Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the *Drosophila* embryo

Osamu Shimmi and Michael B. O'Connor*

Department of Genetics, Cell Biology and Development, Howard Hughes Medical Institute, University of Minnesota, Minneapolis, MN 55455, USA
*Author for correspondence (e-mail: moconnor@mail.med.umn.edu)

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Summary

Dorsal cell fate in *Drosophila* embryos is specified by an activity gradient of Decapentaplegic (Dpp), a homologue of bone morphogenetic proteins (Bmps) 2/4. Previous genetic and biochemical studies have revealed that the Sog, Tsg and Tld proteins modify Dpp activity at the post-transcriptional level. The predominant view is that Sog and Tsg form a strong ternary complex with Dpp that prevents it from binding to its cognate receptors in lateral regions of the embryo, while in the dorsalmost cells Tld is proposed to process Sog and thereby liberate Dpp for signaling. In this model, it is not readily apparent how Tld activity is restricted to the dorsal-most cells as it is expressed throughout the entire dorsal domain. In this study, additional genetic and biochemical assays were developed to further probe the relationships between the Sog, Tsg, Tld and Dpp proteins. Using cell based assays, we find that the dynamic range over which Dpp functions for signaling is the same range in which Dpp stimulates the cleavage of Sog by Tld. In addition, our data supports a role for Tsg in sensitizing the patterning mechanism to low levels of Dpp. We propose that the strong Dpp concentration dependence exhibited by the processing reaction, together with movement of Dpp by Sog and Tsg protein can help explain how Tld activity is confined to the dorsal-most region of the embryo through formation of a spatially dependent positive and negative reinforcement loop. Such a mechanism also explains how a sharp rather than smooth signaling boundary is formed.

Key words: Tld, Dpp, Morphogen, Bmp

Introduction

Dorsoventral (DV) axis formation in *Drosophila* is initially specified by a cascade of maternal gene products that results in the nuclear localization of Dorsal. The Dorsal transcription factor in turn helps establish cell fates by activating and repressing a number of early zygotic genes (St Johnston and Nusslein-Volhard, 1992). Within the dorsal domain, the activity of at least five secreted zygotic gene products is required to specify dorsal ectoderm and the amnioserosa, the two major tissues derived from this region. These products include the Bmp-type ligands Decapentaplegic (Dpp), and Screw (Scw), the metalloprotease Tolloid (Tld), and the two Bmp inhibitors Short gastrulation (Sog) and Twisted gastrulation (Tsg) (Padgett et al., 1987; Shimell et al., 1991; Arora et al., 1994; Mason et al., 1994; Biehs et al., 1996). A similar complement of homologous vertebrate gene products appears to be responsible for specifying the DV axis in both frogs and fish (Blader et al., 1997; Piccolo et al., 1997; Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001).

Among the *Drosophila* zygotic genes, *dpp* is thought to play a pivotal role because, in its absence, all dorsal cells assume ventral lateral fates (Arora and Nusslein-Volhard, 1992). When the expression levels of *dpp* are manipulated by either genetic or physical means, tissue fate along the DV axis is assigned in a dose-dependent manner consistent with Dpp acting as a morphogen (Ferguson and Anderson, 1992; Wharton et al., 1993). Thus, high levels of Dpp specify the dorsalmost amnioserosa, while lower levels instruct development of dorsal ectoderm.

The formation of the Dpp gradient appears to come about through a post-transcriptional/translational mechanism that involves a dynamic interplay of Dpp with the products of the other zygotic DV gene family members. A key component in this process is Sog (Biehs et al., 1996), which is related to vertebrate chordin and contains four cysteine rich (CR) domains. For chordin these CR domains mediate binding to Bmp ligands, and when Bmps are bound to Chordin, they are unable to bind to and activate receptors (Piccolo et al., 1996). As Sog is produced by ventral lateral cells that abut the Dpp expression domain, graded Dpp activity is thought to arise by diffusion of Sog from its ventrolateral site of synthesis into the dorsal domain (Biehs et al., 1996). This produces a gradient in which lateral regions contain relatively high levels of Sog while dorsal regions have low levels. The net effect of the lateral-high to dorsal-low Sog gradient is production of an inverse Dpp activity gradient where lateral cells experience low levels of Dpp, while dorsal cells see the highest levels. The recent direct visualization of the Sog gradient in *Drosophila* blastoderm embryos is consistent with this model (Srinivasan et al., 2002).

Genetic and biochemical experiments suggest that Sog does not act alone to produce the Dpp activity gradient. Both Sog and vertebrate chordin form tripartite complexes with Bmp ligands and Tsg proteins (Oelgeschlager et al., 2000; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). In *Drosophila*, Tsg appears to be necessary for strong binding of Sog to Dpp (Ross et al., 2001), while in vertebrates, chordin alone can bind Bmp4 but its binding is significantly enhanced by Tsg protein (Piccolo et al., 1996; Oelgeschlager et al., 2000; Larrain et al., 2001). The complex of Sog and Tsg is a much stronger inhibitor of Dpp signaling in *Drosophila* than either is alone, and the same appears to be true for the zebrafish counterparts (Ross et al., 2001). In frogs, however, the ratio of Tsg and chordin is crucial for determining the phenotypic outcome (Larrain et al., 2001; Ross et al., 2001). At low concentrations, Tsg enhances the inhibitory action of chordin, whereas at high concentrations it blocks chordin action. This effect has recently been shown to be the result of enhanced degradation of chordin in the presence of high levels of Tsg (Larrain et al., 2001).

One mechanism that contributes to the degradation of chordin and Sog is proteolytic processing by members of the Tld family of metalloproteases. This cleavage results in the liberation of the Bmp ligand such that it is then free to bind and activate receptor. A major distinction between the vertebrate system and *Drosophila* is that cleavage of Sog by Tld is dramatically stimulated by the presence of ligand in *Drosophila*, while, to date, this has not been found to be the case for the vertebrate homologs (Marques et al., 1997). This biochemical difference may be the key to explaining one of the unusual aspects of the *Drosophila* system which is that Sog and Tsg do not act as simple inhibitors to produce a monotonic gradient of Dpp activity. Instead, both are required to generate a peak of Dpp activity in the dorsalmost 8-10 cells that form the amnioserosa. This activity peak is inferred by high level nuclear accumulation of the phosphorylated form of Mad, the primary transducer of the Dpp signal, in dorsal midline cells (Dorfman and Shilo, 2001; Ross et al., 2001). Thus, the Dpp activity gradient is not smooth but instead assumes the shape of a step function with a very sharp transition between cells receiving high and very low signals. In *sog* or *tsg* mutant embryos, this sharp transition does not take place, and instead all dorsal cells receive a moderate level of Dpp signal and do not form amnioserosa (Ross et al., 2001). One model that explains this dichotomy is that Sog and Tsg not only block Dpp signaling laterally, but also help promote its diffusion, through a cyclic binding and cleavage process, from dorsal lateral cells to the dorsal midline (Holley et al., 1995; Marques et al., 1997; Decotto and Ferguson, 2001). According to this model, in the absence of Sog and Tsg, Dpp is not free to diffuse within the dorsal domain as receptor binding would trap it. However, in the presence of Sog and Tsg, Dpp is unable to bind its receptor and could diffuse. The net diffusion of Sog from ventrolateral cells would carry Dpp towards the dorsal side until Tld processes the complex. At the time of processing, the Dpp could either be recaptured by Sog and Tsg, or could bind to its receptor. In dorsolateral regions where the Sog concentration is high, Dpp would be more likely to be recaptured by a second Tsg-Sog complex, further promoting its diffusion. This model has recently received mathematical as well as additional genetic support (Eldar et al., 2002).

Despite the appeal of the transport model, several issues remain to be tested. In particular, this model requires that the rate of Sog cleavage by Tld must be such that it can keep up with the net flux of Sog to establish a sharp transition zone between bound and unbound Dpp. How is this transition zone established? A second issue that needs to be examined is whether the *tsg* mutant phenotype could potentially be explained by another model in which the presence of Tsg, in addition to promoting Sog binding to Dpp, also reduces the kinetics of Sog cleavage. Accordingly, the similarity in the *tsg* and *sog* loss-of-function phenotypes is brought about Tld overdigesting Sog in the absence of Tsg.

We examine in more detail the biochemical and genetic interplay between Sog, Tsg and Dpp. Using double mutants, we find that Tld function is epistatic to Tsg, suggesting that Tsg does not act to downregulate Tld activity. Instead, in vitro biochemical data suggest that Tsg actually enhances the rate of Sog processing at low Dpp concentrations. Furthermore, using proteolysis assays coupled with a cell-based signaling system, we find that both signaling and processing exhibit similar Dpp concentration sensitivities. This suggests that the cell culture model is physiologically relevant to the in vivo situation. We find that within a 10-fold Dpp concentration ranging from 10^{-10} to 10^{-9} M, both signaling and cleavage vary from background to maximum levels. We suggest that this steep Dpp concentration dependence is key to the Dpp transport process in that it provides a positive and negative reinforcement loop that contributes to the formation of a sharp transition zone in the early embryo between cells receiving Dpp signal and those that do not. In this view, as Sog diffusion helps redistribute Dpp from lateral regions to the dorsal side, the rate of Sog cleavage in lateral regions declines resulting in a further increase in Sog concentration and greater inhibition. Simultaneously, as the Dpp concentration rises within the dorsal cells, the rate of Sog cleavage increases and thereby further reduces Sog concentration in these cells enhancing signaling. In this way a sharp signaling transition zone is established. These data also suggest that Tsg acts to sensitize Sog binding and cleavage to low levels of Dpp. This reinforces the robustness of the sharp signaling transition zones that are predicted to occur in the embryo as a result of Dpp transport by the combined action of Tsg, Sog and Tld.

Materials and methods

Fly stocks

UAS-*sog*-HA and UAS-*tsg*-His flies were described previously (Yu et al., 2000). UAS-*atld* and A9-Gal4 flies were described before (Marques et al., 1997). The tsg^{XB86}/FM7, *ftz-lacZ*; tld^{B4}/TM3, *ftz-lacZ* flies were constructed from tsg^{XB86} and tld^{B4} alleles (Marques et al., 1997; Yu et al., 2000).

In situ hybridization

In situ hybridization to whole-mount embryos was performed with digoxigenin-labeled RNA probes and visualized with alkaline phosphatase precipitates as previously described (Nguyen et al., 1994).

Production of recombinant proteins and antibodies

Drosophila S2 cells were used for producing recombinant proteins as described previously (Yu et al., 2000; Ross et al., 2001). For antigen production GST-Sog CR1 [GST-Sog (E^{86}-E^{275})] fusion proteins were expressed in *E. coli* BL21 cells, and inclusion bodies were recovered using 50 mM Tris HCl, pH 7.4, 150 mM NaCl and 6 M Urea. The

solubilized fraction was mixed with an equal volume of complete adjuvant solution and injected into the rabbits subcutaneously. After five injections, the collected serum was precipitated with 50% saturated ammonium sulfate. Affinity purified serum was derived by applying the PBS dialysate to Actigel ALD (Sterogene Bioseparations) beads coupled to GST-Sog (E^{86}-E^{275}). The beads were washed and eluted according to vendor recommendations.

Immunoblotting

Protein samples were heated at 80°C for 10 minutes and electrophoresed on 4-12% gradient NuPAGE gels (Invitrogen) or 10% SDS-PAGE gels, and transferred to a PVDF membrane (Millipore). Blots were pre-incubated with 5% skim milk to block non-specific binding and incubated with the following primary antibodies: anti-HA 12CA5 (Roche), anti-Myc A14 (Santa Cruz), anti-Flag M2 (Sigma), anti-Sog-CR1 or anti-phosphoMad (1:5000 courtesy of P. ten Dijike). The blots were then incubated with the following secondary antibodies: HRP-conjugated goat anti-rabbit IgG for anti-Myc, anti-Sog-CR1 and anti-phosphomad antibodies, or HRP-conjugated goat anti-mouse IgG for anti-HA and anti-Flag antibodies (Jackson Immuno Research Laboratories) and developed using Super Signal (Pierce).

Cell based signaling assay and RNA interference

A cell based assay for Dpp signaling was described previously (Ross et al., 2001). Briefly, purified Dpp protein (R&D systems) was pre-incubated with various combinations of Tld, Sog and Tsg for 6 hours at room temperature. After pre-incubation, the mix of proteins was incubated with *flag-mad* transfected S2 cells for another 3 hours. Samples were then analyzed for Sog cleavage and P-Mad levels by western blots.

For RNA interference, PCR primers for the receptors were designed that carrying the 19 base T7 promoter sequence at the 5′ end. The sequences of the primer are as follows: TAATACGACTCACTATAGGGAAAGCACATCGGCAGCAGAG and TAATACGACTCACTATAGGGATCAGCATAAACACGGACAGGG for Tkv; TAATACGACTCACTATAGGGACTCAATGGCAAGG-AGCTACCG and TAATACGACTCACTATAGGGACGAGCCC-AGTGGATAGTAGTG for Sax; and TAATACGACTCACTATAGGG-AGAGACAACGGGCATCCTGCGC and TAATACGACTCACTA-TAGGGAGCCGCAGGGCTTGCCTGGCTG for Punt. PCR products carrying T7 promoter sequences at both ends were used as templates. In vitro transcribed RNA was produced using the MEGAscript kit (Ambion). The reaction products were annealed to produce double stranded RNA by incubating the products at 65°C for 15 minutes followed by an additional incubation at 37°C for 30 minutes. RNAi treatment was carried out by transfections of 1 μg of *flag-mad* plasmid with or without 10 μg of dsRNA into S2 cells. Cells were transfected at a density of 2×10^6 cells per well of a 12-well plate. After 3 days of transfection, the cells were divided into two fractions. One was used for a Dpp signaling assay by incubating with 10^{-9} M of Dpp for 3 hours at 25°C, and the extracts of the cells were used to detect the phosphomad levels by western blotting. The other half of the sample was used for RT-PCR. RNA was extracted with TRIzol (Invitrogen) and cDNA synthesis was performed using oligo-dT primer in Thermoscript RT-PCR system (Invitrogen). PCR was carried out using cDNA as a template, and the primers are as follows: CTTTGGCTCCATCATCATCTCC and TTCCGAAAATCTCGTC-GTGC for Tkv; CCGGATCAACTGCCCATGATC and CATGTCA-GAGCCGATGAATCC for Sax; CCACGGCAGGGAAACATTCAC and GGTCTTTGATGCCGGGATCTC for Punt; CTGGCACCACA-CCTTCTACAATG and GCTTCTCCTTGATGTCACGGAC for Actin as a control.

In vitro cleavage assays

Mixtures of purified Sog-Myc and Tld-HA were incubated with the indicated amounts of Dpp and Tsg-His for the indicated times at 25°C in the presence of 1× reaction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 μM $ZnCl_2$ and 1× complete™, EDTA-free protease inhibitor cocktail, Roche). Reaction products were analyzed by immunoblotting using anti-Sog-CR1 or anti-Myc antibody.

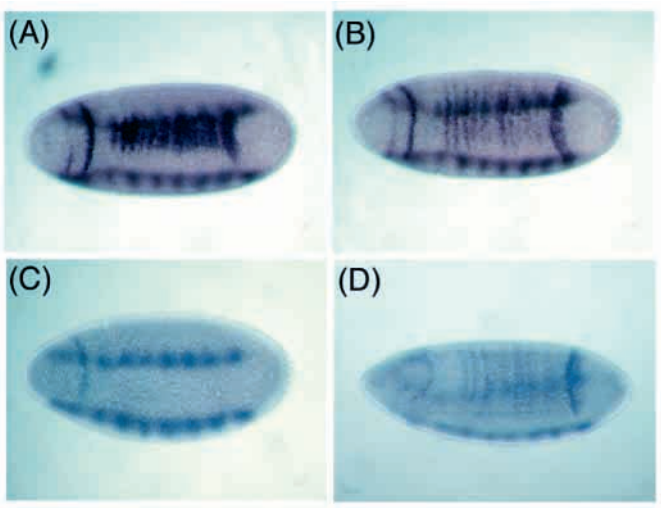

Fig. 1. Tsg is epistatic to Tld. Embryos were hybridized with antisense digoxigenin-labeled *rhomboid* probes. Mutant embryos were identified by lack of hybridization to *lacZ* transcripts produced from the FM7, *ftz-lacZ* and TM3, *ftz-lacZ* balancer chromosomes. Embryos are positioned with their dorsal side upwards and anterior towards the left. (A) Wild-type embryo, (B) *tsg* mutant embryo, (C) *tld* mutant embryo and (D) *tsg*, *tld* mutant embryo. The expression of *rho* on the dorsal side is broader and weaker in *tsg* mutants or *tsg*, *tld* double mutant embryos, than that in the wild type. In the *tld* mutant embryo, *rho* was expressed in its normal ventral domain, but not on the dorsal side, except for a thin anterior stripe.

Results

Tsg and Sog function downstream of Tld

Mutations in *tld*, *sog* and *tsg* all result in loss of amnioserosa, the dorsalmost tissue in *Drosophila* embryos. However, using molecular markers such as *rho*, *zen*, *race* and phosphorylated Mad (P-mad), differences in the phenotypes of these mutations can be ascertained (Yu et al., 2000; Ross et al., 2001) (Fig. 1). These data have suggested that *tsg* mutants are more similar in phenotype to *sog* mutants than they are to *tld* mutants. These observations, together with other genetic arguments, have lead to the suggestion that Tsg is a partner with Sog in a complex whose function is to inhibit Bmp in lateral regions of the embryo and to aid in the diffusion of Dpp to the dorsalmost domain (Ross et al., 2001). Consistent with this view, we have found that Tsg, in a complex with Sog, binds Dpp more effectively than either component alone. Furthermore, this tripartite complex is a more effective inhibitor of Bmp signaling than is either component on its own (Yu et al., 2000; Ross et al., 2001). Similar results have been obtained using the vertebrate homologs of these gene products (Oelgeschlager et al., 2000; Chang et al., 2001; Scott et al., 2001). However, the *tsg* loss-of-function phenotype could be explained in another way. By forming a tight complex with Sog and Dpp, Tsg might slow down the rate of Sog processing by Tld. Thus, in a *tsg*

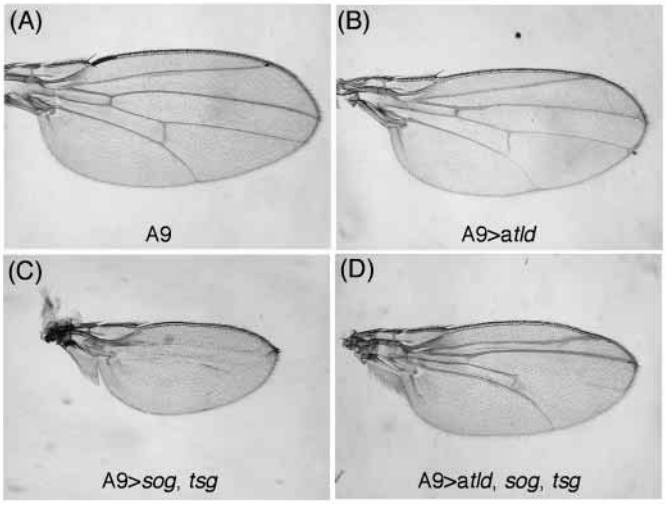

Fig. 2. Wing phenotypes produced by ectopic expression of different combinations of Sog, Tsg and Tld. (A) Wing from a fly expressing the A9-Gal4 driver alone. (B) Wing from a A9-Gal4>UAS-*atld* (activated Tld) fly. There is some loss of margin bristles but all veins form normally. (C) Wing from a A9-Gal4>UAS-*sog*, *tsg* fly. Wings are smaller and most veins are missing. (D) Wing from a A9-Gal4>UAS-*sog*, *tsg*, a*tld* fly. Note that wing size is close to normal and the formation of most veins is recovered.

mutant, Sog would be inactivated at a faster than normal rate thereby leading to abnormally low levels of Sog in the dorsal domain and a *sog* loss-of-function-like phenotype. To distinguish between these two models, we used genetic epistasis tests. Our rationale is that if Tsg normally prevents Tld from hyper-inactivating Sog, then in a *tsg*, *tld* double mutant, the phenotype should be more similar to *tld*. If, however, Tsg is primarily acting together with Sog to inhibit Dpp signaling, then the double mutant phenotype should be more similar to a *tsg/sog* phenotype. As shown in Fig. 1, examination of *rho* expression in the dorsal domain of a *tsg*, *tld* double mutant reveals a *tsg/sog*-like phenotype in which *rho* is expanded laterally rather than being eliminated as is found in a *tld* mutant embryo. Thus, *tsg* function, like *sog* (Marques et al., 1997), is epistatic to *tld*. Therefore we conclude that Tsg does not function to limit inactivation of Sog by Tld but instead acts together with Sog to inhibit Dpp signaling in lateral regions of the early embryo.

Tld regulates the Bmp inhibitory activities of Sog and Tsg in vivo

We also wished to examine whether Tsg altered the ability of Tld to release an active Dpp ligand from the tripartite complex. We previously reported that overexpression of Tsg together with Sog in wing imaginal discs inhibits Dpp signaling producing a small wing with altered patterns of veination (Yu et al., 2000) (Fig. 2C). By contrast, overexpression of an activated form of Tld is able to significantly reverse the small wing phenotype produced by co-expression of Tsg and Sog (Fig. 2D). These results are consistent with our in vitro findings that Tld is still able to cleave and inactivate a Dpp inhibitory complex produced by Sog and Tsg.

Tsg alters the processing of Sog by Tld

In past biochemical studies, we have found that Sog is processed by Tld in at least three positions and Tsg appears to alter the processing patterning producing a new cleavage site within the spacer region between CR1 and CR2 (Marques et al., 1997; Yu et al., 2000). We have re-examined the processing reaction using more highly purified components and a new CR1 specific antibody. Under our present reaction conditions (Fig. 3), we typically see processing at four sites; however, not all fragments can be detected at equivalent molar ratios. Thus, these sites are either differentially cleaved, the products show differential stability or they are differentially recognized by our antibodies. In the absence of Tsg, only two out of the four potential C-terminal fragments are detected (Fig. 3A,B, fragments d,f). Based on size, we infer that fragments d and f correspond to processing at sites I and III (Fig. 3A,B).

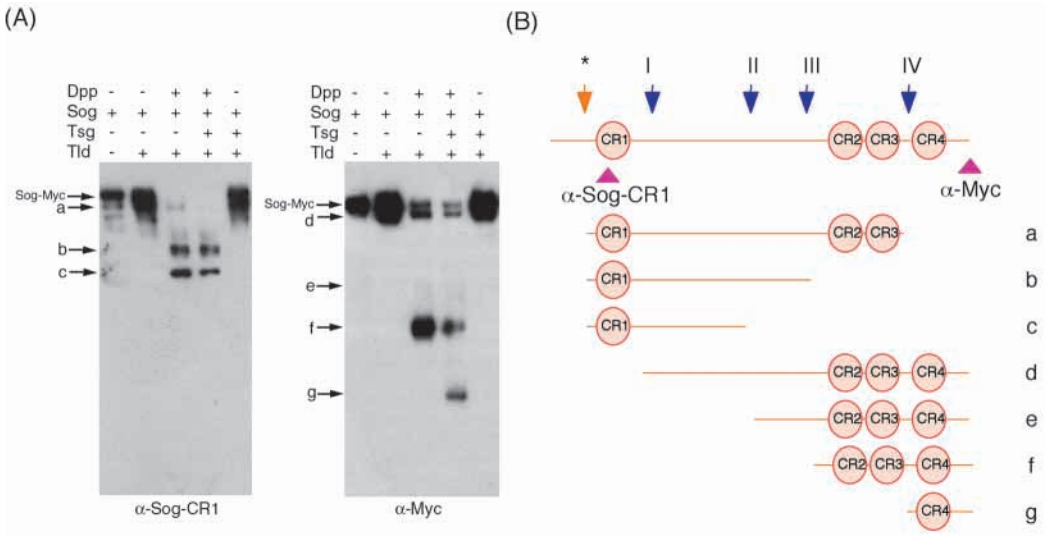

Fig. 3. Sog cleavage by Tld is modulated by Tsg. (A) The indicated combinations of proteins were incubated for 4 hours at 25°C, and Sog fragments were analyzed using an anti-Sog-CR1 antibody or anti-myc antibody. Tld requires Dpp to cleave Sog. In this experiment, the Dpp concentration was 10^{-9} M. The molecular weight of each fragment was calculated relative to markers as: 110 kDa for a, 80 kDa for b, 65 kDa for c, 110 kDa for d, 65 kDa for e, 50 kDa for f and 33 kDa for g. (B) Sog is cleaved in at least five sites. The site marked with an asterisk is cleaved during secretion and was identified to be between R^{79}-H^{80} by protein sequencing. This removes the type II transmembrane domain. The sites I, II, III and IV were cleaved by Tld in a Dpp-dependent fashion. Processing at the C-terminal site IV to produce fragment g was greatly enhanced in the presence of Tsg.

Fragment e produced by processing at a site (II) is weak but is detected on long exposure (e.g. Fig. 5A). We infer that significant processing does takes place at site II from an analysis of the N-terminal pattern of fragments using a new anti-CR1 specific antibody (Fig. 3A). In this case we see three N-terminal fragments which we infer from their sizes to correspond to processing at sites II, III, IV. The N-terminal most fragment of Sog produced by processing at site I (~20 kDa) is barely detectable using the CR1 antibody, although we can detect it using an N-terminal HA tagged form of Sog (data not shown). Interestingly, we see accumulation of substantial amounts of N-terminal fragments corresponding to processing at sites II and III, indicating that once processed at these sites, further cleavage at site I is relatively slow. These fragments should contain the N-terminal CR1 repeat and regions of the spacer region and are therefore very similar in structure to the Supersog-like molecules reported by Yu et al. (Yu et al., 2000). Interestingly, Supersog fragments are also fairly stable and were shown to be functionally resistant to degradation by activated Tld in a *Xenopus* assay (Yu et al., 2000). When Tsg is added to the processing reactions, cleavage at site IV is either enhanced or the fragment is stabilized as fragment g is now readily detectable. Using the CR1 antibody, however, we see no alteration in the ratio of the two prominent N-terminal fragments produced by cleavage at sites II and III (Fig. 3A), as previously reported (Yu et al., 2000). These differences may be due to the more highly purified components used in this set of experiments. Nevertheless, our results still suggest that Tsg can alter site selection or fragment stability (fragment g); however, processing at site IV does not alter production of the Supersog-like fragments which are produced by processing at sites II and III. The significance of the Tsg-induced alteration in C-terminal fragment production remains to be determined.

Dpp shows dose-dependent activities in a cell-based signaling assay that requires the type I receptor Tkv and the type II receptor Punt

To help elucidate the potential biological significance of particular processing reactions, we sought to develop a cell-based signaling assay that could be used in conjunction with in vitro processing experiments. We have previously shown that exogenously added Dpp is able to stimulate phosphorylation of Mad expressed in *Drosophila* S2 cells (Ross et al., 2001). To determine whether this cell-based signaling assay mimics endogenous Dpp signaling, we first asked whether it was dose responsive. As shown in Fig. 4A, varying the concentration of Dpp over a two-log range from 10^{-11} to 10^{-9} molar resulted in a shift from basal levels to saturating levels of phosphorylated Mad (P-Mad) accumulation. Saturation at low Dpp concentrations suggested that phosphorlyation of Mad was not likely to be the result of a non-specific signaling pathway. To examine this issue further, we asked whether this signaling requires the activities of Tkv, Sax and Punt, the known primary receptors for Bmp signals in vivo (Brummel et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998). RNA interference (RNAi) was used to knock down the endogenous expression of each receptor in S2 cells (Caplen et al., 2000; Clemens et al., 2000; Hammond et al., 2000; Ui-Tei et al., 2000) and P-mad accumulation in response to the addition of purified Dpp was

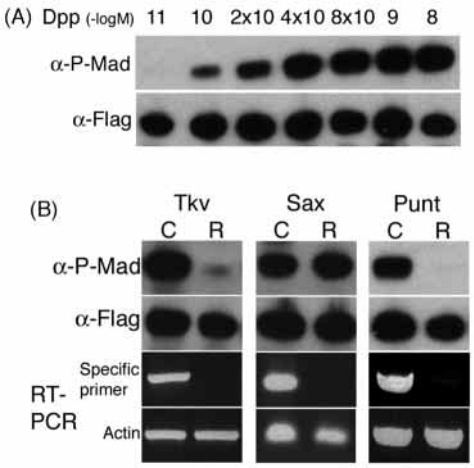

Fig. 4. Characterization of in vitro cell-based Dpp signaling assay. (A) Dose dependency of Dpp in the signaling assay was investigated. S2 cells expressing Flag-Mad proteins were incubated with the indicated amounts of Dpp protein for 3 hours at 25°C, and the cell extracts were blotted and analyzed by western blotting using anti-phosphoMad or anti-Flag antibody. Dpp activity was detected at as low as 10^{-10} M Dpp, and at 10^{-9} M Dpp, the signals were saturated. (B) Dpp signals through Tkv as a type I receptor and Punt as a type II receptor in S2 cells. S2 cells were transfected with Flag-Mad for control (C), or Flag-Mad and dsRNA of *tkv*, *sax*, or *punt* (R). Three days after transfection, cells were collected split into two fractions, one of which was used for a phosphoMad signaling assay and the other was used for RT-PCR.

measured. As shown in Fig. 4B, S2 cells normally express Tkv and Sax as type I receptors and Punt as type II receptor. However, upon transfection with *tkv* dsRNA, the expression of *tkv* is significantly reduced and is no longer detectable by RT-PCR. Consequently, when Dpp was incubated with these RNAi-treated cells, P-Mad accumulation was very low compared with the control cells. Thus, signaling by Dpp in S2 cells requires Tkv. Similar results were obtained when Punt expression was eliminated by RNAi treatment (Fig. 4B). By contrast, Dpp signals were comparable with control levels, when *sax* gene transcripts were knocked down by RNAi treatment (Fig. 4B). These results suggest that in S2 cells Tkv and Punt are the primary mediators of Dpp signals and that Sax is not able to substitute for Tkv. In similar experiments, Gbb and Screw were shown to primarily signal through Sax as a type I receptor (data not shown). This finding is consistent with in vivo genetic analysis, which has suggested that Sax is primarily a receptor for the auxiliary ligands Gbb and Screw and that its signal normally synergizes with that produced by Tkv in response to Dpp (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998).

Tld processing of Sog in vitro is dependent on Dpp dose and occurs in the same molar range as does signaling

We have previously reported that processing of Sog by Tld is dependent on the presence of Dpp (Marques et al., 1997). If ligand dependence of processing is biologically significant, then the Dpp dose that stimulates processing should be in the same concentration range as that which produces effective signals. As shown in Fig. 5A, we found that when Sog was

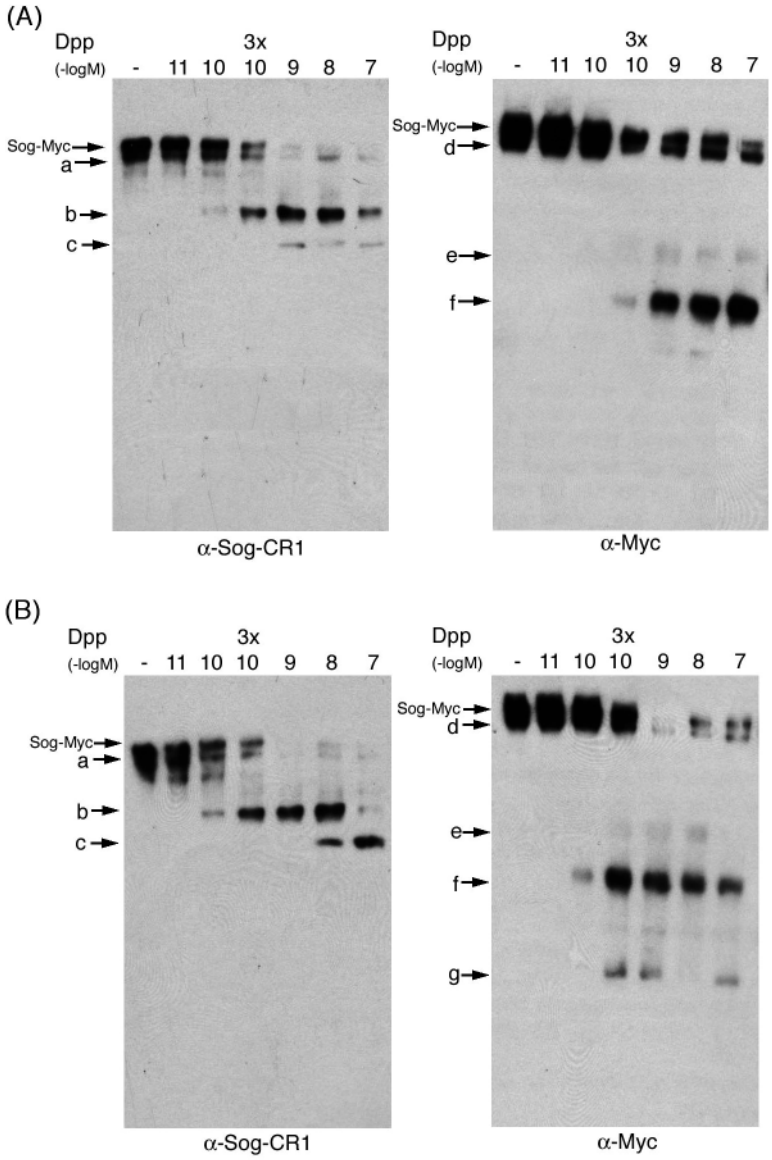

Fig. 5. Sog is cleaved by Tld in a Dpp-dose dependent fashion. (A) Sog, Tld and the indicated amount of Dpp were incubated for 4 hours at 25°C and Sog fragments were analyzed by western blotting using anti-Sog-CR1 or anti-Myc antibody. Processed fragments were detected at 3×10^{-10} M of Dpp. (B) Sog, Tld, Tsg and the indicated amount of Dpp were incubated for 4 hours at 25°C, and Sog fragments were analyzed as above. Tsg was used at the same concentration as Dpp. Cleavage fragments were observed at 10^{-10} M of Dpp. Arrows a-g show the same molecular weight fragments of Sog as shown in Fig. 3.

incubated with Tld and Dpp, strong processing was detected at 10^{-9} M Dpp, less at 3×10^{-10} M, and little processing at 10^{-10} M. This dose-response curve is similar to that elicited by Dpp in the cell-based signaling assay (Fig. 4), suggesting that the in vitro processing reaction probably reflects physiologically relevant conditions for regulating signaling in vivo. We next examined if Tsg influenced the Dpp dose dependence of Sog processing by Tld. When a relatively high amount of Tsg (10^{-7} M) was used in the reaction, a slight inhibitory effect on processing was seen at 10^{-9} M of Dpp or less (data not shown). However, if an equivalent molar ratio of Tsg and Dpp is used in the reactions, then the processing efficiency is significantly increased as illustrated by processing of Sog at 10^{-10} M Dpp (Fig. 5B).

We also examined if Tsg affected the rate of Sog cleavage by Tld. To examine this issue, time course experiments were carried out. When Sog was incubated with Tld in the presence of high concentrations of Dpp (10^{-8} M), cleaved fragments were detected at 15 minutes and the cleavage pattern showed the same time dependency with or without Tsg (data not shown). However, when Sog was incubated with Tld in the presence of 3×10^{-10} M of Dpp, then cleavage fragments are produced very slowly and are just barely detectable after four hours of incubation (Fig. 6A). By contrast, processing was detected in as little at 15 minutes in the presence of Tsg (Fig. 6B). These results demonstrate that the rate of Sog processing is dependent on the Dpp concentration and that Tsg sensitizes the processing of Sog to lower Dpp concentrations.

In vitro processing of Sog by Tld liberates active Dpp

As the cell-based signaling assay appears to mimic endogenous Dpp signaling with respect to the receptor requirements and the in vitro processing reactions show the same Dpp dose dependency as does the signaling assay, we could now ask whether processing of Sog by Tld inactivated the ability of Sog to block Dpp signaling and whether Tsg influenced this process. In particular, we wished to address whether Tsg might aid in the release of these fragments from Dpp as has been proposed in the *Xenopus* system (Oelgeschlager et al., 2000; Larrain et al., 2001). As shown in Fig. 7A (Ross et al., 2001) at a particular concentration relative to Dpp, neither Sog nor Tsg was able to effectively inhibit Dpp signals in S2 cells. However, when combined together at these same concentrations, they form a very potent inhibitory complex (Fig. 7A, compare lanes 3, 5 and 7). When this complex was co-incubated with Tld, signals were restored (Fig. 7A, lane 4). As shown in Fig. 7C, the majority of Sog was found in a processed form, and most N-terminal fragments, including those containing the CR1 domain were degraded and barely detected. Thus, in this cell-based signaling assay, it appears that, Sog N-terminal fragments, once cleaved, are unstable and easily degraded by other proteases.

To determine if Tsg is required to aid in the release of Dpp from the Sog fragments, we used a high concentration of Sog that is able to block Dpp signaling on its own. When this sample is treated with Tld, we find that some signaling is restored even in the absence of Tsg. The level of signal restoration is not as efficient as when Tsg is present (compare Fig. 7A lane 4 with Fig. 7B lane 3). However, we find that in the absence of Tsg, processing is also very inefficient (compare lane 3 in the anti-Myc panel of Fig. 7C with lane 2 in the anti-Myc panel of Fig. 7D). Therefore, it seems that the less than full restoration in signaling can be explained simply by the

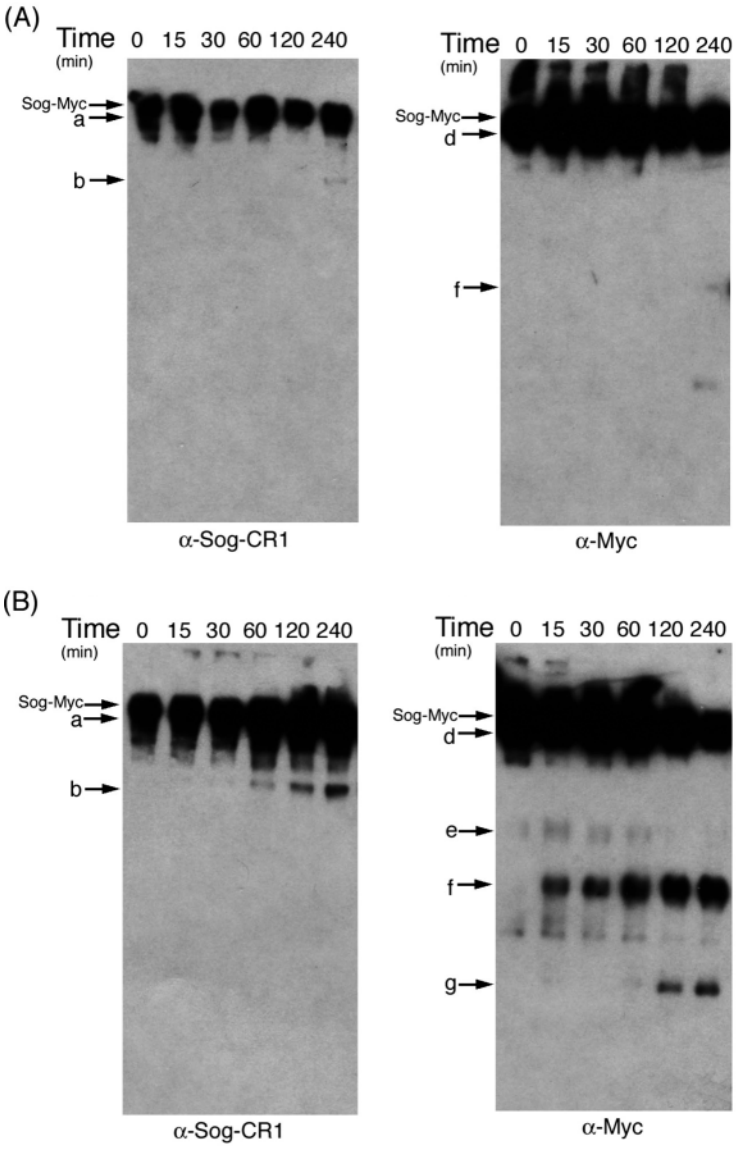

Fig. 6. Time course assay of Sog processing. (A) Sog, Tld and Dpp (3×10^{-10} M) were incubated at 25°C for the indicated time, and Sog fragments were analyzed on western blots using anti-Sog-CR1 or anti-Myc antibodies. The processed fragments were barely seen at 240 minutes. (B) Sog, Tld, Dpp (3×10^{-10} M), and Tsg (3×10^{-10} M) were incubated in the same conditions as above. Processed fragments were detected with as little as 15 minutes of incubation. Arrows a-g indicate the same fragments of Sog shown in Fig. 3.

reduced level of processing and it is not necessary to invoke a requirement for Tsg to displace processed fragments for signaling to occur.

Discussion

Models for how Tsg, Tld and Sog influence DV patterning

In this paper, we report a detailed analysis of the genetic and biochemical interactions among the products of the DV patterning genes Dpp, Sog, Tsg and Tld. Our ultimate goal was to distinguish between various molecular models for one that best explains how these components help establish cell fate within the dorsal tissue. Of particular interest was the determination of how Tld activity is confined to dorsal cells and to further investigate the role of Tsg in the DV patterning process. At present, the prevailing view is that Sog, Tsg and Tld act to create a transport mechanism that helps promote Dpp diffusion from lateral regions of the embryos towards the dorsal side (Holley et al., 1995; Marques et al., 1997; Decotto and Ferguson, 2001; Ross et al., 2001). According to this model, Sog would diffuse into the dorsal domain from its ventral lateral site of synthesis and capture Dpp, thereby preventing it from binding to receptor. Net flux of Sog towards the dorsal side is envisioned to help transport Dpp and thereby increase its concentration in the dorsalmost tissue that is destined to become the amnioserosa. Tld acts to liberate Dpp by cleaving Sog, and Dpp once released, will either be recaptured by another Sog molecule or bound to its receptors.

In order for the transport model to produce a Dpp concentration peak, the proper balance between binding affinities, diffusion rates and proteolytic processing is needed (see Eldar et al., 2002). Tsg has been suggested to have several activities that could influence this balance. In one model, Tsg would act to slow down the intrinsic rate of Sog cleavage by Tld. In this case, loss of Tsg is predicted to result in elevated processing of Sog. This should produce a *sog* loss-of-function phenotype, as is observed when molecular markers are examined (Yu et al., 2000; Ross et al., 2001). In this report, we present data that argues strongly against this possibility. First, we demonstrate that Tsg function is epistatic to Tld. If the *tsg* mutant phenotype was caused by excess Tld activity, then eliminating Tld should produce a *tld* loss-of-function phenotype. However, we observe a *tsg*-like phenotype where there is a general lowering and flattening of the Dpp activity gradient, as assayed by marker gene expression. In addition, biochemical studies reveal that Tsg actually enhances the ability of Tld to cleave Sog. Taken together, we conclude that Tsg does not function during DV patterning to retard Tld proteolytic activity.

A second property that has been attributed to Tsg is that it alters the selection of Tld cleavage sites in Sog thereby producing novel Sog fragments with unique properties (Yu et al., 2000). In particular, a Sog fragment termed Supersog containing the first CR domain and a region of the spacer between CR1 and CR2 appeared to be produced in vitro by the action of Tsg and Tld. Although we continue to see the production of Supersog-like fragments under our present reaction conditions, we do not see any enhancement in their production upon Tsg addition. This may reflect loss of an unidentified component during purification or differences in the sensitivities of the CR1 antibodies used in the two studies. These issues are presently under examination. Whether Supersog-type molecules contribute to DV patterning in vivo is unclear. The fact that overexpression of Supersog can partially rescue *tsg* mutant embryos suggests that they could be important. A full resolution of the role of Supersog will need

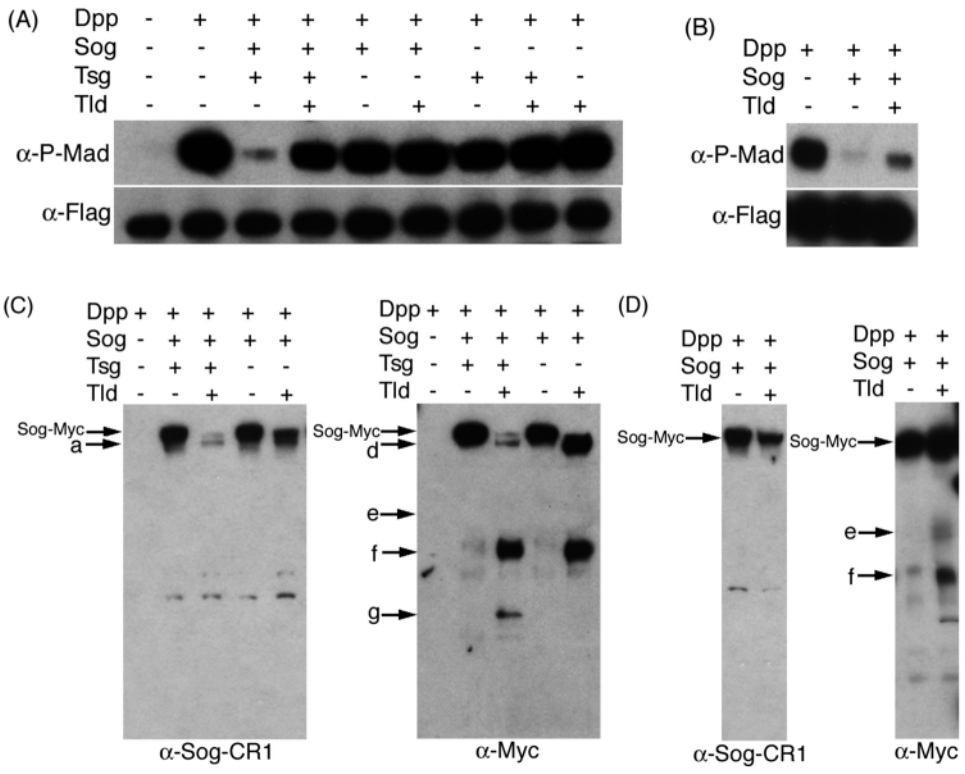

Fig. 7. Tld reverses the Sog and Tsg synergistic inhibition of Dpp signaling in vitro. (A) The indicated combination of proteins, including 10^{-9} M of Dpp, were pre-incubated for 6 hours and further incubated with S2 cells expressing Flag-Mad for 3 hours. The cell extracts were analyzed by anti-phosphoMad antibody for Dpp signals (upper panel) and by anti-Flag antibody for total Mad protein expression (lower panel). Dpp signaling was strongly blocked by Sog and Tsg, but this inhibition was reversed by the co-incubation with Tld. (B) Dpp signal (10^{-10} M Dpp, 1/10 the amount used in A) was blocked by the same concentration of Sog as used in A, and this inhibition was partially reversed by Tld (lane 3). (C) The indicated combination of proteins was incubated as in A, and the collected supernatants were applied to a 4-12% NuPAGE gel and processed fragments were detected by western blotting using anti-Sog-CR1 and anti-Myc antibodies. Full-length Sog is barely detectable in Dpp/Sog/Tsg/Tld. Cleaved C-terminal fragments were detected by anti-Myc antibody, but with the exception of fragment (a) N terminal fragments are not detected by anti-Sog-CR1 antibody, suggesting that, in the presence of cells, these fragments are unstable (compare with Fig. 3A). (D) The supernatants of the indicated protein combinations from the experiment shown in B were analyzed for processing. Sog is partially cleaved by the incubation with Tld, and Dpp signals were partially restored. In all panels, arrows labeled a-g correspond to the fragments illustrated in Fig. 3B.

to await the results of in vivo rescue experiments employing mutants of the different Sog cleavage sites, especially those that lead to the production of Supersog-like fragments.

Tsg sensitizes the DV patterning system to low levels of Dpp

One of the primary findings in this report is that the rate of Sog cleavage is very sensitive to the level of the Dpp protein and varies substantially over a 10-fold range. Interestingly, this is the same Dpp concentration range within which low to maximal signaling occurs in S2 cell culture. We find that Tsg sensitizes the system such that both the binding of Dpp to Sog as well as the rate of cleavage of Sog by Tld is stimulated by Tsg protein. Because in the invertebrate system, the binding of ligand to Sog is required for efficient processing of Sog, it is not surprising that the rate of Sog processing goes up in the presence of Tsg. This follows because, at a given concentration of Sog and Dpp, more complex will be formed in the presence of Tsg leading to a higher substrate concentration for the Tld protease. We speculate that this system evolved in part to enable the embryo to produce a patterning mechanism that functions within the context of a very short developmental window. In *Drosophila*, the time between initial transcription of *dpp* during the early blastoderm stage and assignment of fate required for proper gastrulation is only about 40 minutes. In this short time-window, Dpp concentration must reach an effective signaling level. However, using a genomic Dpp-HA construct, we have been able to visualize Dpp in the early embryo and it is present at much lower levels than in other tissues, such as the epidermis, at later stages of embryogenesis (O.S. and M.B.O'C., unpublished). We propose that under these conditions of low Dpp concentration, the presence of Tsg is required to enable Sog to bind to Dpp and to stimulate Sog cleavage in order to create a cyclic binding and release process that enables Dpp to be carried towards the dorsal midline. Furthermore, we propose that the intrinsic sensitivity of the cleavage reaction to the Dpp concentration is crucial for formation of a sharp signaling boundary. Thus, as illustrated in Fig. 8, as the Dpp concentration drops in the lateral regions as a consequence of Dpp movement towards the dorsal side, the rate of Sog cleavage drops allowing more Sog to enter this region further reducing signaling in lateral regions. The movement of Dpp will simultaneously raise Dpp concentration in the dorsal region further stimulating cleavage and clearance of Sog and thereby reinforcing Dpp signaling at the dorsal midline. This built-in positive and negative reinforcement mechanism should help establish sharp signaling boundaries by formation of steep ligand gradients, instead of the more gradual gradients that would form if Sog cleavage was not sensitive to the Dpp concentration.

Comparison with the vertebrate system

In some vertebrate systems, DV patterning mechanisms have been conserved with respect to the molecules employed, but the polarity of axis over which they act has been inverted (DeRobertis and Sasai, 1996; Holley et al., 1995). Thus, in both amphibians and zebrafish, Bmp ligands specify ventral cell

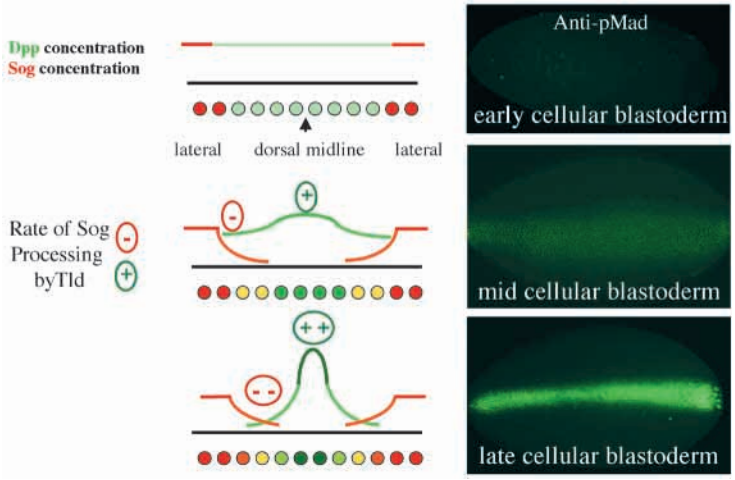

Fig. 8. Tld activity mirrors Dpp concentration helping to create a sharp boundary in signal reception. The graphs illustrate proposed temporal changes within the dorsal half of the embryo in the extracellular concentrations of Dpp and Sog as development proceeds from early to late cellular blastoderm. The green circles below the black line represent cells receiving Dpp signal. The darker green indicates a higher level of cumulative signal received over the entire time span. Yellow and orange represent cells receiving low level of Dpp signal because of early blockage in Dpp signaling by Sog diffusion. Red circles represent Sog-expressing cells. The embryos at the right show P-Mad accumulation with time. Embryos were stained using P-Mad primary antibody at 1/1000 dilution and anti-rabbit HRP at 1/200 dilution. The embryos were developed for 20 minutes.

fates, whereas Bmp inhibitors, such as Chordin, are secreted from dorsal cells. In each of these systems, Tsg- and Tld-like proteins also contribute to axis formation, but the biochemical details of their associations appear different from that of *Drosophila*. Two distinctions are most apparent and these probably have biological significance with respect to the patterning mechanism employed by these organisms. In *Xenopus*, the affinity of chordin for Bmps is significantly higher than Sog for Dpp as Bmps can be co-immunoprecipitated by chordin alone whereas this is not the case for the *Drosophila* components (Piccolo et al., 1996; Ross et al., 2001). In addition, once cleaved by Xolloid, at least some of the CR1 containing fragments of chordin continue to have significant affinity for the Bmp ligand preventing it from signaling (Larrain et al., 2001).

The second major difference between the *Drosophila* and *Xenopus* systems is that in *Drosophila* processing of Sog is dependant on prior binding of Sog to Dpp, while in *Xenopus* this is not the case. Rather, Chordin cleavage by Xolloid appears to be constitutive and is not enhanced by any tested ligand (Piccolo et al., 1997). Without ligand dependent cleavage, net movement of Bmps by Chordin diffusion may not readily occur nor would there be a mechanism to both positively and negatively reinforce the processing reaction. Indeed, recent studies have demonstrated that in the *Drosophila* embryo, Chordin does not have the ability to promote Dpp signaling at a distance, whereas Sog does (Decotto and Ferguson, 2001). As a result, spatially enhanced Bmp concentrations and sharp signaling boundaries that result from net ligand movement by the activities of the Chordin, Xolloid and Tsg proteins may not occur in *Xenopus*. In fact there is no evidence in *Xenopus* that loss of Chordin activity actually results in a reduction in Bmp signaling in select regions of the embryo as occurs in *Drosophila*.

Despite these differences, Tsg may, nevertheless, play both positive and negative roles in modulating Bmp signaling; however, its mechanism is somewhat different. As processed fragments of Chordin still have reasonable affinity for ligand, they may need to be dislodged to allow for signaling. Tsg binding to Bmps appears to help promote this dislodgment and their ultimate degradation (Oelgeschlager et al., 2000; Larrain et al., 2001). In *Drosophila*, as Sog binds poorly to ligand in the absence of Tsg there is no need for Tsg to help promote dissociation of Sog fragments. Rather, it is its ability to help promote association of Sog with Dpp that is key to understanding its function. Tsg appears also to alter the rate of chordin proteolysis (Larrain et al., 2001). Thus, at a high Tsg-to-chordin ratio, Chordin may be degraded and in this way Tsg might help promote signaling (Ross et al., 2001; Larrain et al., 2001). It is possible that some combination of these properties is used in other vertebrates. For example, in zebrafish it has recently been shown that loss of *chordin* can enhance a phenotype that results from haplo-insufficiency for *swirl* a gene that encodes Bmp2b (Wagner and Mullins, 2002). This paradoxical observation, that loss of an inhibitor exacerbates a phenotype resulting from loss of a ligand, is exactly analogous to the case of amnioserosa development in *Drosophila* where loss of Sog (an inhibitor) leads to less Dpp signaling in the dorsal domain. Detailed studies examining the ligand dependence of Chordin cleavage in zebrafish by *minifin*, the gene encoding a Tld homolog, have not been reported. It is possible therefore, that like *Drosophila*, this system may also employ a transport mechanism involving Tsg, Chordin and Tld that acts to boost Bmp signaling in specific tissues. It is interesting to note that the mouse homologs of Tsg, Chordin and Tld also exhibit their own distinct biochemical properties. Thus, a new Tld processing site in Chordin is induced by the presence of Tsg (Scott et al., 2001) but this is not seen when the *Xenopus* components are used (Larrain et al., 2002). Thus, it seems probable that the inherent complexity of this multi-component regulatory mechanism has provided numerous targets for evolutionary change. We speculate that these changes account for the remarkable diversity that this mechanism exhibits with respect to the actual details by which it regulates Bmp signaling in different organisms.

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