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Caenorhabditis elegans genes *sma-2, sma-3, and sma-4* define a conserved family of transforming growth factor β pathway components

(signal transduction/pattern formation/bone morphogenetic protein/multigene family)

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ABSTRACT **Although transforming growth factor β (TGF-β) superfamily ligands play critical roles in diverse developmental processes, how cells transduce signals from these ligands is still poorly understood. Cell surface receptors for these ligands have been identified, but their cytoplasmic targets are unknown. We have identified three *Caenorhabditis elegans* genes, *sma-2*, *sma-3*, and *sma-4*, that have mutant phenotypes similar to those of the TGF-β-like receptor gene *daf-4*, indicating that they are required for *daf-4*-mediated developmental processes. We show that *sma-2* functions in the same cells as *daf-4*, consistent with a role in transducing signals from the receptor. These three genes define a protein family, the dwarfins, that includes the *Mad* gene product, which participates in the decapentaplegic TGF-β-like pathway in *Drosophila* [Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. & Gelbart, W. M. (1995) *Genetics* 139, 1347–1358]. The identification of homologous components of these pathways in distantly related organisms suggests that dwarfins may be universally required for TGF-β-like signal transduction. In fact, we have isolated highly conserved dwarfins from vertebrates, indicating that these components are not idiosyncratic to invertebrates. These analyses suggest that dwarfins are conserved cytoplasmic signal transducers.**

Members of the transforming growth factor β (TGF-β) superfamily of secreted ligands have major regulatory effects on growth and differentiation (1, 2). How cells transduce signals from these ligands is still poorly understood, however. Crosslinking experiments reveal that two transmembrane receptors, type I and type II, bind TGF-β with high affinity (3). These two proteins are related serine/threonine kinase receptors and both are necessary for signal transduction. The receptors for members of the bone morphogenetic protein (BMP) family of TGF-β-like ligands are also type I and type II serine/threonine kinase receptors (4–14). To understand how cells respond to TGF-β-like signals, the cytoplasmic targets for these receptors must be identified and characterized. It is thought that these components are likely to be different from those known to act downstream of tyrosine kinase receptors (15).

Genetic studies in *Drosophila* and in the nematode *Caenorhabditis elegans* have led to the isolation of TGF-β pathway components, including ligands, accessory molecules and receptors, homologous to those identified in vertebrates. In *Drosophila*, the BMP family ligand encoded by the decapentaplegic gene (*dpp*) (16) is important for a variety of developmental decisions, both in embryos and in imaginal discs (17–19). These invertebrate components have sequence similarity to their vertebrate counterparts and are also likely to have functional similarity, since human BMP-4 sequences can substitute for *dpp* in *Drosophila* embryos (20) and DPP can induce ectopic bone formation in mammals (21). Homologs of the TGF-β-like signaling components identified in these model systems are therefore likely to function in TGF-β-like signal transduction in vertebrates as well. In *C. elegans*, two genes, *daf-1* and *daf-4*, encode serine/threonine kinase receptors related to TGF-β receptors (4, 22). DAF-4 is likely a receptor for a member of the BMP family of TGF-β-like ligands, since it binds human BMP-2 and BMP-4 *in vitro* (4). *daf-4* mutant phenotypes reveal multiple roles for TGF-β-like signaling in *C. elegans* development: *daf-4* mutants are dauer-constitutive (23, 24), egg-laying defective (25), and smaller than wild type (4).

We are using *C. elegans* as a model system to dissect the TGF-β-like signal transduction pathway. In addition to the previously described developmental roles for *daf-4*, we have found that *daf-4* is also required for the development and morphogenesis of the male tail. The identification of male tail phenotypes for *daf-4* provided novel criteria for the characterization of components of its signal transduction pathway. Therefore, we examined existing mutant collections to identify loci that share mutant phenotypes with *daf-4*. We report here the characterization of three putative components of the *daf-4* signaling pathway encoded by *sma-2*, *sma-3*, and *sma-4*.¶ These three gene products are related proteins with no obvious functional motifs. They define a gene family, encoding proteins which we call the dwarfins, that includes highly conserved members in *Drosophila* and vertebrates. Our analyses suggest that the dwarfins are cytoplasmic signal transducers in a TGF-β-like pathway.

MATERIALS AND METHODS

Genetic Analyses. Methods for the culturing and handling of *C. elegans* were as described (26). Unless otherwise noted, the following alleles of each gene were used: *daf-4(m72)*, *sma-2(e502)*, *sma-3(e491)*, and *sma-4(e729)*. All experiments were performed at 20°C, except for heat shock experiments. We used strains carrying the extrachromosomal array *mEx11*, which contains a previously described heat shock *daf-4* construct (4). These strains were cultured at 25°C. For heat shock,

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Abbreviations: TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; DH, dwarfin homology region; dpp, decapentaplegic.

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¶The sequences reported in this paper have been deposited in the GenBank database [accession nos. U34778 (*sma-2*), U34902 (*sma-3*), and 34596 (*sma-4*)].

populations of dauers or of eggs and L1 larvae were incubated at 31°C for 1 hr.

The mutation *him-5(e1490)* was used to increase the frequency of males. Individual sensory rays in the *C. elegans* male tail express a variety of identifying features, based on (*i*) anteroposterior position of the ray, (*ii*) dorsoventral position of the sensory ending, and (*iii*) morphology of the ray (27, 28). Ray patterning defects in the mutants described here are identified by alterations in these identifying features and are often accompanied by fusions of the affected rays.

Genetic mosaics were generated from *daf-4*(−) *ncl-1*(−) *unc-36*(−); *qDp3* [*daf-4*(+) *ncl-1*(+) *unc-36*(+)] or *ncl-1*(−) *unc-36*(−) *sma-2*(−); *qDp3* [*ncl-1*(+) *unc-36*(+) *sma-2*(+)] animals by mitotic loss of the free chromosomal duplication *qDp3* (29, 30). *qDp3* contains wild-type *daf-4, sma-2, ncl-1*, and *unc-36* activity. To identify mosaic males, nonSmall nonUncoordinated adult males were scored at ×400 magnification for defects in ray pattern. Animals expressing defects were then scored at ×1000 magnification to identify clones of *ncl-1*(−) cells, which have enlarged nucleoli.

Molecular Cloning and Sequencing. Germline transformation of nematodes to identify rescuing DNA for *sma-3* and *sma-4* was done with *rol-6* as a dominant selectable marker (31). To identify mutations in *sma-2* and *sma-3*, genomic fragments from these genes were amplified by PCR using genomic DNA. PCR amplification, cloning, and sequencing were done as described (10). Mutations were verified by sequencing clones from two independent PCRs.

To isolate vertebrate homologs, degenerate oligonucleotide PCR primers were designed to recognize two conserved motifs in dwarfin homology region 1 (DH1). For the amino acid sequence motif LDGRLQ, the oligodeoxynucleotide primer was 5′-(C/T)TNGA(T/C)GGN(C/A)GN(C/T)TNCA-3′; for CINPYHY, the primer was 5-TG(T/C)AT(T/C/A)AA(T/C)CCNTA(T/C)CA(T/C)TA-3′. The vertebrate clones derive from different genes, since DNA sequence alignments show many third-position changes.

RESULTS AND DISCUSSION

***daf-4* Is Required for Patterning of the *C. elegans* Male Tail.** The *C. elegans* male tail is a complex, fan-shaped structure necessary for copulation (27). Nine bilateral pairs of peripheral sense organs, the sensory rays, form in a precise pattern in the lateral epidermis (27, 28). In the center of the fan is the proctodeum, which houses a pair of needle-like copulatory spicules made by the proctodeal cells (27). We have found that *daf-4* males have defects in both the rays and the spicules, rendering them unable to mate.

The most frequent defects in *daf-4* ray patterning are fusions of rays 7 and 6 and fusions of rays 5 and 4 (compare Fig. 1 *A* and *B*; Table 1). These ray defects do not result from cell lineage changes, since we find that the affected rays have wild-type lineages (data not shown). Rather, the defects are due to transformations of the ray identities. Ray 7 is frequently displaced anteriorly, fusing with ray 6. The sensory ending of ray 7 is not open externally and its shape is conical rather than cylindrical. Thus, ray 7 expresses characteristics more typical of the wild-type ray 6. Similarly, ray 5 defects are seen that are consistent with a shift in ray 5 identity. *daf-4* mutants also have copulatory spicule defects; the spicules are shorter than wild-type and severely crumpled (data not shown).

***sma-2, sma-3*, and *sma-4* Participate in *daf-4*-Mediated Processes.** The identification of male tail phenotypes for *daf-4* provided new criteria for the characterization of components of its signal transduction pathway. Seven genes—*daf-1, daf-3, daf-4, daf-5, daf-7, daf-8*, and *daf-14*—had been proposed to act in a common signaling pathway that regulates dauer development (4, 22, 32, 33), but, other than *daf-4*, none of these genes seem to be required for the regulation of body size or for the development of the rays and spicules (data not shown). Previous studies had shown, however, that *sma-2* and *sma-3* are required for proper body size and male tail development (26, 28, 34). Based on this observation, we screened existing Small and Dumpy mutants for male tail abnormalities and identified three genes—*sma-2, sma-3*, and *sma-4*—that share the *daf-4* Small and male tail phenotypes (Fig. 1 *C–E*; Table 1). Mutations in each of these genes cause crumpled spicules (34) and defects in ray pattern identical to those seen in *daf-4* mutants:

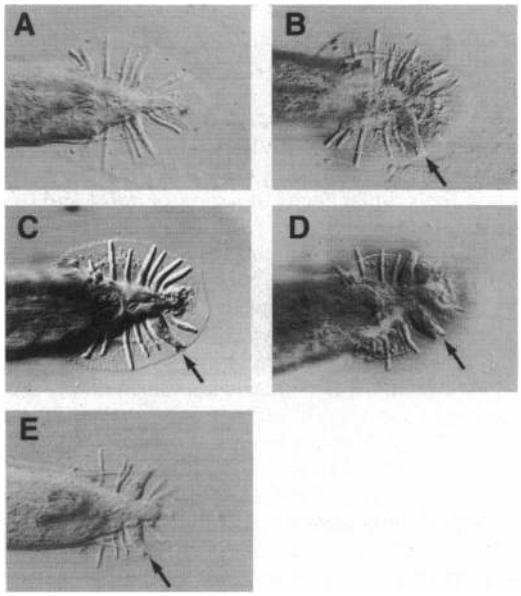

FIG. 1. Male tail phenotypes of *him-5(e1490)*, wild type (*A*), *daf-4(m63);him-5* (*B*), *sma-2(e502);him-5* (*C*), *sma-3(e491);him-5* (*D*), and *sma-4(e729);him-5* (*E*). Photographs of male tails are from Nomarski differential interference contrast microscopy. Arrows indicate fusions of rays 6 and 7 in mutants; the incomplete expressivity of this phenotype can be seen in *B* and *C*, where a fusion is present only on one side. (×260.)

Table 1. Frequency of ray fusions in mutant males

Mutant	Frequency, %				*n*
	Ray 4	Ray 5	Ray 6	Ray 7	
daf-4(e1364)	6	6	56	56	16
daf-4(m44)	17	17	44	44	66
daf-4(m63)	7	11	34	33	84
daf-4(m72)	6	8	58	58	88
daf-4(m76)	14	14	64	64	14
daf-4(m592) at 15°C	3	0	0	0	70
daf-4(m592) at 25°C	41	41	42	42	92
daf-4(sa220)	19	19	42	42	74
sma-2(e172)	13	10	50	51	86
sma-2(e297)	10	10	68	68	117
sma-2(e502)	13	16	45	43	220
sma-2(e1491)	8	11	47	46	358
sma-3(bx77)	18	18	60	60	120
sma-3(e491)	28	28	34	37	67
sma-3(e637)	11	11	74	74	119
sma-3(e958)	5	5	8	8	118
sma-4(e729)	13	14	45	43	220
sma-4(e805)	0	0	0	0	101

The frequency, in percent, at which each ray is fused with another ray in *daf-4, sma-2, sma-3*, and *sma-4* mutants. In addition to fusions of rays 6 and 7 and of rays 4 and 5, other less frequent fusions are seen. The phenotypes are not fully penetrant. n, Number of sides scored.

frequent fusions of rays 7 and 6 and less frequent fusions of rays 5 and 4 (Table 1). The similarity of these mutant phenotypes suggests that *daf-4, sma-2, sma-3,* and *sma-4* act in the same pathway.

Since *sma-2, sma-3,* and *sma-4* showed some mutant phenotypes identical to those of the TGF-β-like receptor gene *daf-4*, we asked whether these genes act in the same cells. We determined the cellular focus of action of *daf-4* and of *sma-2* in establishing ray identity by genetic mosaic analysis (*Materials and Methods*). In mosaic analysis, clones of mutant cells are established in a wild-type background by genetic means (30). We can then determine where the activity of a gene is required for a given phenotypic result and whether gene activity in one cell can influence the development of neighboring cells. For *daf-4*, five mosaic animals with ray 7 defects were observed (Table 2). In all cases, the two neurons of the defective ray 7, which derive from a single ray neuroblast cell, were mutant for *daf-4*. In one case, the cells of the defective ray 7 were the only mutant cells observed. Therefore, *daf-4* is required cell autonomously in each ray 7. For *sma-2*, 13 of 13 animals with ray 7 defects had ray 7 neurons that were mutant for *sma-2*; in 2 cases, ray 7 contained the only mutant cells identified (Table 2). Thus, *sma-2*, like *daf-4*, is required cell autonomously in the ray 7 neuroblast to determine ray identity. This result implies two conclusions: (*i*) SMA-2 does not act as a diffusible factor to influence the identities of neighboring neuroblasts and (*ii*) SMA-2 acts in the same cells as the receptor, DAF-4.

To rule out the possibility that autonomy is due to a requirement of the small (*sma*) genes for *daf-4* expression, we bypassed endogenous regulation of *daf-4* by using a heat shock promoter (4). After heat shock, 6 of 6 *daf-4;mEx11[hs-daf-4(+)]* animals were rescued (nonSmall), and 0 of 10 *sma-2;mEx11*, 0 of 48 *sma-3;mEx11*, and 0 of 13 *sma-4;mEx11* animals were rescued. Thus, *daf-4* expression from this construct rescues the *daf-4* Small and dauer-constitutive phenotypes but cannot rescue *sma-2, sma-3,* and *sma-4* mutants. Therefore, it is unlikely that these genes are necessary for *daf-4* expression, but rather it is likely that they transduce a signal from the receptor, DAF-4, or modify its activity.

Table 2. Mutant phenotypes in genetic mosaics

Left rays	R7	R8	R9	Phso	PLM	Right rays	R7	R8	R9	Phso	PLM
daf-4 mutants											
wt	+	+	+	ND	ND	6-7	−	−	−	ND	ND
wt	+	+	+	ND	ND	6-7	−	−	−	ND	ND
wt	+	+	+	ND	ND	6-7	−	+	+	ND	ND
7	−	−	−	ND	ND	wt	+	+	+	ND	ND
6-7	−	−	−	−	+	wt	+	+	+	+	+
sma-2 mutants											
wt	+	+	+	+	+	7	−	−	−	−	−
wt	+	+	+	+	+	6-7	−	−	−	−	−
wt	+	+	+	+	+	6-7	−	−	−	−	+
wt	+	+	+	+	+	6-7	−	−	−	−	+
wt	+	+	+	+	+	6-7	−	−	−	−	+
wt	+	+	+	+	+	6-7	−	−	−	−	+
wt	+	+	+	+	+	6-7	−	−	−	+	+
6-7	−	+	+	+	+	wt	+	+	+	+	+
6-7	−	+	+	+	+	wt	+	+	+	+	+
6-7	−	−	−	+	+	wt	+	+	+	+	+
6-7	−	−	−	−	+	wt	+	+	+	+	+
6-7	−	−	−	−	+	wt	+	+	+	+	+
6-7	−	−	−	−	+	wt	+	+	+	+	+

Each entry represents an individual mosaic male (29, 30). The ray neurons were scored for their *ncl-1* phenotype (+ or −). To determine the extent of the mutant clones, nearby neurons—specifically, the phasmid socket and touch neurons—were also scored. R*n*, ray *n*; Phso, phasmid socket; PLM, posterior lateral touch neuron; wt, wild type; 6-7, fusion of rays 6 and 7; 7, transformation of ray 7 to ray 6; +, Ncl(+); −, Ncl(−); ND, not determined.

SMA-2, SMA-3, and SMA-4 Are Related Proteins. To characterize their role in TGF-β-like signal transduction, we cloned the genes *sma-2, sma-3,* and *sma-4* (Fig. 2). To identify *sma-2*, we examined cosmid clones and genomic sequence in the *sma-2* region (35–37). We noticed that this interval contains a homolog of a recently described *Drosophila* gene, Mothers against dpp (*Mad*) (38), and we hypothesized that *sma-2* may encode this homolog. *Mad* was identified in genetic screens for modifiers of the activity of the TGF-β-like ligand encoded by *dpp* (39) and is required for *dpp* function at many stages of development (38). We have proven that these two genes are related by sequencing two *sma-2* alleles (Fig. 2). For *sma-3*, we used transformation rescue (31) to show that the cosmid R13F6, which contains a *sma-2* homolog, rescued the Small phenotype of *sma-3* mutants. In addition, we identified a point mutation in one allele. The *sma-4* gene was identified by transformation rescue (Fig. 2*B*). After we obtained *sma-4* cDNA sequence, another *C. elegans* open reading frame related to *sma-2* was identified by the *C. elegans* genome sequencing consortium. We compared this open reading frame with *sma-4* sequences and found them to be the same.

sma-2, sma-3, and *sma-4* therefore encode related, but not functionally redundant, proteins, with no obvious functional motifs (Fig. 3), that are homologous to the *Drosophila Mad* gene product that acts in the *dpp* TGF-β-like pathway. We call this new protein family the dwarfins, to avoid confusion with either MADS-box proteins or proteins encoded by other *sma* genes. Dwarfins contain no potential signal sequences or transmembrane domains, suggesting that they are intracellular proteins, consistent with the cell-autonomous requirement for SMA-2 from our genetic studies. They contain two conserved regions, DH1 (≈110 aa; 45–54% identity between any two family members) and DH2 (≈180 aa; 38–47% identity), separated by a poorly conserved proline-rich linker of ≈90 aa. SMA-4 is the most divergent member of the family, distinguished by a 160-aa N-terminal extension and a 25-aa insert in DH2.

Vertebrate Dwarfins Are Highly Homologous to SMA-2, SMA-3, and SMA-4. We were interested in whether vertebrate dwarfins exist, since these may have a role in vertebrate TGF-β-like signal transduction. We used degenerate PCR to amplify and clone three dwarfins from mice and three from humans (Fig. 3). Dendrogram analysis of dwarfin sequences suggests that *sma-2, sma-3,* and *sma-4* diverged before the common ancestor of nematodes, flies, and vertebrates, predicting the existence of multiple dwarfins in *Drosophila* and other animals. Verification of this hypothesis comes from the identification of a gene similar to *sma-4* in *Drosophila* (P.D. and R.W.P., unpublished data).

Possible Functions of the Dwarfins. The mutant phenotypes of *sma-2, sma-3,* and *sma-4* closely resemble a subset of mutant phenotypes of the TGF-β-like receptor gene *daf-4*. Similarly, mutations in the related *Drosophila* gene *Mad* result in phenotypes like those of the TGF-β-like ligand gene *dpp* (38). This functional and structural similarity between the nematode members of this family and *Drosophila Mad* underscores the association of the dwarfins with TGF-β-like signal transduction. Furthermore, dwarfins are likely downstream signal transducers because (*i*) SMA-2 is required cell-autonomously in the same cells as the TGF-β-like receptor; (*ii*) receptor expression does not require *sma-2, sma-3,* and *sma-4*; and (*iii*) unlike many previously identified TGF-β signaling components, dwarfins have no hydrophobic sequences to specify extracellular or transmembrane localization and so are likely to be cytoplasmic and/or nuclear proteins.

Recent studies on the mechanism of activation of TGF-β and BMP receptors have led to a model of how they respond to the ligand (13, 44). According to this model, type II receptors are constitutively active. Upon ligand binding, the

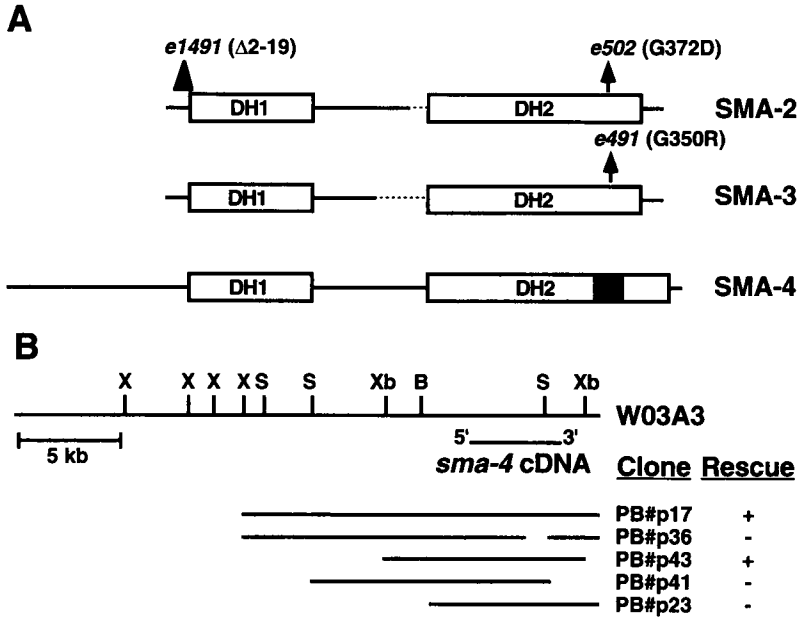

FIG. 2. Structure of *sma-2, sma-3*, and *sma-4*. (*A*) Schematic protein structure of SMA-2, SMA-3, and SMA-4. Highly conserved regions (DH1 and DH2; see Fig. 3) are indicated by boxes. Amino acid deletions or changes are indicated in parentheses. (*B*) Transformation rescue to localize the gene *sma-4*. The upper line represents a partial restriction map of the insert in the cosmid W03A3 that rescues the *sma-4* Small and male tail phenotypes. Restriction sites: B, *Bsi*WI; S, *Spe* I; X, *Xho* I; Xb, *Xba* I. PB#p43 is a 10-kb *Xba* I fragment that rescues the *sma-4* Small, but not the male tail, phenotype.

type I and type II receptors form a complex, and the type II receptor phosphorylates and activates the type I receptor. Working models for the role of the dwarfins within this context are illustrated in Fig. 4. These proteins may form a heteromeric signaling complex (Fig. 4*A*). Alternatively, they may be activated sequentially, in a signaling cascade (Fig. 4*B*). Current data do not allow us to distinguish between these two possibilities. Further biochemical and genetic studies will be needed

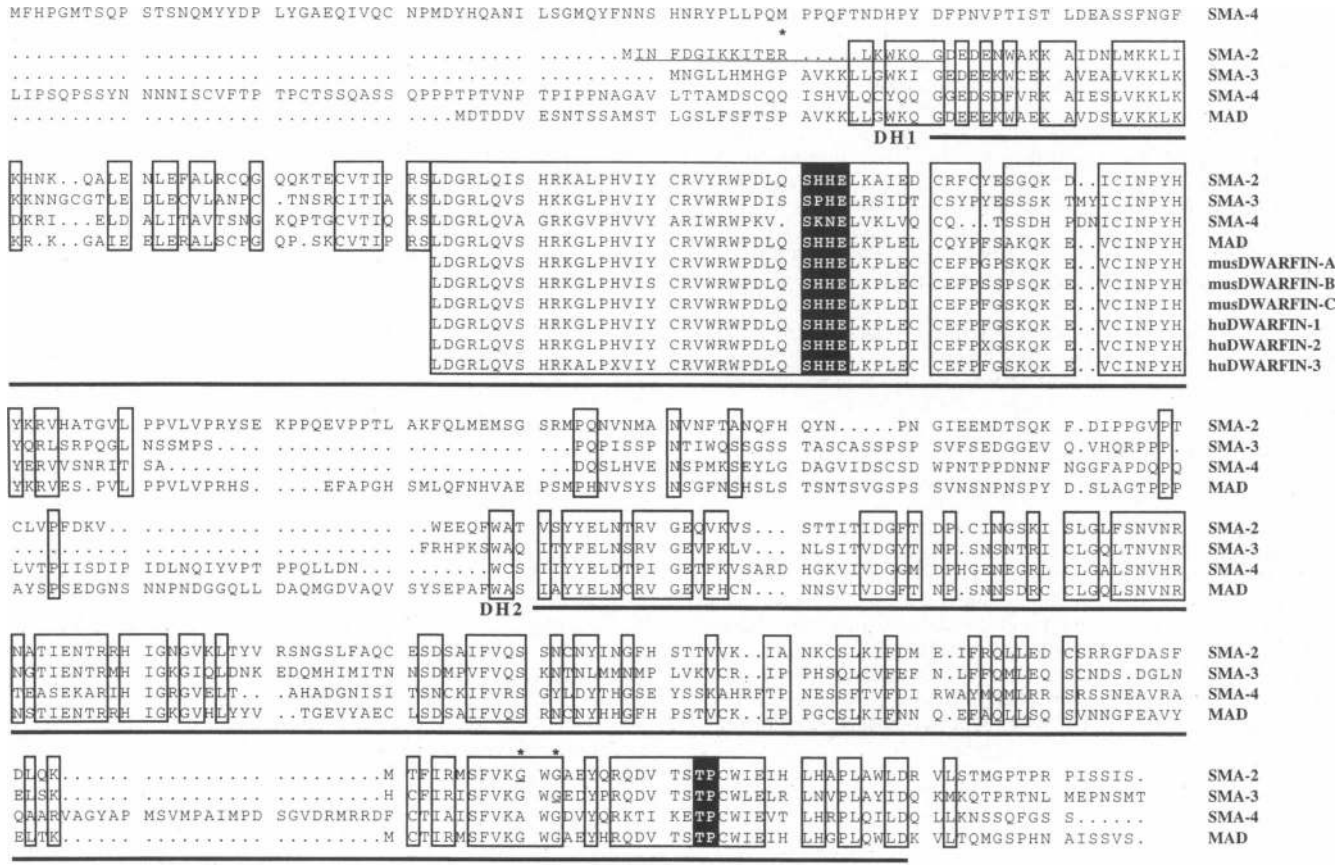

FIG. 3. Sequence of the dwarfins. Protein sequence alignment (40) of the dwarfin family. Dots represent gaps introduced to maximize alignments. Amino acids that are identical in more than half of the sequences shown are boxed. DH1 and DH2 are underlined. Amino acids altered in *sma-2* and *sma-3* alleles are identified by an asterisk and underlined. A short, highly conserved region of DH2 (GWGAEYQRQDVTS in SMA-2) appears to be an important mutational target, since one of two *sma-2*, one of one *sma-3*, and three of three *Mad* (38) alleles alter residues here. Putative Src homology 3-binding sites [PxxP (41)] are present in each of the proteins, but these may be fortuitous, due to the proline-rich nature of the linkers. Two potential phosphorylation sites are conserved in this family (black boxes): a casein kinase II consensus [(S/T)xx(D/E) (42)] in DH1 and a mitogen-activated protein kinase (MAP kinase) minimal core site [(S/T)P (43)] in DH2. The GenBank accession numbers for the nematode sequences are U34778 (*sma-2*), U34902 (*sma-3*), and U34596 (*sma-4*). mus, Mouse; hu, human.

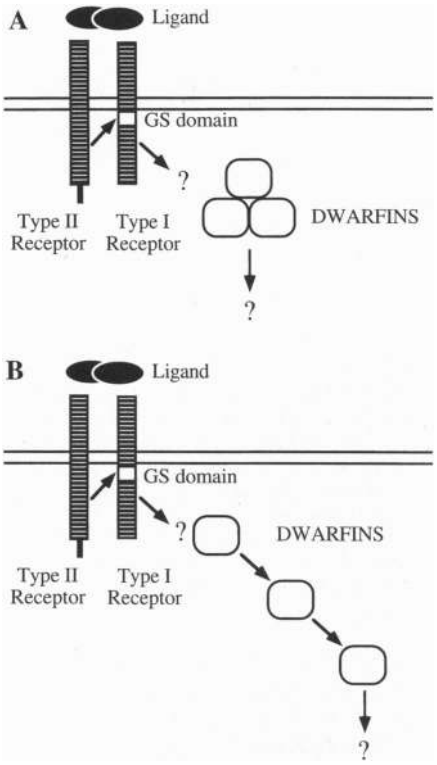

FIG. 4. Models for dwarfin action in TGF-β-like signaling. TGF-β-like receptors are heteromeric complexes of type I and type II transmembrane serine/threonine kinases. Type II receptors typically contain a short C-terminal extension on the intracellular domain. Type I receptors have a conserved motif, the GS domain, immediately upstream of the kinase domain, that is the site of phosphorylation by the type II receptor. We suggest that the dwarfins act downstream of TGF-β-like receptors in one of two possible ways. (*A*) The heteromeric complex model. (*B*) The sequential activation model.

to determine the mechanisms by which the dwarfins participate in TGF-β-mediated signal transduction.

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