

Structure of bone morphogenetic protein 9 procomplex

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Bone morphogenetic proteins (BMPs) belong to the TGF- β family, whose 33 members regulate multiple aspects of morphogenesis. TGF- β family members are secreted as procomplexes containing a small growth factor dimer associated with two larger prodomains. As isolated procomplexes, some members are latent, whereas most are active; what determines these differences is unknown. Here, studies on pro-BMP structures and binding to receptors lead to insights into mechanisms that regulate latency in the TGF- β family and into the functions of their highly divergent prodomains. The observed open-armed, nonlatent conformation of pro-BMP9 and pro-BMP7 contrasts with the cross-armed, latent conformation of pro-TGF- β 1. Despite markedly different arm orientations in pro-BMP and pro-TGF- β , the arm domain of the prodomain can similarly associate with the growth factor, whereas prodomain elements N- and C-terminal to the arm associate differently with the growth factor and may compete with one another to regulate latency and stepwise displacement by type I and II receptors. Sequence conservation suggests that pro-BMP9 can adopt both cross-armed and open-armed conformations. We propose that interactors in the matrix stabilize a cross-armed pro-BMP conformation and regulate transition between cross-armed, latent and open-armed, nonlatent pro-BMP conformations.

Members of the TGF- β family including bone morphogenetic proteins (BMPs) are biosynthesized and processed into complexes between large prodomains and smaller, C-terminal mature growth factor (GF) domains that are separated by proprotein convertase (PC) (furin) cleavage sites. In the original isolation of proteins responsible for BMP activity, bone was first demineralized with 0.5 M HCl. The resulting residual matrix was extracted with 6 M urea or 4 M guanidine HCl (1–3). During subsequent purification under largely denaturing conditions, the GF domains were separated from their prodomains. Therefore, little attention was paid to the potential existence of BMP procomplexes. However, evidence exists that BMP prodomains contribute to maintaining BMP GF domains inactive or latent *in vivo*. For example, early studies showed a 60-fold increase in total BMP activity during the first two purification steps following extraction of the BMP, which was interpreted as purification of BMP away from an inhibitor (2). This finding is consistent with the presence of largely latent complexes between BMPs, their prodomains, and extracellular matrix components in the insoluble residual matrix from which BMPs were purified. In agreement with a regulatory role for the prodomain, mutations of secondary PC sites within the prodomain perturb embryonic development in insects and vertebrates, suggesting that prodomains of several BMPs remain associated with GFs after secretion and regulate the distance over which BMPs signal (4–7). An important role for the prodomain in development is also illustrated by prodomain mutations, including in secondary PC cleavage sites, that cause human diseases (5, 7).

Pro-TGF- β is latent; however, when overexpressed as recombinant proteins, most BMPs are active. Although noncovalently associated with their GF after secretion, the prodomains of most BMPs do not bind strongly enough to prevent GF from binding to receptors and signaling (8, 9). To better understand such differences among members of the TGF- β family, we examine

the structure of pro-BMP9 and compare it to the previously described, cross-armed conformation of pro-TGF- β 1 (10). Although a member of the BMP subfamily and possessing chondrogenic and osteogenic activity, BMP9 is expressed in liver and is required for properly organized blood and lymphatic vascular development (11, 12). Mutations in the prodomain of BMP9, in its receptor Alk1, and in its coreceptor endoglin cause phenotypically overlapping hereditary hemorrhagic telangiectasias (13–15).

Here, we reveal surprising open-armed conformations of pro-BMPs 7 and 9. We propose that binding to interactors in the matrix may regulate transition between open-armed and cross-armed conformations in the TGF- β family and that these conformations regulate GF latency.

Results

Structures of BMP Procomplexes. In marked contrast to the cross-armed, ring-like conformation of pro-TGF- β 1 (10), crystal structures of natively glycosylated pro-BMP9 reveal an unexpected, open-armed conformation (Fig. 1 *A* and *B* and Table S1). All negative stain EM class averages show an open-armed conformation for pro-BMP9 (Fig. 1*C* and Fig. S1) and a similar, although less homogenous, open-armed conformation for pro-BMP7 (Fig. 1*D* and Fig. S2). Crystal structure experimental electron density is excellent (Fig. S3) and allows us to trace the complete structure of each pro-BMP9 arm domain (residues 63–258; Fig. 1*E*). As in pro-TGF- β 1, the arm domain has two β -sheets that only partially overlap. Hydrophobic, nonoverlapping portions of the β -sheets are covered by meandering loops and the α 4-helix (Fig. 1 *E* and *F*). Comparison of pro-BMP9 and pro-TGF- β 1 arm domains defines a conserved core containing two four-stranded β -sheets and the α 4-helix (labeled in black in Fig. 1 *E* and *F*).

One of the BMP9 arm domain β -sheets joins a finger-like β -sheet in the GF to form a super β -sheet (Fig. 1 *A* and *G*). Each GF monomer has a hand-like shape. The two BMP9 GF hands

Significance

Bone morphogenetic protein (BMP) activity is regulated by prodomains. Here, structures of BMP procomplexes reveal an open-armed conformation. In contrast, the evolutionarily related, latent TGF- β 1 procomplex is cross-armed. We propose that in the TGF- β and BMP family, conversion between cross-armed and open-armed conformations may regulate release and activity of the growth factor.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4YCG and 4YCI).

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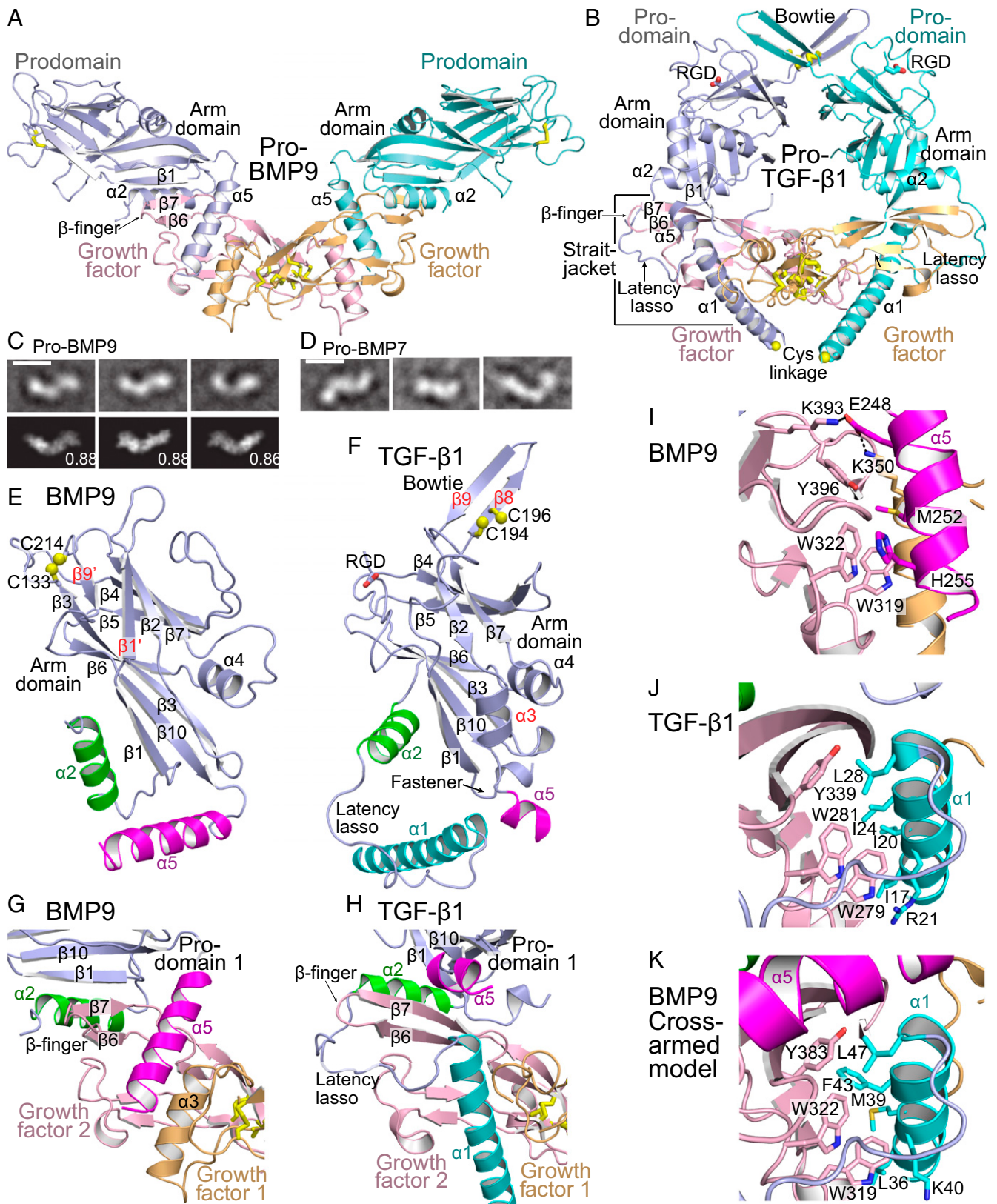
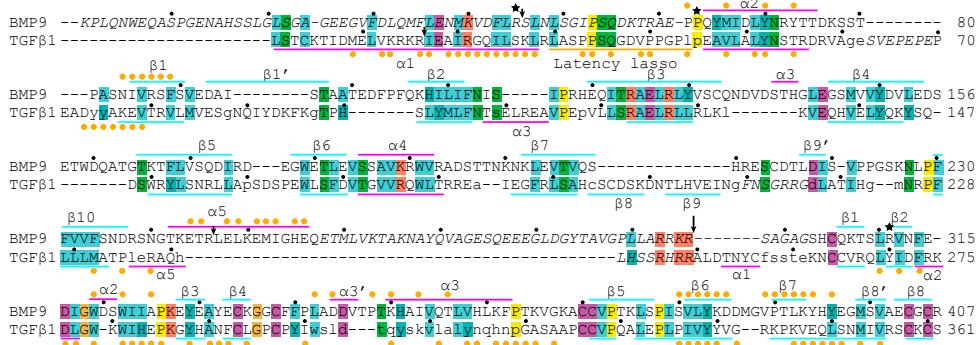


Fig. 1. Structures. (A and B) Cartoon diagrams of pro-BMP9 (A) and pro-TGF- β 1 (10) (B) with superimposition on GF dimers. Disulfides (yellow) are shown in stick. (C and D) Representative negative-stain EM class averages of pro-BMP9 (C) and pro-BMP7 (D). Best correlating projections of the pro-BMP9 crystal structure with their normalized cross-correlation coefficients are shown below class averages. (Scale bars, 100 Å.) (E and F) BMP9 and TGF- β 1 prodomains shown in cartoon after superimposition. Core arm domain secondary structural elements are labeled in black and others in red. Helices that vary in position between cross- and open-armed conformations are color-coded. Spheres show Cys S atoms. (G–K) Prodomain–GF interactions in pro-BMP9 (G and I), pro-TGF- β 1 (H and J), and a model of cross-armed pro-BMP9 (K). Structures are superimposed on the GF monomer. Colors are as in A, B, E, and F. Key residues are shown in stick.

A



B



Fig. 2. Sequence alignments. (A) Sequences aligned structurally with SSM (26). Structurally aligned residues are in uppercase; lowercase residues are not structurally aligned. Residues with missing density are in italics. Decadal residues are marked with black dots. Residues with more than 50% solvent accessible surface area buried in prodomain–GF interfaces are marked with gold dots. Secondary structural elements or named regions are shown with lines. Long black arrow marks cleavage sites between the prodomain and GF; short black arrows mark putative PC cleavage sites after the α 1-helix and in the α 5-helix. Stars mark HHT-like mutation sites. (B) Sequence alignment of representative TGF- β family members in the prodomain α 1-helix (overlined for TGF- β 1).

dimerize in their palm regions (Fig. 1A and Fig. S4) in an interface that buries 1,280 Å² of solvent-accessible surface.

The interface between each BMP9 prodomain and GF monomer buries 1,440 Å². The larger size of the prodomain–GF interface than the GF–GF interface emphasizes its significance, as does the super β -sheet interface between the prodomain and GF and the burial of hydrophobic residues by this interface and by the prodomain α 2-helix (Fig. 1A). A specialization in pro-BMP9 not present in pro-TGF- β 1 is a long α 5-helix (Fig. 1A, B, E, and F) that is a C-terminal appendage to the arm domain and that separately interacts with the GF dimer to bury 750 Å² (Fig. 1A).

Despite markedly different arm domain orientations, topologically identical secondary structure elements form the interface between the prodomain and GF in pro-BMP9 and pro-TGF- β 1: the β 1-strand and α 2-helix in the prodomain and the β 6- and β 7-strands in the GF (Fig. 1A, B, G, and H). The outward-pointing, open arms of pro-BMP9 have no contacts with one another, which results in a monomeric prodomain–GF interaction. In contrast, the inward pointing arms of pro-TGF- β 1 dimerize through disulfides in their bowtie motif, resulting in a dimeric, and more avid, prodomain–GF interaction (Fig. 1A and B).

Twists at two different regions of the interface result in the remarkable difference in arm orientation between BMP9 and TGF- β 1 procomplexes. The arm domain β 1-strand is much more twisted in pro-TGF- β 1 than in pro-BMP9, enabling the β 1- β 10- β 3- β 6 sheets to orient vertically in pro-TGF- β and horizontally in pro-BMP9 in the view of Fig. 1A and B. In addition, if we imagine the GF β 7- and β 6-strands as forefinger and middle finger, respectively, in BMP9, the two fingers bend inward toward the palm, with the β 7 forefinger bent more, resulting in cupping of the fingers (Fig. 1G and H and Fig. S4). In contrast, in TGF- β 1, the palm is pushed open by the prodomain amphipathic α 1-helix, which has an extensive hydrophobic interface with the GF fingers and inserts between the two GF monomers (Fig. 1B) in a region that is remodeled in the mature GF dimer and replaced by GF monomer–monomer interactions (10).

Role of Elements N and C Terminal to the Arm Domain in Cross- and Open-Armed Conformations. A straitjacket in pro-TGF- β 1 composed of the prodomain α 1-helix and latency lasso encircles the GF on the side opposite the arm domain (Fig. 1B). Sequence for putative α 1-helix and latency lasso regions is present in pro-BMP9 (Fig. 2A); however, we do not observe electron density corresponding to this sequence in the open-armed pro-BMP9 map. Furthermore, in the open-armed pro-BMP9 conformation, the prodomain α 5-helix occupies a position that overlaps with the

position of the α 1-helix in the cross-armed pro-TGF- β 1 conformation (Fig. 1A, B, G, and H). The differing twists between the arm domain and GF domains in open-armed and cross-armed conformations relate to the distinct ways in which the prodomain α 5-helix in pro-BMP9 and the α 1-helix in pro-TGF- β 1 bind to the GF (Fig. 1A and B). The strong sequence signature for the α 1-helix in pro-BMP9, which is essential for the cross-armed conformation in pro-TGF- β , suggests that pro-BMP9 can also adopt a cross-armed conformation (*Discussion*).

In absence of interaction with a prodomain α 1-helix, the GF dimer in pro-BMP9 is much more like the mature GF (1.6-Å RMSD for all C α atoms) than in pro-TGF- β 1 (6.6-Å RMSD; Fig. S4). Moreover, burial between the GF and prodomain dimers is less in pro-BMP9 (2,870 Å²) than in pro-TGF- β 1 (4,320 Å²). In the language of allostery, GF conformation is tensed in cross-armed pro-TGF- β 1 and relaxed in open-armed pro-BMP9.

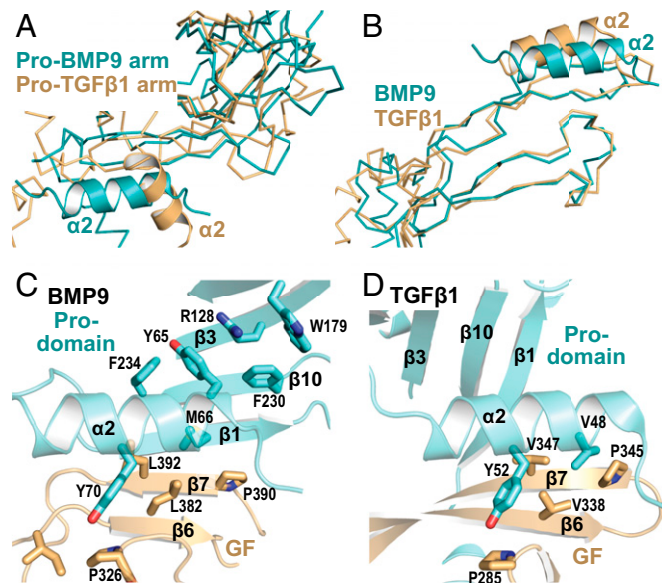


Fig. 3. The prodomain α 2-helix. (A and B) Superimpositions based on the arm domain (A) or on the GF (B), which are shown as C α traces with the α 2-helix in cartoon. (C and D) Prodomain α 2-helix environments in pro-BMP9 (C) and pro-TGF- β 1 (D) shown after superimposition on the prodomain α 2-helix and GF β 6 and β 7-strands. Key interacting residues are shown in stick.

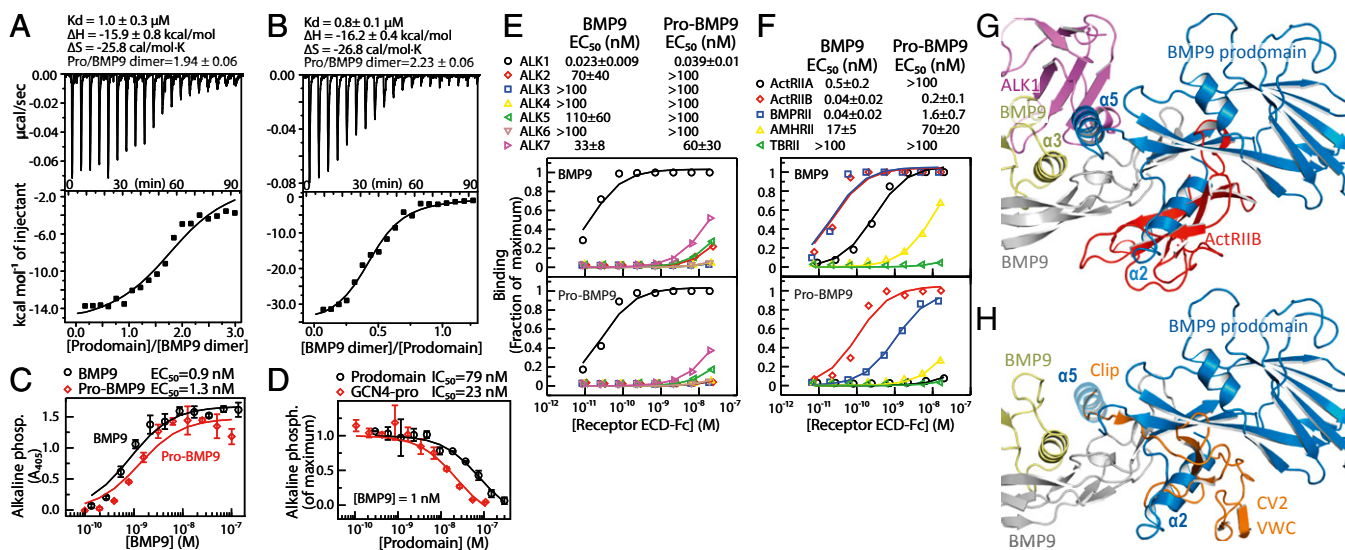


Fig. 4. Binding of BMP9 to the prodomain and receptors. (A and B) Representative ITC data at 15 °C adding material either to the BMP9 dimer (A) or prodomain (B) in the calorimetry cell. (Upper) Baseline-corrected raw data. (Lower) Integrated heats fit to the independent-binding site model. (C and D) Differentiation of C2C12 cells measured by alkaline phosphatase production. (C) Comparison of BMP9 and pro-BMP9. (D) Inhibition of 1 nM BMP9 by GCN4-linked, oligomeric BMP9 prodomain and native prodomain. (E and F) EC₅₀ values of BMP9 and pro-BMP9 for Fc-fused type I (E) or type II (F) receptor ectodomains measured using quantitative ELISA. Data are plotted as the fraction of maximal bound in each experiment and fit to the equation of fractional saturation. Graphs show average of triplicates in one experiment; numerical values show mean EC₅₀ and sd from three such experiments. (G and H) Superimpositions on pro-BMP9 of the BMP9–receptor complex (17) (G) and BMP2–crossveinless2 complex (H) in identical orientations. For clarity, BMP receptor and inhibitor complexes is omitted, and the $\alpha 5$ -helix is transparent in H.

The prodomain $\alpha 2$ -helix covers the interface between the arm and GF domains and acts as a buffer between the open-armed and cross-armed conformations. With superimposition based on arm domains, $\alpha 2$ -helix orientation differs by $\sim 90^\circ$ in pro-BMP9 and pro-TGF- $\beta 1$ (Figs. 1 E and F and 3A), whereas with superimposition based on GF monomers, $\alpha 2$ -helix orientation is similar in pro-BMP9 and pro-TGF- $\beta 1$ (Figs. 1 G and H and 3B). Thus, if we imagine shape-shifting between open- and cross-armed conformations of pro-BMP9 (Discussion), the $\alpha 2$ -helix moves in harmony with the GF rather than the arm domain.

Prodomain $\alpha 2$ -helix association with the GF is stabilized by conserved interactions. Homologous $\alpha 2$ -helix residues Tyr-70 in pro-BMP9 and Tyr-52 in pro-TGF- $\beta 1$ stack against homologous Pro-326 and Pro-285 residues in their GFs (Fig. 3 C and D). Moreover, homologous $\alpha 2$ -helix residues Met-66 in pro-BMP9 and Val-48 in pro-TGF $\beta 1$ are buried in a hydrophobic cavity on their GFs (Fig. 3 C and D).

The $\alpha 2$ -helix in open-armed pro-BMP9 interacts with the arm domain in a way not seen in cross-armed pro-TGF- $\beta 1$. Tyr-65 from the $\alpha 2$ -helix together with Trp-179 and Phe-230 from the arm domain form an aromatic cage (Fig. 3C). Arm residue Arg-128 at the center of this cage forms π -cation interactions with Tyr-65 and Trp-179 (Fig. 3C). Residues for the π -cation cage are well conserved in BMP4, 5, 6, 7, 8, and 10, GDF5, 6, and 7, and GDF15 (Fig. S5). However, in BMP2 and BMP15, Arg-128 is replaced by Gln, potentially weakening association of the prodomain with the GF in the open-armed conformation.

The similar arm domain cores and $\alpha 2$ -helices in the prodomains of BMP9 and TGF- $\beta 1$ are remarkable, given that the prodomains have only 11% identity in sequence and have 12 insertions/deletions (Fig. 2A). This contrasts with the 25% identity between their GF domains (Fig. 2A). Among notable differences, pro-BMP9 lacks the 14-residue bowtie in pro-TGF- $\beta 1$ that disulfide links the two arm domains together and has in its place a $\beta 7$ - $\beta 9'$ loop (Fig. 2A). The two cysteine residues in the TGF- $\beta 1$ arm domain, Cys-194 and Cys-196 (Fig. 1F), form reciprocal interchain disulfide bonds (10). In contrast, our pro-BMP9 structure shows

that the two arm domain cysteines, Cys-133 and Cys-214, form an intrachain disulfide that links the $\beta 3$ strand to the $\beta 7$ - $\beta 9'$ loop (Fig. 1E). The disulfide helps stabilize an extension of the $\beta 3$ -strand in BMP9 and the formation of the $\beta 1'$ - and $\beta 9'$ -strands unique to pro-BMP9 that add onto the $\beta 2$ - $\beta 7$ - $\beta 5$ - $\beta 4$ sheet (Fig. 1 E and F).

The $\alpha 5$ -helix in pro-BMP9 is its most surprising specialization. It is much longer than in pro-TGF- $\beta 1$, orients differently (Fig. 1 E and F), and binds to a similar region of the GF domain as the $\alpha 1$ -helix in pro-TGF- $\beta 1$. However, the prodomain $\alpha 1$ and $\alpha 5$ -helices orient differently on the GF domain (Fig. 1 A, B, G, and H). The BMP9 prodomain $\alpha 5$ -helix inserts into the hydrophobic groove formed by the fingers of one GF monomer and the $\alpha 3$ -helix of the other monomer (Fig. 1A). This association is stabilized by a cluster of specific interactions (Fig. 1I). Glu-248, at the N terminus of the $\alpha 5$ -helix, forms salt bridges with GF residues Lys-393 and Lys-350. In the middle of the $\alpha 5$ -helix, Met-252 plunges into a hydrophobic cavity. At the C terminus, His-255 stacks against GF residue Trp-322 (Fig. 1I). However, GF burial by the pro-BMP9 $\alpha 5$ -helix (750 \AA^2) is less than by the pro-TGF- $\beta 1$ $\alpha 1$ -helix ($1,120 \text{ \AA}^2$) or $\alpha 1$ -helix plus latency lasso ($1,490 \text{ \AA}^2$). Furthermore, when crystals were cryo-protected with a 10% higher concentration of ethanol (3.25-\AA dataset; Table S1), density for the $\alpha 5$ -helix was present in one monomer but not the other (Fig. S6).

Prodomain Functions. We next asked if interactions of the two BMP9 prodomains with the GF dimer are independent or cooperative. Isothermal calorimetry (ITC) showed that, irrespective of whether increasing amounts of prodomain were added to GF or vice versa, heat production showed a single sigmoidal profile (Fig. 4 A and B). Curves fit well to a model in which the two binding sites are independent, and yielded K_D values of $0.8\text{--}1.0 \mu\text{M}$ at pH 4.5, which maintains BMP9 solubility.

A critical question concerning BMP prodomains is whether the BMP9 prodomain inhibits GF signaling and whether making the BMP9 prodomain dimeric as in pro-TGF- $\beta 1$ would provide sufficient avidity to keep the GF latent. Consistent with previous

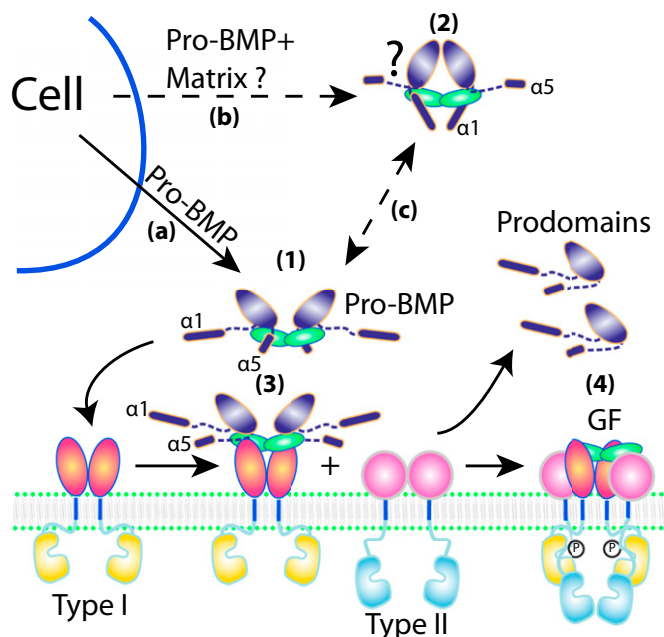


Fig. 5. Models for pro-BMP9 structures and binding to receptors. Models for the open-armed, nonlatent pro-BMP9 conformation characterized here (1), the proposed (dashed lines) pro-TGF- β 1-like cross-armed conformation of pro-BMP9 (2), and stepwise binding (18) to type I (3) and type II receptors (4).

studies (16), pro-BMP9 and BMP9 showed little difference in inducing C2C12 cell differentiation, with EC_{50} values of 1.3 and 0.8 nM, respectively (Fig. 4C). However, by doing the experiment a different way, i.e., by titrating the prod domains into assays with 1 nM BMP9, we found that the prod domain is inhibitory, with $IC_{50} = 79$ nM (Fig. 4D). Thus, a large excess of the BMP9 prod domain over the GF is required to inhibit signaling, whereas at equimolar 1-nM concentrations of prod domain and GF monomers, the BMP9 prod domain has little effect on signaling. We added a cysteine-bearing GCN4 coil at the N terminus of the BMP9 prod domain to disulfide-link BMP9 prod domains into dimers or oligomers (Fig. S7). Multimerization modestly lowered the IC_{50} to 23 nM (Fig. 4D) but did not induce latency at equimolar concentrations.

To determine the basis for the partial inhibition of BMP9 signaling by the prod domain, we compared binding of receptor Fc fusions to pro-BMP9 and BMP9 in quantitative ELISA (Fig. 4E and F). Among the seven type I receptors, ALK1 bound by far the strongest, and there was little difference in affinity and no difference in selectivity between BMP9 and pro-BMP9 (Fig. 4E). In contrast, the prod domain altered both EC_{50} values and selectivity of type II receptors (Fig. 4F). Whereas BMP9 bound ActRIIB and BMPRII similarly and 10-fold better than ActRIIA, pro-BMP9 bound ActRIIB 10-fold better than BMPRII and did not detectably bind ActRIIA (Fig. 4F). The EC_{50} values for type I and II receptors agree with previous affinity measurements with BMP9 (16, 17). Interestingly, the BMP7 prod domain also competes binding of BMP7 to type II but not type I receptors (18), consistent with the open-armed conformation of pro-BMP7 (Fig. 1D).

Discussion

BMPRII and ActRIIA are coexpressed with ALK1 and mediate BMP9 signaling in endothelial cells (12, 19). Furthermore, ALK1, which primarily functions as a receptor for BMP9 and BMP10, has recently been implicated as an important target of antiangiogenic tumor therapy (17). Together with the finding that BMP9 complexed with its prod domain circulates in the

bloodstream at physiologically relevant concentrations (11), our finding that the BMP9 prod domain blocks binding to ActRIIA and alters selectivity for ActRIIB compared with BMPRII has important implications for BMP9 signaling in vivo.

The pro-BMP9 structure explains the selective effect of BMP prod domains on type II receptor binding (18) (Fig. 4G). The prod domain arm domain and α 2-helix occupy the type II receptor binding site. In contrast, only the prod domain α 5-helix blocks the type I receptor binding site (Fig. 4G). Selective displacement of the α 5-helix by the type I receptor with retention of arm domain association is consistent with the relatively weak α 5-helix interface described above. In contrast, arm domain binding to the type II receptor site is strong enough to alter receptor affinity and selectivity.

Antagonists including noggin, gremlins, and chordin bind to BMP GFs and regulate activity. However, little attention has been paid to whether BMP prod domains might prevent antagonist binding. Interestingly, the BMP-inhibiting fragment of the chordin family member crossveinless-2 binds to interfaces on BMP2 (20) similar to those on BMP9 to which the prod domain binds (Fig. 4H). The von Willebrand factor C (VWC) domain binds to a similar site on the GF fingers as the arm domain, whereas an N-terminal appendage called clip binds to the same site as the prod domain C-terminal appendage, the α 5-helix (Fig. 4H). Whether prod domains can protect GF from inhibitors, as well as prevent GF binding to receptors, deserves study.

The crystal structure of pro-BMP9 begins to reveal how prod domains contribute to the tremendous functional diversity among the 33 members of the TGF- β family. Many of these members have prod domains that differ even more than BMP9 and TGF- β , which have only 11% sequence identity. Prod domain divergence may increase the specificity of GF signaling in vivo by regulating pro-complex localization, movement, release, and activation in the extracellular environment.

The open-armed pro-BMP7 and 9 and cross-armed pro-TGF- β 1 conformations differ greatly. Overall learnings from protein families that can adopt multiple conformations, such as tyrosine kinases, integrins, G protein-coupled receptors, membrane channels, and membrane transporters, show that when markedly distinct conformations are glimpsed for individual members, most family members can visit each state, often in a manner that is regulated by other interactors. Thus, we hypothesize that most members of the TGF- β family can visit both cross-armed and open-armed conformations. TGF- β is a later evolving family member; whereas BMPs and activins are found in all metazoans, TGF- β is found only in deuterostomes. Furthermore, TGF- β is the only known member with disulfide-linked arm domains. Thus, trapping pro-TGF- β in a solely cross-armed conformation with disulfides may be a later evolutionary adaptation.

The amino acid sequence of a protein is constrained by its structure, and sequence conservation in evolution is a powerful predictor of protein structure and conformation. The prod domain α 1-helix has an important function in stabilizing the cross-armed conformation but has no function in the open-armed conformation, as shown by lack of electron density and presence of the prod domain α 5-helix in a position that prevents α 1-helix binding. In support of the hypothesis that pro-BMP9 can adopt a cross-armed conformation, the amino acid sequence corresponding to the α 1-helix is highly conserved (44–79% identity at residues 29–47) among human, mouse, zebrafish, and chicken BMP9s. Indeed, the sequence of the α 1-helix is more conserved than the remainder of the prod domain (33–74% identity). Furthermore, the prod domain α 1-helix sequence and its amphipathic signature are also conserved among diverse representatives of the 33-member TGF- β family including BMP7 (Fig. 2B). Importantly, the α 1-helix and its amphipathic signature are highly conserved between pro-TGF- β 1 and pro-BMP9 (Fig. 2). These results support the hypothesis that pro-BMP9 and other TGF- β family members can adopt an α 1-helix-bound, cross-armed conformation similar to that of TGF- β 1.

To more directly test evolutionary support for a cross-armed BMP9 conformation, we made a pro-TGF- β 1-like model of pro-BMP9 that uses the BMP9 conformation of the arm domains, superimposed on the cross-armed orientation of the arm domains in pro-TGF- β 1, and pro-TGF- β 1-like conformations of prodomain α 1- and α 2-helices and GF domains (Movie S1). In addition to the α 1-helix, pro-BMP9 also includes a latency lasso-like sequence, including an identical PSQ sequence (Fig. 2A). There are no clashes between the two pro-BMP9 arm domains in the crossed-arm conformation; notably, the arm domains come close together at their β 4 and β 5-strands, which are on the side of the arm domain conserved between pro-BMP9 and pro-TGF- β 1 (Movie S1). The extensive, amphipathic α 1-helix-GF interface in pro-TGF- β 1 is recapitulated well in the cross-armed pro-BMP9 model, and the long α 5-helix can adopt a conformation similar to the shorter α 5-helix in pro-TGF- β 1 without clashes (Fig. 1K). These results compellingly support a cross-armed conformation for pro-BMP9. A plausible pathway for structural interconversion between open-armed and cross-armed conformations of BMP9 can be described in which crossing of the arms is accompanied by dissociation of the α 5-helix from the GF and its replacement by the α 1-helix and latency lasso (Movie S1).

The strong evolutionary and 3D structural support for a cross-armed conformation of BMP9 (and also BMP7; Fig. 2B) contrasts with our lack of observation of cross-armed BMP7 and BMP9 conformations in EM (Fig. 1C and D). However, this is easily explicable, because it is compatible with a lower energy of the open-armed conformation for the isolated procomplex, and on the other hand, with a lower energy of the cross-armed conformation for the procomplex bound to an interactor. For BMPs in bone, such interactors may be present in the residual matrix, and release from interactors may in part be responsible for the increase in BMP activity found after extraction by denaturants and purification (2).

We hypothesize that cross-armed and open-armed conformations of TGF- β family members correspond to latent and nonlatent states, respectively, and propose a model for conformational regulation of release from storage and latency (Fig. 5). Some family members may be secreted as isolated procomplexes in signaling-

competent, open-armed conformations (Fig. 5, pathway a and structure 1). Other family members may be secreted together with or immediately form complexes after secretion with extracellular matrix components including heparin, proteoglycans, fibrillin, and latent TGF- β binding proteins (8, 9, 21–23). These interactors may stabilize the cross-armed conformation (Fig. 5, pathway b and structure 2), and enable the GF domain, which is very short lived in vivo, to remain latent and reach storage concentrations as high as 100 ng/g in demineralized bone (24). Release from storage in vivo may then yield the open-armed conformation, which is ready for receptor or inhibitor binding (Fig. 5, pathway c).

TGF- β family members with long sequences at the ends of their prodomains that may have α 5-helix-like functions include BMP3, BMP10, BMP15, GDF5, 6, 7, and 9, anti-Müllerian hormone, and nodal (Fig. S5). A number of these, including BMP9 and BMP10, have basic sequences resembling PC cleavage sites (25) in or before the α 5-helix (Fig. 2A and Fig. S5). Moreover, many TGF- β family members have PC or tollid cleavage sites in or after the prodomain α 1-helix that regulate activation or signaling (6, 7, 9, 10, 25) (Fig. S5). Indeed, recombinant pro-BMP9 preparations contain a minor component cleaved at a putative PC site in this region (Fig. 2A and Methods). Thus, potential mechanisms for regulating the switching between open-armed and cross-armed procomplex conformations include removal of the α 1- or α 5-helix by proteases in addition to binding to extracellular matrix components. Our results suggest that the open-armed conformation of pro-BMP9 can readily bind to type I receptors, with displacement of the α 5-helix (Fig. 5, structure 3). The final step in signaling could then be binding to type II receptors, with complete prodomain dissociation, consistent with a previous model of stepwise receptor binding and prodomain displacement (18) (Fig. 5, structure 4).

Methods

Pro-BMP9 and pro-BMP7 were purified from supernatants of CHO and HEK293 cell transfectants, respectively. Crystals formed in 0.15 M zinc acetate, 0.1 M sodium cacodylate, pH 5.8, and 4% (vol/vol) isopropanol. Phases were solved using Zn anomalous diffraction. Complete methods are described in *SI Methods*.

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