Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-β responsive cells

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SUMMARY

The proteins necessary for signal transduction in cells responding to ligands of the TGF-β family are largely unknown. We have previously identified Mad (Mothers against dpp), a gene that interacts with the TGF-β family member encoded by decapentaplegic (dpp) in Drosophila. Assay of Mad’s role in the DPP-dependent events of embryonic midgut development demonstrates that Mad is required for any response of the visceral mesoderm or endoderm to DPP signals from the visceral mesoderm. Replacement of the normal DPP promoter with a heterologous (hsp70) promoter fails to restore DPP-dependent responses in Mad mutant midguts. Experiments utilizing Mad transgenes regulated by tissue-specific promoters show that MAD is required specifically in cells responding to DPP. Immunohistochemical studies localize MAD to the cytoplasm in all tissues examined. Experiments in Xenopus embryos demonstrate that Drosophila MAD can function in the signaling pathway of BMP-4, a vertebrate homolog of dpp. Based on these results, we propose that Mad is a highly conserved and essential element of the DPP signal transduction pathway.

Key words: Mad, intercellular signaling, TGF-β family, midgut morphogenesis, Drosophila

INTRODUCTION

Ligands of the transforming growth factor-β (TGF-β) superfamily are required for numerous developmental events in many organisms (reviewed in Kingsley, 1994). These secreted molecules function through the activation of receptor kinases on the surface of responsive cells (reviewed in Massagué et al., 1994). The activated receptors in turn are thought to propagate the signal through the initiation of intracellular signal transduction cascades. No protein components of these cascades have yet been firmly identified. Here, we report a strong candidate for such a component.

This candidate, the Mothers against dpp (Mad) gene in Drosophila melanogaster, was initially identified by virtue of dominant phenotypic interactions between Mad mutant alleles and specific alleles of the decapentaplegic (dpp) gene (Raftery et al., 1995; Sekelsky et al., 1995). The protein product of the dpp gene, which we refer to as DPP, is a member of the TGF-β family (Padgett et al., 1987) and appears to be the Drosophila homolog of the vertebrate BMP-2 and BMP-4 ligands (Padgett et al., 1993; Sampath et al., 1993). Among its many functions, DPP signaling across germ layers is responsible for the establishment of dorsal mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Studies in Xenopus show that BMP-4 induces ventral mesoderm (reviewed in Harland, 1994), an activity suggested to be analogous by virtue of the hypothesized reversal of the dorsal-ventral axis after the divergence of arthropods and vertebrates (Holley et al., 1995; Schmidt et al., 1995).

Significant progress has been made in characterizing participants in the DPP signaling pathway. Recent reports have identified the DPP type I and II receptors (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995), a potential regulator of DPP processing (tolloid, Shimmel et al., 1991; Finelli et al., 1994), and a transcription factor functioning in BMP-responsive cells (schnurri, Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, the cytoplasmic proteins necessary for transducing the DPP signal from cell surface receptors to nuclear transcription factors are largely unknown. To identify these proteins, our laboratory has conducted several genetic screens (Raftery et al., 1995; Sekelsky et al., 1995).

Mad and dpp display dosage-dependent genetic interactions and homozygous Mad mutant phenotypes show striking parallels with dpp mutant phenotypes. However, the predicted MAD polypeptide contains no identifiable protein motifs, providing no clues to its biochemical function (Sekelsky et al., 1995). Recently, three C. elegans genes (sma-2, sma-3 and sma-4) were shown to have strong sequence similarity to Mad (Sekelsky et al., 1995; Savage et al., 1996). These genes also share aspects of the mutant phenotype displayed by the C. elegans TGF-β type II receptor daf-4. Clonal analysis of cells contributing to the shared mutant phenotype revealed that sma-
Embryo analysis strategies

Mad dpp lines are described as follows: P{GawB}24B - a Gal4 enhancer trap containing an enhancer trap in JS17, which removes Mad, is described in Raftery et al. (1995). The sequenced point mutant null alleles Mad0 and Mad12 and the deficiency Df(2L) C28, which removes Mad, is described in Raftery et al. (1995). The deficiency Df(2L) JS17, which removes Mad, are described in Sekelsky et al. (1995). Strains carrying the β-galactosidase reporter genes are described as follows: lab anterior midgut endoderm/CNS reporter P[3.65 lab] (Chouinard and Kaufman, 1991), dpp visceral mesoderm reporter P{hs-dpp.BP} and MAD in the visceral mesoderm, males from Mad point mutant stocks homozygous for P{UASMad.N} on the X and homozygous for P{GawB}24B on III. 50% of the Mad mutant embryos will have the lab reporter. For embryos expressing MAD in the visceral mesoderm and midgut endoderm, males containing the lab reporter P{3.65 lab} and P{mex1Mad} from the midgut endoderm experiment above were crossed to females homozygous for P{UASMad.N} on the X and homozygous for P{GawB}24B on III. 25% of the Mad mutant embryos will have the lab reporter and P{mex1Mad}. For embryos expressing MAD in the visceral mesoderm and midgut endoderm with P{hs-dpp.BP}, the direction of this cross was reversed allowing the formation of recombinant chromosomes containing P{hs-dpp.BP} and P{mex1Mad}. For P{RD2} in Mad mutant backgrounds with MAD in the visceral mesoderm, males from Mad point mutant stocks homozygous for P{RD2} were crossed to females containing the same Mad allele that were also homozygous for an insertion of P{UASMad.N} on the X and homozygous for P{GawB}24B on III. 100% of the progeny will contain one copy of P{UASMad.N}, P{GawB}24B and P{RD2}. Two insertions of P{UASMad.N} were tested.

Ectopic constructs

P{mex1βgal} was constructed from a 2.15 kb EcoRI-DreIII fragment from genomic clone EcoRlmesG2 (R. Schulz, M.D. Anderson Cancer Center) which corresponds to map position 46 to 48 on the chromosome walk around Eip2829 (Cherbas et al., 1986). This fragment was cloned into the EcoRI and BamHI sites of pCasper-AUG-βgal (Thummler et al., 1988) after the DreIII and BamHI sites were filled in with T4 DNA polymerase. This fragment contains approximately 2 kb of upstream sequence, the promoter and all 5′ untranslated sequence (DreIII cuts the ATG encoding the initiator methionine) for midgut expression I (mex1; Schulz et al., 1991). P{UASMad.N} was constructed from the complete Mad cDNA (Sekelsky et al., 1995). The cDNA was first cloned into pSPORT1 (BRL) and then removed using KpnI and XbaI sites from the pSPORT1 polylinker. The cDNA was then cloned into pUAST (Brand and Perrimon, 1993) using these sites. P{mex1Mad} was constructed from the same KpnI-XbaI fragment containing the Mad cDNA. In this case, the fragment was cloned into P{mex1βgal} which had been cut with KpnI and PstI, after the XbaI and PstI sites were filled in with T4 DNA polymerase. This replaces β-galactosidase sequences with the Mad cDNA. Three independent lines were established for each construct.

Histochemical β-galactosidase analysis

Embryos were collected and dechorionated by standard methods and fixed in 4% formaldehyde in PEM (0.1 M Pipes; 1 mM EGTA; 2 mM MgSO4) at 1:1. Embryos were washed in PEM and analyzed for β-galactosidase activity according to Blackman et al. (1991).

MAD antiserum and western blots

An XhoI-AccI (XA) restriction fragment from the Mad cDNA (bp 704-1524; Sekelsky et al., 1995) was subcloned into Bluescript for the analysis of P{mex1βgal} wild-type expression, three independent insertions were made homozygous. For P{mex1βgal} expression in dpp and Mad mutant backgrounds, mutant stocks homozygous for an insertion on III were created. For P{mex1βgal} expression in lab and Ubx mutant backgrounds, mutant stocks homozygous for an insertion on the X were created.
(Stratagene). This fragment was cut out with XhoI and HindIII, cloned into the pGEX-KG expression vector (Guan and Dixon, 1991) and transformed into E. coli host strain DH5α. The YA-GST fusion protein was induced and purified according to Frangioni and Neel (1993). The XaI restriction fragment from the P{Um1ab} fly was isolated by electrophoresis in an agarose gel and subcloned into pT7Blue (Novagen). The resulting construct, pT7Blue-mycMAD, was transformed into yeast strain STR112 (Clontech) according to manufacturer’s instructions. The XA fragment was then isolated by SDS-PAGE (Laemmli, 1970). The 1% acrylamide gel was briefly stained in Coomassie Blue, the XA band excised and soaked in PBS (342 mM NaCl, 1.7 mM KCl, 3.7 mM KH2PO4, 10.4 mM NaH2PO4) at 4°C. The gel slice was used to immunize two female guinea pigs (890 and 891) according to standard protocols (Pocono Rabbit Farm).

Protein from adult female y w P{UmMad} lab flies was extracted by grinding 20 flies in 100 μl PBS and 100 μl 2x SDS gel loading buffer (Sambrook et al., 1989). The samples were boiled for 5 minutes and approximately 1 fly worth of protein was loaded per lane. The gel was soaked and electroblotted to nitrocellulose according to Thomas and Kiehart (1994). Membranes were blocked in TBS (150 mM NaCl, 10 mM Tris pH 7.5, 0.1% BSA, 0.1% sodium azide)/10% powdered milk for 2 hours at 25°C and then incubated with various dilutions of anti-MAD polyclonal serum or with mouse anti-MYC monoclonal antibodies at a 1:10 dilution in TBST (TBS with 0.1% Triton X-100)/10% normal goat serum (NGS, Sigma) for 1 hour at 25°C. Blots were washed in TBST 3 times for 10 minutes and then incubated with alkaline phosphatase (AP)-conjugated goat anti-guinea pig antibody diluted 1:5000 in TBST/10% NGS for 1 hour at 25°C or with AP-conjugated goat anti-mouse antibodies at a 1:1000 dilution in TBST/10% NGS for 1 hour. All subsequent treatments followed Thomas and Kiehart (1994).

Embryos were collected, fixed as described for histochemical analysis, rehydrated from methanol into PBT and blocked in PBT (PBS with 0.5% Triton X-100, pH 7.0). Salivary glands were dissected from third instar larvae in ice-cold PBS, fixed for 15 minutes in 4% formaldehyde in PEM and washed three times for 10 minutes in PBT (PBS with 0.5% Triton X-100, pH 7.0). Salivary glands were blocked in PBT/10% NGS at room temperature for 1 hour. Following a rinse in PBT/10% NGS, salivary glands were incubated with primary antibody (diluted in this solution) overnight at 4°C. All subsequent treatments followed Thomas and Kiehart (1994).

Antibody analyses of salivary glands and embryos

The primary antibodies are anti-MYC monoclonal antibodies (Oncogene Science 9E10) used on salivary glands at 1:20 and anti-MAD polyclonal serum (guinea pig 891) used on salivary glands and embryos at 1:2000. The secondary antibodies are fluorescein-conjugated goat anti-mouse and biotinylated goat anti-guinea pig (Jackson ImmunoResearch Laboratories) used at 1:200. Salivary glands were dissected from climbing third instar larvae in ice-cold PBS, fixed for 15 minutes in 4% formaldehyde in PEM and washed three times for 10 minutes in PBT (PBS with 0.5% Triton X-100, pH 7.0). Salivary glands were blocked in PBT/10% NGS at room temperature for 1 hour. Following a rinse in PBT/10% NGS, salivary glands were incubated with primary antibody (diluted in this solution) overnight at 4°C. All subsequent treatments followed Thomas and Kiehart (1994).

Embryos were collected, fixed as described for histochemical analysis, rehydrated from methanol into PBT and blocked in PBT/10% NGS/0.5% BSA (bovine serum albumin fraction V, Sigma). Following a rinse in PBT/5% NGS/0.25% BSA, the remaining steps are identical to those for salivary glands. Anti-MYC incubation of salivary glands from P{UmMad} lab larvae was always accompanied by salivary glands from y w larvae as a negative control and 42nM transgenic larvae (expressing myc-tagged P-element transposase; Xu and Rubin, 1993) as a positive control. Analyses with anti-MAD serum were always accompanied by preimmune serum as a negative control.

 Xenopus methods

To generate injectable RNA that encoded Drosophila MAD, the Ncol-Stul restriction fragment from the P[UmMad] plasmid (Sekelsky et al., 1994) was subcloned into the Ncol site of pSP64TEN. This construct, pSP64TEN-mycMAD, was linearized with XbaI and synthetic capped mRNA generated as described by Krieg and Melton (1987). mRNAs encoding Xenopus BMP-4, the BMP-4 type I receptor or the activin type II receptor were synthesized as described (Graff et al., 1994; Hemmati-Brivanlou and Melton, 1992). Embryos were injected, staged and analyzed as described (Graff et al., 1994).

RESULTS

Examining the role of Mad in the DPP pathway

To clarify Mad’s function, we employed dpp’s role in the formation of the embryonic second midgut constriction (reviewed in Bienz, 1994) as our primary assay. In the visceral mesoderm of embryonic parasegment 7 (ps7), the homeotic gene Ultrabithorax (Ubx) initiates dpp expression. Then DPP signals between germ layers to the underlying midgut endoderm. At the same time, within the visceral mesoderm, DPP functions in an autocrine manner to maintain Ubx and thus dpp expression. Within the ps7 midgut endoderm, which does not express dpp, transcription of the homeotic gene labial (lab) is dependent upon the DPP signal. Each of these genes is required for the second constriction. Mad is also required for the second constriction and is transcribed in both cell layers (Sekelsky et al., 1995). A schematic of the known interactions between dpp, Ubx and lab, as well as possible roles for Mad in ps7 is shown in Fig. 1A.

Here we report the results of two classes of experiments. First, using epistasis tests, we determined whether Mad functions with regard to dpp transcription. Second, using tissue-specific expression of MAD in otherwise Mad mutant embryos, we determined which cells require MAD for DPP-dependent responses.

Mad functions downstream of dpp transcription

Expression of a lab reporter gene containing a DPP-independent central nervous system (CNS) enhancer and a DPP-dependent ps7 midgut endoderm enhancer in a wild-type (Mad+) background is shown in Fig. 1B. Comparably staged Mad mutant embryos retain the lab CNS expression but lack lab expression in the midgut endoderm (Fig. 1C). This result is consistent with previous observations that lab RNA in the midgut endoderm was absent in Mad mutant embryos (Sekelsky et al., 1995). To test if the role of Mad is to regulate dpp transcription, we assayed the effect of Mad mutations on lab induction in embryos in which dpp transcription was placed under control of the Drosophila hsp70 promoter (P[hs-dpp.BP]; Twombly et al., 1996). Under these conditions, if Mad functions upstream of dpp transcription, lab expression in the midgut endoderm should occur in Mad mutant embryos. However, the expanded domain of lab midgut endoderm expression expected from using P[hs-dpp.BP] in Mad+ embryos (Fig. 1D; Thuringer and Bienz, 1993) is lacking in embryos that are mutant for Mad (Fig. 1E). Thus, we infer that Mad functions in the DPP pathway between the initiation of dpp transcription and lab expression.

This idea is supported by experiments using the dpp reporter gene P[RD2] which accurately reflects dpp expression in the visceral mesoderm of ps3 and ps7 (Hursh et al., 1993). In ps7, the maintenance of dpp expression is controlled by an autocrine signaling pathway requiring DPP and UBX (Fig. 1A). There is no difference in the initiation of dpp expression between wild-type and Mad mutant embryos in either ps3 or ps7 (Fig. 2A,B). In wild-type embryos, dpp expression
continues at very high levels in both parasegments through very late stages of gut elongation (Fig. 2C,E). In Mad mutant embryos, this is not observed; dpp expression begins to diminish shortly after initiation (Fig. 2D,F). We believe that perduance of β-galactosidase is responsible for residual staining in these mutant embryos. RNA in situ hybridization studies using the dpp cDNA as a probe on Mad mutant embryos revealed only a short period of dpp transcription (data not shown). It appears that maintenance but not initiation of dpp expression requires Mad.

Analyses of Ubx and Sex combs reduced (Scr) visceral mesoderm reporter genes in Mad mutant embryos provides further evidence for placing Mad downstream of dpp transcription initiation. Initiation of Ubx expression in ps7 occurs in wild-type and Mad mutant embryos (Fig. 3A,B). Maintenance of Ubx expression in ps7 does not occur in Mad mutant embryos (data not shown). As in ps7, the maintenance of high levels of dpp expression in ps3 is not seen in Mad mutant embryos (compare Fig. 2C,E with D,F). Failure to initiate dpp expression in ps3 in dpp mutants which lack the visceral mesoderm expression of DPP results in an anterior expansion of Scr expression (Hursh et al., 1993). Similarly, Mad mutant embryos show an anterior expansion of Scr, using a reporter gene assay (Fig. 3C,D). It appears that MAD functions between initiation of dpp transcription and the repression of Scr in the visceral mesoderm of ps3. Thus, in all of its roles in embryonic midgut development, MAD functions downstream of dpp transcription initiation.

**Midgut tissue-specific enhancers**

Further resolution of MAD’s role in the DPP pathway emerges from a determination of whether MAD is required in cells sending or receiving a DPP signal. We have focused on the DPP-dependent induction of lab expression, since the signaling cells are in the visceral mesoderm and the target cells are in the adjacent endoderm. To do this, we used tissue-specific enhancers to drive Mad expression only in the visceral mesoderm or only in the midgut endoderm. We then examined the ability of visceral mesoderm-expressed or midgut endoderm-expressed Mad to rescue lab induction in genetic backgrounds lacking endogenous Mad activity.

At this time, only a limited set of identified enhancer elements or enhancer traps that specifically express in the visceral mesoderm or midgut endoderm are available. Thus, we had to exploit enhancer constructs in special contexts. The properties of the enhancers that we selected are described here. To express MAD specifically in the visceral mesoderm, we utilized a Gal4 enhancer trap (P{GawB}24B; Brand and Perrimon, 1993) to drive expression of a Mad transgene carrying upstream activation sequences regulated by Gal4 (P{UAS-Mad.N}). Early expression from this enhancer trap occurs throughout the presumptive mesoderm while later expression becomes restricted to the somatic mesoderm. Utilizing this enhancer to drive MAD expression in the presumptive mesoderm provides MAD protein, which persists in descendant visceral mesoderm cells.

For midgut endoderm expression, we utilized an enhancer element upstream of midgut expression 1 (mex1; Schulz et al., 1991) which drives expression only in the midgut endoderm. Reporter gene expression from P{mex1}gal in Mad embryos is shown in Fig. 4A,C,E. Expression begins in ps7 and the surrounding midgut endoderm prior to the initiation of the second midgut constriction (at roughly the same time as lab) and continues strongly into the first larval instar. At all times, mex1

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**Fig. 1.** MAD functions between dpp transcription initiation and lab transcription. (A) The known relationships between Ubx, dpp and lab in ps7 of the midgut and four possible functions for Mad. (B,D) Wild-type embryos; (C,E) Mad mutant embryos. (B) Embryos that were not heat shocked; (D,E) Embryos heat shocked to express ubiquitous DPP from P{hs-dpp.BP}. (B) Wild-type expression of a lab reporter gene which contains a DPP-independent central nervous system (CNS) enhancer and a DPP-dependent midgut endoderm enhancer. lab expression in the head and midgut endoderm (square bracket) are clearly seen. (C) CNS expression is unaffected in a Mad mutant embryo but no expression from the lab midgut endoderm enhancer is detectable. (D) Anterior expansion of lab midgut endoderm expression (extended square bracket) in response to P{hs-dpp.BP}. (E) P{hs-dpp.BP} expression has no effect on lab expression in Mad mutant embryos.

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enhancer expression in the midgut endoderm is broader than the lab midgut endoderm enhancer (described in Chouinard and Kaufman, 1991). However, only the anterior and posterior ends of expression from the mex1 enhancer are functional in lab (Fig. 4B), dpp (Fig. 4D) and Mad (Fig. 4F) mutant embryos as well as in Ubx mutants (data not shown). Thus, a construct (P{mex1Mad}) containing this enhancer driving MAD expression, in an otherwise Mad mutant background, can generate an embryo containing Mad+ midgut endoderm cells at the anterior and posterior ends of the mex1 enhancer domain.

MAD is required in DPP-responsive cells

To determine which midgut cells require Mad function, we examined the expression of lab and dpp reporter genes in Mad mutant embryos with only tissue-specific expression of MAD. Expression of MAD in the visceral mesoderm has no effect on lab expression in the midgut endoderm, even in the presence of ubiquitously expressed DPP (Fig. 5A). In these embryos, DPP-independent lab expression in the CNS is evident but no midgut endoderm expression is visible, just as in homogeneous Mad mutant embryos (Fig. 1E). This result suggests that Mad’s role in lab induction does not involve secretion of DPP from the visceral mesoderm or any other process occurring only the signaling cell.

In contrast, expressing MAD solely in the midgut endoderm in the presence of ubiquitous DPP rescues ectopic lab expression in the midgut endoderm as shown by the staining of cells in the elongating gut (Fig. 5C). No midgut endoderm lab expression is seen in homogeneous Mad mutant embryos, even with ubiquitous DPP, at any stage (e.g., Fig. 1E). This limited amount of lab expression is presumably occurring in the lab-independent anterior domain of mex1 expression and requires the heat-shock dpp construct to provide a DPP signal in this domain. Comparing lab expression in embryos with MAD expressed in the visceral mesoderm to embryos expressing MAD in the midgut endoderm (Fig. 5A,C) leads us to conclude that MAD is required in cells receiving a DPP signal.

Examination of dpp expression in embryos with MAD expressed specifically in the visceral mesoderm (Fig. 5B) reveals that the autocrine loop for dpp maintenance in both ps3 and ps7 is partially rescued. Note the well-defined staining of the elongating gastric caeca and midgut, particularly in comparison to wild-type and homozygous Mad mutant embryos (Fig. 2E,F). Our interpretation is that the provision of MAD in the early mesoderm allows sufficient MAD activity to persist into the derived visceral mesoderm to rescue early stages of dpp maintenance during gut elongation.

The expansion of UBX expression in response to heat-shock-induced DPP (Thuringer et al., 1993) permits a broad domain of autocrine signaling in the visceral mesoderm, including cells adjacent to the anterior region of mex1 expression in the endoderm. Thus, Mad mutant embryos expressing MAD in both the visceral mesoderm and midgut endoderm with heat-shock-induced DPP expression exhibit stronger lab induction (compare Fig. 5C and D). Presumably this occurs because the visceral mesoderm expression of MAD allows more DPP to be generated and secreted through the restoration of the autocrine loop, reinforcing the effects of the heat-shock-induced DPP signal. All of our observations support a role for MAD in cells receiving a DPP signal (Fig. 5E).

MAD is a ubiquitously expressed cytoplasmic protein

A very intriguing possibility is that MAD is a component of the signal transduction pathway in these cells. Knowing the subcellular localization of MAD would allow us to evaluate this possibility. Therefore, the subcellular localization of MAD
protein was examined using immunohistochemical techniques. We obtained very similar results with two distinct antibody probes. One probe is anti-MYC monoclonal antibodies directed against a myc-epitope-tagged Mad cDNA transgene (Sekelsky et al., 1995). The other is anti-MAD polyclonal serum generated against a bacterially expressed fusion protein. One myc-epitope-tagged Mad transgenic line overexpresses the transgene in larval salivary glands and their embryonic primordia (data not shown). Their accessibility and large size have made third instar larval salivary glands an excellent tissue for examining subcellular localization of proteins. This tissue also proved very useful for evaluating the specificity of our anti-MAD serum. The western strips in Fig. 6A were generated from extracts of salivary glands from transgenic larvae. Both anti-MYC monoclonal antibodies and anti-MAD polyclonal serum recognize a single protein of 55×10^3 M_r. The identified protein corresponds to the size of the predicted product encoded by the myc-epitope-tagged Mad cDNA transgene carried in this strain. Preimmune serum does not recognize any proteins in these salivary glands. Fig. 6B, C are confocal micrographs of transgenic salivary glands incubated with anti-MYC monoclonal antibodies (Fig. 6B) and anti-MAD serum (Fig. 6C). In this tissue, MAD shows a cytoplasmic subcellular localization though some staining in a few nuclei is seen (Fig. 6C). However, we see no nuclear staining with anti-MYC antibodies or with anti-MAD on embryos (see below) suggesting that the nuclear staining does not accurately reflect MAD’s subcellular localization. The transition in MAD staining, from uniform to punctate, in cells located further from the common duct reflects the accumulation of secretory granules in the cytoplasm (Berendes and Ashburner, 1978). Experiments with salivary glands from wild-type larvae reveal cytoplasmic staining with anti-MAD serum but no signal with anti-MYC monoclonal antibodies (data not shown).

Fig. 3. Ubx and Scr expression in Mad mutant embryos. (A,B) Studies of a Ubx reporter gene. (C,D) Studies of an Scr reporter gene. The left panels show wild-type expression and the right panels expression in Mad mutant embryos. Comparison of A with B shows that the initiation of Ubx expression in ps7, indicated by an arrow, is observed in both embryos. Comparison of C with D shows that the normal ps4 expression of Scr in wild-type embryos (indicated by a horizontal bar) is expanded anteriorly to include ps3 in Mad mutant embryos (indicated by an extended bar).

Fig. 4. Identification of a midgut endoderm enhancer active in Mad mutant embryos. (A,C,E) P[1µgall] wild-type expression; (B,D,F) P[1µgall] expression in dpp, lab and Mad mutant embryos, respectively. (A) Stage 14 embryo expressing β-galactosidase from the mex1 enhancer in the midgut endoderm. The expression is strongest near the second midgut constriction but is also evident anterior and posterior to the constriction (indicated by arrowheads) in a broader band of expression than seen for lab (Chouinard and Kaufman, 1991). (C) A high level of expression, in a short stretch of midgut endoderm, is maintained in stage 17 embryos. (E) High levels of expression (still broader than lab) continue into the first larval instar. (B,D,F) Only the anterior and posterior ends of mex1 expression are seen in the midgut endoderm of dpp, lab and Mad mutant stage 17 embryos, respectively.
Studies with anti-MAD serum on wild-type embryos revealed a ubiquitous tissue distribution (Fig. 6D) during embryonic development. This result is consistent with our RNA in situ hybridization experiments which demonstrated that Mad transcripts are ubiquitous in the embryo (Sekelsky et al., 1995). Fig. 6E shows a high magnification confocal micrograph of the midgut endoderm in ps7 of a wild-type stage 16 embryo indicating that MAD is cytoplasmic in this tissue. Further, the patterns of MAD expression and subcellular localization are unaltered in dpp<sup>+</sup> mutant embryos which specifically lack DPP expression in ps7 (data not shown). This cytoplasmic localization is consistent with MAD’s participation in signal transduction in DPP-responsive cells.

*Drosophila* MAD functions with *Xenopus* BMP-4

The functional conservation and cellular and developmental similarities of DPP and BMP-2 and BMP-4 led us to determine if MAD’s role in DPP signaling is conserved in vertebrates. We conducted a series of experiments using *Xenopus* animal pole explants (Fig. 7A). Different TGF-β family ligands can convert these explants, normally fated to form ectoderm, into one of two easily distinguished types of mesoderm, dorsal or visceral mesoderm. Comparing A with B reveals that MAD in the visceral mesoderm cannot rescue lab expression in the midgut endoderm but rescues dpp maintenance in the visceral mesoderm. To evaluate the extent of dpp maintenance rescued, compare D to wild-type and homogeneous *Mad* mutant embryos in Fig. 6E. (C) MAD in the midgut endoderm rescues lab expression in that tissue; lab-expressing cells (identified by an arrow) are seen in the elongating midgut. (D) The rescue of dpp maintenance by MAD in the visceral mesoderm improves the rescue of lab in an embryo expressing MAD in both tissues. (E) Schematic depiction of the results of the tissue-specific expression experiments.

**Fig. 5.** MAD functions in DPP-responsive cells. (A,C,D) lab expression in Mad mutant embryos with tissue-specific expression of MAD and ubiquitous DPP. (A) MAD in the visceral mesoderm [VM], (C) MAD in the midgut endoderm [ME] and (D) MAD in the visceral mesoderm and midgut endoderm. (B) dpp expression in an embryo with MAD in the visceral mesoderm without ubiquitous DPP. Comparing A with B reveals that MAD in the visceral mesoderm cannot rescue lab expression in the midgut endoderm but rescues dpp maintenance in the visceral mesoderm. To evaluate the extent of dpp maintenance rescued, compare D to wild-type and homogeneous Mad mutant embryos in Fig. 2.E,F. (C) MAD in the midgut endoderm rescues lab expression in that tissue; lab-expressing cells (identified by an arrow) are seen in the elongating midgut. (D) The rescue of dpp maintenance by MAD in the visceral mesoderm improves the rescue of lab by MAD in the midgut endoderm in an embryo expressing MAD in both tissues. (E) Schematic depiction of the results of the tissue-specific expression experiments.

**Fig. 6.** MAD is a ubiquitously expressed cytoplasmic protein. (A) Three strips from a single western blot of protein extracted from P[UmMad] lab third instar larval salivary glands. Lane 1 was incubated with preimmune serum. Lane 2 was incubated with anti-MAD polyclonal serum. Lane 3 was incubated with anti-MYC monoclonal antibodies. The preimmune serum shows no reactivity while the anti-MAD and anti-MYC antibodies both recognize a 55kDa Mr protein. (B) A salivary gland from a P[UmMad] lab third instar larva which was incubated with anti-MYC antibodies revealing a cytoplasmic subcellular localization for MAD. The cells nearest the salivary gland’s common duct are on the upper right. (C) P[UmMad] lab third instar larval salivary gland incubated with anti-MYC antibodies revealing the same subcellular localization, though some staining in a few nuclei is seen. The cells nearest the salivary gland’s common duct are at the lower right. (D) Composite confocal micrograph of a stage 16 wild-type embryo which was incubated with anti-MAD serum showing that MAD is ubiquitously expressed. (E) Confocal micrograph (1500x) of ps7 midgut endoderm, from a wild-type embryo of comparable stage to that shown in D. MAD appears to be a cytoplasmic protein in all tissues.
signals elicit cellular and developmental responses is the ability to manipulate elements of the signaling pathway. Our approach relies on genetic screens for the identification of potential candidates and on further analyses to sift among these, pinpointing those most likely to act in the signal transduction cascade. The MAD protein is a prime candidate for such a signal transduction element.

Loss-of-function Mad mutant phenotypes are remarkably similar to dpp mutant phenotypes, including embryonic dorsal/ventral patterning and midgut defects as well as imaginal disk-derived adult appendage defects (Raftery et al., 1995; Sekelsky et al., 1995). Recently, a role for dpp in the developing eye has been described (reviewed in Heberlein and Moses, 1995). A clonal analysis of strong hypomorphic alleles of Mad in eye disks reveals that Mad mutant clones have the same effect on eye development as dpp mutant clones (Wiersdorff et al., 1996). This result reinforces the proposal that every event that requires dpp also requires Mad.

Our analysis of several reporter genes in Mad mutant backgrounds show conclusively that Mad is not involved in regulating dpp transcription. A function for Mad downstream of dpp transcription is demonstrated for DPP-dependent events in ps3 and ps7 of the embryonic midgut including the induction of lab in the endoderm, the maintenance of dpp and Ubx expression and the repression of Scr in the visceral mesoderm. The Scr results support the suggestion (Hursh et al., 1993) that a DPP-mediated autoregulatory loop exists in the visceral mesoderm of ps3 and ps7. The failure of MAD expression in the visceral mesoderm to rescue lab induction demonstrates that MAD is not required post-transcriptionally to generate the DPP signal. However, the restoration of lab induction when MAD is expressed only in the endoderm shows that MAD expression in cells receiving a DPP signal is sufficient to restore a DPP-dependent response.

Consistent with the absence of secretion or nuclear localization signal sequences (Sekelsky et al., 1995), our immunohistochemical studies indicate that MAD is a cytoplasmic protein whose subcellular distribution is unaltered in dpp mutant genotypes. Graff et al. (1996) detect both nuclear and cytosolic localizations for MAD protein. However, their biochemical assay utilizes embryos which display a functional activation signal sequences (Sekelsky et al., 1995); recently, a role for Mad in regulating DPP signal transduction events. The demonstration that Drosophila MAD functions synergistically with Xenopus BMP-4 to specifically induce ventral mesoderm suggests that MAD’s role in DPP signal transduction is highly conserved.

Prior to this study, the only identified gene product that may contribute to the DPP signal transduction pathway is schnurri (shn, Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, it has yet to be established if all DPP-dependent events require shn function. For example, embryos with complete loss of shn maternal and zygotic function do not resemble dpp null embryos (Grieder et al., 1995).

Given that MAD appears to be a component of the DPP signal transduction pathway, several important questions are immediately raised. Is MAD a general factor involved in the signaling of all TGF-β family members in Drosophila or is...
MAD specific to pathways induced by DPP-receptor activation? While no firm answer can be given, the overall concordance of Mad and dpp mutant phenotypes suggests that MAD may be specific to DPP signaling. Further, aspects of the phenotype elicited by mutations in 60A, another Drosophila TGF-β family member, are not shared by Mad mutants (K. Wharton, personal communication).

The multiplicity of Mad-like genes identified in several non-Drosophila species demonstrate that Mad is a member of a protein family and suggest that Drosophila melanogaster will also have multiple Mad-like genes. Results presented here and in Graff et al. (1996) indicate that members of this protein family participate in the interpretation of TGF-β signals. We propose the name DOT (Downstream of TGF-β) for this protein family.

Our primary interest now is to understand how these various DOT proteins contribute to TGF-β signaling. In Xenopus, two different DOT genes produce qualitatively distinct effects on mesoderm induction (Graff et al., 1996), suggesting that different DOT proteins contribute to different TGF-β signal transduction pathways. Whether the multiplicity of DOT proteins is fully explained as elements downstream of different receptors remains to be determined. Another critical issue in understanding DPP signaling is to elucidate how different levels of signal can produce different biological responses. A graded requirement for DPP has been demonstrated for dorsal-ventral patterning of the Drosophila embryo. Modulation of the level of that signal can lead to the establishment of different fates within the developing dorsal ectoderm (Ferguson and Anderson, 1992; Wharton et al., 1993). From a clonal analysis of saxophone mutations, we have suggestive evidence of a graded requirement for DPP in imaginal disk development as well (M. Singer and W. M. G., unpublished data). What is the contribution of MAD and its relatives to the amount and types of signals that are transduced? For example, different levels of DPP receptor activation might lead to quantitatively distinct levels of MAD activation, or alternatively to activation of different constellations of DOT proteins. It is critical to address these and other cellular aspects of MAD function in order to understand the contributions of TGF-β signaling to development.

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