Dpp-responsive Silencers Are Bound by a Trimeric Mad-Medea Complex*

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Transcriptional regulation by transforming growth factor-β signaling is mediated by the Smad family of transcription factors. It is generally accepted that Smads must interact with other transcription factors to bind to their targets. However, recently it has been shown that a complex of the Drosophila Smad proteins, Mad and Medea, binds with high affinity to silencer elements that repress brinker and bag of marbles in response to Dpp signaling. Here we report that these silencers are bound by a heterotrimer containing two Mad subunits and one Medea subunit. We found that the MH1 domains of all three subunits contributed directly to sequence-specific DNA contact, thus accounting for the exceptionally high stability of the Smad-silencer complex. The Medea MH1 domain binds to a canonical Smad box (GTCT), whereas the Mad MH1 domains bind to a GC-rich sequence resembling Mad binding sites previously identified in Dpp-responsive enhancer elements. The consensus for this sequence, GRCGCN, differs from that of the canonical Smad box, but we found that Mad binding nonetheless required the same β-hairpin amino acids that mediate base-specific contact with GTCT. Binding was also affected by alaniine substitutions in Mad and Med at a subset of basic residues within and flanking helix 2, indicating a contribution to binding of the GRCGCN and GTCT sites. The slight alteration of the Dpp silencers caused them to activate transcription in response to Dpp signaling, indicating that the potential for Smad complexes to recognize specific targets need not be limited to repression.

Transforming growth factor-β signaling pathways activate or repress transcription by means of cooperative action of Smad proteins with a large number of interacting cofactors (1–3). The conserved Smad amino-terminal MH1 domain contributes to target recognition by binding to variations of the short sequence, GTCT (4). Upon activation by signaling, Smads form complexes such that more than one MH1 domain is available to contact DNA (5, 6). With a major portion of target recognition provided by interacting cofactors, DNA contact by a single MH1 domain can be sufficient for regulation in response to signaling (7–10).

In contrast, Smads bind to several Drosophila silencer elements without the assistance of a cofactor (11, 12). These silencers repress the brinker (brk), bag of marbles (bam), and gooseberry (gsb) genes in response to signaling by decapentaplegic (dpp). Repression of brk plays a central role in the dpp pathway (13, 14); in addition to direct activation by Smads. Dpp targets are regulated indirectly through repression by the Brk protein (15–18). Repression of brk occurs through a silencer that binds a complex of the Smad proteins Mothers Against Dpp (Mad) and Medea (Med) (11). Mad is homologous to the BMP2-specific Smad1, Smad5, and Smad8 receptor-activated Smads (rSmads). Med is the homolog of Smad4, the coSmad that forms complexes with rSmads. Genetic and biochemical analyses confirmed that Mad and Medea are both required for repression of brk (11, 19, 20). Repression of brk also requires the large zinc finger protein Schnurri (Shn) (11, 19, 20). Although Shn contains three zinc finger clusters, alone it does not bind to the silencer, and repression requires only a carboxyl-terminal region containing three zinc fingers that confer interaction with the silencer-bound Mad-Med complex (11, 12). In the gonad Dpp signaling and Shn are also required for repression of bam (21, 22), a differentiation factor that must be kept off in germ line stem cells (23). A Dpp-responsive silencer is located in the bam 5‘-untranslated region just 27 bp from the transcriptional start (24). Both silencers consist of paired Mad and Med binding sites (12, 25), and binding of the Mad-Med complex in turn allows binding of Shn.

The ability of Mad-Med complexes to recognize the brk and bam silencers without help from cofactors suggests that Smad complexes are capable of greater DNA binding affinity and specificity than had been observed previously. One means of achieving this would be for a greater number of MH1 domains to interact with DNA in a sequence-specific manner. The number of MH1 domains contained within a Smad complex is determined by the way in which Smad MH2 domains oligomerize (26–29). The tendency of MH2 domains to crystallize as trimers and heterotrimers (30–34) suggests that heterotrimers are the principal heteromeric Smad complex in vivo (34). However, work from several groups has provided evidence that Smads also form other types of complexes, including heterodimers (35–37).

Here we report that Mad and Med bind to the brk and bam silencers through all three MH1 domains of a Mad-Med heterotrimer. The results reveal a mechanism by which Smad complexes bind certain regulatory elements with high affinity and specificity. The arrangement of these sites is critical for silencing, and minor alterations can result in Dpp-responsive transcriptional activation instead of repression.

EXPERIMENTAL PROCEDURES

Plasmids—Mad, Med, and TkvQD effector plasmids for expression in human 293T cells were generated by insertion of full-length cDNAs into the polylinker of a version of the pCI expression vector that had been modified to contain an amino-terminal FLAG, HA, or Myc tag; ShnCT was cloned into epitope tag versions of pCI as a PCR fragment encoding Ser-1884 through Asn-2527. 1XGFPMed was generated by ligating into the XbaI site of pCI an NheI-XbaI fragment from pEGFP-Med (provided by Y. Cai) containing the fused eGFP and Med coding regions. A second copy of eGFP was inserted as a XmaI-BspEI fragment into the BspE1 site of 1XGFPMed to give 2XGFPMed. Substitution of an XbaI-NotI fragment encoding Mad in place of the Med coding region

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2 The abbreviations used are: BMP, bone morphogenetic protein; MH, Mad homology; eGFP, enhanced green fluorescent protein; HA, hemagglutinin.
yielded 1XGFP-Mad and 2XGFP-Mad. Site-directed mutagenesis of Mad and Med effector plasmids was carried out using a previously described method (38). Plasmid constructs and products of site-directed mutagenesis were verified by DNA sequencing. For reporter assays, the Mad, Med, and TkvQD effector plasmids was carried out using a previously described method (38). Plasmid constructs and products of site-directed mutagenesis were verified by DNA sequencing. For reporter assays, the Mad, Med, and TkvQD effector plasmids were transfected in 24-well non-coated plates using Effectene transfection reagent (Qiagen) according to the supplier’s instructions. All reporter experiments were performed in triplicate.

![FIGURE 1. Dpp-responsive silencers are bound by a Mad-Med complex.](image)

A, alignment of the BrkS and BamSE silencer elements with GRCGNC and GTCT sequences underlined. B, gel shift assay showing that purified maltose-binding protein-MadNL domain bound GGGCAG and GTCT probes with similar affinity, whereas maltose-binding protein-MedNL (N = MH1 + linker) bound preferentially to the GTCT probe. For each set 0.1, 0.3, or 1 μl of protein preparation were added to binding reactions as indicated. Complete probe sequences were CATTCTCTGCGAAG and CTTACTGGCGACATTCTG. C, gel shift assays showing that BrkS is bound by a protein complex containing HA-Mad and Flag-Med. Prior to electrophoresis, radioactive DNA probes were incubated with lysates of human 293T cells transiently expressing combinations of TkvQD (all lanes), FlagMad, Med, or MycShnCT, with or without antibodies, as indicated. Position of the Mad-Med-shifted complex is indicated by an arrowhead, free probe is labeled, and a nonspecific band is marked by an asterisk. Probe sequence was AATTCCGACTGCGACATCTGGTCTGCAGCGCC. D, a gel shift assay containing BrkS probe, Flag-Mad, and Med. Addition of FLAG antibody to the binding reaction resulted in two supershifted bands; the upper supershifted band is indicative of two FlagMad subunits in a DNA-bound complex. Asterisk marks background band.

FIGURE 2: Stoichiometry of silencer-bound Mad-Med complexes. A, gel shift assay demonstrating the presence of two Mad subunits in a complex with a BamSE DNA probe. Overexpressed proteins in 293T cell lysates added to binding reactions are indicated above each lane. Compositions deduced for each shifted complex are diagrammed along the sides (orange, Mad; blue, Med; green, eGFP). The left-hand half of the gel shows complexes resulting from fusion of one or two copies of eGFP to the amino terminus of Mad, the right side shows complexes resulting from fusions to Med. An asterisk indicates background band. In addition to the indicated components all transfected cell lysates expressed TkvQD. Probe sequence was AATTCCGACTGCGACATCTGGTCTGCAGCGCC. B, gel shift assay as in A but with the BrkS probe used in Fig. 1.

respectively. Twenty-four hours after transfection, 293T cells were harvested and lysed in 400 μl of ice-cold DNAP buffer (80 mM NaCl, 35 mM KCl, 5 mM MgCl2, 25 mM Tris, pH 7.5, 10% glycerol, 0.1% Nonidet-P40, 1 mM dithiothreitol, 0.1 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 1 μg/ml aprotenin). Lysates were cleared by centrifugation at 14,000 rpm in a microfuge at 4 °C and stored frozen at −80 °C. DNA probes were made by [α-32P]dATP fill-in labeling of annealed oligonucleotides, followed by phenol-chloroform extraction and spin column gel filtration. For each gel shift DNA-binding reaction, 9 μl of cell lysate was mixed with 1 μl of DNA probe diluted to 10−7 M with 1 mg/ml dDNA. Reactions were incubated 30 min on ice, then 2 μl was loaded per well onto a 60 × 80 × 1.5 mm 4% polyacrylamide gel containing 0.5× TBE buffer. Gels were run with 4 °C cooling at 100 V for 40–70 min (run time adjusted according to probe length). Gels were dried and then imaged with a Storm PhosphorImager.

**Reporter Assay—Drosophila S2 cells** were grown at 25 °C in M3 medium supplemented with 12.5% fetal calf serum. Cells were transfected in 24-well non-coated plates using Effectene transfection reagent (Qiagen) according to the supplier’s protocol. Cells were harvested 2 days after transfection, and a chemiluminescent β-galactosidase assay was performed on cell extracts using the GalactoStar assay system (Tropix, Inc.) according to the supplier’s instructions. All reporter experiments were performed in triplicate.

**FIGURE 3:**"
**RESULTS**

_Dpp Silencers Are Bound by a Heterotrimer Containing Mad and Med in a 2:1 Ratio—_The BrkS and BamSE silencers are the only examples in which Smad proteins appear to be solely responsible for target recognition (11, 12, 24, 25). The silencers consist of Smad binding sites separated by five base pairs (Fig. 1A). The silencer consensus sequence was derived from sequence alignment and from determining the effects of base substitutions (12, 25). The left side of the consensus, GRCGNC, resembles the Mad binding consensus, GCCGnCG (41), whereas the right side, GTCT, matches the Smad3/Smad4 consensus for binding of a single MH1 domain (4, 42). Gel shift experiments with purified Mad and Med fusion proteins showed that Mad binds with similar affinity to either site, whereas Med has high affinity only for the GTCT (Fig. 1B) (12, 25). This suggests a simple model in which Mad and Med bind to the left and right side of the silencer, respectively. Gel shift experiments with lysates of transfected cells show that Mad and Med bind the silencers as a complex, with antibody supershifts confirming the presence of both proteins in the complex (Fig. 1C) (11, 12). The binding of the Mad-Med complex allows Shn to be recruited via a carboxyl-terminal region (ShnCT, Fig. 1C) (11, 12). We noticed that when supershifting a complex in which Mad was epitope-tagged, a faint second supershifted band was sometimes visible (Fig. 3).

_Silencer-bound Mad-Med Trimer_

**FIGURE 3.** Mad contacts DNA by means of the β-hairpin. A, alignment of the helix 2 and β-hairpin regions of Mad, Smad1, Med, and Smad4, extrapolated from the crystal structure of the Smad3 MH1 domain. Features of the corresponding Smad3 structure (42) are indicated below the sequences, and solvent-exposed Smad3 residues are colored blue. Mad or Med residues that have been mutated to alanine are colored red. B, gel shift assay comparing the effects of alanine substitutions at Mad and Med helix 2 and β-hairpin residues on formation of Mad-Med complex on a BrkS probe. The mutated residue is indicated above each lane. Binding reactions contained lysates from 293T cells transfected with effector plasmids for TkvQD, Med, and the indicated Mad mutants. Below the gels are shown Western blots detecting amino-terminal FLAG tag for each of the alanine substitution Mad and Med mutants and an HA tag on the wild-type partner at the bottom. None of the mutations had a substantial effect on protein level. C, gel shift assay showing that helix 2 alanine substitutions in Mad (left panel) and Med (right panel) do not affect binding of ShnCT to the Mad-Med-BrkS complex. An asterisk marks the background band.

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*3 S. Gao, J. Steffen, and A. Laughon, unpublished results.*
For BrkS (Fig. 3B), that it caused a less drastic but reproducible reduction in binding affinity makes indirect base contact through water molecules (47), and found that it has been proposed that such sites are bound through sequence-non-

equivalent basic residues, Arg-88, Gln-90, and Lys-95, each of which corresponds to a Smad3 residue whose side chain makes direct base-specific contact in the Smad3-DNA structure (Fig. 3A) (42). Western blots showed that these mutations and all others described below had little or no effect on the level of Mad or Med in whole cell lysates (Fig. 2B, A and B), indicating that the Mad-Med-silencer complex contains only a single Mad subunit.

Mad Contacts GRCGNC by Means of the β-Hairpin—The DNA-binding interface of Smad3 is a β-hairpin structure (42) that is completely conserved in all Smads, with only two amino acid differences in coSmads. This conservation of the DNA-binding interface suggests that all Smads might bind DNA with the same sequence specificity. However, the GC-rich binding sites first described for Mad and later for Smad1 appear to be distinct from the canonical Smad3 GTCT site, and it has been proposed that such sites are bound through sequence-non-specific contact with the lysine-rich helix 2, which lies adjacent to the β-hairpin (46, 47).

Single base pair substitutions in the GRCGNC sites of BrkS and BamSE diminish binding of the Mad-Med complex and repression (12) (data not shown), evidence that binding of the Mad-Med complex to GRCGNC sites is sequence-specific. To investigate the basis for this sequence specificity, we introduced alanine substitutions at three positions within the Mad β-hairpin, Arg-88, Gln-90, and Lys-95, each of which corresponds to a Smad3 residue whose side chain makes direct base-specific contact in the Smad3-DNA structure (Fig. 3A) (42). Western blots showed that these mutations and all others described below had little or no effect on the level of Mad or Med in whole cell lysates (Fig. 3B, bottom). Nonetheless, all three mutations strongly reduced complex formation with BrkS (Fig. 3B) or BamSE (data not shown). As expected, alanine substitutions at the corresponding positions in Med had a similar effect (Fig. 3B). We also tested an alanine substitution for Mad His-93, which corresponds to His-79 in Smad3, a residue that makes indirect base contact through water molecules (47), and found that it caused a less drastic but reproducible reduction in binding affinity for BrkS (Fig. 3B). These results demonstrate that the β-hairpin of Mad is necessary for contact of GRCGNC sites, just as it is for GTCT contact by Med.

Helix 2 Contributes to DNA Binding—Although our results indicated that binding of Mad to the GRCGNC occurs via the β-hairpin, we went on to test whether residues within or adjacent to helix 2 might nevertheless contribute to binding. The lysine-rich stretch of helix 2 has been shown to function as a nuclear localization signal (48, 49) but a series of helix 2 lysine to alanine mutations resulted in little or no change in Mad or Med levels in whole cell lysates of transfected 293T cells, as determined from Western blots (Fig. 3B). Comparing these lysines in gel shift experiments, we found that Mad-Med-BrkS complex formation was strongly disrupted by Mad R58A, a residue that is just carboxyl-terminal to helix 2, with moderate effects caused with Mad K46A and by Med R66A, K74A and D80A (Fig. 3B). The results show that helix 2 and carboxyl-terminally adjacent residues contribute to the DNA binding activity of Med as well as that of Mad, suggesting that helix 2-backbone contact may be a general feature of Smad-DNA interaction and not exclusive to interaction with the GRCGNC site. Together, these results suggest that Mad and Med both contact DNA via the β-hairpin in a configuration that is similar to that of the Smad3 MH1 domain bound to GTCT, possibly with helix 2 tilted somewhat closer to the DNA backbone. Notably, alanine substitutions in helix 2 of Mad and Med had no obvious effect on recruitment of ShnCT (Fig. 3C). Because binding of ShnCT requires precise 5-bp spacing between GRCGNC and GTCT (discussed below), the MH1 domains are likely sites for Shn interaction. These results indicate that any MH1 contact with ShnCT does not depend critically on solvent-exposed helix 2 side chains.

The stretch of basic amino acids that starts in helix 2 extends further in Mad than in Smad3, and we found that one of these additional residues, Arg-58, is required to form the silencer complex. However, in comparison with the Smad3MH1-DNA crystal structure, it is doubtful that Arg-58 would be able to contact the DNA backbone from its position at the end of helix 2. Indeed, the R58A substitution had little effect on binding of purified maltose-binding protein-MadNL (which binds as a monomer) to probes containing GTCT or GGGCAG, whereas R88A dramatically reduced binding to both probes (data not shown), evidence that Arg-58 is not directly involved in DNA contact. Rather than directly contacting DNA, Arg-58 may engage in protein-protein interactions that stabilize the DNA-bound Mad-Med heterotrimer. However, we cannot rule out the possibility that, when bound as a heterotrimer, the Mad MH1 domain adopts a structure in which Arg-58 contacts the DNA backbone.

The β-Hairpin Is Required in Both Mad Subunits—The results described above prove only that the β-hairpin is required in at least one of the two Mad subunits contained within the heterotrimer. To test
whether the β-hairpin is required in both Mad subunits, we coexpressed Mad R88A, Q90A and K95A mutant proteins with wild-type 2xGFP-Mad and performed a gel shift with a BrkS probe. If β-hairpin contact was required in only one of the two Mad MH1 domains, we expected to observe an intermediate band containing 2xGFPMad and MadR88A (or Q90A/K95A). Faint intermediate bands were observed (Fig. 4), but even the strongest of these, Q90A, was much weaker than for wild-type. We interpret these results as a demonstration that an intact β-hairpin is required in both Mad subunits, although presence in just one subunit does allow for some residual binding. Interestingly, Arg-58 was also required in both Mad subunits.

A Mad-Med Heterotrimer Can Activate Transcription—The significance of the GRCGNC(N)_5GTCT topology was investigated with a series of rearrangements (Fig. 5A). Reversal of the GTCT in BrkS (mut1 in Fig. 5A) reduced binding of the Mad-Med-silencer complex formation (Fig. 5B) and essentially eliminated repression (Fig. 5C). In contrast, changing GTCT to GGCG (mut2 in Fig. 5A) had only a modest effect on Mad-Med binding but prevented recruitment of Shn (Fig. 5B), transforming the silencer into a Dpp-responsive activating element (Fig. 5C). Similarly, a 1-bp increase (data not shown) or decrease (mut3 in Fig. 5A) in spacing converted the silencer into an activating element (Fig. 5C), again disrupting Shn binding without severely affecting the binding of Mad and Med (Fig. 5B). In the latter two cases, Mad and Med still bound to the DNA as a heterotrimer with a 2:1 ratio (Fig. 5D). Similar results were obtained with the same alterations of BamSE. These results demonstrate that repression is dependent on a precise (GRCGNC)-(N)_5-GTCT arrangement and that trimeric Smad complexes that cannot recruit Shn behave as transcriptional activators.

DISCUSSION

In this report we provide evidence that Mad and Med bind to Dpp-responsive silencers as a 2:1 heterotrimer and that all three subunits of

![Figure 5. Shn binding and repression require correct spacing and orientation of binding sites within the Dpp-responsive silencers. A, selected mutations in BrkS that demonstrate the importance of binding site sequence and topology for repression. Changed bases in Mut1 and Mut2 are in bold lowercase type. B, gel shift assays showing the effects of topological changes in the BrkS silencer. Compared with wild-type, reversal of the GTCT (Mut1) disrupted binding of the Mad-Med complex. In contrast, changing GTCT to GGCG (Mut2) reduced the Mad-Med complex only slightly but binding of ShnCT was undetectable. Reducing the distance between the Mad and Med binding sites by 1 bp (Mut3) had little effect on binding of the Mad-Med complex but binding of ShnCT was undetectable. DNA binding reactions contained BrkS probe and lysate from 293T cells transfected with effector plasmids for expression of Flag-Mad, HA-Med, or ShnCT, as indicated (all transfected with TkvQD). C, reporter assays in Drosophila S2 cells showing that Smad binding site sequence, orientation and spacing are critical for repression. Repression in response to TkvQD was disrupted by reversal of the Med binding site (Mut1), whereas Mut2 and Mut3 show that changing GTCT to GGCG or reducing the spacing between Mad and Med sites resulted in TkvQD-induced activation. As diagrammed above the graph, the reporter plasmids contained BrkS positioned upstream of a 3xSu(H) binding site array, which allowed for activation in response to cotransfected effector plasmids for Suppressor of Hairless (Su(H)) and an activated version of Notch (N^act) (11, 12, 40). Amounts of effector plasmids used per transfected well (in nanograms) are indicated below the chart. D, gel shift assay using GFP fusion constructs as in Fig. 2 demonstrates that activating Mad-Med complexes (arrowheads) still contain a heterotrimer with a 2:1 Mad:Med ratio. Probes and composition of 293T cell lysates were as indicated above the lanes. The mobility of complexes is more tightly spaced for the Mut3 probe.

![Figure 6. Model of the Mad-Med heterotrimer bound to a Dpp-responsive silencer. The positions of the two Mad MH1 domains in contact with the GRCGNC site are depicted as symmetrical but a tandem arrangement has not been ruled out. Shn is depicted as contacting the MH1 domains because of its sensitivity to spacing between the Mad and Med binding sites. However, Shn also interacts with the Mad and Med MH2 domains (71, 72).]
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this complex make sequence-specific DNA contact by means of the MH1 domain β-hairpin (Fig. 6). Slight alterations in sequence transform these silencers into Dpp-responsive activating elements that continue to be bound by a 2:1 Mad-Med heterotrimer but with a conformation that does not allow recruitment of Shn. This work also demonstrates that Mad binds to GC-rich sites, originally defined as GCCGNC (41) and more recently in the context of silencers as GRCGNC (12), by sequence-specific contact with two subunits. Sequence-specific interaction with GC-rich sites has been demonstrated previously for Smad1 (50) and for Mad (12), but the stoichiometry of these interactions was not documented. Finding that the 6-bp site is contacted by two Mad subunits raises new questions, because it is not clear how the two MH1 domains are arranged on the DNA, nor is it known why GNCG or GNCN (depending on orientation) is recognized. It is not clear how the two MH1 domains are arranged on the DNA, nor is it known why GNCG or GNCN (depending on orientation) is recognized

rather than the GTCT site preferred by Smad3 and Smad4. Previous work involving Smad1-Smad3 chimeras identified Lys-36 and Ser-37 of Smad3 helix 2 versus Asp-35 and Ala-36 in Smad1 as a key difference necessary for the activation of a (CAGA)₉-luc reporter (51). However, we found that mutation of the corresponding residue in Mad (D49A) had little or no effect on binding to BrkS, and thus the structural determinants for the GRCGNC binding site preference of Mad (and by inference, Smad1) remain to be identified.

Our findings contradict a previous report that Mad and Med bind to BrkS as a dimer (12). Although no documentation was provided for how this was determined experimentally, our own attempts to determine stoichiometry by means of antibody supershift experiments suggest a possible explanation for the discrepancy. We found that epitope tags at the Mad amino terminus are only readily accessible to the antibody on one of the two Mad subunits (Fig. 1D), possibly because of crowding by the adjacent DNA-bound MH1 domain of Med. This problem led us to turn instead to the use of protein fusions as a strategy (43) for assessing stoichiometry.

Mad and Med appear to be the only factors that directly contact the bam and brk silencers (11, 12). This conclusion is based on mutational analysis of the BrkS element, which showed that mutations that disrupt silencing also disrupt binding of the Mad-Med complex. The only exceptions were mutation of GTCT to GTGC (12) or to GGGC (Fig. 5) and a 1-bp deletion between the Mad and Med sites (12) (Fig. 5), each of which allowed binding of the Mad-Med complex but disrupted recruitment of Shn. Overexpression of activated Mad-Med complexes is sufficient to generate these gel shift complexes, and therefore it is unlikely that an unknown cofactor is required because it would presumably need to be expressed at high levels endogenously in both Drosophila and human cells. The demonstration that binding of the Mad-Med heterotrimer requires all three MH1 domains also weighs against cofactor involvement because it becomes difficult to explain how GRCGNC could be contacted by a cofactor in addition to two Mad MH1 domains (the Med MH1 contacting GTCT). Nonetheless, it is possible that a cofactor present in Drosophila and human extracts has gone undetected, although the evidence suggests it could not play a direct role in sequence-specific DNA contact.

The apparent ability of a Mad-Med complex to bind silencers without cofactors contrasts with the general reliance of Smads on DNA-binding cofactors for target specificity. brk could be considered a special case because it is negatively regulated by Dpp globally (15–18), whereas other Dpp targets require tissue-specific regulation (41, 52–57). However, bam expression is specific to germ line cells, and thus it is unexpected that DNA contact by Mad and Med would be sufficient for the regulatory specificity of the bam silencer. The existence of a similar Dpp-responsive silencer regulating gooseberry (12) provides further evidence that these novel arrangements of Smad binding sites provide sufficient specificity for regulation in response to signaling. Nonetheless, tissue specificity might be augmented by cooperative interaction of DNA-bound corepressors with Smads bound to BrkS/BamSE-like sites.

The high affinity of Mad and Med for these silencers is explained by the trimeric stoichiometry and involvement of all MH1 domains in directly contacting DNA. The ability of a single Smad complex to engage all three MH1 domains in DNA contact has several implications. The most obvious is that Smad complexes may in some cases make a greater contribution to target recognition than was previously apparent. As in the case of brk, this provides a mechanism by which Dpp, BMP, or even transforming growth factor-β signaling might trigger a general response without the need for a tissue-specific cofactor. Such a response need not be limited to silencing because slight alterations in the silencers transform them into Dpp-responsive activating elements, possibly by allowing CBP to interact with the Mad-Med complex (58) in the absence of Shn. Conserved sites exhibiting the BrkS/BamSE motif have been identified within BMP-response elements for the Id genes (59–61), which as a class are responsive to BMP signaling (62, 63). Conversely, tissue specificity might be conferred upon such tripartite Smad response elements by adjacent binding sites for other transcription factors.

A second implication is that Smad complexes may have greater flexibility in their ability to recognize binding sites than was previously apparent, particularly for moderate affinity sites to which only two MH1 domains make sequence-specific contact. The observed flexibility in spacing between the Mad and Med sites in BrkS and BamSE suggests that DNA binding by Med plus just one Mad MH1 domain might be able to occur in a variety of permutations. However, loss of binding when the Med site was reversed (mut1 in Fig. 5B) shows that the topology of Smad sites has strict limits. A moderate affinity site might also consist of just the GRCGNC Mad binding site without an adjacent Med binding site, as appears to be the case for many Dpp-responsive response elements by adjacent binding sites for other transcription factors.

The third implication is for complexity in the response to BMP signaling. In vertebrates that possess three BMP-responsive rSmads, Smad1, Smad5, and Smad8, BMP signaling might trigger the formation of a variety of trimeric rSmad complexes with Smad4 (e.g. a Smad1-Smad5-Smad4 complex). There is the potential for six such combinations (114, 554, 884, 154, 184, and 584). If such mixed complexes do form, as has been shown for Smad2, Smad3, and Smad4 in activation of p15Ink4B (70), it will be important to determine whether differences exist among them in the range of binding sites that can be bound by means of two or three MH1 domains. The likelihood of differential cofactor interactions by Smad1, Smad5, and Smad8 adds an additional layer of complexity.

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