Composite signalling from Serrate and Delta establishes leg segments in Drosophila through Notch

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SUMMARY

The receptor protein NOTCH and its ligands SERRATE and DELTA are involved in many developmental processes in invertebrates and vertebrates alike. Here we show that the expression of the Serrate and Delta genes patterns the segments of the leg in Drosophila by a combination of their signalling activities. Coincident stripes of Serrate and Delta expressing cells activate Enhancer of split expression in adjacent cells through Notch signalling. These cells form a patterning boundary from which a putative secondary signal leads to the development of leg joints. Elsewhere in the tarsal segments, signalling by DELTA and NOTCH is necessary for the development of non-joint parts of the leg. We propose that these two effects result from different thresholds of NOTCH activation, which are translated into different downstream gene expression effects. We propose a general mechanism for creation of boundaries by Notch signalling.

Key words: Drosophila melanogaster, Serrate, Delta, Notch, Leg development, Signalling

INTRODUCTION

Animal development involves many types of cell communication processes and the molecular bases of some of these processes have been unravelled in recent years. An unexpected finding has been that the same molecular pathways are used to convey messages between cells in vertebrate and invertebrate animals. One of the most pervasive of these signalling pathways is that of Notch. The Notch (N) gene was identified in Drosophila melanogaster on the basis of its requirements in multiple developmental processes. N encodes the transmembrane protein NOTCH (N), which has been shown to act as a receptor during cell communication (reviewed in Fleming et al., 1997b). In its best characterised role in the development of the nervous system, N has been shown to mediate a process of mutual inhibition whereby an excess of cells with a neural potency compete for a smaller number of neural fates. The N ligand or signalling molecule in this process is the product of the Delta (DL) gene (reviewed in Muskavitch, 1994).

In other developmental processes, N receives signals from several ligands (Fleming et al., 1997b). In the induction of a patterning centre at the dorso-ventral (DV) boundary of the presumptive fly wing, the product of the Serrate (Ser) gene acts as a signal to N. In this particular developmental process, the DELTA protein (DL) is involved and also another transmembrane molecule, FRINGE (FNG), which has been proposed to bind N and modulate its signalling (Panin et al., 1997). This multiplicity of ligands stands in contrast with the proposed simpler one-ligand situation during the singling-out of neural precursors (Heitzler and Simpson, 1991; Muskavitch, 1994), but it has been suggested that Ser and the wingless (wg) signalling pathways also have a role in several aspects of neural development (Axelrod et al., 1996; Couso and Martinez Arias, 1994; Zeng et al., 1998). Studies in vertebrates also show a multiplicity of N ligands, with multiple homologous proteins to N and its ligands DL and the SERRATE protein (SER) being involved in a variety of developmental processes. In these cases, amongst them somite development and sensory organ development, a variety of DL-like and SER-like ligands are at work (reviewed in Robey, 1997). Thus, it would seem that in N-mediated processes, a situation where combinations of ligands are present might be the norm and a DL-N lateral inhibition process the exception. An understanding of the molecular basis and the cellular logic behind the activation of N by several ligands would be of wide relevance, but so far N signalling seems to show variety rather than a constant mechanism.

The molecular basis of the transmission of the DL-N signal is beginning to be understood, and in short involves the activation and cleavage of the N protein and the transport to the nucleus of the Suppressor of Hairless protein to act as a transcription factor on target genes like the Enhancer of split (E(spl)) complex (Fleming et al., 1997b; Weinmaster, 1998). However, it is not yet understood how signals from different N ligands are processed by this pathway. In this work we describe the role of N and its ligands SER and DL in the development of the legs of Drosophila melanogaster, and propose a mechanism that might be common to other developmental processes in Drosophila and vertebrates.
During fly leg development the anlage is divided in concentric segments along the proximal-distal axis (Couso and Bishop, 1998). At the boundaries between these segments, a multicellular pattern feature is defined, the articulated joints (Fristrom and Fristrom, 1993). These joints provide functionality to the legs and are one of the distinct and fundamental features of the Arthropod phylum. Here we show that N signalling allocates the presumptive joint areas between segments. Co-expression of Ser and Dl in a stripe of cells proximal to the future position of the joints signals the adjacent row of cells to express members of the E(spl) complex and to become presumptive joint areas. Autonomous self-signalling by Ser and Dl expressing cells is reduced to low levels by the presence of the putative repressor FNG and by possible dominant negative effects of SER and DL. We postulate that, as in the development of the wing margin, N signalling in the presumptive leg joints activates the expression of a secondary signalling process in the target cells. This secondary signalling process then drives the subsequent development of the anlage around it to give rise to the articulated joint.

MATERIALS AND METHODS

Flies

Flies were raised on a standard cornmeal medium at 25°C except in temperature-shift experiments, as indicated. For each genotype, a minimum of 20 adult flies (with a total of 120 legs) were analysed under the dissecting microscope, and a minimum of 60 legs were dissected and analysed under the compound microscope.

Alleles and temperature-sensitive combinations

Oregon-R was used as the wild-type stock. Ser mutant animals were identified as Tb\(^{+}\) progeny from a cross Ser\(^{RX106}\) st e / TM6b × Df(3R)D605 / TM6b (a deficiency for the Ser locus), raised at 18°C (Couso et al., 1995). Dl mutants were identified as Tb\(^{+}\) progeny from a cross of D\(^{683}\)T e / TM6b (temperature-sensitive allele) × D\(^{RF}\) / TM6b raised at 18°C (Parody and Muskavitch, 1993). Dl mutant pupal discs for staining with anti-SER were generated by shifting D\(^{683}\)T e / D\(^{RF}\) larvae from 18°C to 25°C. disco expression in Ser and Dl mutants was detected in Tb\(^{+}\) progeny of the following crosses: disco lacZ / disco lacZ; e D\(^{683}\)T e / D\(^{RF}\) / TM6b at 18°C, and disco lacZ / disco lacZ; e Ser\(^{RX106}\) / TM6b × Df(3R)D605 / TM6b at 18°C. The N temperature-sensitive allele N\(^{11}\) (Schellenberger and Mohler, 1975) was crossed to w\(^{+}\}N^{Se11} / FM7a and shifted from 18°C to 25°C (restrictive temperature) to produce adults with a near total loss of N function (Couso and Martinez Arias, 1994).

dsh function was studied using dsh\(^{1}\) mutants. The dsh\(^{1}\) mutation disrupts the dsh protein domain required for cell polarity (Axelrod et al., 1998). dsh\(^{1}\) mutants were selected as w\(^{+}\}y^{+}\) individuals from the stock v dsh\(^{1}\} / FM7a.

LacZ reporter lines

Expression of disco was detected by monitoring β-gal expression in flies carrying a reporter lacZ construct inserted in the disco gene (Heilig et al., 1991). DI expression was monitored using a lacZ reporter construct in the stock DI lacZ / TM6b (Klein and Martinez Arias, 1998). E(spl) expression was monitored using constructs containing 7 regulatory regions driving the expression of lacZ (Kramatschek and Campos-Ortega, 1994). fng\(^{Y10}\) lacZ is an enhancer trap line inserted at the fng locus (J. P. C. and M. I. Galindo, unpublished), and which faithfully reproduces fng expression in imaginal discs.

Gal4 and UAS lines

The Gal4/UAS system of Brand and Perrimon (1993) was used to express genes ectopically. klu-Gal4 (Klein and Campos-Ortega, 1997) was used to drive ectopic expression of UAS-Ser (Speicher et al., 1994) and UAS-Dl\(^{308}\) (Doherty et al., 1996) using homozygous stocks, UAS-Ser (on 3rd chromosome) and UAS-Dl\(^{308}\) (on 2nd chromosome). In the case of UAS-Ser, pharates and escapers appear at 25°C, whilst with UAS-Dl\(^{308}\) pharates only appear at 18°C.

The expression of disco was assayed in the UAS-Ser and UAS-Dl\(^{308}\) escapers by X-gal staining in the Tb\(^{+}\) progeny of the following crosses: disco lacZ / disco lacZ; klu-Gal4 / TM6b × UAS-Dl\(^{308}\) and disco lacZ / klu-Gal4 UAS-Green Fluorescent Protein (GFP) / TM6b × UAS-Ser, raised at the appropriate temperatures as above.

The construct UAS-Nttra was provided by M. Haenlin and is the same as that used by Klein and Martinez Arias (1998). This construct encodes a constitutively active, truncated form of N. UAS-Nnttra expression was driven in the fourth and fifth tarsal segments by apterous-Gal4 (ap-Gal4) (FlyBase) using the following crosses: y w; ap-Gal4 / CyO × UAS-Nnttra; Ser\(^{RX106}\} / TM6b, pharate adults only appear at 18°C. The construct UAS-ECN (Extra Cellular Notch) encodes a dominant-negative form of N that maintains the extracellular ligand-binding and transmembrane domains, but lacks the intracellular domains required for signal transduction (Jacobsen et al., 1998). Distalless-Gal4 (Dl-Gal4) (Calleja et al, 1996) was used to drive the expression of UAS-fng (Kim et al., 1995) throughout the tarsal region using the following cross: Dl-Gal4 / CyO × UAS-fng / Sm6a-TM6b /Ser\(^{RX106}\} st e.

Immunocytochemistry

Imaginal discs and pupae were staged according to their morphology. Third instar discs were fixed for 10 minutes in cold 4% paraformaldehyde in PBS, and pupal legs were fixed for 15 minutes. These were then stained, using standard procedures, for enzymatic X-gal staining, peroxidase or fluorescence.

Ser expression was detected with a mouse polyclonal anti-SER antibody (Speicher et al., 1994) diluted at 1:50 (fluorescence) or 1:1000 (peroxidase). After paraformaldehyde fixation, a further methanol fixation was carried out for 5 minutes at −20°C. Tissue was embedded in the protocol instead of Triton X-100. DI expression was detected using a mouse anti-DL antibody (Kooh et al., 1993) diluted at 1:10 (fluorescence), also with an additional methanol fixation. For fluorescent double stainings, anti-SER and anti-DL antibodies. Secondary antibodies were from Vector and Jackson. X-gal and peroxidase images were photographed using a Leica confocal microscope, and confocal images were assembled and processed with the Adobe Photoshop program.

RESULTS

It has been described that N and Dl are required for the correct segmentation of the legs in the fly (Parody and Muskavitch, 1993; Schellenberger and Mohler, 1975) and that N is expressed in leg discs (Kidd et al., 1989). In order to ascertain the nature of these requirements, we decided to study the roles of N, Ser and Dl during leg development.
The development of joints and leg segments in Drosophila

The legs of Drosophila are jointed appendages, as in all other arthropod animals. In Drosophila and most insects, the structure of the legs is remarkably constant and is as follows. Every leg carries articulations, or joints, which divide the leg into parts, or segments. These segments are called, from proximal (or close to the body wall) to distal: coxa, trochanter, femur, tibia and five tarsal segments, plus a pre-tarsus, or claw organ, at the tip of the leg (Bryant, 1978). The joints differ from other parts of the leg in that they are devoid of bristles and include a flexible intersegmental membrane and interlocking parts composed of thickened cuticle. The details of the morphology of these parts varies from joint to joint and only the joints between the tarsal segments are identical and composed of a ‘socket’ in the proximal part of the joint and an interlocking ‘ball’ in the distal part (Fig. 1A) (Held et al., 1986). The other joints do not include a ‘ball and socket’ structure but a variety of condyles and cavities (Fig. 1B).

Joints are not obvious in the developing legs until the time of pupation, when the evertting leg discs show a series of constrictions that different authors have identified with the presumptive positions of the final joints (Waddington, 1943; Fristrom and Fristrom, 1993). However, during subsequent metamorphosis, the legs inflate and lose these constrictions although different cell contours can still be seen at positions that seem to correlate with future joints (Fristrom and Fristrom, 1993). The lack of markers other than morphological ones has not allowed an exploration of joint development in more depth.

We have used a marker which seems to correlate faithfully with joints, an enhancer trap inserted into the disconnected (disco) gene (Heilig et al., 1991). The disco gene encodes a protein required for axonal migration and leg development (Heilig et al., 1991) and in the legs its expression is associated with joint development. disco expression is present in the developing leg imaginal disc at 120 hours after egg laying (Fig. 1C). Although it is expressed throughout the presumptive leg region, disco expression is upgraded in a series of rings around the centre of the disc. During the first 4 hours after puparium formation (APF), disco expression can be seen to become more strongly modulated in rings, which correspond with the presumptive leg segments (Fig. 1D). At around 12 hours APF, lacZ expression is restricted to these rings, which in a lateral view of the developing leg appear as stripes about 6 cells wide situated next to but proximal to constrictions in the presumptive tarsal region (Fig. 1E). Staining of adult flies carrying this marker shows disco expression specifically restricted to the joints (Fig. 1F).

Ser signalling controls the development of the leg joints

Null alleles of Ser are lethal but a few mutant flies are formed inside the pupal cases. These animals show a variety of developmental defects (Speicher et al., 1994; Couso et al., 1995), amongst them, leg deformities (Fig. 2C). The legs lack joints between all segments although sometimes a remnant constriction can still be seen. No other leg area is affected and the segment boundaries are still present, as shown by the apical bristles in tarsi and tibia. This mutant phenotype correlates with the pattern of expression of Ser. Using an antibody against
Fig. 2. Ser expression and function in legs.
(A) Third instar leg imaginal disc stained with anti-SER antibody. Ser protein expression forms concentric rings. (B) Ser expression in stripes in a pupal leg 8 hours APF, revealed with an anti-SER antibody. The leg is curved so its proximal end is found to the left and its distal end points down. (C) Legs from a Ser null mutant animal. The joints are completely lost but other areas of the leg are not affected. Tarsal segments 1-5 are indicated and arrows mark the thick, dark apical bristles at the distal end of each segment where the joints are found in the wild type. (D) lacZ expression in the legs of a klu-Gal4 UAS-lacZ fly. klu is expressed in rings in the leg imaginal disc (not shown) and in the adult legs strong expression is seen outside the joint regions. Labels as in Fig. 1F. (E) Detail of the tibia from a klu-Gal4 UAS-Ser leg showing ectopic joint differentiation as constrictions (arrows), and intersegmental naked membrane (arrowhead). (F) Leg from a disco-lacZ; klu-Gal4 UAS-Ser fly. Labels as in Fig. 1F. Ectopic disco expression is seen outside the joint areas where disco expression is restricted in the wild type (compare with Fig. 1F). Note the ectopic joints in the femur (arrowhead), in the folded tibia (brackets) and in the fourth tarsal segment (arrow).

the Ser protein, expression is seen to appear in rings in third instar discs (Fig. 2A; Bachman and Knust, 1998) and later is found close to the presumptive joint areas in pupal legs. Stripes of Ser expression are seen proximal to constrictions in evertting legs (Fig. 2B). Double staining with disco expression shows overlapping expression of disco and Ser (Fig. 3C-C’). However, in the ‘bell-shaped’ distribution of disco, cells with maximum levels of disco are located at the distal edge of the stripe of Ser expressing cells (Fig. 3D-D’).

These results show that Ser is required for the development of joints, and Ser expression adjacent to joint areas suggests that Ser could be directing joint development. If this were the case we would expect that ectopic expression of Ser would lead to the ectopic development of joints, and indeed we find this to be the case. We have used a klu-Gal4 line (Klein and Campos-Ortega, 1997), which is expressed in the legs outside the joints (Fig. 2D), to drive ectopic expression of a UAS-Ser construct. klu-driven expression of Ser leads to the ectopic development of joint-like cuticle, characterised by loss of bristles, cuticle thickenings and inpocketings (Fig. 2E). In leg regions like the tibia, where klu expression has defined boundaries, ectopic constrictions tend to appear. The transformation of interjoint leg regions towards joints is corroborated by the accompanying ectopic expression of disco (Fig. 2F). Reciprocally, in Ser mutants disco expression is lost after 12 hours APF (not shown).

The requirements for Di are more extensive than for Ser
We have revealed Di expression using a Di-lacZ reporter allele and an antibody against the DL protein, and found Di to be expressed throughout the presumptive leg at third instar, but with upgraded expression in rings (Fig. 4A). In pupal legs these rings can be seen to locate proximal to constrictions, and in adult legs lacZ expression is found at low levels throughout the leg, but it is stronger proximal to the joints (Fig. 4B). We have compared the expression of Ser, disco and Di in pupal legs and found that the stripes of high Di expression coincide with cells expressing Ser (Fig. 3A-A”, B-B”).

To study the requirements for Di we have used a viable temperature-sensitive mutant combination of alleles that produces mutant leg phenotypes (Parody and Muskavitch, 1993). Following exposure to the restrictive temperature during the third instar and pupal periods when Di is expressed near the presumptive joints, the Di mutant legs are shortened. The tarsal segments are particularly reduced and have seemingly disappeared, but on close inspection it can be seen that the tarsal apical bristles are still present and sometimes some remnant joint structures as well (Fig. 4C). Because Di and Ser are co-expressed, the joint defects in Di mutants could be indirectly due to a loss of Ser expression in Di mutants, but we examined this and found that the Ser stripes are still present in Di mutant legs (Fig. 4D). Reciprocally, Di expression is still present in Ser mutants (not shown), showing that the expression of Ser and Di are not directly dependent on each other and that their mutant joint phenotypes reflect independent requirements. We interpret these results as showing that although joint areas require Di for their development, the strongest requirement for Di is in the regions located between segmental boundaries. This implies that the requirements for
and fewer rings remain (Fig. 4E,F), a pattern similar to that of Ser expression in Dl mutants. We interpret this disco expression in Dl mutants as a corroboration of the main requirement for Dl being in the intersegmental regions. Failure of these regions to develop causes the absence of non-disco expressing cells between disco stripes so that fewer but wider disco stripes appear in the mutants.

The differences between the requirements and effects of Ser and Dl are again shown by comparison of their ectopic expression. klu-Gal4 UAS-Dl produces no visible phenotype in the interjoint regions of the femur and the tibia, but it expands the area differentiating cuticular joint characteristics, like naked and thick cuticle, in the femoral, tibial and tarsal joints (Fig. 4G). klu-driven Dl expression produces no phenotype in the interjoint regions, presumably because Dl, unlike Ser, is already expressed there and therefore klu-driven Dl expression is not ectopic. Effects are only seen near Ser expressing areas, presumably because a wider co-expression region is created. In the tarsal regions in addition to this joint-extension phenotype a tarsal loss is seen (Fig. 4H) following Dl expression driven by either klu or ap-Gal4 (which drives expression in the presumptive regions of forth and fifth tarsal segments during third instar and pupa). This tarsal reduction does not result from the formation of ectopic structures and thus it is likely to be caused by a negative effect of Dl, since it mimics a Dl or N lack of function phenotype (see also below).

In spite of the differences in the requirements for Dl and Ser, there is an overlap in that they are both required for the development of joints. It is possible that this overlap explains why the requirements of Dl in the joints are weaker than those in the interjoints. We decided to study whether this overlapping requirement is mediated by N.

The requirements for N are a composition of the requirements for Ser and Dl

In spite of their different but partially overlapping requirements, Ser and Dl signalling are thought to be mediated by the same receptor, N, which is expressed ubiquitously (Kidd et al., 1989; Kooh et al., 1993). We studied the requirements for N function in leg development using different temperature-sensitive mutant combinations of alleles that produce adult flies (Shellenbarger and Mohler, 1975; Couso and Martinez Arias, 1994). When exposed to the restrictive temperature during third instar and pupal phases, N mutant flies show a marked reduction in leg length with all areas of the leg segments being affected (Fig. 5A,B). Joints are completely lost but also often apical bristles. The overall length of the segments being affected (Fig. 5A,B). Joints are completely lost but also often apical bristles. The overall length of the segments, and especially of the tarsal region, is more reduced but also often apical bristles. The overall length of the segments being affected (Fig. 5A,B). Joints are completely lost but also often apical bristles. The overall length of the segments, and especially of the tarsal region, is more reduced

**Fig. 3.** Expression of Ser, Dl and disco in pupal legs.

(A-A”) Confocal picture of a double antibody staining of a 4 hour APF pupal leg orientated as in Fig. 1D, showing expression of Dl (red; see also A’) and Ser (green; see also A”). Dl expression is revealed with an antibody against the β-gal protein expressed by the Dl-lacZ reporter gene, whereas Ser expression is revealed by anti-SER antibody. Ser expression is restricted to stripes whereas Dl is expressed at low levels throughout the leg with upgraded stripes that coincide with Ser expression. (B-B”) Expression of Dl and disco revealed with antibodies against the DI protein (green; see also B”) and against the β-gal protein driven by disco (red; see also B’). DI expression is upgraded proximal to disco stripes. (C-C”) Expression of Ser and disco, revealed with anti-SER antibody (green; see also C”) and anti-β-gal antibody (red; see also C’), respectively, in a fly carrying the disco-lacZ reporter gene. Ser expression is proximal to, but overlapping with, disco. (D-D”) High magnification detail of a leg stained as in C-C”. Proximal to the left, distal to the right. The bars mark the 2-3 cell wide stripe of cells with high levels of disco expression which straddles the edge of Ser expression. Cells with lower levels of disco expression can be seen at either side of this stripe. The asterisks label the same cell in these three panels.

Dl are more extensive than those of Ser, and this is corroborated by their different requirements for disco expression. In Ser mutant legs, the stripes of disco expression form but are not maintained properly. However, in Dl mutants, the stripes of disco are not formed correctly and instead wider
These leg phenotypes thus resemble those produced in interjoint regions by loss of $Dl$ function (see Fig. 4C-F).

Expression of a truncated and constitutively activated form of N (UAS-$Nintra$) driven by $klu$-Gal4 is lethal. Using an $ap$-Gal4 driver it can be seen that the fourth tarsal segment becomes hyper-jointed in that double ball joints are formed, similar to the effects of UAS-$Ser$ driven by $ap$-Gal4 or $klu$-Gal4 (Fig. 5D). In addition, the interjoint region is reduced, either as a consequence of its conversion to extra joint tissue, or to an inability to develop the interjoint cell fates which have low, but not high, levels of N activation. These tarsal phenotypes are thus different from those seen with UAS-$Dl$ and UAS-$ECN$, because UAS-$Nintra$ and UAS-$Ser$ do not involve the loss of entire tarsal segments.

Altogether these results suggest that the overlap of $Ser$ and $Dl$ expression and requirements at the joints is mediated by N activation. As a marker of N activity, we have monitored the expression of members of the $E(spl)$ complex (Jennings et al., 1994). Using reporter constructs with the regulatory regions of $E(spl)$ (Kramatschek and Campos-Ortega, 1994), which reproduce the endogenous $E(spl)$ expression in the leg discs (de Celis et al., 1996), it can be seen that $E(spl)m8$ expression is related to joints while $m5$ and presumably $m6$ are not. Expression of the $E(spl)m8$ reporter construct in third instar discs is initially strong in regions undergoing PNS development. In the legs, these correspond to the chordotonal organs in the femur and the tibia (see also Jarman and Ahmed, 1998). In addition, expression near the presumptive joints is seen to appear, and then resolve in the pupa into one-cell wide stripes proximal to the leg constrictions (Fig. 5E), in positions that correlate with cells with maximum levels of $disco$ expression. A similar although much weaker pattern of expression of $E(spl)m¶$ is seen as revealed by the mAb323 antibody (Jennings et al., 1994) (not shown). Another marker of N activity is the expression of N itself, which becomes upregulated in cells where N signalling is being received (de Celis et al., 1997). Using an anti-N antibody, we observe upregulated expression of N immediately proximal to the

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**Fig. 4.** $Dl$ expression and function in the legs. (A) $Dl$-driven lacZ expression in rings in a leg imaginal disc just before pupariation, revealed with an anti-β-gal antibody. (B) Detail of the second to fifth tarsi from an adult fly showing $Dl$-driven lacZ expression. Low expression is seen throughout the leg and upgraded at the proximal side of the joints (arrows). (C) Tarsal region from the adult leg of a $Dl$ temperature-sensitive mutant exposed to the restrictive temperature from the third larval instar until the end of development. Tarsal segments are reduced (remnants of segments 2-4 are indicated by a bracket), and in some cases only the thick, black apical bristles remain (arrow). Joints are reduced as well although in some cases they are not completely lost (see edges of bracket and compare with wild-type legs in Figs 1A and 4I, and $Ser$ mutant legs in Fig. 2C). (D) $Ser$ expression revealed with an anti-SER antibody is still present in a pupal leg from a $Dl$ mutant, sibling of C. The bracket indicates two fused SER stripes. (E) $disco$ expression in a $Dl$ mutant as in C and D. Instead of five wild-type tarsal stripes (see Fig. 1D) only three are seen; the bracket indicates a wider stripe, which results from the fusion of two or more $disco$ stripes. (F) Adult leg of a sibling of E. Fused tarsal segments 2-4 are indicated by the bracket. (G) Detail of a leg from a $klu$-Gal4 UAS-$Dl$ animal. An expansion of the tibia/first tarsus joint region is apparent in that there is an extended region of naked, thickened cuticle (arrow). (H) Leg from a $disco$-lacZ; $klu$-Gal4 UAS-$Dl$ fly. Tarsal segments 2-4 are reduced (brackets), while joint regions around tarsus 1 are expanded, as seen by the expansion of both joint cuticle (arrow) and $disco$ expression (compare with Figs 1F, 4G and 4I). (I) Tarsal segments (1-5) from a wild-type leg. Joints are marked by arrows.
**Fig. 5.** N function in *Drosophila* legs. (A) Legs from a N mutant animal with partial loss of N function (*N^{53e11} N^{ts}* raised at 17°C). Note the lack of joints and apical bristles. (B) Legs resulting from an almost total loss of N function (*N^{53e11} N^{ts}* exposed to 25°C during the third larval instar and pupal periods), showing loss of tarsal segments (bracket). (C) Leg from a klu-Gal4 UAS-ECN fly. Expression of the dominant-negative N protein ECN driven by klu-Gal4 in the interjoint regions produces loss of interjoint tissue. The joints are still present (arrows) but appear fused in some cases. Interjoint loss and joint fusion resemble those produced in *Dl* mutants (see Fig. 4C-F). (D) Distal end of the leg from an ap-Gal4 UAS-Nintr fly. Double ball joints with reversed polarity are formed in tarsus 4 (arrows). (E) E(spl)m8 expression as revealed by the E(spl)m8 2.61-lacZ reporter construct in a leg 4-8 hours APF. Expression is seen in one-cell wide stripes at the proximal side of the constrictions. Asterisk, chordotonal organ. (F) N expression revealed by an antibody against the intracellular domain of N. N expression accumulates in cells immediately proximal to the constrictions (arrowheads).

**Fig. 6.** Repression of joint development by fng and dsh. (A) fng expression, revealed by a lacZ reporter gene, is seen in concentric rings in leg discs just before pupariation. (B) Stripes of fng expression (revealed as in Fig. 6A) in a 4 hour APF pupal leg. (C) Ectopic expression of fng throughout the whole tarsal region in Dll-Gal4 UAS-fng animals only produces a mutant phenotype at the joints, which are reduced (arrows) or completely eliminated (see 4/5 tarsal joint). (D) Tarsi of a dsh mutant adult leg. Ectopic joints with reversed orientation of the ‘ball and socket’ appear proximal to the normal joints. The endogenous joints at both ends of tarsal segment 3 are marked with arrows and the ectopic joint in this tarsal segment is marked by an arrowhead. (E) Dl-driven lacZ staining in the tarsi of a dsh mutant leg. Dl expression is normal (compare with Fig. 4B) but ectopic joints form proximal to the stripe of high Dl expression (arrowhead). Arrows and arrowhead as in D. (F) N expression revealed by anti-N staining in a dsh mutant pupal leg. Proximal to the endogenous stripes of upregulated N expression, ectopic stripes can be seen. Arrows and arrowhead as in D and E.
constrictions in pupal legs (Fig. 5F). At high magnification it can be appreciated that this upregulation is restricted to a single row of cells at this position (not shown), thus confirming that SER and DL are triggering N signalling in these cells.

Other elements in N signalling and allocation of joints

The results presented suggest a model in which the co-expression of Ser and high levels of Dl in a stripe of cells proximal to the future presumptive joints activate N in cells adjacent but distal to this stripe. We wondered whether this specificity could be due to the presence of other factors that would be interfering with Ser and Dl signalling in cells located inside the Ser-Dl stripe or proximal to it. In the DV boundary of the wing, the membrane protein encoded by the gene **fringe** (*fng*) has been postulated to modulate N signalling by interfering with Ser signalling (Fleming et al., 1997a; Panin et al., 1997). In the developing legs, *fng* is expressed in stripes or rings around the positions of presumptive joints (Fig. 6A,B). Using a UAS-*fng* construct (Kim et al., 1995), we misexpressed *fng*. Ectopic expression of *fng* outside the joints driven by klu-Gal4 produces no phenotype. However, uniform tibial and tarsal *fng* expression driven by Dl-Gal4 only affects the joints, which are reduced or disappear, a phenotype reminiscent of that of Ser mutants (Fig. 6C). Thus, *fng* activity in the leg seems to be restricted to a repression of joint development around presumptive joint areas. It is possible that *fng* expression in the wild type is repressing N signalling in cells located in the Ser-Dl stripe or proximal to it, providing the polarity in the joint-promoting function of Ser and Dl. Consistent with this proposal, we also observe in the wings of UAS-*fng* flies margin nicks and thickening of veins, phenotypes characteristic of loss of N signalling in these developmental processes (Couso et al., 1995; de Celis et al., 1997).

Our results also suggest a possible role for other factors in the definition of the polarity of Ser and Dl signalling. **dischevelled** (*dsh*) mutant legs develop ectopic joints (Held et al., 1986) (Fig. 6D). The expression of Dl is normal in dsh mutant legs and it can be seen that the ectopic joints appear proximal to the cells expressing high levels of Dl (Fig. 6E). This ectopic joint differentiation in dsh mutant legs is preceeded by ectopic N activation, as revealed by staining with anti-N antibody (Fig. 6F).

These results are compatible with a role for *dsh* in the repression of N signalling and joint development in cells proximal to the Ser and Dl stripe.

**DISCUSSION**

A combination of signals from Ser and Dl establishes leg segments and joints

Our results suggest a model in which the co-expression of Ser and high levels of Dl in a stripe of cells activate N in cells adjacent but distal to this stripe. Activation of N promotes expression of members of the E(spl) complex and leads to joint formation and disco expression (Fig. 7A).

The only function of Ser in leg development is to promote joint formation, a conclusion supported by the recent findings of de Celis et al. (1998), which are similar to our own. Complete loss of Ser eliminates joints and disco expression, whereas ectopic Ser produces ectopic joints and ectopic disco expression. However, we observe that Dl seems to have more than a simple joint-promoting role in leg development.

Loss of Dl eliminates first the regions between disco/ser-expressing rings, but also, secondly, joints. Since loss of interjoint regions is also seen both in N mutants and following expression of a dominant-negative form of N, we postulate that Dl expression in the interjoint regions produces low levels of activation of N that do not lead to E(spl) expression but which allow cell survival and/or cell proliferation. A requirement for
$N$ in cell growth or survival has been noted in the wing blade where this and other effects of $N$ signalling seem to be mediated by unknown factors other than $E(spl)$ (de Celis et al., 1996). The requirement for $Dl$ in joint development is not mediated by $Ser$ because $Ser$ and $Dl$ expression are not directly dependent on each other. Joint loss in $Dl$ mutants is presumably less severe than interjoint loss because $Ser$ and $Dl$ expression could be synergistic and partially redundant (Michelli et al., 1997; Zeng et al., 1998). The combined and potentially synergistic effects of $Ser$ and $Dl$ would produce a high level of activation of $N$ that would lead to expression of members of the $E(spl)$ complex, upregulation of $N$ expression, and to joint development and disco expression. Thus, we believe that combinations of signalling by $Ser$ and $Dl$ could produce different levels of activation of $N$, which in turn are translated into different downstream effects. As noted in other systems (de Celis et al., 1996) these downstream effects of $N$ signalling should be mediated by more factors than just $E(spl)$, since $E(spl)$ mutant legs have been reported as having a wild-type phenotype (de Celis et al., 1998).

The width of the final joint region is wider than the single row of cells activated by the membrane-tethered $SER$ and $DL$ proteins and visualised by $E(spl)$ expression. In principle it is possible that the cells of the whole final joint all descend from the $E(spl)$ expressing cells, but previous studies have shown that only one or two cell divisions occur in the legs after puparium formation (Graves and Schubiger, 1982). Thus it is likely that in the $E(spl)$ expressing cells another cell signalling molecule is activated, which in a secondary event would define a wider joint presumptive region, just as $N$-induced expression of the secreted signalling wingless protein defines the presumptive wing margin (Couso et al., 1994; Michelli et al., 1997). A reflection of this putative secondary signalling event in the joints can be seen in the expression of disco. disco expression is dependent on $Ser$ but it is wider than the single row of cells where $N$ is activated and thus it cannot be directly reflecting $N$ signalling at the joint. However, the ‘bell-shaped’ distribution of disco might reflect this putative secondary signalling event, with a maximum in cells at the edge of the Ser-Dl stripe. The nature of the joint-promoting putative secondary signal is unknown at the moment, but one possible component is the product of the four-jointed ($ff$) gene. The $ff$ protein is a putative signalling molecule that is expressed and required at the joints (Villano and Katz, 1995). $ff$ expression has recently been shown to depend on $fng$ and $N$ expression during eye development (Papayannopoulos et al., 1998), and it is lost in $N$ mutant legs (S. A. Bishop and J. P. Couso, unpublished observations).

Further elements in joint development
An aspect of joint development which is not explained by the model presented is the polarity with which the stripe of $Ser$ and $Dl$ signals. Lack of signalling in wild-type $Ser$ and $Dl$ expressing cells could be due to an autonomous dominant negative effect of these molecules. Such an effect has been postulated before following ectopic expression experiments (Doherty et al., 1996; Jonsson and Knust, 1996; de Celis and Bray, 1997; Klein et al., 1997), and it has been shown to be present in the wing margin in lack of function conditions (Michelli et al., 1997). Although an interaction of $N$ and $DL$ proteins in the same cell has been recently shown (Jacobsen et al., 1998), the molecular basis of such a negative effect remains unexplained. We find negative effects (tarsal segment loss) following overexpression of $DL$ driven by $klu$ or $ap-Gal4$, and so a putative dominant negative effect could be at work in the $Ser$ and $Dl$ stripes and explain the lack of signalling there. Similar dominant-negative effects of $DL$ and also $SER$ have also been reported by de Celis et al. (1998). However, an autonomous negative effect does not explain why cells adjacent but proximal to the Ser-Dl stripe do not seem to be signalled either.

A possible explanation would be either an asymmetric distribution of $SER$ and $DL$, forming gradients like those seen in the late third instar wing margin (Michelli et al., 1997) and in ectopic expression situations (Doherty et al., 1996; Kim et al., 1995), or a downregulation of $N$ expression as has been noted in the developing wing veins (de Celis et al., 1997). The $SER$ and $DL$ stripes in legs show no apparent asymmetry but $N$ distribution, although ubiquitous and initially uniform (Kidd et al., 1989), becomes upregulated in cells distal to the Ser-Dl stripes. Low availability of $N$ protein could have an effect on the intensity of $N$ signalling, but since upregulation of $N$ is in itself a consequence of $N$ signalling (de Celis et al., 1997), some other factor must polarise the signalling initially. Another explanation would rely on the action of a repressor acting upon cells proximal to the stripe. The phenotypes obtained after ectopic expression of $fng$ are consistent with such a role for $fng$, as postulated in the wing (Fleming et al., 1997a; Panin et al., 1997). The expression of $fng$ in the leg, which has been described as complementary to that of $E(spl)$, that is, present in non-signalled cells but excluded from joint forming ones (de Celis et al., 1998), is also consistent with this hypothesis. Such a function of $fng$ could also repress $Ser$ and $Dl$ signalling in the stripe without recourse, or in addition, to putative autonomous dominant negative effects of $SER$ and $DL$ (Klein and Martínez Arias, 1998; Jacobsen et al., 1998). However, other factors could also be involved, such as the cell polarity pathway (reviewed in Gubb, 1998). Mutant phenotypes for $dsh$ and other members of the cell polarity pathway produce ectopic joints with reversed polarity (Held et al., 1986), which we find to appear just proximal to the position of $Ser$ and $Dl$ stripes. Furthermore in $dsh$ mutants we observe ectopic $N$ activation proximal to the Ser-Dl stripe. Since the $dsh$ protein has been shown to interact with $N$, and $dsh$ has been postulated to inhibit $N$ signalling in this manner (Axelrod et al., 1996), the cell polarity pathway could be involved in repressing $Ser$ and $DL$ signalling to cells proximal to the $Ser$ and $Dl$ stripe (Fig. 7A).

Quantitative and qualitative effects of $N$ ligands
The $SER$ and $DL$ ligands seem to produce the same effects on $N$, and only a synergistic but quantitative effect has been found in most processes examined (Gu et al., 1995; Michelli et al., 1997; Klein and Martínez Arias, 1998; Zeng et al., 1998). Why then are there two $N$ ligands in Drosophila? This could be just a device to increase the amount of $N$ signalling beyond what is achievable by regulating the expression of a single ligand. It might not be possible to either completely eliminate or greatly imbalance $Dl$ expression in certain areas without compromising the process of neurogenesis (Heitzler and Simpson, 1991). The addition of $SER$, a molecule mostly not involved in neural precursor selection (Gu et al., 1995), might
provide for further extremes of N signalling, which could also include dominant-negative effects. This greater quantitative range in the amount of N signalling when induced by the combination of SER and DL (ranging from high to moderate and to low or no signal) could then trespass certain thresholds and be converted into different qualitative gene regulation effects. In these terms a situation with a single ligand, DL, involved in neurogenesis could be the ancestral one, while the evolution of a second ligand, SER, would allow the co-option of the N signalling pathway to other pattern forming processes.

Topological aspects of boundary making by N signalling

Ser, DI and N signalling is used in Drosophila to create boundaries in leg joints, the wing margin, and in the wing veins. In all these cases, the common link is the creation of boundaries between cell populations, sometimes to allow the singling out of boundary cells with new signalling properties. In vertebrates, homologues of Ser, DI and fng are involved in similar processes, as in somite and rhombomere development (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Cohen et al., 1997). In these varied developmental processes, and in the scenario of SER and DL having overlapping and similar effects on N, the precise outcomes of their signalling seem to stem from the different deployments of Ser, DI, N and fng expression (Fig. 7). In leg joint development coincident stripes of cells expressing Ser and DI signal not to themselves, but to cells adjacent to only one side of them (Fig. 7A). Autonomous Ser-DI signalling seems to be blocked by fng, although a dominant negative effect by SER and DL cannot be excluded. Restriction of non-autonomous signalling to one side of the Ser-DI stripe seems due to a combination of repression by fng and dsh plus a modulation of N expression. This situation is similar to somite boundaries in vertebrates where Ser, DI and fng homologues are co-expressed and help to maintain an adjacent somite boundary through N. Alterations of fng, DI or N function result in somite fusion and loss (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Cohen et al., 1997), phenotypes which are reminiscent of the tarsal segment mutant phenotypes presented here.

During Drosophila wing margin development the situation is similar to joint development, with coincident patterns of Ser and DI expression signalling to a boundary of adjacent cells (Fig. 7B). Ser and DI expressing cells do not receive any signalling due to autonomous dominant negative effects (Micchelli et al., 1997). In the developing wing veins during pupal development, autonomous signalling seems to be avoided by exclusion of N expression in the vein stripes (de Celis et al., 1997). Thus a common outcome, the signalling of an adjacent row of cells, is apparently achieved by different mechanisms in the joints, the wing margin and the veins. However, in the wing margin (de Celis and Bray, 1997) and joints, expression of N seems to be regulated as in the veins, being low in Ser-DI stripes but higher in the adjacent signalled cells. Moreover, fng is expressed near the presumptive wing margin and veins (Irvine and Wieschaus, 1994; S. A. Bishop and J. P. Couso, unpublished observations), where it might be repressing N signalling since overexpression of fng produces N-like phenotypes in these places.

N signalling would appear then to be a kit that can be used in different topological and molecular combinations of signalling elements. A general boundary-making activity of N signalling could entail definition of boundaries of cells by: (1) expression of ligands in cells close to the future boundary, (2) activation of N in adjacent cells at the boundary, and (3) repression of signalling in ligand expressing cells (and in some other cells if required) by a combination of ligand dominant negative effects, inhibition by fng and other repressors, and downregulation of N expression.

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