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Drosophila MAD, a Member of the Smad Family, Translocates to the Nucleus upon Stimulation of the *dpp* Pathway

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Smads are a novel group of proteins which act to mediate signaling by members of the TGF-β superfamily. Seven vertebrate Smad genes, which fall into three classes, have been reported. Members of the Class I Smads have been shown to bind to the cytoplasmic portion of the TGF-β like receptors, where they become phosphorylated and translocate to the nucleus. Once in the nucleus they may function as transcriptional activators. We wondered if translocation to the nucleus is a general property of the Smads and whether it was evolutionarily conserved. We examined the subcellular localization of *Drosophila* MAD and found that it is capable of nuclear translocation, in *Drosophila* S2 cells, when the *dpp* pathway is stimulated. To prove the functional conservation of receptor/Smad interactions, we used the mouse BMP type I receptor *ALK6* to stimulate the pathway and found that it is capable of sending MAD to the nucleus. These results show that cytoplasmic localization with translocation to the nucleus upon stimulation is a feature of the Smads that is conserved through evolution.

The transforming growth factor-β (TGF-β) superfamily consists of secreted molecules that include TGF-βs, bone morphogenetic proteins (BMPs) and activins. This growth factor family is found in vertebrates as well as invertebrates and is involved in regulating cell proliferation, differentiation and development. These processed secreted ligands bind serine-threonine kinase receptors (type II receptors) which in turn recruit related serine-threonine kinase (type I receptors) receptors to form a complex (1). The constitutively active type II receptor phosphorylates the type I receptor on the GS domain, allowing transmission of the signal (1). The first molecules discovered, which function downstream of the serine-threonine receptor kinases, were identified in *Drosophila* and *C. elegans*. *Drosophila Mad* was identified in genetic screens for modifiers of *decapentaplegic* (*dpp*), a TGF-β-like ligand (2, 3), soon followed by the cloning of *sma-2*, *sma-3*, and *sma-4* from *C. elegans* (4). Since then, seven distinct vertebrate Smads have been characterized, which can be classified into three groups based upon sequence and functional similarity (Fig. 1) (4-17). A unified nomenclature has been adopted for this protein family: Smad, a term derived from the *sma* and *Mad* genes. Two human Smads appear to be tumor suppressor genes and are implicated in colorectal and pancreatic cancers (7,9).

The three classes of Smads show no similarity with any other proteins and thus define a new family. They possess two conserved domains, Domain 1 and Domain 2, separated by a variable linker region. Domain 1 binds DNA (18) and Domain 2 functions as a transcriptional activator (12,19). Smad6, an exception, lacks Domain 1 (20). Class I Smads contain characteristic carboxyl terminal serines which become phosphorylated in response to an activated type I receptor, whereas Class II and Class III do not contain these sequences (21,22). Recently, the Class III Smad, Smad7, has been shown to inhibit phosphorylation of the Class I Smads (17).

Much attention has focused on how the Smads transduce the signal from the ligand bound receptor complex to target genes within the nucleus. Cytoplasmic homodimeric Class I Smads form heterodimers with a Class II Smad, Smad4/DPC4, in response to stimulation by the type I receptor (16,22,23). Stimulation of the BMP or the TGF-β pathways results in Smad1 or Smad2, respectively, translocating to the nucleus. Translocation of the other Smads has not been reported.

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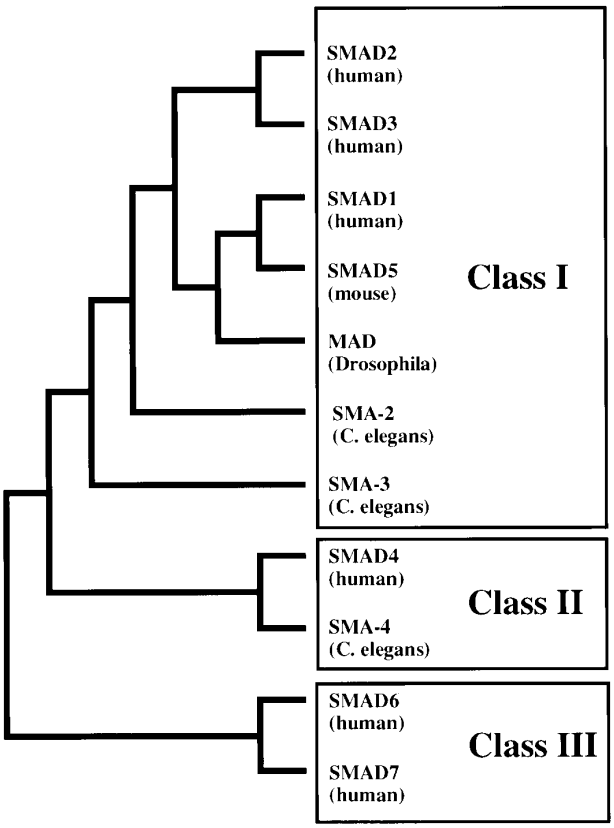

FIG. 1. Smad family dendrogram. The seven published Smads fall into three classes. Two members of Class I have been shown to translocate to the nucleus.

Here we demonstrate that MAD, like vertebrate Smad1 and Smad2, translocates to the nucleus upon stimulation of the *dpp* pathway. Activated type I receptors were cotransfected with *Mad* into *Drosophila* S2 cells and analyzed for nuclear localization. The number of cells showing nuclear accumulation differed depending upon which type I receptor, *thick veins* (*tkv*) or *ALK6*, was transfected. These results further support the notion that both structure and function of the Smads have been conserved between animal phyla.

MATERIALS AND METHODS

An epitope tagged version of *Mad* (N-terminal Flag), an activated *tkv* receptor and an activated *ALK6* receptor were cloned into the plasmid vector pMK33 (24), which allows for inducible expression by copper under the control of the metallothionein promoter. Constitutively active forms of the two receptors were generated by substitution of an amino acid near the GS domain (25). These receptors were cotransfected into cells to stimulate TGF-β-like signals.

Drosophila S2 cells were maintained in Schneider's Insect Medium (Sigma) with 12.5% Fetal Calf Serum (FCS) (Sigma). Transient transfections were performed using a cationic liposome technique previously described (26). S2 cells were washed once, resuspended in serum free media and plated at 80% confluency. Either 5 or 10 μg of total DNA was mixed with DDAB/DOPE (Sigma) liposomes in serum free media and incubated 20 minutes at room temperature. The DNA/lipid mixture was then dispersed onto the plated cells and allowed to incubate 4-5 hours at room temperature. The serum free media with DNA and lipid was removed and replaced with 5 mls of media containing 12.5% FCS. Twenty-four hours post-transfection, cells were induced with 0.7mM CuSO4, harvested 8-12 hours later and assayed by immunofluorescence. Cells were washed once with PBS and fixed with 2% paraformaldehyde for 10 minutes. Before and after antibody incubations, cells were washed 3 times with PBS containing 0.1% saponin and 1% BSA. M2 or M5 Flag primary antibody (Kodak) was used at 0.02 μg/ml. Secondary fluorescent mouse CY3 antibody (Jackson Laboratories) was used at a 1:500 dilution. Both antibodies were incubated for 1 hour at room temperature. Cells were resuspended in 50 μl PBS and analyzed with a fluorescent microscope.

RESULTS AND DISCUSSION

It has been demonstrated that vertebrate Smad1 and Smad2 become phosphorylated in the cytoplasm and translocate to the nucleus upon stimulation of the pathway. In order to test whether or not the subcellular localization of Smads is conserved across animal phyla we examined *Drosophila* MAD. Since MAD is of the same class as Smad1 and 2, it might also respond to receptor stimulation similarly and therefore accumulate in the nucleus. *Drosophila* S2 cells were transiently transfected with Flag-*Mad* with or without stimulation. Figure 2A shows that MAD remains cytoplasmic when transfected alone. We cotransfected *Mad* with activated *tkv*, a *dpp* type I receptor, and observed nuclear localization of MAD (Fig. 2B). In four separate experiments, we find that 29% of the cells cotransfected with activated *tkv* and *Mad* show nuclear localization of MAD protein while only a 1.5% nuclear accumulation is evident when *Mad* is transfected alone. Upon stimulation, we see a 19-fold increase in nuclear localization.

Similar experiments with Smad1 and Smad2 showed that translocation to the nucleus increases approximately 10-fold (12,21). This is similar to the results we obtained in the *Drosophila* cell culture system. Since the Smads are not translocated to the nucleus in most cells, it suggests that the pathway is being partially stimulated or that there are other proteins necessary for translocation that are limiting. In our experiments, we stimulated the pathway using an activated type I receptor. This approach produces receptors with only partial activity, which may not stimulate all cells enough to translocate significant amounts of the transfected Smads. If only a fraction of the Smad translocated to the nucleus, it would not be detected.

Because nuclear translocation is a conserved feature of Class I Smads, we wondered if the vertebrate bone morphogenetic protein (BMP) receptor, *ALK6*, could substitute for *Drosophila tkv*. The *Drosophila dpp* pathway is most similar to the vertebrate BMP pathway. *Drosophila* S2 cells were cotransfected with *Mad*

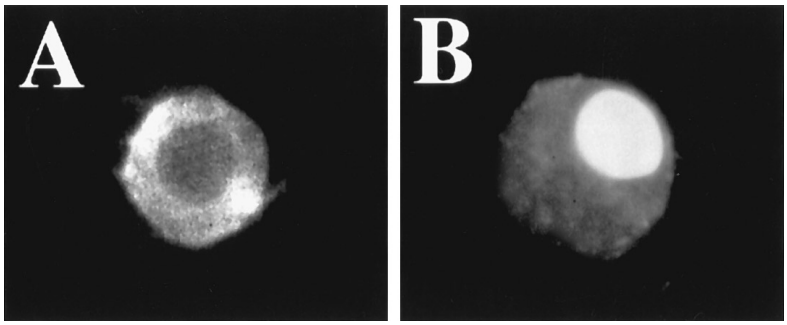

FIG. 2. Translocation of MAD to the nucleus upon stimulation by activated TKV. *Drosophila* S2 cells were transfected with (A) Flag-*Mad* or (B) Flag-*Mad* and activated *tkv*. MAD is found primarily in the cytoplasm of unstimulated cells and translocates to the nucleus in response to stimulation by activated TKV.

and an activated *ALK6* type I receptor. In this instance, MAD was able to translocate to the nucleus when stimulated by the activated ALK6 receptor but to a lesser extent than activated TKV (Fig. 3). Nuclear accumulation of MAD was found in 7% of the cells cotransfected with activated *ALK6* as compared to 29% when cotransfected with activated *tkv*.

Although stimulation of MAD by the BMP type I receptor is not as extensive as that of TKV, it is still interesting to note that conserved functional features are present among the invertebrate and vertebrate pathways. Both receptor kinases appear to function similarly as is evident by translocation of MAD to the nucleus but what makes one receptor more efficient than the other remains unclear. It is possible that the Smad docking site of ALK6 is not as efficient in MAD binding as the docking site found in TKV. It is interesting to note that partial rescue of *dpp* null embryos by a chimeric *dpp*/BMP4 transgene has been observed (27). Partial rescue may be due to a lower affinity of BMP4 for the *Drosophila* receptor, therefore causing it to be less effective in sending its signal and rescuing the mutant phenotype (27). MAD might be unable to efficiently recognize and bind the BMP receptor, therefore affecting its phosphorylation and translocation to the nucleus.

Swapping experiments between the various cytoplasmic regions of the receptors, TKV and ALK6, might identify which portions are required to transmit the signal to the Class I Smad effector molecules. In addition, similar swapping experiments between MAD and other Class I Smads could reveal important interaction domains. From these experiments the requirements for receptor-effector interactions would be better understood. Class II Smads have been shown to have transcriptional activity in the nucleus (12). How Class II Smads translocate to the nucleus is unclear. Once this translocation event is characterized, it will be of interest to see if there is conservation of this event between vertebrates and invertebrates.

Taken together, these results show that another aspect of the TGF-β signaling pathway is conserved. The *Drosophila* Smad is mostly cytoplasmic, and upon stimulation, moves to the nucleus. Further, the mouse receptor, ALK6, can also stimulate translocation of the *Drosophila* Smad, suggesting that there is conservation of the Smad binding sites. Future experiments are aimed at defining the sequences in-

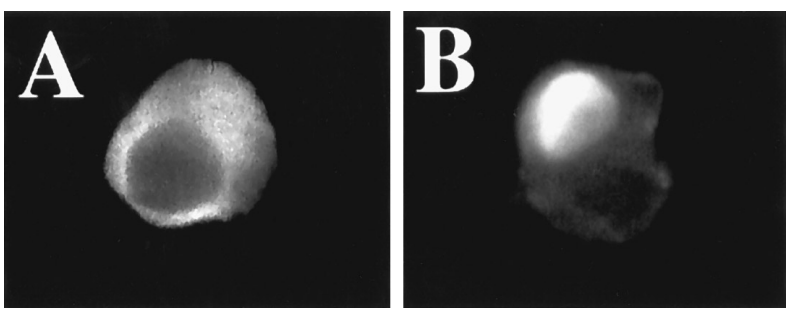

FIG. 3. Activated BMP type I receptor, ALK6, causes nuclear accumulation of MAD. *Drosophila* S2 cells were transfected with (A) Flag-*Mad* or (B) Flag-*Mad* and activated *ALK6*. Translocation of MAD to the nucleus is evident when stimulated by activated ALK6, but to a lesser extent than that observed with activated TKV.

volved in receptor-Smad interactions and nuclear translocation.

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