SURVEY

Signal Transduction by Bone Morphogenetic Proteins

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Bone morphogenetic proteins (BMPs) are multifunctional cytokines, which are members of the transforming growth factor-β (TGF-β) superfamily. Activities of BMPs are extracellularly regulated by BMP-binding proteins, Noggin and Chordin. BMPs bind to two different types of serine-threonine kinase receptors, type I and type II. Two BMP type I receptors and a BMP type II receptor have been identified in mammals. Intracellular signals are transduced by Smad proteins. Smad1, Smad5 and probably MADH6, are activated by BMP receptors, form heteromorphic complexes with Smad4, and translocate into the nucleus where they may activate transcription of various genes. Smad6 and Smad7 are inhibitory Smads, and may act as autocrine switch-off signals. In Drosophila melanogaster, Decapentaplegic (Dpp) is a homologue of mammalian BMPs. In this review, mechanism of action of Dpp will be discussed in comparison with that of BMPs.

Key words: Bone morphogenetic protein · Serine-threonine kinase receptor · Signal transduction · Smad · Drosophila.

Bone morphogenetic proteins (BMPs) were originally identified as molecules that induce bone and cartilage formation when implanted at ectopic sites in rats [1–3]. In accordance with their in vivo effects, BMPs regulate growth and differentiation of chondroblast and osteoblast lineage cells in vitro. However, BMPs have been shown to be multifunctional proteins with a wide range of biological activities on various cell types, including monocytes, epithelial cells, mesenchymal cells, and neuronal cells. BMPs regulate growth, differentiation, chemotaxis, and apoptosis of these cells, and play pivotal roles in morphogenesis of various tissues and organs [4].

BMPs belong to the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins/inhibins, and Müllerian inhibiting substance (MIS) [5]. Activins were originally identified as proteins that induce secretion of follicle stimulating hormone. Importantly, activins induce dorsal mesoderm when injected into Xenopus embryos. In contrast, BMP-4, a member of BMPs, induces ventral mesoderm in the Xenopus animal cap assay. Thus, activins and BMPs have opposing effects in the mesoderm induction. BMPs also inhibit neural induction of ectodermal cells and induce epidermis in Xenopus embryos [4]. It is noteworthy that many important findings regarding the action of activins and BMPs have been obtained by studies using the Xenopus assay.

BMP-like molecules have been identified in various species, including Drosophila melanogaster and Caenorhabditis elegans. Drosophila BMP homologues, i.e. gene products of decapentaplegic (dpp), 60A and Screw (Scw), play important roles in morphogenesis during embryonal development. Drosophila Dpp induces bone and cartilage in mammals [6] and mammalian BMP-4 can rescue genetic defects by dpp mutations [7]; thus, mammalian and Drosophila BMPs are functionally interchangeable. Studies on Drosophila Dpp have provided valuable information for understanding the roles of BMPs in mammals, and those on mammalian BMPs have been valuable for Drosophila research. In this review, we discuss the mechanism of action of mammalian BMPs by comparing their receptors and signal transduction system with those for Drosophila Dpp.

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BMPs IN MAMMALS AND DROSOPHILA

BMPs are disulfide-linked dimeric proteins. Most of the BMP proteins have seven cysteine residues in each monomer, which are conserved in the members of the TGF-β superfamily. Thus, the three-dimensional structures of BMPs are similar to the other members in the TGF-β superfamily. BMPs are produced as large precursor proteins; after proteolytic processing at the characteristic RXRR consensus site, the C-terminal regions form mature BMPs.

Thus far, more than 15 BMPs have been identified in mammals (Fig. 1). They can be classified into several subgroups by their amino acid sequence similarity. BMP-2 and BMP-4, which have 83% amino acid sequence identity with each other, are the best-studied members in the BMP family. BMP-2 and BMP-4 have potent bone and cartilage-inducing activity in vivo. They play important roles in early embryonic development; null mutation of the BMP-4 gene resulted in defects in extraembryonic and posterior/ventral mesoderm formation, and the embryos died between embryonic day 6.5 (E6.5) and E9.5 [8]. BMP-2 also plays a critical role in extraembryonic and embryonic mesoderm development; BMP-2-deficient mice had amnion/chorion malformation and defect in cardiac development, and died between E7.5 and E9.0 [9]. BMP-2 and BMP-4 are structurally most similar to the dpp gene product of Drosophila, which plays critical roles, e.g., in dorsal-ventral patterning, gut morphogenesis and wing vein formation. Thus, the members in the BMP-2/4 group have a wide range of biological activity in vivo, and they play pivotal roles in embryonal development.

Osteogenic protein-1 (OP-1; also termed BMP-7), OP-2 (OP-8), BMP-8B (OP-3), BMP-5, and BMP-6 (also termed Vgr-1) form another subfamily. OP-1/BMP-7 has a potent bone- and cartilage-inducing activity in vivo. Mice with null mutation in the OP-1/BMP-7 gene died shortly after birth with defects in the morphogenesis of kidneys and eyes [10, 11]. Moreover, the mutant mice exhibited defects in skeletal patterning in the hind limbs, the skull and the rib cage. BMP-8B is present in mice but not in human; BMP-8B-deficient mice were shown to have abnormalities in the spermatogenesis in early puberty as well as in the adult [12]. Mice known as the short ear have mutations in the BMP-5 gene; the mice have abnormalities in the skull and axial parts of the skeleton, e.g., ears, sternum, ribs and vertebral processes [13]. Thus, animals which lack members of the OP-1/BMP-7 subgroup are not embryonic lethal, but many of them have defects in various tissues including skeletal tissues. Drosophila 60A gene product is similar to OP-1/BMP-7 and other members in this subgroup. Phenotypes in dpp mutants can be rescued by dpp but not by 60A, indicating that they have distinct and specific effects in Drosophila development [14].

Growth/differentiation factor-5 (GDF-5) was also isolated as cartilage-derived morphogenetic protein-1 (CDMP-1) [15, 16]. GDF-5 is structurally similar to GDF-6 (CDMP-2) and GDF-7. GDF-5 is produced in cartilage and joints [16, 17], and it acts in a relatively specific manner for chondrogenesis when assayed using rat limb bud cells [18]. GDF-5, GDF-6 and GDF-7 were also shown to induce tendon and ligament formation when implanted at ectopic sites in vivo [17]. Mice with mutations in the GDF-5 gene are known as the brachypodism mice, which have short limbs and reduced numbers of bones in the digits [15]. Mutations in the human GDF-5/CDMP-1 gene result in abnormalities similar to the mice brachypodism known as acromesomelic chondrodysplasia, Hunter-Thompson type (CHTT) [19] and chondrodysplasia Grebe type (CGT) [20]. The limb shortening and dysmorphogenesis in CGT is much more severe than those in CHTT, because the mutant GDF-5/CDMP-1 protein in CGT may form heterodimers with other BMPs, and affect the functions of these proteins. Moreover, a recent study has shown that heterozygous mutations in the GDF-5/CDMP-1 gene are responsible

![Figure 1](image-url). Members of the BMP family. Amino acid sequences of the mature regions of BMPs in mammals and Drosophila [D] are compared. Phenotypes of functionally null mice and human (underlined) are shown.
BMP Receptors and Signal Transduction

EXTRACELLULAR ANTAGONISTS OF BMPs

TGF-βs are secreted as latent high molecular weight complexes from producer cells. In order for TGF-βs to exhibit their activities, latent TGF-β should be activated. In contrast, members of the BMPs appear to be secreted as active forms, and they do not require the activation process. However, there are proteins that regulate the functions of BMPs by forming inactive complexes before binding of BMPs to their receptors.

The noggin gene product has been identified as a molecule produced by Spemann organizer in Xenopus embryos. Noggin antagonizes the action of BMPs, and induces neural tissue and dorsalizes ventral mesoderm. Noggin directly binds BMP-4 and BMP-2 with a high affinity, and prevents the BMP-4 binding to the receptors [28]. Noggin binds OP-1/BMP-7 with a lower affinity, but not TGF-β1.

The chordin gene product in Xenopus, and its Drosophila homologue short gastrulation (sog), also act as antagonists of BMPs. Similar to Noggin, Chordin directly binds BMP-4 and BMP-2, but not TGF-β1 or activin, and interfere with the binding to the receptors [29]. Although the affinity of Chordin to BMP-4 (K_D = 20 nM) is lower than that of Noggin (K_D = 300 pM), Chordin is more potent than Noggin in neural induction in Xenopus, suggesting the difference in their in vivo action [29]. Noggin and Chordin have been originally identified in Xenopus, but their homologues appear to exist in mammals.

Follistatin binds activins with a high affinity and serves as an activin antagonist. Although the affinity is much lower, follistatin was shown to bind OP-1/BMP-7 [30] as well as BMP-4 [31]. Mice with null mutation in the follistatin gene had multiple defects e.g. in skin, diaphragm, hard palate, and ribs, and died shortly after birth. Since the phenotypic defects are more widely observed than in the activin-deficient mice, follistatin may regulate the activities of not only activins but also other members in the TGF-β superfamily [32].

BMP RECEPTORS

Activation of serine-threonine kinase receptors

Members in the TGF-β superfamily bind to two different types of serine-threonine kinase receptors, termed type I and type II receptors [33]. Both receptor types are required for the signal transduction. In the TGF-β and activin receptor systems, ligands bind directly to the type II receptors, whereas the type I receptors can bind ligands only in the presence of the type II receptors. Overall structures of the type II and type I receptors are similar; they have a relatively short extracellular domain with some conserved cysteine residues, a single transmembrane domain, and an intracellular domain containing serine-threonine kinase region. At the N-terminal to the serine-threonine kinase domains, type I receptors, but not type II receptors, have the GS domains, which have a characteristic repeat of serines and glycines. Thus far, seven type I receptors and five type II receptors have been identified in mammals (Fig. 2A).

Mechanism of activation of the serine-threonine kinase receptors has been most intensively studied in the TGF-β receptor system. The TGF-β type II receptor (T/βR-II), and probably the type I receptor (T/βR-I), exist as homologmers in the absence of ligands. The T/βR-II kinase, but not the T/βR-I kinase, is constitutively active without ligand stimulation [34]. After ligand binding, T/βR-II and T/βR-I form a hetero-oligomeric complex, most likely to be composed of two molecules of each of T/βR-II and T/βR-I. The serine-threonine kinase of T/βR-II transphosphorylates the GS domain as well as certain other amino acids in the intracellular part of T/βR-I, which leads to the activation of the T/βR-I kinase. Consequently, signals are transduced from the T/βR-I serine-threonine kinase.

Mutations in Thr-204 in T/βR-I and corresponding amino acid residues (Gln in BMP type I receptors; see below) to acidic amino acids, i.e. aspartic acid or glutamic acid, lead to constitutively active forms of type I receptors [35]. The constitutively active type I receptors transduce signals even in the absence of ligands and type II receptors, which suggests that the type I receptors act as downstream components of type II receptors in the signal transduction pathway. Although most of the signals appear to be transduced by constitutively active type I receptors, a possibility still remains that certain signals are transduced via type II receptors.
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Figure 1. Amino acid sequences of the kinase domains of serine-threonine kinase receptors in mammals and Drosophila (D) are compared. Phenotypes of functionally null mice and human (underlined) are shown. Null mutations in the TβR-II and MISR-II genes were reported in Refs. 116 and 117, respectively. Mutations in the human ALK-1 gene were reported in Ref. 119. (b) Comparison of the amino acid sequences of the L45 loop of type I receptors. Parts of the kinase subdomains IV and V are also shown. Amino acid residues conserved in more than 4 receptor molecules are boxed.

Figure 2. Serine-threonine kinase receptors. (A) Amino acid sequences of the kinase domains of serine-threonine kinase receptors in mammals and Drosophila (D) are compared. Phenotypes of functionally null mice and human (underlined) are shown. Null mutations in the TβR-II and MISR-II genes were reported in Refs. 117 and 118, respectively. Mutations in the human ALK-1 gene were reported in Ref. 119. (B) Comparison of the amino acid sequences of the L45 loop of type I receptors. Parts of the kinase subdomains IV and V are also shown. Amino acid residues conserved in more than 4 receptor molecules are boxed.

Yeast two-hybrid screening using the TβR-I intracellular domain as a bait led to the identification of FKBP12 as a protein that interacts with type I receptors [36–39]. FKBP12 is a binding protein for immunosuppressants such as FK506 and rapamycin. Through binding to the Leu-Pro sequence in the GS domain of type I receptors, FKBP12 negatively regulates the activity of type I receptors. Since type I and type II receptors have intrinsic affinity with each other even in the absence of ligands, FKBP12 prevents spontaneous activation of type I receptors by type II receptors in vivo [38, 39].

**Type II receptors for BMPs**

A type II receptor for BMPs (BMPR-II) has been identified by a polymerase chain reaction (PCR)-based approach [40, 41] as well as by yeast two-hybrid screening using the TβR-I intracellular domain as a bait [42, 43]. To different variants of BMPR-II have been reported. The long form is comprised of 1038 amino acid residues, which has a long C-terminal tail rich in serine and threonine residues after the kinase domain. The short form, which lacks the long C-terminal tail, may occur by alternative splicing of mRNA [44], but it is rare in most cell types. It was reported that the long and short forms of BMPR-II were functionally indistinguishable when assayed in Xenopus embryos [45], but it is possible that they have different in vivo functions.

BMPR-II is expressed in many different tissues, including skeletal muscles, heart and brain [40, 42]. BMPR-II is also expressed in cultured cell lines, such as ROB-C26 osteoprogenitor cells, mink lung epithelial cells and COS cells [40, 46]. The type II receptor for TGF-β or activin binds ligands with a high affinity by itself. In contrast, BMPR-II binds BMP-2, BMP-4 and OP-1/BMP-7 only weakly in the absence of type I receptors, and the binding is facilitated by the type I receptors [see below; Refs. 40, 41, 43]. These data suggest the presence of coordination for the optimal binding of BMP type I and type II receptors to their ligands.

In addition to BMPR-II, which is specific for BMPs, type II receptors for activin (ActR-II and ActR-IIB) bind certain members of BMPs, e.g. OP-1/BMP-7 and BMP-2 [30, 47]. However, binding affinities for BMPs to ActR-II and ActR-IIB were lower than those for activins. Binding of OP-1/BMP-7 to ActR-II and ActR-IIB was also enhanced by the type I receptors [30]. Truncated forms of ActR-II and ActR-IIB have been used as antagonists of activins in various studies, but since they may antagonize activins as well as BMPs, the experimental data should be carefully interpreted.

Fetuin was suggested to act as an antagonist of both
TGF-β and BMPs [48]. Binding affinity of fetuin to BMPs was higher than that to TGF-β. A 19 amino acid sequence in fetuin, which forms a disulfide loop, showed a weak homology to the extracellular domain of TβR-II, and this part was shown to bind to BMPs and, with a lower affinity, to TGF-β. However, this part is not similar to BMPR-II or ActR-IIs, suggesting that the antagonist effect of fetuin to BMPs is not due to the competition with BMPs to the type II receptors.

Some of the ActR-II-deficient mice had hypoplasia of mandible and other skeletal and facial abnormalities [49], although most of the mice did not show such defects. In addition, suppression of the secretion of follicle stimulating hormone (FSH) and reproductive defects were observed in the ActR-II-deficient mice. Null mutation of ActR-IIB gene resulted in complicated cardiac defects, disturbance of lateral asymmetry, and abnormal patterning of the vertebrae, indicating that ActR-IIB plays a pivotal role in the patterning of the anteroposterior and left-right body axes [50]. The phenotypes of ActR-II- or ActR-IIB-deficient mice are different from those of activin A- or activin B-deficient mice [51], indicating that ActR-II and ActR-IIB bind various TGF-β superfamily members.

Punt was originally identified as a Drosophila activin type II receptor, Atr-II, since it binds activin A when transfected into mammalian cells [52]. Punt was then shown to bind Dpp and BMP-2 in the presence of Drosophila Dpp type I receptors and function as a type II receptor for Dpp in vivo [53, 54]. Thus, similar to mammalian activin type II receptors, Punt may be shared by Drosophila BMP homologues and activin homologues.

**Type I receptors for BMPs**

In mammals, two different type I receptors specific for BMPs, termed BMP type IA (BMPR-IA; also termed activin receptor-like kinase-3 or ALK-3) and type IB receptors (BMPR-IB; also termed ALK-6), have been isolated [55–57]. The two BMP type I receptors are most similar to each other in their structures; in the kinase domain, they have 85% amino acid sequence identity. Thus far, functional difference in the intracellular domains between BMPR-IA and BMPR-IB is not fully determined, but they activate Smad proteins, at least in part, in a different fashion [Ref. 58 and data not shown], suggesting that they have different in vivo functions.

BMPR-IA is expressed in various cultured cells, including MC3T3-E1 osteoblasts, human foreskin fibroblasts [55], and C2C12 myoblasts [59]. In contrast, BMPR-IB expression is limited to certain cell types, e.g., glioblastoma cell lines and ROB-C26 cells [46]. During embryogenesis, BMPR-IA is ubiquitously expressed [60]. BMPR-IB is seen only in brain in adult tissues, but it is expressed in various tissues during embryogenesis [6]. However, expression profile of BMPR-IB is more restricted than that of BMPR-IA. BMPR-IB is expressed in glioblastoma tissues, and its expression correlates with the grade of malignancy of glioblastoma, suggesting that BMPs may play an important role in the progression of this type of tumor [61].

Binding affinities to BMPR-IA and BMPR-IB are quite different among the members in BMPs. Both BMPR-IA and BMPR-IB bind BMP-4 with similar affinities. OP-1/BMP-7 binds more strongly to BMPR-IB than to BMPR-IA [55]. In contrast, GDF-5 preferentially binds to BMPR-IB, but not to BMPR-IA [46]. In addition to the two BMP-specific type I receptors, one of the activin type I receptors, ActR-I (also termed ALK-2 or Tsk-7L), binds to OP-1/BMP-7 and probably other BMPs [55]. Thus, ActR-I is shared by activins and BMPs. However, most of the signals for activins are transduced by ActR-IB; physiological ligands and in vivo function of ActR-I remain to be determined.

In contrast to the TGF-β receptor systems, BMP type I receptors bind ligands even in the absence of type II receptors; binding to type I receptors is facilitated in the presence of type II receptors [40, 41, 43, 55, 56]. Similar binding properties have been demonstrated in Drosophila Dpp type I receptors, Thick veins (Tkv) and Saxophone (Sax) [62, 63]. Although the binding properties are different from those of the TGF-β and activin receptors, both type I and type II receptors are required for the signal transduction for BMPs; BMP type II receptors activate type I receptors, and signals are mediated through the type I receptors. Specificity of the signals is thus determined by type I receptors. In contrast, type II receptor kinases are less specific with regard to the signaling specificity; studies using chimeric receptors revealed that BMP or activin type II receptors can efficiently activate TβR-I kinase [64, 65].

Soluble form of TβR-II without the transmembrane and cytoplasmic domains can bind ligand independently and blocks the activity of TGF-β [66]. In contrast, the extracellular domain of BMPR-IA binds BMP-4 with a relatively high affinity in the absence of type II receptors, but not activin A or TGF-β1 [67]. Therefore, it may be useful as an antagonist of BMPs.

Truncated forms or kinase inactive forms of BMPR-IIs have been used to investigate various functions of BMPs in vitro and in vivo. BMPs inhibit the differentiation of C2C12 myoblasts to myotubes and converts their differentiation to osteoblast lineage; however in the presence of the truncated form of BMPR-IA, the cells differentiated into myotubes [59]. In Xenopus embryos, the truncated BMPR-IA converted ventral mesoderm to dorsal mesoderm, which showed the important role of BMPs in ventral mesoderm induction [57, 68]. In the chick limbs, truncated forms of BMPR-IA and BMPR-IB induced the web formation in the interdigital zone, indicating that BMPs induce apoptosis in interdigital tissues [69, 70].

Gene targeting of BMPR-IA showed that homozygous mutants were smaller than the normal mice at E7.0 and died by E9.5 due to a defect in mesoderm formation during gastrulation [71], indicating that, similarly to its ligands BMP-2 and -4, BMPR-IA plays an important role in mesoderm formation.
In *Drosophila*, two type I receptors for Dpp, Tkv and Sax have been identified. Both act as type I receptors for Dpp together with the type II receptor Punt, but there appears to be certain differences in their *in vivo* functions. High concentrations of Dpp activate both Tkv and Sax, but intermediate levels of Dpp activate only Tkv [72]. It was recently reported that a region in the kinase sub-domain IV and V, termed “L45 loop”, is most important for the determination of signaling specificity of type I receptors [73]. The most divergent eight amino acid sequence of the L45 loop is identical between BMPR-IA and BMPR-IB except for one amino acid, but quite different from that of other mammalian type I receptors (Fig. 2B). The L45 loop of Tkv is similar to BMPR-IA and BMPR-IB, whereas that of Sax is more similar to ALK-1 and ActR-I, suggesting that the kinases of Tkv and Sax may transduce different signals. Further studies, e.g. interaction with Smad proteins, will be important for the elucidation of the signaling activity of Tkv and Sax.

**INTRACELLULAR SIGNALLING BY SMAD PROTEINS**

Identification of members of the Smad family

Various strategies have been undertaken to search for signaling components located downstream of the type I receptor kinases, which has led to the isolation of different classes of molecules. A yeast genetic screen for mitogen-activated protein kinase kinase (MAPKKK) resulted in the cloning of TGF-β activated kinase (TAK1) [74]. TAK1 stimulates the expression of plasminogen activator inhibitor-1 (PAI-1), one of the TGF-β-inducible genes. The kinase activity of TAK1 is stimulated not only by TGF-β but by BMP-4. Further studies will be required to elucidate the precise role of TAK1 in the signal transduction of the TGF-β superfamily.

A breakthrough toward the understanding of the signaling pathway of the TGF-β superfamily has come from genetic screens conducted in two different organisms. *Mothers against dpp* (Mad), along with Medea, was isolated in an attempt to identify enhancers of dpp mutations in *Drosophila* [75, 76]. Maternally, Mad mutations exacerbated the embryonal lethality caused by dpp mutations. Mad mutations also enhanced various dpp phenotypes associated with appendages such as wings, eyes and claws in zygotes. The protein sequence of Mad, without any known functional motifs, served no clue then to understand the function of the protein.

Daf-4 is a type II serine-threonine kinase receptor in *Caenorhabditis elegans*, and binds BMP-2 and BMP-4 *in vitro*. Mutants in this gene exhibit a small body size and abnormalities in the tail. Sma-2, 3 and 4 were identified as genes whose mutations show identical phenotypes to the daf-4 mutations [77]. The sequence analysis of these genes revealed that all of them are homologous to *Drosophila* Mad. From genetic mosaic analysis, it was shown that Sma-2 and Daf-4 are cell-autonomous; Sma-2 is not a secreted factor, but acts in the same cells as Daf-4. Moreover, Daf-4 expression failed to rescue Sma mutations, suggesting that Sma proteins act downstream of the receptor.

These independent findings in evolutionarily distant organisms strongly suggested that Mad and Sma proteins belong to a novel family of molecules that mediate signals of the TGF-β superfamily. Mad was then shown to be specifically required in cells responding to Dpp, and Mad mutation blocked the expression of a Dpp-responsive gene, *labial* [78]. More direct evidence that Mad functions downstream of Dpp came from observations that null mutations of Mad suppress signals of the constitutively active Tkv [47, 79].

Intensive efforts to find vertebrate homologues of Mad and Sma have rapidly expanded the members of this family. “Smad” was coined from the merger of Sma and Mad to unify the nomenclature of the vertebrate newcomers [80]. Smads have been identified either as a candidate tumor suppressor (Smad4/DPC4) [81], as a mesoderm inducer (mouse Smad2) [82], or, mostly, according to their sequence similarities to the original members of the Smad family. “Smad” now represents all of the members of this family from invertebrates to vertebrates.

**Structure-function relationship of Smad proteins**

Eight mammalian, two *Xenopus*, two *Drosophila* and three *C. elegans* Smads have been reported and more are found in GenBank (Fig. 3A). Structural differences of Smads correlate well with the differences of their roles in signaling. All Smad proteins except Smad6, Smad7 and *Drosophila* Daughters against dpp (Dad) share a conserved structure consisting of the N-terminal homology domain-1; MH1) and C-terminal (MH2) domains that are connected by a proline-rich linker region with variable lengths and sequences (Fig. 3B). Smad1, 2, 3, 5 and MADH6 contain the Ser-Ser-X-Ser (SSXS) motif at the C-terminal end, the last two serines of which are the direct phosphorylation sites by type I receptors [83, 84]. Smads in this class transduce ligand-specific, or pathway-restricted signals. Smad 1, 5 and, most likely, MADH6, belong to the same structural subgroup, and mediate BMP signals [47, 85–88], whereas Smad2 and 3 transduce TGF-β and activin signals [89, 90]. Smad4 lacks the SSXS motif and is not a direct substrate of type I receptors. Smad4 rather acts as a common component essential to all signaling pathways [90, 91]. *Medea* is a Smad4 homologue identified in *Drosophila* [RW Padgett and P Das, Rutgers Univ]. Smad6, Smad7 and Dad have MH2 domains, but lack the C-terminal SSXS motif and diverge from the rest of the Smads in the N-terminal region. These three oppose to the other Smad proteins in that they play an inhibitory action in signaling [58, 92–94].

Contrasting actions of Smad1 and Smad2 were first demonstrated using the *Xenopus* system. Injection of Smad1 RNA into *Xenopus* embryos induced ventral
Figure 2. Amino acid sequences of the Smad proteins in mammals and Drosophila [D] are compared. In addition to these Smads, three C. elegans and two Xenopus Smads have thus far been reported. (B) Schematic representation of the structure of ligand-specific Smad. The C-terminal SSXS motif is found only in the ligand-specific Smads, and MH1 domain is found in the ligand-specific and common mediator Smads, but not in the inhibitory Smads.

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mesoderm along with the expression of ventral markers, while Smad2 injection caused dorsalization. Smad1 also suppressed neural differentiation [95, 96]. Thus, Smad1 exerted BMP-like activities and Smad2 exhibited activin-like effects. Smad5 showed the same effects as Smad1 [87]. In contrast, Smad4 induced both dorsal and ventral markers [91, 97]. Drosophila Mad, which is similar to Smad1 and Smad5, also induced ventral mesoderm when injected into Xenopus embryos [78]. BMP-4 induces concentration-dependent patterning of Xenopus ectoderm, suggesting that it can act as a morphogen in this system. Increasing dose of Smad1 mimicked the effect of BMP-4 [86].

The MH2 domain of Smad1, when fused to a heterologous DNA binding protein, exerted constitutive transactivation activity, whereas the full-length Smad1 transactivated the reporter only after BMP stimulation [98]. These results suggested that Smad proteins possibly act as transcription factors; MH2 is the effector domain, while MH1 negatively regulates the activity of the MH2 domain. Consistently, the N-terminally truncated Smad2 was more effective in mesoderm induction in Xenopus than the full-length Smad2 [82].

Recently, the three-dimensional structure of the MH2 domain of Smad4 was determined [99]. This domain alone forms a trimeric complex, which is in consistence with the previous observation that Smad4 forms a homotrimer [91]. Notably, most of the mutations of Smad4 and other Smads found in tumors or in Drosophila or C. elegans development are located, in the Smad4 trimeric model, at the interface between two Smad4 molecules. The GS mutation (Gly-to-Ser change in the conserved GG motif) documented both in Mad and Sma [76, 77] resides in the L3 loop, which is possibly involved in the physical interaction of Smad4 with ligand-specific Smads. Thus, molecular interactions of Smads seem to be crucial to their functions. Importantly, MH1 domain regulates the function of MH2 through physical interaction [100]. Characterization of a mutation in the MH1 domain of Smad4 (Arg-100 to Cys) and the equivalent mutation in Smad2 (Arg-133 to Cys) demonstrated that these mutations enhance the affinity between MH1 and MH2 [100].

Activation of Smads

Phosphorylation of Smads by the receptors

As described above, ligand-specific Smads are direct substrates of type I receptor kinases (Fig. 4). Smad2 is recruited to TβR-I upon ligand stimulation, undergoes phosphorylation, and is immediately released from the receptor [83]. The interaction of Smad2 with TβR-I requires activation of the type I receptor by the type II receptor. As Smad2 associates with TβR-I transiently, the interaction escapes detection by co-immunoprecipitation studies under physiological conditions. Indeed, the interaction can be demonstrated only when the phosphate transfer is blocked either by the kinase-negative mutation of TβR-I or by modifications of the C-terminal phosphorylation sites of Smads [83, 101]. Direct binding of Smad1 or Smad5 to BMP receptors has not yet been reported, suggesting that the interaction may be even
more unstable in this combination. Smad4 does not bind to the receptor or undergo ligand-dependent phosphorylation [90], clearly distinguishing its role from those of the ligand-specific Smads. The domain of Smads responsible for the interaction with the receptors may be the MH2 domains since Smad6 and Smad7 lacking the MH1 domains bind to the type I receptors [58, 92, 93].

Mad is phosphorylated upon Dpp stimulation through Tkv [102]. It is not known whether Sax can also phosphorylate Mad. Since Drosophila has another type I receptor, Atr-I, which is structurally similar to the mammalian ActR-IB and binds activin in vitro, there may be other, yet unidentified, ligand-specific Smads in Drosophila.

Heteromeric complex formation

Smad4 was shown to exist as a homo-oligomer by co-immunoprecipitation experiments [90, 91, 103]. The complex seems to be a trimer as was further confirmed by the three-dimensional structure study and column chromatography [99]. Ligand-specific Smads can also form homo-oligomeric complexes [100], which are probably trimers as Smad4. Upon phosphorylation by type I receptors, ligand-specific Smads associate with Smad4, which has been demonstrated for Smad1 after phosphorylation by BMPR-I [91], and Smad2 [91] and Smad3 [101] by TβR-I or ActR-IB. Thus, Smad4 acts as a common partner for different ligand-specific Smads (Fig. 4). Although direct evidence has not been shown, a model is proposed that the phosphorylation-induced complex might be a heterohexamer [99]. It is also shown that TβR-I induces complex formation of Smad2 and Smad3 [101]. Likewise, Smad1, Smad5, and possibly MADH6, may form a heteromeric complex with each other. Thus, phosphorylation-induced complexes may contain multiple ligand-specific Smads in addition to Smad4.

It was shown in Smad2 and Smad4 that the MH2 domain is most important for homo-oligomerization with minor contribution of the MH1 domain and the linker region [97, 100, 103]. In contrast, Smad2 and Smad4 associate with each other through their MH2 regions and, perhaps, the linker region of Smad4, but the MH1 domain of either Smad is inhibitory [97, 100, 104]. The MH1 domain thus serves as a negative regulatory domain, as discussed above.

Some of the naturally occurring mutations of Smads have proved to have dominant-negative effects. One of the lethal alleles of Mad, Mad12, has a termination instead of Gln-417 [76]. Another mutation of Smad4 found in pancreatic cancers, PX101, also has a termination at Arg-515 [81]. These two mutations are located at neighboring sites in the consensus sequence and cause almost an identical truncation of the C-terminus of each Smad. In the three-dimensional structure of Smad4 [99], this premature stop causes elimination of the C-terminal half of the L3 loop, β-sheet 11, and z-helix 5. Smad4 forms a trimer in the head-to-tail fashion through two separate domains, “the loop/helix region” and “the helix bundle” that includes z-helix 5. The L3 loop may possibly mediate hetero-oligomerization. Thus, this mutation presumably destroys one of the two interfaces in the homo-oligomer as well as the hetero-oligomerization domain [91, 103], which might be the basis for the dominant-negative effect of this mutation. In the PAI-1 reporter assay, Smad2, Smad3, and Smad4 with mutations in the corresponding regions, inhibited transcriptional activation by ligand, receptors or Smads [Refs. 90, 91, and data not shown]. Moreover, the dominant-negative

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**Figure 4.** Mechanism of the activation of BMP receptors and signal transduction by Smads. Smad4, and probably other Smads, exist as homotrimers.
Smad4 inhibited mesoderm induction by activin, Smad2, or the wild type Smad4 in *Xenopus* embryos [91].

**Nuclear targets of Smad proteins**

Following hetero-oligomerization, Smad complexes translocate from the cytoplasm into the nucleus (Fig. 4). Smad2 and Smad3 translocate to the nucleus together with Smad4 upon TGF-β stimulation [83, 90], whereas Smad1 does upon BMP stimulation [47, 85, 98]. In *Drosophila*, however, Mad was detected only in the cytoplasm in tissues responding to endogenous Dpp, although overexpression of Dpp induced nuclear translocation of Mad [102]. Undetectable level of nuclear translocation might be sufficient for Mad to transduce signals *in vivo*. Although the N-terminally truncated Smad2 was shown to accumulate in the nucleus even in the absence of activin [82], the nuclear localization signal of Smads has not been determined.

A major, if not the sole, function of the TGF-β superfamily is transactivation of responsive genes. The target genes include *PAI-1* [105], and cyclin-dependent kinase inhibitors such as *p15* and *p21* for TGF-β [106, 107] *goosecoid* for activin [108], and *collagen X* for BMPs [109]. One of the best characterized promoters is that of *PAI-1*. TGF-β and constitutively active TβR-I activate the PAI-1 reporter in transient assays. Smad2, Smad3 or Smad4 alone activates PAI-1, but only inefficiently. Combination of Smad2 and Smad4, or that of Smad3 and Smad4 greatly enhances the PAI-1 expression, demonstrating that Smad2 or Smad3 cooperate synergistically with Smad4 in the transcriptional activation. The result is consistent with the fact that ligand-specific Smads form complexes with Smad4. Gene induction by Smad1 or Smad5 has been shown in *Xenopus* embryos as discussed above, but not reported yet in mammalian cells.

The direct link of Smads to the target genes was first demonstrated in the *Xenopus* system. *Mix.2* is an immediate-early response gene activated by activin. The activin responsive element in the *Mix.2* promoter and the protein that binds to this element, FAST-1, were identified [110]. It was shown that Smad2 associates with FAST-1 in the transactivation complex. Smad4 was later shown to be incorporated in the complex [111].

In *Drosophila*, Mad was shown to bind directly to DNA [112]. *Vestigial* is required for wing formation and its expression is induced by Dpp. Mad directly binds to the “quadrant” enhancer of the *vestigial* gene. Interestingly, Mad binds to DNA through its MH1 domain, and the MH2 domain appears to negatively regulate the binding. Thus, in certain cases, MH1 domain, instead of MH2 domain, acts as a functional domain, and the MH2 domain regulates the function of MH1. Smads may regulate transcription of the target genes either through association with other DNA binding proteins or their binding to DNA.

Schurri (Shn) was identified as a downstream component of Dpp through a genetic approach using *Drosophila* [113–115]. Shn mutants share strikingly similar phenotypes with those of *dpp*, *tkv* and *punt*. Shn is also required for the expression of Dpp-responsive genes. Shn is homologous to mammalian zinc-finger transcription factors including MBP-1/PRDII-BF1/HIV-EP1 and MBP-2/HIV-EP2, and may serve as a nuclear target of Dpp. It would be interesting to study whether transcription factors of this family are involved in signaling of BMPs and/or other ligands in mammalian cells. It would be also important to examine whether Mad interacts with Shn.

**Inhibitory Smads**

Smad6 [58], Smad7 [92, 93] and *Drosophila* Dad [94] comprise the third class of Smads. Their N-terminal regions significantly differ from the canonical MH1 domain conserved among the other Smads, while their overall sequences are relatively similar to each other, suggesting that Smad6, Smad7, and Dad share a common function. Indeed, Smad6 and Smad7 were shown to inhibit signaling of TGF-β, and Dad functions as an inhibitor of Dpp signals. Smad6 and Smad7 both bind to TβR-I; the association requires phosphorylation of TβR-I by TβR-II as in Smad2 and Smad3. Smad6 and Smad7, however, are not phosphorylated by TβR-I. Rather, they stably bind to the receptor, in contrast to Smad2 and Smad3 that are immediately released from the receptor after phosphorylation. The inhibitory action of Smad6 and Smad7 seems to be exerted at the receptor level, since Smad7 was shown to compete with Smad2 in binding to TβR-I [92]. Consequently, Smad6 and Smad7 suppress phosphorylation of Smad2, hetero-oligomerization of Smad2 with Smad4, and transactivation of the TGF-β-responsive genes. Smad6 was also shown to bind to BMPR-IB and inhibit phosphorylation of Smad1 by BMPR-IB, suggesting that BMP signaling is also interfered with by this class of the Smad members.

Expression of Smad6 and Smad7 is regulated by physiological and pathological conditions. In vascular endothelial cells, Smad6 and Smad7 are induced by laminar shear stress [116]. Smad6 and Smad7 are also induced by certain members of the TGF-β superfamily [Ref. 93, and data not shown]. Thus, Smad6 and Smad7 may act as autoregulatory switch-off signals. In *Drosophila*, Dad was identified as a Dpp-inducible gene, indicating that Dad also acts as a mediator of negative feedback signal. Since proteins in the TGF-β superfamily must act under a tight spatial and temporal control, such negative feedback systems may be required to tune the signals of the TGF-β superfamily members.

**CONCLUSION**

There are many members in the BMP family. Gene targeting of these BMPs revealed that some of the members are expressed only in certain tissues, and exert their specific effects. However, certain BMPs, e.g. BMP-2 and BMP-4, have important roles in early development, and null mutant embryos died at early embryonic stages. Con-
ditional gene targeting may be important to understand their roles in different tissues in vivo. BMPs can be classified into several subgroups based on their amino acid sequences, but their specific functions are still not understood. Studies on the receptors for different BMPs may be important to understand the functions of these BMPs. Moreover, BMP receptors, such as the extracellular domain of BMPR-1A, may be useful to design new strategies for diagnosis and treatment of various clinical disorders. Studies on the Smad proteins revealed the signal transduction pathway of the members in the TGF-β superfamily. However, it is still unclear how specific signals are transduced by different Smads. Identification of nuclear targets of BMPs may be an important question in the future. In addition, other signaling pathways may exist in the serine-threonine kinase receptor system.

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