Bone Morphogenetic Protein 1 Prodomain Specifically Binds and Regulates Signaling by Bone Morphogenetic Proteins 2 and 4*

Highly purified fractions of bone extracts capable of inducing ectopic bone formation have been reported to contain peptides corresponding to the mature active regions of the TGF-β-like bone morphogenetic proteins (BMPs) 2–7, and to the prodomain region of the metallocproteinase BMP1. Co-purification of BMPs 2–7 with BMP1 prodomain sequences through the multiple biochemical steps used in these previous reports has suggested the possibility of interactions between the BMP1 prodomain and BMPs 2–7. Here we demonstrate that the BMP1 prodomain binds BMPs 2 and 4 with high specificity and with a \( K_D \) of \(~11 \text{ nm} \), in the physiological range. It is further demonstrated that the BMP1 prodomain is capable of modulating signaling by BMPs 2 and 4 \textit{in vitro} and \textit{in vivo}, and that endogenous BMP1 prodomain-BMP4 complexes exist in cell culture media and in tissues.

Highly purified fractions of bone morphogenetic activity isolated from bone extracts contain peptides corresponding to bone morphogenetic proteins (BMPs)\(^2\) 1–7 (1, 2). BMPs 2–7 are all TGF-β superfamily members and are involved not only in formation of bone, but in the formation of various tissues, including the nervous system, heart, and kidneys (3). BMPs 2 and 4 in particular have also been shown to play central roles in formation of various tissues, including the nervous system, heart, and kidneys (3). BMPs 2 and 4 in particular have also been shown to play central roles in formation of bone, but in the formation of various tissues, including the nervous system, heart, and kidneys (3).

In the present study, it is demonstrated that the BMP1 prodomain binds active BMP2 and BMP4 with high specificity and high avidity (\( K_D = 10.9 \text{ nm} \)); that it is capable of modulating signaling by these BMPs \textit{in vitro} and \textit{in vivo}; and that BMP1 prodomain-BMP4 complexes are found in cell culture media and within human tissues.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—A construct for expression of mutated BMP1, in which the prodomain is impervious to SPC activity, was generated via PCR using reverse primer 5′-TCGGGACGTCGCCCTGCTGGCTACTGATCTACTCCTCCTCA-3′, which encodes proBMP1 sequences in which the subtilisin-like proprotein convertase recognition site RSRR were converted to sequences encoding subtilisin-like proprotein convertase recognition site RSRR.
residues SSQQ. ProBMP1 sequences within vector pBluescript II KS+ (Stratagene) were used as template, and T3 primer was used as the forward primer. The resulting amplimer was digested with AflII and AatII and used to replace the corresponding wild-type fragment in the original proBMP1/pBluescript clone. Subsequently, an AflII-Apal fragment containing the mutation was excised from the pBluescript construct and exchanged for the corresponding wild-type fragment in full-length proBMP1 sequences previously placed within vector pcDNA4/TO-His (Invitrogen), such that mutant proBMP1 produced by the resultant construct would have a COOH-terminal His tag.

To create recombinant baculovirus for expression of the BMP1 prodomain, PCR amplification was performed, using primers 5′-GAGTAGATCTAGCCTAGCCGACTACCC-TATGACCT-3′ (forward) and 5′-GAGTGTAGTACCAGCCGGGCTACGGGATCTA-3′ (reverse), and using wild-type proBMP1 sequencse in pcDNA4/TO as the template. The resulting amplimer was restricted with BglII and NheI, and then inserted between the BamHI and XbaI sites of the pAcGP67.coco baculovirus transfer vector (11) (kind gift of Deane F. Mosher, University of Wisconsin-Madison). The pAcGP67.coco vector is a modification of vector pAcGP67A (BD Biosciences Pharmingen), with sequences encoding the thrombin cleavage site (LVPRGS) and a six-histidine tag (His6 tag) added immediately 3′ to the multiple cloning site. Recombinant baculovirus was generated by co-transfection with Baculogold-linearized AcNPV viral DNA (BD Biosciences Pharmingen) into SF9 cells (Invitrogen). Recombinant baculovirus was propagated, and protein was expressed and purified as described by Mosher et al. (11).

To create a construct for expression of a BMP1 prodomain–Fc fusion protein, a KpnI-NgoMI cDNA fragment encoding BMP1 prodomain sequences fused to BM40 signal peptide sequences (12) was inserted within a KpnI site and a BamHI site that had been blunt-ended with Klenow fragment in a construct that contained enterokinase cleavage site sequences, followed by sequences encoding the human IgG1, Fc domain, in a pcDNA3 backbone (13). The enterokinase site/Fc domain construct was the kind gift of Carl P. Blobel (Hospital for Special Surgery, Cornell University).

To generate mRNA for injection into zebrafish embryos, BMP1 prodomain sequences were PCR-amplified with primers 5′-CTGAGAATTTCCAGGACCATGAGGGC-3′ (forward) and 5′-GAGTTGACCTAGCCTAGCCGGGCTACGGGATCTA-CCT-3′ (reverse) and inserted between the EcoRI and XbaI sites of vector pcPS2+. To generate mRNA for expression of a BMP1 prodomain/CD-2 fusion protein, prodomain sequences were PCR-amplified with primers 5′-CTGAGAATTTCCAGGACCATGAGGGC-3′ (forward) and 5′-GACTGAGATTTCTAGCCGGGCTACGGGATCTA-CCT-3′ (reverse). The resulting amplimer, which contained AvrII and EcoRI, sites in reverse primer sequences, was digested with BamHI and EcoRI and was inserted between corresponding sites of pcPS2+. The resulting construct was restricted with AvrII and EcoRI, for placement of an AvrII-EcoRI cDNA fragment encoding rat CD-2 transdomain and partial exodomain sequences (kind gift from Gary Struhl, Columbia University).

**Protein Biochemistry**—For expression of recombinant proteins, constructs were transfected into T-REx 293 cells using Lipofectamine™ (Invitrogen). Recombinant proteins with His tags or Fc domains were purified from conditioned serum-free medium using Ni-NTA resin (Qiagen) or protein A-Sepharose (Amersham Biosciences), respectively. FLAG-tagged murine Chordin and wild-type human BMP1 were produced, purified, and analyzed for purity on zinc-stained SDS-PAGE gels as previously described (12). Concentrations of purified proteins were calculated by comparing intensities of Coomassie Blue-stained bands from serial dilutions of each sample to those of serially diluted bovine serum albumin of known concentrations. Procollagen and Chordin cleavage assays were performed as previously described (12). Immunoprecipitations of recombinant proteins were performed as previously described (14), using equimolar amounts of purified proteins. For the competition experiments, 5 nM proBMP1SSQQ was incubated 30 min at 37 °C with 5 nM BMP4 in the presence of excess BMP2, BMP5, EGF, or TGF-β1 (with the extent of excess noted in the text) and immunoprecipitated. Similarly, mBMPR-1A/Fc was incubated with BMP4 under conditions similar to those described above, was competed with equimolar amounts of Chordin or BMP1 Prodomain, and complexes were immunoprecipitated with protein A-Sepharose. For immunoprecipitations from MG-63 cultures, 80% confluent MG-63 cells were washed twice with phosphate-buffered saline (PBS), incubated 15 min with serum-free Dulbecco’s modified Eagle’s medium at 37 °C, followed by two PBS washes. Cells were then incubated 48 h in serum-free Dulbecco’s modified Eagle’s medium, 40 μg/ml soybean trypsin inhibitor, 2 ng/ml TGF-β1 (R&D Systems). Conditioned medium was harvested and cells were washed twice with ice-cold PBS, followed by lysing in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 1 mM ZnCl2, 1 mM CaCl2, 1 mM MgCl2, 1% Triton X-100, 1% CHAPS, 10% glycerol; with clearing of lysates by centrifugation. Cell lysate or conditioned medium, with Triton X-100 and CHAPS added to 0.1% final concentrations, was precleared with protein A-Sepharose at 4 °C. Anti-BMP1 prodomain antibodies or preimmune serum was then added, followed by incubation with protein A-Sepharose. Immunoprecipitates were subjected to six 10-min washes with PBS/0.5% Triton X-100, followed by elution of samples with SDS sample buffer containing 100 mM dithiothreitol, and separation of samples on 4–20% acrylamide SDS-polyacrylamide electrophoresis gels (Bio-Rad). Samples were transferred to nitrocellulose membranes and probed with anti-BMP1 prodomain antibodies or monoclonal anti-BMP4 antibody (R&D systems).

Separate dermal and epidermal extracts were obtained from neonatal foreskin, essentially as described by Bruckner-Tuderman et al. (15), except that dermal extraction was performed in the absence of reducing agents. Briefly, artificial epidermolysis was performed in 1 M NaCl, 0.05 M Tris/HCl, pH 7.4, 20 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, 10 mM ε-aminoepropionic acid (buffer 1) for 72 h at 4 °C. Subsequently, epidermis was peeled off, and the dermis was extracted twice for 30 s at 95 °C with 0.125 M Tris-HCl, pH 6.8, 8 M urea, 2% SDS, 20 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, 10 mM ε-aminocap-
BMP1 Prodomain Binds BMP2/4

RESULTS

ProBMP1, but Not Mature BMP1, Specifically Binds BMPs 2 and 4—To begin characterizing possible functional roles of the BMP1 prodomain, site-directed mutagenesis was employed to alter the proBMP1 furin recognition site RSRR to SSQQ (Fig. 1A), to enable comparison of the properties of pro and mature BMP1. When expressed in transfected T-Rex-293 cells, only
full-length proBMP1SSQQ was detected in conditioned medium, with no evidence of processing to mature BMP1 (Fig. 1B). The proBMP1SSQQ displayed no detectable activity for processing BMP1 substrates type I procollagen (Fig. 1C) and Chordin (Fig. 1D). Moreover, proBMP1SSQQ was found to interfere with cleavage of procollagen and Chordin by mature/active wild-type BMP1 (Fig. 1, C and D). There is evidence that BMP1-like proteinases do not form oligomers (22). Thus, the apparent dominant negative effect of proBMP1SSQQ likely results from binding to, and sequestering of substrate. The ability of proBMP1SSQQ to bind substrate, suggests that it is correctly folded.

In an initial attempt to ascertain the ability of BMP1 prodomain sequences to interact with TGF-β-like BMPs, mature BMP1, and proBMP1SSQQ were separately incubated with BMP4, and then immunoprecipitated with anti-BMP1 antibodies (26). Blots were cut and the separate pieces were incubated with anti-BMP1 or anti-BMP4 antibodies. 8, proBMP1SSQQ was incubated with a fixed amount of BMP4 (5 nM) alone, or in the presence of increasing amounts of BMP2 (molar ratios of BMP4:BMP2 of 1:1, 1:2, 1:5, and 1:10) followed by immunoprecipitation with anti-BMP1 antibody (26) and immunoblotting with anti-BMP4 or anti-BMP2 monoclonal antibodies. As a control for pull-down efficiency, the anti-BMP4 blot was stripped and re-probed with anti-BMP1 antibodies to detect immunoprecipitated proBMP1SSQQ. C, purified BMP1 prodomain, produced in a baculovirus system, was visualized by staining with Coomassie Brilliant Blue R-250 (lane 2). Numbers to the left of the gel correspond to the approximate sizes in kDa of molecular mass markers (lane 1). D, Western blot is used for characterization of anti-BMP prodomain antibodies, which at a 1:20,000 dilution detect 6 ng of BMP1-Fc fusion protein (lane 1), but do not detect 6 ng of Fc domain (lane 2).
sequence homology and evidence of similar binding of the two proteins to previously described BMP-binding proteins (23, 24). In contrast, 10-fold molar excesses of the unrelated growth factor EGF, or even other TGF-β-like growth factors such as TGF-β1 (36% sequence identity to BMP4) or BMP5 (54% sequence identity to BMP4) did not compete with BMP4 for binding to proBMP1 SSQQ (data not shown).

**BMP1 Prodomain Sequences Specifically Bind BMP2/4 with High Affinity**—To more closely explore the ability of BMP1 prodomain sequences to interact with TGF-β-like BMPs, BMP1 prodomain, free of other sequences other than a COOH-terminal His tag, was produced in a baculovirus system (Fig. 2C). In addition, for such studies antibodies were raised against the recombinant prodomain, capable of detecting 6 ng of prodomain at a 1:20,000 antibody dilution (Fig. 2D). Upon incubation of the His-tagged BMP1 prodomain with BMP4, pull down of the His-tagged prodomain with nickel-charged resin was found to co-precipitate BMP4 (Fig. 3A). Similarly, pull down of the prodomain with the anti-BMP1 prodomain antibody shows that BMP1 prodomain-BMP4 complexes in tissues. Because BMP1 is expressed at relatively high levels in skin (26, 27), and BMP signaling is important to the development and homeostasis of skin and hair follicles (28, 29), skin was assayed for the possible presence of BMP1 prodomain-BMP4 complexes. Toward this end, immunoprecipitations with the anti-BMP1 prodomain antibodies were performed on skin extracts followed by immunoblot analysis for detection of the BMP1 prodomain and for detection of co-precipitated TGF-β-like molecules. As can be seen (Fig. 5A), an immunoblot probed with anti-BMP1 prodomain antibody shows that BMP1 prodomain sequences persist in dermis primarily as isolated prodomains, but also, in lesser amounts, as uncleaved proBMP1 bodies also co-precipitated BMP2 (Fig. 3B). Thus, BMPs-2 and 4 are bound by the BMP1 prodomain. The specificity of this interaction is demonstrated in Fig. 3C, in which a 10-fold molar excess of BMP2, but not of TGF-β1, EGF, or BMP5, effectively competed with BMP4 for binding to the BMP1 prodomain.

To compare the affinity of the BMP1 prodomain for BMP4 to that of Chordin, a known in vivo BMP4-binding partner (23), an equilibrium binding experiment was performed. This experiment entailed incubating BMP4 with an equimolar amount of soluble BMP receptor IA (ALK-3), fused to an Fc domain, in the presence of 1:1, 2:1, or 5:1 molar ratios of either BMP1 prodomain or Chordin. As can be seen (Fig. 3D), BMP1 prodomain, like Chordin, was able to effectively compete with the BMPR-IA-Fc fusion protein for BMP4 binding, and both proteins were able to essentially block BMP4 binding to the BMPR-IA-Fc fusion protein at a molar ratio of 5:1. Thus, affinity of the BMP1 prodomain for BMP4 is shown to be of the same order of magnitude as the affinity of Chordin for this ligand.

**BMP1 Prodomain Sequences Bind BMP2 with an Apparent KD of 11 nM**—To quantitate the strength of the interaction between the BMP1 prodomain and BMP2, binding affinity was determined via surface plasmon resonance (BIAcore) analysis. Toward this end, BMP2 was immobilized on the surface of a sensor chip, and the strength of binding was separately determined for different concentrations of BMP1 prodomain. Toward this end, a BMP1 prodomain-Fc fusion protein was produced and purified (Fig. 4A), the Fc domain was removed, and the prodomain region was repurified (Fig. 4B). For comparison, the strength of binding to immobilized BMP2 was also determined for different concentrations of Chordin. From the kinetic association and dissociation rates, the equilibrium dissociation constants (apparent $K_D$) for binding of BMP1 prodomain (Fig. 4C) and Chordin (Fig. 4D) to BMP2 were calculated to be ~11 and 7 nM, respectively. The apparent $K_D$ for murine Chordin calculated here, is comparable to the $K_D$ of ~12 nM previously calculated for murine Chordin via surface plasmon resonance (25). Furthermore, the $K_D$ calculated here for BMP1 prodomain binding of BMP2 is clearly in the same range as the $K_D$ values of proteins like Chordin and Crossveinless 2 (25), which are thought to bind BMP2 and 4 in vivo and modulate their signaling under physiological conditions.

**Endogenous BMP1 Prodomain-BMP4 Complexes Are Found in Skin**—The strength and specificity of BMP1 prodomain-BMP4 interactions, and the co-purification of BMP1 and TGF-β-like BMPs from bone (2) prompted an attempt to identify BMP1 prodomain-BMP4 complexes in tissues. Because BMP1 is expressed at relatively high levels in skin (26, 27), and BMP signaling is important to the development and homeostasis of skin and hair follicles (28, 29), skin was assayed for the possible presence of BMP1 prodomain-BMP4 complexes. Toward this end, immunoprecipitations with the anti-BMP1 prodomain antibodies were performed on skin extracts followed by immunoblot analysis for detection of the BMP1 prodomain and for detection of co-precipitated TGF-β-like molecules.

As can be seen (Fig. 5A), an immunoblot probed with anti-BMP1 prodomain antibody shows that BMP1 prodomain sequences persist in dermis primarily as isolated prodomains, but also, in lesser amounts, as uncleaved proBMP1
BMP1 Prodomain Binds BMP2/4

on cell lysates and conditioned medium of MG-63 osteosarcoma cells, previously shown to express both BMP1 and BMP4 (9, 31). As can be seen (Fig. 5C), anti-BMP1 prodomain antibody co-precipitated BMP4 from conditioned medium, but not from cell lysates of MG-63 cultures, consistent with the conclusion that BMP1 prodomain-BMP4 complexes form in the extracellular space. It should also be noted that free BMP1 prodomain was detected in conditioned medium, but not in cell extracts (Fig. 5C). This indicates that, at least in some cells, excision of the BMP1 prodomain occurs concurrent with or subsequent to secretion, and is not an intracellular event.

**BMP1 Prodomain Sequences Can Modulate BMP4 Induction of Osteoblastic Differentiation**—We next sought to determine the ability of BMP1 prodomain sequences to affect BMP4 signaling. Toward this end, we compared the abilities of proBMP1SSQQ and isolated His-tagged BMP1 prodomain to that of Chordin in affecting the ability of BMP4 to induce osteoblastic differentiation of M2–10B4 stromal stem cells; a process dependent upon BMP signaling (21). Both proBMP1SSQQ and His-tagged BMP1 prodomain were found to be comparable to Chordin in ability to inhibit the BMP signaling-dependent osteoblastic differentiation of M2–10B4 cells (Fig. 6A). In a similar assay, mature active BMP1 was found to have no effect on BMP4-induced M2-10B4 cell osteoblastic differentiation (Fig. 6B). Thus, BMP1 prodomain sequences, but not other BMP1 sequences, are capable of blocking BMP4 signaling.

**Tethered BMP1 Prodomain Sequences Induce Dorsalization in Developing Zebrafish Embryos**—Extracellular antagonists of signaling by BMPs 2 and 4, such as Chordin, induce dorsalization when overexpressed in early zebrafish and *Xenopus* embryos (32, 33). Thus, we overexpressed the BMP1 prodomain in zebrafish embryos to determine whether these prodomain sequences might be similarly capable of modulating BMP signaling *in vivo*. In initial attempts, injection of 1–2-cell embryos with 1 ng of mRNA encoding isolated BMP1 prodomain, which had effectively blocked BMP4 signaling in cell culture assays (Fig. 6A), only mildly dorsalized 6 of 56 surviving injected embryos (Table 1). In contrast, 250 pg of Chordin mRNA strongly dorsalized 24 of 32 surviving embryos. Interestingly, the observation that BMP1 prodomain overexpression does not ventralize embryos is consistent with the probability that prodomain and Chordin binding sites on BMP2/4 molecules do not coincide.

Excision of the BMP1 Prodomain and Formation of BMP1 Prodomain-BMP4 Complexes Are Extracellular Events—To determine whether endogenous BMP1 prodomain-BMP4 complexes are intracellular or extracellular, immunoprecipitations with anti-BMP1 prodomain antibodies were performed (lane 3). The same blot also shows small amounts of proBMP1, but no isolated BMP1 prodomain, to be detectable in epidermis (lane 4). An immunoblot probed with anti-BMP4 antibody (Fig. 5B), demonstrates that prodomain sequences in dermis pulled down, and are thus contained in complexes with, BMP4 (lane 3). In contrast, immunoblots show that BMP1 prodomain sequences did not pull down BMP5, TGF-β1, or the unrelated growth factor EGF. Similarly, there was no detectable pull down of BMP2 or BMP6 by BMP1 prodomain sequences in such assays (data not shown). The finding of BMP1 prodomain-BMP4 complexes, but lack of detectable endogenous BMP1 prodomain-BMP2 complexes in dermis may be explained by the fact that the two factors are expressed in different compartments of the skin: BMP2 appears to be expressed primarily in the epithelial compartment, whereas BMP4 is expressed primarily in the mesenchymal/dermal compartment (30). In fact, skin BMP2 expression is primarily associated with hair follicles (30), which are absent in foreskin samples, such as that used in Fig. 5, A and B.

**FIGURE 4. The BMP1 prodomain binds BMP2 with a Kd of 10.9 nM.** Coomassie Brilliant Blue R-250-stained SDS-PAGE gels are shown for molecular mass standards (lane 1) and purified BMP1 prodomain-Fc fusion protein (lane 2) (A) or molecular mass standards (lane 1) and purified BMP1 prodomain minus the Fc domain (lane 2) (B). Numbers to the left of gels correspond to the approximate sizes in kDa of molecular mass markers. BMP1 prodomain (C) and murine Chordin (D) bind to BMP2 with Kd's of 10.9 ± 4.7 nM and 6.7 ± 1.0 nM, respectively.
BMP1 Prodomain Binds BMP2/4

It has previously been shown that the BMP4-binding protein twisted gastrulation (Tsg) is unable to induce secondary axis formation upon overexpression in Xenopus embryos, whereas a membrane-tethered form, created via fusion of Tsg to the integral membrane protein CD2, has a strong dorsalizing activity similar to that of Chordin (34). Presumably, this difference in effects is caused by the normally highly diffusible nature of Tsg, which renders it incapable of accumulating to a high enough localized concentration to establish an organizer-like activity (34). To examine whether a less diffusible, membrane-tethered form of BMP1 prodomain would have modified/enhanced dorsalizing activity, a chimeric protein composed of BMP1 prodomain fused to the CD2 transmembrane domain was overexpressed in 1–2-cell stage zebrafish embryos. As can be seen (Table 1), microinjection of 1 ng or 500 pg of mRNA for the membrane-tethered form of BMP1 prodomain yielded dorsalization of 100% of surviving embryos, and microinjection of 250 pg resulted in 78% dorsalization. Moreover, the majority of embryos dorsalized by injection with 1 ng or 500 pg of tethered BMP1 prodomain mRNA were severely dorsalized and could be rated as having class 4/5 dorsalization defects, according to the classification scheme of Mullins et al. (35). Thus, as with Tsg (34), a form of BMP1 prodomain designed to accumulate to high, localized concentrations within embryos acts like known in vivo antagonists of BMP signaling. Embryos injected at the two-cell stage with 500 pg of prodomain-CD2 mRNA are shown in Fig. 7, B and D at 12- and 24-h postfertilization (hpf), respectively. At 12 hpf such embryos become ovoid, rather than round just before the bud stage, a characteristic of dorsalized gastrulating embryos (35, 36). A representative 24 hpf-injected embryo is shown with a class 4 dorsalized phenotype, in which the trunk axis twists around itself, reminiscent of homozygous recessive snail-house (snh) mutations (35). Consistent with a dorsalized phenotype, in situ hybridization analysis of injected embryos at 70% epiboly demonstrated expansion of the expression domain for the dorsal marker chd, in 22/24 embryos observed (Fig. 7F), and markedly reduced expression, in 32/32 embryos observed, of gata2 (Fig. 7H), a marker for ventral ectoderm and hematopoetic cells in the ventral mesoderm. In contrast, the expression domain for goosecoid, an early marker gene associated with formation of the organizer, was unchanged in injected embryos. These data are consistent with the interpretation that dorsalization occurred downstream of organizer induction, as a consequence of disrupted BMP signaling during gastrulation.

BMP2 appears to be the primary ventralizing factor in early zebrafish dorsoventral patterning (4), in contrast to Xenopus and mouse, in which BMP4 appears to play this role (37, 38). Thus, results in the zebrafish (Fig. 7 and Table 1) and M2-10B4 (Fig. 6) systems are together consistent with the interpretation that the BMP1 prodomain is capable of binding and modulating signaling by both BMPs 2 and 4.

**Discussion**

Here a cogent explanation is provided for the previously observed co-purification of BMP1 prodomain sequences and TGFβ-like BMPs through multiple and varied biochemical purification steps. We demonstrate that the BMP1 prodomain binds BMP2/4 with high specificity and with high affinity, with a $K_d$ similar to that of Chordin, in the physiological nM range. Indeed, the affinity and specificity of binding to BMP2/4 within organisms is such that overexpression of a tethered form of the BMP1 prodomain induces a severely dorsalized phenotype in zebrafish embryos that closely resembles the phenotype achieved by Chordin overexpression. Importantly, it is demonstrated here that endogenous free BMP1 prodomain not only persists in tissues, but that it is found bound to endogenous BMP4 in such tissues. The latter results, obtained from dermal
samples, combined with the previous findings of BMP1 prodomain persistence in bone and co-purification with TGF-β-like BMPs (2), build a strong case for in vivo roles for BMP1 prodomain-BMP2/4 interactions in these tissues, and perhaps in other tissues as well. Other results presented herein strongly suggest that BMP1 prodomain-BMP4 interactions occur in the extracellular space, rather than intracellularly.

BMP1 prodomain-BMP2/4 interactions appear to have been conserved at least over the span of evolutionary time that separates humans and zebrafish, as human BMP1 prodomain sequences are shown in the current study to be capable of dorsalizing zebrafish embryos. It should also be noted that we have recently isolated zebrafish BMP1 sequences (39), and have found CD2-tethered zebrafish BMP1 prodomain sequences to induce severe dorsalization of zebrafish embryos in a manner indistinguishable from the effects of the CD2-tethered human BMP1 prodomain sequences.3 In regard to the specificity of interactions of BMP1 prodomain-BMP2/4 interactions, we have found the prodomains of the other mammalian BMP1-like proteinases, mammalian Tolloid-like 1 and 2 (mTLL1 and mTLL2), to lack the ability to bind BMP2/4.3 It remains to be determined whether the prodomains of these other proteinases have specificities for binding other growth factors.

Despite the demonstrated ability of the BMP1 prodomain to bind BMP2/4 and to inhibit BMP2/4 signaling in cell culture assays and zebrafish embryos, and despite the isolation of BMP1 prodomain-BMP4 complexes from tissues, the normal developmental and physiological consequences that result from BMP1 prodomain-BMP2/4 complex formation await further elucidation. However, genetic approaches toward such elucidation are hampered by the possibility that manipulation of BMP1 prodomain sequences and/or expression levels may also affect the physiological roles of mature BMP1, thus interfering with the interpretation of results. For example, ablation of BMP1 prodomain sequences could lead to ventralization because of loss of prodomain dorsalizing activity, but such an effect might also be ascribed to an increase in the ventralizing activity of mature BMP1, which normally acts to clear the BMP antagonist Chordin from the extracellular space (40). In regard to the latter possibility, it has previously been shown that deletion of the prodomain

3 D. S. Greenspan, unpublished data.
sequences of the BMP1-related Drosophila proteinase Tolloid results in a superactivated form of the proteinase (41).

It is likely instructive that overexpressed BMP1 prodomain, like overexpressed Tsg, only induced embryo dorsalization when in a membrane-tethered form. This suggests that the BMP1 prodomain, like Tsg, may normally be highly diffusible in tissues, and thus unable to accumulate to a high enough localized concentration to establish an organizer-like activity, upon overexpression. Consistent with the latter possibility is the finding that cleaved BMP1 prodomains are found in conditioned medium, but not associated with cell layers of cultured cells (Fig. 5C), suggesting that prodomain sequences do not bind to extracellular matrix or cell surface components. The suggestion of high diffusibility, in turn suggests the possibility that BMP1 prodomain sequences may serve a carrier function that increases the diffusibility, and perhaps affects the half-life, of BMP2/4 in tissues.

Tsg can serve a linker function, as it binds both BMP2/4 and Chordin in a tripartite complex (14, 34, 42). The possibility remains open that BMP1 prodomain sequences may serve to link BMP2/4 to other molecules, and it will thus be of interest to determine whether the BMP1 prodomain binds molecules other than BMP2/4. However, regardless of whether BMP1 prodomain serves a linker function, it is highly probable that BMP 2/4 bound to BMP1 prodomain sequences have different properties than do unbound BMP2/4, and that BMP1 prodomain sequences therefore modulate BMP2/4 signaling. In fact, BMP1 prodomain sequences may serve to modulate BMP signaling in a complex fashion, as do Tsg and another BMP-binding protein Crossveinless 2, both of which appear able to either enhance or inhibit BMP signaling according to varying in vivo conditions (14, 25, 34, 42). Indeed, it seems unlikely that the BMP1 prodomain would serve only to inhibit BMP2/4 signaling, since mature BMP1 serves to activate BMP2/4 via cleavage of Chordin (5). However, the possibility that the BMP1 prodomain modulates BMP2/4 signaling in a complex way, perhaps serving to refine signaling gradients in response to extracellular levels of other proteins, may further serve to hamper elucidation of its in vivo roles.

Acknowledgments—We thank Elisabeth Schwarz (Institut für Biotechnologie, Martin-Luther-Universität Halle-Wittenberg) for providing recombinant BMP2, and Guy G. Hoffman for excellent technical support.

REFERENCES

1. Celeste, A. I., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A., and Wozney, J. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9843–9847
2. Wozney, J. M., Rosen, V., Celeste, A. I., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) Science 242, 1528–1534
3. Hogan, B. L. (1996) Genes Dev. 10, 1580–1594
4. Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M., and Schulte-Merker, S. (1997) Development (Cambr.) 124, 4457–4466
5. Ge, G., and Greenspan, D. S. (2006) Birth Defects Res. C Embryo Today 78, 47–68
6. Ge, G., and Greenspan, D. S. (2006) J. Cell Biol. 175, 111–120
7. Ducy, P., and Karsenty, G. (2000) Kidney Int. 57, 2207–2214
8. Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S., and Wozney, J. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9484–9488
9. Lee, S., Solow-Cordero, D. E., Kessler, E., Takahara, K., and Greenspan, D. S. (1997) J. Biol. Chem. 272, 19059–19066
10. Leighton, M., and Kadler, K. E. (2003) J. Biol. Chem. 278, 18478–18484
11. Mosher, D. F., Huwiler, K. G., Misenheimer, T. M., and Anns, D. S. (2002) Methods Cell Biol. 69, 69–81
12. Scott, I. C., Blitz, I. L., Pappano, W. N., Imamura, Y., Clark, T. G., Stelgitz, B. M., Thomas, C. L., Maas, S. A., Takahara, K., Cho, K. W., and Greenspan, D. S. (1999) Dev. Biol. 213, 283–300
13. Lum, L., Reid, M. S., and Blobel, C. P. (1998) J. Biol. Chem. 273, 26236–26247
14. Scott, I. C., Blitz, I. L., Pappano, W. N., Maas, S. A., Cho, K. W., and Greenspan, D. S. (2001) Nature 410, 475–478
15. Bruckner-Tuderman, L., Schnyder, U. W., Winterhalter, K. H., and Bruckner, P. (1987) Eur. J. Biochem. 165, 607–611
16. Hillger, F., Herr, G., Rudolph, R., and Schwarz, E. (2005) J. Biol. Chem. 280, 14974–14980
BMP1 Prodomain Binds BMP2/4

17. Detrich, H. W., 3rd, Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., Pratt, S., Ransom, D., and Zon, L. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10713–10717

18. Miller-Bertoglio, V. E., Fisher, S., Sánchez, A., Mullins, M. C., and Halpern, M. E. (1997) Dev. Biol. 192, 537–550

19. Schulte-Merker, S., Hammerschmidt, M., Beuchle, D., Cho, K. W., De Robertis, E. M., and Nüsslein-Volhard, C. (1994) Development 120, 843–852

20. Schulte-Merker, S., Lee, K. J., McMahon, A. P., and Hammerschmidt, M. (1997) Nature 387, 862–863

21. Jasuja, R., Allen, B. L., Pappano, W. N., Rapraeger, A. C., and Greenspan, D. S. (2004) J. Biol. Chem. 279, 51289–51297

22. Hojima, Y., van der Rest, M., and Prockop, D. J. (1985) J. Biol. Chem. 260, 15996–16003

23. Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996) Cell 86, 589–598

24. Zimmerman, L. B., De Jesús-Escobar, J. M., and Harland, R. M. (1996) Cell 86, 599–606

25. Rentzsch, F., Zhang, J., Kramer, C., Sebald, W., and Hammerschmidt, M. (2006) Development 133, 801–811

26. Amano, S., Scott, I. C., Takahara, K., Koch, M., Champliaud, M. F., Gerecke, D. R., Keene, D. R., Hudson, D. L., Nishiyama, T., Lee, S., Greenspan, D. S., and Burgeson, R. E. (2000) J. Biol. Chem. 275, 22728–22735

27. Fukagawa, M., Suzuki, N., Hogan, B. L., and Jones, C. M. (1994) Dev. Biol. 163, 175–183

28. Botchkarev, V. A., and Sharov, A. A. (2004) Differentiation; Res. Biol. Divers. 72, 512–526

29. Ming Kwan, K., Li, A. G., Wang, X. J., Wurst, W., and Behringer, R. R. (2004) Genesis 39, 10–25

30. Botchkarev, V. A. (2003) J. Investig. Dermatol. 120, 36–47

31. Kawai, S., and Sugiura, T. (2001) Bone 29, 54–61

32. Blader, P., Rastegar, S., Fischer, N., and Strähle, U. (1997) Science 278, 1937–1940

33. Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K., and De Robertis, E. M. (1994) Cell 79, 779–790

34. Chang, C., Holtzman, D. A., Chau, S., Chickering, T., Woolf, E. A., Holmgren, L. M., Bodorova, J., Gearing, D. P., Holmes, W. E., and Brivanlou, A. H. (2001) Nature 410, 483–487

35. Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haftter, P., Heisenberg, C. P., Jiang, Y. J., Kelsch, R. N., and Nüsslein-Volhard, C. (1996) Development 123, 81–93

36. Solnica-Krezel, L., Stemple, D. L., and Driever, W. (1995) Bioessays 17, 931–939

37. Steinbeisser, H., Fainsod, A., Niehrs, C., Sasai, Y., and De Robertis, E. M. (1995) EMBO J. 14, 5230–5243

38. Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995) Genes Dev. 9, 2105–2116

39. Jasuja, R., Voss, N., Ge, G., Hoffman, G. G., Lyman-Gingerich, J., Pelegri, F., and Greenspan, D. S. (2006) Mech. Dev. 123, 548–558

40. Pappano, W. N., Steiglitz, B. M., Scott, I. C., Keene, D. R., and Greenspan, D. S. (2003) Mol. Cell Biol. 23, 4428–4438

41. Marqués, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W., and O’Connor, M. B. (1997) Cell 91, 417–426

42. Oelgeschläger, M., Larrain, J., Geissert, D., and De Robertis, E. M. (2000) Nature 405, 757–763