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Mesoderm Induction by BMP-4 and -7 Heterodimers

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Bone morphogenetic proteins (BMPs) are peptide growth factors belonging to the TGF-β superfamily. A large number of these ligands, including BMP-2, -4 and -7 is expressed during early embryogenesis in the vertebrate embryo. In this study, we demonstrate that BMP-7 has ventralizing activity both in ectodermal explants as well as in whole embryos. As it was the case for BMP-2 and BMP-4, BMP-7 is a very poor inducer when provided as a homodimer protein. Because of this weak mesoderm inducing activity, it has been suggested that mesoderm induction by BMPs might represent an artifact of overexpression. We provide evidence demonstrating that unlike the homodimers of BMP-4 or BMP-7, the purified recombinant heterodimer of *Xenopus* BMP-4 and BMP-7 (BMP-4/7) has a potent mesoderm inducing activity at physiological concentrations. These results provide the first evidence for an embryonic function of BMP-4/7 heterodimers in the vertebrate embryo.

Bone morphogenetic proteins (BMPs) are secreted proteins which constitute a subgroup of the TGF-β superfamily of growth factors. In vertebrate embryos, BMPs display a very dynamic pattern of expression and have been associated with a variety of embryological functions (1). In the amphibian embryo, BMP-2, BMP-4 and ADMP (a BMP-3 related gene) have been shown to have both inductive and patterning activities in the context of mesoderm. Induction of ventral mesoderm in embryonic explants by BMPs, however, requires the injection of approximately 1 ng of RNA. On the other hand, 1 pg of activin RNA, another TGF-β factor, is sufficient to induce a comparable amount of mesoderm. This observation has led to speculations that BMP ligands is not involved in mesoderm induction (2). The fact that a non physiological amount of these homodimer proteins (about 1 μg/ml) is required to induce ventral tissues from embryonic explants seems to support this view (3). An alternative to this scenario is one in which these proteins are necessary but not sufficient to fulfill their inductive functions and require the presence of cofactors and partners. In support of the latter hypothesis, heterodimers of BMPs have been shown to act synergistically in a variety of assays (1). In this report we first demonstrate that BMP-7, another member of the BMP family expressed maternally in *Xenopus* embryos (4), can induce ventral mesoderm from ectodermal explants in a similar fashion as BMP-2 and -4. In addition, we provide direct evidence demonstrating that while both BMP-4 and BMP-7 are poor mesoderm inducers as homodimers, a BMP-4/7 heterodimer protein is a potent ventral mesoderm inducer at physiological range. This provides the first evidence for a potential synergism between BMPs during early embryonic development.

MATERIALS AND METHODS

Embryo manipulations. *Xenopus* embryos were obtained and staged as previously described (5). Synthetic mRNAs were transcribed from pSP64T vectors carrying *Xenopus* BMP genes (4). Ectodermal explants were cultured in 1XSteinberg's solution with 1 mg/ml crystallized bovine serum albumin and 100 μg/ml kanamycin (5). bFGF was purchased from Boehringer Mannheim.

RT-PCR assay. RT-PCR assays were performed as previously described (5). Primer sequences and PCR condition are as follows: goosecoid (forward primer, 5′-ACTACTATGGACAGTTGCACG-3′; reverse primer, 5′-TTCTGATTCCTCTGATGAAGATC-3′; 27 cycles); α-globin (forward primer, 5′-TTGCTCTGTGGGGCAAAATC-3′; reverse primer, 5′-AGGTTGTAGGCATGCAGGTCA-3′; 21 cycles); Histone H4 (forward primer, 5′-ATAACATCCAGGGCATCACC-3′; reverse primer, 5′-ACATCCATAGCGGTGACGGT-3′; 21 cycles); Xwnt8 (forward primer, 5′-GTTCAAGCATTACCCCGGAT-3′; reverse primer, 5′-CTCCTCAATTCCATTCTGCG-3′; 24 cycles); Xbra (forward primer, 5′-GAATCATCTTCTCAGCGCTGTGGA-3′; reverse primer, 5′-GTTGTCGGCTGCCACAAAGTCCA-3′; 21 cycles). Other primers are described previously (5).

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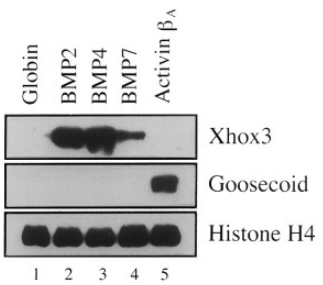

FIG. 1. BMP-7 induces ventral mesoderm in ectodermal explants. At early gastrula stage, the expression of Xhox3 is induced in the explants derived from embryos injected with BMP-7 mRNA. 2 ng of BMP-2, -4, -7 and globin RNA and 1 pg of activin βA RNA were used.

RESULTS AND DISCUSSION

Xenopus BMP-7 mRNA Induces Mesoderm and Has Ventralizing Activity

In order to assess the activity of *Xenopus* BMP-7, we performed experiments in ectodermal explants and in the whole embryo. Embryos at the two cell stage were injected with RNA encoding BMP-7 in the animal pole and ectodermal explants (animal caps) were removed at early blastula stage (stage 8.5). These animal caps were cultured to appropriate stage and assayed by RT-PCR for a variety of molecular markers. Activin RNA was used in these experiments both as positive control and as comparison for the amount of RNA required for mesoderm induction. Figure 1 shows that while a low amount of activin RNA (1 pg) induces a dorsal mesoderm marker (goosecoid), high amounts of BMP-7 RNA (2 ng) as well as BMP-2 and BMP-4, can induce early ventral mesoderm markers such as Xhox3. From this observation, we conclude that in a fashion similar to BMP-2 and BMP-4, BMP-7 can act as a ventral mesoderm inducer, albeit at high RNA concentrations.

We next assessed the activity of BMP-7 in whole embryos followed by phenotypical analysis using molecular markers. BMP-7 mRNA was microinjected into either dorsal or ventral blastomeres at the 4 cell stage, and injected embryos were allowed to develop to tadpole stages. While injection in the ventral side did not show any phenotypical abnormality when compared to the sibling uninjected embryos, expression of BMP-7 in the dorsal blastomeres lead to ventralization. Figure 2A shows that embryos which received 2 ng of BMP-7 mRNA in the dorsal blastomeres have reduced anterior structures such as eyes and cement glands. The most severely affected embryos completely lacked dorsal axial structures such as notochords and neural tubes. This phenotype resembles the one reported for embryos injected with BMP-4 mRNA. Examination of both early and late molecular markers confirmed the conclusions derived from the phenotype and histological analysis (data not shown). Figure 2B shows that when examined at early gastrula stages, the expression of the ventral marker Xhox3 was enhanced while the expression of the dorsal marker goosecoid was inhibited. Figure 2C shows that at tadpole stages, injection of BMP-7 mRNA resulted in the inhibition of expression of both a marker of axial mesoderm, cardiac muscle actin, and an anterior ectoderm marker, XAG-1. In contrast, expression of the ventral mesodermal marker α-globin was enhanced by BMP-7 mRNA. These results are in agreement with our observations made in animal caps and suggest that BMP-7 can act as a ventralizer. However, we note again that a large amount of RNA is required to unveil this activity.

Synergistic Effect of BMP-4 and BMP-7 mRNAs on Mesoderm Induction of Xenopus Embryos

We subsequently addressed the possibility that BMP-7 and BMP-4 coordinately act on mesoderm induction of *Xenopus* embryos. Animal pole explants were excised from embryos injected with each or both of the RNAs and the expression of mesodermal markers were analyzed at gastrula stage. As shown in Figure 3, injection of BMP-4 or BMP-7 RNA alone shows weak mesoderm induction at a low amount of RNA (200 pg), (lanes 4 and 5). If 100 pg each of both RNAs was co-injected together, however, the expression of mesoderm markers was strongly induced (lane 6). Quantification of the RT-PCR bands suggests that this effect is synergistic rather than additive (data not shown). This result suggests that BMP-4 and BMP-7 synergistically act on mesoderm induction of *Xenopus* embryos, raising the possibility that heterodimer of BMP-4 and BMP-7 might be involved in this process.

The Heterodimeric Protein BMP-4/7 Has a Potent Mesoderm Inducing Activity

Members of the TGF-β superfamily are synthesized as monomeric precursors which subsequently form dimers. This dimerization step is required for production of physiologically active ligands. In the case of activins, both homodimers (A and B) as well as heterodimer (AB) have been purified in vivo. Most of the previous studies, performed in the embryo, have focused on the mode of action of BMP homodimers, with little investigation into the possible role of heterodimeric complexes during early development. The fact that mesoderm inducing activity of BMP-2, -4 and -7 required unusually high amount of RNA and protein (Figure 1) and that synergistic effects of BMP heterodimers were previously documented (6), we were prompted to investigate whether these heterodimers could have stronger effects in our embryonic assays. We started our investigation by comparing the mesoderm inducing activities

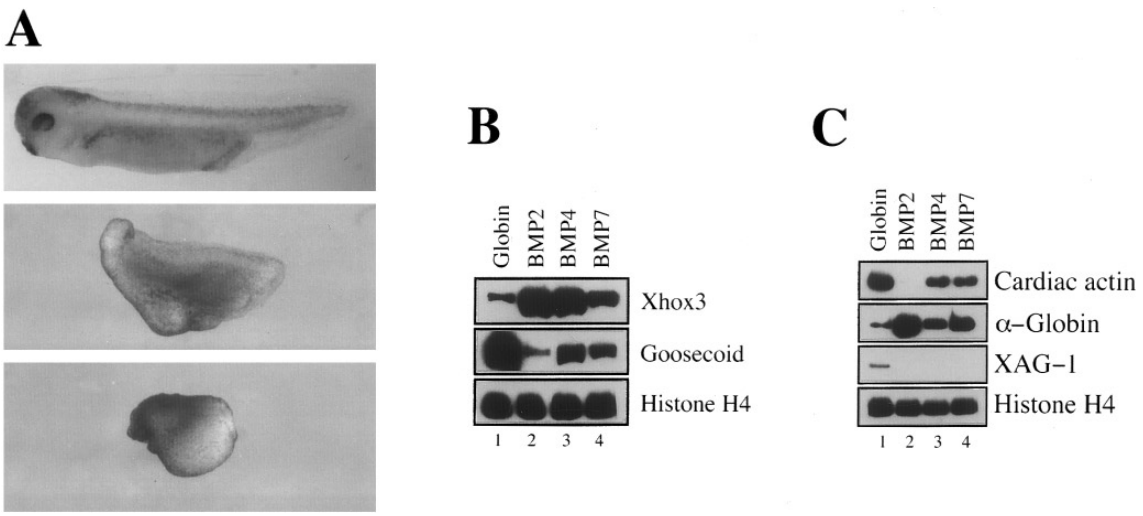

FIG. 2. BMP-7 ventralizes *Xenopus* embryos. (**A**) Embryos injected with BMP-7 RNA are ventralized. Upper panel, control embryo injected with 2 ng globin RNA; middle and lower panels, embryos injected with 2 ng BMP-7 RNA. Total RNAs purified from 10 embryos were pooled and assayed by RT-PCR at early gastrula (**B**) and tadpole stages (**C**). Two nanograms of RNA was also used for globin, BMP-2, -4 and -7. Histone H4 was used as loading control.

of BMP-4 and BMP-7 when presented to the explants as either homodimers or as a heterodimer of BMP-4 and BMP-7 (BMP-4/7). We used recombinant *Xenopus* BMPs which were produced by means of the baculovirus expression system (7). Purified *Xenopus* BMP proteins produced in this manner have been shown to dimerize correctly and to have full biological activity in bone-inducing assays (6, 7). We tested the purified homodimers of BMP-2, -4, or -7, along with BMP-4/7 proteins for their mesoderm inducing activities in ectodermal explant assays. Explants isolated at blastula stage were cultured in medium containing BMPs until sibling controls reached early gastrula stages. Expression of marker genes was analyzed by RT-PCR assay. Figure 4 shows that, in agreement with previous reports, BMP-2, -4, -7 homodimers, or a mixture of BMP-4 and -7 homodimers could barely induce the expression of Xhox3, Xbra, or Xwnt-8 even when used at very high doses (1 μg/ml, data not shown). Interestingly, however, the BMP-4/7 heterodimer induced mesoderm at

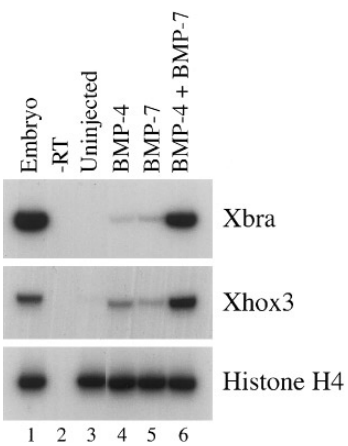

FIG. 3. Synergistic effect of BMP-4 and BMP-7 on mesoderm induction of *Xenopus* embryo. Two hundred picograms of BMP-4 or BMP-7 RNAs were injected separately or co-injected together (100 pg of each) into animal pole of 2-cell embryos. Animal pole explants were excised at blastula stage and cultured until control embryos reached gastrula stage. Xbra and Xhox3 were used as early mesodermal markers. Uninjected explants were used for control and absence of reverse transcriptase in the RT-PCR reaction is shown as -RT.

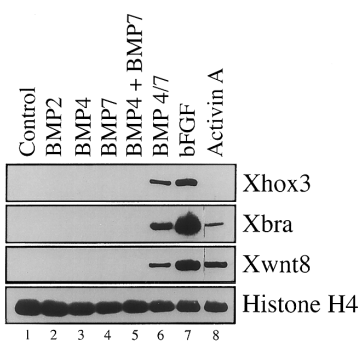

FIG. 4. *Xenopus* BMP-4/7 heterodimer induces mesoderm. Ectodermal explants from blastula stage embryos were cultured in 1XSteinberg's solution containing recombinant *Xenopus* BMP homodimers (BMP-2, -4, and -7) or a heterodimer of BMP-4 and BMP-7 (BMP-4/7) as indicated. Lane 1, 1XSteinberg's solution only; lanes 2-4, 50 ng/ml of BMP-2, -4 and -7, respectively; lane 5, combination of 50 ng/ml each of BMP-4 and BMP-7; lane 6, 50 ng/ml of BMP-4/7; lane 7, 100 ng/ml of bFGF; lane 8, 10 ng/ml of activin A. The explants were cultured until control embryos developed to early gastrula stage, and the expression of early mesodermal marker gene was examined by RT-PCR assay using a total RNA equivalent to 2.5 explants.

doses 20 times (50 ng/ml) below what was required for homodimer proteins. This is comparable to doses of bFGF protein required to induce mesoderm. This result suggests for the first time that heterodimers of BMPs might be involved in mesoderm induction and not only patterning during gastrula stages as it was previously suggested. Both BMP-4 and BMP-7 transcripts are expressed ubiquitously in the embryo maternally and during the time of mesoderm induction and patterning, supporting the possibility of heterodimer formation (8, 9). While this type of heterodimerization can generate multiple biological activities from limited numbers of subunit proteins, it remains to be seen if this capacity is used more globally to also operate within the ectoderm during epidermal and neural specification (3).

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