Regulation of Bone Morphogenetic Protein-4 Activity by Sequence Elements within the Prodomain*

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Bone morphogenetic protein-4 (BMP-4) is synthesized as a large precursor protein, which undergoes proprotein convertase-mediated proteolytic maturation along the secretory pathway to release the active ligand. Pro-BMP-4 is initially cleaved at a consensus furin motif adjacent to the mature ligand domain (the S1 site), and this allows for subsequent cleavage at an upstream motif (the S2 site). This sequential cleavage liberates a small, evolutionarily conserved, prodomain fragment (the linker peptide) of unknown fate and function. Here we show that the linker domain is essential for proper folding, exit from the endoplasmic reticulum, and thus cleavage of the BMP-4 precursor when overexpressed in Xenopus oocytes and embryos but not in cultured mammalian cells. Mature BMP-4 synthesized from a precursor in which the S1 site is non-cleavable, such that the linker domain remains covalently attached to the ligand, has little or no activity in vivo. Finally, analysis of folding, cleavage, and bioactivity of chimeric precursors containing the BMP-7 prodomain and BMP-4 mature domain, or vice versa, with or without the BMP-4 linker domain revealed that the linker domain is only functional in the context of the BMP-4 prodomain and that differential cleavage around this domain can regulate the activity of a heterologous ligand.

Bone morphogenetic protein-4 (BMP-4),³ a member of the transforming growth factor-β (TGF-β) superfamily, was first isolated for its ability to induce ectopic cartilage and bone formation. Subsequently, BMP-4 was shown to be involved in a wide variety of biological processes including cell proliferation and differentiation, apoptosis, and cell fate determination. BMP-4 has been established as a morphogen that plays essential roles during embryonic development (1). The mature BMP-4 ligand is a functional dimer harboring seven cystine residues, six of which are involved in intramolecular disulfide linkage, and the seventh of which is responsible for dimer formation (2), BMP-4 is synthesized as a large inactive precursor that is proteolytically cleaved following a multibasic motif (-RXKR-, termed the S1 site) to generate the active C-terminal ligand (3). Cleavage is carried out by specific member(s) of the proprotein convertase (PC) family of serine proteases (4, 5). The best characterized member of this family, furin, recognizes the preferred consensus sequence, -RX(K/R)R-, but can also cleave following the minimal sequence -RXXR- (6).

We have shown that BMP-4 undergoes a second cleavage at a minimal furin consensus motif (-RXXR-, the S2 site) located upstream of the S1 site within the prodomain (7). Paradoxically, although cleavage of pro-BMP-4 occurs sequentially (S1 and then S2), and the initial cleavage at the S1 site releases the mature ligand, subsequent cleavage at the S2 site regulates the activity and the signaling range of mature BMP-4 (7). In Xenopus embryos, BMP-4 synthesized from exogenous precursor in which the S2 site is non-cleavable is less active, signals over a shorter range and accumulates at lower levels than does BMP-4 cleaved from native precursor. Conversely, pro-BMP-4 in which the upstream site is mutated to an optimal furin motif is rapidly and simultaneously cleaved at both sites and this generates a ligand that is more active and signals over a greater range than does BMP-4 cleaved from native precursor.

Biochemical analysis of Pro-BMP-4 maturation in Xenopus oocytes suggests that the precursor is first cleaved at the S1 site in the trans–Golgi network (TGN), at which stage the prodomain remains non-covalently associated with the mature ligand (8). This complex then traffics to a post-TGN compartment, where the more acidic environment unmasks and facilitates cleavage at the second site. S2 cleavage breaks the non-covalent association between mature BMP-4 and the prodomain, and the free ligand is stable and able to signal over long range. If cleavage at the S2 site does not occur, the prodomain/ligand complex is targeted to the lysosome for degradation. As a result, mature BMP-4 in complex with the prodomain signals only at short range, to nearby cells. Cleavage at the S2 site therefore determines how much BMP-4 is available for signaling.

Analysis of mice carrying a point mutation that prevents S2 processing has shown that cleavage of the S2 site is essential for normal development and, more importantly, suggests that this site might be selectively cleaved in a tissue-specific fashion (9). Specifically, these mice show severe loss of BMP-4 activity in some tissues, such as testes and germ cells, whereas other tissues that are sensitive to BMP-4 dosage, such as the limb, dorsal vertebrae, and kidney, develop normally. In a haploinsufficient background, inability to cleave the S2 site leads to embryonic and postnatal lethality because of defects in multiple organ systems. These data support the hypothesis that differential use of the S2 site provides a mechanism for tissue specific

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³ The abbreviations used are: BMP-4, bone morphogenetic protein 4; HA, hemagglutinin; TGF, transforming growth factor; ER, endoplasmic reticulum; DMZ, dorsal marginal zone; VMZ, ventral marginal zone; TGN, trans-Golgi network; Endo H, endoglycosidase H; PNGase, N-glycosidase F; DAI, dorsoanterior index.
regulation of BMP-4 activity, although biochemical analysis of endogenous pro-BMP-4 cleavage products will be required to substantiate this theory.

Sequential cleavage of pro-BMP-4 liberates a small prodomain fragment (the linker peptide) of unknown fate and function. Primary and upstream furin cleavage motifs are present in all known vertebrate BMP-2 and BMP-4 precursor proteins, and in their Drosophila ortholog, DPP (illustrated in Fig. 2A), and the primary amino acid sequence is more highly conserved across species within the linker domain than within the remainder of the prodomain. Thus, the linker domain may play an essential role in pro-BMP-4 maturation, possibly by providing the signals that direct intracellular trafficking of the cleaved ligand to either degradatory or secretory/recycling pathways. On the other hand, BMP-7 and other family members have only a single furin consensus site and lack a linker domain. This raises the possibility that this region functions merely as a spacer between the two cleavage sites in BMP-4 and is dispensable, or even inhibitory for ligand formation. Consistent with this possibility, a mutant form of pro-BMP-4 in which the linker domain is rapidly removed by simultaneous cleavage of the S1 and S2 sites is more active than that generated by sequential cleavage of the precursor, or by cleavage at the S1 site alone (7).

In the current study we perform structure-function analyses to examine the role of the BMP-4 linker domain. Our results show that the linker domain functions in concert with upstream sequences within the prodomain to ensure proper folding and thus cleavage of pro-BMP-4.

EXPERIMENTAL PROCEDURES

cDNA Constructs—A cDNA encoding Xenopus BMP-7 containing a FLAG tag in the prodomain was obtained from Dr. Ken Cho (10). Sequence encoding a Myc epitope tag was inserted into the mature domain of BMP-7, 26 amino acids downstream of the cleavage site, using a PCR-based approach. cDNAs encoding HA- and Myc-tagged native and S1 cleavage mutant forms of pro-BMP-4 have been described previously (8). cDNAs encoding deletion mutant and chimeric precursors were made using the splicing by overlap extension method (11) or by PCR-mediated introduction of appropriate restriction sites.

Embryo Culture and Manipulation—Xenopus eggs were obtained, embryos injected with synthetic capped RNA and cultured as described (12). Embryonic stages are according to Nieuwkoop and Faber (13). For animal cap assays, RNA was cultured as described (12). Embryonic stages are according to Nieuwkoop and Faber (13). For animal cap assays, RNA was cultured as described (12). Embryonic stages are according to Nieuwkoop and Faber (13). Nieuwkoop and Faber (13).

RNA Isolation and Analysis—Total RNA was isolated and Northern blots were hybridized with antisense riboprobes as described previously (14). Radiolabeled bands were visualized using a Molecular Dynamics phosphorimager and quantified using the Macintosh IP lab gel program.

Protein Expression in Oocytes—Oocytes (stage VI) were isolated, injected, and cultured as described earlier (8). The precursor and cleavage products were immunoprecipitated from oocyte lysates and/or culture media, by incubating with 12Ca5 (HA), M2 (FLAG), or 9E10 (Myc) antibodies along with protein A-Sepharose beads overnight at 4 °C. Beads were washed and bound protein denatured by boiling in presence of 0.5% SDS with or without 1% β-mercaptoethanol followed by deglycosylation with endoglycosidase H (Endo H), or peptide: N-glycosidase F (PNGase F). Radiolabeled proteins were resolved on an 11% SDS-polyacrylamide gel, fixed in 15% methanol, 7.5% acetic acid, dried, and exposed either to a phosphorimager screen or Kodak Biomax MS film at −70 °C.

Transient Transfection and Western Blot Analysis—Cells were cultured in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum. BMP constructs were expressed in HEK293 cells by transient transfection of the plasmids harboring them (2 μg) using Lipofectamine 2000 as per the manufacturer’s protocol. Cells were cultured for 60–70 h in OptiMEM. Proteins were precipitated from conditioned media using 10% trichloroacetic acid. The cells were lysed in radioimmunoprecipitation assay buffer (50 mm Tris–Cl, pH 8, 150 mm NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) with protease inhibitors, cleared by centrifugation and protein estimated by BCA assay (Pierce). Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Membranes were probed with anti-Myc (9E10) antibodies and immunoreactive proteins were detected using Enhanced Chemiluminescence reagent (Pierce).

In Vitro Cleavage with Furin—[^35S]Met/Cys labeled pro-BMP was synthesized using rabbit reticulocyte lysates and proteins immunoprecipitated using antibodies specific for the tags in the protein. Digestion was carried out using recombinant human furin at neutral pH (7.0) as described (8). All results were confirmed in a minimum of three independent cleavage reactions.

Alkaline Phosphatase Assay—C2C12 myoblastic cells were plated in a 24-well plate in Dulbecco’s modified Eagle’s medium +10% fetal bovine serum. Cells were allowed to grow for 12–18 h. The medium was subsequently replaced with Dulbecco’s modified Eagle’s medium containing 0.25% fetal bovine serum and 1 μm all-trans-retinoic acid (Sigma) in the presence or absence of conditioned media from HEK cells transfected with cDNAs encoding wild type or linker deletion mutant BMP-4. After 48 h, cells were lysed by adding alkaline phosphatase extraction buffer containing 0.1 m Tris, pH 9, 0.2 m NaCl, 0.2% Nonidet P-40, 0.2% Triton X-100, and 1 mm MgSO4 with 10 μg/ml aprotinin and 1 mm phenylmethlysulfonyl fluoride. The dish was incubated at 4 °C with shaking for 30 min. Cleared lysate (0–150 μl) was incubated with 250 μl of substrate (1 mg/ml p-nitrophenyl phosphate, Sigma) for 30 min at room temperature. The reaction by stopped by adding 50 μl of 3 N NaOH per 200 μl of reaction mix, and absorbance was measured at 405 nm.

RESULTS

The Linker Domain Is Essential for Proper Folding and Cleavage of pro-BMP-4 in Vivo—To determine whether the highly conserved sequence between the S1 and S2 cleavage sites is required to generate functional BMP-4, a cDNA encoding an epitope-tagged deletion mutant form of pro-BMP-4 lacking the 31 amino acids comprising the linker domain as well as the S2 site (BMP-4(ΔS2L)) was constructed (illustrated above Fig. 1A).
Structure-Function Analysis of the BMP-4 Prodomain

We have previously shown that the HA tag in the prodomain and the Myc tag in the mature domain have no effect on the biological activity of the encoded ligand (8). Pro-BMP-4(ΔS2L) contains an optimal furin motif at the S1 site (RSKR), which, if cleaved, will produce wild-type mature BMP-4.

To test whether deletion of the linker domain affects the biological activity of BMP-4, we carried out animal cap assays in Xenopus embryos. Two cell embryos were injected with RNA (600 pg) encoding pro-BMP-4 or pro-BMP-4(ΔS2L) and cultured until stage 8 (blastula), at which point the ectodermal layer (animal cap) was removed. RNA was isolated from explants when sibling embryos reached stage 10.5 and expression of the BMP-4 target gene Xbra was analyzed by Northern blot hybridization. Xbra expression was detected in animal caps isolated from embryos expressing wild-type pro-BMP-4 but not pro-BMP-4(ΔS2L) (Fig. 1A). Thus, the linker domain is required to generate functional BMP-4 in vivo.

To ask whether amino acid residues adjacent to the cleavage site and contained within the linker domain are required for furin to recognize its consensus motif, we assayed the ability of furin to cleave pro-BMP-4(ΔS2L) in vitro. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysates, immunoprecipitated using an antibody to the Myc tag in the mature domain and subjected to in vitro cleavage with recombinant human furin. As shown in Fig. 1B, the linker deletion mutant precursor was cleaved in vitro, in the presence of recombinant furin.

To determine whether the linker domain is required for proper dimerization, folding, and/or cleavage of pro-BMP-4 in vivo, we examined maturation and processing of wild-type and linker deletion mutant forms of pro-BMP-4 in Xenopus oocytes. Oocytes were injected with capped mRNA encoding pro-BMP-4 or pro-BMP-4(ΔS2L) (5 ng) along with [35S]Met/Cys. Precursor and mature ligand were immunoprecipitated from oocyte lysates using antibodies against the Myc epitope, deglycosylated and analyzed by SDS-PAGE under non-reducing conditions (Fig. 1C, upper panel). As a control, oocytes were injected with [35S]Met/Cys, in the absence of RNA and treated as above. No endogenous radiolabeled protein was pulled-down under these conditions (data not shown). Carbohydrate residues that are transferred onto proteins in the ER are sensitive to digestion with Endo H, but when further modified in the Golgi network become Endo H-resistant and sensitive to PNGase F. Thus, Endo H resistance/PNGase F sensitivity is a hallmark of pro-

FIGURE 1. The linker domain is required for proper folding of pro-BMP-4 in Xenopus. A, schematic of Myc and HA epitope-tagged wild type and linker mutant forms of pro-BMP-4. Ectodermal cells were explanted from Xenopus embryos made to express native or linker deletion mutant forms of pro-BMP-4 and cultured to stage 10.5. Expression of the BMP-4 target gene Xbra was analyzed by Northern blot hybridization. The filter was rehybridized with a probe for odc as a loading control. Levels of Xbra transcripts in animal caps, normalized to levels of odc transcripts, are expressed as percent of that detected in BMP-4-expressing explants below each lane. The values are the mean of duplicate determinants with the experiment having been performed three times. B, radiolabeled wild type or linker mutant forms of pro-BMP-4 were synthesized in rabbit reticulocyte lysates and incubated with recombinant furin. Aliquots were removed and analyzed by SDS-PAGE at the indicated times. The position of precursor, S1- and S2-cleaved prodomains are illustrated schematically to the right. C, RNA encoding wild type or linker mutant forms of pro-BMP-4 was injected into oocytes together with [35S]Met/Cys. BMP-4 precursor and cleavage products were immunoprecipitated from lysates 24 h later using a Myc-specific (top) or HA-specific (bottom) antibody. Samples were treated with or without Endo H or PNGase F prior to SDS-PAGE under non-reducing or reducing conditions, as indicated. Bands corresponding to Endo H-sensitive/PNGase F-resistant mature ligand (hatch) are indicated. Diffuse bands representing misfolded dimers are labeled with asterisks. D, Western blots of lysates and media from HEK293 cells that were not transfected (UT) or were transfected with cDNAs encoding wild type or linker deletion mutant pro-BMP-4 were probed with a Myc-specific antibody. E, C2C12 cells were exposed to equivalent amounts of mature BMP-4 (as assayed by Western blot) in conditioned medium of HEK293 cells transfected with wild type or deletion mutant pro-BMP-4. Cell lysates were analyzed for alkaline phosphatase activity using pNPP as a substrate. Activity is assayed by Western blot (left) and measured as absorbance at 405 nm (right). The values are the mean of duplicate determinants with the experiment having been performed three times. The position of precursor, S1- and S2-cleaved prodomains are illustrated schematically to the right.
pro-BMP-4/ΔS2L) was biologically active, we added conditioned medium from transfected HEK293 cells to C2C12 mesenchymal progenitor cells and measured levels of the osteogenic marker, alkaline phosphatase, as an indicator of BMP activity. Mature BMP-4 cleaved from the wild-type precursor induced significantly more alkaline phosphatase activity than an equivalent or greater amount of mature BMP-4 cleaved from the linker deletion mutant (Fig. 1E and data not shown). These results were confirmed in three independent experiments. The simplest explanation for these results is that the high levels of precursor protein produced in transient transfection assays can exceed the capacity for quality control in the ER, allowing misfolded pro-BMP-4/ΔS2L dimers to escape the ER where they can be cleaved by resident PCs in the TGN to generate a partially misfolded, and thus less active ligand. By contrast, when expressed at lower levels in oocytes, misfolded pro-BMP-4/ΔS2L dimers, are retained in the ER (Fig. 1C). These dimers are thus inaccessible to candidate convertases, which are first active in the TGN, leading to a complete loss of bioactivity.

The Primary Amino Acid Sequence of the Linker Domain Is Essential for Its Function—To determine whether the primary amino acid sequence within the linker domain is important for its function we generated a cDNA encoding a precursor (pro-BMP-4(W → T)) in which a single tryptophan residue that is conserved in the linker of all known vertebrate BMP-2 and BMP-4 precursor proteins and in the Drosophila ortholog, decapentaplegic (DPP) (boxed W in Fig. 2A), was substituted with a threonine. The biological activity of mature BMP-4 cleaved from pro-BMP-4(W → T) was analyzed in a Xenopus animal cap assay as described above. The level of Xbra induced in ectodermal explants isolated from embryos injected with RNA (600 pg) encoding pro-BMP-4(W → T) was severely reduced relative to that induced in explants made to express the same amount of native precursor (Fig. 2B). This reduction in biological activity was not due to an inability of furin to recognize the cleavage motif in pro-BMP-4(W → T) because pro-BMP-4(W → T) was cleaved efficiently in vitro (Fig. 2C). Analysis of cleavage in vivo in three independent experiments, however, revealed that less mature BMP-4 was generated in Xenopus oocytes made to express pro-BMP-4(W → T) than in those expressing native precursor (Fig. 2D). Furthermore, whereas native pro-BMP-4 dimers migrated predominantly as a tight, Endo H-resistant/PNGase F-sensitive band (arrowhead), the intensity of this band was severely reduced in oocytes expressing pro-BMP-4(W → T). Pro-BMP-4(W → T) dimers instead migrated predominantly as a diffuse, fully Endo H-sen- sitive band (asterisk) that most likely corresponds to protein that is misfolded and cannot exit the ER to be cleaved. An additional, higher molecular weight precursor band is reproducibly detected in oocytes made to express pro-BMP-4(W → T) (dot). This same band is present at much lower levels in oocytes made to express native precursor, and most likely represents a higher molecular weight oligomer. The W-T point mutation appears to favor formation of these oligomers. Thus, the primary sequence of the linker domain is critical for its function in promoting proper folding of pro-BMP-4.

Cleavage of the S1 Site of pro-BMP-4 Is Required to Generate a Bioactive Ligand—To determine whether the S2 site can be cleaved independent of the S1 site to generate a bioactive ligand that includes the linker domain, we analyzed maturation of a precursor (pro-BMP-4/S1G), illustrated above Fig. 3A) in which the furin motif at the S1 site had been disrupted. Previous studies have shown that the S2 site of this precursor is efficiently cleaved by furin in vitro (8). To determine whether the same is true in vivo, RNAs (5 ng) encoding HA- and Myc-tagged wild type or S1 cleavage mutant forms of pro-BMP-4 were
injected into Xenopus oocytes together with [35S]Met/Cys, and precursor and cleavage products were immunoprecipitated from lysates after 20 h of culture. As shown in Fig. 3A, equivalent levels of Endo H-sensitive (arrow) and Endo H-resistant/PNGase F-sensitive (arrowheads) forms of pro-BMP-4 and pro-BMP-4(S1G) dimers were detected in oocyte lysates. Thus, blocking cleavage at the S1 site does not prevent the precursor from dimerizing or trafficking out of the ER. Furthermore, we observed two Myc-immunoreactive bands (asterisks, Fig. 3A) corresponding to mature BMP-4 in lysates of oocytes made to express pro-BMP-4(S1G). The two bands do not result from differential N-glycosylation and are not disulfide bond conformers because they are present even following deglycosylation under reducing conditions (Fig. 3B, upper panel). The relative molecular mass ($M_r$) of mature BMP-4 dimers generated by cleavage at the S2 site alone is predicted to be ~6 kDa larger than that generated by cleavage at both sites under non-reducing conditions. Based on molecular weight calculation by plotting $R_f$ values against the log of their molecular weights, we predict that the minor, lower molecular weight band detected on PNGase F treatment of BMP-4(S1G) precursor (Fig. 3A) may correspond to linker-attached mature BMP-4.
determine whether the dominant, higher molecular weight Myc-reactive mature BMP-4 band might result from cryptic activation of an upstream cleavage site, which should also generate an additional prodomain species, we examined prodomain fragments generated by cleavage of pro-BMP-4(mS1G). Duplicate samples were immunoprecipitated using HA-specific antibodies and subjected to deglycosylation as above. Following deglycosylation, a single band corresponding to the S2-cleaved prodomain was observed in lysates of oocytes made to express the wild type or S1 cleavage mutant precursor (Fig. 3B, bottom panel). Thus, the additional mature BMP-4 species is not generated by cleavage of pro-BMP-4(S1G) at an upstream site within the prodomain. These data were reproduced in at least three experiments and show that mutation of the S1 site does not prevent dimerization, folding, or S2 cleavage of pro-BMP-4 in vivo. Cleavage of the S2 site, however, generates an altered form(s) of mature BMP-4.

To determine whether mature BMP-4 cleaved from pro-BMP-4(mS1G) is biologically active, or whether it can function as a dominant mutant to block the activity of endogenous BMPs as has previously been reported for similar S1 cleavage mutant forms of pro-BMP-4 and pro-BMP-2 (10, 15), we assayed its activity in whole Xenopus embryos and explants. BMPs are required for ventral patterning in vivo and thus up- or down-regulation of BMPs in whole animals causes a loss, or duplication of dorsal structures, respectively. RNA (400 pg to 1 ng) encoding pro-BMP-4 or pro-BMP-4(S1G) was injected near the dorsal marginal zone (DMZ) or ventral marginal zone (VMZ) of four-cell embryos. At the tailbud stage, embryos were scored for BMP-mediated loss of dorsal structures using the dorsoanterior index (DAI) scale (16) in which five signifies normal patterning (low BMP activity) and zero signifies complete loss of all dorsal and anterior structures (high BMP activity). Whereas embryos injected with RNA encoding pro-BMP-4 showed a dose-dependent ventralization, embryos injected with pro-BMP-4(S1G) showed only a minimal loss of dorsal structures at the same doses (Table 1). Conversely, embryos were assayed for the ability of pro-BMP-4(S1G) to dominantly interfere with endogenous BMP signaling in ventral cells, by scoring for induction of a secondary dorsal axis. As a positive control for BMP inhibition, 1 ng of RNA encoding a dominant mutant truncated BMP receptor (tBR) was injected near the VMZ, and this was sufficient to induce a secondary axis in 84% of embryos (Table 1). By con-
TABLE 1
Effect of DMZ or VMZ injection of BMP RNAs on *Xenopus* embryos

| RNA     | Dose | Injection site | DAI (range) | Secondary axis | n |
|---------|------|----------------|-------------|----------------|---|
| BMP-4   | 400 pg | DMZ          | 2.2 (0–4)   | 0              | 68 |
| BMP-4S1G| 400 pg | DMZ          | 4.9 (4–5)   | 0              | 58 |
| BMP-4  | 1 ng  | DMZ          | 0.2 (0–2)   | 0              | 72 |
| BMP-4S1G| 1 ng  | DMZ          | 4.0 (3–5)   | 0              | 66 |
| tBR    | 1 ng  | VMZ          | NA          | 84             | 78 |
| BMP-4  | 1 ng  | VMZ          | 3.7 (1–5)   | 0              | 88 |
| BMP-4S1G| 1 ng  | VMZ          | 4.9 (4–5)   | 0              | 70 |
| BMP-4S1G| 4 ng  | VMZ          | 3.9 (3–5)   | 0              | 54 |

As a more quantitative and sensitive assay for BMP activity, we injected RNA (600 pg) encoding pro-BMP-4 or pro-BMP-4S1G into two cell embryos and compared levels of expression of *Xbra* in *Xenopus* animal pole explants. As shown in Fig. 3C, *Xbra* induction was barely detectable in animal caps isolated from embryos made to express a precursor that cannot be cleaved at the S1 site relative to the native precursor. Taken together, these data demonstrate that pro-BMP-4 that cannot be cleaved at the S1 site generates a ligand in which the linker domain remains covalently attached to the N terminus, and this ligand has minimal biological activity when overexpressed in *Xenopus* embryos.

**The BMP-4 Linker Domain Cannot Regulate BMP-4 Ligand Activity When Present in the Context of a Heterologous Prodomain**—Having shown that the linker domain is required for proper folding of pro-BMP-4, and that cleavages around the linker domain can regulate the activity of the mature ligand, we next asked whether these functions require sequences within the BMP-4 prodomain, the mature domain, or both. To do so, we generated chimeric constructs encoding the prodomain of BMP-7, which has only a single, minimal furin consensus cleavage motif, fused to the BMP-4 mature domain in the presence or absence of the linker region and one or both cleavage sites (Fig. 4A). As shown in Fig. 4B, the chimeric precursors were all efficiently cleaved by furin in *vitro*. We then compared the activity of mature BMP-4 generated by each chimeric precursor to that generated by native pro-BMP-4. RNAs (600 pg) encoding native BMP-4 or chimeric precursor proteins were injected into two cell embryos and levels of expression of *Xbra* were analyzed in *Xenopus* animal pole explants by Northern blot hybridization. Chimeras that included the BMP-4 linker domain together with the BMP-7 prodomain did not generate biologically active BMP-4 regardless of whether they contained two cleavage sites (BMP-7/4), which would potentially allow the linker region to be removed during maturation, or only a single cleavage site (BMP-7/S2G/4), which would leave the linker attached to the BMP-7 prodomain (Fig. 4C). By contrast, robust BMP-4 activity was detected in embryos made to express pro-BMP-7ΔS2L/4, which lacks the linker domain and includes the optimal furin motif normally found at the S1 site. In three separate experiments, the level of *Xbra* induced in embryos made to express pro-BMP-7ΔS2L/4, relative to that induced by wild-type pro-BMP-4 ranged from 64–100% (Fig. 4C and data not shown). Expression of *Xbra* was induced at lower, but still appreciable levels, in embryos made to express an analogous chimeric precursor (BMP-7ΔS1L/4) that contains a minimal (-RSVR-) rather than an optimal (-RSKR-) furin motif at its cleavage site. These data demonstrate that the linker domain is not required, and in fact inhibits the generation of active BMP-4 when present in the context of the BMP-7 prodomain.

To begin to determine why the presence of the linker domain interferes with the production of bioactive BMP-4 in the context of a heterologous prodomain, we examined the maturation of chimeric precursor proteins *in vivo*. RNAs (5 ng) encoding Myc-tagged native or chimeric precursors were injected into *Xenopus* oocytes together with [35S]Met/Cys. Proteins were immunoprecipitated using Myc antibody, subjected to Endo H/PNGase F treatment and separated by SDS-PAGE under non-reducing conditions. In lysates of oocytes made to express BMP-4, BMP-7 or chimeric precursors lacking the linker domain [BMP-7ΔS1L/4 or BMP-7ΔS2L/4], tight protein bands corresponding to Endo H-sensitive (Fig. 4C, arrow) as well as Endo H-resistant/PNGase F-sensitive (arrowhead) forms of the dimerized precursor can be detected. By contrast, in oocytes made to express pro-BMP-7/4 and pro-BMP-7S2G/4, both of which include the linker domain downstream of the BMP-7 prodomain, the tight band is absent and only a diffuse, fully Endo H-sensitive band is observed (asterisk). These data suggest that the presence of the BMP-4 linker domain in the context of the BMP-7 prodomain prevents the precursor from folding properly such that it cannot exit the ER to be cleaved. Consistent with this possibility, mature BMP-4 is not detected in oocytes made to express chimeric precursors that include the linker domain. Mature BMP-4 is present in oocytes made to express precursors that lack the linker domain, albeit at levels significantly below that observed in oocytes made to express native precursors. The discrepancy between the low levels of BMP-4 ligand generated by cleavage of pro-BMP-7ΔS2L/4 in oocytes and its high activity in the *Xbra* induction assay in embryos may indicate that the ligand cleaved from the chimeric precursor is more potent. Alternatively, this may reflect intrinsic differences in maturation of these precursors in oocytes relative to embryos. These data were reproducible in three independent experiments. Taken together, our results suggest that the BMP-7 prodomain contains all of the necessary information to correctly fold the heterologous BMP-4 ligand and that the presence of the BMP-4 linker domain in this context inhibits the proper folding and cleavage of the precursor.

**The BMP-4 Linker Domain Can Function with the BMP-4 Prodomain to Regulate the Activity of a Heterologous Ligand**—To test whether the BMP-4 linker domain is required in the context of the BMP-4 prodomain to generate a bioactive heterologous ligand, we generated chimeric constructs encoding the BMP-4 prodomain, with or without the linker region and one or both cleavage sites, fused to the BMP-7 mature domain (Fig. 5A). As shown in Fig. 5B, furin recognized and cleaved all S1 and/or S2 sites present in chimeric precursors *in vitro*. *Xenopus* animal cap assays were performed to compare the activity of mature BMP-7 generated from each chimeric precursor to that generated from native pro-BMP-7. RNAs (600 pg) encoding BMP-7 or chimeric precursor proteins were
injected into two-cell embryos, ectoderm was isolated at the late blastula stage and expression of Xbra was analyzed by Northern blot hybridization at early gastrula stages. The level of Xbra induction in embryos made to express pro-BMP-4/7, which includes the linker domain and both the S1 and S2 cleavage sites, was less than (data not shown) or equivalent to that observed in embryos expressing native pro-BMP-7. Barely detectable levels of Xbra were induced in explants expressing pro-BMP-4(S2G)/7, in which the S2 site cannot be cleaved. This suggests that sequential cleavage of the BMP-4 prodomain

FIGURE 4. The BMP-4 linker domain inhibits folding and cleavage of a chimeric precursor containing the BMP-7 prodomain. A, schematic illustration of BMP7/4 chimeras. B, radiolabeled BMP-7 or BMP-7/4 chimeric precursor proteins were incubated in vitro with furin for the times indicated. Cleavage products were separated by SDS-PAGE and analyzed by autoradiography. C, ectodermal cells were explanted from Xenopus embryos made to express BMP-7 or BMP-7/4 chimeras and cultured to stage 10.5. Expression of Xbra was analyzed by Northern blot hybridization. The filter was rehybridized with a probe for odc as a loading control. Levels of Xbra transcripts in animal caps, normalized relative to levels of odc transcripts, are expressed as percent of that detected in BMP-4-expressing explants below each lane. The values are the mean of duplicate determinants with the experiment having been performed three times. D, oocytes were injected with RNA encoding BMP-4, BMP-7, or BMP-7/4 chimeras along with [35S]Met/Cys. Oocytes were harvested 20 h postinjection and precursor proteins and mature BMP-4 were immunoprecipitated from lysates using antibodies specific for the Myc epitope. Samples were treated with or without Endo H and PNGase F prior to SDS-PAGE under non-reducing conditions, as indicated. Bands corresponding to Endo H-sensitive (arrow) or Endo H-resistant/PNGase F-sensitive (arrowhead) dimers are indicated. Asterisks are located above misfolded, ER-retained precursor dimers.
The BMP-4 linker domain can function with the BMP-4 prodomain to regulate the activity of a heterologous ligand. A, schematic illustration of BMP4/7 chimeras. B, radiolabeled BMP-7 or BMP-4/7 chimeric precursor proteins were incubated in vitro with furin for the times indicated. Cleavage products were separated by SDS-PAGE and analyzed by autoradiography. C, ectodermal cells were explanted from Xenopus embryos made to express BMP-4 or BMP-4/7 chimeras and cultured to stage 10.5. Expression of Xbra was analyzed by Northern blot hybridization. The filter was rehybridized with a probe for odc as a loading control. Levels of Xbra transcripts in animal caps, normalized relative to levels of odc transcripts, are expressed as percent of that detected in BMP-7-expressing explants below each lane. The values are the mean of duplicate determinants with the experiment having been performed twice. D, oocytes were injected with RNA encoding BMP-4, BMP-7, or BMP-4/7 chimeras along with [35S]Met/Cys. Oocytes were harvested 20 h postinjection and precursor proteins and mature BMP-4 were immunoprecipitated from lysates using antibodies specific for the FLAG epitope in the prodomain (top panel) and Myc epitope in the mature domain (bottom panel). Samples were treated with or without Endo H and PNGase F prior to SDS-PAGE under non-reducing conditions, as indicated. Bands corresponding to Endo H-sensitive (arrow) or Endo H-resistant/PNGase F-sensitive (arrowhead) dimers are indicated. Asterisks are located above misfolded, ER-retained precursor dimers.
Structure-Function Analysis of the BMP-4 Prodomain

at the S1, then S2 sites can regulate the activity of a heterologous ligand in a fashion identical to that observed for native BMP-4. Chimeras that lacked the BMP-4 linker domain did not generate biologically active BMP-4 regardless of whether they contained an optimal (BMP-4(ΔS2L)/7) or a minimal (BMP-4(ΔS1L)/7) furin consensus cleavage motif. Thus, the linker domain is an essential component of the BMP-4 prodomain and sequential cleavage of both the S1 and the S2 sites surrounding this domain is required to generate maximal ligand activity regardless of whether the ligand is BMP-7 or BMP-4.

To further examine the mechanism by which the BMP-4 linker domain regulates the activity of BMP-7, we examined maturation of each chimeric precursor in Xenopus oocytes in three independent experiments, as described previously. In lysates of oocytes made to express BMP-4, BMP-7, or chimeric precursors containing the linker domain, tight protein bands corresponding to properly folded forms of the dimerized precursor can be detected (arrows, arrowheads). By contrast, in oocytes made to express pro-BMP-4(ΔS2L)/7 or pro-BMP-4(ΔS1L)/7, both of which lack the linker domain, the tight band is absent and only a diffuse, fully Endo H-sensitive band is observed (asterisk). Furthermore, mature BMP-7 is not detected in oocytes made to express chimeric precursors that lack the linker domain, demonstrating that this region is essential to direct proper folding, and thus cleavage, of precursors proteins even if they contain a heterologous mature domain. Cleaved prodomain (upper panel) and mature BMP-7 (lower panel) was detected in oocytes expressing pro-BMP-4/7 but was barely detectable or undetectable in oocytes expressing pro-BMP-4(S2G)/7 despite the presence of relatively equivalent levels of properly folded precursor protein (arrowheads). These data demonstrate that the linker domain functions together with sequences present in the N-terminal portion of the BMP-4 prodomain to regulate precursor protein folding. Furthermore, our results suggest that failure to cleave the S2 site within the prodomain of BMP-4 can promote targeted degradation of a heterologous ligand.

**DISCUSSION**

The structure and function of the highly conserved ligand domain of TGF-β family proteins have been well characterized while that of the prodomain is less well understood. With exception of nodal (17), the prodomain is believed to be essential for the proper dimerization and folding of all TGF-β precursor proteins (18). The observation that heterologous prodomains can direct proper folding of divergent TGF-β ligands (18–21) suggests that structural features of this domain may be conserved among different family members, despite the relatively low sequence identity.

In addition to functioning as an intramolecular chaperone, TGF-β family prodomains have been shown to affect the activity of the mature ligand by stabilizing it, maintaining it in an inactive, latent state and/or by targeting it to the extracellular matrix (ECM). Following cleavage of TGF-β, for example, the prodomain remains non-covalently associated with the mature ligand in a small latent complex that cannot interact with its receptor (22). This complex is targeted to the ECM via an interaction between the prodomain and members of the latent TGF-β-binding protein (LTBP) family of matrix proteins. Receptor activation requires removal of the prodomain from the complex through the action of thrombospondins, integrins, and other proteins. Recent studies have identified a distinct ECM protein, emilin, which interacts with pro-TGF-β and prevents it from being proteolytically activated until after it is secreted from the cell (23). The prodomain of BMP-7, like that of TGF-β, remains non-covalently associated with the mature domain following cleavage, but in this case the complex appears to be capable of binding to and activating its receptor (24).

Rather than conferring latency, the prodomain of BMP-7 targets the mature ligand to the ECM via binding to fibrillins (25). Finally, the uncleaved prodomain of nodal has been proposed to stabilize the ligand, so that it can travel in the extracellular space in a precursor form to distal sites of action (17). Following cleavage, the nodal prodomain remains bound to the mature domain and may target it for degradation (5).

The prodomain of BMP-4 is unique in its ability to bind to, and target the mature domain for lysosomal degradation following an initial cleavage, but to be released, along with a small peptide fragment, following a subsequent, upstream cleavage. Like BMP-7, mature BMP-4 is signaling competent when complexed with its prodomain, but it signals over a shorter distance because of increased lability. The current studies were designed to investigate the fate and function of the small C-terminal prodomain fragment that is released following sequential cleavage of pro-BMP-4. Our results clearly show that this peptide is essential for proper folding, and thus cleavage, of pro-BMP-4, but this early function precludes analysis of potential later roles. It is possible, for example, that the peptide contains sequence information that directs intracellular trafficking to the lysosome, or that the peptide remains associated with free, mature BMP-4 following S2 cleavage and facilitates diffusion or transport in the extracellular space. Unfortunately, we have been unable to generate antibodies that recognize the cleaved linker peptide so its fate following S2 cleavage is currently unknown.

Surprisingly, although the linker domain is absolutely essential for proper folding of pro-BMP-4 in Xenopus oocytes and embryos, such that a linker deletion mutant is completely retained in the ER and cannot be cleaved to generate even an inactive, misfolded ligand, the same is not true in mammalian cultured cells. We find that the same linker deletion mutant is efficiently processed in HEK293, COS, or CHO cells, although the ligand cleaved from this precursor is less active than that cleaved from native precursor, suggesting that it may be partially misfolded. We have noted similar differences in maturation of other mutant or chimeric forms of pro-BMP-4 in cultured cells versus Xenopus or mouse embryos. For example, in Xenopus, mature BMP-4 generated from a precursor that cannot be cleaved at the S2 site is targeted for lysosomal degradation and thus accumulates at much lower levels than does the same ligand cleaved from native precursor (8). By contrast, inability to cleave the S2 site has no effect on steady-state levels of mature BMP-4 in any mammalian cell line that we have tested, including cell lines derived from embryonic rat calvaria.

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4 L. Berg and J. L. Christian, unpublished data.
or chondrocytes that normally produce and respond to endogenous BMPs. The reason for this discrepancy is unclear although it may be caused by the higher levels of expression achieved in transiently transfected mammalian cells, which saturate the lysosomal trafficking machinery thereby masking any differences in endocytic trafficking of the two proteins. Alternatively, it is possible that accessory proteins expressed in embryos but not cultured cells are required to regulate BMP-4 maturation and/or endocytic trafficking. Regardless of the underlying cause, these discrepancies suggest that maturation of proproteins in transiently transfected cell lines does not always accurately reflect that in whole animals.

The observation that the S1 cleavage mutant form of pro-BMP-4 efficiently dimerizes and is cleaved to produce an inactive ligand, yet does not block the function of endogenous BMPs when overexpressed is surprising. By contrast with our results, previous studies have shown that a similar S1 mutant form of pro-BMP-4 is not cleaved in oocytes and functions as a dominant negative mutant in Xenopus embryos (10). An analogous mutant form of BMP-2 has also been reported to function as a dominant mutant in Xenopus embryos (15). In both of these studies, all four amino acids upstream of the S1 cleavage site were mutated, which may have disrupted proper folding of the precursor, whereas we introduced only a single amino acid substitution. Although this might account for discrepancy in cleavage, it is difficult to explain why neither our S1 mutant precursor nor the linker deletion mutant possess inhibitory activity. Many different naturally occurring or genetically engineered mutations in TGF-β family members have been identified that prevent the formation of an active ligand, yet allow for dimerization with endogenous precursors and thus inhibit their activity (19, 26–28). It is possible, although unlikely, that our deletion and cleavage mutant precursors can only dimerize with overexpressed mutant forms, and not with endogenous wild-type BMPs, or that the minimal activity generated by the S1 cleavage mutant is sufficient to substitute for endogenous BMP signals. Curiously, when expressed in HEK293 cells, the S1 cleavage mutant is either inefficiently cleaved or the cleavage products are rapidly degraded. Furthermore, this mutant destabilizes or prevents cleavage of wild-type BMP-4 when the two precursors are co-expressed, suggesting that in cultured cells, it can function as a dominant mutant. Whether this reflects differences in the repertoire of convertases expressed in the two cell types, or other factors, remains to be investigated.

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