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The *Drosophila sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis

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SUMMARY

We have identified and characterized a *Drosophila* gene, which we have named *sugarless*, that encodes a homologue of vertebrate UDP-glucose dehydrogenase. This enzyme is essential for the biosynthesis of various proteoglycans, and we find that in the absence of both maternal and zygotic activities of this gene, mutant embryos develop with segment polarity phenotypes reminiscent to loss of either Wingless or Hedgehog signaling. To analyze the function of Sugarless in cell-cell interaction processes, we have focused our analysis on its requirement for Wingless signaling in different tissues. We report that *sugarless* mutations impair signaling by Wingless, suggesting that proteoglycans contribute to the reception of Wingless. We demonstrate that overexpression of Wingless can bypass the requirement for *sugarless*, suggesting that proteoglycans modulate signaling by Wingless, possibly by limiting its diffusion and thereby facilitating the binding of Wingless to its receptor. We discuss the possibility that tissue-specific regulation of proteoglycans may be involved in regulating both Wingless short- or long-range effects.

Key words: *Drosophila*, Wingless, proteoglycan, *sugarless*, cell signalling

INTRODUCTION

Drosophila Wingless (Wg) belongs to a family of highly conserved proteins, the Wnt family, which have been implicated in both embryonic development and the regulation of cell proliferation in diverse species (reviewed by Nusse and Varmus, 1992; McMahon, 1992). Wnt proteins are putative secreted glycoproteins that serve as signaling molecules in intercellular communication processes to control cell differentiation and proliferation. During *Drosophila* embryonic development, Wg plays a critical role in patterning the entire segmental unit to specify ventral naked cuticle and cell type diversity in the epidermis. In addition, Wg is required for other developmental processes including segmental patterning of the midgut epithelium, development of Malpighian tubules, formation of the stomatogastric nervous system, specification of a subset of neuroblasts and imaginal disc patterning (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994).

Biochemically, Wnt family proteins possess a putative signal peptide, consensus sites for the attachment of N-linked oligosaccharides and 22 highly conserved cysteine residues, whose spacing is conserved. All the Wnt proteins examined to date enter the secretory pathway and are modified with N-linked oligosaccharides (Burrus and McMahon, 1995). Wnt proteins are poorly secreted into the culture medium and levels of the proteins are increased by addition of the polyanions heparin or suramin (Burrus and McMahon, 1995), suggesting that the majority of secreted Wnt proteins are associated with the cell surface or the extracellular matrix (ECM). It has been demonstrated that both Wg and mouse Wnt-1 are tightly associated with the cell surface (Papkoff and Schryver, 1990; van den Heuvel et al., 1993; Reichsman et al., 1996) and the ECM (Bradley and Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993; Reichsman et al., 1996). The nature of this association is likely due to the interaction of the Wg/Wnt-1 proteins with heparan sulfate proteoglycans (HSPGs), since both of these proteins can be released by addition of exogenous heparin (Bradley and Brown, 1990; Reichsman et al., 1996). Further, heparin can directly bind to purified Wnt-1 (Bradley and Brown, 1990) and Wg protein (Reichsman et al., 1996). The association of Wg protein with the cell surface and the ECM has been shown to be important for its signaling activity, since Wg signaling can be inhibited by removal of the heparan sulfate with heparinase or by treatment of cells with sodium perchlorate, a competitive inhibitor that blocks the sulfation of proteoglycans (PGs) (Reichsman et al., 1996). Thus, HSPGs may play an important role in both the localization of Wg protein and its signaling activity during development.

While biochemical studies of Wnt signaling have been hampered by the difficulty of isolating active Wnt proteins, genetic studies in *Drosophila* have successfully identified some of the molecules involved in Wg signaling. Phenotypic and genetic interaction studies have identified four genes, *zeste white* 3 (*zw*3, also know as *shaggy*), *dishevelled* (*dsh*), *armadillo* (*arm*) and *porcupine* (*porc*), which act in Wg signaling (reviewed by Perrimon, 1996). The current model is that Porc is required for the secretion of Wg protein, whereas

in the receiving cells, Wg initiates a signaling cascade through Dsh, which inactivates Zw3 kinase. Inactivation of Zw3 results in the accumulation of cytoplasmic Arm protein, which in turn regulates many downstream effectors of Wg, including the transcription factor *engrailed* (*en*) in the embryonic epidermis and genes of the *achaete scute complex* at the wing margin (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994). Recently, compelling evidence has been obtained in tissue culture cells that *Drosophila Frizzled2* (*Dfz2*) functions as a Wg receptor in cultured cells (Bhanot et al., 1996). Dfz2 is a member of the Frizzled family of proteins, which possess a putative signal sequence, an extracellular domain composed of a conserved region of 120 amino acid residues with an invariant pattern of ten cysteines, seven putative transmembrane domains and a short cytoplasmic domain (Wang et al., 1996).

The role of *porc*, *zw3* and *dsh* in Wg signaling was originally recognized following examination of the segmentation phenotypes of embryos that lack both maternal and zygotic activities. These mutations were identified as the result of systematic screening of zygotic lethal mutations in germline mosaics (Perrimon et al., 1989). We have recently identified several new segment polarity mutants using this approach (Perrimon et al., 1996; and unpublished), and in this study we describe the identification and characterization of one of them that we have named *sugarless* (*sgl*). We show that the *sgl* mutation impairs signaling by Wg, and encodes a homologue of vertebrate UDP-glucose dehydrogenase, an enzyme essential for the biosynthesis of proteoglycans (PGs). We find that overexpression of Wg can bypass the requirement for *sgl*. Our results are consistent with a model suggesting that PGs facilitate the interaction of Wg with its receptor Dfz2 by regulating the diffusion of secreted Wg.

MATERIALS AND METHODS

Genetics of *sgl*

A single *P[lacZ, ry$^+$]* element insertion *l(3)08310* (Spradling et al., 1995), located at 65D04-05, was identified in a screen for maternal effects of zygotic lethal mutations (Perrimon et al., 1996) that was associated with a fully penetrant maternal effect phenotype. The association of lethality with this insertion was confirmed in two ways. First, *sgl* mutants do not complement the deficient line *Df(3L)W5.4* (Anderson et al., 1995) that covers the region from 65A to 65E. Second, we mobilized the P element associated with the *sgl* mutation using the *y w; Δ2-3, Sb/TM6* strain (Robertson et al., 1988). Out of 108 excision lines, 67 were homozygous viable.

Maternal effect phenotype of *sgl*

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype *sgl FRT^{2A}/TM3, Sb* were mated with males of the genotype *y w FLP^{22}/+, FRT^{2A} P[ovo^{D1}]/TM3, Sb*. The resulting progeny were heat-shocked at 37°C for 2 hours at the larval stages, and *y w FLP^{22}/+; sgl FRT^{2A}/P[ovo^{D1}]FRT^{2A}* females carrying *sgl* homozygous germline clones were selected.

Introduction of a paternal wild-type copy rescues the *sgl* maternal effect phenotype partially (Perrimon et al., 1996). While *l(3)08310*, null embryos exhibit a severe segment polarity phenotype, approximately 30% of the paternally rescued embryos exhibit weak segment polarity phenotypes (partial fusion of denticle bands) and do not hatch.

Antibody staining and in situ hybridization

Fixation of embryos and antibody staining procedures were performed as described (Patel, 1994). Anti-Wg serum was a gift from S. Cumberledge and used at 1:500 dilution. Anti-En mAb4D9 was used at 1:300 dilution and obtained from Developmental Studies Hybridoma Bank (Patel et al., 1989). Antibody against the Crumbs protein was used at 1:50 dilution and obtained from E. Knust (Tepass and Knust, 1993).

In situ hybridization of whole-mount embryos was done with PCR-generated digoxigenin-labeled DNA probe (Lehmann and Tautz, 1994). *wg* digoxigenin-labeled DNA probe was prepared from a *wg* cDNA subcloned in the p^{sp65} plasmid.

Molecular biology

Genomic DNAs flanking the *sgl* P-element insertion were obtained by plasmid rescue in *E. coli* (Cooley et al., 1988). To isolate *sgl* cDNAs, we screened a 0- to 4-hour embryonic cDNA library (Brown and Kafatos, 1988) with genomic DNA fragments obtained from plasmid rescue. Several 2.3 kb *sgl* cDNAs encompassing the entire coding region were isolated. DNAs were sequenced by Taq-polymerase cycle sequencing and an automatic sequencer. To define the P-element genomic insertion site, the rescued plasmid from *sgl* flies was also sequenced using a primer derived from the P-element.

Northern blots of total RNA or poly(A)$^+$ RNA were carried out by standard procedures (Sambrook et al., 1989). Probes used were as follows: 1.87 kb *Sst*I-*Xho*I fragment of the *sgl* cDNA (see Fig. 3A for sites) and 0.6 kb *Hin*dIII-*Eco*RI DNA fragment of ribosomal gene rp49 (O'Connell and Roshbash, 1984). Sequence alignments were produced using 'DNA star' software.

RNA injection rescue

To mark embryos for RNA injection rescues, the *sgl* mutant chromosome was recombined with a *trachealess* (*trh*) mutant (Wilk et al., 1996; Isaac and Andrew, 1996) located at 61 C1-2. *trh* mutants exhibit defective Filzkörper, which are easily scorable cuticle markers. Females carrying *trh sgl* homozygous germline clones were generated and mated with *sgl/trh* transheterozygous males. 50% of the embryos resulting from this cross are homozygous for the *sgl* mutation and exhibit strong segment polarity phenotypes, while the other 50% are paternally rescued and easily identifiable by their defective Filzkörper.

RNAs were produced by in vitro transcription (Sambrook et al., 1989) from full-length cDNA plasmids containing an SP6 promoter using cap analog GpppG (Stratagene). Transcribed RNA was resuspended in DEPC-treated water at a concentration of 0.2 μg/μl. Embryos generated from the cross above were collected and microinjected as described (Anderson and Nüsslein-Volhard, 1984). Injected embryos were allowed to complete development for 2 days at 18°C prior to preparation. Scoring of cuticles was as described (Wieschaus and Nüsslein-Volhard, 1986). Of 800 injected embryos derived from females with *trh sgl* germline clones, 168 *sgl* mutant embryos (*trh sgl/sgl*) developed scorable cuticle structures, and 30% of them were rescued.

Misexpression experiments

The *pairedGAL4/TM3* (*prdGAL4*) line used in this study is described in Yoffe et al. (1995). *prdGAL4* was recombined with the *sgl FRT^{2A}* chromosome to a *sgl prdGAL4 FRT^{2A}* chromosome that was used to generate homozygous germline clones. The *UASwg^{ts}* (*M7-2.1*) line is located on the third chromosome and was described previously (Wilder and Perrimon, 1995; Yoffe et al., 1995). This insertion is homozygous viable. The *UASwg^{ts}* was recombined with the *sgl* mutation to generate *sgl UASwg^{ts}*. *UAS wg* was a gift of Henry Krause (unpublished). *UAS arm^{s10}* was obtained from Mark Peifer (Pai et al., 1997). The insertion used is homozygous viable and located on the second chromsome. *UAShh* was obtained from. P. Ingham (Ingham and Fietz, 1995).

RESULTS

sgl is a novel segment polarity gene

The mutation in *sgl* was originally identified in a large screen to characterize the maternal effects of zygotic lethal mutations (see Perrimon et al., 1996; and Materials and methods for details). Homozygous *sgl* mutant animals derived from heterozygous mothers die at the third instar larval or early pupal stages. In contrast, homozygous *sgl* mutant embryos derived from females lacking germline *sgl* activity (referred to as *sgl* null embryos throughout the text) die with segmentation defects that resemble the phenotypes of mutants in segment polarity genes (Fig. 1B). The *sgl* maternal effect is partially paternally rescuable (see Perrimon et al., 1996; and Materials and methods for details) indicating that the gene is expressed at least during both oogenesis and early embryonic development.

The cuticle phenotype of *sgl* is reminiscent of the phenotypes exhibited by mutations in either *wg* or *hedgehog (hh*), suggesting that it may be involved in either or both of these signaling pathways. To further determine the involvement of *sgl* in Wg or Hh signaling, we examined the expression of *wg* mRNA, Wg protein and En protein in *sgl* null mutant embryos. In the ventral embryonic epidermis, Wg signaling is required for maintenance of *en* transcription at stage 10 (DiNardo et al., 1988; Yoffe et al., 1995). Subsequently, En, through a signaling pathway mediated by Hh (Ingham et al., 1991; Lee et al., 1992), is required for the maintenance of *wg* transcription (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991). As shown in Fig. 1, *wg* mRNAs fade in *sgl* (Fig. 1E) mutants at stage 9. Similarly, *en* expression is affected, since En protein disappears from the epidermis by stage 10 (Fig. 1F). These observations are reminiscent of other segment polarity mutants (DiNardo et al., 1988; van den Heuvel et al., 1993; Yoffe et al., 1995; Manoukian et al., 1995; Alcedo et al., 1996) and suggest a role for *sgl* in either Wg and/or Hh signaling. Double stainings for both En and Wg protein indicate that when En protein begins to fade during late stage 9 in *sgl* mutants, Wg protein has almost completely decayed (Fig. 1H). *wg* mRNA disappearing from the epidermis significantly earlier than En has also been observed in *porc* mutants and interpreted as being characteristic for genes acting upstream of *wg*. This observation is consistent with a role for *sgl* in an upstream part of the Wg signal transduction pathway.

sgl encodes UDP-glucose dehydrogenase

To identify the transcript associated with the *sgl* mutation, a 2.9 kb fragment of genomic DNA flanking the P-element insertion was cloned following P-element rescue. This fragment was subsequently used to screen a *Drosophila* genomic DNA library in order to obtain a continuous stretch of genomic DNA representing the region of the P-element insertion. A screen for cDNAs, using genomic DNA fragments encompassing approximately 5 kb on both sides of the P-element insertion, identified a single cDNA of 2.3 kb mapping in the immediate vicinity of the P-element insertion point. The length of the cDNA corresponds well with the length of a single signal obtained in a northern blot analysis using the cDNA as a probe (data not shown).

Several lines of evidence suggest that the isolated cDNA identifies the gene responsible for the *sgl* mutant phenotype. First, the P-element was found to be inserted into the 5′-untranslated leader region of the putative transcript 106 bp upstream of the putative ATG start codon (Fig. 2A). Second, northern blot analysis showed a complete loss of *sgl* maternal transcripts in 0-1.5 hour embryos derived from females with *sgl* germline clones, indicating that the P-element insertion disrupts the *sgl* transcript (Fig. 2B). Third, RNA transcribed in vitro using the isolated cDNA as a template was shown to be able to rescue the *sgl* mutant phenotype when injected into mutant embryos at the syncytial blastoderm stage (Fig. 3B,C).

Analysis of the nucleotide sequence of the *sgl* cDNA revealed a continuous open reading frame of 477-amino-acid residues (Fig. 2C). Comparison of the putative protein sequence derived from this reading frame with sequences represented in the databases revealed striking homology with bovine UDP-glucose dehydrogenase (Fig. 2C). The overall identity between Sgl and bovine UDP-glucose dehydrogenase is 67.7%. This enzyme provides the only pathway in all animals to convert UDP-glucose into UDP-glucuronic acid, an essential substrate for the biosynthesis of glycosaminoglycans (GAG) such as chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate (HS) (Hempel et

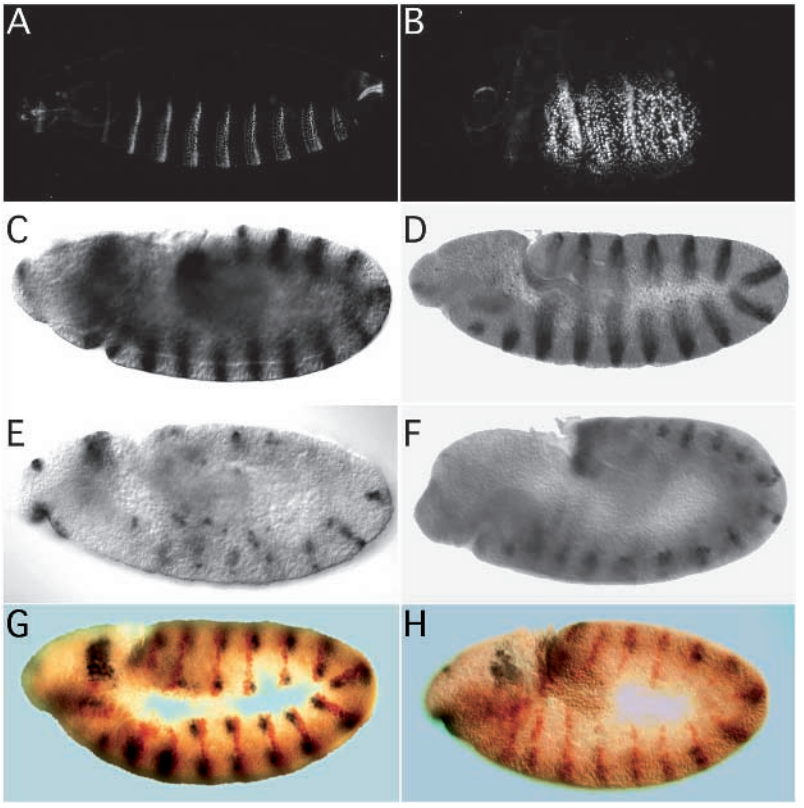

Fig. 1. Segment polarity phenotype associated with the *sgl* mutation The cuticle phenotypes of wild type (A) and *sgl* (B) null embryos are shown; Transcription of *wg* mRNA is shown at stage 9 in wild type (C) and *sgl* (E) embryos; the expression of En protein is shown at stage 10 in wild type (D) and *sgl* (F) null embryos. The expression pattern of both Wg (blue/black) and En protein (brown) is shown at stage 10 in wild type (G) and at late stage-9 *sgl*(H) null embryos.

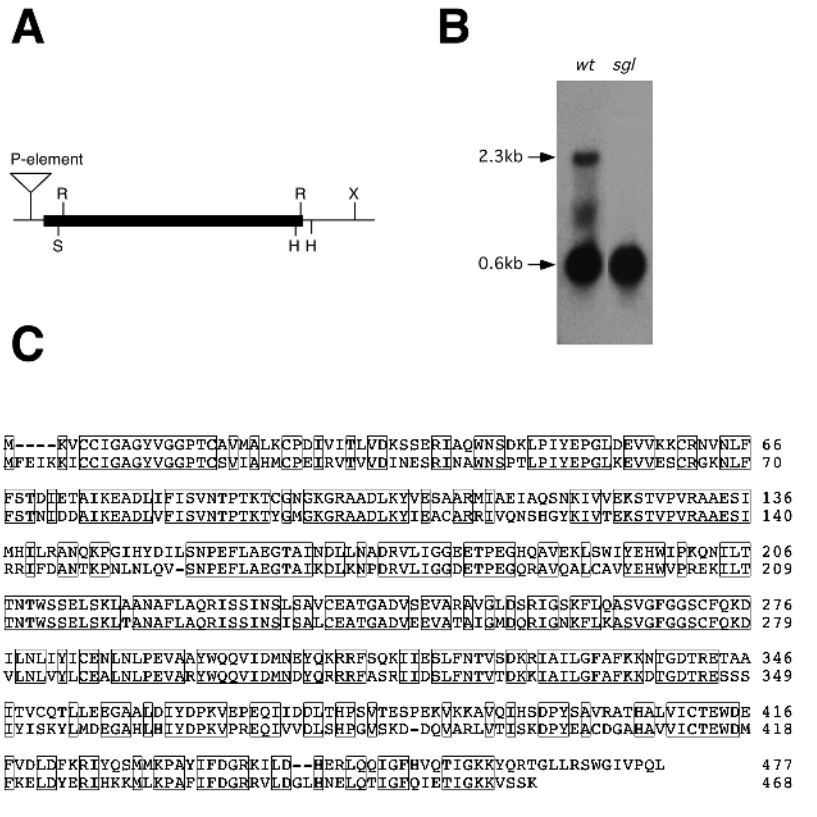

Fig. 2. Molecular characterization of *sgl* and northern blot analysis. (A) Restriction map of the *sgl* cDNA. The insertion of the P-element *l(3)08310* is indicated and corresponds to nucleotide 113 of the untranslated region of the *sgl* cDNA (106 bp upstream of the putative ATG start codon). The position of the open reading frame encoding the Sgl protein is indicated by the heavy line. H, *Hin*d III; R, *Eco*RI; S, *Sst*I; X, *Xho*I. (B) Northern blot analysis of *sgl* on RNA from 0-1.5 hour wild-type embryos and embryos derived from females with *sgl* germline clones. About 2 μg of poly(A)$^+$ RNA were loaded. The blot was probed with ^{32}P-labeled 1.87 kb *Sst*I-*Xho*I fragment of the *sgl* cDNA (see Fig. 2A for sites) and a probe specific for ribosomal protein gene rp49, which served as loading control. The 2.3 kb mRNA corresponds to the *sgl* transcript and the 0.6 kb mRNA to the ribosomal protein gene 49 transcript. (C) Putative amino acid sequence of Sgl (*Drosophila*) and comparison of the amino acid sequences of Sgl protein (*Drosophila*) with Bovine UDP-glucose dehydrogenase (Bovine). Identical residues are boxed.

al., 1994). Consistent with its role in the biosynthesis of GAGs, *sgl* is expressed both maternally and ubiquitously throughout embryonic development (data not shown).

Sgl activity is required for Wg signaling in various tissues

The molecular nature of Sgl predicts that the biosynthesis of PGs is disrupted in *sgl* mutants (see Discussion for details). Since HS/heparin has been demonstrated to be important in Wg signaling in tissue culture experiments (Reichsman et al., 1996), it is anticipated that Wg signaling is affected in *sgl* mutants and that the cuticle phenotype seen in *sgl* mutants reflects a role of HS/heparin, and possibly other PGs, in Wg signaling. Studying the requirement of PGs for Wg signaling in the epidermis is complicated because of the codependence of Wg and Hh expression (Ingham et al., 1991; Lee et al., 1992). Thus, to determine the dependence of Wg signaling on the presence of intact PGs, we examined the phenotype associated with the *sgl* mutation in other tissues in which Wg is required for pattern formation but where its role can be distinguished from that of Hh.

One of these tissues is the anlage of the stomatogastric nervous system (SNS) (González-Gaitán and Jäckle, 1995). The SNS arises during stage 10 from a distinctive region of the invaginating foregut. During stage 11, three invaginations form in the dorsal epithelium of the foregut (Fig. 4A). Later these invaginations will form vesicles that migrate dorsally towards the brain to form the SNS. In *wg* mutant embryos only one of the three invaginations forms (Fig. 4B). Virtually identical phenotypes, i.e. only one invagination, are observed in embryos mutant for genes that have previously been shown to be involved in Wg signaling like *dsh* and *arm* (González-Gaitán and Jäckle, 1995). In contrast, *zw3* mutant embryos form additional invaginations, which is consistent with the opposite phenotypes of *wg* and *zw3* mutants in the ventral cuticle (Siegfried et al., 1992). Altogether, the Wg signaling pathway in the ventral epidermis and in the development of the SNS appears to involve the same set of genes (González-Gaitán and Jäckle, 1995). In contrast to *wg* mutants, *hh* mutants, like wild-type embryos, form three SNS invaginations. Although *hh* is expressed in the SNS anlage, we could not detect any defects specifically associated with the SNS in *hh* mutants (data not shown). For this reason the stomatogastric nervous system provides a system with which to determine whether Sgl activity is required for Wg signaling.

In order to examine the role of *sgl* in the development of the SNS anlage, *sgl* null mutant embryos were stained with an antibody against the Crumbs protein, which labels the apical surfaces of epithelial cells throughout the embryo (Tepass and Knust, 1993). In contrast to the phenotypes seen in genes that have previously been implicated in transducing the Wg signal, we did not observe the one-invagination phenotype in *sgl* mutant embryos. Instead, 2-3 invaginations that are fused at the base are usually detected (Fig. 4C). This phenotype is reminiscent of a weak *wg* mutant phenotype, as observed in a weak *arm* allele (see Fig. 8I in González-Gaitán and Jäckle, 1995). However, in contrast to the ventral epidermis, where the phenotype of *sgl* resembles an amorphic *wg* phenotype, defects in the SNS are less severe, suggesting that Wg signaling in the SNS is not completely abolished. Interestingly, in contrast to the ventral epidermis of *sgl* mutant embryos, where Wg expresssion has disappeared at stage 10, Wg expression in the SNS anlage persists beyond this stage (Fig. 4E, also see Fig.

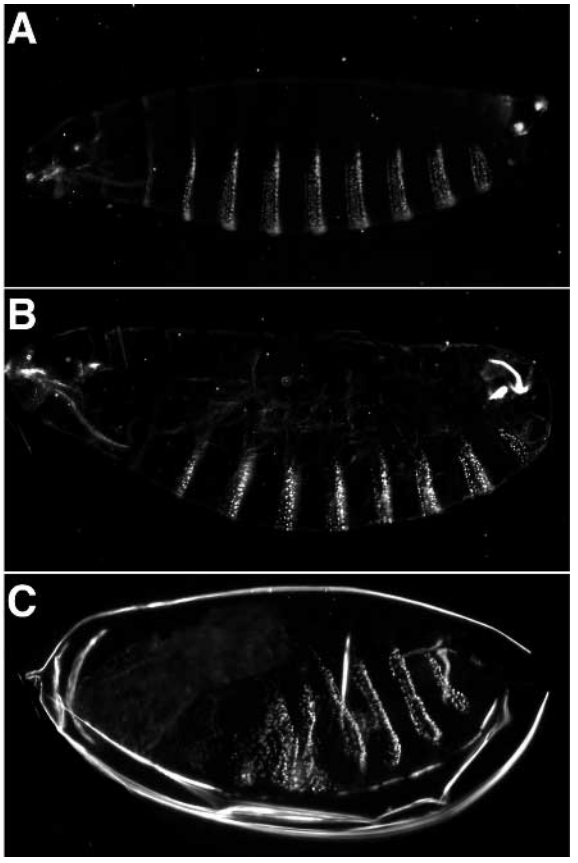

Fig. 3. Rescue of the *sgl* maternal effect phenotype by RNA injection. (A) Cuticle phenotype of a paternally rescued *sgl* embryo marked with a *trh* mutation (see Materials and methods for details). Note the defective Filzkörper. (B) and (C) are two examples of *sgl* null embryos derived from germline clones injected with *sgl* RNAs transcribed from the 2.3 kb *sgl* cDNA. Note that (B) is a fully rescued embryo in which the ventral denticle bands as well as head and tail structures are fully restored; (C) shows a partially rescued embryo.

1E). This observation suggests that the difference in severity of phenotypes between the SNS and the ventral epidermis may originate from the different modes of Wg regulation deployed in the respective tissues.

We have also examined the effect of loss of Sgl activity on the formation of the Malpighian tubules and the midgut constrictions. Both tissues require Wg activity for their development. In *wg* mutants only two short Malpighian tubules form instead of four (Skaer and Martinez-Arias, 1992) and the second constriction of the midgut does not form (Bienz, 1994). *sgl* null embryos develop four very short Malpighian tubules, a phenotype that would be expected to result from a partial loss of *wg* activity (data not shown). In addition, as observed in *wg* mutant embryos, *sgl* null embryos do not form the second midgut constriction. This is likely due to loss of expression of the homeobox gene *Ultrabithorax* (*ubx*), which is dependent on Wg signaling (Bienz, 1994) (Fig. 4G).

In summary, the observation of *wg*-like phenotypes in at least four tissues that depend on Wg inputs for their development further substantiates the requirement of *sgl* activity in Wg signaling. Based upon the severity of the mutant phenotypes, it is apparent that in some tissues the requirement for Sgl is not absolute.

Ectopic expression of Wg can bypass the requirement for Sgl

Studies in the SNS and the Malpighian tubules suggest that Sgl is not absolutely essential in all tissues to implement the Wg signal, but instead may be involved in modulating the strength of the cellular response to Wg. To test this hypothesis we examined the effect of ectopic expression of Wg in the ventral epidermis of *sgl* null mutant embryos. We reasoned that if Sgl promotes the cellular response to the Wg signal, ectopic expression of various amounts of Wg should elicit dosage-dependent effects. We used the GAL4/UAS system of targeted gene expression (Brand and Perrimon, 1993) to misexpress various forms of Wg in *sgl* mutant embryos. A prdGAL4 line was used that drives the expression of a UAS-target gene in the *paired* (*prd*) pair-rule expression domain from stages 8 to 13 (Yoffe et al., 1995).

We first examined whether ectopic expression of Wg^{ts} effectively restores the naked cuticle as it does in *wg* and *porc* mutant embryos (Yoffe et al., 1995; Manoukian et al., 1995). In wild-type embryos, ectopic expression of Wg is associated with ectopic *en* as well as generation of naked cuticle (Yoffe et al.,

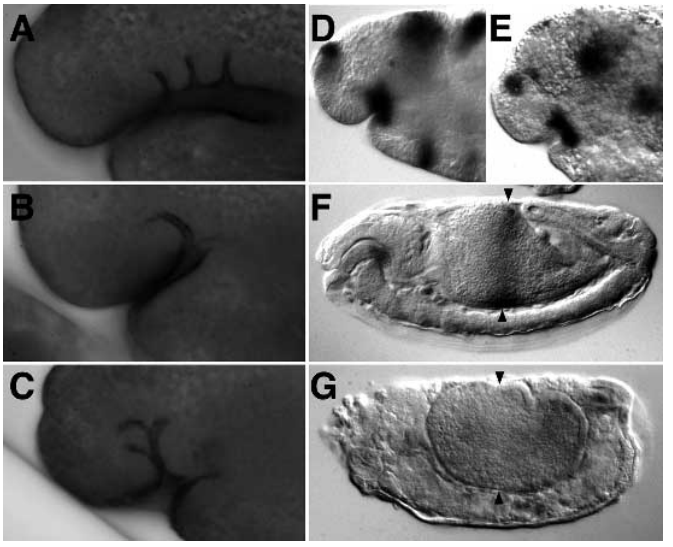

Fig. 4. Development of the stomatogastric nervous system (SNS) in *sgl* mutants. (A-C) Embryos are stained with anti-Crumbs antibody: wild type (A), *wg* (B) and *sgl* (C) null embryos are shown. The SNS is derived from three invaginations forming in the dorsal epithelium of the developing foregut of wild-type embryos at stage 10 (A). In a *wg* mutant embryo only one invagination is formed (B). In *sgl* (C) mutant embryos three invaginations are formed and fused at the base. This phenotype is reminiscent of *wg* hypomorphs and suggests that in *sgl* mutants Wg activity is reduced but not completely abolished. (D,E) Expression of Wg protein at stage 10 in wild type (D) and *sgl* (E) null embryos. In contrast to *wg* expression in the ventral epidermis, which is lost (see Fig. 1E,H), *wg* expression in the SNS anlage persists in *sgl* (E) mutants. (F,G) Expression of labial (lab) protein in a wild type (F) and a *sgl* (G) null embryo at stage 15. Labial staining, marking the position where the second midgut constriction will form in a wild-type embryo (F), is absent from a *sgl* (G) null embryo. Arrowheads mark the positions in which the constriction will form (F and G).

1995). As shown in Fig. 5, in *sgl* mutant embryos, expression of *prdGAL4/UASwg^{ts}* does not generate naked cuticle (Fig. 5A), while in control experiments, *prdGAL4/UASwg^{ts}* induced the expansion of En stripes in *sgl* zygotic mutant embryos with maternal supply (data not shown), as was shown previously (Yoffe et al., 1995). This result demonstrates that *sgl* is required for implementation of the Wg signal.

In light of the requirement of Sgl activity for Wg signaling during SNS formation, we reasoned that the inability of Wg^{ts} to generate naked cuticle in *sgl* mutant embryos may be due to the low activity of Wg^{ts} under these experimental conditions. These experiments were conducted at 16°C, which is the permissive temperature for Wg^{ts} (van den Heuvel et al., 1993; Wilder and Perrimon, 1995). At this temperature, GAL4 is not as efficient as at higher temperatures (Brand et al., 1994), and the activity of Wg^{ts} may be relatively low. Thus, we repeated the same misexpression experiment in the embryonic epidermis using a *UAS-wild type wg* construct. In wild-type embryos, expression of wild-type Wg protein in the paired expression domain results in deletion of denticle bands when embryos are allowed to develop at either 25°C (Fig. 5B) or 16°C (Fig. 5C). Wild-type Wg protein has stronger activity than Wg^{ts}, since expression of Wg^{ts} under identical conditions only causes the deletion of one row of denticles (Yoffe et al., 1995). Strikingly, at 25°C, expression of *prdGAL4/UASwg* in *sgl* mutant embryos induces naked cuticle (Fig. 5D), demonstrating that Wg signaling can occur in the absence of *sgl* activity. However, we observed only a weak effect of ectopic Wg, indicated by the formation of narrow regions of naked cuticle when the same experiment was conducted at 16°C (Fig. 5E). These results indicate that ectopic expression of Wg can rescue the defects of cuticle patterning in *sgl* in a dose-dependent manner.

We also examined the effect of misexpression of Hh in the paired domain in *sgl* null mutant embryos. As shown in Fig. 5F, *prdGAL4/UAShh* can effectively induce naked cuticle in *sgl* null mutant embryos at 16°C (not shown) or 25°C. Strikingly, in embryos mutant for *sgl*, Wg protein is maintained/induced in the paired domain where ectopic Hh is expressed at stage 11 (Fig. 5G). This result is consistent with the Wg misexpression experiments since the effect of Hh is mediated by *wg*.

To further determine at what level Sgl is required for Wg signaling, we examined the epistatic relationships between Sgl and Arm by misexpressing a gain of function Arm protein (Arm^{s10}) in which 54 N-terminal amino acid residues of Arm are deleted (Pai et al., 1997). As shown in Fig. 5H, expression of this constitutively active, Wg-independent form of Arm under the control of *prdGAL4*, in *sgl* null embryos leads to the formation of naked cuticle in the paired domain. Restoration of naked cuticle by expression of constitutively active Arm confirms that it is in fact loss of Wg signaling that leads to the *sgl* mutant phenotype, and that furthermore, *arm* is epistatic to *sgl*-activity. The formation of naked cuticle is most likely the result of ectopic expression of *wg*, triggered by ectopic *en* and *hh*, which occurs in response to ectopic expression of Arm^{s10} (Pai et al., 1997).

DISCUSSION

Sgl is involved in Wg signaling

We have identified and characterized a novel segment polarity gene, *sgl*, which encodes an enzyme involved in proteoglycan (PG) biosynthesis. Embryos that develop in the absence of both maternal and zygotic *sgl* gene products exhibit cuticle defects and defects in gene expression in the ventral epidermis that are identical to those observed in *wg* or *hh* mutant embryos (Fig. 1). To demonstrate that normal expression of *sgl* is required for proper Wg signaling, we analyzed the requirement for *sgl* in

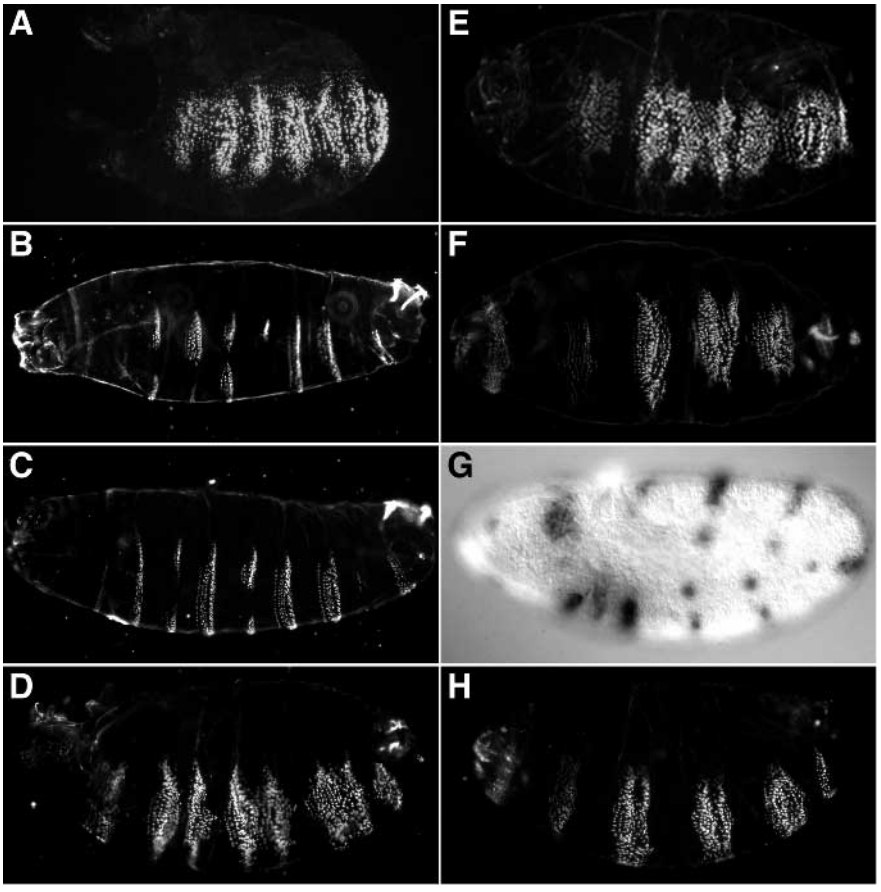

Fig. 5. Misexpression of *wg^{ts}*, *wg*, *hh* and *arm^{s10}* in *sgl* mutants. Except for (B) and (C), which are controls of *UASwg/prdGAL4* in wild-type backgrounds, all panels show embryos derived from females with *sgl* germline clones. The cuticle phenotypes shown are: *sgl prdGAL4/sgl UASwg^{ts}* (A), *UASwg/+; prdGAL4/+* (B,C), *UASwg/+; sgl prdGAL4/sgl* (D,E), *UAShh/+; sgl prdGAL4/sgl* (F). *UASarm^{s10}/+; sgl prdGAL4/sgl* (H), Embryos shown in (A) and (E) developed at 16°C. All the others developed at 25°C. (G) Wg protein expression at stage 11 in a *UAShh/+; sgl,prdGAL4/sgl* embryo. Note that while stripes of endogenous Wg protein fade completely, new stripes of Wg protein are induced by ectopic expression of Hh.

SNS formation, where *wg* expression does not depend on Hh activity (Fig. 4). Interestingly, we found that Wg signaling is only partially blocked in *sgl* null mutant embryos during SNS formation. We have also examined the effect of loss of Sgl activity on the formation of the Malpighian tubules and the midgut constrictions, two tissues that require Wg activity for their development (Fig. 4). In *sgl* mutants, the second midgut constriction does not form, as is observed in *wg* mutants, and four short Malpighian tubules are present, a phenotype reminiscent to a partial loss of *wg* function. Altogether, these observations suggest that the requirement for Sgl activity in Wg signaling is tissue specific, since implementation of the Wg signal can occur to some extent in some tissues in the total absence of Sgl. Our misexpression studies of *wg* in *sgl* mutants are consistent with this view because we demonstrate that high levels of ectopic Wg can bypass the requirement for Sgl activity.

Our study illustrates the critical requirement for PGs during embryogenesis, and we demonstrate that at least the Wg signaling pathway is affected in *sgl* mutants. However, it is likely that other signaling pathways that involve PGs are perturbed in the absence of Sgl activity. *sgl* mutant embryos do not exhibit cuticle phenotypes reminiscent to loss of cadherins or EGF receptor tyrosine kinase or TGF-β signaling pathways; however, preliminary results indicate that FGF signaling is affected in *sgl* mutants (X. Lin, A. Michelson and N. Perrimon, unpublished data). Finally, the requirement for Sgl in Hh signaling remains to be determined. We demonstrate that misexpression of Hh can effectively induce/maintain ectopic Wg and induce naked cuticle in *sgl* mutants, supporting a notion that Sgl is not involved in Hh signaling. Nevertheless, since we have not tested whether misexpression of a weaker Hh protein could signal in a *sgl* mutant background, there is a formal possibility for a dose-dependent requirement of *sgl* in Hh signaling, as observed for Wg.

In conclusion, based upon examination of the *sgl* mutant phenotypes, we propose that PGs are required for efficient Wg signaling. Their requirement appears to be specific to particular signaling pathways, since we do not observe a general disruption of the cellular context but instead disruption of specific signaling activities such as Wg.

sgl encodes an enzyme involved in proteoglycan biosynthesis

PGs are ubiquitous macromolecules associated with the cell surface and the ECM of a wide range of cells that play central roles in morphogenesis, neurite outgrowth, angiogenesis and tissue repairs (reviewed by Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). The biological function of PGs is mostly attributed to the highly negatively charged glycosaminoglycan (GAG) chains that interact with positively charged side chains of proteins.

GAGs are polymers of disaccharide repeats, which are often highly sulfated and negatively charged. Except for the nonsulphated polysaccharide hyaluronan, which occurs in the form of free GAG chains, all other GAGs identified, including heparin, heparan sulfate (HS), chondroitin sulfate and keratan sulfate, are covalently bound to a protein core to form a PG (reviewed by Kjellén and Lindahl, 1991). With the exception of keratan sulfate, all GAGs require D-glucuronic acid as a substrate for biosynthesis of their polysaccharide chains. The molecular analysis of *sgl* reveals that it encodes a homologue of bovine UDP-glucose dehydrogenase, which converts UDP-D-glucose to UDP-D-glucuronic acid. We therefore predict that except for keratan sulfate, biosynthesis of all other GAGs is disrupted in *sgl* null mutant embryos. Consistent with its role in biosynthesis of many proteoglycan polysaccharides, *sgl* is expressed both maternally and ubiquitously throughout embryonic development.

We propose that, in the absence of Sgl protein, Wg signaling is perturbed due to lack of or abnormal biosynthesis of PGs. If the general synthesis of PGs is perturbed in *sgl* mutants, then it is expected that other signaling pathways, which utilize PGs as coreceptor(s), may also be disrupted in the absence of Sgl proteins. Consistent with this hypothesis, we have observed that mesoderm migration in *sgl* mutants is defective (X. Lin, A. Michelson and N. Perrimon, unpublished) and similar to embryos carrying mutations in *heartless*, a *Drosophila* homologue of the FGF receptor (Beiman et al., 1996; Gisselbrecht et al., 1996). The availability of *sgl* mutants will allow us to examine the roles of PGs in signaling mediated by other growth factors, including FGFs.

Proteoglycans and Wnt signaling

Biochemically, there is ample evidence for the importance of PGs in Wnt-1/Wg signaling. It has been demonstrated that both mouse Wnt-1 and Wg are associated with the cell surface (Papkoff and Schryver, 1990; van den Heuvel et al., 1993; Reichsman et al., 1996) and the ECM (Bradley and Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993; Reichsman et al., 1996). The association of Wnt-1/Wg with the cell surface or the ECM can be released by addition of exogenous HS or heparin, and heparin can directly bind to purified Wnt-1 and Wg protein (Bradley and Brown, 1990; Reichsman et al., 1996), suggesting that the association of Wnt-1/Wg proteins with the cell surface or the ECM is likely due to the direct interaction of Wnt-1/Wg protein with HSPGs. Further, recent tissue culture experiments have shown that HSPGs can modulate both extracellular localization of the Wg protein and Wg signaling (Reichsman et al., 1996). Treatment of Wg-responsive cells with heparinase, or blocking HS sulfation with perchlorate, results in the reduction of Wg activity. Consistent with these results, our studies demonstrate that loss of a component required for biosynthesis of PGs results in defects in Wg signaling and establishes a critical role for PGs in signal transduction in vivo.

In addition to Wnt-1/Wg protein, other Wnt family proteins have also been shown to be associated with the cell surface and to be effectively released into culture medium by the highly negatively charged polyanions heparin or suramin (Burrus and McMahon, 1995). Given the fact that the Wnt family of proteins are highly conserved, it is reasonable to suggest that PGs are also likely to be required for signaling by other, if not all, Wnt family members. In support of this view, recent experiments demonstrate that PGs, most likely HSPGs, are required for maintenance of Wnt-11 expression in the ureter tips (Kispert et al., 1996).

A model for the role of PGs in Wg signaling

Among the growth factors that interact with PGs, members of the FGF family have been particularly well studied. It has been proposed that HSPGs are required for dimerization of FGF receptors (Spivak-Kroizmon et al., 1994; Schlessinger et al., 1995). In an alternative model, HSPGs have been proposed to reduce the dimensionality of ligand diffusion from three to two dimensions (Schlessinger et al., 1995). Our genetic experiments demonstrate that in *sgl* mutant embryos, overexpression

of Wg protein can bypass the requirement for PGs to transduce the Wg signal to receiving cells. Based on these results, we propose that the function of PGs is to increase the local concentration of Wg ligand for its receptor. Binding of Wg protein to PGs on the cell surface or the ECM reduces the diffusion of the Wg ligand so that the Wg molecules are more likely to bind to the less abundant Wg receptor. In the absence of PGs, the concentration of Wg protein presented on the cell surface may be lower than its threshold concentration, and the efficiency of Wg signaling will be reduced. Overexpression of ectopic Wg protein can compensate for the loss of Wg protein on the cell surface and therefore bypass the requirement for PGs.

This model is consistent with our observation that in the SNS and in the Malpighian tubules Wg signaling is not completely abolished in *sgl* mutants (Fig. 4). In the ventral epidermis, however, the situation appears to be different as the loss of Sgl activity mimics a complete loss of Wg activity (Fig. 1). This observation most likely reflects the fact that in the ventral epidermis, the maintenance of *wg* transcription requires Wg signaling itself, either directly in an autocrine pathway, or via the Hh-mediated feedback loop. The reduction in Wg efficiency caused by lack of PGs is amplified through the feedback loop and rapidly leads to a complete loss of *wg* transcription, resulting in the amorphic phenotype.

Growth factors and HSPGs

Accumulating evidence has demonstrated that coreceptors, such as HSPG, function as indispensable components for signaling by a number of growth factors (Schlessinger et al., 1995; Massagué, 1996). For example, FGFs require HSPGs as coreceptors for signaling via a tyrosine kinase receptor. TGF-β binds to a serine/threonine kinase receptor in association with the membrane protein betaglycan (Schlessinger et al., 1995; Massagué, 1996). Either GDNF or CNTF require GPI-anchored proteins to mediate signaling (Massagué, 1996; Stahl and Yancopoulos, 1993). Binding of growth factors to co-receptors such as cell surface HSPGs will not transmit a signal, but will modulate the ability of growth factors, or the signaling receptors, to generate a biological response.

While the function of these molecules has been proposed to limit ligand diffusion from the cell surface, and to initiate dimerization of receptors, we speculate that these molecules may mediate other functional aspects of growth factors. For instance, Wg protein functions both as a short-range inducer in the ventral epidermis (van den Heuvel et al., 1989; DiNardo et al., 1988; Vincent and Lawrence, 1994) and as a long-range organizer in imaginal discs (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1995). Recent evidence suggests that Wg can act directly and at long range as a gradient morphogen (Zecca et al., 1996). If HSPG is a major limiting factor for diffusion of Wg protein, one would expect that dynamic changes of the expression of HSPGs may regulate the diffusion of Wg and eventually control the signaling range of Wg protein. Interestingly, a Glypican-related HSPG, named Dally, was identified, and *dally* mutants show wing notching with loss of wing margin structures (Nakato et al., 1995), an effect seen in *wg* and *dsh* mutants (Couso et al., 1994), suggesting a potential involvement of Dally in Wg signaling. A *Drosophila* homologue of vertebrate Syndecans has also been characterized (Spring et al., 1994). Syndecan is a transmembrane HSPG and represents the major source of HSPGs in epithelial cells. Syndecan has been demonstrated to function as a coreceptor for FGF signaling (Bernfield et al., 1992; David, 1993). Further genetic and biochemical studies should reveal whether Dally and/or Syndecan play a direct role in Wg signaling.

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Note

The DNA sequence of UDP-glucose dehydrogenase has been submitted to GenBank and has been assigned the accession number: AF009013.

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Note

The gene we call *sugarless* was characterized by Binari et al. and Härry et al., who named the gene *Kiwi* and *Suppenkasper*, respectively. Following discussion with Dr. M. Ashburner, it was agreed to refer to the gene as *sugarless* (*sgl*) in subsequent publications.