Distinct and collaborative roles of Drosophila EXT family proteins in morphogen signalling and gradient formation

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Summary

Heparan sulfate proteoglycans (HSPG) have been implicated in regulating the signalling activities of secreted morphogen molecules including Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp). HSPG consists of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. The formation of HS GAG chains is catalyzed by glycosyltransferases encoded by members of the EXT family of putative tumor suppressors linked to hereditary multiple exostoses. Previous studies in Drosophila demonstrated that tout-velu (ttv), the Drosophila EXT1, is required for Hh movement. However, the functions of other EXT family members are unknown. We have identified and isolated the other two members of the Drosophila EXT family genes, which are named sister of tout-velu (sotv) and brother of tout-velu (botv), and encode Drosophila homologues of vertebrate EXT2 and EXT-like 3 (EXTL3), respectively. We show that both Hh and Dpp signalling activities, as well as their morphogen distributions, are defective in cells mutant for ttv, sotv or botv in the wing disc. Surprisingly, although Wg morphogen distribution is abnormal in ttv, sotv and botv, Wg signalling is only defective in botv mutants or ttv-sotv double mutants, and not in ttv nor sotv alone, suggesting that Ttv and Sotv are redundant in Wg signalling. We demonstrate further that Ttv and Sotv form a complex and are co-localized in vivo. Our results, along with previous studies on Ttv, provide evidence that all three Drosophila EXT proteins are required for the biosynthesis of HSPGs, and for the gradient formation of the Wg, Hh and Dpp morphogens. Our results also suggest that HSPGs have two distinct roles in Wg morphogen distribution and signalling.

Key words: Heparan sulfate proteoglycans, Exostosin (EXT), tout-velu (ttv), botv of tout-velu (botv), sister of tout-velu (sotv), Wingless (Wg), Hedgehog (Hh), Decapentaplegic (Dpp), Drosophila

Introduction

Secreted signalling molecules of the Wnt/Wingless (Wg), Hedgehog (Hh) and transforming growth factor β (TGF-β)/Decapentaplegic (Dpp) families function as organizers to control growth and pattern formation of tissues during animal development (Cadigan, 2002; Gurdon and Bourillot, 2001; Lawrence and Struhl, 1996; Telem et al., 2001; Vincent and Dubois, 2002). Studies in Drosophila have demonstrated that Wg, Hh and Dpp act as morphogens to specify positional information during wing development. In the wing disc, Wg is required for dorsal (D)/ventral (V) patterning and wing margin specification, whereas Hh and Dpp are responsible for anterior (A)/posterior (P) patterning. In the DV axis, Wg is expressed in a narrow stripe of cells at the DV border and emanates from the DV border to form an extracellular gradient (Strigini and Cohen, 2000). Wg acts in a concentration-dependent manner to directly trigger a graded transcriptional response of its target genes (Neumann and Cohen, 1997; Zecca et al., 1996). In the AP axis, Hh and Dpp function as short- and long-range morphogens, respectively, to organize the anteroposterior patterning of the wing. Hh protein is exclusively expressed in the P compartment and moves into a stripe of A cells adjacent to the AP border to induce expression of its target genes including dpp (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Posakony et al., 1990; Tabata and Kornberg, 1994). Dpp moves bi-directionally into both the A and P compartments, and functions as a long-range morphogen to activate the expression of its target genes in a concentration-dependent manner (Entchev et al., 2000; Lecuit et al., 1996; Nellen et al., 1996; Tanimoto et al., 2000; Telem and Cohen, 2000). Although the functions of Wg, Hh and Dpp as morphogens are well established, little is known about how their extracellular gradients are generated and how their morphogen concentrations are interpreted into their signalling outputs.

In the past few years, genetic studies in Drosophila have demonstrated the crucial roles of heparan sulfate proteoglycans (HSPG) in signalling events controlled by secreted Wg, Hh and Dpp morphogens (Lander and Selleck, 2000; Lin and Perrimon, 2000; Nybakken and Perrimon, 2002; Perrimon and Bernfield, 2000). HSPGs consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are
attached (Bernfield et al., 1999; Esko and Selleck, 2002; Perrimon and Bernfield, 2000). The biosynthesis of HS GAG chains is initiated by the formation of a GAG-protein linkage region consisting of a tetrasaccharide (\(-\text{GlcA}1-3\text{Gal}1-3\text{Gal}1-4\text{Xyl}1\beta-0\)-) attached to specific serine residues in a proteoglycan core protein (Bernfield et al., 1999; Esko and Selleck, 2002). Following the transfer of \(\alpha\)-GlcNAc as the first N-acetylatedhexosamine unit to this linkage region, HS co-polymerases add alternating 1-4-linked GlcA and \(\alpha\)-4-linked GlcNAc residues, generating HS GAG chains of 100 or more sugar units in length (Esko and Selleck, 2002). Biochemical studies have demonstrated that both the attachment of the first \(\alpha\)-GlcNAc to the GAG-protein linkage region and the subsequent polymer formation are catalyzed by members of the hereditary multiple exostoses (EXT) gene family of tumor suppressors (Esko and Selleck, 2002; Zak et al., 2002). In vertebrates, the EXT gene family consists of EXT1, EXT2, and three EXT-like genes designated EXTL1, EXTL2 and EXTL3 (Zak et al., 2002). Human mutations in EXT1 and EXT2 are associated with hereditary multiple exostoses (HME), a benign bone tumor characterized by multiple cartilage-capped outgrowths of various bones (Ahn et al., 1995; Stickens et al., 1996). However, three EXT-like genes have not been demonstrated to be linked to genetic disorder(s). A number of biochemical studies have shown that EXT1 and EXT2 function as HS co-polymerases involved in HS polymerization (Lind et al., 1998; McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000). Recent biochemical studies also demonstrated that both EXT2 and EXT3 proteins possess enzymatic activities that can transfer \(\alpha\)-GlcNAc to the GAG-protein linkage region and to intermediates of chain polymerization, suggesting roles for these proteins in initiation and polymerization reactions (Kim et al., 2001; Kitagawa et al., 1999; Zak et al., 2002). Despite intensive biochemical studies of the EXT family proteins in the HS GAG biosynthesis, their relationship in HS GAG biosynthesis and, in particular, their respective in vivo roles in development are largely unknown.

The Drosophila genome contains three EXT family members. Previous studies have shown that Tout-velu (Ttv), the Drosophila homologue of mammalian EXT1, is required for Hh signalling (Bellaiche et al., 1998; The et al., 1999). Hh movement in the wing disc is defective in cells mutant for ttv. Further study demonstrated that only cholesterol-modified Hh (Hh-Np), and not cholesterol-unmodified (Hh-N), is dependent on Ttv function for its movement (The et al., 1999). Consistent with this observation, a recent study showed that the movement of large punctate structures containing Hh-Np across cells is contingent upon the activity of Ttv (Gallet et al., 2003). The involvement of Ttv in HS GAG biosynthesis was also demonstrated. HS GAG is strikingly reduced, but not completely eliminated, in the \(\text{ttv}^{\text{null}}\) mutant embryo (The et al., 1999). Biochemical analysis further showed that HS GAG is markedly reduced in \(\text{ttv}^{\text{null}}\) mutant larvae (Toyoda et al., 2000). Together, these studies have demonstrated that Ttv is required for Hh movement and involved in the HS GAG biosynthesis. Interestingly, it was shown that Ttv is required specifically for Hh, but not for Wg and Fgf signalling (The et al., 1999), raising the question of whether the other two Drosophila EXT members play partially redundant roles with Ttv in signalling pathways other than Hh.

To understand the molecular mechanisms by which Wg, Hh and Dpp morphogen gradients are regulated during wing development, we have conducted a genetic screen for mutations associated with specific wing patterning defects (Belenkaya et al., 2002). In this paper, we report the identification and characterization of sister of tout-velu (sotv; \(\text{ttv}^{\text{botv}}\)) and brother of tout-velu (botv), encoding Drosophila homologues of mammalian EXT2 and EXTL3, respectively. We show that Hh signalling and its distribution are defective in either sotv or botv mutant cells. We further demonstrate that all three Drosophila EXT proteins (\(\text{ttv}^{\text{botv}}\) and sotv) are essential for Dpp signalling and its morphogen distribution. Surprisingly, although all three Drosophila EXT proteins are required for the proper extracellular Wg distribution, Wg signalling is only defective in botv mutant or \(\text{ttv}^{\text{botv}}\) double mutant cells, but not in \(\text{ttv}^{\text{botv}}\) mutant cells. We provide further biochemical evidence that Ttv and Sotv form a complex and are co-localized in vivo. Our results provide new insights into the functions of the EXT family proteins in morphogen signalling during development.

Materials and methods

Genetic screen, mapping and identification of botv and sotv mutations

tout-velu (ttv), brother of tout-velu (botv) and sister of tout-velu (sotv) were isolated from a F1 generic screen (Belenkaya et al., 2002). Approximately 200,000 F1 flies were screened, leading to the isolation of 9 \(\text{ttv}^{\text{botv}}\), 23 botv and 4 sotv alleles. \(\text{ttv}^{\text{botv}}\) is a putative null, containing a C to T transition, which leads to a nonsense mutation at 22AR and therefore a deletion of most of the protein. botv was mapped to the cytological interval 56A-56C by using deficiencies Df(2R)P34 and Df(2R)PC4. Mutations of botv alleles in CG15110 were subsequently identified by sequencing DNA from homozygous botv larvae. botv
ttv is a putative null and has a C to T transition, resulting in a nonsense mutation at 169Q. sotv failed to complement deficiencies Df(2R)Jp8 and Df(2R)Jp4, and was mapped to 52F. Mutations in CG8433 were identified from sotv alleles by sequencing. sotv is a putative null, containing a C to T transition, which leads to a nonsense mutation at 169Q. sotv failed to complement deficiencies Df(2R)Jp8 and Df(2R)Jp4, and was mapped to 52F. Mutations in CG8433 were identified from sotv alleles by sequencing. sotv has a G to A transition at the start codon of CG8433. The second Met (ATG) of CG8433 in sotv is at AA195, and is unlikely to be an alternative start codon for a functional protein as it is located after the signal peptide and the transmembrane domain. Therefore sotv is considered as a null. Consistent with this, cuticle defects associated with sotv null embryos are as severe as those of sotv/Df(2R)Jp8 or sotv/Df(2R)Jp4.

Generation of marked clones for phenotypic analysis

Females with germline clones were generated using the autosomal ‘FLP-DFS’ technique (Chou and Perrimon, 1996) as described (Belenkaya et al., 2002; Hacker et al., 1997). Imaginal disc clones of mutant cells were generated as described (Belenkaya et al., 2002; Hacker et al., 1997). To induce the expression of dsRed marker, third-instar larvae were subsequently subjected to a second heat shock for 90 minutes at 37°C and allowed to recover for 5 hours at room temperature before fixation and immunostaining. Below, we list the genotypes used in our analyses.

(1) \(\text{ttv}^{\text{botv}}\), sotv and botv clones marked by the absence of GFP (Figs 3, 4; Fig. 5B-D’, Fig. 6B-H’):

\[\text{y w hspx70-flp}^+/+ \text{ or Y; FRT}^{\text{G13}} \text{ ubiquitin-GFP M(2)58F / FRT}^{\text{G13}} \text{ ttv}^{\text{botv}};\]

\[\text{y w hspx70-flp}^+/+ \text{ or Y; FRT}^{\text{G13}} \text{ ubiquitin-GFP M(2)58F / FRT}^{\text{G13}} \text{ sotv};\]

\[\text{y w hspx70-flp}^+/+ \text{ or Y; FRT}^{\text{G13}} \text{ ubiquitin-GFP M(2)58F / FRT}^{\text{G13}} \text{ botv}^{\text{botv}}.\]

(2) \(\text{ttv}^{\text{botv}}\)-sotv clones marked by the absence of GFP (Fig. 6I-J):
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Antibody and X-gal staining

Fixation of embryos and imaginal discs as well as antibody staining procedure were performed as described (Belenkaya et al., 2002; Hacker et al., 1997). For antibody staining in Drosophila Schneider’s S2 cells, cells were fixed in PBS with 2% formaldehyde for 15 minutes and then subjected to the same antibody staining procedure as for disc staining. X-gal staining was as described (Sullivan et al., 1996). For antibody staining in embryos, the nuclei were stained with X-gal and then subjected to the same antibody staining procedure as for disc staining. Antibody staining was performed as described (Baeg et al., 2001; Strigini and Cohen, 2000). Primary antibodies were used at the following dilutions: rabbit anti-pMad (PS1) at 1:500 (Persson et al., 1998; Tanimoto et al., 2000); rabbit anti-Wg at 1:500 (Reichsman et al., 1996); rat anti-En at 1:10 (Mozny and Holmgren, 1995); rabbit anti-Hh at 1:2000 (Taylor et al., 1993); mouse anti-Dll at 1:2000 (Taylor et al., 1993); rabbit anti-Hh at 1:2000 (Taylor et al., 1993); mouse anti-Dll at 1:500 (Duncan et al., 1998); mouse anti-Engrailed 4D9 at 1:500 (Iowa Developmental Studies Hybridoma Bank; IDSHB); mouse anti-Wg 4D4 at 1:3 (IDSHB); rabbit anti-GFP Alexa Fluor 488 at 1:1000 (Molecular Probe); rabbit anti-DsRed at 1:4000 (Clontech); mouse anti-β-Actin 5G10 at 1:1000 (Iowa Developmental Studies Hybridoma Bank); rat anti-HA 3F10 at 1:1000; mouse anti-Myc 9E10 at 1:500 (Roche Molecular Biochemicals); mouse anti-α-Tubulin (Novex); and mouse anti-Drosophila Golgi at 1:100 (Calbiochem).

Molecular biology

The botv full-length cDNA was isolated by screening a 0-4 hour Drosophila embryonic cDNA library (Brown and Kafatos, 1988). The coding region of botv cDNA with three haemagglutinin (HA) tags in-frame at its C terminus was then cloned into the pUAST vector to generate pUAST-botv-HA. The sotv cDNA was obtained from EST cDNA clone GH02288 (Invitrogen). The V5-tagged Sotv construct was obtained by cloning the coding region into the Kpl-EcorV site of pAc5.1 V5-His C vector (Invitrogen), in-frame with the V5 tag. To generate the pAc5.1-ttv-myc construct, the ttv-myc fragment was amplified by PCR from the genomic DNA of the UAS-ttv-myc line (The et al., 1999) and subcloned into the pAc5.1 V5-His A vector. HS-DsRed was generated by cloning the full-length (BamHI-SpeI) DsRed T1 coding region from pDsRed expression vector (Clontech) into BglII-XhoI sites of the pCasper-Rhs vector.

Immunoprecipitation and western blotting

Drosophila Schneider’s S2 cells (1×10^7) were transfected with 10 μg of corresponding expression vectors by the calcium phosphate precipitation method. For the induction of Botv-HA cloned in pUAST vector, 10 μg pUAST-botv-HA and 10 μg of pArmadillo-Gal4 (Klug et al., 2002) were co-transfected. Cells were harvested 60 hours later, and lysed in 1.5 ml of 20 mM Tris-HCl (pH 7.4), 2% Triton X-100, 150 mM NaCl and 7.5 ml protease inhibitor tablet (Roche Molecular Biochemicals) on ice for 20 minutes. After clearance, one half of each lysate was used for immunoprecipitation with 1 μg antibodies for 3 hours at 4°C and then incubated for an additional 1.5 hours in the presence of 12.5 μl bed volume of protein G sepharose (Amersham Pharmacia). Immunoprecipitates were washed three times with 10 mM Tris-HCl (pH 7.4), 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA and 1 μl protease inhibitor (Sigma), and twice with 10 mM Tris-HCl (pH 7.4). Western blotting was carried out as described (Belenkaya et al., 2002).

Results

brother of tout-velu (botv) and sister of tout-velu (sotv) are two new segment-polarity genes

To identify novel genes involved in the signalling events mediated by the Wg, Hh and Dpp morphogens during wing patterning, we conducted a genetic screen using a wing specific FLPase/FRT system (Belenkaya et al., 2002). Three independent loci on the second chromosome were recovered and exhibit similar wing defects, including fusion of longitudinal veins L3 and L4, vein deletions (Fig. 1A-D) and wing nicks (data not shown). One of loci was identified as tout-velu (ttv) (Fig. 1B) (see Materials and methods). On the basis of similar wing phenotypes associated with these loci, we named other two loci as brother of tout-velu (botv) and sister of tout-velu (sotv).

Previous studies have demonstrated that Ttv is required for Hh signalling during embryogenesis and development of the wing (Bellaiche et al., 1998; The et al., 1999). To further characterize botv and sotv, we examined the embryonic cuticle patterns associated with these mutants using null alleles (see Materials and methods). Animals zygotically mutant for either botv or sotv survive until the third instar larval stage and appear to have normal cuticle patterning (data not shown). However, homozygous mutant embryos derived from females lacking germline botv activity (referred to as botv null embryos) die with a strong segment polarity phenotype (Fig. 1G). Homozygous mutant embryos derived from females lacking germline sotv activity (referred to as sotv null embryos) also die with segment polarity phenotypes, albeit relatively weak and variable (Fig. 1H1). In the ventral embryonic ectoderm, Wg and Hh signalling are required for normal expression of engrailed (en) and wg (Hatini and DiNardo, 2001). In botv and sotv null embryos, stripes of En and Wg expression were lost from the ectoderm by stage 11 (Fig. 1K,L,N,O). Taken together, these results suggest that both botv and sotv are segment-polarity genes, and are likely to be involved in Hh and/or Wg signalling.

botv and sotv encode the Drosophila homologues of mammalian EXTL3 and EXT2, respectively, and are required for HS GAG biosynthesis

botv and sotv were subsequently mapped to the cytological positions 56A-56C and 52F, respectively (see Materials and methods). Searches of annotated Drosophila genome databases identified a Drosophila EXT-like gene (CG15110) in 56A-56C and a Drosophila EXT2 (DEXT2; CG8433) in 52F. The Drosophila genome contains three EXT genes including ttv, CG15110 and CG8433. Based on the similarities of botv and sotv with ttv in both wing and embryonic cuticle defects, we suspected that the CG15110 and CG8433 transcripts may encode Botv and Sotv, respectively. Two lines of evidence strongly suggest that this is indeed the case. First, we used the RNA interference (RNAi) method (Kennerdell and Carthew, 1998) to perturb...
CG15110 and CG8433 transcripts. Embryos injected with either CG15110 or CG8433 double-stranded RNA showed segment-polarity defects (data not shown). Second, all the sequenced alleles of botv and sotv have mutations in the CG15110 and CG8433 genes, respectively (selected alleles are shown in Fig. 2A).

We generated a phylogenetic tree of the EXT family members among humans, mouse and Drosophila based on their amino acid sequences (Fig. 2B). Ttv is most similar to EXT1, whereas Botv and Sotv are more closely related with EXT2 and EXTL3, respectively. Ttv is 31.4% and 33.5% identical to Botv and Sotv proteins, respectively. In particular, all three Drosophila EXT members shared high amino acid identity in their C-terminal regions (Fig. 2C).

Biochemical studies have demonstrated that EXT family proteins are required for HS GAG biosynthesis (Esko and Selleck, 2002; Zak et al., 2002). Biosynthesis of HS GAG chains is strikingly reduced in ttv mutant embryos (The et al., 1999) and larvae (Toyoda et al., 2000). We analyzed levels of HS GAG chains in clones mutant for botv, botv and sotv in the wing disc. In wild-type cells, HS GAG staining was found in punctate particles as well as on the membrane (Fig. 3). Consistent with previous studies in embryos, HS GAG staining in ttv mutant clones was strikingly reduced (Fig. 3A,A'), suggesting that Ttv is required for HS GAG biosynthesis in the wing disc as well. We also observed similar reductions in HS GAG staining in cells mutant for sotv (Fig. 3B,B') or botv (Fig. 3C,C'). On the basis of these observations, we conclude that all three Drosophila EXT proteins are indispensable for the biosynthesis of HS GAG chains.

**Botv and Sotv are required for Hh signalling in the wing disc**

Previous studies have demonstrated the essential role of Ttv in Hh signalling in the wing disc (Bellaiche et al., 1998). Hh movement is blocked in A compartment cells mutant for ttv (Bellaiche et al., 1998). Two lines of evidence support the idea that Botv and Sotv are also required for Hh signalling. First, the level of Cubitus interruptus (Ci) is stabilized by Hh signalling in about 8-10 cells in the AP border (Aza-Blanc et al., 1997; Methot and Basler, 1999; Motzny and Holmgren, 1995). However, Ci stabilization is strikingly reduced in anterior cells mutant for sotv (Fig. 4B,B') or botv (Fig. 4C,C'). Second, whereas Hh proteins are present as punctate particles in anterior cells at the AP border, these punctate particles are absent within sotv (Fig. 4E,E') and botv clones (Fig. 4F,F'), except in the first row of cells facing the P compartment. These results suggest that similar to Ttv, Sotv and Botv activities are required for Hh movement in its receiving cells. In the absence of either Sotv or Botv, Hh can only move into the first row of cells immediately adjacent to the Hh-expressing cells, and fails to move further.

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**Fig. 1.** Identification of botv and sotv as two novel segment-polarity genes. (A-D) Wings are oriented proximal to the left, anterior up. (A) A wild-type wing. (B-D) Wings bearing somatic clones of ttv (B), botv (C) and sotv (D). Adult wings with clones of these mutations exhibit a variety of phenotypes, including vein loss, vein fusion, blister, narrowed wing and wing notching (data not shown). (E-I) Cuticle preparations of a wild-type embryo (E), a hh homozygous embryo (F), and embryos derived from mutant germ-line clones of botv (G) and sotv (H). All embryos are oriented anterior to the left. Embryos lacking both maternal and zygotic activities of botv or sotv exhibit typical segment-polarity phenotypes; however, the defects of sotv mutants can be relatively weak (I). (J-L) En staining of a stage 10 wild-type embryo (J), and embryos derived from mutant germ-line clones of botv (K) and sotv (L). (M-O) Wg staining of a stage 10 wild-type embryo (M), and embryos derived from mutant germ-line clones of botv (N) and sotv (O).
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Ttv, Sotv and Botv are required for Dpp signalling and its gradient distribution

In the wing disc, Dpp functions as a long-range morphogen to control the growth and patterning of cells in the AP axis (Lecuit et al., 1996; Nellen et al., 1996). Dpp signalling is shown to activate its downstream signalling component Mad in a concentration-dependent manner (Tanimoto et al., 2000). Recently, the Dpp morphogen gradient has been visualized directly using GFP-Dpp fusion proteins that retain signalling activity (Entchev et al., 2000; Teleman and Cohen, 2000).

Fig. 2. Sotv and Botv are members of the Drosophila EXT family of tumor suppressors. (A) Structures and mutations of Sotv and Botv. Sotv and Botv are putative type II transmembrane proteins. Three mutations of botv and sotv are shown in green bars (except sotv 44, which harbors a G to A transition at the start codon). (B) Phylogenetic tree of Drosophila, mouse (m), and human (h) EXT family proteins. Ttv, Sotv and Botv are the Drosophila EXT1, EXT2 and EXT3 proteins, respectively. (C) Sequence comparison of Ttv, Sotv and Botv. Identical residues are highlighted (light red) and consensus residues are boxed. A conserved nucleotide sugar-binding motif DXD is found at the C-terminal portion of all three proteins and is boxed (blue). The sequence alignment and phylogenetic tree were generated using Lasergene software with the Jotun Hein method.

Ttv, Sotv and Botv are required for Dpp signalling and its gradient distribution

In the wing disc, Dpp functions as a long-range morphogen to control the growth and patterning of cells in the AP axis (Lecuit et al., 1996; Nellen et al., 1996). Dpp signalling is shown to activate its downstream signalling component Mad in a concentration-dependent manner (Tanimoto et al., 2000). Recently, the Dpp morphogen gradient has been visualized directly using GFP-Dpp fusion proteins that retain signalling activity (Entchev et al., 2000; Teleman and Cohen, 2000).

Vein deletions are the most striking defects associated with wing-bearing clones mutant for ttv, sotv and botv (Fig. 1B,C,D). As Dpp signalling is required for vein formation (de Celis et al., 1996; Ray and Wharton, 2001), we investigated whether Dpp signalling and its gradient distribution were defective in clones mutant for ttv, sotv and botv. We first examined Dpp signalling activity by visualizing the activated form of Mad (p-Mad), which is phosphorylated by the activated Dpp receptor Thickveins (Tkv) in response to Dpp signalling (Tanimoto et al., 2000). In the wild-type wing disc (Fig. 5A), p-Mad levels were high in the central region of the wing disc and gradually decline towards the A and P distal cells. p-Mad levels were lower at the AP boundary owing to the reduced expression of tkv (Tanimoto et al., 2000). p-Mad levels were lower at the AP boundary.
levels were strikingly reduced in either A or P cells mutant for \(ttv\), \(sotv\) or \(botv\) (Fig. 4B-D'), providing evidence that all three \(Drosophila\) EXT proteins are required for Dpp signalling. We further tested whether the EXT proteins control Dpp signalling by regulating Dpp morphogen distribution. For this purpose, we expressed GFP-Dpp in the endogenous \(dpp\) expression domain using \(Dpp\) GAL4 and analyzed GFP-Dpp distribution in clones mutant for \(ttv\), \(sotv\) or \(botv\). In the wild-type background, GFP-Dpp exhibits a gradient pattern in both the A and P compartment (Fig. 5E). However, levels of GFP-Dpp are reduced in clones mutant for \(ttv\), \(sotv\) or \(botv\) (Fig. 5F-H'). Together, these results argue that Ttv, Sotv and Botv promote Dpp signalling by modulating its morphogen distribution.

**Distinct roles of Botv from those of Ttv and Sotv in Wg signalling in the wing disc**

In the wing disc, Wg forms a long-range gradient and acts both at short and long range to regulate the expression of...
several target genes in different spatial domains (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996). The homeodomain protein Distal-less (Dll) (Neumann and Cohen, 1997; Zecca et al., 1996) and the zinc-finger protein Senseless (Sens) (Nolo et al., 2000; Parker et al., 2002) are the long- and short-range targets of the Wg morphogen, respectively (Fig. 6A, A’). We have previously shown that HSPGs are required for Wg short- and long-range signalling, as well as for its extracellular distribution in the wing disc (Baeg et al., 2001; Lin and Perrimon, 1999). However, a previous study on Ttv demonstrated that Ttv is not involved in Wg signalling during embryogenesis and in the wing disc, suggesting that Ttv selectively participates in morphogen signalling (The et al., 1999). To evaluate the specificity of the three Drosophila EXT genes in morphogen signalling in the wing disc, we examined Wg short- and long-range signalling activities, as well as its morphogen distribution in clones mutant for ttv, sotv and botv.

We observed reductions in the ranges of Dll expression in ttv, sotv and botv mutant cells (Fig. 6B, C, D). These defects were fully penetrant, suggesting that all three EXT proteins are normally required for Wg long-range activity. Interestingly, we found that Dll levels were not reduced in regions close to the DV boundary within the clones of ttv and sotv mutant cells; however, they were significantly reduced in the same region within the botv mutant clone (Fig. 6B, C, D). These results suggest that both the range of Wg action and its signalling are affected in the botv mutant clone; however, only the range of Wg action, and not its signalling per se, is reduced in the ttv or sotv clones. Consistent with this, we found that Sens expression was diminished in botv clones, but not in ttv nor sotv clones, confirming that Wg signalling is defective only in the botv mutant, and not in ttv or sotv mutant (Fig. 6B’, C’, D’). The observed defects in the ranges of Dll expression in ttv, sotv and botv mutant clones could be due to reduced levels of Wg morphogen. We tested this by staining extracellular Wg. The result was in agreement with the Dll data (Fig. 6F–H’). On one hand, the range of extracellular Wg distribution was reduced...
Fig. 6. Involvement of the Drosophila EXT genes in Wg signalling. In all discs carrying mutant clones, the clones are marked by the absence of GFP and are outlined with dots. (A-D”) Dll and Sens staining in a wild-type wing disc (A-A”), and in wing discs carrying mutant clones of \(ttv^{63}\) (B-B”), \(sotv^{44}\) (C-C”) and \(botv^{103}\) (D-D”). Dll expression appears to be a gradient with the peak at the DV boundary. The ranges of the Dll gradient in the mutant clones of \(ttv^{63}\) (B), \(sotv^{44}\) (C) and \(botv^{103}\) (D) are reduced. Note that the level of Dll expression close to the DV boundary within the mutant clones of \(ttv^{63}\) and \(sotv^{44}\) is comparable with that of wild-type cells; however, it is significantly reduced in the \(botv^{103}\) mutant clone. Wg-dependent Sens expression in the wing disc is in two narrow stripes abutting Wg expressing cells. This expression is unaffected in the mutant clones of \(ttv^{63}\) (B) and \(sotv^{44}\) (C”), but is diminished in the \(botv^{103}\) mutant clone (D’). Note that the Wg-independent expression of Sens in \(botv^{103}\) mutant clone is still maintained. (E-H’) Extracellular Wg distribution is shown in a wild-type wing disc (E), and in discs carrying mutant clones of \(ttv^{63}\) (F-F”), \(sotv^{44}\) (G-G”) and \(botv^{103}\) (H-H”). Extracellular Wg is almost completely lost in the \(botv^{103}\) clone except at the surface of Wg expressing cells, whereas it is maintained at some level around the DV boundary in the mutant clones of \(ttv^{63}\) and \(sotv^{44}\). (I-J’) Sens staining in discs bearing clones of \(ttv^{63}-sotv^{44}\) double mutant cells. The Wg-dependent Sens expression is diminished in the double mutant clones.
in clones mutant for \textit{ttv}, \textit{sotv} and \textit{botv}. On the other hand, extracellular Wg was maintained at some levels in the regions close to DV boundary in the \textit{ttv} or \textit{sotv} clone, however, it was absent in the same regions within the \textit{botv} clone, except at the surface of Wg-expressing cells. Together, these results suggest that all three \textit{Drosophila} EXT proteins are required for the long-range distribution of Wg protein. However, Wg signalling is only defective in the \textit{botv} mutant, and not in \textit{ttv} or \textit{sotv} mutant cells.

One apparent explanation for this is that Ttv and Sotv are functionally redundant in Wg signalling. To test this, we examined Sens expression in clones of \textit{ttv-sotv} double mutants and found that, indeed, Sens expression was diminished (Fig. I-J'). A virtually identical result in Sens expression was observed in \textit{ttv-sotv-botv} triple mutant cells (data not shown). Taken together, our findings suggest that all three EXT proteins are required for proper extracellular Wg distribution. However, whereas Botv is independently required for Wg signalling, Ttv and Sotv are redundant in Wg signalling.

**Biochemical interactions and subcellular localization of the \textit{Drosophila} EXT proteins**

The distinct roles of Botv from those of Ttv and Sotv in Wg signalling suggest that they may have unique functions in the biosynthesis of HS GAG chains. In vertebrate, direct enzymatic assay for vertebrate EXTL3 demonstrates that its GlcNAc transferase activities are involved in both the initiation and polymerization reactions (Kim et al., 2001), whereas several biochemical studies suggested that EXT1 and EXT2 may function as a HS GAG co-polymerase in which both EXT1 and EXT2 serve as subunits essential for the activity (McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000; Zak et al., 2002). As Ttv and Sotv are most similar to the vertebrate EXTL1 and EXTL2, respectively, we anticipated that Ttv and Sotv may function as a HS GAG co-polymerase whose full activity requires both Ttv and Sotv. By contrast, Botv, a homologue of vertebrate EXTL3, may participate in the initiation step of HS GAG biosynthesis, which is distinct from the role of Ttv and Sotv.

To determine whether Ttv and Sotv function as subunits for the HS copolymerase, we performed a co-immunoprecipitation experiment to examine whether Ttv and Sotv form a complex in cells. Myc-tagged Ttv and V5-tagged Sotv were expressed either individually or in combination in \textit{Drosophila} S2 cells. Upon immunoprecipitation of Myc-tagged Ttv from the cellular lysate of transfected cells, the Sotv protein could be detected by western blotting in the immunoprecipitate (Fig. 7A). Interestingly, we did not observe an interaction between Ttv and Sotv when cellular lysates from individually transfected cells were mixed and immunoprecipitated (Fig. 7A, lane labeled m), indicating that Ttv and Sotv cannot associate ex vivo. We further conducted similar experiments to examine the association of Botv with Ttv or Sotv (Fig. 7B,C). In these cases, no interactions were detected. These data suggest that Ttv and Sotv are physically associated in vivo, but that they do not form complexes with Botv.

We also conducted an experiment to determine the subcellular localization of Ttv, Sotv and Botv in transfected \textit{Drosophila} S2 cells. Epitope-tagged protein constructs were transfected individually or in combination into \textit{Drosophila} S2 cells. Consistent with previous result in embryos (The et al., 1999), Myc-tagged Ttv proteins were present in both endoplasmic reticulum (ER) and Golgi (Fig. 7D–E”). Sotv and Botv were also localized in both ER and Golgi (data not shown) in cells transfected with either V5-tagged Sotv or HA-tagged Botv. Interestingly, in cells transfected with all three EXT proteins (Fig. 7F–H”), we found that Ttv and Sotv protein staining were virtually identical, and that they were concentrated in certain compartment(s). However, Botv protein staining appeared to be more uniform than that of Ttv or Sotv. This data is consistent with the results obtained from the co-immunoprecipitation experiments, providing further evidence that Ttv and Sotv are present in a complex(es) in vivo.

We further tested whether overexpression of Ttv can replace the function of Sotv and Botv. For this purpose, we ectopically expressed Ttv in the hairy expression domain by using hairyGal4 IJ3 in either \textit{sotv} or \textit{botv} null mutant embryos. Ectopic expression of Myc-tagged Ttv fully rescued the cuticle patterning of the \textit{tv} mutant embryo in the \textit{hairy} domain (Fig. 7H), but was not able to rescue cuticle defects associated with \textit{sotv} or \textit{botv} null mutant embryos (Fig. 7I,J). This result further indicates that individual EXT members play indispensable roles in HS GAG biosynthesis.

**Discussion**

Biochemical studies have demonstrated essential roles of the EXT family proteins as glycosyltransferases required for HS GAG biosynthesis. However, their respective in vivo roles during development are largely unknown. \textit{Drosophila} contains three EXT family proteins. Although previous studies demonstrated an essential role for Ttv, the \textit{Drosophila} EXT1, in Hh movement and its signalling, the functions of the other two EXT proteins are unclear. Our results demonstrate essential functions of all three \textit{Drosophila} EXT family proteins in signalling events mediated by the Wg, Hh, and Dpp morphogens. We provide strong evidence that all three \textit{Drosophila} EXT proteins are involved in HS biosynthesis and are required for the proper distributions of the morphogen molecules Wg, Hh, and Dpp. Interestingly, we found that Wg signalling is defective only in \textit{botv} mutant or \textit{ttv-sotv} double mutant cells, but not in \textit{ttv} nor \textit{sotv} mutant cells, which suggests partially redundant roles for Ttv and Sotv in Wg signalling. Our results are consistent with a model in which Ttv and Sotv collectively function as a co-polymerase required for the biosynthesis of HS GAG chains, whereas Botv is likely to be involved in distinct step(s), possibly in the initiation of HS GAG biosynthesis. Our results also suggest that HSPGs have two distinct roles in regulating Wg morphogen activity: a function in maintaining the extracellular Wg protein and a co-receptor role in Wg signalling.

**Roles of the \textit{Drosophila} EXT family proteins in Hh and Dpp morphogen signalling**

Previous studies have demonstrated that Ttv is involved in Hh movement (Bellaiche et al., 1998; Gallet et al., 2003; The et al., 1999). Our results in this work suggest that like Ttv, both Sotv and Botv are also required for Hh movement. Interestingly, we found that Hh is detectable in the first row of mutant cells immediately adjacent to its posterior-producing cells. We propose that specific HSPGs modified by EXT family proteins are required for the movement of Hh from its
expressing cells into the anterior-receiving cells. In the absence of EXT activities, Hh can be carried into the first row of mutant cells, but fails to move further. Our results are consistent with previous work that demonstrated that the first row of ttv mutant cells can still transduce Hh signalling and can activate the expression of its downstream target gene patched (ptc) (Bellaiche et al., 1998). It is important to note that, while HSPGs modified by EXT family members are likely to be required for the movement of Hh, they may also be involved in preventing Hh from being degraded on the cell surface. In
the absence of EXT proteins, Hh may be degraded and therefore it cannot reach the wild-type cells anterior to the clones of EXT mutant cells. It remains to be determined whether both mechanisms are involved in Hh transport.

We provide strong evidence for the involvement of the three EXT proteins in Dpp signalling and its morphogen distribution. Our results suggest that specific HSPG(s) modified by the three EXT proteins may promote Dpp morphogen signalling by modulating levels of Dpp morphogen ligands in Dpp-receiving cells. Previous studies have implicated a role for Dally, a Drosophila glypican member of HSPGs, in Dpp signalling in the development of imaginal discs (Fujise et al., 2003; Jackson et al., 1997). Elevated expression of Dally in the wing disc can promote Dpp signalling, as assayed by p-Mad levels (Fujise et al., 2003). It was proposed that Dally may act as a co-receptor for Dpp in activating its signalling. Our results in this work suggest that HS GAG chains control Dpp signalling by modulating levels of Dpp morphogen in its receiving cells. We propose that the three EXT proteins are required for the biosynthesis of HS GAG chains on the Dally protein and that at least one of the roles of Dally in Dpp signalling is to modulate levels of the Dpp morphogen on its receiving cells.

Role of HSPGs in Wg morphogen distribution and its signalling

Perhaps one of the most interesting findings in this work is the differential roles of the three EXT proteins in Wg morphogen signalling. Although mutations in any of the three EXT genes lead to reduced ranges of extracellular Wg distribution, we found that Wg signalling was defective only in botv mutant cells, and not in either ttv or sotv mutant cells. However, ttv-sotv double mutants showed virtually identical defects in Wg signalling to those of botv mutants. This qualitative difference between Botv and Ttv or Sotv in Wg signalling is unexpected as mutations in all three genes led to striking reductions in Hh and Dpp signalling.

Previous studies have implicated HSPGs in both Wg signalling and its morphogen gradient formation. Embryos null for either sugarless or sulfateless, exhibit defects in Wg signalling in various tissues (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). The Drosophila glypican Dally has been shown to be required for Wg signalling (Fujise et al., 2003; Lin and Perrimon, 1999; Tsuda et al., 1999). A reduction of dally activity can block the Wg signalling activity induced by overexpression of the Wg receptor Drosophila frizzled 2 (fz2) in the wing disc (Lin and Perrimon, 1999). Interestingly, recent studies have demonstrated that Notum (Wingful), a putative Drosophila pectin acetylenase, can inhibit Wg signalling activity by modulating the activity of the heparin sulfate proteoglycans Dally-like (Dly) and Dally (Gerlitz and Basler, 2002; Giraldes et al., 2002). Overexpression of Notum can block the signalling activity of the Wg protein tethered to the cell surface by a transmembrane domain from Neurotactin, suggesting that Notum inhibits the function of proteoglycans that are involved in Wg signalling (Gerlitz and Basler, 2002). The function of HSPGs in Wg morphogen gradient distribution has also been demonstrated. In the wing disc, cells mutant for sulfateless showed a reduction in the levels of extracellular Wg (Baeg et al., 2001). It was shown that ectopic expression of Dly leads to the accumulation of extracellular Wg protein (Baeg et al., 2001). Furthermore, loss of Notum function leads to increased Wingless activity by altering the shape of the Wingless protein gradient (Giraldes et al., 2002).

Taken together, our results, along with previous studies, suggest that HSPGs are involved in both Wg signalling and in extracellular Wg morphogen distribution in the wing disc. We suggest that HSPGs have at least two distinct functions in the wing disc: (1) in the distribution of the extracellular Wg protein; and (2) as a co-receptor for Wg signalling. In this regard, Botv is required both for Wg signalling and for its morphogen gradient formation, whereas Ttv and Sotv are only required for the distribution of extracellular Wg protein; they are functionally redundant in Wg signalling. Consistent with our observations, a previous report on ttv showed that, during embryogenesis, Wg signalling in the stomatogastric nervous system (SNS) is not defective in ttv mutant embryos (The et al., 1999). It remains to be determined whether botv mutants and sotv-ttv double mutants show affected Wg signalling in the SNS and in other tissues as well.

Mechanisms and specificities of Ttv, Sotv and Botv in cell signalling

Previous analysis of Ttv has demonstrated its specificity in cell signalling (The et al., 1999). Although Hh signalling is defective in the ttv null mutant, neither Wg nor Fgf signalling is altered (The et al., 1999). It was proposed that Ttv is required for the synthesis of an Hh-specific HSPG. Consistent with the previous report, our results demonstrated that Wg signalling is only defective in the ttv-sotv double mutant, and is not altered in either ttv or sotv single mutants. However, we also observed striking defects in both Dpp signalling and the range of extracellular Wg protein distribution in ttv and sotv mutants. Therefore, the previous view that Ttv is involved only in Hh signalling should be revised.

To understand the molecular mechanisms by which the Drosophila EXT proteins play distinct and collaborative roles in cell signalling, we performed biochemical experiments to analyze their interactions and subcellular localization. We found that Ttv and Sotv physically associate with each other to form a complex, and that they have virtually identical subcellular localizations. However, neither Ttv nor Sotv physically interact with Botv. Botv also has a more diffusive staining in cells than Ttv and Sotv. Consistent with our results, biochemical studies in vertebrates showed that vertebrate EXT1 and EXT2 also physically associate with each other to form a complex. Biochemical studies have further demonstrated that both EXT1 and EXT2 have GlcNAc and GlcA transferase activities when expressed independently, although EXT1 has a more robust activity than does EXT2 (Lind et al., 1998; McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000). However, co-expression of EXT1 and EXT2 has a synergistic effect on enzyme activities (McCormick et al., 2000; Senay et al., 2000). These results led to a proposal that the fully active HS co-polymerase may be a complex containing EXT1 and EXT2, in which both subunits are essential for the activity (Zak et al., 2002). The functions of EXT-like proteins have also been investigated. EXTL3 appears to be a bifunctional αGlcNAc transferase that can transfer GlcNAc to both the linkage region and to intermediates during chain polymerization, suggesting that EXTL3 is involved in both the
initiation and polymerization of HS GAG chains (Kim et al., 2001). Importantly, a recent biochemical study demonstrated that Botv has αGalNAc transferase activities that can transfer GalNAc to both the linkage region and to intermediates in chain polymerization (Kim et al., 2002).

On the basis of our genetic evidence and previous biochemical studies, we propose that Ttv and Sotv are likely to function as co-polymerases required for HS GAG polymerization, whereas Botv is likely to be involved in the initiation of HS GAG and is possibly involved in HS GAG polymerization as well. In the absence of Botv, no HS GAG chains are initiated and therefore mutations in Botv disrupt all the functions of HS GAG chains. However, in the absence of either Ttv or Sotv, initiation of HS GAG biosynthesis occurs, and the residual activity of HS GAG polymerase(s), carried out by another member (either Sotv or Ttv) together with Botv, may synthesize relatively short HS GAG chains that could act as co-receptors for Wg signalling, but have less capacity for maintaining the levels of secreted Wg, Hh and Dpp morphogen proteins. When the activities of both Ttv and Sotv are removed in the ttv-sotv double mutant, HS GAG polymerization may not occur because of the lack of αGalNAc transferase activity, even though Botv is present. In support of this view, previous biochemical studies demonstrated that short HS GAG oligosaccharides have the capacity to form Fgf-Fgfr-HS complexes and to stimulate Fgf signalling (Pellegrini, 2001). In this regard, in the absence of Ttv, both Wg and Fgf signalling may occur. Consistent with this view, a previous study demonstrated that Fgf signalling is not affected in ttv mutant embryos (The et al., 1999). Biochemical studies on the activities of Ttv and Sotv as HS co-polymerases will further validate this view. An alternative model is that fewer intact HS GAG chains of Ttv and Sotv as HS co-polymerases will further validate this view. An alternative model is that fewer intact HS GAG chains are synthesized in the absence of either Ttv or Sotv. Although this is less likely to be the case, our current results can not exclude this possibility.

EXT proteins and HME disease

Human mutations in EXT1 and EXT2 are associated with hereditary multiple exostoses (HME), which is a autosomal dominant disorder characterized by the formation of multiple cartilage-capped tumors (exostoses) of various bones. Our results demonstrate the essential functions of both Ttv (EXT1) and Sotv (EXT2) in regulating the activities of the three secreted morphogen molecules Hh, Wg and Dpp. Wg and Dpp are the homologues of human WNT and bone morphogen protein (BMP) molecules, respectively. As both WNT and BMP family proteins have been shown to be essential for bone growth and differentiation, our results suggest possible roles for WNT and BMP signalling in the generation of HME diseases associated with EXT1 and EXT2. Our new findings together with previous work on the role of Ttv in Hh movement may provide new insights into the molecular mechanism(s) associated with HME disease.

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