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Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in *Drosophila*

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SUMMARY

The BMP-like signaling mediated by the ligands Dpp and Gbb is required to reinforce the development of most veins in the *Drosophila* wing. However, the formation of the cross veins is especially sensitive to reductions in BMP-like signaling. We show here that the formation of the definitive cross veins occurs after the initial specification of the longitudinal veins in a process that requires localized BMP-like activity. Since Dpp and Gbb levels are not detectably higher in the early phases of cross vein development, other factors apparently account for this localized activity. Our evidence suggests that the product of the *crossveinless 2* gene is a novel member of the BMP-like signaling pathway required to potentiate Gbb of Dpp signaling in the cross veins. *crossveinless 2* is expressed at higher levels in the developing cross veins and is necessary for local BMP-like activity. The Crossveinless 2 protein contains a putative signal or transmembrane sequence, and a partial Von Willebrand Factor D domain similar to those known to regulate the formation of intramolecular and intermolecular bonds. It also contains five cysteine-rich domains, similar to the cysteine-rich domains found in Chordin, Short Gastrulation and Procollagen that are known to bind BMP-like ligands. These features strongly suggest that Crossveinless 2 acts extracelluarly or in the secretory pathway to directly potentiate Dpp or Gbb signaling.

Key words: BMP, Dpp, Gbb, Sog, Chordin, Kielin, Vein formation, Imaginal discs, *crossveinless 2*, *Drosophila*

INTRODUCTION

During development, the dorsal and ventral epithelia of the *Drosophila* wing form a precisely patterned array of veins (Fig. 1A). The specification and maintenance of vein cells requires the cooperation and interaction between several different signaling pathways. The roles played by two such pathways have been relatively well characterized: the MAPK signaling mediated by the *Drosophila* EGF Receptor (Egfr) stimulates vein formation, while Notch signaling inhibits and refines vein formation. Both these pathways are active from the earliest stages of vein formation, at mid-late third instar, and are required to maintain and refine vein fates until at least 30 hours after pupariation (a.p.) (Shellenbarger and Mohler, 1975; Diaz-Benjumea and Garcia-Bellido, 1990; Sturtevant and Bier, 1995; Martin-Blanco et al., 1999). The mechanisms that localize Egfr signaling to the veins are not completely understood, but involve regulating ligand expression, ligand activation and the sensitivity of cells to active ligand (Sturtevant et al., 1993; Simcox et al., 1996; Guichard et al., 1999; Martin-Blanco et al., 1999; Wessells et al., 1999; Vervoot et al., 1999; Mohler et al., 2000). The Notch ligand Delta is expressed along the veins, apparently in response to high Egfr signaling, induces Notch target gene expression and inhibits vein formation in neighboring cells (Sturtevant and Bier, 1995; de Celis et al., 1997; Huppert et al., 1997).

A third pathway, the BMP-like signaling mediated by the ligands Decapentaplegic (Dpp) and Glass Bottom Boat (Gbb, also known as 60A), also plays a role in vein specification. In wing discs, *dpp* is expressed just anterior to the anteroposterior compartment boundary, between L3 and L4, and plays a role setting up the axes of the wing. It has not yet been determined whether the reception of BMP-like signals initiates vein specification, although high BMP activity has been detected near the fourth longitudinal vein (LV) at late third instar (Tanimoto et al., 2000). However, at later pupal stages, *dpp* expression is lost from the compartment boundary and rises along the veins; this expression is required for maintaining the fate of most veins and ectopic signaling can induce ectopic veins (Posakony et al., 1990; Yu et al., 1996; de Celis, 1997).

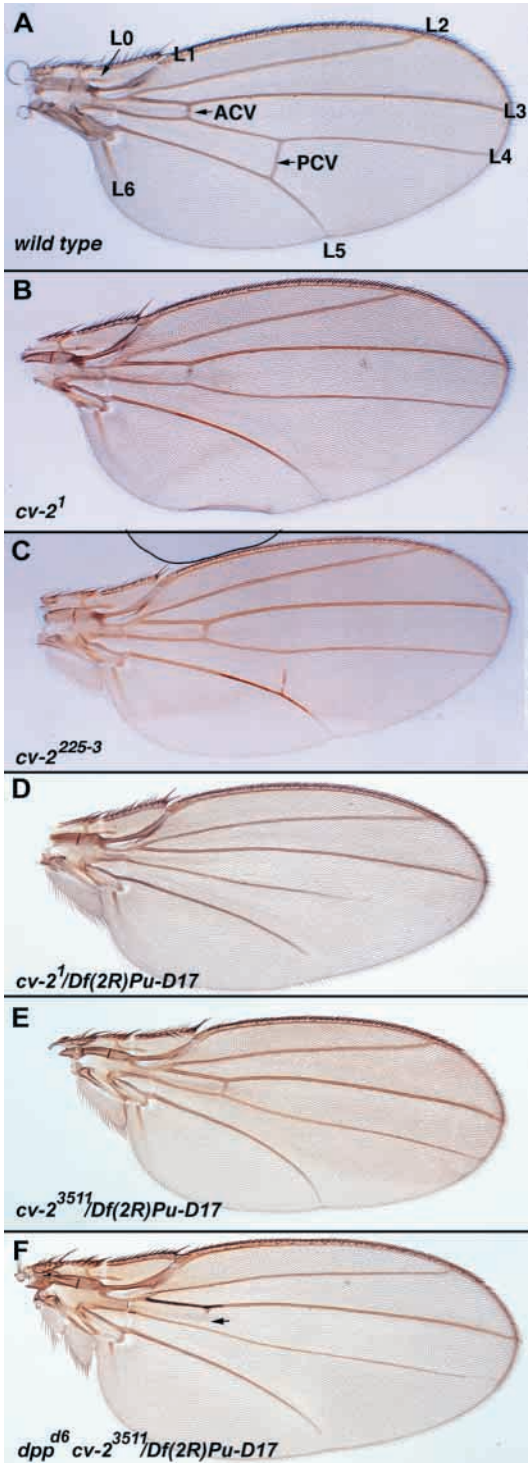

Fig. 1. Adult wings. (A) Wild-type wing, showing the location of the ACV, PCV and the LVs (L0-L6); (B) *cv-2*^{1}; (C) *cv-2*$^{225–3}$; (D) *cv-2*^{1}*/Df(2R)Pu-D17.* This is an extreme example; note loss of the entire ACV and the ends of some LVs. (E) *cv-2*^{3511}*/Df(2R)Pu-D17.* Note presence of the entire ACV. (F) *dpp*^{d6} *cv-2*^{3511}*/Df(2R)Pu-D17.* Note additional losses in the ACV (arrow) and ends of some LVs.

Although Gbb expression is not higher in pupal veins (see below), it also helps maintain vein formation, either by raising general levels of signaling or by interacting with localized modulators of signaling (Doctor et al., 1992; Khalsa et al., 1998). Both Dpp and Gbb vein signals may be mediated largely by the type I receptor Thickveins (Tkv), rather than the alternate type I receptor Saxophone (Sax). Cells lacking Tkv do not form veins (Burke and Basler, 1996), but removal of Sax does not reliably remove veins (Singer et al., 1997).

However, not all veins are equally sensitive to reductions in Dpp and Gbb signaling. The hypomorphic *gbb*^{4} mutation shows complete loss of the cross veins (CVs), but only slight loss of the ends of the LVs (Khalsa et al., 1998). Sog encodes a Chordin-like molecule that inhibits BMP-like signaling; both Sog and Chordin are thought to bind to and sequester ligands, preventing the activation of receptors (Francois et al., 1994; Holley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Piccolo et al., 1996; Larrain et al., 2000). Overexpressing Sog in the wing specifically blocks formation of the CVs and the ends of the LVs (Yu et al., 1996, 2000). The secreted Tolloid proteases, similar to vertebrate BMP1s, can increase BMP signaling by cleaving and inactivating Chordin or Sog (Shimell et al., 1991; Piccolo et al., 1997; Marques et al., 1997; Scott et al., 1999). Loss of *tolkin* (also known as *tolloid-related*) blocks formation of the CVs and the tips of the LVs (Nguyen et al., 1994; Finelli et al., 1995). Overexpressing a dominant negative form of Sax again induces a similar phenotype (Haerry et al., 1998).

Such phenotypes are very reminiscent of the *crossveinless* class of mutations in *Drosophila* (reviewed in Garcia-Bellido and de Celis, 1992). As we will show here, strong reductions in *crossveinless 2* (*cv-2*) function remove the posterior CV (PCV), the anterior CV (ACV), and the ends of the LVs (Fig. 1B-E). However, despite the possibility that the *crossveinless* genes encode novel players in BMP-like signaling, none have been characterized and the sensitivity of CVs to BMP-like signaling has not been explained.

We will present evidence that *cv-2* encodes a novel member of the BMP-like signaling pathway, expressed in and required for high levels of BMP-like signaling in the developing cross veins. The Cv-2 protein contains five cysteine-rich domains similar to those known to bind BMP-like ligands, strongly suggesting that Cv-2 directly modulates Dpp or Gbb activity.

MATERIALS AND METHODS

Drosophila stocks

The original *cv-2*^{1} *cn bw*/*CyO* stock (kindly provided by A. Garcia-Bellido) was homozygous lethal, but outcrossing yielded a homozygous viable stock. All the analyses here used the viable stock, except that shown in Fig. 1C. *cv-2*$^{225–3}$ was obtained from *P[lacW]* stocks kindly provided by A. Laughon and S. Carroll, while *cv-2*^{3511} is the *P[lacW]* insertion *l(2)k03511*. *gbb*^{4} was kindly provided by K. Wharton. *gbb*^{4} *cv-2*^{3511} and *dpp*^{d6} *cv-2*^{3511} chromosomes were created by recombination. The *lacZ* enhancer traps *rho*^{AA69} (Nambu et al., 1990; kindly provided by S. Spencer), *argos*^{rJ472} (kindly provided by M. Freeman), *wg*^{en11} (Kassis et al., 1992), *dpp*^{10638} and *tkv*^{lacZ} (Tanimoto et al., 2000; kindly provided by T. Tabata) were used to monitor gene expression. UAS-*sog* was kindly provided by E. Bier (Yu et al., 1996).

Immunohistology

Dissection and staining with rat anti-DSRF, Promega mouse anti-β-gal and Cappell rabbit anti-β-gal, and guinea pig anti-Dl were

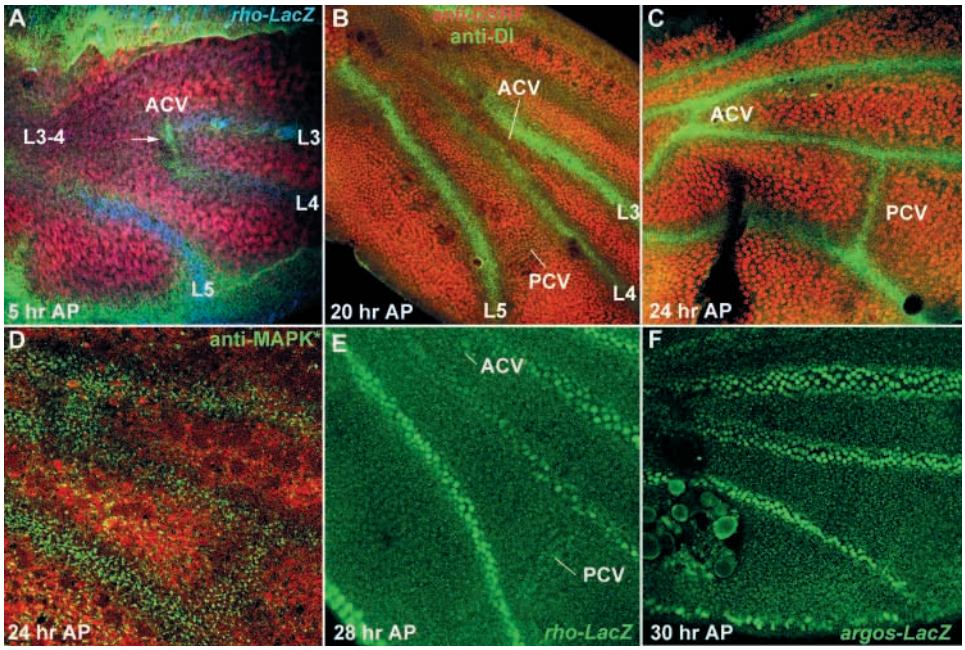

Fig. 2. Development of the CVs. (A) Anti-DSRF (red) and anti-Dl (green) staining in a *rho-lacZ* (blue) 5 hour a.p. wing. Note the ACV-like provein between L3 and L4; high *rho-lacZ* marks the cells of the ACV campaniform sensillum (arrow). Separate L3 and L4 veins are poorly defined proximal to the ACV-like provein. (B,C) Anti-DSRF (red) and anti-Dl (green) staining in 20 hour (B) and 24 hour (C) a.p. wings. (B) Anti-DSRF staining is very slightly reduced in the ACV and PCV, but anti-Dl staining is absent. (C) Note strong reductions of anti-DSRF and gains in anti-Dl staining in the CVs. (D) Anti-DSRF (red) and anti-MAPK* (green) staining in the veins of a 24 hour a.p. wing. (E) Anti-β-gal staining (green) in a 28 hour a.p. *rho-lacZ* wing; note the initiation of very faint staining in the CVs. (F) Anti-β-gal staining (green) in a 30 hour a.p. *argos-lacZ* wing; note the lack of staining in the CVs.

performed as previously described (Blair and Ralston, 1997; Micchelli et al., 1997; Blair 2000). Rabbit anti-p-Mad (kindly provided by T. Tabata and P. ten Dijke) was used at 1/2,000 overnight, and rabbit anti-Asense (kindly provided by A. Jarman; Brand et al., 1993) at 1/4,000. For anti-MAPK* staining, tissues were fixed overnight in PBS with 4% formaldehyde, dissected, stored in cold methanol for 2-4 hours and washed with PBS containing 0.1% Tween-20; wings were incubated overnight with 1/200 Sigma mouse anti-MAPK* in PBS-Tween.

In situ hybridization

Tissue was prepared as described by Cadigan et al. (1998), except that the initial fix of partly dissected pupae in PBS-formaldehyde was performed overnight, the wings were fully dissected prior to the next fixation and formaldehyde was not used in the final methanol-PBS-formaldehyde step. Probes were digoxigenin-labeled single-stranded DNA from either the 5′ and 3′ ends of the *cv-2* open reading frame (ORF), PCR amplified from cDNA template with a tenfold excess of one primer.

Molecular analyses

Plasmid rescue and inverse PCR techniques used have been described elsewhere (Wilson et al., 1989; Rehm, http://www.fruitfly.org/EST/). *cv-2* sequence and cDNAs were isolated from a λgt10 imaginal disc library (kindly provided by G. Rubin). Initial sequence was obtained after PCR amplification of the library using nested primers. The cDNAs were isolated using two probes. Template was PCR amplified from genomic DNA from a 200 bp region immediately upstream of the predicted ORF and from a 250 bp region lying 30 bp further downstream, within the ORF; these were gel purified, sequenced and used to generate digoxigenin probes. Each probe was hybridized against duplicate filters according to standard protocols (Sambrook et al., 1989).

RESULTS

The timing of cross vein formation

Morphologically, the LVs first become visible from 4-8 hours after pupariation (a.p.) (Waddingon, 1940; Mohler and Swedberg, 1964; Murray et al., 1984, 1995; Fristrom et al., 1993). The dorsal and ventral surfaces of the everting wing disc come together to form the wing blade, and LV ‘proveins’ form as broad gaps or lacunae between the dorsal and ventral epithelia. The pattern of LV proveins differs from the mature vein pattern: the veins are broader and somewhat incomplete, and proximally the L3 and L4 proveins fuse into a central, single provein. Molecular markers of veins, such as *rhomboid-lacZ* (*rho-lacZ*) (Nambu et al., 1990; Sturtevant et al., 1993), *argos-lacZ* (Sawamoto et al., 1994; Schweitzer et al., 1995), anti-Delta (anti-Dl) (Huppert et al., 1997), and an antibody against the activated form of MAPK (anti-MAPK*; Gabay et al., 1997), or of interveins, such as anti-*Drosophila* Serum Response Factor (DSRF, also known as Blistered) (Montagne et al., 1996), define the LV proveins as early as mid-late third instar. However, even with these markers, distinct L3 and L4 proveins were difficult to detect in the proximal region, where L3 and L4 fuse (Fig. 2A). The provein lacunae are lost from 8-16 hours a.p. as the wing inflates, separating the dorsal and ventral surfaces. Only as the dorsal and ventral surfaces of the wing reappose, at approximately 16-26 hours a.p., are the definitive veins visible. MAPK activity remains high in the definitive veins, but is lost from the veins and become high in the intervein regions after 30 hours a.p. (Gabay et al., 1997; Guichard et al., 1999; Martin-Blanco et al., 1999; S. S. B., unpublished observations).

The development of the ACV and PCV differ; a temporary, ACV-like provein is formed during the formation of the LV proveins, but no PCV provein is visible (Waddington, 1940). The L3 and L4 proveins join at the future site of the ACV, as shown by heightened *rho-lacZ* expression in the cells of the ACV campaniform sensillum (Fig. 2A). Anti-Dl and anti-DSRF detected a distinct, CV-like structure extending between L3 and L4 in this region, but no PCV-like gene expression could be detected at 5-6 hours a.p. (Fig. 2A). However, the ACV-like provein was no longer visible with

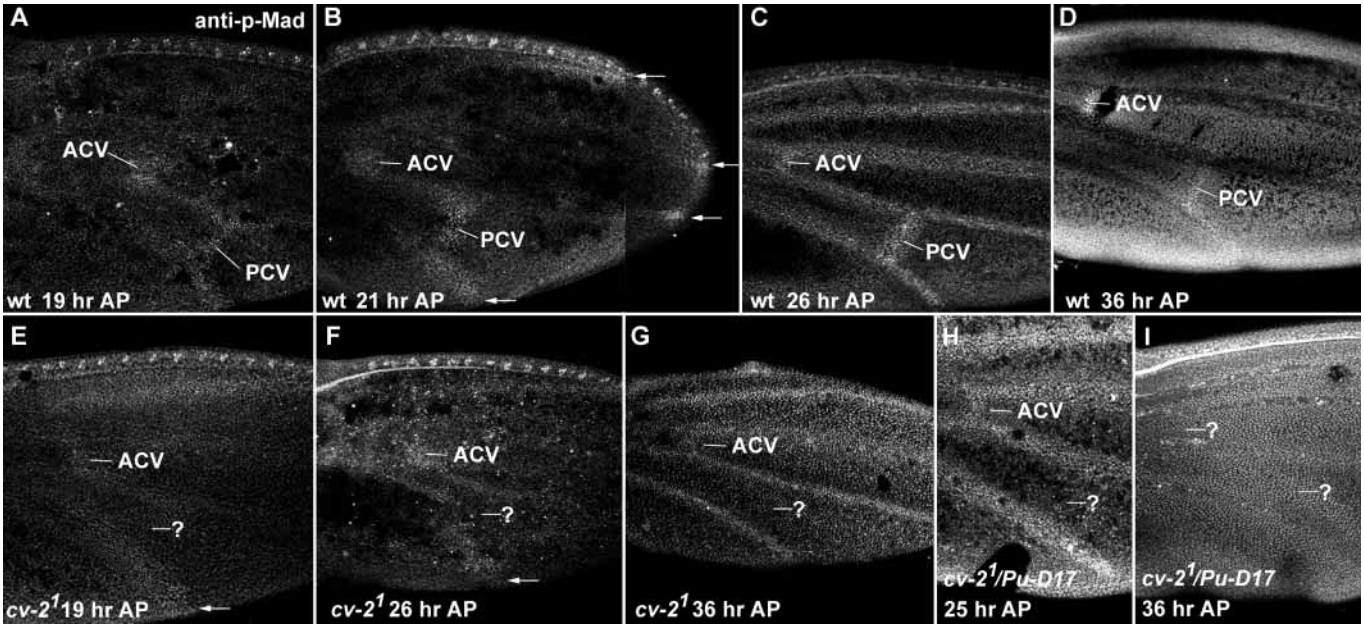

Fig. 3. Anti-p-Mad staining in normal (A-D) and mutant (E-I) pupal wings. At all stages staining is higher in the axons and nuclei of the neurons of the anterior margin. (A) 19 hours a.p. Note the stronger nuclear staining in regions in and around the CVs and the end of L5, and lower staining in L4 between the ACV and the PCV. (B) 21 hours a.p. Composite of two images of the same wing, taken in slightly different focal planes. Note the strong staining in the CVs and in the ends of all the LVs (arrows). (C) 26 hours a.p. Strong staining is now present along all the LVs, but is still higher in the CVs. (D) 36 hours a.p. Staining is present throughout the wing, but is still higher in the CVs. (E-G) *cv-2*^{1} homozygotes at 19 (E), 26 (F)and 36 (G) hours a.p. Staining is lost from normal site of the PCV (?) but, as expected from the adult phenotype, is still present in the ACV and the end of L5 (arrow). (H,I) *cv-2*^{1}*/Df(2R)Pu-D17* wings at 25 (H) and 36 (I) hours a.p. Staining is lost from the normal site of the PCV at 25 hours a.p., but from the sites of both CVs at 36 hours a.p. (?).

anti-Dl at 16-20 hours a.p., even though the LVs can be identified at these stages (Fig. 2B).

The definitive ACV and PCV first became visible at 19-22 hours a.p. as broad regions with slightly reduced anti-DSRF staining (Fig. 2B,C); this reduction was only rarely observed at 19-20 hours a.p. The DSRF suppression begins during the morphological formation of the definitive CVs (Mohler and Swedberg, 1964). By 23-26 hours a.p. narrow, well-defined CVs were visible with anti-Dl, anti-DSRF (Fig. 2C) and anti-MAPK* (Fig. 2D; also see Martin-Blanco et al., 1999). Although, in our hands, the reduction of anti-DSRF in the CVs preceded detectable levels of anti-MAPK* staining, DSRF is suppressed in a cell-autonomous manner by Egfr signaling (Roch et al., 1998) so its reduction may correspond to increased Egfr activity. *rho-lacZ* and *argos-lacZ* did not appear in the CVs until 28-32 hours a.p. (Fig. 2E,F). *wingless-lacZ* was expressed in the developing CVs (Phillips and Whittle, 1993; Blair, 1994), beginning at 25-26 hours a.p. (not shown). *wingless* plays only a weak role in the formation of the CVs (C. A. C. and S. S. B., unpublished data).

Mad is activated in the developing cross veins

To follow the signaling mediated by Dpp and Gbb, we used an antiserum specific to the phosphorylated, activated form of Mothers Against Dpp (Mad), the receptor-activated Smad in *Drosophila* (anti-p-Mad; Persson et al., 1998; Tanimoto et al., 2000). We did not detect any patterned anti-p-Mad staining during early pupal stages (5-6 hours a.p.), when the temporary ACV-like provein is formed. However, at 19 and 21 hours a.p., we observed nuclear staining in broad regions at the future sites of the CVs and in the tips of the LVs; some weaker staining was also observed in the portion of the LVs near the CV attachment sites (including the portion of L4 between the ACV and PCV) and along L2 (Fig. 3A,B). Interestingly, the CV anti-p-Mad staining typically preceded the reduction of anti-DSRF staining in the CVs, suggesting that Mad activation precedes the suppression of DSRF expression mediated by Egfr. At 24 and 26 hours a.p. anti-p-Mad-stained nuclei along all the LVs, but staining was still stronger near the CVs (Fig. 3C). At approximately 36 hours a.p., staining was observed throughout the wing, but again was stronger in the CVs (Fig. 3D). Strong staining was also observed in both the nuclei and axons of the PNS of the wing at 18-36 hours a.p.

To confirm that Mad activation was induced by BMP-like signaling, we overexpressed Sog, which can inhibit BMP-like signaling in pupal wings (see Yu et al., 1996). *en*-Gal4 was used to drive UAS-*sog* in the posterior of the wing. Adult wings lacked the ACV and the PCV, but these levels of Sog were not sufficient to block formation of the ends of the LVs; as expected, heightened anti-p-Mad staining was not detected near the PCV in 19-20 hours a.p. pupal wings, while staining in the ACV was reduced and in the LVs was largely normal (not shown).

Localized ligand expression cannot apparently account for the early stages of Mad activation in the CVs. *dpp* is first expressed along the LVs at 18-20 hours a.p., but is not detected in the CVs until some time after 24 hours a.p., as shown by in situ hybridization (Yu et al., 1996; de Celis, 1997; Martin-Blanco et al., 1999) and *dpp*^{lacZ} enhancer traps (not shown). As in imaginal discs, anti-Gbb staining (Doctor et al., 1992;

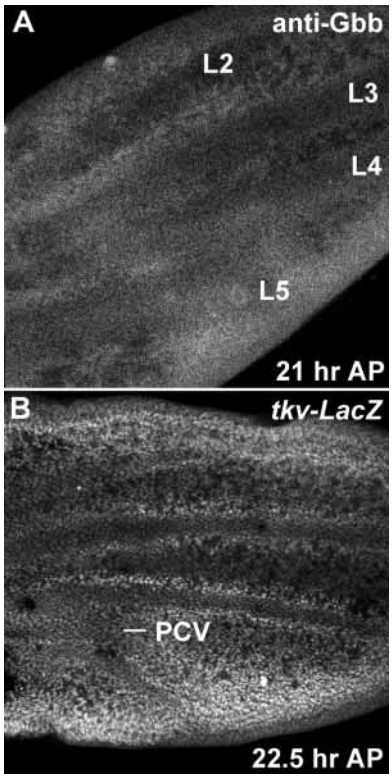

Fig. 4. (A) Anti-Gbb staining in a 21 hour a.p. wing. Staining is slightly higher in the interveins, but not higher near the CVs. (B) Anti-βgal staining in a *tkv-lacZ* wing at 22.5 hours a.p. Staining is slightly reduced in the LVs and the PCV.

Khalsa et al., 1998) appeared largely uniform at 21 and 24 hours a.p., and was not higher in the CVs (Fig. 4A). The localized activation of Mad is apparently not due to localized receptor expression. Expression of the BMP receptor *tkv* is ubiquitous during pupal stages, but is lower in the veins and higher in the cells immediately flanking the veins; the lowered expression is thought to be mediated by Dpp signaling (de Celis, 1997). *tkv-lacZ* expression was slightly reduced in the CVs at 22.5 hours a.p. (Fig. 4B).

cv-2 alleles

cv-2^{1} was isolated by Nicoletti (Lindsley and Zimm, 1992); it failed to complement *Df(2)AA21*, *Df(2)Pu-D17*, *Df(2R)PI12*, *Df(2R)PK1*, *Df(2)PL3* and *l(2)57DEFb-1 57*, localizing it to chromosome bands 57D11-E1. Two *P(lacW)* enhancer trap element insertions were also isolated with recessive CV-less phenotypes. Both were mapped to the 57D region by in situ hybridization on polytene chromosomes and failed to complement *cv-2*^{1}. A majority of the excisions generated by remobilizing the P elements reverted the phenotypes in both lines, indicating that the P-element insertions were responsible for the mutations.

The *cv-2*^{1} allele generated the strongest wing phenotype; wings typically lacked most or all of the PCV and, in some cases, the posterior of the ACV (Fig. 1B). *cv-2*$^{225-3}$ flies typically lacked part or all of the PCV (Fig. 1C). *cv-2*^{3511} overlapped wild type (not shown). Phenotypes of all three alleles were strengthened when placed over a deficiency; *cv-2*^{1}*/Df(2)Pu-D17* always lacked the PCV, variably lacked some or all of the ACV and, on occasions, lacked the ends of the LVs (Fig. 1D). A small percentage of lethal P-element excisions were also generated, but none had appreciably stronger wing phenotypes than their parent P-element lines. No other defects were detected in adults or embryos in any of these lines.

cv-2 affects Dpp and Gbb signaling

As discussed above, *dpp* and *gbb* mutations both disrupt CV formation. We found that weak *cv-2* alleles were strengthened by *dpp* and *gbb* loss-of-function mutations. *cv-2*$^{225-3}$*/cv-2*^{3511} flies never lacked the entire PCV, but 50% of *gbb*^{4} *cv-2*$^{225-3}$*/cv-2*^{3511} flies lacked the entire PCV. Similarly, *cv-2*^{3511}*/Df(2R)Pu-D17* only rarely disrupted the ACV, but *dpp*^{d6} *cv-2*^{3511}*/Df(2R)Pu-D17* commonly did (Fig. 1E,F). However, *cv-2* cannot dominantly enhance earlier *dpp*-dependent patterning in the wings: *dpp*^{d5} *Df(2R)Pu-D17/dpp*^{hr4} wings looked no worse than *dpp*^{d5}*/dpp*^{hr4} wings (not shown).

To provide a more direct link between *cv-2* and Dpp and Gbb signaling, we examined Mad activation in mutant pupal wings. In *cv-2*^{1} adults, the PCV was more reliably disrupted than the ACV; the anti-p-Mad staining normally found near the PCV in 19, 22, 26 and 36 hours a.p. wings was lost or disrupted in *cv-2*^{1} homozygotes (Fig. 3E-G), as was the reduction of anti-DSRF in the PCV. In adults of the stronger allelic combination *cv-2*^{1}*/Df(2R)Pu-D17*, the ACV was also often lost along with the ends of some of the LVs. Interestingly, we did not detect disruption of the ACV or LV anti-p-Mad staining *cv-2*^{1}*/Df(2R)Pu-D17* pupal wings at 21 or 25 hours a.p. (Fig. 3H); only at 36 hours a.p. was staining lost from the ACV (Fig. 3I). This indicates that *cv-2* is required not only to initiate Mad activity in the PCV, but also to maintain that activity in the ACV.

Genomic region

We used plasmid rescue and inverse PCR to isolate genomic DNA flanking the two P-element insertions; these mapped within a fully sequenced 190 kb contig generated by the Berkeley *Drosophila* Genome Project from P1 DS01261 and BACR48K03. A map of the *cv-2* region of this contig is shown in Fig. 5A. The two P elements lie 6.8 kb from one another, 12.2 kb (3511) and 5.4 kb (225-2) upstream of a novel transcript. This transcript is likely to encode *cv-2*; as shown below it is expressed in CVs and other tissues in a pattern similar to that seen in the enhancer traps, and its expression is lost or reduced in *cv-2* mutants.

Other neighboring genes are unlikely to contribute to the *cv-2* wing phenotype. 9-10 kb proximal to the putative *cv-2* sequence is a gene highly similar to *Drosophila antigen 5-related*, encoding a protein thought to act as an allergen or antimicrobial agent (Kovalick et al., 1998). 3.2-6.7 kb distal to the 3511 insertion is a gene encoding a protein highly similar to GTP-binding mitochondrial and bacterial Eftu elongation factors, and a partially sequenced EST (LD27358) with limited similarity to GTP-binding proteins that is predicted by Fgenesh (Salamov and Solovyev, unpublished; see http://genomic.sanger.ac.uk/) to correspond to the 3′ end of the elongation factor. 0.2-6.1 kb distal to the elongation factor lie two orthologs of the peroxisomal acyl-CoA oxidases (*Dmel\acox57Dp* and *Dmel\acox57Dd*). Gene prediction programs identified no other likely coding regions in this region.

Four *Drosophila* lines contain EP insertions (Rorth, 1996) just distal to the *cv-2*^{3511} insertion (*EP(2)1103*, *EP(2)2395*; *EP(2)2634*; *EP(2)0796*), and another contains the partially male-sterile *ms(2)05235* (*trail mix*) P-element insertion (Castrillon et al., 1993). The EP lines are homozygous viable

and have wild-type wings; *EP(2)1103* and *ms(2)05235* complement *cv-2*.

cv-2 message and protein

Fgene (Solayev et al., 1995; see http://genomic.sanger.ac.uk/) predicted that the putative *cv-2* sequence contains six exons (Fig. 5A); a strong TATA box is found 550 bp upstream and a predicted poly(A) site 1.2 kb downstream of the coding sequence. The predicted splice sites and the translation start and stop sites were first confirmed by PCR sequencing directly from an amplified imaginal disc cDNA library. This library was also screened using probe from the 5′ end of the predicted sequence. A cDNA was isolated which, when sequenced, confirmed the predicted open reading frame.

The amino half of the putative Cv-2 protein (Fig. 5B) contains five adjacent cysteine-rich domains (CRs), similar to those found in a number of secreted and transmembrane proteins, including vertebrate Chordin, Sog and Procollagens (Sasai et al., 1994; Francois et al., 1994), the mammalian and *C. elegans* CRIM1 proteins (Kolle et al., 2000), and *Xenopus* Kielin (Matsui et al., 2000). The CRs in Chordin and Procollagen IIA bind BMPs and can mediate Chordin-like biological activity during axis formation in *Xenopus* embryos (Zhu et al., 1999; Larrain et al., 2000). The spacing of cysteines is especially well conserved in the CxxCxC and CCxxC motifs (Fig. 5C). The second Cv-2 CR is the most divergent in terms of cysteines, lacking two. The Chordin and Sog CRs also contain a conserved tryptophan (Francois and Bier, 1995) that is shared by the second and fifth Cv-2 CRs. The CRs are similar to Von Willebrand Factor Type C domains (VWFc), but most regions in these CRs are shorter than the equivalent regions in the canonical VWFc (e.g. Fig. 5D), and the first and second Cv-2 CRs only weakly match VWFc domains.

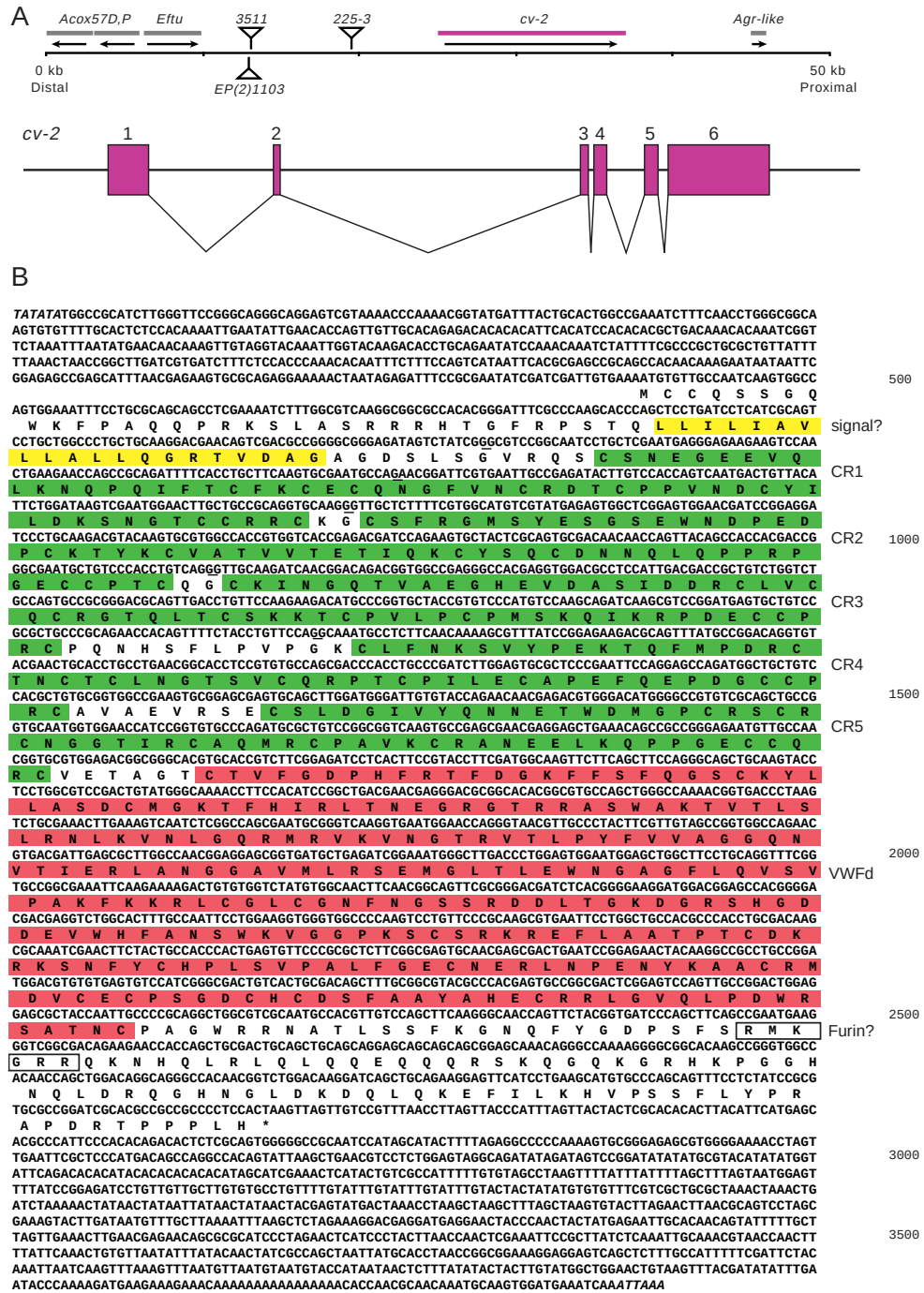

Fig. 5

The portion of Cv-2 carboxy terminal to the CRs contains a region highly similar to the amino two thirds of the Von Willebrand Factor Type D domain (VWFd; Fig. 5B,E). VWFd domains are found in a number of secreted proteins, including Von Willebrand Factors and Mucins, and are involved in the regulation of disulfide bonds and the formation of protein multimers (see Discussion). The similarity ends at Cys^{646}; the remaining portion of Cv-2 shares no significant similarity with any known protein, but does contain a potential Furin cleavage site (Fig. 5B; Nakayama, 1997).

The amino terminus of Cv-2 contains a hydrophobic region predicted to act as either a signal peptide, with a likely cleavage site between amino acids 53 and 54, or as an uncleaved transmembrane anchor (Nielsen et al., 1997). Cv-2 does not contain any obvious secretory retention signals, such as KDEL, suggesting that this protein acts extracellularly, being either

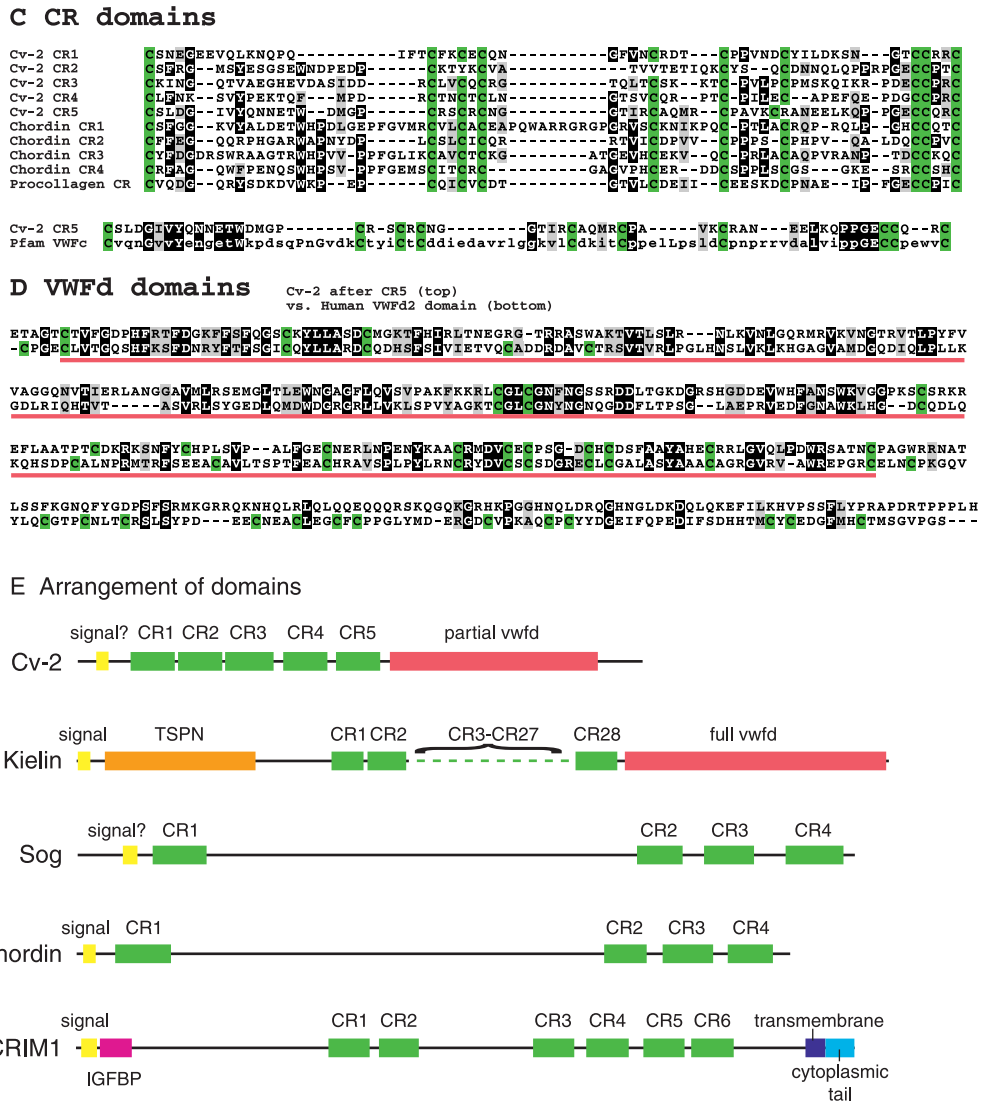

Fig. 5. (A) Top: Genomic organization and P-element insertions near *cv-2*. Arrows show direction of transcription. Bottom: Exon-intron structure of *cv-2*. B. Sequence of *cv-2*, starting at the predicted TATA box, ending at the predicted poly(A) site. The putative hydrophobic signal sequence (yellow), cysteine-rich domains (CR, green), partial VWFd domain (red) and potential Furin cleavage site (box) are marked. Exon splice sites are underlined. Sequence shown is based on identical genomic sequences from the Berkeley *Drosophila* Genome Project (AC007175) and Adams et al. (2000) (AE003453) (see Genbank accession number AF284429). Sequence derived from the cDNA contained three amino acid differences, changing A^{432} to S, N^{482} to D and C^{572} to R (see Genbank accession number AF288223). (C,D) Alignments of Cv-2; cysteines are shaded green, while amino acids shaded black and grey are conserved or similar, respectively, in half or more of the sequences. (C) Comparison of Cv-2 CRs with Chordin and Procollagen CRs known to bind BMPs (aligned using MAP; Huang et al., 1994), and of Cv-2 CR5 with the canonical Pfam VWFc domain (aligned using Pfam, Bateman et al., 2000). Less well conserved amino acids in the canonical Pfam VWRc sequence are shown in lower case. (D) Comparison of the Cv-2 VWFd domain with the VWFd2 domain from human VWF (aligned using ClustalW; Thompson et al., 1994). Cv-2 matches only a portion of the VWFd2 domain (underlined red) and a similar region in other VWFd domains (not shown). (E) Schematic comparison of Cv-2 with Kielin, Sog, Chordin and CRIM1. Kielin has a region similar to the amino terminal domain of Thrombospondin (TSPN), while human and mouse (but not *C. elegans*) CRIM1s have a region similar to a insulin-like growth factor binding-protein domain (IGFBP).

secreted or membrane bound. This, and the presence of domains typical of secreted proteins, makes it likely that Cv-2 acts either extracellularly or in the secretory pathway.

Despite sharing similar CR domains, Cv-2 is not an obvious ortholog of Chordin, Sog or CRIM1. The number and arrangement of CRs differs, and the portion of Cv-2 outside the CRs is not similar to Chordin, Sog or CRIM1, as these lack a VWFd domain (Fig. 5E). Cv-2 is most similar to the *Xenopus* Kielin protein, in that Kielin contains a region of repeated CR domains, followed by a VWFd domain (Matsui et al., 2000; Fig. 5E). Kielin is much longer than Cv-2, however, having 28 CR domains, a full rather than a partial VWFd domain, and an amino-terminal region, similar to the amino-terminal domain of Thrombospondin, that is lacking in Cv-2 (Fig. 5E). Nonetheless, BLAST analyses indicate that Cv-2's VWFd domain is more similar to the VWFd domain of Kielin than to other VWFd domains in the database, suggesting that the two might share a common ancestor (see Discussion).

cv-2 RNA and enhancer trap expression in normal and mutant flies

To provide further evidence that this transcript is *cv-2*, we generated probes to the 5′ and 3′ ends of the putative *cv-2* open reading frame, as well as to the neighboring EST (LD27358) and elongation factor coding regions, and performed in situ hybridization. LD27358 and the elongation factor were expressed uniformly in the wing disc (not shown). However, the putative *cv-2* transcript was expressed in a dynamic pattern, including expression in and near the developing CVs, that is identical to a combination of the *lacZ* expression patterns seen in both of the *cv-2* enhancer trap lines. Moreover, this expression was lost or reduced in *cv-2* mutants.

In late third instar wing discs, both enhancer trap lines showed weak general expression and slightly heightened expression along the anteroposterior (AP) boundary in the region of *dpp* expression (Figs 6A, 7A). The *cv-2*^{3511} line also showed strong expression in three distinct regions just distal to the prospective notum, as identified by the

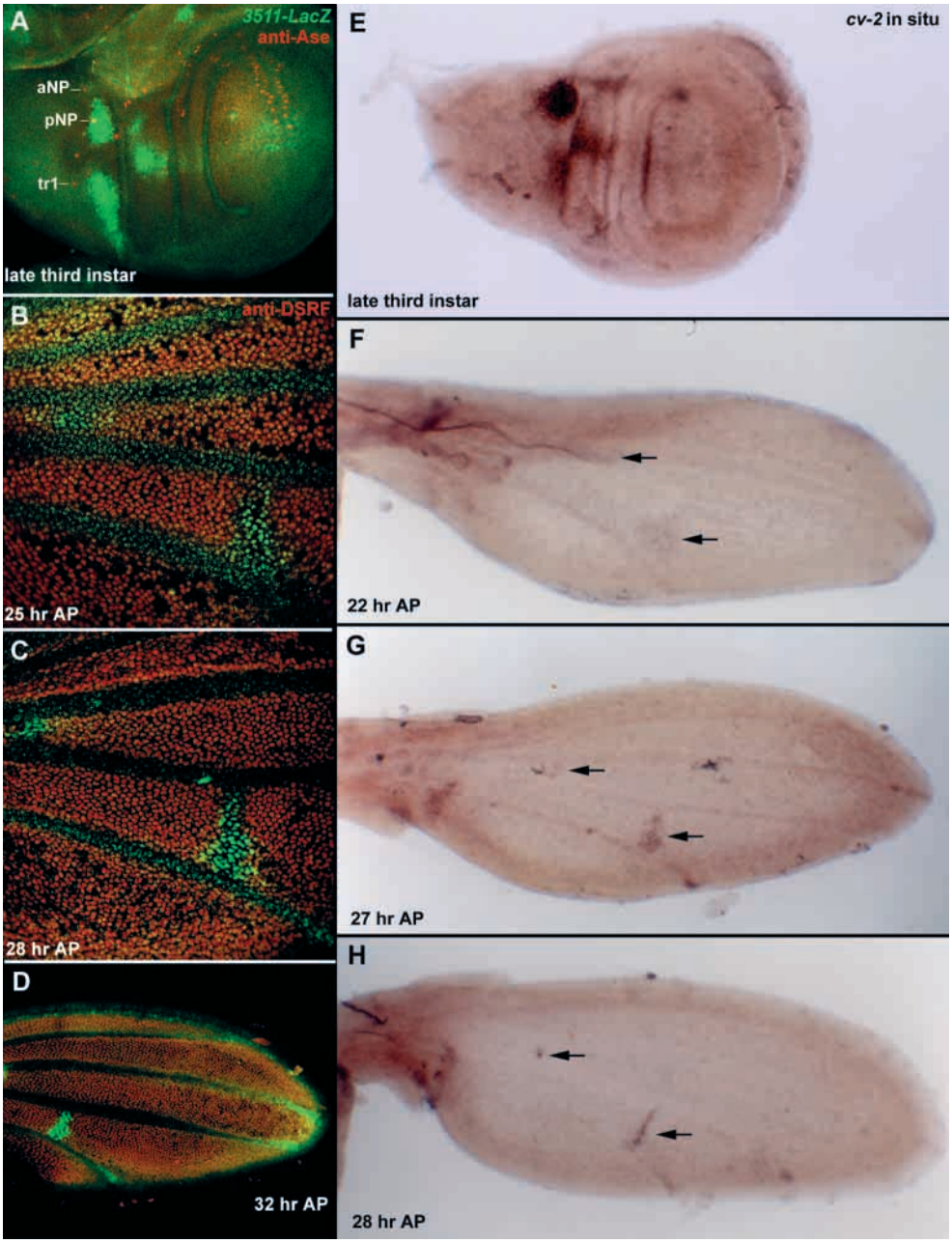

Fig. 6. (A-D) Anti-β-gal staining (green) in the *cv-2*^{3511} enhancer trap. (A) A late third instar wing disc, counterstained with anti-Asense (red) to identify bristle precursors. Note the anti-β-gal staining in three regions of notum. The anterior-proximal notal region of *cv-2*^{3511} staining contains and lies distal to the posterior notopleural bristle (pNP), and lies posterior to the anterior notopleural bristle (aNP). The posterior-proximal region of *cv-2*^{3511} staining lies distal to the trichoid sensillum (tri1). Faint staining is also observed along the AP boundary in the prospective wing blade. B-D. Pupal wings counterstained with anti-DSRF (red). Staining in and around the CVs is shown at 25 (B) and 28 (C) hours a.p. Staining in the ends of the LVs is shown at 32 hours a.p. (D), but is also present at earlier stages. E-H. In situ hybridization with *cv-2* probe. (E) Late third instar wing disc. Expression is similar to that in the *cv-2*^{3511} enhancer trap. (F-H) Staining in pupal wings. Faint diffuse staining is visible around the CVs (arrows) and the ends of the LVs at 22 hours a.p. (F), and becomes stronger and more tightly defined at 27 (G) and 28 (H) hours a.p.

position of notal bristle precursors (Fig. 6A). In pupal wings, *cv-2*^{3511} was expressed generally at low levels, but was also found at the tips of the LVs and, beginning at 25 hours a.p., in a broad region surrounding the CVs (Fig. 6B-D). *cv-2*$^{225-3}$ retained a slight emphasis along the AP boundary in pupal wings, but lacked CV expression (not shown).

The putative *cv-2* transcript was expressed in a pattern resembling a combination of the enhancer traps. There was weak general expression at all stages. Expression in late third instar wing discs was also strong in the three regions near the prospective notum, and weakly emphasized the AP boundary (Fig. 6E). At 19-21 hours a.p. expression was stronger in broad regions in and around the developing CVs; this expression resolved into narrower regions by 28 hours a.p. (Fig. 6F-H). Heightened expression was also observed at the tips of the LVs in pupal wings. This pattern is thus very similar to that of anti-p-Mad staining at these stages.

The *cv-2* enhancer traps expressed *lacZ* in other imaginal tissues (Fig. 7B,C). The pattern in leg and antennal discs was similar to that of *dpp*; expression in both was higher along the dorsal or lateral sides, respectively, of the AP boundary. Fainter expression was also observed in eye discs posterior to the morphogenetic furrow.

Expression of the *cv-2* message was altered in *cv-2*^{1} and *cv-2*$^{225-3}$ lines (Fig. 8). At late third instar, the heightened expression observed in the three regions near the notum was largely lost in *cv-2*$^{225-3}$ and consistently reduced in *cv-2*^{1} (Fig. 8B,C). Mutant adults have no obvious defects in the notum or hinge, so this loss is unlikely to be a secondary effect of a structural change. Expression near the CVs was also reduced

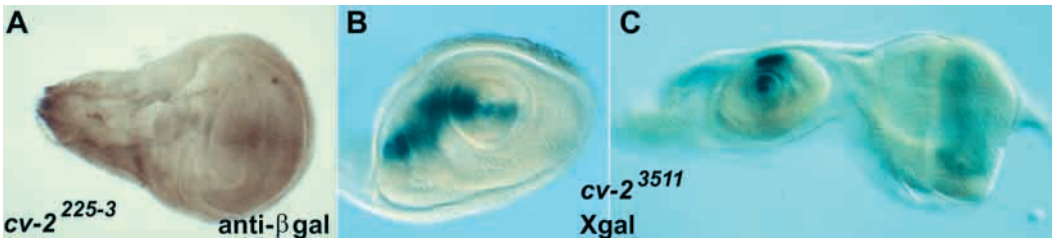

Fig. 7. (A) Anti-βgal staining in a late third instar *cv-2*$^{225–3}$ enhancer trap. Note the faintly emphasized staining along the AP boundary. (B,C) X-gal staining of late third instar leg (B) and eye-antennal (C) discs in the *cv-2*^{3511} enhancer trap. Note staining along the AP boundary in leg and antenna; dorsal expression is stronger in both. Also note the fainter staining posterior to the morphogenetic furrow in the eye.

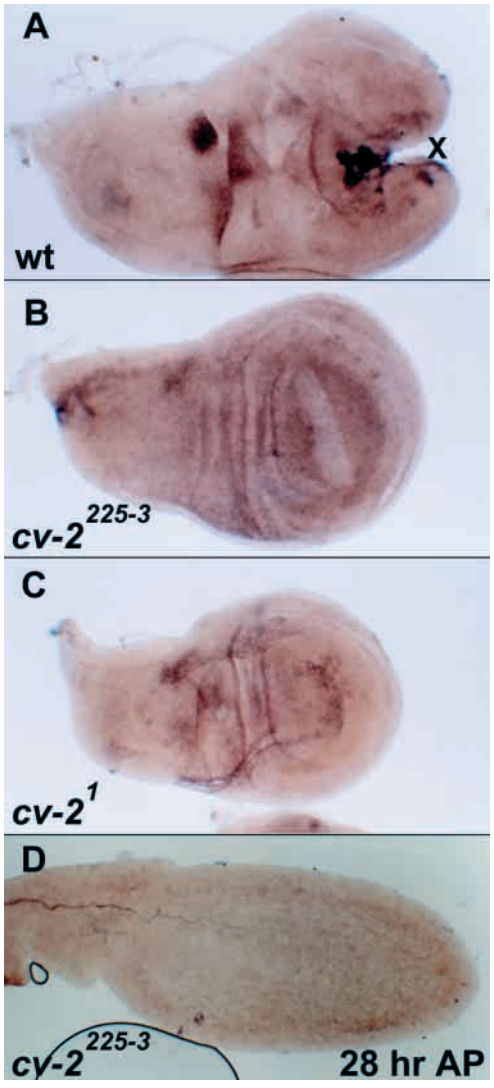

Fig. 8. In situ hybridization with a *cv-2* probe in normal mutant wing discs and pupal wings. (A-C) Late third instar wings discs. (A) Wild-type wing disc; control discs were stained in the same tubes as the mutant discs and marked with a cut (X). (B) *cv-2*$^{225–3}$; notal staining is reduced or absent. (C) *cv-2*^{1}; notal staining is consistently reduced. D. 28 hours a.p. *cv-2*$^{225–3}$ pupal wing; CV staining is reduced or absent.

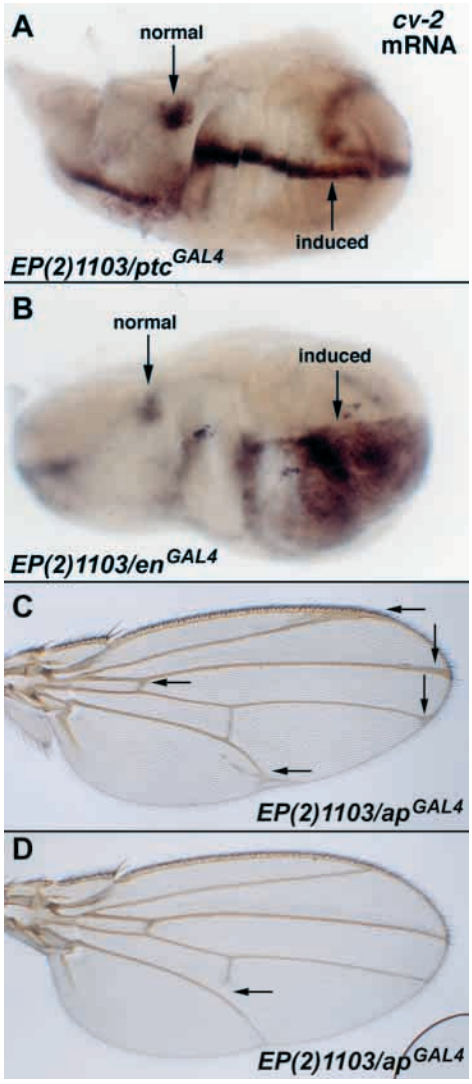

Fig. 9. Misexpression of *cv-2* using EP(2)1103. (A,B) In situ hybridization with a *cv-2* probe in wing discs in which the *EP(2)1103*-driven misexpression of *cv-2* was induced using *patched*-Gal4 (A) or *engrailed*-Gal4 (B). Levels of message in regions of ectopic expression (induced) were equal to or greater than levels in the regions of normal notal expression (normal). Adult wings from such flies were normal (not shown). (C,D) Adult wings in which *cv-2* misexpression was induced using the stronger *ap*-Gal4 driver. (C) Note ectopic venation at the tips of the LVs and the thickening of the ACV (arrows). (D) Note partial loss of the PCV (arrow); this phenotype was rare.

in both these lines (Fig. 8D). However, general expression was not totally lost from either *cv-2*$^{225-3}$ or *cv-2*^{1}, consistent with their hypomorphic phenotypes.

Misexpression of *cv-2*

EP elements contain multiple UAS sequences coupled to a heat-shock promoter; once inserted into the genome, these can be used in conjunction with Gal4 enhancer traps to drive the expression of neighboring genes (Rorth, 1996). As noted above, several EP lines contain EP insertions upstream of *cv-2*, close to the insertion site of the *cv-2*^{3511} P element. Crossing the *EP(2)1103* line to either *patched-Gal4* or *engrailed-Gal4* lines resulted in strong misexpression of the *cv-2* transcript at levels equal to or greater than endogenous levels (Fig. 9A,B). Adults from these crosses were fully viable and no wing phenotypes were apparent (not shown). This suggests that wild-type levels of Cv-2, while required for CV formation, are not normally sufficient to induce ectopic venation. However, the stronger *ap-Gal4* driver did generate a range of wing phenotypes; the most severely affected wings had ectopic venation near L2, vein deltas at the tips of the LVs, thickening of the ACV and, rarely, the partial loss of the PCV (Fig. 9C,D).

DISCUSSION

As shown both morphologically (Waddington, 1940; Mohler and Swedburg, 1964) and using molecular markers (see above), the definitive CVs are not formed until long after the initial specification of the LVs. The CVs therefore must form within territory that has already been specified as intervein. The CVs must also interconnect with existing LVs at a time when the Delta expressed by the LVs is thought to inhibit vein formation in adjacent cells (de Celis et al., 1997; Huppert et al., 1997). Mechanisms must exist that override both intervein specification and the lateral inhibition of veins, allowing the formation of continuous, interconnected vein tissue.

Our results show that BMP-like signaling plays a special role in the formation of the CVs from within intervein territory. BMP-like signals also help maintain the connections between the LVs and the margin of the wing. *cv-2* is a critical factor in these processes, as it is expressed more highly in the CVs and the ends of the LVs and is required for the high levels of BMP-like signaling observed in these regions. The structure of the Cv-2 protein strongly suggests that these effects are direct, and that Cv-2 is a novel player in the BMP-like signaling pathway.

Mad signaling and the formation of cross veins

While MAPK signaling appears to play the primary role in the initial specification of most or all of the LV proveins in third instar discs, BMP-like signaling helps maintain the LVs during later stages. After the first day of pupal development, *dpp* is lost from the L3-L4 intervein and is expressed along the LVs and, later, along the CVs; this expression is required to reinforce vein formation (Posakony et al., 1990; Yu et al., 1996; de Celis, 1997). We observed that Mad is indeed activated in all of the veins during pupal stages. However, Mad activity was higher and appeared earlier in the CVs and the ends of the LVs, the regions that are especially sensitive to reductions in BMP-like signaling. Mad activation may play the initial role during CV formation, preceding the activation of MAPK. Higher Mad activity was detected in the CVs before the loss of DSRF expression which is thought to be mediated by MAPK activity (Roch et al., 1998). Ectopic Dpp signaling is capable of inducing vein formation in regions lacking high MAPK activity (Yu et al., 1996; de Celis, 1997).

CV formation is sensitive to reductions in Gbb and Dpp expression (Posakony et al., 1991; Khalsa et al., 1998), and the genetic interactions of both *dpp* and *gbb* with *cv-2* mutations shown above indicate that both Gbb and Dpp play a role in the local activation of Mad in the CVs. However, localized expression of *gbb* or *dpp* cannot apparently account for the initial activation of Mad near the CVs. At the stage when Mad activity was first detected in the CVs, *dpp* is expressed along the LVs but is not detectable in the CVs (Yu et al., 1996; de Celis, 1997; Martin-Blanco et al., 1999), and anti-Gbb staining did not obviously emphasize the CVs (Fig. 4A). While it is possible that other BMP-like ligands locally activate Mad, Screw, the only other known BMP-like ligand known in *Drosophila*, has not been detected in pupae (Arora et al., 1994). It is therefore likely that other localized factors potentiate Mad activity in the CVs. Our results suggest that Cv-2 is such a factor.

A similar situation occurs during axis formation in the embryo, as extracellular modulators are thought to help localize BMP-like signaling (reviewed in Podos and Ferguson, 1999). Mad activity is highest on the extreme dorsal side of the embryo, but Dpp is expressed in a much broader dorsolateral domain and the other BMP-like ligand Screw is expressed ubiquitously. Ventrally expressed Sog inhibits Screw activity and thus provides a further dorsal bias in signaling (Neul and Ferguson, 1998; Nguyen et al., 1998). Sog may also act as a long-range activator of signaling in a process that requires cleavage by the secreted, dorsally expressed Tolloid protease (Zusman et al., 1988; Ashe and Levine, 1999). The dorsally secreted Twisted gastrulation protein (Tsg) also modulates signaling (Mason et al., 1994, 1997), perhaps by altering the cleavage of Sog by Tolloid (Yu et al., 2000), or more directly by binding ligands and potentiating signaling downstream of Sog (Oelgeschlager et al., 2000).

Cv-2 and vein formation

cv-2 is expressed at higher levels in the developing CVs and the ends of the LVs, and is required for Mad activation in these regions. The structure of the Cv-2 protein is consistent with a direct role in Dpp or Gbb signaling. Cv-2 contains a putative signal or transmembrane region, and CR and VWFd domains typical of secreted proteins, suggesting that it acts either extracellularly or in the secretory pathway. Moreover, the strong similarity between the five closely apposed CR domains in Cv-2 and the CR domains in Chordin, Sog and Procollagen (Fig. 5C) suggest that Cv-2 may directly bind Dpp or Gbb. Chordin and Procollagen CRs bind BMPs and can mediate Chordin-like inhibition of BMP signaling (Zhu et al., 1999; Larrain et al., 2000). *Xenopus* Tsg contains a partial CR that can also bind BMPs (Oelgeschlager et al., 2000).

As Sog overexpression induces *cv-2*-like phenotypes (Yu et al., 1996), Sog and Cv-2 have opposing effects on vein formation. Sog is expressed in the interveins during CV formation and is likely to diffuse to non-expressing cells (Yu et al., 1996). To raise BMP-like activity, localized factors may have to overcome the Sog-mediated inhibition of signaling.

Thus, one possible role for Cv-2 is to protect or release Gbb or Dpp from Sog. This is similar to the role proposed for *Xenopus* Tsg (Oelgeschlager et al., 2000). While clones lacking *sog* do not induce ectopic CVs (Yu et al., 1996), such clones might be rescued by the diffusion of Sog from outside the clone. Sog can act over long distances; overexpression of Sog in the anterior compartment of the wing can block PCV formation in the posterior (not shown).

Another possible role for Cv-2 is in the activation or stability of the ligands themselves. BMPs are initially expressed as inactive precursors that must be cleaved and stabilized during secretion (reviewed in Constam and Robertson, 1999). Interestingly, Thrombospondin, which also contains a Chordin-like CR region, is required for the activation of a cleaved but latent form of TGFβ1 in vivo (Crawford et al., 1998).

Cv-2 also contains a partial VWFd domain. VWFd domains are found in a number of secreted proteins, including Von Willebrand Factors (VWFs) and Mucins, and both directly bridge protein multimers and regulate the formation of intermolecular and intramolecular disulfide bonds (reviewed in Sadler, 1998; Perez-Vilar and Hill, 1999). Cv-2 lacks the portion of the VWFd domain that has been proven to directly bridge VWFs or Mucins. Cv-2 does, however, contain a CGLCG motif, which in VWFs and Mucins is required for the formation of protein multimers; this region is similar to a motif found in disulfide isomerases, and thus may regulate the formation of disulfide bonds (Mayadas and Wagner, 1992; Azuma et al., 1993; Dong et al., 1994; Perez-Vilar and Hill, 1998).

The presence of multiple CR domains and a VWFd domain makes Cv-2 different from Chordin, Sog, Tsg or CRIM1, but similar to the recently identified *Xenopus* protein Kielin which, like Cv-2, can regulate BMP-dependent patterning (Fig. 5E; Matsui et al., 2000). Kielin and Cv-2 are not identical, however (Fig. 5E). The much longer 2,327 amino acid Kielin has 28 CR domains instead of the five found in Cv-2, followed by a full rather than a partial VWFd domain, and has an amino-terminal region, similar to the amino-terminal domain of Thrombospondin, which is lacking in Cv-2. Thus, while it is possible that these proteins are homologs, one or both would have to have been severely modified from its original form.

Cv-2 and Kielin also differ functionally. While Cv-2 is required for high levels of BMP-like signaling in the CVs, ectopic Kielin inhibits some (but not all) forms of BMP-like signaling during axis formation in *Xenopus* (ibid). This functional difference may be caused by the structural differences between these proteins. Alternatively, other factors may regulate the ability of these two proteins to either inhibit or potentiate signaling. This would not be surprising in a situation where multiple ligand-binding proteins compete for available ligand. The cleavage of Chordin or Sog by Tolloid apparently lowers their affinity for ligand. In the *Drosophila* embryo, this is thought to convert Sog from an inhibitor to an activator of signaling, as Sog is required for the highest levels of BMP-like activity in a process that requires Tolloid (Zusman et al., 1988; Ashe and Levine, 1999). *Xenopus* Tsg potentiates BMP signaling, possibly by protecting ligand that would otherwise be sequestered by the cleaved form of Chordin; however, Tsg may also sequester ligand by forming a complex with full-length Chordin (Oelgeschlager et al., 2000).

The activity of Cv-2 thus may be highly context dependent and, indeed, our evidence suggests that other locally expressed factors are required for Cv-2's activity. While *cv-2* is necessary for Mad activity surrounding the CVs, misexpression of *cv-2* message at levels at or above normal levels had little effect on vein formation or other types of Mad-dependent development. Flies that had misexpressed *cv-2* throughout embryogenesis, larval and pupal life were viable and appeared normal. Only the very high levels of wing expression driven by *ap*-Gal4 induced ectopic vein phenotypes, and these were limited to the regions near where Mad activity is normally high. It is therefore unlikely that Cv-2 alone is responsible for localizing Mad activation. Other extracellular regulators of Mad activity are similar in this regard: for instance, ectopic Tsg expression has little effect on the *Drosophila* embryo (Mason et al., 1997), and overexpression in the wing causes a mild phenotype similar to that observed after misexpression of Cv-2 (Yu et al., 2000). The remaining *crossveinless* loci provide obvious candidates for the missing factors.

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