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TGF-β Family Signal Transduction in *Drosophila* Development: From *Mad* to Smads

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The transforming growth factor-β (TGF-β) superfamily encompasses a large group of soluble extracellular proteins that are potent regulators of development in both vertebrates and invertebrates. *Drosophila* TGF-β family members include three proteins with homology to vertebrate bone morphogenetic proteins (BMPs): Decapentaplegic (Dpp), Screw, and Glass bottom boat-60A. Genetic studies of Dpp signaling led to the identification of Smad proteins as central mediators of signal transduction by TGF-β family members. Work in mammalian tissue culture has elucidated a biochemical model for signal transduction, in which activation of receptor serine–threonine kinase activity leads to phosphorylation of specific Smad proteins and translocation of heteromeric Smad protein complexes to the nucleus. Once in the nucleus Smad proteins interact with other DNA binding proteins to regulate transcription of specific target genes. Dissection of Dpp-response elements from genes expressed during embryonic mesoderm patterning and midgut morphogenesis provides important insights into the contributions of Smad proteins and tissue-specific transcription factors to spatial regulation of gene expression. Genetic studies in *Drosophila* are now expanding to include multiple BMP ligands and receptors and have uncovered activities not explained by the current signal transduction model. Identification of more ligand sequences and demonstration of a functional *Drosophila* activin-like signal transduction pathway suggest that all TGF-β signal transduction pathways are present in flies. © 1999 Academic Press

Key Words: **Smad; Dpp; TGF-β; BMP; activin; wing patterning; midgut patterning; transcription factors.**

INTRODUCTION

Finely tuned signals between cells are necessary to coordinate all aspects of development, from patterning of the embryonic body axes to homeostasis of adult tissues. The transforming growth factor-β (TGF-β) superfamily is a large group of secreted proteins that mediate such signals. Members of this family have been identified in sea urchins, nematodes, flies, and vertebrates, with as many as 24 family members in a single mammalian species. TGF-β superfamily members can direct a wide range of cellular responses, including proliferation, changes in cell shape, apoptosis, and specification of cell fate. In both vertebrates and invertebrates, TGF-β family members play fundamental roles in early axial patterning, in inductive interactions during organogenesis, and in tissue homeostasis. The range of biological activities is reflected by the variety of names given to members of the family: bone morphogenetic proteins (BMPs), growth and differentiation factors, and activin are all structurally related to the prototypical TGF-β proteins. Two members of the TGF-β superfamily, activin and *Drosophila* Decapentaplegic (Dpp), can function as morphogens, in that each protein can specify multiple cell fates in a concentration-dependent manner (Neumann and Cohen, 1997).

The past 4 years have been exciting for the TGF-β field, due to the identification of a class of proteins that transduce the signal from receptor to nucleus. The intracellular signal transduction pathway eluded biochemical analyses until a candidate transducer was identified through genetic screens in *Drosophila:* the novel protein product of a gene called *Mothers against dpp* or *Mad.* The identification of Mad-related, or Smad, proteins from several organisms facilitated the rapid progress of the field. Multidisciplinary efforts in the fly *Drosophila melanogaster,* the nematode *Caenorhabditis elegans,* the frog *Xenopus laevis,* and mammalian tissue culture cells have culminated in a molecular model for Smad-mediated signal transduction for two

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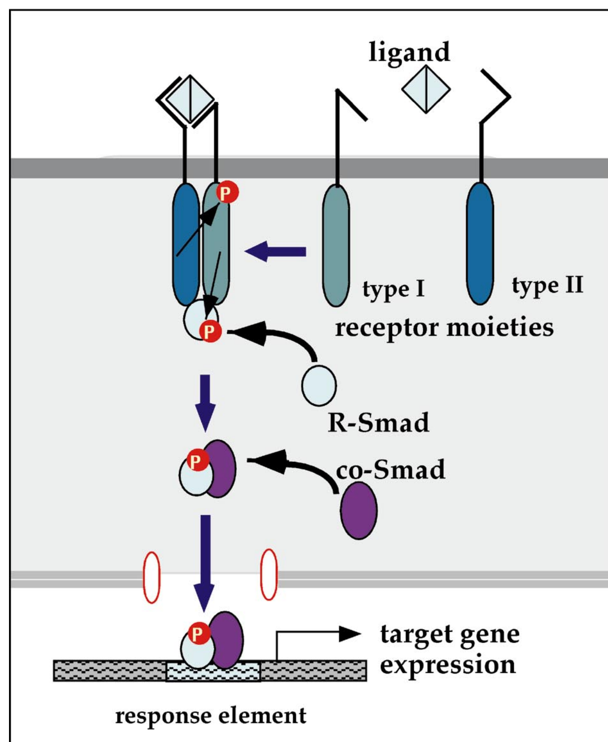

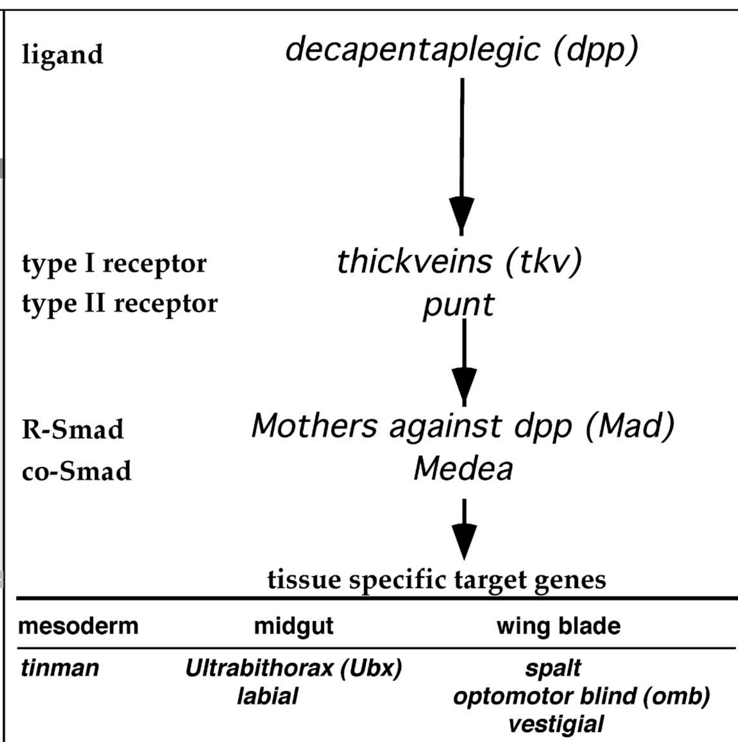

FIG. 1. Simple representation of the biochemical and genetic pathways for Dpp signal transduction. The ordering of the genes was tested by overexpression of Dpp in receptor or Smad mutant backgrounds (Newfeld *et al.*, 1997; Ruberte *et al.*, 1995; Wisotzkey *et al.*, 1998) and by expression of constitutively active Tkv receptor in a Smad mutant background (Hoodless *et al.*, 1996; Hudson *et al.*, 1998; Newfeld *et al.*, 1997; Wiersdorff *et al.*, 1996). These are simplified pathways, the reality is more complex.

groups of TGF-β family ligands, one comprising the Dpp/BMP2/BMP4 subfamily and a second comprising activin and the TGF-βs.

The task now facing the field is to determine whether this molecular model is sufficient to describe endogenous signal transduction in multiple developmental contexts. Detailed studies of specific Dpp responses in *Drosophila* support a Smad-centered signal transduction pathway (Fig. 1), but indicate that the spatial pattern of target gene expression depends on additional tissue-specific transcription factors. Studies of other BMP ligands and receptors raise the possibility that the molecular model for BMP signal transduction is incomplete. Identification of an activin-like signal transduction pathway expands the range of TGF-β family functions that are present in *Drosophila.* The sophisticated genetics of this model organism will provide important tools to sort out the network of signal transduction pathways used by this potent family of extracellular ligands.

I. OVERVIEW OF A CORE DPP SIGNALING PATHWAY

The prototypical Smad, Mad, was identified through genetic screens in *Drosophila* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995). Mutations in two genes, *Mad* and *Medea,* were recovered in a screen to isolate dominant mutations that, if present in the mother, exacerbated the *dpp* phenotype of the zygote. Both *Mad* and *Medea* are required for maximal *dpp* function during dorsal–ventral patterning of the embryo and again during subsequent development of the adult appendages (Raftery *et al.*, 1995). Mutations in the *Medea* gene had previously been isolated and characterized as genes required for growth of the imaginal disks (Gatti and Baker, 1989; Shearn and Garen, 1974). In a number of developmental assays, mutations in *Mad* or in *Medea* cause a terminal phenotype very similar to that of mutations in *dpp* (Das *et al.*, 1998; Hudson *et al.*, 1998; Hursh *et al.*, 1993; Irish and Gelbart, 1987; Raftery *et al.*, 1995; Raftery and Wisotzkey, 1996; Spencer *et al.*, 1982; Wisotzkey *et al.*, 1998). Thus, both Mad and Medea are required for the final phenotypic outcome of Dpp signaling in multiple tissues.

Molecular cloning of the *Mad* gene identified a novel protein product, with homology to three predicted polypeptides from the *C. elegans* genome (Sekelsky *et al.*, 1995). The *C. elegans* sequences were subsequently found to correspond to three *sma* genes, which were implicated in a TGF-β family signaling pathway that regulates nematode body size and male tail development (Savage *et al.*, 1996).

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Simultaneously, vertebrate Mad-related proteins were identified and shown to be involved in BMP, activin, and TGF-β signal transduction (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Hoodless *et al.*, 1996; Lechleider *et al.*, 1996; Liu *et al.*, 1996; Yingling *et al.*, 1996; Zhang *et al.*, 1996). The vertebrate proteins were named Smad proteins, a fusion of *Mad* and *sma,* to avoid confusion with unrelated proteins bearing the same name (Derynk *et al.*, 1996).

Mad and Medea are central components of the Dpp signal transduction pathway (Fig. 1). *Mad* is required downstream of both Dpp and the BMP type I receptor, Thick veins (Tkv), in responding cells (Newfeld *et al.*, 1996; Wiersdorff *et al.*, 1996), and is required for expression of Dpp target genes (Kim *et al.*, 1997; Newfeld *et al.*, 1996). *Medea* also encodes a Smad protein that functions downstream of both Dpp and Tkv in responding cells (Das *et al.*, 1998; Hudson *et al.*, 1998; Wisotzkey *et al.*, 1998). Mad and Medea are each more similar in sequence and in function to specific vertebrate Smads than they are to each other (Das *et al.*, 1998; Newfeld *et al.*, 1997; Wisotzkey *et al.*, 1998). The family of Smad proteins is now broken into subfamilies of proteins with distinct functions in signal transduction.

A simplified version of the biochemical pathway is presented in Fig. 1. Mad is a substrate for phosphorylation by BMP receptors and thus belongs to the receptor-regulated class of Smads, or R-Smads. Medea shows signal-dependent association with R-Smads, thus it belongs the common-mediator class of Smad proteins (co-Smads). Smad proteins show signal-dependent localization to the nucleus, where they can directly bind DNA and regulate transcription. The current biochemical model for Smad function in signal transduction is more complex; we present this model in the next section.

II. SMAD PROTEINS ARE CENTRAL IN THE MODEL FOR TGF-β FAMILY SIGNAL TRANSDUCTION

Subsequent studies of Smad protein function in multiple systems contributed to the current model for signal transduction (Fig. 2). First, the specificity of the R-Smad:type I receptor binding interaction appears to determine the specificity of signal transduction for BMP ligands versus TGF-β and activin ligands. Second, co-Smads do not appear to have specificity for different ligand signals. Third, the presence of co-Smad and R-Smad proteins in the nucleus of cultured cells is signal dependent. Finally, a class of antagonistic Smad proteins (anti-Smads) has been identified. We give an overview of the working model for signal transduction in this section. For a comprehensive review of the vertebrate TGF-β family signal transduction literature, refer to Massagué (1998). We briefly review the ligands and receptors, before discussing the roles of Smad proteins.

TGF-β Ligands Cluster into Subfamilies

TGF-β family members are synthesized as large proproteins, which are proteolytically processed to form the biologically active ligands (reviewed in Massagué *et al.*, 1994). The proteins dimerize prior to secretion, so that heterodimeric ligands can form if two family members are expressed in the same cell. Once secreted, the ligands interact with a growing array of extracellular proteins. Several different types of extracellular proteins bind the ligand and make it unavailable to receptors (Biehs *et al.*, 1996; Ferguson and Anderson, 1992; Holley *et al.*, 1995; Hsu *et al.*, 1998; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Other types of extracellular proteins release the sequestered ligand or otherwise facilitate its binding to receptors (Jackson *et al.*, 1997; López-Casillas *et al.*, 1993; Marques *et al.*, 1997; Nakato *et al.*, 1995). Although this is an exciting area of research, it is beyond the scope of this review. We will consider ligands only in terms of the receptors and signal transduction pathways they activate.

Ligands within the TGF-β superfamily cluster into groups of closely related sequences, or subfamilies. Dpp and the vertebrate proteins BMP2 and BMP4 form one BMP subfamily, the Dpp/BMP4 subfamily. Dpp is a functional ortholog of BMP2 and BMP4: BMP4 ligand domain sequences can substitute for Dpp ligand sequences in flies (Padgett *et al.*, 1993) and Dpp can induce endochondral bone formation in mammals (Sampath *et al.*, 1993), the hallmark activity of mammalian BMPs (Ozkaynak *et al.*, 1990; Sampath and Reddi, 1981; Wozney *et al.*, 1988). The fly ligands Glass bottom boat-60A (Gbb-60A) and Screw, and the vertebrate ligands BMP5, BMP6, BMP7, and BMP8, form the Gbb-60A/BMP7 subfamily (Burt and Law, 1994). It is not clear whether members of the Gbb-60A/BMP7 subfamily are functional orthologs, although it has been proposed that all vertebrate BMP ligands overlap in function (Dudley *et al.*, 1995, 1997; Katagiri *et al.*, 1998; Luo *et al.*, 1995; Lyons *et al.*, 1995). Two additional ligands have been reported in *Drosophila* (Table 1); it is likely that more will be identified through the *Drosophila* genome project. Studies of Smad function in *Drosophila* have focused on Dpp signal transduction. Studies of vertebrate Smads have predominantly focused on signal transduction in response to three ligand subfamilies: TGF-β, activin, and BMP2/BMP4.

The Type I Receptor Moiety Is an Inducible Serine–Threonine Kinase

An active TGF-β family receptor is a ligand-induced transient complex of two distantly related transmembrane serine–threonine kinases, called the type I and type II receptors (reviewed in Massagué, 1998; ten Dijke *et al.*, 1996). The type II receptor is a constitutively active kinase, whereas the type I receptor kinase becomes activated when it is phosphorylated by the type II receptor. The active receptor complex includes two type I and two type II receptors, presumably due to recruitment by a dimeric ligand (Yamashita *et al.*, 1994). Mutant forms of both type I

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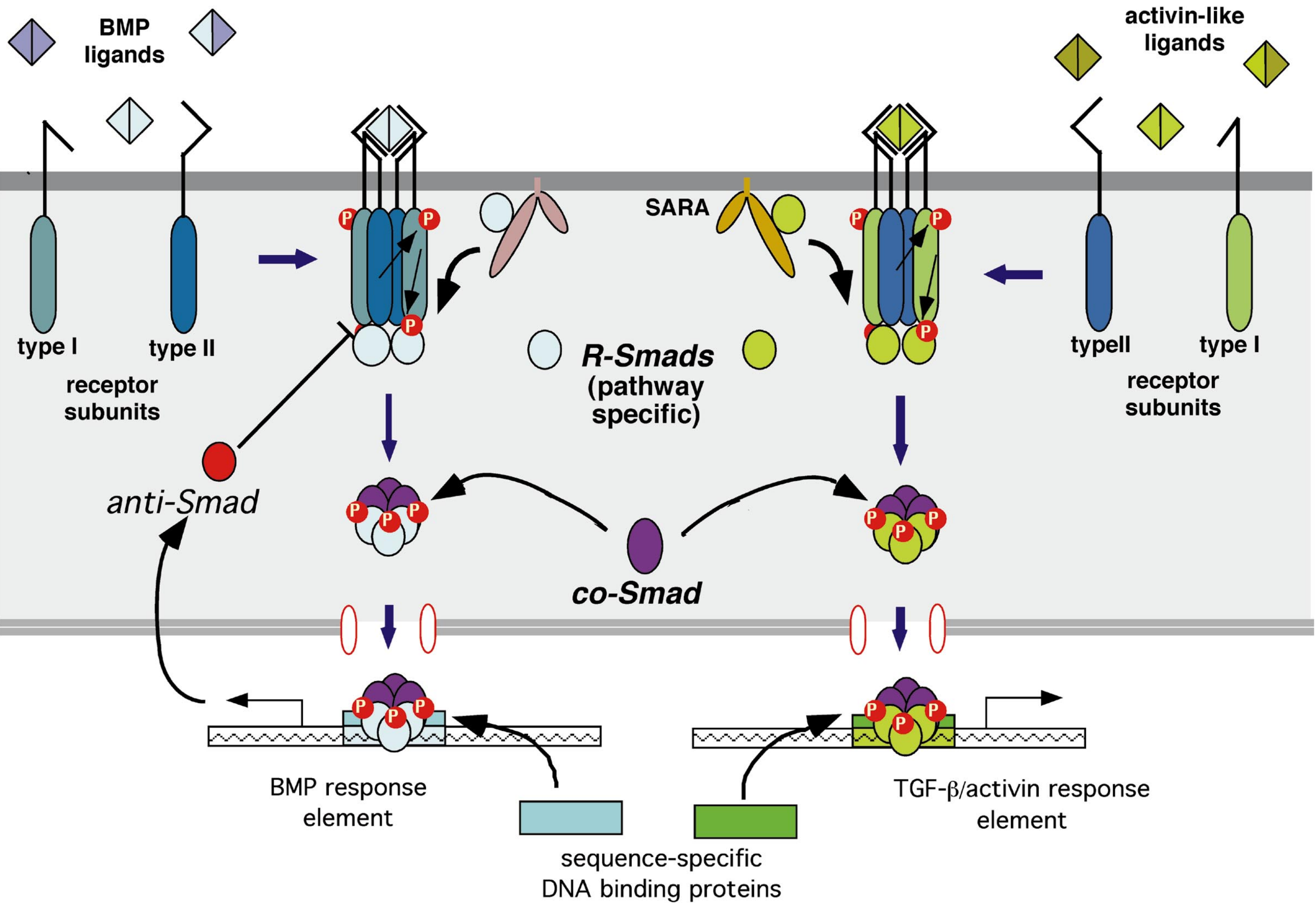

FIG. 2. Model for central role of Smads in two specific pathways for TGF-β family signal transduction. This signal transduction pathway is a working model that relies on data from flies, frogs, and mammalian tissue culture cells. To date, biochemical analyses have involved the use of epitope-tagged Smad proteins overexpressed in tissue culture cells. R-Smads are specific for either the BMP or the TGF-β/activin pathway. In contrast, the co-Smads function in both pathways. Vertebrate TGF-β/activin R-Smads are recruited to the receptor by SARA, which associates with the membrane (Tsukazaki et al., 1998). In Drosophila the PUNT type II receptor may function in both DPP and activin-like pathways (Letsou et al., 1995; Ruberte et al., 1995; Simin et al., 1998). The composition of functional Smad complexes is under debate.

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TABLE 1
TGF-β Family Members in *Drosophila*

Fly ligand	Vertebrate relatives
Decapentaplegic (Dpp)[a]	BMP2/BMP4
Glass bottom boat-60A (Gbb/60A)[b]	BMP5/BMP6/BMP7/BMP8
Screw[c]	BMP5/BMP6/BMP7/BMP8[d]
dActivin[e]	Activin
EST DS07149[f]	Müllerian inhibiting substance[g]

[a] Padgett *et al.* (1987), Spencer *et al.* (1982).
[b] Chen *et al.* (1988a), Doctor *et al.* (1992), Khalsa *et al.* (1998), Wharton *et al.* (1991).
[c] Arora *et al.* (1994).
[d] Burt and Law (1994).
[e] Kutty *et al.* (1998).
[f] Berkeley *Drosophila* genome project, unpublished data. URL: http://www.fruitfly.org/.
[g] Bootstrap phylogeny analysis of the ligand domain sequences suggests that Berkeley *Drosophila* Genome Project EST DS07149 may be more closely related to mammalian Müllerian inhibiting substance and *Caenorhabditis elegans* DAF7 than to other family members (R. G. Wisotzkey and L.A.R., submitted), but the correlation is not strong enough to predict an evolutionary clade (Hillis and Bull, 1993).

and type II receptors that lack the kinase domain have dominant negative activity (Brand *et al.*, 1993; Haerry *et al.*, 1998) and are widely used. However, the mechanism of action is unknown; these mutant receptors may sequester ligand or they may sequester other receptors into nonfunctional complexes.

The type I receptor serine–threonine kinase is the key signal-transducing activity. A single amino acid mutation confers constitutive serine–threonine kinase activity in the type I receptors (Wieser *et al.*, 1995). Mutationally activated TGF-β type I receptor is sufficient to recapitulate the effects of ligand stimulation in a cell line that lacks both the TGF-β type I and type II receptors (Wrana *et al.*, 1994b). Tests of mutationally activated type I receptors in BMP, activin, and TGF-β signal transduction assays consistently indicate that this receptor is sufficient for signal transduction.

There are many fewer receptor family members than ligand family members, suggesting that multiple ligands share a given receptor. In addition, different type I and type II receptors pair to bind different ligands. For example, vertebrate activin and BMPs can each bind to several different type I and type II receptors (summarized in ten Dijke *et al.*, 1996). The overlap in binding specificity has led to some confusion in the vertebrate receptor nomenclature. *In vivo* verification of functional receptor:ligand relationships generally relies on the similarity of loss of function mutant phenotypes, the similarity of effects of added ligand to effects of mutationally activated receptor, and the ability of either loss of receptor or a dominant negative receptor mutant to block the effects of added ligand.

Three type I receptors and two type II receptors have been genetically characterized in *Drosophila* (Table 2). Tkv is an essential type I receptor for Dpp (Brummel *et al.*, 1994; Nellen *et al.*, 1994; Penton *et al.*, 1994; Ruberte *et al.*, 1995). Initial studies of the Saxophone (Sax) type I receptor demonstrated low-affinity binding to the Dpp ortholog BMP2 (Brummel *et al.*, 1994); thus Sax was proposed to boost the

TABLE 2
TGF-β Family Receptors in *Drosophila*

	Fly receptor	Ligands bound	Vertebrate relatives[a]
Type I	Thickveins (Tkv)[b]	Dpp, BMP2[c]	BMPR-IA, BMPR-IB
	Saxophone (Sax)[d]	BMP2[e]	ALK-1, ALK-2[f]
	Baboon (Babo, also Atr-I)[g]	Activin[h]	ActR-IB, TβR-I, ALK-7[f]
Type II	Punt[i]	BMP2, activin[j]	ActR-II, ActR-IIB
	Wishful thinking (Wit)[k]	?	BMPR-II, AMHR
	STK-D[l]	?	?

[a] Evolutionary relationships between vertebrate and fly receptors are analyzed in Newfeld *et al.* (1999).
[b] Brummel *et al.* (1994), Nellen *et al.* (1994), Penton *et al.* (1994), Terracol and Lengyel (1994).
[c] Penton *et al.* (1994).
[d] Brummel *et al.* (1994), Nellen *et al.* (1994), Penton *et al.* (1994), Schüpbach and Wieschaus (1989), Twombly (1995), Xie *et al.* (1994).
[e] Brummel *et al.* (1994).
[f] The function of these activin-like kinase receptors (ALKs) is not well understood (Massagué, 1998).
[g] Brummel *et al.* (1999), Childs *et al.* (1994).
[h] Wrana *et al.* (1994a).
[i] Jürgens *et al.* (1984), Letsou *et al.* (1995), Ruberte *et al.* (1995), Simin *et al.* (1998).
[j] Letsou *et al.* (1995), Wrana *et al.* (1994a).
[k] *wit* mutants die as pupae (G. Marques and M. B. O'Connor, personal communication).
[l] Ruberte *et al.* (1995). It is not known if Wit and STK-D are the same type II receptor or if STK-D represents a third *Drosophila* type II receptor.

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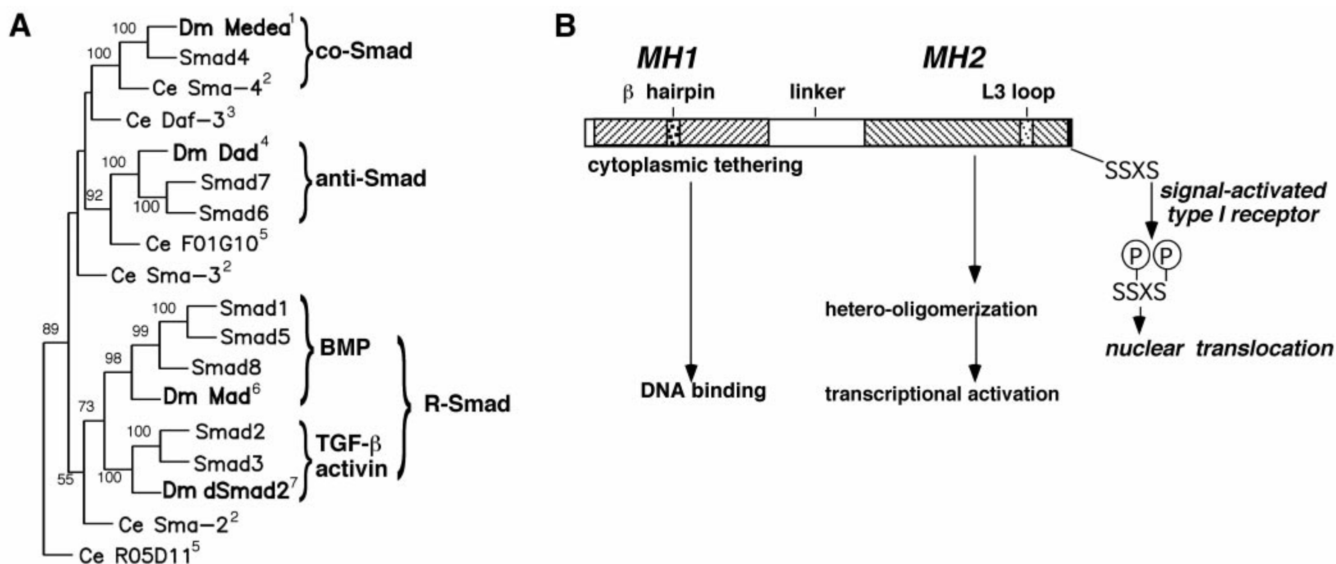

FIG. 3. The Smad family. (A) Phylogenetic tree of human, fly (Dm), and nematode (Ce) Smad protein sequences. Functional classes of Smad proteins cluster together on the tree, as indicated by the brackets on the right. At each node, the number indicates how many times the sequences to the right of the node clustered together in bootstrap analysis ($n = 100$) (Wisotzkey *et al.*, 1998); the tree is unrooted. Values greater than 70 indicate a reliable relationship (Hillis and Bull, 1993). All sequences are from GenBank. (B) Schematic of the functions associated with conserved domains of Smad proteins. Two domains within R-Smads and co-Smads contain homology to *Drosophila* Mad, these are called the Mad homology domains MH1 and MH2. The β hairpin of the MH1 domain directly contacts DNA (Shi *et al.*, 1998). Evidence that the N terminus is required to sequester Smads in the cytoplasm comes from Baker and Harland (1996). Specificity of binding to the appropriate type I receptor is determined by amino acids in the L3 loop of the MH2 domain (Chen *et al.*, 1998b; Lo *et al.*, 1998). Additional information is provided in the text. [1]Das *et al.* (1998); Hudson *et al.* (1998); Inoue *et al.* (1998); Raftery *et al.* (1995); Wisotzkey *et al.* (1998); Xu *et al.* (1998). [2]Savage *et al.* (1996). [3]Patterson *et al.* (1997). [4]Tsuneizuni *et al.* (1997). [5]Both F01G10 and R05D11 loci are from the *C. elegans* sequencing consortium (Wilson *et al.*, 1994). [6]Raftery *et al.* (1995); Sekelsky (1993). [7]Brummel *et al.* (1999); Henderson and Andrew (1998).

intracellular level of Dpp signal transmitted by Tkv (Nellen *et al.*, 1994; Singer *et al.*, 1997). However, experiments with dominant negative receptors suggest that Dpp is not a physiologically relevant ligand for Sax (Haerry *et al.*, 1998; Neul and Ferguson, 1998; Nguyen *et al.*, 1998). Instead, Sax appears to predominantly serve as a receptor for two other BMP ligands, Screw (Neul and Ferguson, 1998; Nguyen *et al.*, 1998) and Gbb-60A (Haerry *et al.*, 1998; Khalsa *et al.*, 1998). These ligands may also activate Tkv. The third type I receptor, Baboon (Babo; Brummel *et al.*, 1999), can bind activin (Wrana *et al.*, 1994a). The Punt type II receptor can bind either activin or BMP2, depending on the associated type I receptor (Letsou *et al.*, 1995; Wrana *et al.*, 1994a). The second type II receptor, Wishful thinking, is poorly understood (Wit; M. B. O'Connor, personal communication).

Two Classes of Smad Proteins Are Required for Signal Transduction

The central role for Smad proteins in TGF-β family signal transduction has its roots in two observations: Mad is essential for all Dpp signal transduction events examined to date and specific Smad proteins are the only known substrates for type I receptor serine–threonine kinase activity. Vertebrate and *Drosophila* Smad proteins fall into three classes based both on phylogenetic relationships and on functional assays (Fig. 3). *C. elegans* Smad proteins are more divergent, and only Daf-3 has been characterized for molecular function (Thatcher *et al.*, 1999). Two classes, the receptor-regulated Smads (R-Smads) and the common-mediator Smads (co-Smads), have central roles in signal transduction. A third class, the anti-Smads, antagonizes signal transduction. Some aspects of Smad protein function are shared by all Smad proteins and others are specific to one or two classes. The two classes of signal-transducing Smad proteins share sequence homology in two unrelated regions, Mad homology domains MH1 and MH2 (Fig. 3). These domains are separated by a poorly conserved, proline-rich linker region of variable length. The N-terminal MH1 domain can bind DNA, as discussed further below. The C-terminal MH2 domain can act as a transcriptional activator (Baker and Harland, 1996; Howell and Hill, 1997; Liu *et al.*, 1996) and mediates formation of oligomeric complexes (Shi *et al.*, 1997). Here we focus mainly on the

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literature for the *Drosophila* Smad proteins, Mad and Medea.

R-Smads. The receptor-regulated Smads are at the crux of signal transduction because these are substrates for type I receptor phosphorylation. Two serines in a C-terminal SSXS sequence are phosphorylated; mutation of the serines prevents signal-dependent phosphorylation (Kretzschmer *et al.*, 1997b; Macías-Silva *et al.*, 1996; Souchelnytskyi *et al.*, 1997). Blocking phosphorylation also prevents two other signal-dependent activities of R-Smads: translocation to the nucleus and association with co-Smads (Wisotzkey *et al.*, 1998).

Studies of vertebrate Smad function suggest that there are two distinct signal transduction pathways for TGF-β family signals, one pathway that regulates BMP responses and another pathway that regulates both TGF-β and activin responses (Baker and Harland, 1996; Graff *et al.*, 1996). *Drosophila Mad* is a BMP pathway-specific R-Smad, which is phosphorylated in cultured cells in response to BMP2 (Newfeld *et al.*, 1997). *Drosophila* dSmad2 is a TGF-β/activin pathway-specific R-Smad. It is specifically phosphorylated by the *Drosophila* activin type I receptor Babo and can mediate induction of TGF-β/activin-specific gene expression in vertebrate tissue culture cells (Brummel *et al.*, 1999). The specificity of R-Smad:type I receptor binding defines the specificity of signal transduction. Discrete structural elements of R-Smads (Fig. 3) and type I receptors determine the specificity of binding (Chen *et al.*, 1998b; Lo *et al.*, 1998; Persson *et al.*, 1998).

Co-Smads. The proposed functions of common mediator Smads, or co-Smads, are based on the properties of Smad4 and Medea when overexpressed in tissue culture cells. Smad4, which is encoded by the human tumor suppressor gene Deleted in pancreatic cancer 4 (Hahn *et al.*, 1996), is the only known vertebrate co-Smad, just as Medea is the only known co-Smad in *Drosophila* (Das *et al.*, 1998; Hudson *et al.*, 1998; Wisotzkey *et al.*, 1998). Co-Smads are thought to be important for all TGF-β family signal transduction, because vertebrate Smad4 can enhance signaling by both BMP-specific R-Smads and TGF-β/activin-specific R-Smads (Lagna *et al.*, 1996). Furthermore, a dominant negative form of Smad4 can antagonize both BMP and activin signaling in *Xenopus* mesoderm patterning (Lagna *et al.*, 1996). Medea and Smad4 can substitute for each other in cell biological assays (Wisotzkey *et al.*, 1998) and in genetic assays (Hudson *et al.*, 1998), indicating that Medea is a Smad4 ortholog. The essential role of *Medea* in *dpp* genetic pathways supports a central role for co-Smads in BMP signaling (Das *et al.*, 1998; Hudson *et al.*, 1998; Wisotzkey *et al.*, 1998). However, the specific requirements for Medea function to regulate individual Dpp response genes vary (Wisotzkey *et al.*, 1998), as discussed below in section V.

The co-Smad Medea shows signal-dependent association with phosphorylated R-Smads. Medea lacks the SSXS motif for type I receptor phosphorylation and does not appear to be phosphorylated in response to BMP signals (Wisotzkey *et al.*, 1998). However, Medea can associate with phosphorylated Mad or dSmad2 (Brummel *et al.*, 1999; Wisotzkey *et al.*, 1998). Mutations in Medea that prevent signal-dependent association with Mad in mammalian cultured cells confer strong mutant phenotypes *in vivo* (Wisotzkey *et al.*, 1998). Thus, the ability of a co-Smad to associate with R-Smads is important for *in vivo* function.

Smad oligomerization. Two lines of evidence indicate that Smads function as oligomeric complexes. The first line of evidence is the signal-dependent association of co-Smads with R-Smads, discussed above. The second is based on the crystal and solution structures of the isolated C-terminal MH2 domain of the vertebrate co-Smad, Smad4 (Shi *et al.*, 1997). This domain forms a stable homotrimer, at least when separated from the N-terminal portion of the protein. All Smads share the ability to form trimers, for the amino acids of the homomeric contact faces are highly conserved. Furthermore, a number of inactivating mutations in both R-Smads and co-Smads alter amino acids on this contact face. Thus, the capability to form trimers appears to be essential for function.

The composition of functional Smad complexes is under debate. Shi *et al.* (1997) suggested that both R-Smads and co-Smads normally exist as homotrimers and that signal-dependent Smad complexes are heterohexamers. In contrast, the requirement for three Smad proteins in each of the two *C. elegans* TGF-β family signal transduction pathways has been interpreted to indicate that Smads function in heterotrimeric complexes of three different Smad proteins (Krishna *et al.*, 1999). A third group has found that R-Smads exist as monomers in the absence of signal and only associate in homomeric complexes in the presence of signal (Inoue *et al.*, 1998; Kawabata *et al.*, 1998).

Translocation of Smads to the Nucleus Is Signal Dependent

When Smad proteins are overexpressed in cultured cells, regulated translocation to the nucleus is a key event for induction of gene expression. The R-Smad Mad is cytoplasmically localized in tissue culture cells in the absence of signal, but is predominantly located in the nucleus in the presence of actively signaling receptors (Inoue *et al.*, 1998; Maduzia and Padgett, 1997). Mutation of the sites for type I receptor phosphorylation prevents translocation to the nucleus (Wisotzkey *et al.*, 1998). Although phosphorylation triggers both association with co-Smads and transport to the nucleus, association with a co-Smad is not necessary for Mad to move to the nucleus (Wisotzkey *et al.*, 1998).

The presence of co-Smads in the nucleus is also signal dependent in tissue culture assays, due to their signal-dependent association with phosphorylated R-Smads. Activated Tkv does not drive Medea into the nucleus in the absence of similar levels of an R-Smad (Das *et al.*, 1998; Wisotzkey *et al.*, 1998). However, in the presence of both Mad and activated receptors, Medea is found in the nucleus (Das *et al.*, 1998; Wisotzkey *et al.*, 1998). Mutations in

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Medea that prevent signal-dependent association with Mad also prevent translocation to the nucleus in mammalian cultured cells (Wisotzkey *et al.*, 1998).

Other signals may modulate TGF-β family signal activity through regulation of Smad nuclear translocation. Activated MAP kinase can negatively regulate BMP signals by blocking Smad accumulation in the nucleus of specific types of cultured cells (Kretzschmer *et al.*, 1997a). However, little is known about *in vivo* regulation of Smad accumulation in the nucleus, because Smad proteins have not been detected in the nucleus under endogenous signal levels. Mad is detected only in the cytoplasm in fly tissues, even in cells in which Mad is required to regulate Dpp-response gene expression (Newfeld *et al.*, 1996, 1997). It is thought that the level of Mad necessary to induce gene expression responses is below the limit of detection. Overexpression of Dpp *in vivo* drives Mad into the nucleus, consistent with data from Smad overexpression in cultured cells (Newfeld *et al.*, 1997). Given the possibility that Smad subcellular localization is modulated by other extracellular signals, tools to detect nuclear accumulation of endogenous Smad proteins would be valuable.

A Third Class of Smad Proteins Feeds Back to Down-regulate Signal Transduction

A third class of Smads antagonizes signal transduction, the anti-Smads (Fig. 3). These distantly related Smad proteins retain the MH2 domain, but lack the DNA binding MH1 domain as well as the SSXS motif required for phosphorylation by type I receptors (Massagué, 1998). Ectopic expression of the *Drosophila* anti-Smad Daughters against Dpp (Dad) in the developing wing blade antagonizes the effects of ectopic Mad (Tsuneizuni *et al.*, 1997). Studies of Dpp-dependent maintenance of the female germ line also support a role for Dad in down-regulating Dpp signal activity (Xie and Spradling, 1998).

We present the current working model for anti-Smad function in Fig. 2. Dad shares the properties of vertebrate anti-Smad proteins when overexpressed in tissue culture cells. Epitope-tagged Dad associates with Tkv in a stable complex, whereas the wild-type epitope-tagged Mad–Tkv complex cannot be detected (Inoue *et al.*, 1998). Coexpression of Dad with Mad antagonizes both Tkv-dependent phosphorylation of Mad and Mad translocation to the nucleus. Thus the presence of Dad can block Mad activity. However, recent studies of a vertebrate anti-Smad have suggested that binding to type I receptors may be an artifact of anti-Smad overexpression in tissue culture systems (Hata *et al.*, 1998). These authors suggest that at physiological levels of expression, an anti-Smad binds to specific phosphorylated R-Smads, thus preventing association with the co-Smad. This controversy over the mechanism for anti-Smad function emphasizes the importance of studying the function of Smads at physiological levels *in vivo.*

Dad is involved in a feedback loop to moderate Dpp signaling during wing blade patterning (Tsuneizuni *et al.*, 1997). Expression of an enhancer trap reporter in the *dad* gene is dependent on Dpp signaling, so that in the developing wing blade Dad expression overlaps with expression of other Dpp target genes. Similarly, vertebrate anti-Smad expression is signal dependent (Massagué, 1998). Thus Dad expression creates a feedback loop that moderates the level of intracellular signal during prolonged exposure to Dpp.

III. SMAD PROTEINS DIRECTLY REGULATE TRANSCRIPTION

The key function of Smad proteins in TGF-β family signal transduction is transcriptional regulation. Biochemical evidence indicates that Smad proteins can bind DNA; genetic evidence indicates that some of the bound sites are essential for Dpp-responsive gene expression *in vivo.* In addition, Smads complex with other transcription factors in the absence of DNA binding and have transcriptional activator function. Thus, receptor regulation of Smad protein function directly leads to altered gene expression.

The MH1 domains of R-Smads and co-Smads can directly bind DNA. This was first demonstrated with an enhancer from the *Drosophila vestigial* gene, which is expressed in the wing imaginal disk (Kim *et al.*, 1997). Reduced levels of Dpp signaling cause reduced expression from the *vestigial* "quadrant" enhancer (Kim *et al.*, 1996). DNase I footprinting with the bacterially expressed Mad MH1 domain identified a single 12-bp Mad binding site within the quadrant enhancer. Mutation of this site severely reduced *lacZ* reporter expression *in vivo* (Kim *et al.*, 1997). These authors identified Mad binding sites in Dpp-response elements from the *labial* and *Ultrabithorax* (*Ubx*) genes as well. A GC-rich consensus binding sequence, GCCGnCG, was deduced from alignment of five Mad binding sites from the four genes. More recently, eight GC-rich Mad binding sequences were identified in the Dpp-response element from the *tinman* gene (Fig. 4) (Xu *et al.*, 1998).

The crystal structure has been solved for an R-Smad MH1 domain, from vertebrate Smad3, bound to its optimized DNA sequence, GTCT (Shi *et al.*, 1998). An unusual β hairpin structure in the MH1 domain contacts three bases within the DNA major groove. The three amino acids that make nucleotide-specific contacts are invariant among all Smads, which led the authors to suggest that BMP R-Smads and TGF-β/activin R-Smads have little difference in intrinsic DNA binding specificity. If this is the case, then the different promoter specificity of BMP R-Smads and TGF-β/activin R-Smads must be due to distinct interactions with other sequence-specific DNA binding proteins. However, studies of the *Drosophila tinman* promoter indicate that the co-Smad Medea cannot bind all sites that are bound by the R-Smad Mad (Fig. 4). R-Smads and co-Smads have conserved differences in a few amino acids of the DNA binding β hairpin (Shi *et al.*, 1998; Wisotzkey *et al.*, 1998), which may confer subtly different DNA binding specificity.

Smad proteins may be in an inactive conformation in the

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FIG. 4. Diagram of protein binding sites in the dorsal mesoderm enhancer from the *tinman* gene. This diagram is based on the work of Xu *et al.* (1998). The 349-bp dorsal mesoderm enhancer *tinD* lies 3′ to the tinman coding region. Essential regions of the dorsal mesoderm enhancer *tinD* were defined by deletion analysis, and protein binding sites were identified by DNase I footprinting and gel shift assays. Eight Mad binding sites were identified (ovals), four of these also bind Medea (Mad/Medea sites, gray ovals). Two clusters of Smad binding sites are *necessary* for reporter expression (brackets). Only one of these, *tin D3,* is *sufficient* to drive reporter expression *in vivo,* when present in five copies. *tinD3* contains additional sequences (triangles) that are necessary for function, but are not required for Mad or Medea binding. Tinman binding sites are indicated by squares, the leftmost site overlaps with a site that appears to be required for repression in the ectoderm.

absence of signal, so that the two conserved domains interact to inhibit DNA binding and transcriptional activator functions. The C-terminal half of Smad proteins can serve as a transcriptional activator when separated from the MH1 domain and fused to a heterologous DNA binding domain (Liu *et al.*, 1996, 1997). The MH1 domain appears to inhibit transcriptional activator function of the MH2 domain, and specific mutations in the MH1 domain enhance this autoinhibition (Hata *et al.*, 1997). Conversely, the Smad MH2 domain appears to block the DNA binding activity of the MH1 (Kim *et al.*, 1997; Liu *et al.*, 1997), although this is under debate for co-Smads (e.g., Dennler *et al.*, 1998; Xu *et al.*, 1998). It is possible that signal-dependent association of MH2 domains into oligomeric Smad complexes causes an allosteric change that permits the MH1 domain to bind DNA.

Vertebrate Smad proteins interact with other proteins that can contribute to both DNA binding and transcriptional activation. The DNA binding specificity of endogenous *Xenopus* activin response factor is determined by the forkhead DNA binding domain of FAST-1 (Chen *et al.*, 1996). Other protein–protein interactions may be required for general transcriptional function, such as interactions between vertebrate R-Smads and the transcriptional activator/histone acetylase CBP/p300 (Feng *et al.*, 1998; Janknecht *et al.*, 1998). The Schnurri zinc finger transcription factor has been implicated in Dpp signaling pathways in *Drosophila,* but whether it has a direct role in Dpp-response factor complexes is unknown (Arora *et al.*, 1995; Grieder *et al.*, 1997; Staehling-Hampton *et al.*, 1995). It is likely that the set of proteins recruited into response factor complexes will determine both tissue specificity and the sensitivity of gene expression responses to the effective signal level; two examples are discussed below.

IV. DPP-RESPONSE ELEMENTS *IN VIVO:* ADDITIONAL TRANSCRIPTION FACTORS ARE REQUIRED

Dpp directs patterning and morphogenesis in many tissues. Dpp is required for dorsal–ventral patterning of embryonic ectoderm (Ferguson, 1996; Rusch and Levine, 1996), inductive patterning of the embryonic dorsal mesoderm (Frasch, 1995; Staehling-Hampton *et al.*, 1994), foregut morphogenesis (Pankratz and Hoch, 1995), inductive patterning of the embryonic midgut (Bienz, 1994), establishment of embryonic imaginal disk placodes (Goto and Hayashi, 1997), tracheal cell migration (Vincent *et al.*, 1997), morphogenetic movements of the embryonic ectoderm (Noselli, 1998), patterning of the optic lobes in the larval brain (Kaphingst and Kunes, 1994), events in the morphogenetic furrow during eye development (Chanut and Heberlein, 1997; Heberlein *et al.*, 1993; Penton *et al.*, 1997; Pignoni and Zipursky, 1997; Wiersdorff *et al.*, 1996), growth and patterning of the adult appendage primordia (Brook *et al.*, 1996; Gelbart, 1989), wing vein formation (de Celis, 1997; Segal and Gelbart, 1985; Yu *et al.*, 1996), patterning of the eggshell (Twombly *et al.*, 1996), and maintenance of the female germ line during adult life (Xie and Spradling, 1998). Dpp signaling induces distinct cellular and gene expression responses in each tissue (Fig. 1).

Smad proteins confer a general response to Dpp signals; however, Smad proteins alone are not sufficient for induction of gene expression. Detailed characterization of Dpp-response elements from three *Drosophila* genes indicates that other transcription factors are required to tailor tissue-specific responses to the Dpp signal.

Direct Regulation of tinman Expression by Smad Proteins

DNA binding by the co-Smad Medea, and probably also by the R-Smad Mad, is essential for spatially regulated expression of the Nkx homeobox gene *tinman* (Xu *et al.*, 1998). Dpp is expressed in the early embryonic dorsal ectoderm and induces the adjacent dorsal mesoderm to acquire visceral mesoderm and cardiac mesoderm fates (Frasch, 1995; Staehling-Hampton *et al.*, 1994). In particular, Dpp signaling maintains dorsal mesoderm expression of *tinman* (Frasch, 1995). The dorsal mesoderm enhancer from *tinman,* called *tinD,* is sufficient to drive *lacZ* expression in the spatial pattern of the endogenous gene. The *tinD* enhancer contains eight Smad binding sites; two pairs of

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sites are essential for expression of the *tinD* reporter (*tinD3* and *tinD6,* Fig. 4). The *Medea* gene is necessary for *in vivo* expression of the *tinD* reporter; *Mad* has not been tested.

Interdependent Regulation of tinman Expression by Smads and Tissue-Specific Transcription Factors

A smaller region of *tinD* can act alone to drive *lacZ* reporter expression, but it lacks the correct spatial pattern (Xu *et al.*, 1998). This 42-bp element, *tinD3,* drives Dpp-responsive *lacZ* expression, but in an abnormal pattern: strong in the dorsal ectoderm and weak in the dorsal mesoderm. *tinD3* contains two Smad binding sites that are essential for function, one Medea/Mad binding site and one Mad binding site (Fig. 4). A distinct repeated sequence is also essential, but is not required for Smad binding in gel shift assays. An additional sequence-specific DNA binding protein probably binds these sites in an intimate complex with the Smad proteins (Derynck *et al.*, 1998). It is striking that the additional sequence, CAATGT, resembles the site bound by the *Xenopus* activin response factor protein, FAST-1 (Chen *et al.*, 1996; Xu *et al.*, 1998). The *tinD3* element represents the minimal portion of the enhancer that is sufficient for regulation by Dpp.

The correct spatial pattern of *tinman* expression requires additional tissue-specific factors (Xu *et al.*, 1998). Mesoderm expression is conferred by two Tinman binding sites present in the full-size *tinD* enhancer (Fig. 4). One of these sites may also be required to prevent expression in the ectoderm. Tinman protein is present prior to the onset of Dpp regulation of the gene (Yin *et al.*, 1997) and synergizes with the Dpp response factor to refine and maintain of its own expression. Such a positive autoregulatory loop may be a common theme in tissue inductive responses, for the Labial homeodomain protein is similarly involved in regulating expression of its own gene in the visceral endoderm (Grieder *et al.,* 1997; Tremml and Bienz, 1992).

Function of the Dpp-Response Element from labial Requires CRE Sites

Studies of Dpp-dependent gene expression in the developing midgut indicate that Mad binding sites can be dispensable for Dpp-dependent gene expression (Szüts *et al.*, 1998). Localized *dpp* expression in parasegment 7 of the visceral mesoderm patterns a subdomain of the embryonic midgut (Bienz, 1994). The mesodermal Dpp signal enhances and maintains expression of the endodermal homeobox transcription factor gene *labial* and induces an adjacent constriction of the gut tube, called the second constriction (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990). In addition, Dpp signaling acts in a positive feedback loop to maintain expression of the visceral mesoderm homeobox transcription factor gene *Ubx* and the *dpp* gene itself (Hursh *et al.*, 1993). These three gene expression markers are frequently used as assays for Dpp-dependent responses (Letsou *et al.*, 1995; Nellen *et al.*, 1994; Newfeld *et al.*, 1996; Ruberte *et al.*, 1995; Wisotzkey *et al.*, 1998). However, Dpp regulation of *labial* and *Ubx* occurs primarily through CRE sequences.

Although Mad binding sites are present in the Dpp response region for *labial* (Kim *et al.*, 1997), four CRE sequences are essential for Dpp responsiveness (Eresh *et al.*, 1997). CRE sequences can mediate responses to either cAMP or Ras signaling (de Groot *et al.*, 1993; Galien *et al.*, 1991; Kanei-Ishii and Ishii, 1989). Strikingly, Dpp induces expression of the EGF receptor ligand Vein (Szüts *et al.*, 1998), which probably signals through the Ras pathway (Schnepp *et al.*, 1996). Ectopic Dpp and activated Ras strongly synergize to induce ectopic *labial* expression (Szüts *et al.*, 1998). A precise mutational analysis of the CRE sequences and Mad binding sites within the enhancer revealed that only the CRE sequences are essential for Dpp responsiveness (Szüts *et al.*, 1998). Thus Dpp indirectly regulates *labial* expression through a secondary receptor tyrosine kinase signal.

This is a surprising observation, because *Mad* is essential in the endoderm for expression from the *labial* enhancer (Newfeld *et al.*, 1996), and *Medea* is important for full expression of the *labial* gene (Wisotzkey *et al.*, 1998). The simplest explanation is that the only essential role for Dpp in regulation of *labial* expression is to induce the secondary signal Vein, which then induces *labial* expression via the CRE sequences. A second possibility is that these CRE sequences are bound by a transcription factor complex that includes Smad proteins, but the Smads are not necessary for the DNA binding activity of the complex. Studies of the Dpp-response element from *Ubx* are consistent with predominantly indirect regulation through a CRE sequence, although the data are not as conclusive (Szüts *et al.*, 1998).

V. THRESHOLD RESPONSES TO A MORPHOGEN GRADIENT INVOLVE DISTINCT CONTRIBUTIONS OF THE R-SMAD MAD AND THE CO-SMAD MEDEA

Studies of embryonic dorsal–ventral patterning and limb patterning have emphasized the role of Dpp as a morphogen (Neumann and Cohen, 1997). Recent studies of BMP signaling suggest that in both tissues the erstwhile Dpp gradient is in fact a gradient of different levels of intracellular signal induced by at least two BMP ligands signaling through two BMP type I receptors, Tkv and Sax (Haerry *et al.*, 1998; Neul and Ferguson, 1998; Nguyen *et al.*, 1998). The molecular model for TGF-β family signal transduction predicts that the concentration of R-Smad:co-Smad complexes in the nucleus is the direct readout of the effective intracellular signal level. Unfortunately, currently available reagents are insufficient to test this prediction (Newfeld *et al.*, 1996, 1997). However, studies of threshold gene expression responses during wing patterning indicate that there are differential requirements for the R-Smad Mad and the

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co-Smad Medea (Lecuit *et al.*, 1996; Wisotzkey *et al.*, 1998). These observations suggest that different types of Smad complexes may contribute to the intracellular response at different signal levels. First we review the evidence that a morphogen gradient induces threshold gene expression responses during wing patterning.

A BMP Morphogen Gradient Induces Nested Gene Expression Responses

A Dpp morphogen gradient is involved in patterning the wing blade (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). The *dpp* gene is expressed in a narrow stripe at the center of the wing imaginal disk (Masucci *et al.*, 1990; Posakony *et al.*, 1991; Sanicola *et al.*, 1995); its expression at this site is required for growth and patterning of the entire wing blade (Posakony *et al.*, 1991). Within the developing wing blade primordium, the genes *optomotor blind* (*omb;* Grimm and Pflugfelder, 1996) and *spalt* (de Celis *et al.*, 1996) are expressed in a nested pattern with the stripe of *dpp* expression at the center. Local ectopic expression of Dpp induces nearby cells to express ectopic *spalt* and *omb* (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). Low levels of Dpp are sufficient to induce *omb,* whereas higher levels are needed to induce *spalt.* The Dpp type I receptor Tkv is required for expression of both genes (Nellen *et al.*, 1996). Furthermore, local expression of activated Tkv causes cell-autonomous expression of the two target genes, indicating that induction of gene expression at a distance from the site of *dpp* gene expression does not involve induction of a secondary extracellular signal. These data indicate that Dpp can travel from the site of expression to directly specify different cell fates at different concentrations.

The full morphogen gradient involves additional BMP ligands (Haerry *et al.*, 1998; Khalsa *et al.*, 1998; Singer *et al.*, 1997). While the Tkv type I receptor is essential for Dpp responses, the Sax type I receptor boosts the level of intracellular signal uniformly throughout the wing blade primordium (Singer *et al.*, 1997). Recent studies suggest that Gbb-60A contributes to the gradient by signaling through Sax (Haerry *et al.*, 1998; Khalsa *et al.*, 1998). However, a detailed comparison of *gbb-60A* and *sax* mutant phenotypes in the adult wing margin suggests that Gbb-60A is not the ligand that activates Sax in this region (Khalsa *et al.*, 1998; Singer *et al.*, 1997).

Differential Requirements for Mad and Medea in Specific Gene Expression Responses

Studies of gene expression in genetically mosaic wing imaginal disks have revealed differences in the requirements for the R-Smad Mad and the co-Smad Medea. Expression of *spalt* is lost and expression of *omb* is severely reduced in all cells with reduced *Mad* function (Lecuit *et al.*, 1996). Thus the R-Smad, Mad, like the type I receptor Tkv (Lecuit *et al.*, 1996), appears essential for Dpp-dependent gene expression in this tissue. Surprisingly, *Medea* appears to be essential for *omb* expression only in cells that receive low levels of Dpp signal. *omb* expression is retained in *Medea* mutant cells at or near the site of *dpp* expression, whereas it is lost in more distant mutant cells (Wisotzkey *et al.*, 1998). This observation suggests that at high levels of Dpp signal, Dpp-response factor complexes that lack Medea are active. This may indicate that another Smad can partially replace Medea function in Dpp-response factor complexes or that Dpp-response factor complexes that contain only Mad are active. Two additional observations suggest that Mad can transduce a signal independent of Medea: Mad can move to the nucleus independent of stoichiometric levels of a co-Smad (Wisotzkey *et al.*, 1998) and the crystal structure indicates that each Smad MH1 domain binds DNA independently (Shi *et al.*, 1998). In contrast to the *omb* gene, *spalt* expression appears to require *Medea* at all positions in the developing wing blade (D.J.S. and L.A.R., unpublished data). These data indicate that the two genes have differential sensitivity to the loss of the co-Smad Medea.

Assuming that *omb* and *spalt* are directly regulated by Dpp-response factor complexes, then the differential sensitivity of the two genes lies in the promoters. We envision two models to explain the differences. In one model, there are two classes of Dpp-response factors with different DNA binding specificity; one class requires both Mad and Medea, the other requires Mad but not Medea. In this case, the differences between the two promoters would be in the types of Smad binding sites present. The *omb* promoter may contain sites that allow binding of both classes of Dpp-response factors, perhaps similar to the two types Smad binding sites in the *tinman* enhancer (Fig. 4). In the second model, there is a single class of Dpp-response factor, which is fully active with Medea and weakly active without. In this case, the ability to respond to DPP signaling in the absence of Medea would be conferred by cooperative interactions between the weakly active DPP-response factor and a coactivator that binds the *omb* promoter and not the *spalt* promoter. Such a mechanism is involved in threshold gene expression responses to the Dorsal morphogen gradient in the early *Drosophila* embryo, in which sensitive Dorsal target genes are induced through cooperative binding of Dorsal and bHLH proteins (Jiang and Levine, 1993). Detailed characterization of Dpp-response elements from the *omb* and *spalt* genes will be important to distinguish these models.

VI. SYNERGISTIC RECEPTOR INTERACTIONS: IS THE MOLECULAR SIGNAL TRANSDUCTION MODEL INCOMPLETE?

Synergistic genetic interactions between two BMP type I receptors, Sax and Tkv, do not fit easily into the current model for TGF-β family signal transduction. The evidence for synergy is compelling and comes from studies of both

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embryonic dorsal–ventral patterning and of wing patterning (Haerry *et al.*, 1998; Neul and Ferguson, 1998; Nguyen *et al.*, 1998). Similar synergistic interactions are observed for the ligands Dpp and Screw in the early embryo and the ligands Dpp and Gbb-60A in the developing wing, consistent with proposals that Screw and Gbb-60A are ligands for Sax.

Evidence for synergy between the receptors comes from the observation that activated Sax and activated Tkv cause strong phenotypes when expressed together, whereas the equivalent amount of either activated receptor causes little or no phenotype when expressed alone (Haerry *et al.*, 1998; Neul and Ferguson, 1998; Nguyen *et al.*, 1998). For dorsal–ventral patterning in the embryo, the effective level of signal is measured by the amount of dorsal ectoderm in the terminal cuticular pattern and by the amount of differentiated amnioserosa present at an earlier stage (Neul and Ferguson, 1998; Nguyen *et al.*, 1998). For wing blade patterning, the effective level of signal is measured by the amount of overgrowth in the wing imaginal disk and by the area of ectopic expression of the Dpp-response genes *omb* and *spalt* (Haerry *et al.*, 1998).

It is possible that synergy simply causes elevated signaling through the established Dpp signal transduction pathway. One such mechanism would involve cooperative interactions between type I receptors at the cell surface; there is evidence that active TGF-β family receptors associate in tetrameric complexes (Yamashita *et al.*, 1994). Other possible mechanisms for synergy between the receptors require that one of the two receptors can signal through an independent pathway. Any additional signal-transducing activity is likely to reside in Tkv, and not in Sax, because the Tkv intracellular domain can replace the function of the Sax intracellular domain (Haerry *et al.*, 1998). A larger variety of mechanisms fit under this model, such as increased retention of Mad or Medea in the nucleus, activation of additional Smads, or modified activity of other proteins in Dpp-response factor complexes. A candidate to mediate synergy is the TGF-β-activated kinase TAK-1, which can act downstream of BMP, activin, and TGF-β in vertebrates (Shibuya *et al.*, 1996, 1998; Yamaguchi *et al.*, 1995).

VII. MORE LIGANDS, MORE SMAD PATHWAYS?

Genetic analysis of additional ligands and receptors is now under way in *Drosophila.* Mutant phenotypes for Gbb-60A suggest that this BMP ligand can both cooperate with and antagonize Dpp. Characterization of an activin-like type I receptor, Babo, indicates that all components of a functional activin-like pathway are present in this organism.

Two Mechanisms for Gbb-60A Signaling?

Gbb-60A is similar in sequence to a group of vertebrate BMPs that includes BMP7 (Table 1). Studies of both wing and midgut patterning suggest that Dpp and Gbb-60A signaling have overlapping, perhaps even synergistic, effects in one region of the tissue and antagonistic effects in another (Chen *et al.*, 1998a; Haerry *et al.*, 1998; Khalsa *et al.*, 1998). Given that Gbb-60A is likely to signal through the type I receptor Sax, it is likely that overlapping and synergistic functions of Gbb-60A and Dpp are mediated by the interactions between Sax and Tkv. It has been proposed that overlapping requirements for vertebrate homologs of these BMPs are due to the formation of heterodimers in cells that express both ligands (Israel *et al.*, 1996; Nishimatsu and Thomsen, 1998; Suzuki *et al.*, 1997). However, imaginal disk cells that express Dpp exhibit reduced levels of Gbb-60A protein, suggesting that heterodimers are not a major component of the ligand population in this tissue (Khalsa *et al.*, 1998).

Gbb-60A and Dpp have antagonistic effects on gene expression in the developing midgut, raising the possibility that Gbb-60A can signal through an additional mechanism. Gbb-60A is required in the anterior midgut, where it maintains expression of the *Antennapedia* gene and induces formation of the first midgut constriction (Chen *et al.*, 1998a). Dpp appears to antagonize Gbb-60A signaling in this region of the midgut, because ectopic Dpp blocks both *Antennapedia* expression and formation of the first constriction (Staehling-Hampton and Hoffmann, 1994). Formation of the first constriction does not require Tkv or Sax (Nellen *et al.*, 1994), Mad (Newfeld *et al.*, 1996), or Medea (Wisotzkey *et al.*, 1998). Opposing responses have been reported for BMP7 homodimers versus BMP7/BMP4 heterodimers during *Xenopus* mesoderm patterning (Nishimatsu and Thomsen, 1998; Suzuki *et al.*, 1997; Yamashita *et al.*, 1995). Understanding the complex activities of Gbb-60A will be important for understanding the roles of this poorly understood subfamily of BMPs.

An Activin-like Signal Transduction Pathway

Evidence for a functional TGF-β/activin-like pathway in *Drosophila* comes from studies of the activin-like type I receptor Babo (Brummel *et al.*, 1999). Babo can bind activin in association with the Punt type II receptor (Wrana *et al.*, 1994a). A candidate ligand, dActivin, has been identified by sequence homology (Kutty *et al.*, 1998). The *Drosophila* activin-like pathway parallels the vertebrate TGF-β/activin pathway (Brummel *et al.*, 1999). The R-Smad dSmad2 is phosphorylated by activated Babo in tissue culture cells and can mediate expression from vertebrate TGF-β and activin-response elements. Medea can form complexes with dSmad2 in response to activated Babo signaling. Although dSmad2 is similar in sequence to both vertebrate TGF-β/activin Smads (Fig. 3), it may be functionally more similar to vertebrate Smad3, because it lacks an insert present in the vertebrate Smad2 MH1 domain (Brummel *et al.*, 1999).

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This insert prevents binding to DNA (Yagi *et al.*, 1999), so that Smad3 and Smad2 show different activity in promoter studies (Labbe *et al.*, 1998).

babo is required for cell proliferation during optic lobe development in the larva and for a small component of the cell proliferation in imaginal disks (Brummel *et al.*, 1999). Although *babo* mutant phenotypes in these tissues overlap with those of *dpp, babo* does not regulate Dpp-response genes in imaginal disks. At present only one phenotype distinguishes activin-like signaling from Dpp signaling: *babo* mutant larvae have swollen anal pads, associated with fluid accumulation (Brummel *et al.*, 1999). *Medea* mutant larvae have a similar phenotype (J. Peterson and L.A.R, unpublished observations); dActivin and dSmad2 have not been characterized genetically. Further analysis of this signal transduction pathway will be facilitated by identification of Babo target genes.

VIII. SUMMARY AND PROSPECTIVE

Tremendous progress has been made in the understanding of TGF-β family signal transduction in the few years since Smad proteins were first identified. Central roles have been established for the R-Smad Mad and the co-Smad Medea during many Dpp-dependent developmental events. The anti-Smad Dad participates in a feedback loop to modulate Dpp signaling. However, it is not clear whether this complement of Smads is sufficient to explain threshold gene expression responses. Promoter studies suggest that tissue-specific transcription factors are essential components of Dpp-response factors, but none have been identified. Characterization of sensitive and insensitive Dpp-response elements from multiple tissues is needed to address these issues.

The signal transduction model presented in Fig. 2 is a working model. Genetic analysis of Dpp signaling in *Drosophila* has provided *in vivo* exceptions that challenge the model. Observations of synergy between the Tkv and the Sax type I receptors suggest that BMP signal transduction is more complex. Are there additional signal transduction mechanisms that regulate the function of Mad or Medea? Is an additional signal transduction mechanism activated at high signal levels? Studies of threshold gene expression responses in the developing wing suggest that such a mechanism would be independent of the co-Smad Medea.

Studies in mammalian tissue culture cells indicate that nuclear localization of Smad complexes is a key regulatory step in signal transduction, but the mechanism for regulation of subcellular localization is unknown. The model predicts that nuclear levels of Smad proteins are the intracellular measure of effective signal levels. A sensitive methodology to visualize nuclear localization of Smad proteins would provide a powerful tool to study signal transduction *in vivo.* Alternatively, endogenous signal activity might be assayed with antibodies specific to phosphorylated R-Smads, similar to the diphospho-Erk antibodies that are now used to identify sites of active Ras pathway signaling (Gabay *et al.*, 1997). It will also be important to determine whether all cellular responses require nuclear localization of Smads or whether some involve only cytoplasmic signal transduction.

Are there more Smad proteins in *Drosophila?* Each of the TGF-β family signal transduction pathways characterized in *C. elegans* requires three Smad proteins (Larsen *et al.*, 1995; Malone *et al.*, 1996; Patterson *et al.*, 1997; Savage *et al.*, 1996) (J. Thomas, personal communication). Only two Smads are known to act in the *Drosophila* activin signal transduction pathway; perhaps additional Smads act in this pathway. If a *Drosophila* ligand is functionally similar to Müllerian inhibiting substance or Daf-7 (Table 1), then additional Smads will likely be required for a third signal transduction pathway.

The complement of ligands and receptors now identified in *Drosophila* suggest that all vertebrate TGF-β family signal transduction pathways are represented in this organism. *Drosophila* is well suited to sort out the network of signal transduction pathways used by these ligands, due to the low level of genetic redundancy combined with the powerful tools available for manipulation of gene activity. Detailed analyses of mutant phenotypes have already challenged the model for signal transduction and will lead the way to understanding signal transduction *in vivo.*

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Note added in proof. To induce expression of *omb* and *spalt*, Dpp must repress expression of the negative regulator *brinker*, which may be a transcriptional repressor (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999). Mad and Medea may not regulate expression of *omb* by direct binding to its promoter; however, regulation of *spalt* may involve both direct and indirect mechanisms.

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