A function of CBP as a transcriptional co-activator during Dpp signalling

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CBP/p300 is a transcriptional co-activator that is recruited to enhancers by various DNA-binding proteins, including proteins whose activity is controlled by extracellular signals. Here, we report that *Drosophila* CBP loss-of-function mutants show specific defects which mimic those seen in mutants that lack the extracellular signal Dpp or its effector Mad. Furthermore, we find that CBP loss severely compromises the ability of Dpp target enhancers to respond to endogenous or exogenous Dpp. Finally, we show that CBP binds to the C-terminal domain of Mad. Our results provide evidence that CBP functions as a co-activator during Dpp signalling, and they suggest that Mad may recruit CBP to effect the transcriptional activation of Dpp-responsive genes during development.

Keywords: CBP/Dpp/Mad/TGF- β /transcription

Introduction

The transforming growth factor- β (TGF- β) family includes a large number of structurally related secreted polypeptides which control cell growth, patterning and differentiation during animal development (reviewed by Kingsley, 1994). During some of these events, TGF- β proteins have morphogenetic properties, i.e. they act across long distances and confer positional information in a concentration-dependent way (Green and Smith, 1990; Ferguson and Anderson, 1992; Green *et al.*, 1992; Gurdon *et al.*, 1994; Lecuit *et al.*, 1996; Nellen *et al.*, 1996). One of the best studied examples is the *Drosophila* TGF- β homolog Decapentaplegic (Dpp) (Padgett *et al.*, 1987). *dpp* is required for several aspects of fly development such as oogenesis (Twombly *et al.*, 1996), dorsoventral axis formation in the young embryo (Irish and Gelbart, 1987), and patterning and growth of adult appendages (Spencer *et al.*, 1982; Lecuit *et al.*, 1996; Nellen *et al.*, 1996). Moreover, *dpp* plays a critical role during mesoderm (Staehling-Hampton *et al.*, 1994; Frasch, 1995) and endoderm induction in fly embryos (Bienz, 1997).

In the visceral mesoderm, *dpp* is expressed in para-segment (ps) 7 under the control of the homeotic gene *Ultrabithorax* (*Ubx*) (Sun *et al.*, 1995). In this cell layer, *dpp* stimulates its own expression and the expression of *Ubx* (Hursh *et al.*, 1993; Thüringer and Bienz, 1993). *dpp* also stimulates the expression of *wingless* (*wg*), an extracellular signalling molecule of the Wnt family, in the

neighbouring ps8 (Immerglück *et al.*, 1990). *wg* in turn feeds back to stimulate *Ubx* (Thüringer and Bienz, 1993) and *dpp* expression in ps7 (Yu *et al.*, 1996). Thus, *dpp* is part of a paracrine feedback loop by which *Ubx* maintains its own expression indirectly through controlling *dpp* and *wg* (Bienz, 1997). Dpp also diffuses from its mesodermal source through the endodermal cell layer of the embryonic midgut where it stimulates the expression of *D-Fos* (Riese *et al.*, 1997a) and of the homeotic gene *labial* (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990). These inductive steps ultimately specify the differentiation of distinct cell types in the larval midgut epithelium (Hoppler and Bienz, 1994, 1995).

During the past few years, several highly conserved components of the TGF- β signalling pathway have been identified. Genetic screens in *Drosophila* for modifiers of *dpp* phenotypes have led to the identification of the genes *Mothers against dpp* (*Mad*) and *Medea* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995). *Mad* is the founding member of the Smad protein family. Smad family members are characterized by two conserved Mad homology domains, MH1 (N-terminal) and MH2 (C-terminal), separated by a linker region of variable sequence and length (Hoodless *et al.*, 1996). Based on structural and functional evidence, Smad proteins have been classified into three groups (Heldin *et al.*, 1997). Class I Smads, such as *Mad*, are direct substrates of the TGF- β receptor kinases. Class II Smads, such as *Medea*, participate in signalling by associating with class I Smads. Finally, class III Smads, such as *Dad* in *Drosophila*, are negative regulators of TGF- β signalling.

Evidence from various experimental systems suggests the following model for TGF- β signalling (Massagué, 1998). In the absence of signalling, Smad proteins remain inactive in the cytoplasm due to an inhibitory interaction between their MH1 and MH2 domains. TGF- β ligands signal by promoting the formation of transient complexes between two types of transmembrane serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor (encoded by *punt* in *Drosophila*) activates the type I receptor (*saxophone* or *thick veins*), which in turn phosphorylates conserved serine residues in the MH2 domain of a ligand-specific class I Smad. The phosphorylated Smad then interacts with class II Smads, and the heteromeric complex translocates to the nucleus. Smad complexes finally activate the transcription of TGF- β target genes by binding directly to their enhancers. DNA-binding proteins such as Fast-1 may be involved in their recruitment to DNA. Although the C-terminal domains of Smad1 and Smad2 activate transcription in a ligand-independent way when fused to GAL4 DNA-binding domains (Liu *et al.*, 1996, 1997), the mechanism of transactivation by Smad proteins remains unclear.

In order to understand the mechanism by which *dpp*

stimulates transcription, we have previously characterized a short enhancer fragment of *Ubx*, called *Ubx* B, which contains response sequences for *dpp* and *wg* signalling in the embryonic midgut (Thüringer *et al.*, 1993; Eresh *et al.*, 1997; Riese *et al.*, 1997b; Szüts *et al.*, 1998; Yu *et al.*, 1998). The *dpp* response sequence of this enhancer is bipartite, consisting of a tandem repeat of Mad binding sites and a cAMP response element (CRE) (Eresh *et al.*, 1997; Szüts *et al.*, 1998). The presence of the latter raised the question whether the co-activator CBP (CREB-binding protein, binding to CREs) might participate in *dpp*-induced transcriptional activation. CBP was originally identified as a co-activator of CREB and is highly related to the protein p300 (Arias *et al.*, 1994; Eckner *et al.*, 1994; Kwok *et al.*, 1994). CBP/p300 was shown to bind to and function as a co-activator of a large number of transcription factors, including transcription factors whose activity is regulated by extracellular signals (Goldman *et al.*, 1997). The ability of CBP to bind to multiple signal-activated transcription factors has led to the proposal that CBP integrates positive and negative inputs from various signalling pathways (Eckner *et al.*, 1996; Janknecht and Hunter, 1996; Kamei *et al.*, 1996).

Our knowledge of how CBP functions is mostly derived from biochemical *in vitro* assays and from co-transfection experiments. However, genetic analyses have recently revealed new *in vivo* functions of CBP. In man, CBP loss-of-function is associated with the development of Rubinstein-Taybi syndrome, characterized by multiple developmental defects, severe mental retardation and increased tumour incidence (Petrij *et al.*, 1995). Moreover, somatic mutations in *p300* and different chromosomal translocations involving *CBP* or *p300* have been associated with cancer, suggesting that *CBP* and *p300* could be tumour suppressor genes (Borrow *et al.*, 1996; Muraoka *et al.*, 1996; Ida *et al.*, 1997; Sobulo *et al.*, 1997). Knock-outs of *CBP* and *p300* in the mouse have revealed that these genes are required in a dose-dependent manner for embryonic development and for cell proliferation (Yao *et al.*, 1998). In *Drosophila*, *CBP* loss-of-function mutations cause embryonic lethality and various pattern formation defects (Akimura *et al.*, 1997a). Specifically, *Drosophila* CBP, also called *nejire* (*nej*), was shown to be a co-activator for Cubitus interruptus during *hedgehog* signalling, and also for Dorsal (Akimura *et al.*, 1997a,b). Finally, in contrast to CBP's well established role as a co-activator, we have recently discovered that *Drosophila* CBP acts as a repressor and antagonizes Wg signalling by inhibiting the binding between TCF (T cell factor), the transcription factor activated by Wg, and its co-activator Armadillo (Waltzer and Bienz, 1998). During the course of this work, we observed some phenotypes that suggested an activating role of CBP during Dpp signalling.

We therefore decided to explore further this putative role of CBP during Dpp signalling. Here, we report that *nej* mutants show *dpp*- and *Mad*-like phenotypes in multiple developmental contexts, that *nej* interacts genetically with a weak allele of *dpp*, and that it acts downstream of Dpp itself and of the Dpp receptor Sax. Furthermore, we show that the transcriptional response to *dpp* of two distinct enhancers is compromised in *nej* mutants. Finally, we show that *Drosophila* CBP binds to the MH2 domain of Mad. Our results strongly suggest

that CBP is a co-activator of Mad, mediating *dpp*-induced transcriptional stimulation.

Results

The function of Drosophila CBP in the embryonic midgut

The embryonic midgut of *nej* mutants (whose CBP function is reduced) show phenotypes related to *wg* gain-of-function phenotypes such as increased *labial* expression in the endoderm, and derepression of the *Ubx* B enhancer in the visceral mesoderm (Waltzer and Bienz, 1998; Figure 1, compare B with A). These phenotypes do not resemble those seen in *dpp* or *Mad* mutants: in *Mad*^{l2} mutants, *labial* expression is strongly reduced (Newfeld *et al.*, 1996), and so is the β -galactosidase (*lacZ*) staining mediated by the *Ubx* B enhancer in the middle midgut (Figure 1C). However, we also noticed that the narrow band of *lacZ* staining normally visible in the visceral mesoderm of the gastric caeca (in ps3; Figure 1A and D, arrowheads) is absent in *nej*³ embryos (Figure 1B and E, open triangles). Indeed, closer inspection revealed that the gastric caeca frequently fail to elongate in these mutants (Figure 1B and E). A similar phenotype is observed in *Mad*^{l2} (Figure 1C) and in *dpp* mutants (Panganiban *et al.*, 1990). Thus *nej*, like *dpp*, is required for the formation of the gastric caeca, and also for the activity of the *Ubx* B enhancer in the caecal primordia. Note that the activity of this enhancer in these primordia coincides with Dpp expression and depends on *dpp* function (Thüringer and Bienz, 1993).

We also observed that the formation of the first midgut constriction is often impeded (not shown; see Figure 2B in Waltzer and Bienz, 1998). While this could reflect overactive Wg signalling, it also mimics loss of *glass bottom boat* (*gbb*) signalling: *Gbb* is a Dpp homolog which is expressed in the visceral mesoderm and whose function is required for the formation of the first midgut constriction (Doctor *et al.*, 1992; Khalsa *et al.*, 1998; K. Wharton, personal communication).

CBP loss-of-function reduces the activity of a dpp-responsive enhancer in the visceral mesoderm

We decided to test the hypothesis that CBP is a co-activator of *dpp*-induced transcription by examining the Dpp response of the *Ubx* enhancer in *nej* mutants. Because we expected the repressive effect of CBP on this enhancer to mask a possible activating effect of CBP in cells in which the enhancer is stimulated by Wg signalling (Waltzer and Bienz, 1998), we resorted to a mutant version of *Ubx* B, called B4, whose positive response to Wg is abolished (Riese *et al.*, 1997b). B4 activity in the midgut is reduced compared with the wild-type enhancer; however, B4 still contains a fully functional *dpp*-response sequence and can be efficiently stimulated by ectopic Dpp (Riese *et al.*, 1997b). B4 can thus be used to monitor selectively the stimulation of *Ubx* by Dpp in the visceral mesoderm.

We found that the activity of *Ubx* B4 is significantly reduced in *nej*³ mutants (Figure 2, compare B with A). *LacZ* staining is particularly weak in ps6/7 (near the Dpp source), but also in ps10, and is barely detectable in the gastric caeca (open triangles in Figure 2B). Furthermore, in *nej*¹ embryos derived from *nej*¹ mutant germlines (*nej*¹ GLC), *lacZ* staining mediated by B4 is even weaker than

Fig. 1. CBP function in the gastric caeca. Dorsal views of ~14 h old embryos bearing the *Ubx* B enhancer, stained with antibody against lacZ. (A and D) Wild-type; (B and E) *nej*³ hemizygote; (C) *Mad*^{l2} homozygote; (D and E) high magnification views of the anterior midgut showing the gastric caeca (arrowheads). *Ubx* B activity is enhanced in the midgut visceral mesoderm of *nej*³ mutant embryos [arrows in (B)], but strongly reduced in the incipient gastric caeca [compare open triangles in (B and E) with arrowheads in (A and E)]. Enhancer activity is drastically reduced throughout the midgut in *Mad*^{l2} mutants [open triangles in (C)], and the gastric caeca do not form properly. Anterior to the left.

in the zygotic *nej*³ mutants (Figure 2C): although these *nej*^l GLC embryos are somewhat variable in terms of their phenotypes (Waltzer and Bienz, 1998), the most severely mutant embryos show lacZ staining in only a few cells in the ps8 region. Similarly, in *Mad*^{l2} mutant embryos, lacZ staining is much reduced, with some staining remaining in ps6 and ps8 (Figure 2D). This implies that CBP, like Mad, is required for the Dpp response of the *Ubx* B4 enhancer.

We also examined the response of B4 to GAL4-mediated ectopic Dpp in *nej* mutant embryos. If Dpp is expressed throughout the mesoderm, B4-mediated lacZ staining is increased and detectable throughout the midgut mesoderm (Riese *et al.*, 1997b; Figure 2E). In *nej*³ mutants, this

response of B4 to ectopic Dpp is strikingly disabled (open triangles in Figure 2F): there is barely any lacZ staining in the anterior midgut, and only a moderate increase of lacZ staining in the ps8/9 region indicating a residual Dpp response in this region. These results strongly support our conclusion that CBP is required for the transcriptional response of the *Ubx* enhancer to Dpp signalling. They argue that CBP functions downstream of the Dpp signal.

CBP is required for the activity of a Dpp-responsive blastoderm enhancer

In the early blastoderm embryo, *dpp* mediates the subdivision of the dorsal ectoderm into two embryonic tissues,

Fig. 2. CBP function in the Dpp response of the *Ubx* midgut enhancer. Side views of ~14 h old embryos, bearing the *Ubx* B4 enhancer (A–C) or *Ubx* B4 as well as 24B.Gal4/UAS.Dpp (E and F), stained as in Figure 1. (A and E) Wild-type; (B and F) *nej*³ hemizygotes; (C) *nej*¹ GLC; (D) *Mad*¹² homozygote. *Ubx* B4 activity is reduced in *nej* (B, C) and *Mad* (D) mutants (open triangles), and its response to ectopic Dpp is disabled in these mutants [open triangles in (F); not shown]. Anterior to the left, dorsal up.

the amnioserosa and the dorsal epidermis (Irish and Gelbart, 1987). High Dpp levels in the dorsal-most cells specify amnioserosa while lower Dpp levels in dorsolateral regions specify epidermis (Ferguson and Anderson, 1992). Expression of the gene *Race* (*related to angiotensin converting enzyme*; the earliest known marker for the amnioserosa) in the dorsal blastoderm embryo depends on *dpp* signalling (Tatei *et al.*, 1995). We thus asked whether the activity of the *Race* enhancer (Rusch and Levine, 1997) depends on CBP function. As previously shown (Rusch and Levine, 1997; Figure 3A), this enhancer mediates lacZ staining in the presumptive amnioserosa and in the anterior midgut primodium, whereby the former but not the latter staining requires *dpp*. We found that, in *nej*¹ GLC embryos, there is no detectable lacZ staining in the presumptive amnioserosa (open triangles in Figure 3B) while staining remains, and is even slightly enhanced in the head and in the anterior midgut primordium (arrows in Figure 3A and B). This demonstrates that the *Race* enhancer depends on an activating function of CBP exclusively in a subset of the blastoderm cells, namely in the dorsal-most cells of the embryonic trunk. It suggests that CBP is required for the response of this enhancer to *dpp*.

A *dpp*-like phenotype of CBP loss-of-function in the developing tracheae

To see whether CBP may be required in other developmental contexts in which *dpp* functions, we decided to examine the developing tracheae in *nej* mutant embryos. The tracheal system develops from segmentally repeated clusters of ectodermal cells, the tracheal placodes. These cells undergo a complex process of migration and fusion to generate the final branched structure of the tracheal system. *dpp* signalling plays a crucial role in this process, and has been implicated in the dorsoventral migration of certain tracheal branches (Affolter *et al.*, 1994; Vincent *et al.*, 1997). For example, in *punt* or *thick veins* mutants, the branches that normally migrate towards dorsal or ventral (the dorsal and ganglionic branches, respectively) fail to develop whereas the branches which grow out anteriorly (the dorsal trunk and the visceral branches) are essentially not affected (Affolter *et al.*, 1994).

We examined the tracheae in *nej*³ mutant embryos using an antibody which stains the lumina of the tracheal trees (2A12; Affolter *et al.*, 1994). We found that the dorsal trunk and the visceral branches are essentially normal in these mutants (Figure 4A and B). We occasionally saw a lack of staining in the dorsal trunk, but

Fig. 3. CBP function in the amnioserosa of the blastoderm embryo. Side views of late blastoderm embryos, bearing the Dpp-responsive *Race* enhancer, stained as in Figure 1. (A) Wild-type; (B) *nej^l GLC*. Enhancer activity in the presumptive amnioserosa (where it is *dpp*-responsive; Rusch and Levine, 1997) is undetectable in *nej* mutants (open triangles) while this activity remains strong in the head and in the anterior midgut primordium in these mutants (arrows).

inspection under Nomarski optics clearly revealed that this trunk was always fully formed; in other words, the lack of 2A12 antibody staining in these cases is likely to be due to an effect of the *nej* mutation that is subsequent to tracheal migration. However, in most *nej³* mutant embryos (11/14), we see branching defects: usually, one or two dorsal branches fail to form at each side, and ganglionic branches fail to fuse (Figure 4B, open triangles). Essentially the same defects were also seen in *nej^l GLC* embryos (not shown). These defects resemble those found in *punt* hypomorphs (Vincent *et al.*, 1997) and in *Mad^{l2}* mutant embryos, although the most apparent defects in the latter mutants are the fusion defects in their ganglionic branches (Figure 4E, open triangles). Once again, the similarity of the tracheal phenotypes between *nej* mutants on one hand, and *dpp*, *punt* and *Mad* mutants on the other hand suggest that CBP may be required during Dpp signalling.

***dpp*-related functions of CBP in the developing wing**

dpp promotes vein development during pupal stages, and a subclass of *dpp* mutant alleles cause loss of veins (Segal and Gelbart, 1985; St Johnston *et al.*, 1990). In particular, in *dpp^{SI}* homozygous flies, vein 4 fails to reach the margin (Figure 5, compare B with A). We exploited this weak *dpp* allele to see whether there is a genetic interaction between *dpp* and *nej*.

Indeed, while *nej³* heterozygosity on its own shows no abnormality whatsoever in the wing (not shown), this condition clearly enhances the vein phenotype of *dpp^{SI}* homozygotes: in many of the wings from flies of this

genetic constitution, neither vein 4 nor vein 2 reaches the margin (Figure 5C). This synergy in the wing between *nej* and *dpp* loss-of-function alleles is consistent with our notion that CBP functions during Dpp signalling.

To clarify the position of CBP in this Dpp response in the wing, we asked whether the mild *dpp* overactivation phenotype due to overexpression of a constitutively active form of Sax (*Sax**), a Dpp type I receptor, depends on *nej* gene dosage. Expression of *Sax** under the control of engrailed.GAL4 induces ectopic venation and overgrowth of the posterior part of the wing (Das *et al.*, 1998; Figure 5D). Moreover, removal of one copy of genes required for Sax signalling such as *Mad* or *Medea* suppresses this phenotype (Das *et al.*, 1998; Figure 5F). Likewise, we find that *nej³* heterozygosity suppresses the wing phenotypes caused by *Sax** to a considerable extent (Figure 5E). Note that this suppression cannot be attributed to a general effect of CBP loss on GAL4-mediated phenotypes since *nej³* heterozygosity enhances the wing phenotypes that are produced by GAL4-mediated overexpression of Armadillo (Waltzer and Bienz, 1998; unpublished results). Therefore, our result is consistent with CBP being required for Sax signalling, and it indicates that CBP functions downstream of this Dpp receptor.

CBP binds to the MH2 domain of Mad

As Mad mediates transcriptional activation by *dpp* (Kim *et al.*, 1997; Szüts *et al.*, 1998; Xu *et al.*, 1998) and appears to be a transcription factor required for every aspect of *dpp* signalling (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995; Wiersdorf *et al.*, 1996; Newfeld *et al.*, 1997), we asked whether CBP might be recruited by Mad as a transcriptional co-activator. To test whether CBP might bind to Mad, we used the yeast two-hybrid system. When we began our binding studies, *Drosophila* CBP had not yet been discovered. So we used fragments of mouse CBP to test whether these might bind to Mad, assuming that a putative interaction between the two proteins would be conserved. Indeed, there is a strong degree of homology between mouse and *Drosophila* CBP (Akimura *et al.*, 1997; Figure 6A). We used a set of fragments of mouse CBP that cover the whole protein and fused these to a transcriptional activation domain ('prey'). This series of prey was tested in two-hybrid assays in yeast with full-length Mad protein fused to the LexA DNA-binding domain ('bait'). We found that the C-terminal domain of mouse CBP (CBP1678 to 2441) interacted specifically with Mad in this assay (Figure 6B).

We confirmed this interaction using a similar set of prey with fragments from the *Drosophila* CBP protein which we tested against a series of baits containing different Mad domains. This revealed that a fragment of *Drosophila* CBP that spans amino acids 2240–2608 (which overlaps the above mentioned C-terminal domain of mouse CBP; Figure 6A) interacted specifically with the MH2 domain of Mad (Mad219–455). These specific interactions in the yeast two-hybrid assay between CBP fragments and Mad almost certainly reflect direct binding since yeast does not encode any proteins homologous to either of these. We note that the interactions between CBP fragments and Mad were significantly stronger if the N-terminal domain of Mad was removed, suggesting that MH1 inhibits

Fig. 4. CBP function in the tracheal system. Dorsolateral (A and B) and ventrolateral (C–E) views of 12–15 h old embryos, stained with 2A12 antibody which visualizes tracheal lumina. (A and C) Wild-type; (B and D) *nej*³ hemizygotes; (E) *Mad*¹² homozygote. *nej* mutants fail to develop some of the dorsal branches [open triangles in (B)] and show fusion and migration defects in the ventral ganglionic branches [open triangles in (D)]; *Mad* mutants show defects primarily in the ganglionic branches [open triangles in (E)]. The formation of the dorsal trunk (dt) was not affected in these mutants (note that not all tracheal branches can be shown in the same focal plane; some of the apparent interruptions in staining thus do not signify defects). Anterior to the left.

the binding of CBP to MH2. Inhibitory interactions between MH1 and MH2 have been described previously (e.g. Liu *et al.*, 1996).

To confirm these results, we tested direct binding between Mad and CBP *in vitro* with pull-down assays. In these assays, we used [³⁵S]methionine-labelled Mad domains and various fragments from *Drosophila* CBP expressed as GST fusion proteins and immobilized on GST–Sepharose beads. Our results show that either full-length Mad or its MH2 domain bind to the same *Drosophila* CBP fragment that interacted with Mad in the yeast assay while Mad's MH1 domain does not bind CBP (Figure 6D). Interestingly, deletion of the C-terminal of Mad's MH2 domain (amino acids 372–455) abolished CBP interaction, demonstrating that the C-terminal 84 amino acids of Mad are required for binding to CBP. The linker domain (L) between MH1 and MH2 seems to be dispensable for Mad interaction with CBP. We also observed weak binding of full-length Mad to CBP2 (CBP2240–2507; Figure 6D), the highly conserved domain of *Drosophila* CBP that overlaps the Mad-binding fragment of CBP, however, the significance of this binding is uncertain as we could not detect this binding activity in the reciprocal assay nor in yeast. Finally, none of the *in vitro* translated Mad proteins showed any significant

binding to GST alone nor to CBP1 (CBP896–1050), another highly conserved domain of *Drosophila* CBP (Figure 6A and D). These results fully confirmed those obtained in yeast.

Finally, to characterize more precisely the mutual binding domains within Mad and *Drosophila* CBP, we performed the reciprocal experiment using [³⁵S]methionine-labelled C-terminal fragments of CBP and various GST–Mad fusion proteins. As found previously, CBP bound to GST–MH2, but not to GST alone, nor to GST–MH1+L fusion proteins which include extended MH1 fragments (Mad1–241), nor to GST–MH2ΔC fusion protein in which the last 84 amino acids of Mad's MH2 domain are deleted.

Deletion mapping of the C-terminal region of CBP revealed a minimal fragment of CBP (CBP2413–2608) that is sufficient for binding to Mad (Figure 6E). This domain partially overlaps the highly conserved CBP2 domain but, as mentioned above, in most of our binding assays, CBP2 by itself was not sufficient for binding to Mad (Figure 6C and D).

Altogether, these experiments demonstrate that CBP and Mad bind to each other, and that the stretch between amino acids 2507 and 2640 within *Drosophila* CBP is critical for CBP's binding to the MH2 domain of Mad.

Fig. 5. CBP function in the wing. (A) Wild-type wing; longitudinal veins L2–L5 are labelled. (B) *dpp^{S1}/dpp^{S1}* (open triangle indicates defect in L4). (C) *nej³/+; dpp^{S1}/dpp^{S1}* (open triangle indicates defect in L4 and L2). (D–F), UAS.Sax*/engrailed.GAL4 in a wild-type (D), *nej³/+* (E) or *Mad¹²/+* (F) genetic background; venation and growth defects caused by Sax* are suppressed by *nej* and by *Mad* heterozygosity. Anterior up.

Discussion

In the last few years, there has been rapid progress in our understanding of the TGF- β signalling pathway. The identification of the Smad protein family has laid the basis for the dissection of the mechanisms of intracellular signalling induced by TGF- β -like proteins. For example, it is reasonably well understood how Smad proteins are activated in the cytoplasm by the signalling. However, it is less clear how activated Smads stimulate transcription. Here, we have examined the function of the transcriptional co-activator CBP in *dpp* signaling. We provide evidence that Mad recruits CBP as a co-activator to effect transcriptional stimulation of *dpp* target genes.

CBP is required for *dpp* signalling during development

We have found that CBP loss-of-function mimics Dpp loss-of-function at multiple stages of fly development. In the early embryo, CBP appears to be involved in *dpp*-mediated dorsoventral patterning as indicated by the apparent inability of the amnioserosa-specific *Race* enhancer to respond to *dpp* in *nej* mutants. Later in development, CBP is required for *dpp*-induced stimulation of the *Ubx* enhancer in the visceral mesoderm. At this stage, the embryonic midgut of *nej* mutants shows mutant phenotypes that mimic loss of *dpp* (in the gastric caeca)

or loss of the *dpp*-related ligand *gbb* (in the first midgut constriction). Loss of CBP also affects the *dpp*-dependent migration of the tracheal branches along the dorsoventral axis. Finally, loss of CBP worsens the vein phenotype of a weak *dpp* allele in the wing and alleviates the mutant wing phenotype due to a constitutively active Dpp receptor.

This strong correlation between *nej* and *dpp* phenotypes in multiple developmental contexts indicates a function of CBP in Dpp signalling. However, we are aware that many if not all of the developmental processes we have examined are not only controlled by *dpp*, but also by other signalling pathways. In particular, signalling through receptor tyrosine kinases controls some of the processes that we have described. In the embryonic midgut, there is an intimate link between Dpp and EGF receptor signalling (Szűts *et al.*, 1998). These synergize in transcriptional stimulation of the *Ubx* enhancer, and their loss produces similar mutant phenotypes, including the ones observed in *nej* mutants (namely lack of the first constriction and of the gastric caeca; Szűts *et al.*, 1998; unpublished results). Likewise, vein formation in the wing depends critically on EGF receptor signalling (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; Díaz-Benjumea and Hafen, 1994). On the basis of the midgut and wing phenotypes alone, it would be difficult to discriminate between a requirement for CBP in Dpp versus EGF receptor signalling. However, in the

Fig. 6. Binding between CBP and Mad. **(A)** The structural organization of mouse and *Drosophila* CBP (mCBP and dCBP) is shown, with the most conserved regions indicated (including CBP1, CBP2 and the histone acetyltransferase domain, HAT). **(B)** Interaction tests between different fragments of mouse CBP and full-length Mad in the yeast two-hybrid assay, revealing that the C-terminal domain of mouse CBP interacts with Mad. **(C)** Interaction tests between different fragments of *Drosophila* CBP and different domains of Mad in the yeast two-hybrid assay, showing that a C-terminal fragment of CBP interacts specifically with the MH2 domain of Mad (the equivalent interaction between mouse CBP and MH2 is indicated, as a comparison); the strength of interactions (ranging from negative, -, to strongly positive, +++) is based on semiquantitative estimates of lacZ activity from culture spots on galactose plates (prey protein expressed; no lacZ activity was detected on control glucose plate). **(D and E)** Mapping of CBP and Mad binding domains by *in vitro* pull-down assays. Equimolar amounts of the different GST fusion proteins were incubated with *in vitro* translated [³⁵S]methionine-labelled protein fragments as indicated; 20% of the total reactions was loaded in the input lanes (I).

tracheal system, whose formation not only depends on EGF but also on fibroblast growth factor (FGF) receptor signalling, the processes that are affected by these receptors are distinct from those affected by *dpp*: while the latter, like *nej*, controls dorsoventral migration of the dorsal and ganglionic branches (Affolter *et al.*, 1994; Vincent *et al.*, 1997), the former control anteroposterior migration, dorsal trunk and visceral branch formation (Klambt *et al.*, 1992; Sutherland *et al.*, 1996; Llimargas and Casanova, 1997;

Wappner *et al.*, 1997), all of which are unaffected by CBP loss. Also, there is no known requirement for tyrosine kinase receptor signalling in the presumptive amnioserosa where we see a requirement for CBP. Therefore, the latter two requirements of CBP argue for a specific requirement for CBP in Dpp signalling. This is strongly supported by the results from our biochemical analysis which demonstrate that CBP binds to Mad (see below).

Previous analyses of *nej* mutants have implicated CBP

in *hedgehog* and *Wg* signalling (Akimura *et al.*, 1997a; Waltzer and Bienz, 1998), and also in ventral specification of the early embryo (Akimura *et al.*, 1997b). As far as we are aware, none of the mutant phenotypes that we have described here can be explained by any of these other functions of CBP.

CBP is required for the Dpp response of different enhancers

We have found that the activity of different *dpp*-responsive enhancers is strongly reduced in *nej* mutants. Given that CBP has been shown to be a co-activator for a large number of transcription factors in mammals (Goldman *et al.*, 1997), and a co-activator for Cubitus interruptus and Dorsal in *Drosophila* (Akimura *et al.*, 1997a,b), one might suspect that a decreased enhancer activity might reflect a general positive requirement of CBP for transcriptional activation.

However, several lines of evidence argue against this. First, the activity of the *Race* enhancer is reduced in the presumptive amniosera, but remains high (or is even slightly derepressed) at the same embryonic stage in adjacent cells in the head. Thus, there is a clear region-specific effect of CBP loss which correlates with the region in which *dpp* regulates the activity of the enhancer (Rusch and Levine, 1997). Secondly, while the activity of the mutant *Ubx* B4 enhancer (lacking the *wg* response sequence) is reduced in *nej* mutants, the activity of the wild-type enhancer is increased in these mutants. Furthermore, this derepression of the wild-type enhancer is only observed in the middle midgut region (around the *Wg* signalling source; Waltzer and Bienz, 1998) while its activity is reduced in the anterior midgut (in the gastric caeca; Figure 1), once again revealing a region-specific effect of CBP loss.

Clearly, CBP loss does not always lead to loss of enhancer activity. Since the regions in which it does so correlate with regions of endogenous (or exogenous) Dpp expression, and with regions whose development is controlled by *dpp*, this strongly suggests that the observed activation function of CBP in these regions participates in the *dpp*-induced transcriptional stimulation of these enhancers.

CBP is a co-activator for Mad

Our biochemical experiments showed that CBP binds to Mad. This strongly supports our conclusions from the genetic analysis that implicates CBP in Dpp signalling. In particular, it is consistent with our conclusion that CBP acts downstream of Dpp and of a constitutively active Dpp receptor. Taken together, these lines of evidence suggest that Mad recruits CBP to Dpp-responsive enhancers, and that, once recruited, CBP stimulates the transcription of the linked Dpp target genes.

This close functional relationship between Mad and CBP seems to be conserved: it was shown recently by biochemical and co-transfection analysis that mammalian CBP binds to and is a co-activator for Smad2 and Smad3 (Feng *et al.*, 1998; Janknecht *et al.*, 1998; Topper *et al.*, 1998). As in flies, the region of the mammalian Smad proteins which binds to CBP is the MH2 domain. This could explain why this domain is absolutely required for Smad proteins to transduce the TGF- β signal and why

MH2 has transactivation potential when targeted to a gene by an heterologous DNA-binding domain. Nonetheless, conflicting results have been obtained concerning the requirement for the C-terminal amino acids in the MH2 domain. On one hand, we found that deleting the last 84 amino acids of Mad abolishes its binding to CBP, in agreement with the data of Feng *et al.* (1998) who showed that the last 35 amino acids of Smad3 are required for its binding to CBP. This may be significant as this part of the MH2 domain contains the site phosphorylated by activated receptors (Massagué, 1998). On the other hand, Janknecht *et al.* (1998) observed binding between mouse CBP and Smad3 protein lacking its 83 C-terminal amino acids. The reasons for this discrepancy are unknown. In any case, all three sets of domain mapping experiments identify MH2 as the domain which binds to CBP.

Regarding the portion of CBP that binds to Smads, our results agree with those obtained by Janknecht *et al.* (1998) and Feng *et al.* (1998). All three sets of experiments identify a region of CBP critical for binding to Smads which abuts the highly conserved CBP2 (or C/H3) domain at its C-terminal. CBP2 itself however is neither required nor sufficient for binding. Surprisingly, the minimal Mad-interacting region within CBP does not show any detectable primary sequence conservation.

Thus, our results and the results from mammalian systems strongly suggest that type I Smads recruit CBP as a co-activator to effect the transcription of TGF- β target genes. This is a significant step forward in our understanding of the mechanism of how TGF- β signalling stimulates transcription since it links the type I Smad proteins to the transcriptional machinery. However, we consider it likely that Smads act together with other transcription factors to recruit CBP to TGF- β target genes. This is indicated by the observation that TGF- β response elements are often bipartite and include not only Smad binding sites, but also sequence motifs to which CBP-recruiting proteins bind such as CREB or AP1 (Eresh *et al.*, 1997; Yingling *et al.*, 1997; Szüts *et al.*, 1998). Moreover, it has recently been found that Smads cooperate with AP1 proteins during TGF- β -mediated transcriptional activation (Zhang *et al.*, 1998). CBP-binding proteins may thus assist Mad in recruiting CBP.

Finally, Smads are not likely to be involved in the initial recruitment of CBP to TGF- β target genes. Bear in mind that Smads become nuclear only after phosphorylation by the ligand-activated TGF- β receptors. On the other hand, it is plausible that enhancer complexes are preassembled by architectural factors such as TCF on signal-responsive genes which are thus poised for subsequent activation upon signalling (Bienz, 1998). TCF may recruit CBP to genes like *Ubx*, but in the absence of signalling, CBP inhibits TCF rather than functioning as its transcriptional co-activator (Waltzer and Bienz, 1998). As a result of Dpp signalling, Mad becomes nuclear and presumably part of this preassembled enhancer complex where it may prise away (with putative partner proteins, see above) CBP from TCF. As a result, inhibition of TCF by CBP may be relieved, and transcriptional activation of the linked target gene may become possible. This scenario assumes that it is the specific interaction between CBP and its recruiting DNA-binding proteins which determines whether CBP can link up with the basal transcription

machinery to effect transcription. Switching CBP from repressing to activating functions by distinct CBP-recruiting proteins might be an important aspect of the much discussed signal-integrating function of CBP.

Material and methods

Fly strains

The following mutant alleles or transformants were used: *nej³*, *nej¹* (Akimura *et al.*, 1997a,b), *Mad¹²* (Sekelsky *et al.*, 1995), *dpp^{SL}* (St Johnston *et al.*, 1990), UAS.Dpp (Staehling-Hampton and Hoffmann, 1994), 24B.GAL4 (Brand and Perrimon, 1993), UAS.Sax* (Das *et al.*, 1998), engrailed.GAL4 (Sanson *et al.*, 1996), *Ubx B* (Bhz, Thüringer *et al.*, 1993), *Ubx B4* (Riese *et al.*, 1997b), *Race.lacZ* (Rusch and Levine, 1997). To express the activated Sax receptor (Sax*) in the wing, we generated recombinant chromosomes between UAS.Sax* and engrailed.GAL4. Females carrying germline clones of *nej¹* were generated as described by Akimura *et al.* (1997b) using the FLP-DFS system (Chou *et al.*, 1993).

Phenotypic analysis

Standard crosses were set up and embryos were collected at 25°C. All mutants were identified either by their midgut phenotypes or by the use of 'blue' balancer chromosomes. The following monoclonal antibodies were used: anti-lacZ (Promega), 2A12 (Affolter *et al.*, 1994). Antibody stainings and analysis of wings were done as described (Waltzer and Bienz, 1998).

In vitro binding assays

Various domains of Mad and *Drosophila* CBP were cloned into pGEX-2T and pT7βlink using standard cloning procedures. GST fusion proteins were produced in *Escherichia coli* BL21 and purified by affinity chromatography on GST-Sepharose beads (Pharmacia). *In vitro* translated and [³⁵S]methionine-labelled proteins were incubated with immobilized GST fusion proteins for 2 h at 4°C in buffer A (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 300 mM KCl, 0.1% Triton X-100). After extensive washing in buffer A, bound proteins were eluted in SDS-loading buffer, separated by SDS-PAGE and visualized by autoradiography.

Yeast two-hybrid assays

Interaction assays in yeast were performed as described previously by Gyuris *et al.* (1993). Different domains of Mad were cloned by PCR into the bait vector pEG202, and various portions of *Drosophila* and mouse CBP were cloned into the prey vector pJG4-5. The yeast strain EGY148-*lacZ* which contains an integrated LexA-*lacZ* reporter gene was transformed with different combinations of prey and bait plasmids. β-gal activity of the resulting transformants was assayed on galactose (prey expressed) or glucose (prey not expressed) plates. Details of the plasmids used are available upon request.

Acknowledgements

We thank Bill Gelbart for the Mad cDNA, Marek Mlodzik, Markus Affolter, Richard Padgett and Michael Levine for fly strains, Marta Llimargas for the 2A12 antibody, and Fiona Townsley and Matthew Freeman for helpful comments on the manuscript. L.W. is supported by an EMBO long-term fellowship.

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Received December 18, 1998; revised and accepted January 22, 1999