Direct competition between Brinker and *Drosophila* Mad in Dpp target gene transcription

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Brinker is a nuclear protein that antagonizes Dpp signalling in *Drosophila*. Its expression is negatively regulated by Dpp. Here, we show that Brinker represses *Ultrabithorax* (*Ubx*) in the embryonic midgut, a HOX gene that activates, and responds to, the localized expression of Dpp during endoderm induction. We find that the functional target for Brinker repression coincides with the Dpp response sequence in the *Ubx* midgut enhancer, namely a tandem of binding sites for the Dpp effector Mad. We show that Brinker efficiently competes with Mad *in vitro*, preventing the latter from binding to these sites. Brinker also competes with activated Mad *in vivo*, blocking the stimulation of the *Ubx* enhancer in response to simultaneous Dpp signalling. These results indicate how Brinker acts as a dominant repressor of Dpp target genes, and explain why Brinker is a potent antagonist of Dpp.

INTRODUCTION

Signalling by Dpp and other TGF- β family proteins affects phosphorylation of cytoplasmic Smad proteins. As a result, activated Smads translocate to the nucleus and stimulate the transcription of TGF- β target genes (Massagué, 1998). Smad-induced transcriptional switches determine multiple cell fates during normal animal development (Raftery and Sutherland, 1999). In mice and man, Smads and TGF- β receptors also function as tumour suppressors (Massagué *et al.*, 2000).

Recently, a nuclear protein called Brinker (Brk) has been discovered that antagonizes the Dpp response in *Drosophila* (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a,b; Minami *et al.*, 1999). Brinker negatively regulates Dpp target genes in multiple developmental contexts and is itself repressed by Dpp signalling. This repression is mediated by Schnurri, a nuclear zinc finger protein whose expression is induced by Dpp near Dpp signalling sources (Marty *et al.*, 2000). Hence, the transcriptional activation of Dpp target genes reflects both a

positive and a double-negative event, namely the activation of the Mad agonist and the removal of the Brinker antagonist by the Schnurri repressor.

Among the direct Dpp target genes are the HOX genes *Ultrabithorax* (*Ubx*) and *labial* (Tremml and Bienz, 1992; Hursh *et al.*, 1993; Thüringer and Bienz, 1993), which partake in *Drosophila* endoderm induction. This process is initiated in the visceral mesoderm by *Ubx*, which activates, and also responds to, the localized expression of Dpp and Wingless (Wg), while *labial* is the ultimate target of these extracellular signals in the subjacent endoderm (Bienz, 1996). The midgut enhancers of *Ubx* and *labial* contain binding sites for Mad (Kim *et al.*, 1997) that mediate the response to Dpp stimulation (Szüts *et al.*, 1998).

Here, we show that Brinker represses *Ubx* in the embryonic midgut. The functional target for Brinker repression within the *Ubx* midgut enhancer coincides with tandem Mad binding sites. We find that Brinker efficiently competes with Mad *in vitro* for binding to these sites and that it prevents activated Mad from stimulating Dpp target enhancers *in vivo*. These findings indicate a mechanism by which Brinker could dominate over activated Mad to block the activity of Dpp target genes.

RESULTS

The control of *Ubx* by Dpp and Wg signalling has been studied by functional dissection of a minimal midgut enhancer called *Ubx* B (Thüringer *et al.*, 1993). This enhancer directs expression of a linked β -galactosidase (*lacZ*) gene in parasegments (ps) 6–9, and also in ps3, of the midgut mesoderm as a result of stimulation by Dpp and Wg, which are expressed in or near these regions (Figure 1A). This stimulation requires distinct Dpp and Wg response sequences (DRS and WRS) within *Ubx* B (Eresh *et al.*, 1997; Riese *et al.*, 1997; Szüts *et al.*, 1998) (Figure 1B). In addition, *Ubx* B is repressible by high Wg levels near the Wg signalling source (Yu *et al.*, 1998), and is also repressed in the

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Fig. 1. Gene expression in the midgut and signal-responsive sequence from *Ubx*. (A) Expression of HOX proteins (top), Wg (Wg) and Dpp (Bienz, 1996) in the midgut mesoderm in relation to estimated domains of *brk* expression (Jazwinska *et al.*, 1999b); midgut limits, positions of the gastric caeca (in ps3) and of the gut constrictions are indicated above. Critical regulatory interactions between the genes in the middle midgut are shown (arrows, stimulatory; barred line, repressive). (B) Top, signal-responsive sequence from *Ubx* B; underneath, mutant enhancer and L-CRE sequences, as indicated. Three perfect matches to the Brk consensus binding site are given at the bottom, and their presence in the mutant enhancers is indicated on the right. The ability of Brinker to bind to, or to repress, a mutant enhancer correlates with the presence of Brk bs1 or 2 (highlighted in yellow). nd, not done.

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absence of Wg signalling in cells remote from the source (Riese *et al.*, 1997; Waltzer and Bienz, 1998). The former repression is mediated by the WRS-R, a sequence coinciding with the Mad binding sites within the DRS, the latter by the WRS, a binding site for dTCF (*Drosophila* T cell factor) (Figure 1B).

Ubx is repressed by Brinker in the embryonic midgut

Since *Ubx* is a Dpp target gene in the embryonic midgut, we asked whether this HOX gene might be under *brk* control. Thus, we stained *brk* mutant embryos with an antibody against *Ubx* and found weak ectopic *Ubx* staining in the posterior midgut mesoderm of these mutants (Figure 2A and B). Normally, the HOX protein Abdominal-A represses *Ubx* in the posterior midgut (Bienz and Tremml, 1988) (Figure 1A), but evidently this is not sufficient to keep *Ubx* repressed in the absence of *brk*. However, we did not detect any *Ubx* derepression in the anterior midgut of *brk* mutants, probably because of the silencing of *Ubx* in this region by *Polycomb* (Immerglück *et al.*, 1990). But we did find derepression in the anterior and posterior midgut of *brk* mutant embryos when examining lacZ expression conferred by an extensive *Ubx* midgut enhancer called RP9 (Figure 2C and D), the expression of which closely resembles *Ubx* expression in the midgut (Thüringer and Bienz, 1993). These stainings show that *brk* represses *Ubx* in the embryonic midgut.

To determine the target for *brk*-mediated repression within *Ubx*, we asked whether the minimal *Ubx* B midgut enhancer is also under *brk* control. Indeed, we found that *Ubx* B is strongly derepressed at both ends of the midgut in *brk* mutant embryos, approximately in ps2 and 12 (Figure 2E and F). We note that each of these ps overlaps a domain of *brk* expression (Jazwinska *et al.*, 1999b) (Figure 1A), indicating that Brinker represses *Ubx* and its reporters cell-autonomously at both midgut ends.

Next, we tested a series of mutant versions of *Ubx* B that carry nested point mutations (Figure 1B). Most of these were still derepressed in *brk* mutants, e.g. BM1 (Figure 2G and H), which has a mutated MadB site. However, three mutant enhancers were no longer derepressed: B4, which has a mutated dTCF binding site (Figure 2I and J); B4R8, which carries a mutation in a conserved sequence motif (Figure 2K and L); and BM2, in which both Mad binding sites are mutated (Figure 2M and N). Formally, each of these mutations could define a target for Brinker repression. Alternatively, they define sequences that are essential for enhancer activation, in particular for ectopic activation at the midgut ends. This is a clear possibility since B4, BM2 and B4R8 are each considerably less active than *Ubx* B and other mutant enhancers such as BM1.

Brinker binds to the Mad binding sequence within *Ubx* B

We expressed full-length and various fragments of Brinker (Figure 3A) as glutathione *S*-transferase (GST) fusion proteins in bacteria, in order to test whether these fusion proteins can bind to the signal-responsive sequence from *Ubx* B (residues shown in Figure 1B, plus 32 additional 3' residues) in gel shift assays. This revealed that full-length Brinker, or its N-terminus alone, can bind to this sequence, whereas the C-terminus cannot (Figure 3B, lanes 3–5). This is consistent with the suggestion that

Fig. 2. Expression of *Ubx* and *Ubx* reporters in *brk* mutants. Dorsolateral views of 12–14-hour-old wild-type and *brk* mutant embryos, bearing *Ubx* reporter genes as indicated, stained with α -*Ubx* (A and B) or α -lacZ antibody (C–N). Arrows point to derepression in the anterior and posterior midgut of *brk* mutants. Anterior to the left, dorsal up.

the N-terminus contains a putative DNA binding domain similar to the homeodomain (Jazwinska *et al.*, 1999a) (Figure 3A). Indeed, a minimal fragment spanning this domain (BRK44–99) binds to the probe as well as full-length Brinker (Figure 3B, lanes 2 and 5).

Next, we tested Brinker binding to mutant DNA probes (Figure 1B). Of these, BM2 and BM0 were the only mutants that

Fig. 3. Direct binding of Brinker to the MadA binding site in *Ubx* B. (A) Map of Brinker, with DNA binding domain (black) and CtBP-recruiting motif PMDLS indicated (see Jawinska *et al.*, 1999a). (B–D) Gel shift assays with GST–Brinker fusion proteins as indicated (B; 10 µg per assay), or with the DNA binding domain of Brinker (Brk44–99; 1 µg) (C and D). Wild-type (B and D) or mutant sequences (C) as indicated above lanes were used as probes; in (D), an excess of unlabelled wild-type or BM2 mutant probe was used as competitor. (E) Gel shift assays, with 15 µg of the DNA binding domain of Mad (MH1+L) per assay; competitors as in (D). (F) Competition between Mad and Brinker; 15 µg of Mad (MH1+L) and increasing amounts of Brk44–99 (0, 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 µg) were used. (G) Quantitative evaluation of two independent experiments [(F) and not shown] showing competition between Brinker and Mad.

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no longer showed any binding to Brinker (Figure 3C). Likewise, Brinker binding to DNA can be competed with an excess of unlabelled wild-type probe, but not with mutant BM2 probe (Figure 3D). This shows that Brinker binds to *Ubx* B in a sequence-specific manner, and that the residues mutated in BM2 and BM0 are critical for Brinker binding.

While our manuscript was under review, Sivasankaran *et al.* (2000) published a consensus site for Brinker binding, GGCG C/T C/T, to which we find three perfect matches in *Ubx* B (Figure 1B). These are adjacent to one another, and each of them is mutated in BM2. Our results with BM1 indicate that the first of these matches (Brk bs1) is sufficient for Brinker function *in vivo* and *in vitro*. However, Brk bs3 alone is unlikely to be sufficient for function, given that Brinker cannot bind to the mutant probe BM0 (Figure 3C). Finally, our results indicate that Brk bs2 (perhaps together with bs3) can substitute for Brk bs1 and provide full function: BC2 is repressible by *brk* *in vivo* (also see below), and Brinker binds to BC2, BC and BM01 mutant probes (Figure 3C), all of which lack Brk bs1.

Interestingly, the three Brinker binding sites completely overlap the two Mad binding sites within the DRS (Figure 1B). Indeed, our previous work indicated that the Dpp response critically depends on MadA (Szűts *et al.*, 1998); MadA fully overlaps Brk bs1, which we find to be sufficient for Brinker function *in vitro* and *in vivo*. We thus asked whether Brinker might be able to compete with Mad for DNA binding. We performed competitive DNA binding experiments using bacterially expressed DNA binding domains of Brinker and Mad. This revealed that the former is capable of competing successfully with the latter for DNA binding at a molar ratio of 1:150, and Brinker almost completely blocks Mad binding at a ratio of 1:15 (Figure 3F and G). Note that full-length Mad binds to DNA ~100× less efficiently than its isolated DNA binding domain (Kim *et al.*, 1997), indicating that Brinker would be able to compete even more successfully with full-length Mad. Thus, Brinker can block Mad binding to DNA *in vitro* in the presence of a considerable molar excess of Mad.

Ectopic Brinker targets the Mad binding sequence *in vivo*

To confirm that the above Brinker binding sites within *Ubx* are functional targets *in vivo*, we expressed Brinker throughout the midgut mesoderm with the GAL4 system (Brand and Perrimon, 1993). This revealed that expression of *Ubx* in the middle midgut is nearly eliminated in Brinker-overexpressing embryos (Figure 4A and B). Instead, many of these embryos show an endodermal bulge in the middle midgut (Figure 4B, arrowhead) that is also observed in *Ubx* mutants (Tremml and Bienz, 1989). Furthermore, the first and second midgut constrictions are rudimentary at best, and often missing altogether (Figure 4E and F). Again, loss of the second constriction is indicative of mutations of *Ubx* and *dpp* (Bienz and Tremml, 1988; Panganiban *et al.*, 1990), while loss of the first may reflect mutation of the *dpp*-related gene *gbb* (Wharton *et al.*, 1999). Finally, ectopic Brinker also drastically reduces *dpp* and *wg* expression in the middle midgut (Grieder *et al.*, 1995; Marty *et al.*, 2000; Figure 5E–H and data not shown), which is expected since their expression depends on *Ubx*. This indicates that Brinker, by virtue of

Fig. 4. Ectopic Brinker represses *Ubx* and *Ubx* reporters. Dorsolateral views of 12–14-hour-old embryos, bearing *Ubx* reporters as indicated, stained with α-*Ubx* (A and B) or α-lacZ antibody (C–H); note that the GAL4 driver in (H) was 48Y.GAL4 instead of 24B.GAL4. Open triangles point to missing (B and D) or reduced expression (F); arrowhead points to endodermal bulge in the Brinker-overexpressing embryo (B and D). These also lack the first and second gut constriction [(F); vertical bars in (E) mark constrictions in the wild type]. The staining marked by an arrow in (H) reflects full activity of L-CRE in the visceral mesoderm, while its endodermal activity is undetectable. Anterior to the left, dorsal up.

repressing *Ubx*, is capable of blocking the whole process of endoderm induction that depends on this HOX gene.

LacZ staining from *Ubx* B was virtually eliminated in Brinker-overexpressing embryos, except for two patches of strong staining in the head and some staining in the dorsal epidermis (Figure 4C and D). Of the mutant enhancers, we found BM2 to be the least affected by ectopic Brinker (Figure 4E and F): in this case, we observed individual cells in ps8 and 9 of the visceral mesoderm that retained strong lacZ staining. In contrast, other mutant enhancers such as B4, B4R8 and BM1 behave like *Ubx* B itself, at best showing traces of lacZ staining in Brinker-overexpressing embryos (not shown). Thus, BM2 is unique in being refractory to Brinker repression, indicating that the Brinker binding sites that are mutated in this enhancer are functional *in vivo*.

We confirmed this by testing a minimal construct derived from *Ubx* B, called L-CRE, which spans the WRS, and MadA/Brk bs1 and bs2 (Figure 1B), and which is signal-responsive in both the visceral mesoderm and the endoderm (Riese *et al.*, 1997). If we overexpress Brinker in the endoderm only (to avoid repressing *wg* and *dpp* in the visceral mesoderm), L-CRE is completely repressed in this germ layer, while it remains active in the visceral mesoderm (Figure 4G and H). From this and our DNA

implies that Brinker is a potent repressor that can block activated Mad *in vivo*.

DISCUSSION

We have shown that Brinker binds to a specific sequence within the *Ubx* midgut enhancer, and that this sequence is a functional Brinker target *in vivo*. Ectopic Brinker represses *Ubx* in the middle midgut, and also blocks the expression of other Dpp target genes in this region, including *dpp* itself and *wg*. Our results indicate that Brinker is a direct repressor of *Ubx*, and thus a potent antagonist of the Dpp-dependent process of endoderm induction. We note that Brinker is expressed in 'signal-free' zones bordering the anterior and posterior limits of the midgut (Figure 1A). Its presence in these zones may have a barrier function, helping to block the spread of the Dpp response beyond the midgut limits.

Interestingly, the critical Brinker target site within *Ubx* B overlaps MadA, a functional Mad binding site that is required for the stimulation of this enhancer by Dpp signalling. Furthermore, Brinker competes effectively with Mad in binding to this site *in vitro*, and blocks activated Mad from stimulating *Ubx* B *in vivo*. This indicates that the mechanism by which Brinker repression dominates over stimulatory Dpp inputs is based on direct competition for binding to Dpp target enhancers. Given that most, if not all, Dpp signalling is mediated by Mad (Raftery and Sutherland, 1999), it seems likely that this competition-based mechanism of Brinker repression is widespread and extends to genes that are Dpp targets in other developmental contexts.

Notably, MadA is also the target sequence for repression of *Ubx* B in response to high Wg levels in the middle midgut (Yu *et al.*, 1998). MadA is thus a pivotal enhancer sequence that gauges and integrates positive inputs from Dpp and negative inputs from Brinker and Wg. Wg-mediated repression in the middle midgut is mediated by the zinc finger protein Teashirt (Waltzer *et al.*, 2001) and can be overridden by simultaneous Dpp stimulation (Yu *et al.*, 1998). In contrast, Brinker-mediated repression dominates over simultaneous Dpp stimulation (Figure 5). It thus appears that Brinker is a more potent repressor than Teashirt, and is designed to function as a signal-antagonist even in the presence of high levels of Dpp signalling.

Jazwinska *et al.* (1999a) noticed that Brinker contains the sequence PMDLS (Figure 3A), which resembles the P-DLS motif through which a number of transcription factors recruit the co-repressor dCtBP (Schaeper *et al.*, 1995; Nibu *et al.*, 1998a,b; Poortinga *et al.*, 1998). Indeed, using *in vitro* pull-down assays, we found that dCtBP binds to full-length Brinker as well as to an N-terminal Brinker fragment that contains the PMDLS motif (not shown). This suggests that Brinker may recruit dCtBP to repress Dpp target genes in the embryo. Interestingly, dCtBP assists various transcription factors, such as Knirps, Snail and Krüppel, that act at short-range to repress their target genes (Nibu *et al.*, 1998a,b). These short-range repressors bind to autonomous enhancers to quench nearby bound transcriptional activators, which prompted Levine and colleagues to suggest that dCtBP may be specifically designed to quench (Nibu *et al.*, 1998b). Therefore, this quenching ability of dCtBP could enable Brinker to not only compete efficiently with activated Mad in the binding of DNA, but also out-compete the activity of nearby transcription factors such as activated dTCF.

Fig. 5. Competition between Brinker and activated Mad *in vivo*. Dorsolateral views of 12–14-hour-old embryos, bearing *Ubx* B or the *dpp* reporter BMPX as indicated, stained with α -lacZ antibody. Lack of lacZ staining and endodermal bulges due to ectopic Brinker are marked by open triangles and arrowheads, respectively; vertical bars point to the gut constrictions that form under the various conditions.

binding studies we conclude that MadA/Brk bs1 and bs2 are direct targets for Brinker repression in the embryonic midgut.

Brinker successfully competes with activated Mad in the embryo

Our DNA binding experiments indicated that Brinker can prevent Mad from binding to functional target sites. Furthermore, we found that ectopic endodermal Brinker repressed L-CRE in the endoderm, implying that this repression occurs in the presence of unimpaired signalling by Dpp and Wg.

To test directly whether Brinker repression can overcome Dpp- and Mad-mediated target gene activation, we monitored *Ubx* B after simultaneous overexpression of Brinker and Dpp in the mesoderm. If Dpp is overexpressed alone, this leads to substantial stimulation of *Ubx* B expression in most regions of the midgut (Thüringer *et al.*, 1993) (Figure 5A and B). However, *Ubx* B was repressed by ectopic Brinker whether or not Dpp was present (Figure 5C and D). Essentially the same was observed if a Dpp-responsive *dpp* enhancer called BMPX (Sun *et al.*, 1995) was tested in this way: BMPX was strongly stimulated by ectopic Dpp, but only in the absence of co-expressed Brinker (Figure 5E–H). These results demonstrate that Brinker can repress Dpp-responsive targets even in the presence of Dpp stimulation. This confirms our results from the DNA binding studies *in vitro*, and

METHODS

Fly strains and embryo staining. The loss-of-function allele *brk^{F124}* (Lammel *et al.*, 2000) was used for analysis. The following transformants were used: RP9 (Thüringer and Bienz, 1993); *Ubx* B (also called Bhz; Thüringer *et al.*, 1993); B4 (Riese *et al.*, 1997); BM1, BM2 and BC2 (Szüts *et al.*, 1998); B4R8 (Yu *et al.*, 1998); B24.GAL4 (Brand and Perrimon, 1993); 48Y.GAL4 (Martin-Bermudo *et al.*, 1997); UAS.Brk (Lammel *et al.*, 2000); UAS.Dpp (Staehling-Hampton *et al.*, 1994). Mutant embryos and embryos overexpressing various proteins were identified by blue balancers or by their gut phenotypes.

Embryos were fixed and stained as described (Yu *et al.*, 1996). The following mouse monoclonal antibodies were used: α -Ubx (White and Wilcox, 1984), α -Wg (Brook and Cohen, 1996) and α -lacZ (Promega).

GST fusion proteins, gel shift and pull-down assays. Full-length Brinker and Brinker fragments were subcloned into pGEX-2TK (Pharmacia) using standard PCR cloning procedures and expressed in *Escherichia coli* BL21 as described (Waltzer and Bienz, 1998). The plasmid encoding the DNA-binding fragment of Mad (MH1+L or Mad1-241) was also used (Waltzer and Bienz, 1998; see also Kim *et al.*, 1997). All fusion proteins were purified by affinity chromatography and eluted from the GST-agarose beads in glutathione elution buffer (0.01 M reduced glutathione in 50 mM Tris-HCl pH 8.0). Note that the preparations of Mad (MH1+L) were variable; only two of six preparations gave satisfactory binding results (Figure 3E–G).

For band shifts, a probe spanning the signal-responsive sequence from *Ubx* B (see above and Figure 1B) was labelled with [α^{32} P]dATP and [α^{32} P]dCTP by PCR. Binding reactions were typically performed with 10 μ g (or as indicated in the figure legend) of purified GST fusion proteins incubated with 0.2 ng of probe in 20 μ l of buffer containing 5 mM HEPES pH7.5, 10% glycerol, 70 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 1 mM EDTA, 2 μ g poly(dI-dC), 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors for 20 min at room temperature. Electrophoresis was carried out with 5% polyacrylamide gels/0.5x TBE containing 2.5% glycerol.

Pull-down assays were performed as described (Waltzer and Bienz, 1998).

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