for the heparan sulfate binding sequence (Ruppert et al., 1996). These authors suggest that the exogenous heparin might potentiate receptor activity perhaps by stabilizing receptor complexes or conformations. A third possibility is that GAG breakdown products have been implicated in direct modulation of some transcription factors and, in one recent case, in directly modulating a cell cycle control protein by binding to it (Grammatikakis et al., 1995). Thus, GAGs may be able to exert their functions inside the cell as well as outside. If so, lack

Fig. 6. *ska* affects heparan sulfate side chain synthesis. (A) Extract from wild-type and *ska*^{P8310} germ-line mutant embryos were immunoblotted from a 7% denaturing acrylamide gel and incubated with a monoclonal antiserum against heparan sulfate. Several robust bands are detected in the wild-type lane but not in the *ska* mutant lane. (B) Analysis of the same blot using an antiserum against the heparan sulfate-containing protein Syndecan. The extract from wild-type embryos reacts with a smear containing two or three major bands that migrate higher than 200 kD (bracket). These bands are missing in extracts from *ska* germ-line mutant embryos while a new band running around 125×10³ M_r appears (arrow). A band migrating at about 115×10³ M_r is common to both wild-type and mutant extracts. (C) Control staining of the blot shown in A and B with an antiserum against the *mothers against dpp* (*mad*) gene product showing that equal amounts of proteins were loaded and no protein degradation has occurred.

of GAGs could potentially directly modulate intracellular signaling events.

Because of a mutually supportive positive feedback loop in the late germ-band-extended embryo, it is difficult to distinguish the role of GAGs in Wg versus Hh signaling at this stage (Martinez Arias, 1993). By-passing this loop by ectopic expression of one or the other factor does not resolve the two events because Wg signaling seems also to be required for Hh-induced expansion in *wg* expression (Hooper, 1995). Our studies of Arm accumulation and the late appearance of partial En stripes in *ska* mutants suggest that Wg signaling is greatly reduced but still partially functional in *ska* loss-of-function animals. Therefore, the lack of *wg* expansion in *ska* mutants after *hh* overexpression could be explained by the absence of sufficient Wg signaling to provide the autocatalytic requirement for Wg, or by a role for GAGs in Hh signaling, or by an additive effect of partial reduction in both Wg and Hh signaling. The reduction of *dpp* expression in the morphogenetic furrow of the eye disc while *hh* expression is readily detectable suggests some reduction of Hh signaling may be occurring in *ska* mutants. Interestingly, *ska* expression in the morphogenetic furrow is stronger than in the rest of the eye disc.

Although our data suggest a role for GAG chains in modulating growth factor signaling, other functions for GAGs during *Drosophila* development are also possible. GAG chains have been implicated in mediating numerous other biological processes most notably cell-cell and cell matrix interactions (Jackson et al., 1991). Remodeling of the matrix has long been speculated to be important for patterning since, depending on the components, the matrix could limit or enhance differential cell migration. The fact that gastrulation and germ-band extension appear normal in *ska* germ-line mutant embryos implies that, at least in *Drosophila*, these early cell movements do not require GAG polymers.

The apparent non autonomy of *ska* mutants

The lack of *ska* phenotypic effects in imaginal clones was surprising since we imagined that GAG chains might be required for Wg signaling. Several possibilities might account for this observation. Firstly, it is possible that the enzyme or the proteoglycans and/or GAGs are relatively stable and thus perdure long after clone induction. An alternative possibility is that UDP-glucuronate (or a downstream metabolite) can be exchanged between cells perhaps via gap junctions. Finally, it is formally possible that a proteoglycan coreceptor or recycled oligosaccharides could be passed between cells. Whatever the mechanism, it is striking that a defect in a general and fundamental step of GAG chain biosynthesis can have such specific effects on growth-factor-dependent developmental events in vivo.

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