Molecular Determinants of Xolloid Action in Vivo*

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Xld (Xolloid) is a member of the Tolloid family of metalloproteases found in embryos of the frog *Xenopus laevis*. It cleaves Chordin, an inhibitory binding protein for BMP2/4, releasing fragments with reduced affinity for these important ventralizing signals. As a consequence, increasing Xld activity ventralizes *Xenopus* embryos. We have used this phenotype as an assay to determine the requirement for the C-terminal, nonprotease component of Xld for in vivo activity. This part of the protein is composed of five complement C1r/C1s-sea urchin epidermal growth factor-BMP1 (CUB) and two epidermal growth factor domains, which are thought to be involved in protein-protein interactions and may confer substrate specificity. Our results show that the protease coupled to CUB1 and CUB2 is the minimum domain structure required to ventralize *Xenopus* embryos and to block the dorsal axis-inducing activity of Chordin. Xld-CUB1-CUB2 cleaves Chordin, and a protease-inactive version co-precipitates Chordin. Our results indicate that the first and second CUB domains bind Chordin and present it to the protease domain. Protease-inactive Xld blocks the cleavage of Chordin by wild-type Xld and dorsalizes injected *Xenopus* embryos. We find that protease-inactive Xld-CUB1-CUB2 does not share this activity and that all of the C-terminal domains are required to generate the dorsalized phenotype.

In amphibian embryos, the development of dorsal tissues, such as the nervous system, notochord, and somites, is dependent upon the Spemann organizer, a signaling center that is localized to the dorsal marginal zone of gastrulae (1–3). Grafting this region to the opposite side of the embryo produces a second dorsal axis in which most of the tissues, including the nervous system, are derived from the host (1, 4). Several transcripts have been identified that are localized to the dorsal marginal zone and exhibit organizer activity when injected into ventral regions of the embryo. They encode secreted proteins, such as Chordin, Noggin, and Follistatin (5–7). Depletion studies have shown that all three proteins are required for the development of dorsal tissues (8), although depleting Chordin alone is sufficient to block induction of a second dorsal axis by the organizer (9). Chordin, Noggin, and Follistatin bind bone morphogenetic proteins (BMPs) in the extracellular space and prevent them from activating their receptors (10–12). BMPs are important ventralizing signals in amphibian embryos (13–16), and inhibiting their function is necessary for dorsal development (8, 17, 18).

The BMP inhibitory activity of Chordin is regulated by members of the Tld (Tolloid) family of metalloproteases (19). Members of this family cleave Chordin at two locations (20, 21), generating fragments with reduced affinity for BMP4 (22). As a consequence, BMP signaling increases when these proteases are overexpressed, and the resulting embryos are ventralized (20, 23, 24). The phenotype, reduced head and notochord coupled with expansion of ventral tissues, is identical to that of Chordin-depleted embryos (9). Inhibiting these proteases produces dorsalized embryos with an enlarged head and notochord and reduced ventral tissues, presumably because uncleaved Chordin accumulates in the embryo and reduces BMP signaling (20, 25–27). Thus, the Tld family regulates dorsal-ventral patterning by modulating activity levels of ventralizing BMPs (28). A similar mechanism operates during the cellular blastoderm stage of *Drosophila* development, where Tld is required for dorsal, rather than ventral, development (29). *Drosophila* Tld regulates development by cleaving SOG (short gastrulation), an orthologue of Chordin (30, 31).

Tlds are complex proteins with several modules in addition to the protease domain. They are synthesized with an N-terminal signal peptide and proregion, which must be removed for biological activity (32). The active protease domain is coupled to C-terminal CUB and EGF-like domains with the arrangement CUB1-CUB2-EGF1-CUB3-EGF2-CUB4-CUB5 (19, 33). BMP1, a major splice variant of vertebrate Tld, has the arrangement protease-CUB1-CUB2-EGF1-CUB3. These C-terminal domains appear to be involved in binding substrates, with strongest binding requiring both CUB and EGF domains (34). The full complement of CUB and EGF domains is not required for cleavage of substrates in vitro by mammalian Tlds, with CUB1 and/or CUB2 often sufficient (35–37). Indeed, efficient cleavage of probiglycan has been reported for the protease domain alone for both BMP1 and mTLL1 (mammalian Tld-like 1) (37). However, the full complement of CUB and EGF domains is required for cleavage of SOG by *Drosophila* Tld (38).

Xld (Xolloid) is a member of the Tld family found in embryos of the frog *Xenopus laevis* (23). It ventralizes

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* The abbreviations used are: BMP, bone morphogenetic protein; CUB, complement-sea urchin epidermal growth factor-BMP1; EGF, epidermal growth factor; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol; HA, hemagglutinin.
CUB Domain Specificity of Xld

injected Xenopus embryos, blocks the dorsalizing activity of co-injected Chordin, and cleaves Chordin in vitro (20, 23). Dominant negative versions block Xld-dependent cleavage of Chordin and dorsalize injected Xenopus embryos (20, 25). We have used these in vivo assays to determine the role of the C-terminal CUB and EGF domains in Xld activity. Our results show that only CUB1 and CUB2 are required for the ventralizing activity of Xld, indicating that the most distal domains stabilize interactions with this substrate. Stable interactions between Xld and Chordin were demonstrated by co-immunoprecipitation.

EXPERIMENTAL PROCEDURES

Xenopus Embryos—Female X. laevis were induced to lay eggs by subcutaneous injection of human chorionic gonadotrophin (Chorulon, Intervet). Eggs were fertilized with a macerated testis and dejellied with 2% Ficoll (Sigma), with capped synthetic mRNAs and defects scored at early tailbud stages. Animal caps (20 per experiment) were isolated from late blastulae, in normal amphibian medium, and then incubated in a microcentrifuge tube for 6 h (at 18 °C) in 25 μl of calcium- and magnesium-free modified Barth’s saline (39). Animal caps do not undergo wound healing under these conditions, allowing secreted proteins to diffuse into the incubation medium. Both media and cells were collected for Western blot analysis. For co-precipitation experiments, injected late blastulae (5 embryos/experiment) were bisected and incubated in 500 μl of calcium- and magnesium-free modified Barth’s saline for 6 h (at 18 °C).

DNA Constructs—PCR was used to make Xld deletion constructs, using pCS2+ Xld-Myc (23) as a template. The entire plasmid was copied with Pfu turbo polymerase (Stratagene) for 16 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 18 min), excluding domains to be deleted, using the following primers: C-term-F, 5'-TTCAGGATCCCTTAC-3'; C2-F, 5'-TGGGGAGGAGACATCAAAAAAGACTCTGG-3'; C3-F, 5'-TGGGGTTTTATTACACAAC-3'; MP-R, 5'-TGGTGGGCAATTATAGGTTTCAC-3'; C1-R, 5'-TATAGCTCTATATGGCTGCAGCA-3'; C2-R, 5'-TTAAAAAATGCGAGAAA-3'; E1-R, 5'-TGCGGCTTACACATT-3'; C3-R, 5'-TTCACTCTTATCAAGAAAA-3'; C4-R, 5'-ACCAAGCCCTTTACTACGTCTAGG-3'.

Each construct (Fig. 1) was sequenced to confirm the accuracy of the deletion. Protease-inactive versions were generated by deleting the Sphl-Notl fragment of pCS2+ XldY296N (20), which encodes the C-terminal CUB and EGF domains, and replacing it with the Sphl-Notl fragment of pCS2+ Xld-Myc deletion constructs. This strategy was not possible for pCS2+ Xld'Y296N'C2, which was created by PCR using pCS2+ Xld'Y296N'C1-C2 as a template. Constructs were linearized with Notl and transcribed with SP6 polymerase using the mMessage mMachne in vitro transcription kit (Ambion). Synthetic mRNA was purified on RNeasy spin columns (Qiagen) and stored in RNase-free water (Sigma).

Protein Analysis—Animal caps and embryos were homogenized (100 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, protease inhibitor mixture (Roche Applied Science)) and then microcentrifuged (10 min at 13,000 rpm) to remove yolk platelets. Cell extracts from one animal cap and half the culture medium were loaded onto Novex 4–12% BisTris gradient gels (Invitrogen) for SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare) using a semidry blotter (Bio-Rad). Xld-Myc and Chordin-HA were detected by ECL, according to the manufacturer’s instructions (GE Healthcare), using either anti-Myc (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-HA (3F10; Roche Applied Science) antibodies. For co-precipitation of Xld and Chordin, 50 μl of modified Barth’s saline, 50 μl of 10 mg/ml bovine serum albumin, and 2.5 μl of 9E10 antibody (Santa Cruz Biotechnology) were added to 500 μl of culture medium and incubated overnight at 4 °C. Antibodies were pulled down by adding 50 μl of protein L-agarose (Santa Cruz Biotechnology) and incubating for 2 h (at 18 °C) while rotating. Samples were washed at least four times and released from the bead by adding SDS-PAGE sample buffer (+β-mercaptoethanol) and boiling. All of the immunoprecipitate and one-quarter of an embryo of cell extract were used for SDS-PAGE.

RESULTS

Secretion Analysis of Xld Deletion Mutants—A series of deletion mutants were constructed for Xld, removing specific C-terminal CUB and EGF domains (Fig. 1). Six Myc tags were added to the C terminus to enable detection on Western blots. Since Xld is an extracellular protease, we first analyzed the effect these deletions had on secretion by Xenopus embryonic cells. Embryos were injected with Xld mRNAs, and animal caps were isolated from late blastulae (Fig. 2A). They were cultured in dissociation medium that allows the accumulation of secreted proteins. Western blots of cell extracts showed that all mRNAs were translated, producing proteins that were larger, because of glycosylation (23), than predicted from the amino acid sequence (Fig. 2B). Smaller proteins were also detected for Xld, XldC2, and XldC1–C3, which we believe are N-terminally processed and active proteases. Most of the deletion mutants were also found in the culture medium, the exceptions being

![FIGURE 1. Domain structure of wild-type and truncated Xld. Shown are schematic diagrams of wild-type and C-terminally truncated versions of Xld used in this study. P, proregion; MP, metalloprotease domain, C1–5, CUB domains; E, EGF domains (×2); M, Myc tag, 9E10 epitopes.](image-url)
XldC3–C5 and XldC1+C3–C5. For Xld, XldC2, and XldC1–C3, the predominant protein in the medium was the shorter N-terminally processed form. The longer, unprocessed, form predominated in the medium of animal caps expressing XldC1 and XldC1-C2, but shorter forms were also detected. The preponderance of N-terminally processed forms in the culture medium is consistent with cleavage late in the secretory pathway, as previously demonstrated for human BMP1 (32). Our results show that the distal CUB and EGF domains are not required for either secretion or N-terminal processing of Xld.

**CUB1 and CUB2 Are Required for the Ventralizing Activity of Xld**—To test the activity of the Xld deletion mutants we used an in vivo assay in which synthetic mRNA was injected into Xenopus embryos (Fig. 3A). Injection of full-length xld mRNA caused mild ventralization of Xenopus embryos, which displayed a reduced head size and an expansion of ventral-posterior structures (Fig. 3C). Previous studies have shown that this is caused by a mild ventralization of the mesoderm, with loss of the notochord and expansion of the ventral blood island (20–23). The same phenotype was observed for embryos injected with either xldC1–C3 or xldC1-C2 mRNA (Fig. 3, D and E), whereas embryos injected with either xldC1, xldC2, xldC3–C5, or xldC1+C3–C5 mRNA were always normal (Fig. 3, G and H). The absence of secretion and N-terminal processing can explain the lack of activity associated with XldC3–C5 and XldC1+C3–C5 but not XldC1 or XldC2. Inactive XldC2, for example, is processed and secreted as efficiently as active XldC1–C3 (Fig. 2B). All of the active proteins included the pro tease domain and at least the first two CUB domains, whereas the inactive proteins lacked one or more of these domains. Our results show that the first and second CUB domains are the minimal C-terminal domains required for the ventralizing activity of Xld.

**CUB1 and CUB2 Are Required to Counteract Chordin**—Our second in vivo assay took advantage of the ability of full-length Xld to counteract the dorsal axis-inducing activity of Chordin (20). Chordin is normally expressed on the future dorsal side of Xenopus gastrulae, and injection into ventral blastomeres induces a second dorsal axis, albeit lacking a head (7). We found that injection of chordin mRNA induced a second dorsal axis in 58% of cases (Fig. 4B), an activity that was completely blocked by coinjecting xld mRNA (Fig. 4C). Axis induction by chordin was also completely blocked by coinjecting either xldC1–C3 or xldC1-C2 mRNAs (Fig. 4, D and E) but not by coinjecting either xldC1, xldC2, xldC3–C5, or xldC1+C3–C5 mRNAs (Fig. 4, F–H). These results demonstrate that only ventralizing mutants, containing both CUB1 and CUB2, can block the dorsalizing activity of Chordin. As a further test of the activity of Xld deletion constructs, we used the animal cap assay (Fig. 2A) to look at cleavage of Chor-
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FIGURE 4. CUB1 and CUB2 are required for Xld to block Chordin. Xenopus embryos were co-injected (two ventral blastomeres at the 4-cell stage) with 800 pg of chordin mRNA and 800 pg of xld mRNAs and incubated until sibling controls (A) had reached stage 26. B, injecting chordin (Chd) alone induces a second dorsal axis (*) that lacks a head. (58%, n = 139). Axis induction by Chordin is blocked by coinjecting either xld (0%, n = 77) (C), xldC1–C3 (0%, n = 74) (D), or xldC1–C2 (0%, n = 74) (E) mRNAs but not by coinjecting either xldC1 (45%, n = 82) (F), xldC2 (69%, n = 35) (G), or xldC3–C5 (28%, n = 76) (H) mRNAs.

din. Animal caps were isolated from embryos co-injected with chordin and xld mRNAs, and Western blots were used to detect Chordin protein. A protein of 120 kDa was detected in cell extracts and media for all injections, whereas a shorter protein of 96 kDa was only detected in the medium of animal caps co-injected with either xld or xldC1–C2 mRNAs (Fig. 5B). XldC1–C3 was not tested in this assay, and XldC1 and XldC2 did not produce the 96-kDa protein. We note that the 120-kDa protein is always less intense in medium for animal caps co-injected with xld, but the 96-kDa protein is never detected even after extreme overexposure. Xld cleaves Chordin at two sites (20), after the first and third cysteine-rich domains (Fig. 5A). Cleavage at the first site will generate the 96-kDa fragment observed in this study, whereas cleavage at the second site will generate a 16-kDa fragment that was never observed, probably because it is too small for the gel system we used. The results show that only ventralizing Xld mutants cleave Chordin at the N-terminal cleavage site and that the first and second CUB domains are the minimal C-terminal domains required for chordinase activity.

CUB1 and CUB2 Are Required for Xld to Bind Chordin—Our results suggest that the first and second CUB domains are required for Xld to interact with its substrate Chordin. To test this, we introduced a point mutation, Y296N, that inactivates the metalloprotease domain (Fig. 6A). We reasoned that this would permit more stable interactions with Chordin than active Xld, which would cleave Chordin. This might allow co-precipitation of Xld and Chordin, as demonstrated in Drosophila for protease-inactive Tld and its substrate SOG (40). Embryos co-injected with chordin and xld mRNAs were incubated in dissociation medium for 6 h, when both medium and cell extracts were collected. Western blots of cell extracts showed that all mRNAs were translated, producing proteins of the expected size (Fig. 6B). Anti-Myc antibodies were used to immunoprecipitate Xld constructs from the media and Chordin detected on Western blots with anti-HA antibodies. As expected, Chordin was co-precipitated by XldY296N, XldY296NC1–C3, and XldY296NC1–C2, but not by XldY296NC1 (Fig. 6B). Thus, CUB1-CUB2 constitute the minimum domain required for Xld to interact with Chordin.

CUB1 and CUB2 Are Not Sufficient for Dominant Negative Xld Activity—Previous studies have shown that full-length XldY296N acts as a dominant negative mutant, inhibiting cleavage of Chordin by active Xld and dorsalizing Xenopus embryos (20). Injected embryos have expanded dorsal-anterior structures at the expense of ventral-posterior ones (Fig. 7B), the opposite phenotype to wild-type Xld. We have used this phenotype as an assay to measure the requirement for CUB and EGF domains. As expected, XldY296NC1, XldY296NC2, and XldY296NC1+C3–C5 had no discernible effect on Xenopus development (data not shown), consistent with lack of activity in the preceding assays. Surprisingly, XldY296NC1–C2 and XldY296NC1–C3 also had no discernible effect on injected embryos, despite having the minimal structure required for binding Chordin (Fig. 6B). This was not because of poor translation, since Western blot analysis showed that all constructs were translated efficiently (see Fig. 6B). Thus,
Although the first and second CUB domains are sufficient for the ventralizing activity of wild-type Xld, they are not sufficient for the dorsalizing activity of the Y296N mutant. We next made a construct in which only the fifth CUB domain was deleted and introduced the Y296N point mutation. Injecting mRNA for this construct had no discernible effect on Xenopus development, demonstrating that all of the C-terminal domains are required for the dorsalizing activity of XldY296N.

Finally, we have looked to see if XldY296N deletion constructs can act as dominant negatives, using the axis-inducing assay. Chordin mRNA was injected into ventral-vegetal blastomeres at the 8-cell stage, inducing a second dorsal axis in 58% of cases (Fig. 8). As expected, including xldC1–C5 mRNA in the injection reduced the frequency of duplicated axes to just 11%. The frequency of secondary axes was increased to 66% by adding a third mRNA, xldY296NC1–C5, to the injection, consistent with the reported dominant negative activity of this mutant (20). In contrast, none of the deletion constructs we tested, XldY296NC1-C2, XldY296NC1–C3, and XldY296NC1–C4, increased the frequency of secondary axes when coexpressed with Chordin and XldC1–C5 (Fig. 8). Although the first and second CUB domains are sufficient for Xld to bind Chordin, they are not sufficient to confer dominant negative activity on XldY296N.

**DISCUSSION**

To determine the minimal structure required for Xld activity, we have used in vivo assays in which Xld is overexpressed in Xenopus embryos. Injection of full-length xld mRNA into each blastomere at the 2-cell stage weakly ventralizes Xenopus embryos, which display a reduced head and enlarged posterior (20, 23). Internally, dorsal tissues are either absent (notochord) or reduced (nervous system), and the ventral blood island is expanded. Our results show that only the protease and the first and second CUB domains are required for this phenotype, with constructs lacking either of these domains having no discernible effect on embryos. One explanation for the lack of activity observed with mutants lacking either CUB1 or CUB2 is that these domains are required for the secretion of this extracellular protease, as suggested by a report that CUB1 is required for...
secretion of human BMP1 (35). Indeed, we find that XldC3–C5 and XldC1+C3–C5, which are inactive in the ventralization assay, are not secreted by animal cap cells (Fig. 2B). However, the inactive mutants XldC1 and XldC2 and all of the active mutants are secreted by animal cap cells. Hence, lack of secretion cannot account for lack of activity in all cases. A second explanation is that either CUB1 or CUB2 is required for N-terminal processing, which is necessary for the protease activity of Tlds (32). Yet all of the secreted mutants appear to be N-terminally processed, including the inactive mutants XldC1 and XldC2. Thus lack of N-terminal processing cannot account for lack of activity.

To date, the only known substrate for Xld is Chordin, although studies on its mammalian homologues suggest that there may be many more (33). Chordin is a secreted protein that directly binds and inhibits BMP2 and BMP4 (10), two molecules required for the development of ventral-posterior fates in Xenopus embryos (16). Xld cleaves Chordin at two locations (20), releasing fragments with reduced affinity for BMPs (22). As a consequence, Xld increases BMP signaling, providing an explanation for the ventralized phenotypes of Xld-injected embryos (20, 23). Consistent with this view, identical phenotypes are obtained following injection of xld mRNA, low concentrations of BMP4 mRNA (13, 15), and injection of antisense morpholino-oligonucleotides that block translation of chordin mRNA (9). Chordin is normally synthesized in the dorsal mesoderm of early gastrulae, and expression in ventral mesoderm causes the formation of a second dorsal axis (7). This phenotype can be blocked by co-expressing full-length Xld (20), and we have used this as a second in vivo assay to measure the activity of Xld deletion constructs. Our results were entirely consistent with the ventralization assay, with protease-CUB1-CUB2 being the minimum domain structure required to block axis induction by Chordin. We used the animal cap assay to show that this is also the minimum domain structure required for normal cleavage of Chordin by Xld. Surprisingly, Chordin levels were consistently reduced by XldC1, although the specific 96-kDa cleavage product was not detected. Recently, Wermter et al. (41) have shown that BMP1 lacking all the C-terminal domains initially cleaves procollagen VII at the same site as full-length BMP1. However, proteolysis then continues until the substrate is completely degraded. Perhaps something similar is occurring here with XldC1. Whatever the explanation, degradation of Chordin by XldC1 must be specific to in vitro conditions, since XldC1 does not block the dorsaling activity of Chordin in vivo.

CUB and EGF domains are thought to be involved in protein-protein interactions and may confer substrate specificity to the attached protease (19). For example, BMP1 lacking all of the C-terminal domains can cleave proteins that are not substrates for the full-length protease (41). The C-terminal domains of mammalian Tld have been shown to bind procollagen I, with greatest affinity requiring both the CUB and EGF domains (34). Binding is highest for the more C-terminal domains, which may also explain why mammalian Tld interacts with the ECM to a greater extent than its shorter splice variant BMP1 (42). In Drosophila, the C-terminal CUB and EGF domains appear to regulate the reaction kinetics of Tld and its homologue Tlr (Tld-related). Both proteases cleave SOG at similar sites, but Tld has much faster kinetics (40). When the protease domains are swapped, the faster kinetics are associated with the C-terminal domains. The C-terminal domains of Tld may present SOG to the protease domain more efficiently than those of Tlr. Domain swaps have also shown that the C-terminal domains of BMP1 are sufficient to confer chordinase activity on mTLL2 (36), which normally does not have this activity (21). All of this suggests that the first and second CUB domains of Xld should be sufficient to confer binding to its substrate Chordin. We have shown that this is true by co-precipitating Chordin with a catalytically inactive mutant coupled to CUB1 and CUB2. Presumably, the catalytically inactive form was necessary because it forms more stable interactions with Chordin than wild-type Xld, since the latter cleaves Chordin.

To generate catalytically inactive Xld, we used mutagenesis to replace tyrosine 296 with asparagine (20). Tyrosine 296 is located within the protease domain and is conserved in all members of the Tld family. This mutation generates a strong antimorphic allele in Drosophila Tld (43, 44) and dominant negative activity in Xld (20). Full-length XldY296N blocks the chordinase activity of co-expressed wild-type Xld and dorsalizes Xenopus embryos. Injected embryos display an enlarged head, nervous system, and notochord as well as reduced ventral blood island and posterior structures. The most probable explanation is that XldY296N forms stable complexes with Chordin, blocking access to the endogenous wild-type protease. We have used this dorsalized phenotype as an assay to measure the requirement of the C-terminal CUB and EGF domains for the function of Xld in vivo. To our surprise, we found that all of the C-terminal domains were required for the dorsalizing activity of XldY296N. Presumably, the first and second CUB domains are sufficient for stable interactions with Chordin but not sufficient to prevent access to the wild-type protease. Consistent with this, we find that all of the CUB domains are necessary for XldY296N to act as a dominant negative. One explanation is that the most C-terminal CUB and EGF domains increase the binding affinity for Chordin, enabling full-length Xld to displace the truncated XldY296N constructs from this substrate. This is supported by a study on the binding affinities of the CUB and EGF domains of human Tld for its substrate procollagen I (34). Binding affinity was greatest for constructs that included the most C-terminal CUB and EGF domains. A second explanation is that the most C-terminal CUB and EGF domains are required for interactions with an additional protein that might enhance the chordinase activity of Xld. Enhancer proteins, which lack intrinsic enzymatic activity, have been described for the C-proteinase activity of Tlds, but they have no effect on their chordinase activity (36, 45). PCPE1 (procollagen C-proteinase enhancer 1) binds both procollagen I and mTLL1, an interaction that requires the full complement of C-terminal CUB and EGF domains (37). The full complement of C-terminal CUB and EGF domains is also required for PCPE1 to enhance the C-proteinase activity of BMP1 (36). A candidate for an enhancer of the chordinase activity of Xld is Tsg (twisted gastrulation), which can ventralize Xenopus embryos and inhibit axis duplication by Chordin (46, 47, 48). Tsg does not possess an intrinsic protease activity but forms a ternary complex with Chordin and BMP4 and enhances the cleavage of Chordin by...
Xld (49). Further studies will be required to clarify the requirement of the most C-terminal CUB and EGF domains for the dominant negative activity of Xld<sup>296N</sup>.

In summary, our results show that the first and second CUB domains of Xld metalloprotease are required for interactions with its substrate Chordin. However, a much larger domain structure is required for the dominant negative activity of protease-inactive Xld, indicating that the distal CUB and EGF domains may increase the binding affinity for Chordin and/or be required for interactions with other proteins.

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