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Mothers against dpp participates in a DPP/TGF-β responsive serine-threonine kinase signal transduction cascade

Stuart J. Newfeld[1], Arun Mehra[2], Matthew A. Singer[1], Jeffrey L. Wrana[3], Liliana Attisano[2] and William M. Gelbart[1,]*

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA
[2]Department of Anatomy and Cell Biology, University of Toronto, Toronto, ON, Canada, M5S 1A8
[3]Program in Developmental Biology, The Hospital for Sick Children, Toronto, ON, Canada, M5G 1X8

*Author for correspondence: (email: gelbart@morgan.harvard.edu)

SUMMARY

***Mothers against dpp* (*Mad*) is the prototype of a family of genes required for signaling by TGF-β related ligands. In *Drosophila*, *Mad* is specifically required in cells responding to Decapentaplegic (DPP) signals. We further specify the role of *Mad* in DPP-mediated signaling by utilizing tkv^{Q199D}, an activated form of the DPP type I receptor serine-threonine kinase *thick veins* (*tkv*). In the embryonic midgut, tkv^{Q199D} mimics DPP-mediated inductive interactions. Homozygous *Mad* mutations block signaling by tkv^{Q199D}. Appropriate responses to signaling by tkv^{Q199D} are restored by expression of MAD protein in DPP-target cells. Endogenous MAD is phosphorylated in a ligand-dependent manner in *Drosophila* cell culture. DPP overexpression in the embryonic midgut induces MAD nuclear accumulation; after withdrawal of the overexpressed DPP signal, MAD is detected only in the cytoplasm. However, in three different tissues and developmental stages actively responding to endogenous DPP, MAD protein is detected in the cytoplasm but not in the nucleus. From these observations, we discuss possible roles for MAD in a DPP-dependent serine-threonine kinase signal transduction cascade integral to the proper interpretation of DPP signals.**

Key words: *Mad*, TGF-β family, DPP signal transduction, midgut morphogenesis, nuclear accumulation, *Drosophila melanogaster*

INTRODUCTION

Intercellular signaling by ligands of the transforming growth factor-β (TGF-β) superfamily are required for control of many aspects of cell growth, patterning and differentiation in many organisms (Kingsley, 1994). Receptor serine-threonine kinases on the surface of responsive cells are activated by these secreted molecules and thereby initiate an intracellular signal transduction cascade (Massagué et al., 1994). Recent studies have begun to identify components of these cascades that relay the signal to the nucleus and elicit appropriate transcriptional responses.

The secreted processed product of the *decapentaplegic* (*dpp*) gene in *Drosophila melanogaster* (Padgett et al., 1987) initiates one of the genetically best characterized TGF-β signaling pathways. The DPP pathway requires both type I and type II receptors (*saxophone*, *thick veins*, *punt*: Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995) and at least one transcription factor has been suggested as an immediate target of the pathway (*schnurri*: Arora et al., 1995; Grieder at al., 1995; Staehling-Hampton et al., 1995). We have been focusing on the characterization of another signal transduction cascade component, the MAD protein encoded by *Mothers against dpp* (Raftery et al., 1995; Sekelsky et al., 1995).

Mad loss-of-function mutants exhibit numerous phenotypes with similarities to those of various *dpp* mutants and partial reduction in *Mad* activity exacerbates phenotypes associated with specific *dpp* mutant genotypes (Raftery et al., 1995; Sekelsky et al., 1995). These genetic parallels and interactions form the basis for our suggestion that *Mad* encodes a component of a DPP-responsive signal transduction pathway. This idea is further supported by our studies in the embryonic midgut demonstrating that *Mad* is required in DPP-target cells (Newfeld et al., 1996).

Analyses of *Mad* activity in adult appendages have shown that heterozygosity for a *Mad* mutation can suppress gain-of-function phenotypes due to expression of a constitutively active form of the DPP type I receptor kinase *thick veins* (tkv^{Q199D}: Hoodless et al., 1996). However, inferences about epistatic relationships derived from genotypes in which a gene product is quantitatively reduced but not eliminated are open to multiple interpretations. In the adult appendages, *Mad* null clones do not proliferate (Wiersdorff et al., 1996) so that epistasis experiments utilizing homozygous *Mad* null genotypes cannot be conducted. By focusing on DPP signaling in the embryonic midgut, we are able to examine epistatic relationships between *Mad* and *dpp* in genotypes that are functionally null for *Mad*. Here we report that tkv^{Q199D} constitutively induces several DPP-dependent

responses during midgut development. These responses are blocked by homozygosity for a *Mad* null mutation and appropriate tkv^{Q199D} signaling is restored by providing MAD in specific DPP-target cells.

While the predicted MAD polypeptide contains no identifiable motifs (Sekelsky et al., 1995), a series of related genes define a protein family with extensive conserved domains (Smad: Massagué, 1996; Wrana and Attisano, 1996). Transfection studies in mammalian cells determined that cytoplasmic Smad proteins are phosphorylated (Hoodless et al., 1996; Lechleider et al., 1996; Yingling et al., 1996) and accumulate in the nucleus (Hoodless et al., 1996; Liu et al., 1996) in response to ligand. Here we report the ligand-dependent phosphorylation of endogenous MAD in *Drosophila* cell culture, the nuclear accumulation of cytoplasmic MAD in response to DPP overexpression and efforts to detect nuclear accumulation of MAD in response to endogenous DPP at several developmental stages. Our results are consistent with a pivotal role for MAD in a DPP-dependent serine-threonine kinase signal transduction cascade.

MATERIALS AND METHODS

Reporter gene experiments

All *Drosophila* strains are described in Newfeld et al. (1996) except for P{UAS-*tkv*.Q199D} (Hoodless et al., 1996) and P{Gal4-*Hsp70*.PB} (a gift from Andrea Brand via Elizabeth Noll and Norbert Perrimon). Analyses of reporter genes in wild-type, *Mad* null backgrounds and *Mad* null backgrounds with tissue-specific expression of MAD are described in Newfeld et al. (1996). For analysis of P{UAS-*tkv*.Q199D} in wild type and *Mad* null embryos, strains were constructed which are homozygous for P{UAS-*tkv*.Q199D} on the X chromosome and P{Gal4-*Hsp70*.PB} on chromosome 3, with and without Mad^{12}/*CyO* P{*wg*-βgal}. Females from both of these strains were crossed to males hemizygous for the *lab* reporter gene P{3.65*lab*66A}, heterozygous for *Df(2L)JS17* (which deletes *Mad*)/*CyO* P{*wg*-βgal} and homozygous for P{hs-*dpp*.BP} on chromosome 3. The *lab* reporter gene contains a DPP-independent CNS enhancer, which acts as a marker of that chromosome. Females from both strains were also crossed to males heterozygous for Mad^{12}/*CyO* P{*wg*-βgal} who were either homozygous for *dpp* midgut reporter gene P{RD2} or carried an insertion of *Scr* anterior/posterior midgut reporter gene P{HZR+0.8X/H} on TM6B maintained over *Sb* gl^3. For comparison of the effects of P{UAS-*tkv*.Q199D} and heat-shock induced DPP on *Scr*, females homozygous for P{hs-*dpp*.BP} on chromosome 3 were crossed to males heterozygous for the *Scr* reporter gene. For analysis of P{UAS-*tkv*.Q199D} effects on *lab* in *Mad* null background embryos with tissue-specific expression of MAD, females homozygous for P{UAS-*tkv*.Q199D} on the X chromosome and P{Gal4-*Hsp70*.PB} on chromosome 3 and heterozygous for Mad^{12}/*CyO* P{*wg*-βgal} were crossed to males hemizygous for the *lab* reporter, heterozygous for *Df(2L)JS17*/*CyO* P{*wg*-βgal} and heterozygous for P{hs-*dpp*.BP} and P{mex1*Mad*} on chromosome 3. Embryos carrying P{mex1*Mad*} were identified by observation of an unambiguous positive result, rescue of reporter gene expression in the appropriate tissue predicted from the results of Newfeld et al. (1996). For analyses of P{UAS-*tkv*.Q199D} effects on *dpp* and *Scr* in *Mad* null background embryos with tissue-specific expression of MAD, males hemizygous for P{UAS-*tkv*.Q199D}, heterozygous for Mad^{12}/*CyO* P{*wg*-βgal} and carrying *dpp* or *Scr* reporter genes were crossed to females homozygous for P{UAS*Mad*.N} on the X chromosome and P{GawB}24B on chromosome 3 that were also heterozygous for Mad^{12}/*CyO* P{*wg*-βgal}. Embryos carrying P{UAS *tkv*.Q199D} were identified by observation of an unambiguous positive result, rescue of reporter gene expression in the appropriate tissue predicted from the results of Newfeld et al. (1996). All crosses were reared at room temperature (21°C) to avoid low level expression of P{hs-*dpp*.BP} and P{Gal4-*Hsp70*.PB} at 25°C. 37°C induction of P{hs-*dpp*.BP} and P{Gal4-*Hsp70*.PB} and subsequent analyses of β-galactosidase activity were performed according to Newfeld et al. (1996).

Phosphorylation experiments

Drosophila melanogaster wing imaginal disk cell lines MLDmD8 and MLDmD12 (Ui et al., 1987) were grown at pH 6.5 in M1 media (Sigma) with 166.0 mg/ml yeastolate, 5.0 mg/ml bactotryptone, 10 μg/ml insulin and 12.5% fetal calf serum at 27°C. For in vivo phosphate labeling, the cell lines were treated as described (Wrana et al., 1994). Briefly, cells were incubated at pH 6.5 for 2 hours at 27°C in phosphate-free modified Eagle's medium containing 20 mM Hepes and [^{32}P]phosphate at 1 mCi/ml in the presence or absence of 10 nM human recombinant BMP2 in the last 30 minutes of labeling (unless otherwise indicated). Cells were lysed in TNE (50 mM Tris pH 7.4, 150 mM NaCl and 1 mM EDTA) containing 0.5% Triton X-100 in the presence of protease and phosphatase inhibitors (10 μg/ml antipain, 50 μg/ml aprotinin, 100 μg/ml benzamidine hydrochloride, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 5 μg/ml RNAse A, 25 mM sodium flouride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 100 μg/ml soybean trypsin inhibitor). Cell lysates were immunoprecipitated with anti-MAD antibodies or preimmune sera (Newfeld et al., 1996) followed by adsorbtion to protein A sepharose (Pharmacia). Immunoprecipitated complexes were washed twice in TNE containing 0.1% Triton X-100, followed by two washes in RIPA (150 mM NaCl, 1% NP-40, 0.5% deoxychloate, 0.1% sodium dodecyl sulfate in 50 mM Tris at pH 8.0), followed by a final wash in TNE containing 0.1% Triton X-100. Lysate proteins were separated by SDS-PAGE (8%) and visualized by autoradiography. For metabolic labeling, cells were incubated with 100 μCi/ml [^{35}S]methionine for 2 hours at 27°C in methionine-free modified Eagle's medium containing 20 mM Hepes at pH 6.5. MAD was immunoprecipitated and analyzed as described for phosphate labeling.

MAD subcellular localization experiments

Embryos

For DPP overexpression, a strain containing six copies of P{hs-*dpp*.BP} and its parental strain *y* w^{67c23} were tested in parallel. Embryos from developmental stages corresponding to midgut constriction formation were heat-shocked and analyzed by confocal microscopy as described in Newfeld et al. (1996). For endogenous DPP, *y* w^{67c23} blastoderm embryos and midgut formation stage embryos were analyzed as in Newfeld et al. (1996).

Disks

For endogenous DPP, imaginal disks from the strain P{BS3.0} (Blackman et al., 1991), which carries a dpp^{disk} region reporter gene were tested. Early to mid-third instar larvae were dissected in PBS (20 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) and fixed in 2% EM-grade formaldehyde (Polysciences) in PEM (100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.95) for 30 minutes. Tissue was incubated 18 hours at 4°C in a 1:2000 dilution of rabbit anti-β-galactosidase antibodies and/or 1:1000 dilution of guinea pig anti-MAD polyclonal sera. Subsequently, tissue was incubated for 3 hours in a 1:1200 dilution of goat anti-rabbit LSRC and/or a 1:100 dilution of donkey anti-guinea pig-FITC (Jackson Immuno Research). Tissue was placed into 30% glycerol mountant (in 50 mM Tris pH 8.8, 150 mM NaCl, 0.02% NaN_3) with 0.5 mg/ml p-phenylenediamine (Sigma) and analyzed by confocal microscopy.

RESULTS

Mad functions downstream of the kinase activity of a DPP type I receptor

To clarify *Mad*'s function in DPP-responsive cells, we have continued to focus on the embryonic midgut. Here we are able to utilize genotypes that are null for *Mad* to examine the relationship between *Mad* and *dpp*. In the midgut, *dpp* is expressed in the visceral mesoderm of parasegments (ps) 3 and 7 (Fig. 1). In response to DPP signals, cells expressing *dpp* in ps3 repress the expression of the homeotic gene *Sex combs reduced (Scr)*. DPP signals are also required to maintain *dpp* expression in ps3 through an autocrine feedback loop. However, cells in ps4 do not appear to be affected by DPP signals; *Scr* is expressed while *dpp* is not (Hursh et al., 1993). In ps7, the homeotic gene *Ultrabithorax* (*Ubx*) initiates *dpp* expression. Subsequently, DPP functions in an autocrine manner to maintain *Ubx* and thus *dpp* expression. In ps7, DPP also signals between germ layers to the underlying endoderm. Within the midgut endoderm, which does not express *dpp*, expression of the homeotic gene *labial* (*lab*) is dependent upon DPP signals (Bienz, 1994).

Proper response to DPP requires a heteromeric complex of transmembrane receptors. In the receptor complex, the type I receptor, acting downstream of the type II receptor, initiates the intracellular signaling cascade (Wrana et al., 1994). The cytoplasmic protein MAD is also required for appropriate responses to DPP signals (Newfeld et al., 1996). For our analyses, we have utilized a constitutively active DPP type I serine-threonine kinase receptor, *tkv*^{Q199D}, that functions in a cell autonomous manner (Hoodless et al., 1996). We have examined *Mad* function with regard to *tkv*^{Q199D} in the induction of *lab* in ps7 midgut endoderm, in *dpp* maintenance in ps7 and repression of *Scr* in ps3 visceral mesoderm. If indeed *Mad* is an essential part of the DPP signal transduction apparatus, the absence of MAD should block any phenotypes due to a constitutive signal from *tkv*^{Q199D}.

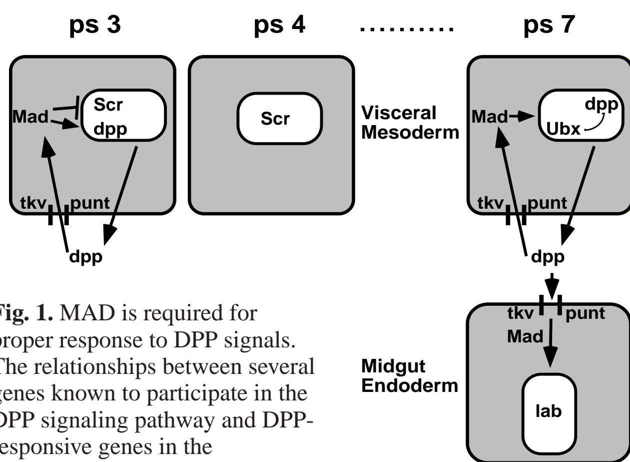

Fig. 1. MAD is required for proper response to DPP signals. The relationships between several genes known to participate in the DPP signaling pathway and DPP-responsive genes in the embryonic midgut are depicted. Genes in the DPP pathway include *dpp*, the receptors *tkv* and *punt*, and *Mad*. In ps3 visceral mesoderm, the proper response to DPP signals is the maintenance of *dpp* expression and the repression of *Scr* expression. In ps7 visceral mesoderm, *Ubx* is also involved in the DPP pathway and the proper response to DPP signals is the maintenance of *Ubx* and *dpp* expression. In ps7 midgut endoderm, the proper response to DPP signals is the induction of *lab* expression.

Expression of a *lab* reporter gene containing a DPP-independent central nervous system enhancer and a DPP-dependent ps7 midgut endoderm enhancer in a *Mad*$^{+}$ background is shown in Fig. 2A. Fig. 2B,D show the anterior expansion of *lab* midgut endoderm expression in response to ubiquitously expressed *tkv*^{Q199D} in early and late stage embryos, respectively, an effect also seen with ubiquitous DPP (Newfeld et al., 1996). Comparably staged *Mad* null *tkv*^{Q199D} embryos (Fig. 2C,E) lack any *lab* expression in the midgut endoderm, strongly suggesting that *Mad* is epistatic to *tkv*^{Q199D}. When we express MAD solely in the midgut endoderm using P{mex1*Mad*}, in an otherwise *Mad* null embryo, in the presence of *tkv*^{Q199D} we observe ectopic *lab* expression in that tissue, as shown by the staining of cells in the elongating gut (Fig. 2F). The cells showing *lab* expression derive solely from the *lab*-independent anterior domain of expression of the *mex1* enhancer driving MAD in the endoderm. This result is also seen in a comparable embryo expressing ubiquitous DPP instead of *tkv*^{Q199D} (Newfeld et al., 1996). Thus, we infer that *Mad* functions in the DPP signal transduction cascade downstream of the serine-threonine kinase activity of *tkv* but upstream of *lab* induction.

Further experiments utilize the *dpp* reporter gene P{RD2}, which accurately reflects *dpp* expression in the visceral mesoderm of ps3 and ps7 (Hursh et al., 1993). In both regions, the maintenance of *dpp* expression is controlled by an autocrine signaling pathway requiring *dpp*, *tkv* and *Mad*. In early stage *Mad*$^{+}$ *tkv*^{Q199D} embryos, *dpp* expression is expanded to include all of the intervening parasegments (Fig. 3A). This result is also seen in embryos expressing DPP throughout the mesoderm (Staehling-Hampton and Hoffmann, 1994). In early stage *Mad* null *tkv*^{Q199D} embryos, the initiation of *dpp* expression is unaffected. *dpp* ectopic expression in ps4 through ps6, mediated by the activity of *tkv*^{Q199D} in *Mad*$^{+}$ embryos, does not occur in *Mad* null embryos (Fig. 3D). In late stage *Mad*$^{+}$ *tkv*^{Q199D} embryos (Fig. 3B), the expanded domain of *dpp* expression is maintained at very high levels. In late stage *Mad* null *tkv*^{Q199D} embryos, this is not observed (Fig. 3E). *dpp* expression begins to diminish shortly after initiation and we believe that the perdurance of β-galactosidase is responsible for residual staining in these embryos. In mid-stage *Mad* null background *tkv*^{Q199D} embryos that also express MAD specifically in the visceral mesoderm (Fig. 3C), there is maintenance of *dpp* expression in ps3 and ps7 and ectopic expression of *dpp* in the intervening parasegments (due to *tkv*^{Q199D}). In a comparable embryo lacking *tkv*^{Q199D}, only the maintenance of ps3 and ps7 *dpp* expression occurs (Fig. 3F). Thus, we infer that *Mad* functions in the DPP signal transduction cascade downstream of the serine-threonine kinase activity of *tkv* but upstream of elements required for maintenance of *dpp* expression in the visceral mesoderm of ps3 and ps7. *Mad* also functions downstream of the serine-threonine kinase activity of *tkv*^{Q199D} but upstream of ectopic *dpp* expression in the intervening parasegments.

Analysis of an *Scr* visceral mesoderm reporter gene in *Mad* null *tkv*^{Q199D} embryos provides further evidence that *Mad* functions downstream of *tkv*^{Q199D}. *Scr* is normally expressed in ps4 visceral mesoderm (Fig. 4A) immediately posterior to the *dpp*-expressing cells in ps3. Failure to initiate *dpp* expression in ps3 in certain *dpp* mutants results in an anterior expansion of *Scr* expression into ps3 (Hursh et al., 1993), indi-

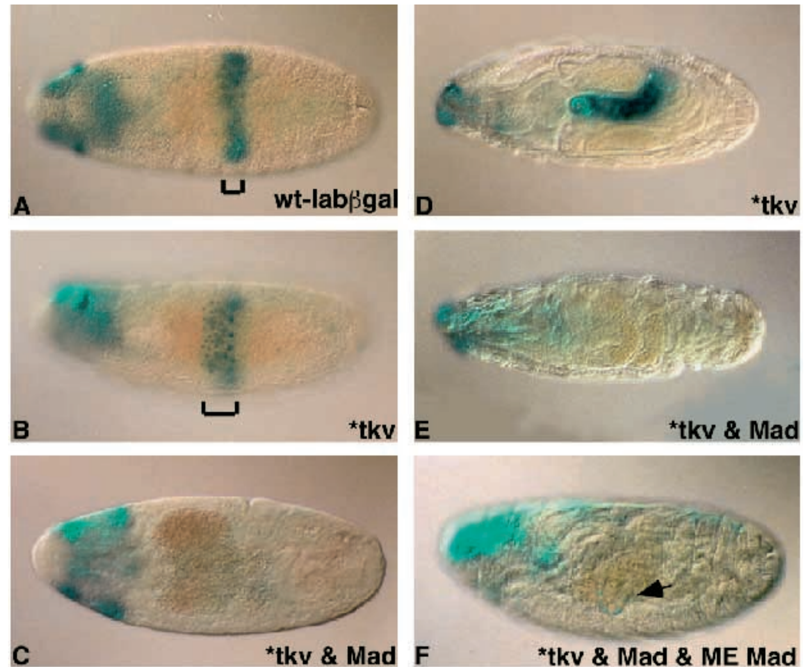

Fig. 2. MAD functions downstream of tkv^{Q199D} in *lab* induction. (A) Wild-type expression of a *lab* reporter gene that contains a DPP-independent central nervous system (CNS) enhancer and a DPP-dependent midgut endoderm enhancer in a stage 13 Mad^{+} embryo. *lab* expression in the head and midgut endoderm (square bracket) are clearly seen. (B) Anterior expansion of *lab* midgut endoderm expression (extended square bracket) in response to P{Gal4-*Hsp70*.PB} driven P{UAS-*tkv*.Q199D} in a stage 13 Mad^{+} embryo. The effect of tkv^{Q199D} on *lab* is virtually indistinguishable from that seen with P{hs-*dpp*.BP} (Newfeld et al., 1996). (C) *lab* CNS expression is unaffected while no expression from the *lab* midgut endoderm enhancer is detectable in a stage 13 *Mad* null tkv^{Q199D} embryo expressing heat-shock *dpp*. (D) Expanded expression from the *lab* midgut endoderm enhancer continues in a Mad^{+} stage 17 embryo expressing tkv^{Q199D}. (E) tkv^{Q199D} expression has no effect on *lab* midgut endoderm expression in a *Mad* null stage 17 embryo. (F) Providing MAD only in the midgut endoderm (ME) in an otherwise *Mad* null embryo rescues the induction of *lab* expression by tkv^{Q199D} in that tissue. *lab*-expressing cells (arrow) are seen in the elongating midgut of a stage 17 embryo.

cating a role for DPP in repressing *Scr* in ps3. *Mad* null embryos show the same anterior expansion of *Scr* (Newfeld et al., 1996) demonstrating that *Mad* is required for this activity of DPP. Alternatively, ubiquitous expression of tkv^{Q199D} or DPP represses *Scr* in ps4 (Fig. 4B,C). In an *Mad* null tkv^{Q199D} embryo, the expression of *Scr* (Fig. 4E) clearly resembles that seen in the *Mad* null embryo (Fig. 4D) and not the tkv^{Q199D} embryo (Fig. 4B). *Mad* function is epistatic to tkv^{Q199D} in the repression of *Scr* in the visceral mesoderm of ps3. This result is supported by examination of *Scr* expression in *Mad* null background tkv^{Q199D} embryos that also express MAD specifically in the visceral mesoderm. These embryos display an initial anterior expansion of *Scr* into ps3 comparable to a *Mad* null embryo (data not shown). The expansion likely occurs prior to the accumulation of sufficient MAD to allow tkv^{Q199D} to repress *Scr* expression. In older *Mad* null background tkv^{Q199D} embryos expressing MAD specifically in the visceral mesoderm (Fig. 4F), upon restoration of tkv^{Q199D} activity by visceral mesoderm-specific MAD, there is clear repression of *Scr* in both ps3 and ps4. Thus, we infer that *Mad* functions in the DPP signal transduction cascade downstream of the serine-threonine kinase activity of *tkv* but upstream of the repression of *Scr* in the visceral mesoderm of ps3. In summary, proper responses to DPP signals during midgut

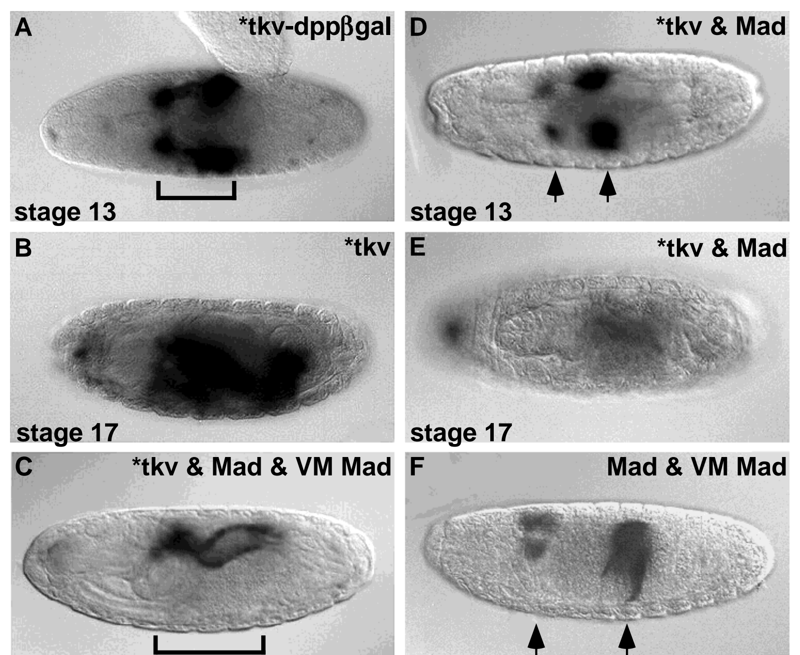

Fig. 3. MAD functions downstream of tkv^{Q199D} in *dpp* maintenance. (A) Expression of a *dpp* reporter gene which contains visceral mesoderm enhancers expressed in ps3 and ps7, in response to P{Gal4-*Hsp70*.PB} driven P{UAS-*tkv*.Q199D} in a stage 13 Mad^{+} embryo. High levels of expression in ps3 and ps7 as well as ectopic expression in the intervening parasegments (extended square bracket) are clearly seen. (D) Initiation of *dpp* expression in ps3 and ps7 (arrowheads) is unaffected in a comparably staged *Mad* null tkv^{Q199D} embryo as expected since the initiation of *dpp* expression is the result of direct activation by UBX. The ectopic expression of *dpp* in the intervening parasegments, due to tkv^{Q199D}, is not seen in this embryo. Comparison of B with E shows that *dpp* expression is maintained for an extended period at very high levels throughout the ps3 to ps7 region in response to tkv^{Q199D} in a Mad^{+} embryo (B) but not in a *Mad* null stage 17 embryo (E). The *Mad* null background embryo in C is expressing P{UAS*Mad*.N} and tkv^{Q199D} in the visceral mesoderm (VM) in response to P{GawB} 24B. The comparable *Mad* null background embryo in F does not contain P{UAS-*tkv*.Q199D}. (C) Ectopic expression of *dpp* between ps3 and ps7 in response to tkv^{Q199D} is restored by providing MAD in the VM. Providing MAD in the VM without tkv^{Q199D} does not result in ectopic expression of *dpp* (F).

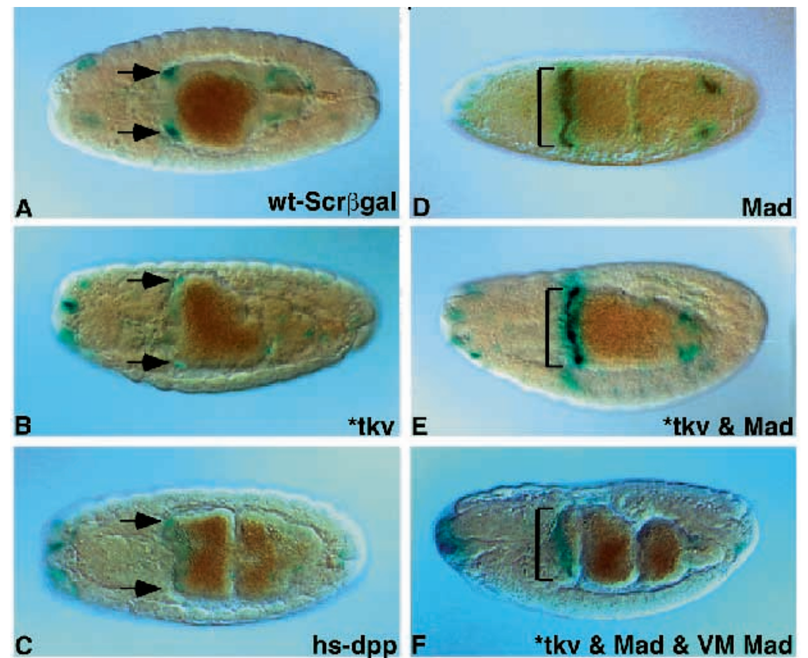

Fig. 4. MAD functions downstream of tkv^{Q199D} in *Scr* repression. (A) Wild-type expression of an *Scr* reporter gene that contains a DPP-responsive anterior midgut enhancer in a stage 14 embryo. *Scr* expression in ps4 visceral mesoderm (arrows) is clearly seen. (B) Repression of *Scr* ps4 expression in response to P{Gal4-*Hsp70*.PB} driven P{UAS-*tkv*.Q199D} in a stage 15 embryo. A distinctly reduced amount of *Scr* expression is seen (arrows). (C) The effect of tkv^{Q199D} on *Scr* is virtually indistinguishable from that seen with P{hs-*dpp*.BP}. (D) *Scr* expression is expanded anteriorly to include ps3 (square bracket) in a stage 15 *Mad* null embryo. This result is also seen in embryos lacking *dpp* ps3 expression (Hursh et al., 1993). (E) A stage 15 *Mad* null tkv^{Q199D} embryo. The anterior midgut expression of *Scr* resembles the pattern detected in *Mad* null embryos (anterior expansion – square bracket) and not the pattern seen in tkv^{Q199D} embryos (reduced ps4 expression). The *Mad* null background embryo in F is expressing P{UAS*Mad*.N} and tkv^{Q199D} in the visceral mesoderm (VM) in response to P{GawB}24B. In this stage 16 embryo, the anterior expression of *Scr* presumably occurred prior to the accumulation of sufficient MAD to allow tkv^{Q199D} to reduce *Scr* expression. The repression of ectopic *Scr* expression (square bracket), by visceral mesoderm-specific MAD and tkv^{Q199D} is evident when comparing F with E.

development require MAD activity in a DPP-dependent signal transduction cascade.

Ligand-dependent phosphorylation of endogenous MAD

Studies using transiently transfected cell lines have shown that mammalian Smad proteins become phosphorylated upon induction of TGF-β signaling pathways. For example, Smad1 becomes phosphorylated upon BMP2 addition and Smad2 is phosphorylated in response to TGF-β (Hoodless et al., 1996; Eppert et al., 1996). Thus, we sought to determine whether endogenous *Drosophila* MAD might be regulated in a similar manner. Since MAD appears to mediate DPP signaling in imaginal disks (Sekelsky et al., 1995), we analyzed two wing imaginal disk cell lines (MLDmD-8 and MLDmD-12: Ui et al., 1987). We stimulated DPP signaling in these cells using human BMP2, a homolog of DPP (Padgett et al., 1993; Sampath et al., 1993) and the cell lines responded to this treatment.

To determine whether MAD phosphorylation is regulated by ligand addition, we immunoprecipitated endogenous MAD from MLDmD-8 and MLDmD-12 cell lines using anti-MAD sera (Newfeld et al., 1996). Examination of immunoprecipitates from cells metabolically labeled with [^{35}S]methionine revealed a protein of approximately 50×10^3 M_r (Fig. 5A lower panels) that is close to the predicted size of MAD (50.5×10^3 M_r). This protein was absent in immunoprecipitates prepared using preimmune sera, thus confirming its identity as MAD. In both imaginal disk cell lines, MAD protein levels were unaffected by treatment of cells with BMP2 prior to lysis. However, treatment with BMP2 for 30 minutes prior to lysis yielded a MAD protein that displayed a slightly slower mobility on SDS-PAGE gels (MAD*), a characteristic often observed for phosphorylated proteins.

To directly examine MAD phosphorylation, MAD was immunoprecipitated from [^{32}P]phosphate-labeled cells treated with or without BMP2. Phosphorylation of MAD was almost undetectable in immunoprecipitates in the absence of BMP2 treatment (Fig. 5A upper panels). Treatment of cells with BMP2 prior to lysis induced phosphorylation of MAD. Ligand-induced phosphorylation of MAD was observed in both cell lines. To characterize the kinetics of MAD phosphorylation, [^{32}P]phosphate-labeled MLDmD-12 cells were treated with BMP2 for varying times. Phosphorylation of MAD was detected within 15 minutes of BMP2 addition, appeared to plateau at 30 minutes and remained elevated for at least 60 minutes (Fig. 5B). Together these findings indicate that MAD is rapidly phosphorylated in response to BMP2 and that MAD is a direct downstream component of the DPP signal transduction pathway.

MAD dramatically alters its subcellular distribution in response to DPP overexpression

In *Drosophila* embryos and salivary glands, staining with anti-MAD sera showed that endogenous MAD protein maintains a predominantly cytoplasmic subcellular distribution (Newfeld et al., 1996). Transfection studies in mammalian cell lines demonstrated that providing ligand or an activated type I receptor causes epitope-tagged Smad proteins to become completely or predominantly nuclear (Hoodless et al., 1996; Liu et al., 1996). We examined embryos containing six copies of a heat-shock-inducible *dpp* transgene to determine if endogenous MAD was altered in subcellular distribution in response to DPP overexpression.

MAD appears unaffected by the presence of the heat-shock-inducible *dpp* transgenes at a noninductive temperature (Fig. 6A) and is indistinguishable from the control embryo (Fig. 6F). The midgut endoderm of transgenic embryos stained with anti-MAD sera immediately after a one hour heat-shock display a uniform distribution of MAD throughout the cell, staining both the nucleus and cytoplasm (Fig. 6B). A comparable control

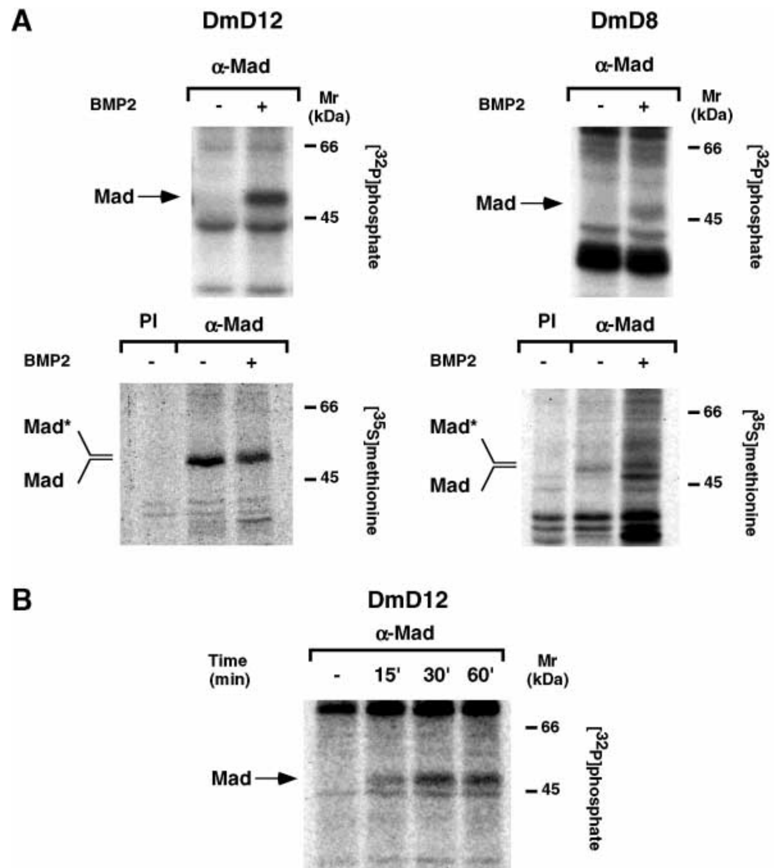

Fig. 5. BMP2-dependent in vivo phosphorylation of endogenous MAD.(A) Ligand-dependent phosphorylation of MAD. Cells from DmD12 (left panels) or DmD8 (right panels) imaginal disk cell lines were labeled with either [^{32}P]phosphate (upper panels) or [^{35}S]methionine (lower panels) and incubated in the presence (+) or absence (−) of human BMP2. Lysates were immunoprecipitated with anti-MAD antibodies (α-MAD) or preimmune sera (PI). The migration of phosphorylated MAD is indicated and the approximate positions of molecular mass markers (×10^{-3}) are shown. Note that phosphorylated MAD (MAD* in the lower panels) migrates slightly more slowly than MAD. (B) Kinetics of MAD phosphorylation. DmD12 cells were labeled with [^{32}P]phosphate and stimulated with BMP2 for increasing times as indicated. Labeling of MAD is detected in the first sample and appears complete by 30 minutes of stimulation.

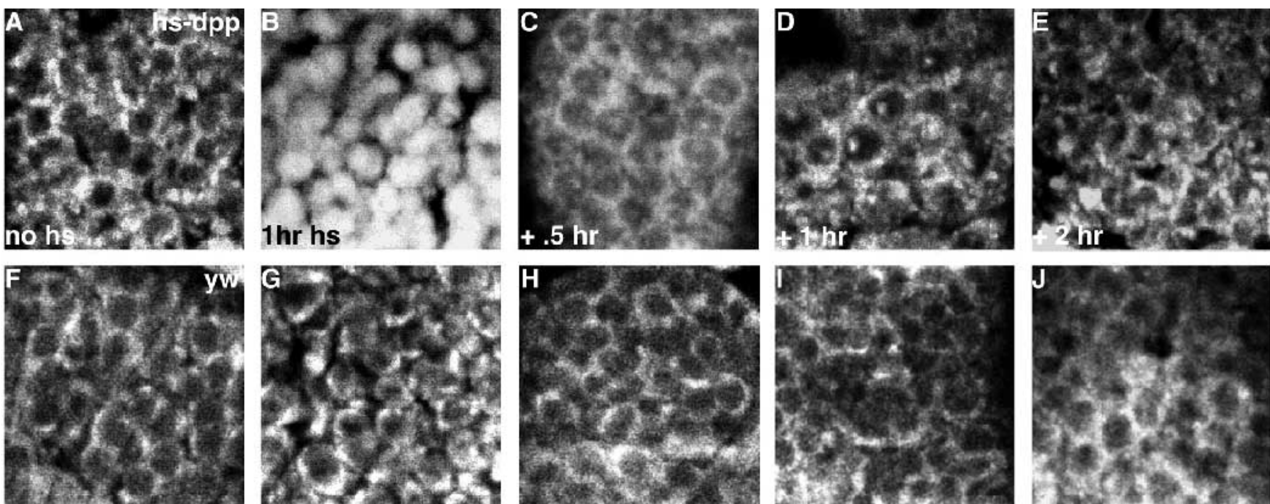

Fig. 6. DPP overexpression causes dynamic changes in MAD subcellular distribution in the midgut endoderm. (A-E) Midgut endoderm from stage 16 embryos carrying six copies of P{hs-*dpp*.BP} stained with MAD polyclonal sera. (F-J) Similarly staged and stained embryos without P{hs-*dpp*.BP}. (A,F) Embryos have not been heat-shocked and MAD appears to be a predominantly cytoplasmic protein in both. (B,G) Embryos stained immediately after a one hour heat-shock (a standard method for expressing transgenes under the control of the *hsp70* promoter). (B) MAD appears to be uniformly distributed throughout the cell, staining the nucleus and cytoplasm equally. (G) No change in MAD subcellular distribution. (C,H) Embryos after one-half hour (+.5 hours) room temperature recovery from a one hour heat-shock. (C) Predominantly cytoplasmic anti-MAD staining. However, the distinction between nuclear and cytoplasmic MAD staining is not as pronounced as in the preheat-shock embryo of the same genotype (A) or the comparable *y w* embryo (H). In addition, bright spots of anti-MAD staining are visible within the nucleus that are not visible in the preheat-shock embryo of the same genotype (A) or the comparable *y w* embryo (H). (D,I) Embryos after a one hour (+1 hour) room temperature recovery. (D) Predominantly cytoplasmic anti-MAD staining. The ratio of nuclear to cytoplasmic staining is indistinguishable from the preheat-shock embryo of the same genotype (A) and the comparable *y w* embryo (I). However, the bright spots of nuclear staining are still visible. (E,J) Embryos after a two hour (+2 hours) room temperature recovery. In both of these embryos, MAD appears indistinguishable from preheat-shock embryos.

embryo shows no change in MAD subcellular localization in response to the heat-shock (Fig. 6G). After a half-hour recovery from heat-shock, the experimental embryo again shows predominantly cytoplasmic anti-MAD staining (Fig. 6C). However, the distinction between nuclear and cytoplasmic staining is not as pronounced as in the preheat-shock embryo of the same genotype (Fig. 6A) or the comparable control embryo (Fig. 6H). In addition, a single bright spot of anti-MAD staining is visible within each nucleus (Fig. 6C), unlike either the preheat-shock embryo (Fig. 6A) or the post-heat-shock control (Fig. 6H). After a one hour recovery, the experimental embryo shows predominantly cytoplasmic anti-MAD staining (Fig. 6D) and the ratio of nuclear to cytoplasmic staining is indistinguishable from the preheat-shock condition (Fig 6A) and the comparable control embryo (Fig. 6I). The bright spots of anti-MAD nuclear staining are still visible. After a two hour recovery (Fig. 6E), MAD immunolocalization is indistinguishable from preheat-shock embryos (Fig. 6A) and the comparable control embryo (Fig. 6J). MAD's subcellular distribution changes dramatically in response to DPP overexpression and upon cessation of the overexpressed signal returns to its presignaling state.

No alteration in MAD subcellular distribution detected in response to endogenous DPP

To ascertain if MAD nuclear accumulation occurs in response to endogenous levels of DPP, we analyzed several developmental stages characterized by DPP-dependent events. Based upon our studies of DPP overexpression in the midgut endoderm, we examined additional embryos from the time of midgut constriction formation. Using the constrictions as landmarks for DPP-target cells, we cannot detect MAD nuclear accumulation (data not shown). We also examined blastoderm-stage embryos and third instar wing imaginal disks. In these stages of development, clearly defined morphogenetic gradients of DPP activity have been documented providing morphological and molecular landmarks for cells exposed to the highest levels of endogenous DPP. For our analysis of blastoderm-stage wild-type embryos, we used confocal microscopy to generate a series of optical sections along the dorsal-ventral axis (Fig. 7A-E). Here the highest level of DPP activity occurs in the dorsalmost cells (Ferguson and Anderson, 1992; Wharton et al., 1993) and one might predict the nuclear accumulation of MAD in these cells. However, MAD subcellular localization revealed no alterations in MAD's primarily cytoplasmic distribution in any cell type.

We also analyzed early to mid-third instar wing imaginal disks from a strain carrying the dpp^{disk} reporter gene P{BS3.0}. We double-labeled disks with anti-MAD polyclonal sera and for *dpp* expression. Consistent with our demonstration that MAD is ubiquitously expressed in embryos (Newfeld et al., 1996), MAD is expressed in all cells of the wing disk (Fig. 8A). At this stage, the highest level of DPP activity occurs in the region expressing DPP (Nellen et al., 1996; Lecuit et al., 1996; Singer et al., 1997) and one might expect nuclear accumulation of MAD in the DPP-expressing cells. However, MAD appears to be cytoplasmic in all cells of the disk. Even upon closer examination of double-labeled cells, those expressing DPP and potentially exposed to the highest levels of endogenous DPP (Fig. 8B,C), the cytoplasmic subcellular distribution of MAD appears unaltered.

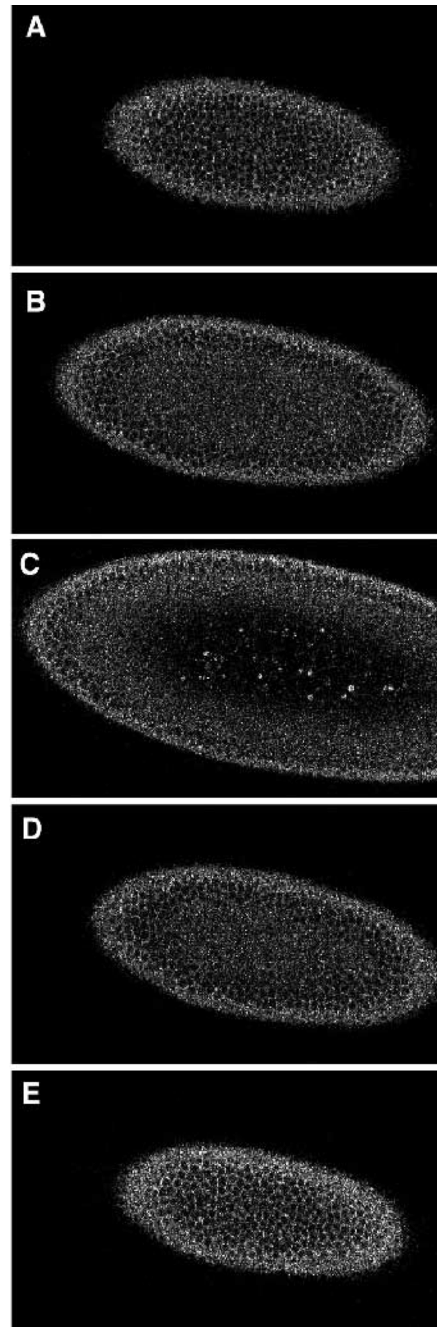

Fig. 7. MAD nuclear accumulation is undetectable in cells responding to endogenous DPP signals in blastoderm embryos. (A-E) A series of optical cross-sections along the dorsal-ventral axis of a blastoderm-stage wild-type embryo stained with anti-MAD polyclonal sera. The highest level of DPP activity at this stage occurs in the dorsalmost cells (Ferguson and Anderson, 1992; Wharton et al., 1993). Thus, the two surfaces of this embryo (A,E) depict cells exposed to maximum and minimum levels of endogenous DPP signals. No alteration in MAD's primarily cytoplasmic subcellular localization is seen.

DISCUSSION

Embryonic midgut formation provides a tractable system for placing *Mad* in the DPP signaling pathway. Unlike *Mad*'s con-

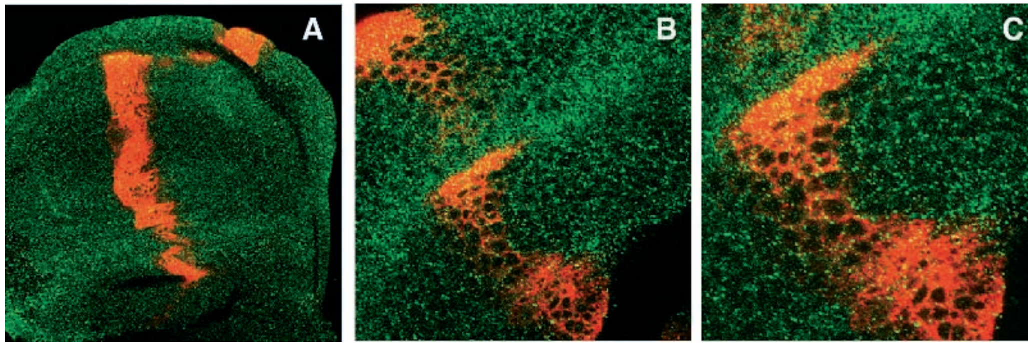

Fig. 8. MAD nuclear accumulation is undetectable in cells responding to endogenous DPP signals in wing imaginal disks. (A-C) Two different early to mid-third instar wing imaginal disks from a strain carrying the dpp^{disk} reporter gene P{BS3.0}. Disks are stained with anti-MAD polyclonal sera (green) and for *dpp* expression (red; visualized with anti-β-galactosidase). The highest level of DPP activity at this stage occurs in the region expressing *dpp* (Nellen et al., 1996; Lecuit et al., 1996; Singer et al., 1997). In the wing disk shown at low magnification (A), note the ubiquitous expression of MAD in all cells of the disk. In the other wing disk, shown at two magnifications (B,C), note the primarily cytoplasmic subcellular localization of MAD. MAD appears cytoplasmic even within and adjacent to cells expressing DPP (yellow), that ought to be receiving the highest levels of endogenous DPP signals.

tribution to DPP signaling in dorsal-ventral patterning of the blastoderm embryo, *Mad*'s role in midgut development is based purely upon zygotic expression. Thus, *Mad* null mutant genotypes can be readily analyzed. Also, investigation of one of the roles of visceral mesoderm DPP signaling (induction of *lab* in the midgut endoderm) permits a clear distinction between signaling cells and target cells.

By examining various aspects of embryonic midgut formation in genetic backgrounds containing a constitutively signaling *tkv* mutation, we are able to establish a clear epistatic relationship between MAD and the TKV type I DPP receptor. We demonstrate that tkv^{Q199D} cannot induce *lab* expression in the endoderm, cannot repress *Scr* or maintain *dpp* in the visceral mesoderm in *Mad* null embryos, unambiguously placing MAD downstream of the serine-threonine kinase activity of TKV. Adding a transgene expressing MAD only in the endoderm restores the ability of tkv^{Q199D} to induce *lab* expression and supplying MAD only in the mesoderm restores tkv^{Q199D} effects on *Scr* and *dpp*. These observations place MAD upstream of additional functions necessary for *Scr* repression, *dpp* maintenance and *lab* induction. Taken together, these observations are completely consistent with a role for MAD in mediating signal transduction in DPP-responsive cells.

These results beg the question of how MAD contributes to signal transduction. Is MAD modified during DPP signaling? Consistent with observations in mammalian cell lines (Hoodless et al., 1996; Lechleider et al., 1996; Yingling et al., 1996), in *Drosophila* cell culture MAD is rapidly phosphorylated in a ligand-dependent manner. Is MAD's subcellular distribution altered during DPP signaling? As seen in vertebrate studies (Hoodless et al., 1996; Liu et al., 1996), a heat-shock promoter driven *dpp* transgene causes a substantial fraction of the normally cytoplasmic MAD protein to accumulate in the nucleus of cells in the midgut endoderm. Recent observations that MAD accumulates in the nucleus of follicle cells in response to ectopic DPP (L. Dobens and L. Raftery, personal communication) suggests that our observations of MAD subcellular translocation are not specific to the endoderm but represent a general feature of MAD's role in DPP signal transduction. Thus, data for MAD are consistent with the current working hypothesis for the function of proteins of the Smad family, developed largely through studies of cultured mammalian cells. Upon activation of the DPP receptor, MAD is in turn activated through phosphorylation and translocates to the nucleus where it serves to regulate directly or indirectly the transcription of target genes (Chen et al., 1996, Macías-Silva et al., 1996). However, it should be noted that, in all of the experiments reporting nuclear accumulation, signals are likely to be at levels far higher than maximal endogenous signaling.

To date, in living animals, we have only observed the nuclear accumulation of MAD in response to DPP overexpression. We have looked extensively for evidence of MAD nuclear accumulation in three different tissues known to have active DPP signaling: the blastoderm embryo in which dorsal-ventral fates in the dorsal half of the embryo are being established according to a graded DPP signal, the midgut endoderm during stages when *lab* induction is occurring and third instar wing imaginal disks in which anterior-posterior fates are being established according to a graded signal emanating from a stripe of DPP expression bisecting the disk. In each case, we observe an identical pattern of MAD subcellular distribution in DPP-responsive and nonresponsive cells: a primarily cytoplasmic distribution. Further, single-labeling experiments were conducted on ovarian follicle cells from a *myc-Mad* transgenic strain expressing a spatially restricted DPP-dependent enhancer trap. *myc*-epitope-tagged MAD cannot be detected in nuclei of the follicle cell subpopulation that expresses the MAD-dependent DPP target gene (L. Dobens and L. Raftery, personal communication).

The absence of nuclear MAD under native signaling conditions may be explained in two ways. The possibility that we favor is that there is a considerable reservoir of cytoplasmic MAD, even under conditions of maximal endogenous signaling, such that the fraction of MAD translocated to the nucleus is too small to be detected by our techniques. The alternative, however, is that MAD nuclear accumulation is an artifact of overstimulation of the signaling pathway and that MAD's biological role is independent of the observed subcellular translocation. This situation needs to be addressed by

more sensitive methods such as the development of antisera specific to the phosphorylated form of MAD.

Changes to the subcellular distribution of Smad proteins upon cessation of ligand overexpression have not been examined in vertebrate studies. After withdrawal of DPP overexpression, MAD's subcellular distribution returns to its presignaling state through a transitional phase characterized by a bright spot within each nucleus. How is the cytoplasmic localization restored? Perhaps MAD is recycled out of the nucleus to the cytoplasm for further use or nuclear MAD is targeted for rapid degradation. Consistent with several features of *Mad*, we favor the second possibility. First, *Mad* RNA and protein are ubiquitously expressed (Sekelsky et al., 1995; Newfeld et al., 1996) suggesting ongoing production of MAD in every cell. Further, the MAD polypeptide contains motifs characteristic of rapid protein turnover. In the proline-rich region between the highly conserved N-terminal and C-terminal domains, there are small islands of amino acid identity between subsets of Smad proteins. One of these, a hexaamino acid block (TPPPA/GY: *Mad* #220-225; Sekelsky et al., 1995) is found in several human and *Xenopus* Smads (Graff et al., 1996; Zhang et al., 1996) but not in *C. elegans* (Savage et al., 1996) nor human Smad4 (Hahn et al., 1996). In all Smad proteins that contain this hexaamino acid block, it is located in a potential PEST sequence. PEST sequences are signals for rapid intracellular proteolysis and PEST-FIND scores above 5 are considered significant (Rechsteiner and Rogers, 1996). In MAD, the PEST sequence (amino acid #191-272) has a PEST-FIND score of 6.3. In addition, the PEST sequence in MAD is similar to the PEST sequence which controls signal-independent degradation of unbound Cactus protein in *Drosophila* (Belvin et al., 1995). Both sequences contain several casein kinase II phosphorylation sites. When the casein kinase II sites of the Cactus mammalian homolog IκBα are mutated, the protein is twice as stable as wild-type (Schwartz et al., 1996).

During the animal's lifetime, individual cells and their descendants are subjected to multiple rounds of DPP signaling. For example in the wing imaginal disk, DPP signals are required for cell proliferation and/or cell survival (Burke and Basler, 1996; Singer at al., 1997), for anterior/posterior patterning (Posakony et al., 1991; Singer at al., 1997) and for vein formation (Sturtevant and Bier, 1995). Perhaps the restoration of MAD's cytoplasmic localization reflects a DPP-target cell's return to non-signaling status. This suggests that, in DPP-responsive cells, there is an equilibrium of MAD between the nucleus and cytoplasm. Observations of Smad2 subcellular localization in unstimulated versus ligand-stimulated mammalian cells led Macías-Silva et al. (1996) to propose a similar model of MAD nuclear-cytoplasmic equilibrium. From this perspective, it is tempting to speculate that a gradient of MAD nuclear accumulation is the basis for how individual cells interpret the morphogenetic gradient of DPP which impacts their cell surfaces. Is MAD a direct readout of the DPP gradient such that certain thresholds of nuclear MAD elicit distinct responses or does MAD nuclear accumulation simply relay the level of receptor activation for interpretation by other signaling pathway components? Addressing this question will require additional experiments including the detection of MAD nuclear accumulation in response to endogenous DPP signaling.

Other aspects of DPP signal transduction currently under investigation are whether there is a cytoplasmic sequestering activity holding MAD and its potential transcriptional partners outside the nucleus in the absence of DPP signals and what are the transcriptional partners for MAD? Schnurri (Arora et al., 1995; Grieder at al., 1995; Staehling-Hampton et al., 1995) and Extradenticle (Mann and Abu-Shaar, 1996) are two potential transcriptional partners for MAD. Our laboratory is currently conducting genetic screens that may identify molecules that participate in both of these aspects.

In summary, our data on MAD are consistent with a dynamic role in a DPP-dependent serine-threonine kinase signal transduction cascade. An attractive possibility is that one function of MAD is to interpret the morphogenetic gradient of DPP signals. A nuclear-cytoplasmic equilibrium model of MAD subcellular localization provides a mechanistic basis for DPP's ability to induce multiple changes in a cell's fate throughout development.

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