A Complete Domain Structure of Drosophila Tolloid Is Required for Cleavage of Short Gastrulation

Elizabeth G. Canty, Laure Garrigue-Antar, and Karl E. Kadler

From the Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom

The tolloid/bone morphogenetic protein 1 (BMP-1)2 metalloproteinases are fundamental to the normal development of animals because they cleave antagonists of the BMP subfamily of transforming growth factor β signaling molecules (1). For example, Drosophila tolloid cleaves Short gastrulation (Sog), thereby augmenting the activity of decapentaplegic (DPP), a transforming growth factor β superfamily growth factor, by cleaving its antagonist Short gastrulation (Sog). Similarly, the activity of BMP-2/4 (vertebrate homologues of DPP) is augmented by cleavage of chordin. However, whereas TLD is an effective Sogase, mTLD is a poor chordinase and is functionally replaced by its smaller splice variant BMP-1, which lacks the most C-terminal epidermal growth factor (EGF)-like and CUB domains of mTLD. Moreover, the minimal chordinase activity resides in the N-terminal half of BMP-1. This study showed that the proteolytic activity of TLD is considerably enhanced by Ca2+ and tested the hypothesis that the Sogase activity of TLD resides in the N-terminal half of the proteinase. Unexpectedly, it was found that TLD lacking the CUB4 and CUB5 domains and/or the EGF-like domains was unable to cleave Sog. Loss of function mutations have been reported in the tld gene that result in amino acid substitutions at E835K (in CUB4), S915L (in CUB5), and N760I (in EGF2) in TLD. The CUB mutants were found to be ineffective Sogases, but the activity of the EGF2 mutant was unchanged. The results show that substrate recognition and cleavage by Drosophila tolloid and mTLD are different despite their identical domain structure and homologous functions in patterning. The result that the N760I mutant has full Sogase activity suggests that novel substrates for TLD exist.

The tolloid/bone morphogenetic protein 1 (BMP-1)2 metalloproteinases are fundamental to the normal development of animals because they cleave antagonists of the BMP subfamily of transforming growth factor β signaling molecules (1). For example, Drosophila tolloid cleaves Short gastrulation (Sog), thereby augmenting the activity of decapentaplegic (DPP) and Screw (SCW) in dorsal-ventral patterning of the fly (2–4). Similarly in vertebrates, chordin, which is a functional homologue of Sog and sequesters BMPs, is cleaved by tolloid family members (5–7). On the face of it, the mechanism of proteolytic activation of the BMP-antagonist complex by tolloid proteinases would appear to be identical in all animals, from flies to man. However, evidence from in vitro and in vivo studies suggests that there are fundamental differences in the way tolloids recognize their substrates in flies and vertebrates.

An obvious difference is that Drosophila only has two tolloid genes, which encode two distinct proteins, whereas vertebrates have three tolloid genes and at least five tolloid and tolloid-like proteins (Fig. 1). The Drosophila genes code for tolloid (TLD) (8) and tolloid-related (TLR), also known as tolloid-related 1 and tolkin (9–11). These proteins contain a signal peptide (which directs the newly synthesized protein to the endoplasmic reticulum), a prodomain (which is cleaved by dibasic pro‐protein convertases in the secretory pathway), a zinc‐binding astacin‐like metalloproteinase domain, and five CUB domains (numbered 1–5) that are interspersed with two EGF-like domains between CUB2 and CUB3 and between CUB3 and CUB4. The three tolloid genes in vertebrates code for proteins with identical domain structure, namely mammalian tolloid (mTLD), tolloid like-1 (mTLL-1), and tolloid like-2 (mTLL-2) (7, 12, 13). However, the gene encoding mTld also gives rise to two smaller splice variants that lack the EGF2-CUB4-CUB5 domains of the molecule. One molecule, BMP-1, contains a unique short sequence at its C terminus and the other, BMP-1His, contains a sequence rich in histidine residues.

Comparison of the fly and vertebrate tolloids highlights further differences. For example, mTLD is a poor chordinase, whereas its smaller splice variant, BMP-1, is a highly effective chordinase (7). Furthermore, the chordinase activity of mTLD can be greatly enhanced by genetically engineering a mTld protein that lacks the EGF-like domains (14). The differences in mechanism are further apparent because TLD will only cleave Sog in the presence of DPP or SCW (2, 3), whereas vertebrate tolloid proteinases do not require complexed BMPs to cleave chordin. Vertebrate tolloids also have additional developmental functions in that they are involved in the biosynthetic processing of extracellular matrix macromolecules (15).

It is intriguing that BMP-1, which is the shortest tolloid proteinase in vertebrates and lacks the most C-terminal CUB and EGF-like domains that are present in mTLD, is a highly effective chordinase. This implies that the Sogase activity of TLD could be increased by removal of the C-terminal CUB domains, i.e. a fly equivalent of BMP-1 could be a more effective Sogase. However, mutations E835K (in CUB4) and S915L (in CUB5) disrupt dorsal-ventral patterning during Drosophila embryogenesis (16, 17), implying that these domains have an important function. The effect of tolloid mutations on patterning is similar to, but less severe than, the effect of mutations in the app gene (18).

In this study we have shown that the complete domain structure of TLD is required for Sogase activity. Mutant TLD proteins lacking the EGF-like domains or truncated to remove the C-terminal CUB and EGF domains were ineffective proteases. Furthermore, Sogase activity was significantly reduced for the E835K and S915L mutants. These results demonstrate the importance of the C-terminal CUB domains for TLD activity.
EXPERIMENTAL PROCEDURES

Source of Materials—Plasmids encoding full-length Drosophila Sog (pBSsog) and tolloid (pNBtld) and the plasmid SKAsc2 (pBluescript II SK+/H11006 modified to contain two AscI sites in the multiple cloning site) were a kind gift from Dr. Hilary Ashe (University of Manchester).

cDNA Manipulations—The tolloid cDNA was subcloned from plasmid pNB40 (19) into pSKAsc2 using HindIII and NotI (to give pSKTld). Subsequent site-directed mutagenesis was then used to convert the stop codon to an XbaI restriction site (to give pSKTldXbaI). The tolloid cDNA cassette was transferred to pAc5.1/V5-HisA (Invitrogen) using KpnI and XbaI such that the 3'-end of the tolloid cDNA was in-frame with the downstream V5 tag provided by the expression vector (to give pAcTld). The Sog cDNA had been previously subcloned from pNB40 into Bluescript SK+/H11006 to give pBSsog. The stop codon was similarly removed, and the Sog cDNA subcloned into pAc5.1/V5-HisA (Invitrogen).

Site-directed Mutagenesis—A two-step strand overlap PCR strategy, or a single PCR amplification, using Expand High Fidelity polymerase was used as described under "Experimental Procedures."
Mutational Studies of Drosophila Tolloid

A

TLD

Sog

100 kDa

CR1

CR2

CR3

CR4

20 kDa

100 kDa

45 kDa

20 kDa

EDTA (5 mM)

CaCl2 (mM)

0

0.1

1

5

B

C

FIGURE 2. Expression of recombinant Sog and TLD in S2 cells confirms that the proteins are secreted and that the Sogase activity of TLD is enhanced by calcium ions. A, domain structure of epitope-tagged Sog and tolloid proteins. CR, cysteine-rich domain; VS, VS epitope tag; His, 6 × histidine epitope tag. The cleavage sites and observed fragment sizes obtained on digestion of recombinant VS-tagged Sog with TLD are indicated. The observed molecular mass of the Sog fragments appears to depend both on the electrophoresis system and the molecular mass markers used. B, expression of recombinant Sog and TLD. S2 cells were transfected with vectors encoding the Sog or tolloid cDNAs. Cell culture medium was collected as described under "Experimental Procedures." Samples were separated by electrophoresis under reducing conditions and recombinant proteins detected by Western blotting using the anti-V5-HRP antibody. Recombinant Sog and tolloid have a similar molecular mass under these conditions. Recombinant tolloid migrates as two distinct bands that correspond to the latent (black star) and mature (black circle) forms of the enzyme. C, the Sogase activity of Drosophila tolloid is enhanced by calcium ions. S2 cells were transfected with vectors encoding the Sog and tolloid cDNAs. The conditioned medium containing VS-tagged Sog was subjected to dialysis and the conditioned medium containing VS-tagged TLD passed over a PD10 desalting column. The resulting samples in TBS were incubated together, in a 10:1 ratio, with the addition of CaCl2, in the presence of DPP and protease inhibitors. Cleavage of Sog is indicated by the appearance of 100-, 45-, and 20-kDa cleavage products after electrophoresis and Western blotting. EDTA abolishes protease activity both by chelating Ca²⁺ and by removal of Zn²⁺ from the active site of the metalloproteinase domain.

(Stop)

Mutational Studies of Drosophila Tolloid

with BpuA1R. These PCR products were then gel purified and amplified separately using Bsu36IF and BpuA1R prior to TA cloning. There are two BpuA1 sites in pAc5.1/V5-His. To circumvent this problem, the fragment was first subcloned into pSKTldXbaI along with the ΔEGF1 fragment for the double deletion mutant, and the entire cDNA cassette was transferred to pAc5.1/V5-HisA using KpnI and XbaI. HA-tagged tolloid was produced using a single PCR reaction with the primers Bsu36IF and HA+STOP+XbaIR. After TA cloning and sequencing, Bsu36I and XbaI restriction enzymes were used to obtain the full-length HA-tagged tolloid cDNA in pAc5.1/V5HisA. For the HA-tagged tolloid mutants, the initial PCR reactions were performed using Bsu36IF with N760IR, E835KR, or S915LR and N760IF, E835KF, or S915LF with HA+STOP+XbaIR. The second round of PCR was performed using Bsu36IF with HA+STOP+XbaIR and the appropriate gel-purified first-round PCR products. Digestion with Bsu36I and XbaI was used to transfer the cDNA fragments containing the HA tags and mutations into pAcTld. For the EGF1 mutations, the initial PCR reactions were performed using BstEIIF with D581ER or D581AR and D581EF or D581AF with Bsu36IR. These PCR products were then gel purified and amplified separately using BstEIIF and Bsu36IR prior to TA cloning. The resulting cDNA fragments were subcloned into pAcTld using BstEI and Bsu36I restriction enzymes to produce the required constructs.

Protein Expression—The Drosophila Expression System (Invitrogen) was used to express recombinant proteins in Drosophila melanogaster S2 cells according to the manufacturer’s instructions. Briefly, S2 cells were maintained at 24 °C in Schneider’s Drosophila medium supplemented with 10% fetal calf serum and 1% Pen-Strep. Calcium phosphate-based transient transfections were carried out using 5 or 19 μg of DNA (without any observable difference in expression levels) per 35-mm plate. Expression of recombinant tolloid constructs was analyzed by Western blotting at 24, 48, and 72 h post-transfection. The expression of V5-tagged Sog was investigated further to maximize the amount of substrate obtained from each transient transfection and was found to continue up to 7 days post-transfection. Depending on the volume to be processed and where required, the medium was dialysed into 50 mM Tris-HCl, pH 7.4, 100 mM NaCl or loaded onto a HiTrap or disposable PD10 desalting column (Amersham Biosciences) and eluted with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, and 0.01% NaN₃ (TBS).

Preparation of Medium and Cell Lysates—S2 cells in suspension were separated from the cell culture medium by centrifugation at 1000 × g for 3 min. The medium was decanted and the cells washed by resuspension in phosphate-buffered saline. The cells were resuspending the supernatant discarded. The cell pellet was lysed in ice-cold Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) and the cell lysates clarified by centrifugation at 13,000 × g for 1 min.

Electrophoresis and Western Blotting—Samples were resolved by electrophoresis on 8 or 12% gels by SDS-Page or on NuPAGE Novex 4–12% Bis-Tris gels using NuPAGE MES or MOPS running buffer (Invitrogen) under reducing conditions and subjected to Western immunoblotting. The mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-V5 antibody (Invitrogen) or the mouse monoclonal HRP-conjugated 3F10 antibody directed against the HA tag (Roche Applied Science) was used as appropriate. The signal was detected by enhanced chemiluminescence using ECL (Amersham Biosciences) or SuperSignal West Dura Extended Duration Substrate (Pierce). Where necessary, the intensity of the bands corresponding to the active forms of the enzymes, or of the Sog cleavage products, was quantified by densitometry using AIDA 2.0 software.
**Assay of Sogase Activity**—Digestions were performed in TBS or in cell culture medium in the presence of protease inhibitors at 25 °C overnight or for the indicated times. A concentrated stock solution of protease inhibitors was made using EDTA-free Protease Inhibitor Mixture tablets (Roche Applied Science). Digests also contained recombinant DPP (0.5 ng/H9262l, 33 nM) (R&D Systems), CaCl2 (0.1–5 mM), and EDTA (5 mM) as required and where indicated. The V5-tagged Sog cleavage products were detected by electrophoresis and Western blotting. The use of V5-tagged enzymes did not affect the detection of V5-tagged Sog fragments because they were present at least an order of magnitude less than the substrate in the assay.

**Location of Exon/Intron Junctions within the Tolloid-related Domain Structure**—The genomic organization and sequence for tolkin was obtained from Flybase (flybase.bio.indiana.edu). The entire genomic sequence was translated in all reading frames using the ExPasy proteomics server translate tool (us.expasy.org/tools/dna.html). CDNA sequences are also available on GenBank™ (accession numbers U34777 and U12634). Comparison of the conceptual translation products, the cDNA and genomic sequences, the amino acid sequence of tolkin, and the location of the domains allowed the identification of the location of the introns on the tolkin domain structure.

**RESULTS**

**V5-His-tagged Sog and Active V5-His-tagged Tolloid Are Expressed and Secreted by D. melanogaster Schneider S2 Cells**—Full-length tolloid and Sog cDNAs were subcloned in-frame into the vector pAc5.1V5HisA (Invitrogen) and used to transfect Drosophila S2 cells. Western blot analysis of the cell culture medium using the anti-V5 antibody showed that TLD-V5-His and Sog-V5-His (Fig. 2A) were readily secreted from the S2 cells (Fig. 2B) and that cleavage of Sog-V5-His by TLD-V5-His required the addition of exogenous DPP (not shown) (2). Hence, the addition of two tandem epitope tags at the C termini of the molecules does not abolish the secretion of the proteins from S2 cells or the ability of TLD to cleave Sog.

**The Sogase Activity of Drosophila Tolloid Is Substantially Promoted by Exogenous Calcium Ions**—It was previously shown that procollagen C proteinase, purified from chick embryo tendons, required 10 mM calcium chloride for maximal cleavage of the C-propeptides from type I procollagen and that the enzyme was inactive in the absence of exogenous calcium ions (20). Subsequent studies showed that procollagen C proteinase activity is exhibited by BMP-1 and mTLD (21, 22) as well as mammalian tolloid-like I (7). These results
have been used to imply that all members of the tolloid family require relatively high concentrations of Ca\textsuperscript{2+} for proteolytic activity. TLD is able to cleave Sog in the presence of exogenous DPP in Schneider’s cell culture medium where the concentration of CaCl\textsubscript{2} is 5.41 mM and in M3 insect medium where the concentration is 7 mM (2, 11, 23, 24). To determine whether Drosophila tolloid requires Ca\textsuperscript{2+}, the cell culture medium was subjected to dialysis or gel filtration prior to the digestion assay. TLD exhibited negligible Sogase activity in the absence of exogenous Ca\textsuperscript{2+} ions but could be recovered by the addition of 0.1–5 mM CaCl\textsubscript{2} (Fig. 2C).

A Drosophila-equivalent BMP-1 Does Not Cleave Sog—The Drosophila tolloid gene contains six short introns that are thought to facilitate rapid transcription and splicing during the short cell division cycles of the developing Drosophila embryo (10, 16, 17, 25). Tolloid-related plays a crucial role later in development, and its gene contains larger and more numerous introns (10). No shorter tolloid or tolloid-related splice variants have been reported in D. melanogaster (10, 26). Alternative splicing events between the exons coding for the CUB3 and EGF2 domains in the mammalian tolloid gene are responsible for the production of the shorter BMP-1 and BMP-1His isoforms (Fig. 1). BMP-1 lacks the C-terminal EGF2, CUB4, and CUB5 domains and is a more effective C-proteinase and chordinase. To test the hypothesis that a shorter splice variant of tolloid would be a more effective Sogase, we produced a BMP-1-like tolloid molecule lacking the EGF2, CUB4, and CUB5 domains (Fig. 3A). This molecule was constructed to contain a C-terminal V5-His tag and expressed in S2 cells. The Drosophila equivalent BMP-1 (truncated tolloid) was found in both the latent (i.e. retaining the prodomain) and mature (prodomain deficient) forms in the cell culture medium (Fig. 3B).
Mutational Studies of Drosophila Tolloid

TABLE 2

Tolloid alleles resulting in disruptions to dorsal ventral patterning in the Drosophila embryo

The allele strength given is as described in the published work. @, mutation that resulted in a stop codon.

| Strength               | Allele names | Amino acid change | Domain     | References | Notes       |
|------------------------|--------------|-------------------|------------|------------|-------------|
| Very weak              | tld^{A}, tld^{B}, tld^{E}356 | M487K             | CUB2       | (17, 18)   | Antimorph   |
|                        | tld^{E}, tld^{F}, tld^{G}349 | R253K             | CUB2       | (17, 18)   | Antimorph   |
| Weak, incompletely penetrant | tld^{C2}, tld^{C4}, tld^{D}899 | E899K             | CUB4       | (16, 18)   |             |
| Weak, completely penetrant | tld^{C2}, tld^{C4}, tld^{G}349 | D894V             | CUB4       | (16, 18)   |             |
|                        |              | D728Y             | CUB3       | (17, 18)   |             |
|                        |              | E517K             | CUB3       | (17, 18)   |             |
|                        |              | N760L             | EGF2       | (16)       |             |
|                        | tld^{E6}     | V21 M & D728N     | SS & CUB3  | (16)       |             |
|                        | tld^{E6}     | D A234-H241       | Protease   | (16)       |             |
| Moderate to severe     | tld^{B3}     | S284F             | Protease   | (17, 18)   | Antimorph   |
|                        | tld^{E}, tld^{F}, tld^{E}28 | Splice site mutation in intron 3 | Protease | (17, 18)   | Antimorph   |
|                        | tld^{B7}     | C272Y             | Protease   | (17, 18)   | Antimorph   |
|                        | tld^{E}, tld^{E}4, tld^{E}241 | Y272N             | Protease   | (17, 18)   | Antimorph   |
|                        | tld^{E2}     | E835K             | Protease   | (16)       |             |
|                        | tld^{F}      | Q506@             | Protease   | (16)       |             |
|                        | tld^{L4}     | D317V             | Protease   | (16)       |             |
|                        | tld^{L3}, tld^{L4}, tld^{F}241 | Y722N             | Protease   | (17)       |             |
| Strong                 | tld^{E7}     | Q35@              | CUB1       | (16)       |             |
|                        | tld^{E9}     | Q604@             | CUB1       | (16)       |             |
|                        | tld^{F}      | K240E             | CUB1       | (16)       |             |
| Null                   | tld^{A}, tld^{D}, tld^{E}7941, tld^{G}71 | Q478@             | CUB2       | (17, 18)   | Antimorph   |
|                        | tld^{C4}, tld^{C}8, tld^{D}695 | S276F             | CUB2       | (17, 18)   | Antimorph   |
|                        | tld^{C4}     | Q44@              | CUB2       | (17, 18)   | Antimorph   |
|                        | tld^{D}6, tld^{D}10, tld^{D}102 | Splice site mutation in intron 4 | CUB3       | (17, 18)   | Antimorph   |
|                        | tld^{G}657    | Q440@             | CUB1       | (17)       |             |

a Effectively C193G.

b Replaces last two amino acids of CUB1 with three different residues and then terminates.

medium (Fig. 3B). Presumably, the proprotein was cleaved by a furin-like proprotein convertase to produce the mature protein. On assaying the recombinant tolloid its was found that the short form of tolloid did not cleave Sog (Fig. 3C).

Removal of the EGF-like Domains from Drosophila Tolloid Affects the Secretion and Activity of the Enzyme Produced by S2 Cells—It has been shown that removal of the EGF-like domains from mTLD converts it to a chondinase and more effective C-proteinase (14). To investigate the role of the EGF-like domains in Drosophila TLD, a series of deletion mutants were generated that lacked either, or both, of the EGF-like domains (Fig. 4A). cDNA constructs encoding these proteins were transfected into S2 cells and the cell lysates and cell culture medium analyzed by Western blotting from 24 to 72 h post-transfection (Fig. 4B). Removal of the EGF-like domains markedly reduced the amount of enzyme secreted from S2 cells.

To normalize the amounts of each enzyme and compare the relative Sogase activity for equivalent amounts of enzyme, a range of volumes of cell culture medium obtained 48 h post-transfection were analyzed by Western blotting using the anti-V5 antibody (Fig. 5A). The relative position of the immunoreactive bands indicates that the enzymes were present in both the latent and the mature (prodomain deficient) forms in the cell culture medium. By using equivalent amounts of the active forms of each enzyme in the Sogase assay we were able to show that only the ΔEGF1 TLD enzyme retained any activity, whereas proteins lacking the EGF2 domain were unable to cleave Sog (Fig. 5B).

Sogase Activity Is Reduced by Point Mutations at Conserved Residues in CUB4 and CUB5, But Not by Mutations in EGF1 or EGF2—To investigate further the role of the CUB and EGF domains in TLD, the range of chemically induced mutations in the tolloid gene that affect dorsal-ventral patterning in the Drosophila embryo were collated from published data (16–18) and from Flybase (flybase.bio.indiana.edu) (Table 2). These mutations affect residues throughout the prodomain and regulatory domains, and many of the mutations introduce premature stop codons into the N-terminal half of tolloid (see “Discussion”). It was of particular interest to determine the effects of the single amino acid substitutions located in the C-terminal EGF2, CUB4, and CUB5 domains in light of the observation that these domains are crucial for the Sogase activity of TLD. There are eight substitutions in the CUB domains and one in EGF2. The positions of the substitutions within the CUB domain structure were identified (Fig. 6A) and superimposed onto the crystal structure of a single CUB domain from the spermadhesin porcine seminal plasma PSP-1/PSP-II heterodimer (Fig. 6B). This indicated that the CUB domain mutations cluster in a region that appears to be involved in calcium ion binding. The single mutation in EGF2 affects an asparagine (N) residue that is part of the consensus sequence for Ca^{2+} binding (Fig. 8A).

The substitutions N706I in EGF2, E839K in CUB4, and S915L in CUB5 were engineered into Drosophila tolloid along with a C-terminal HA tag in place of V5-His (Fig. 7A). The E835K mutation in CUB4 was chosen because it is associated with a stronger phenotype than the D884V and D839K mutations in the same domain (Table 2) and it directly affects a conserved Ca^{2+} binding residue (Fig. 6). Furthermore, the equivalent mutation in CUB2 (E517K in TLD) was found to abolish procollagen C proteinase activity when it was introduced into BMP-1 (E483K in BMP-1) (27). cDNA constructs encoding these proteins were transfected into S2 cells and the cell culture medium analyzed by Western blotting using the anti-HA HRP antibody (Fig. 7B). All the enzymes were secreted from S2 cells; however, conversion of the latent N706I and S915L mutants to their mature forms was impaired. To evaluate the relative activity of these enzymes, equivalent amounts of the mature forms of the HA-tagged recombinant proteins were assayed for Sogase activity. Unexpectedly, we discovered that there was no obvious difference between the activity of the N706I mutant compared with TLD-HA.
FIGURE 6. Location of single amino acid substitutions that affect dorsal-ventral patterning in the Drosophila embryo within the CUB domain structure. A, amino acid sequence alignment of the CUB domains from human mammalian tolloid (mTld) and Drosophila tolloid (dTld) was performed using Clustal W (1.82) multiple sequence alignment (www.ebi.ac.uk/clustalw). The β-strand (1–10) and loop (a–i) regions were assigned by direct comparison with Ref. 31. The locations of amino acids whose substitution results in a developmental phenotype are shown in red bold type and underlined. The locations of amino acids involved in Ca$^{2+}$ binding are boxed (32). B, the crystal structure of PSP-1 was used as a template to predict the locations of the amino acid substitutions (arrows) and amino acids involved in calcium ion binding (circles). Substitutions that were subsequently engineered into TLD are boxed.
FIGURE 7. Expression of HA-tagged tolloid in S2 cells and the effect of mutations in EGF2, CUB4, and CUB5 on Sogase activity. A, locations of single amino acid substitutions (numbered) within the HA-tagged tolloid domain structure. HA, HA epitope tag. B, S2 cells were transfected with vectors encoding HA-tagged tolloid constructs containing single amino acid substitutions in the C-terminal EGF and CUB domains. The cell culture medium was analyzed by Western blotting using the anti-HA-HRP antibody 72 h post-transfection. Latent (black star) and mature (black circle) forms of each enzyme were identified. The levels of the mature forms were normalized by densitometry as described under “Experimental Procedures.” C, V5-His-tagged Sog was digested with equal amounts of prodomain-deficient HA-tagged tolloid enzymes with or without mutations in the EGF, CUB4, or CUB5 domains or with an empty vector control (–). The full-length and digested Sog fragments were detected by Western blotting using the anti-V5-HRP antibody. The digests were performed in triplicate at different time points and the activities compared with TLD-HA by the relative amounts of the 45-kDa Sog digestion product present after Western blotting. The 45-kDa product was quantified in preference to the 100- or 25-kDa products because it was readily resolved by electrophoresis and was more consistently observed when enzyme activity was reduced. A graphic representation of the percentage Sogase activity relative to TLD-HA is shown. Closed diamond, TLD-HA; closed square, N760I; closed triangle, E835K; cross, S915L; star, empty vector (–).

DISCUSSION

This study has shown that the C-terminal EGF2, CUB4, and CUB5 domains of TLD are required for cleavage of Sog by TLD. However, these domains are not present in BMP-1, which is a more effective procollagen C proteinase and chordinase than mTLD. In fact, the chordinase activity of BMP-1 only requires one CUB regulatory domain (28). The presence of a tolloid isoform with increased activity in Drosophila could be detrimental by both increasing DPP signaling locally and preventing long-range DPP/SCW signaling (by restricting the diffusion of the intact Sog/DPP/SCW complex). Furthermore, alternative splicing may take longer to complete and result in the production of insufficient quantities of active protein during the short embryonic developmental time frame.

A number of the mutant tolloid alleles (Table 2) also result in premature truncation of the tolloid protein. The engineered truncated tolloid construct, which was longer than any of these predicted gene products (terminating after CUB3), was efficiently secreted and its prodomain removed in cultured S2 cells, but it had no Sogase activity. The alleles are neither weak nor antimorphic, indicating that the mutations cause a significant loss of enzyme activity. If the enzymes are secreted it appears that they are inactive but have no adverse effect on the activity of the normal full-length enzyme produced from the normal allele. Alternatively, these enzymes could be misfolded, retained, and degraded or the introduction of a premature stop codon could result in mRNA destabilization and nonsense-mediated mRNA decay.
Mutational Studies of Drosophila Tolloid

In contrast to the BMP-1-like truncated TLD, the molecules lacking the EGF-like domains were inefficiently secreted from S2 cells. It is likely that removal of these domains results in misfolding and subsequent degradation within the cell. This result is in agreement with the observation that removal of the EGF-like domains from mTLD causes a reduction in secretion (14). However, the reduction was greater for ΔEGF TLDs secreted from S2 cells than for ΔEGF mTLDs secreted from 293 cells. The low levels of secretion meant that it was necessary to compare the activity of these mutants to much lower amounts of TLD than were present in previous assays. Under these conditions, only the 45-kDa Sog cleavage product was observed, and residual activity was only observed for the ΔEGF1 TLD. The enzymes lacking the EGF2 domain had no detectable activity. The EGF2 domain may be important for the Sogase activity of TLD, or the small amounts of ΔEGF2 TLD found in the cell culture medium have escaped the cellular quality control mechanisms that operate in the endoplasmic reticulum (29) and might not be folded into a conformation that allows substrate cleavage. In direct contrast, for mTLD, removal of the EGF-like domains increased enzyme activity despite reducing secretion.

The requirement for Drosophila toolloid to have a complete domain structure to cleave Sog might be explained by a number of differences between the mammalian and Drosophila systems. First, BMPs are not required for BMP-1 to cleave chordin but TLD is unable to cleave Sog unless DPP is present. It has been postulated that the role of DPP is to cause a conformational change within Sog that facilitates proteolysis (11). It might also be the case that DPP or SCW binds to the TLD regulatory domains and sequesters the enzyme into the signaling complex, thereby presenting the active site to the appropriate cleavage sites on Sog. Second, the fact that the domain structures of vertebrate and Drosophila toolloids are identical, yet the mechanism of substrate cleavage appears to be different, suggests that the enzymes could have a markedly different positioning of the CUB domains with respect to each other and to the metalloproteinase domain. Furthermore, the relative contributions of the metalloproteinase domain and the CUB domains to substrate recognition and binding might not be identical. Third, chordin is processed at fewer sites than Sog (2, 7, 24). The amino acid sequences of the cleavage sites identified in chordin are not conserved in Sog (7) though the two sites are in a similar location.3 Therefore it is possible that the three-dimensional structures of chordin and Sog may also not be directly comparable.

This study has confirmed the importance of calcium ions for the Sogase activity of TLD. Ca2+ might be involved in substrate or cofactor binding. Alternatively, Ca2+ could have a structural role in the allosteric promotion of enzyme activity. The mutations that were engineered into the CUB and EGF domains changed residues relevant to Ca2+ binding (Figs. 6B and 8A), but only the mutations in the CUB domains resulted in a loss of enzyme activity. The reduction in activity of the E835K mutant is consistent with the moderate loss of function phenotype of the tldB2 allele, whereas both inefficient removal of the prodomain and some loss of enzyme activity of the S915L mutant probably account for the moderate phenotype of the tldH10 allele.

Activation of TLD, which depends on cleavage by furin or furin-like proprotein convertases between the pro- and metalloproteinase domains, is affected by mutations in CUB5 and in EGF2. This implies that these domains could be close to the pro- and metalloproteinase domains of toolloid in the folded enzyme, activation could depend on the binding of the proprotein convertases to these domains, or the mutations could affect toolloid trafficking and co-localization of the enzymes in the late secretory pathway. It is unlikely that the presence of the enzyme proforms in our assays could exert a dominant negative effect on the activity of the mature enzyme, because the N760I mutation in EGF2 was characterized by inefficient prodomain removal but still retained full activity (Fig. 7). It is unclear whether the weak phenotype of this allele (tldE4) could be accounted for by the slower kinetics of prodomain removal. A large proportion of the toolloid found in embryos is in the latent form (2), indicating that only small amounts of active TLD are required to modulate DPP/SCW signaling during embryonic patterning. However, subtle perturbations to this balance could result in pattern defects. It is possible that TLD has other substrates in vivo, the cleavage of which could be affected by mutations in the EGF-like domains. The phenotype associated with null alleles of toolloid-related also indicates that novel substrates for the toolloid family of enzymes are yet to be identified in Drosophila (11).

The EGF-like repeats of toolloid contain a Ca2+ binding sequence that is conserved except for the replacement of aspartic acid by glutamic acid in the first EGF repeat of mammalian toolloid. Alteration of the equivalent residue in the 21st Ca2+ binding EGF-like repeat of fibrillin-1 results in an inherited connective tissue disorder, manifested by skeletal and ocular defects, known as Marfan syndrome (30). Mutation of this residue to glutamic acid or alanine in TLD had no effect on enzyme activation or substrate cleavage, and the N760I mutation in the second EGF-like repeat also had no effect on Sog cleavage. These results imply that Ca2+ binding to the EGF-like repeats is not crucial for Sogase activity. In support of this conclusion, it has been shown that mammalian

---

3 M. B. O’Connor, University of Minnesota, personal communication.
tolloid lacking the EGF-like repeats still requires calcium ions to cleave type I procollagen (14).

It has recently been shown that the CUB and EGF-like domains specify the activity of the tolloid metalloprotease domains. For example, the relative rates of cleavage of Sog by tolloid and tolloid-related are determined not by the metalloprotease domains but by the CUB and EGF-like repeats (11). Furthermore, mammalian tolloid-like 2 can be converted to a procollagen C proteinase by replacement of its CUB domains with the corresponding domains from BMP-1 (28). These studies have now been extended to show that the most C-terminal CUB domains with the corresponding domains from BMP-1 (28).

Acknowledgments—The Sog and tolloid cDNAs were a generous gift from Dr. Hilary Ashe, University of Manchester, UK. We thank Maria Scott for help with cDNA manipulations and protein expression and Tobias Starborg for assistance with CUB domain visualization.

REFERENCES

1. Balemans, W., and Van Hul, W. (2002) Dev. Biol. 250, 231–250
2. Marques, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W., and O’Connor, M. B. (1997) Cell 91, 417–426
3. Nguyen, M., Park, S., Marques, G., and Arora, K. (1998) Cell 95, 495–506
4. Shimmi, O., Urmulis, D., Othmer, H., and O’Connor, M. B. (2005) Cell 120, 873–886
5. Blader, P., Rastegar, S., Fischer, N., and Strahle, U. (1997) Science 278, 1937–1940
6. Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E. M. (1997) Cell 91, 407–416
7. Scott, I. C., Blitz, I. L., Pappano, W. N., Inamura, Y., Clark, T. G., Steiglitz, B. M., Thomas, C. L., Maas, S. A., Takahara, K., Cho, K. W., and Greenspan, D. S. (1999) Dev. Biol. 213, 283–300
8. Shimell, M. J., Ferguson, E. L., Childs, S. R., and O’Connor, M. B. (1991) Cell 67, 469–481
9. Nguyen, T., Jamal, J., Shimell, M. J., Arora, K., and O’Connor, M. B. (1994) Dev. Biol. 166, 569–586
10. Finelli, A. L., Xie, T., Bossie, C. A., Blackman, R. K., and Padgett, R. W. (1995) Genetics 141, 271–281
11. Serpe, M., Ralston, A., Blair, S. S., and O’Connor, M. B. (2005) Development (Camb.) 132, 2645–2656
12. Takahara, K., Lyons, G. E., and Greenspan, D. S. (1994) J. Biol. Chem. 269, 32572–32578
13. Takahara, K., Brevard, R., Hoffman, G. G., Suzuki, N., and Greenspan, D. S. (1996) Genomics 34, 157–165
14. Garrigue-Antar, L., Francois, V., and Kadler, K. E. (2004) J. Biol. Chem. 279, 49835–49841
15. Greenspan, D. S. (2005) Top. Curr. Chem. 247, 149–183
16. Finelli, A. L., Bossie, C. A., Xie, T., and Padgett, R. W. (1994) Development (Camb.) 120, 861–870
17. Childs, S. R., and O’Connor, M. B. (1994) Dev. Biol. 162, 209–220
18. Ferguson, E. L., and Anderson, K. V. (1992) Genet. 31, 360–362
19. Scambler, G. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 93, 5127–5130
20. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) Science 271, 360–362
21. Li, S. W., Sieron, A. L., Fertala, A., Hujima, Y., Arnold, W. V., and Prockop, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5127–5130
22. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) Science 271, 360–362
23. Yu, K., Srinivasan, S., Shimmi, O., Piehs, B., Rashka, K. E., Kimelman, D., O’Connor, M. B., and Bier, E. (2000) Development (Camb.) 127, 2143–2154
24. Shimmi, O., and O’Connor, M. B. (2003) Development (Camb.) 130, 4673–4682
25. Rothe, M., Pehl, M., Tauritz, H., and Jackle, H. (1992) Nature 359, 156–159
26. Takahara, K., Lee, S., Wood, S., and Greenpan, D. S. (1995) Genomics 29, 9–15
27. Hartigan, N., Garrigue-Antar, L., and Kadler, K. E. (2003) J. Biol. Chem. 278, 18045–18049
28. Petropoulou, V., Garrigue-Antar, L., and Kadler, K. E. (2005) J. Biol. Chem. 280, 22616–22623
29. Kleizen, B., and Braakman, I. (2004) Curr. Opin. Cell Biol. 16, 343–349
30. Kainulainen, K., Karttunen, L., Puhakka, L., Sakai, L., and Peltonen, L. (1994) Nat. Genet. 6, 64–69
31. Sieron, A. L., Tretiakova, A., Jameson, B. A., Segall, M. L., Lund-Katz, S., Khan, M. T., Li, S. W., and Stocker, W. (2000) Biochemistry 39, 3231–3239
32. Gregory, L. A., Thielens, N. M., Arlaud, G. J., Fontecilla-Camps, J. C., and Gaboriaud, C. (2003) J. Biol. Chem. 278, 32157–32164
33. Rees, D. J., Jones, I. M., Handford, P. A., Walter, S. J., Essnouf, M. P., Smith, K. J., and Brownlee, G. G. (1988) EMBO J. 7, 2053–2061
34. Handford, P. A., Mayhew, M., Baron, M., Winship, P. R., Campbell, I. D., and Brownlee, G. G. (1991) Nature 351, 164–167