Evidence for a Role of Smad6 in Chick Cardiac Development

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Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are obligatory growth factors for early embryogenesis and heart formation. SMAD proteins transduce signals of the TGF-β superfamily. We isolated chicken Smad6 (cSmad6), a member of inhibitory SMADs, and found its expression to be remarkably restricted to the developing heart, eyes, and limbs. cSmad6 expression was detected in the cardiogenic region of stage 5 embryos and overlapped Nkx2-5 and bmp-2, -4, and -7 expression. Throughout development, cSmad6 was expressed strongly in the heart, primarily in the myocardium, endocardium, and endocardial cushion tissue. Myocardial expression of cSmad6 was stronger in the forming septum, where highly localized expression of bmp-2 and -4 was also observed. Ectopically applied BMP-2 protein induced the expression of cSmad6, a putative negative regulator of BMP-signaling pathway, in anterior medial mesoendoderm of stage 4–5 embryos. In addition, blocking of BMP signaling using Noggin downregulated cSmad6 in cardiogenic tissue. cSmad1, one of the positive mediators of BMP signaling, was also expressed in cardiogenic region, but was not BMP-2 inducible. Our data suggest that cSmad6 has a role in orchestrating BMP-mediated cardiac development. We propose the possible mechanism of action of cSmad6 as modulating BMP signal by keeping a balance between constitutively expressed pathway-specific cSmad1 and ligand-induced inhibitory cSmad6 in the developing heart. © 1999 Academic Press

Key Words: bone morphogenetic protein; inhibitory SMAD; Smad6; Smad1; cardiac development; chick embryo.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) gene family and play multiple roles during morphogenesis and pattern formation in vertebrate embryos (Lyons et al., 1990; Jones et al., 1991; reviewed in Hogan, 1996). Recently SMAD proteins have been identified as signal transducers of the TGF-β superfamily (reviewed in Heldin et al., 1997; Kretzchmar and Massagué, 1998; Whitman, 1998). To date, 10 different Smad genes have been isolated (Heldin et al., 1997; Kretzchmar and Massagué, 1998; Whitman, 1998; LeSueur and Graff, 1999) that have been categorized into three subgroups (reviewed in Heldin et al., 1997).

The first subgroup encodes pathway-specific signal-transducing SMADs, which consists of Smad1, Smad5, and Smad8 that mediate BMP signaling and Smad2 and Smad3 that mediate TGF-β and activin signaling. These positive mediators are phosphorylated at a carboxy-terminal SSXS consensus motif by ligand-bound type I receptor (Kretzschmar et al., 1997; Macias-Silva et al., 1996) and subsequently form a heterodimer with Smad4 (Lagna et al., 1998; Zhang et al., 1996, 1997). Smad4 is a member of the second subgroup of SMAD proteins and binds to all pathway-specific SMADs. Heterodimers formed between pathway-specific SMADs and Smad4 translocate into the nucleus (Kretzschmar et al., 1997; Macias-Silva et al., 1996) and transactivate specific target genes (Liu et al., 1996). The third subgroup of the SMAD family is composed of inhibitory SMADs, which antagonize the function of the pathway-specific SMADs (reviewed in Whitman, 1997). In vertebrates, Smad6 and Smad7 function as inhibitors of BMP, TGF-β, and activin signaling (Imamura et al., 1997; Topper et al., 1997; Hata et al., 1998; Kleeff et al., 1999). Whereas Smad7 acts as an inhibitor of signaling by all
TGF-β family members (Nakao et al., 1997; Hayashi et al., 1997; Bhushan et al., 1998), Smad6 appears to be an inhibitor of BMP signaling (Hata et al., 1998). TGF-β/BMP signaling is inhibited by Smad6 and Smad7 because these proteins bind to type I receptors with greater affinity than the pathway-specific SMADs and thereby prevent phosphorylation of the pathway-specific SMADs (Imamura et al., 1997; Hayashi et al., 1997; Nakao et al., 1997). Moreover, Smad6 competes with Smad4 for binding to receptor-activated Smad1, yielding an inactive Smad1–Smad6 complex (Hata et al., 1998).

Even though BMP and TGF-β proteins are clearly involved in embryogenesis (reviewed in Hogan, 1996), little is known about the expression pattern and the roles of the various SMADs during embryogenesis (reviewed in Heldin et al., 1997; Whitman, 1998). In the mouse embryo Smad1 and Smad2 are expressed at high levels in a variety of developing organs at sites where epithelial–mesenchymal interactions occur (Dick et al., 1998). A brief description of the pattern of expression of Smad6 was reported for Xenopus embryos (Nakayama et al., 1998a). Xenopus Smad6 (xSmad6) transcripts are found in parts of the dorsal neural tube, the dorsal part of the eye, the otocyst, and the heart anlage (Nakayama et al., 1998a). Deletions of thebmp-2 and bmp-4 genes in mice result in early embryonic death. In the case of bmp-2−/− mice, amnion and chorion formation and cardiac development are abnormal (Zhang and Bradley, 1996). bmp-4−/− and ALK3 (BMP type I receptor)−/− mice exhibit little or no mesodermal differentiation (Winner et al., 1995; Mishina et al., 1995). Recently Smad2, Smad4, and Smad5 genes have been knocked out in the mouse with the result of early embryonic lethality. Homozygous Smad4 mutants fail to gastrulate, do not express mesodermal markers, show abnormal visceral endoderm development, and die before E8.5 (Yang et al., 1998; Siraard et al., 1998). Mice that lack Smad2 also fail to undergo gastrulation and do not make normal mesoderm (Waldrup et al., 1998; Nomura et al., 1998; Weinstein et al., 1998). Smad5 knockout mice showed a less severe phenotype than mutant mice lacking BMPs, Smad2, or Smad4, but they eventually died at E9.5−11.5 with cardiac defects similar to bmp-2 knockout mice or impaired vasculogenesis/hematopoesis (Chang et al., 1999). Taken together, these findings suggest that BMPs and their downstream signal transducers, the SMADs, are required for gastrulation and mesoderm formation.

The heart is among the first functional organs that arise from mesoderm. Drosophila tinman is a mesoderm-specific homeobox gene (Bodmer et al., 1990) that is required for midgut and heart formation (Azpiazu and Frasch, 1993; Bodmer, 1993). Decapentaplegic (Dpp), a Drosophila homolog of bmp-2 and -4, is required for the expression of tinman and is thought to determine which cells of the mesoderm become competent to develop into visceral mesoderm and the heart (Frasch, 1995). The 3′-flanking region of the tinman gene contains binding sites for the M ectoderm and Mad proteins, the Drosophila homologs of Smad4, and of pathway-specific SMADs, respectively (Xu et al., 1998). Medea mutant embryos show significant reduction of tinman expression in prospective cardiac mesoderm indicating a requirement for Medea to activate tinman expression in response to Dpp. A vertebrate homolog of tinman, Nkx2-5 (reviewed in Harvey, 1996), is the earliest known marker of mesoderm fated to give rise to myocardium (Schultheiss et al., 1995). Murine Nkx2-5 null mutants form a rudimentary heart tube that subsequently fails to loop and form a four-chamber heart. Therefore, these embryos die of cardiac failure by E10−11.5 (Lyons et al., 1995; Tanaka et al., 1999). This cardiac phenotype is similar to that seen in bmp-2−/− mouse embryos that develop to E9.5 (Zhang and Bradley, 1996). In addition, BMP-2 ectopically applied to noncardiogenic region of early chick embryos induces Nkx2-5 as well as other cardiac marker genes such as GATA4 and MHC (Schultheiss et al., 1997; Andreé et al., 1998). These findings in Drosophila and vertebrates implicate the importance of BMP signaling in vertebrate heart development but the potential role of SMAD proteins in cardiac development has not yet been addressed in vertebrates and is the focus of the present study.

We have isolated a chicken Smad6 cDNA as well as partial cDNAs of several other chicken Smad genes using RT-PCR and determined their expression patterns during early chick embryogenesis. We found that chicken Smad6 (cSmad6), one of the inhibitory SMADs, was expressed strongly in the developing heart, eyes, and limbs. In particular, during the period of cardiac induction, cSmad6 expression overlapped the expression of bmp-2, -4, and -7. We found that locally released BMP-2 protein induced cSmad6 in anterior medial mesoderm, a region that responded to BMP-2 by expressing cardiac marker genes (Nkx2-5, GATA4, MHC; see Schultheiss et al., 1997; Andreé et al., 1998). BMP-pathway-specific cSmad1 was also expressed in cardiogenic region, but was not BMP-2 inducible. Incubation of cardiogenic mesodendoderm with Noggin, an antagonist of BMP signaling (Zimmerman et al., 1996), decreased the level of cSmad6 expression. These data suggest an important role of cSmad6 in orchestrating BMP-mediated cardiac development. We propose the possible mechanism of action of cSmad6 as modulating BMP signal by keeping a balance between constitutively expressed pathway-specific cSmad1 and ligand-induced inhibitory cSmad6 in the developing heart.

**MATERIALS AND METHODS**

**Isolation of a cSmad6 cDNA**

Total RNA was isolated from stage 18–21 (Hamburger and Hamilton, 1951) chicken embryos. First-strand cDNA was synthesized using random primers and AMV reverse transcriptase. PCR was carried out using the degenerate oligonucleotides 5′-AGTAACAGTTTCTGATCTGTTCCGA-3′ (forward) and 5′-CCAGCCCTTGCGCAAGCTGATGC-3′ (reverse) (arrows in Fig. 1A). The PCR conditions were 94°C, 4 min and then 35 cycles of
94°C, 20 s; 55°C, 30 s; 72°C, 60 s; and final extension by 72°C, 10 min. PCR products were run on a 1% agarose gel, isolated, and subcloned into pCRscript-SK(+) vector (Stratagene). The 590-bp PCR fragment generated was used to screen one million plaques from a lambda-ZAP phage cDNA library prepared from stage 14–17 chick embryos. Isolated clones were sequenced using an AmpliTaq-FS dye terminator cycle sequencing kit (Perkin Elmer) and an ABI-Prism377 sequencer (PE-Applied Biosystems). Partial cDNAs (cSmad1, cSmad5, cSmad6) were isolated by RT-PCR (Accession Nos.: cSmad1, AF143239; cSmad5, AF143240; cSmad6, AF165889).

Expression Analysis by in Situ Hybridization

Chick embryos at stage 4–30 were fixed in MEMFA fixative (0.1 M Mops, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) and stored at −20°C until use as described (Albrecht et al., 1997). Synthesis of digoxigenin-labeled RNA probes corresponding to cSmad6, cSmad1, cSmad5, Nkx2-5 (Schulteiss et al., 1995), BMP-2 (Fransis et al., 1994), BMP-4 (Roberts et al., 1995), and BMP-7 (Oh et al., 1996) was performed with a Stratagene RNA labeling kit as described previously (Albrecht et al., 1997). All probes were alkaline hydrolyzed to the size of ~300 bp in order to enhance probe penetration (Angerer and Angerer, 1992) and whole-mount in situ hybridization was carried out following the protocol described in Albrecht et al. (1997) except that polyvinyl alcohol was included to increase signal (Barth and Ivarie, 1994). Following color development, 10-μm sections were cut from paraffin-embedded whole mounts. In situ hybridization on paraffin sections was carried out using 32P-labeled riboprobes as described (Albrecht et al., 1997). Seven-micrometer sections were hybridized at 58°C overnight, and stringency washes were performed at 67°C. Slides were dipped in NTB-2 emulsion and exposed for 2–3 days. Tissue was visualized by Hoechst dye staining of nuclear DNA. Silver grains were visualized in the dark field were represented in false colors in figures.

BMP-2 Application

Rat bone matrix particles obtained from Genetics Institute (Cambridge, MA) or heparin–acrylamide beads (Sigma H-5263) were soaked in 1 μl of 30–90 μg/ml solution of human recombinant BMP-2 (Genetics Institute) overnight at 4°C in a moist chamber. Beads soaked in carrier protein (0.1% bovine serum albumin) were used as a control. Stage 5 embryos were placed ventral side up on albumin–agar plates (Sundin and Eichele, 1992), and BMP-2 carriers were implanted to the anterior medial area of the embryos as previously described (Schulteiss et al., 1997). Embryos were incubated for 4–9 h, fixed with MEMFA as described above, and processed for whole-mount in situ hybridization.

Explant Cultures

Tissues were dissected from chick embryos with tungsten needles (Sugi et al., 1993) and cultured in type I collagen gels (Becton Dickinson, Bedford, MA) (Schulteiss et al., 1997; Selleck et al., 1998) in wells of a Lab-Tek chamber slide (Nunc No. 178599). Anterior medial explants from stage 5–6 embryos were used for the BMP-2 treatment. Explants were cultured in M 199 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin with or without BMP-2 protein. Anterior lateral mesoendoderm from stage 4–5 embryos was used for the Noggin experiments.

Noggin-conditioned medium was harvested from CHO cells stably transfected with Xenopus noggin (Lamb et al., 1993). Control conditioned medium was prepared from parental CHO cell line. Noggin and control media were added to explants and supplemented with fetal bovine serum to a final serum concentration of 10%. Explants were cultured in a humidified incubator at 37°C and 5% CO2 until evaluation.

Reverse Transcription (RT)-PCR Analysis

RT-PCR analysis was performed as described previously (Schulteiss et al., 1995; Yatskievych et al., 1997). RNA was harvested from explant cultures by using RNaseasy kit (Qiagen, Valencia, CA). RT reactions were performed in a 40-μl volume using random hexamers and 200 U of Superscript reverse transcriptase (Life Technologies). PCR reactions were carried out in a volume of 25 μl, using PCR buffer (Perkin Elmer, Foster City, CA), 1.25 U of Taq polymerase (Perkin-Elmer), 1.5 mM MgCl2, 0.2 mM each dNTP (Boehringer Mannheim), 0.4 μM of each primer, and one-tenth of reverse transcribed cDNA. The primers for the genes to amplify GAPDH and Nkx2-5 were those described by Schulteiss et al. (1995). The primer pair for cSmad6 was 5′-CCAGAGTCCGCTGCTCC-3′ (forward) and 5′-CCTTCTGCTCAGAGGCTGACTGTTATCG-3′ (reverse) corresponding to nucleotides 922–948 and 1597–1623 of chicken Smad6, designed to amplify a 702-bp PCR product. Amplification was carried out by a shortened program (Mai et al., 1998) as follows: (1) denaturation at 94°C for 2 min; (2) cycles of denaturation (94°C, 2 s), annealing (56°C for 5 s), and extension (72°C, 5 s); and (3) final extension at 65°C for 10 min. The cycle number for each primer pair was chosen to fall within the linear range of amplification using positive control RNA samples. To test for the absence of contaminating genomic DNA, RNA samples, which were not reverse transcribed, were simultaneously processed. For visualization, one-third of each PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide. Alternatively, PCR was performed with 0.1 μl of [32P]dCTP, and products were separated on a 6% polyacrylamide gel. Dried gels were exposed overnight to X-ray film.

RESULTS

Isolation and Characterization of the Chicken Smad6 cDNA

A partial cSmad6 cDNA was obtained using a RT-PCR based strategy, and subsequently this fragment was used to screen a chick cDNA library (see Materials and Methods). The longest isolated cDNA was 1967 nucleotides (nts) in length that included 1296 nts of an open reading frame encoding 431 amino acids (Fig. 1A), 306 nts of 5′-untranslated region, and 365 nts of the 3′-untranslated region. BLAST search revealed that the protein encoded by this cDNA had a significant similarity to Smad6 and Smad7 proteins of other species. The highest degree of homology was observed with human Smad6 (hSmad6) (P(N) = e−107), mouse Smad6 (mSmad6) (P(N) = e−108), and Xenopus Smad6 (xSmad6) (P(N) = e−118), followed by human Smad7 (P(N) = e−107). The overall identity of the chicken protein amino acid sequence was 64% with hSmad6, 62%
with mSmad6, and 69% with xSmad6, followed by 46–47% with Smad7 proteins of other species. This suggests that the chicken cDNA encodes the chick homolog of Smad6.

Further examination of chicken Smad6 (cSmad6) protein revealed conserved SMAD structural domains (Fig. 1B). SMAD proteins typically consist of three regions: the highly conserved N- and C-terminal domains termed MH1 and MH2 and a less-conserved linker (reviewed in Heldin et al., 1997). Although the MH1 domain of the putative cSmad6 shared only 44–46% identity with mSmad6 and hSmad6 (Fig. 1B), the first 18 amino acids of the avian MH1 domain were 100% identical to those of the human and mouse Smad6 proteins. Residues 19 to 93 were poorly conserved, but the following amino acids extending from Glu 94 to the C-terminus of MH1 (Pro 206) were highly conserved (56–73% identity). The linker region of the putative cSmad6 (Glu 207 to Ser 263) had no similarity with pathway-specific SMADs or with Smad4, but this region was conserved amongst the Smad6 proteins of different species (Fig. 1B). In particular, the ESP(P/T)PPYXR motif located at the N-terminus of the linker region was identical to that in all other Smad6 and Smad7 proteins. The MH2 domain of the putative cSmad6 (His 264 to C-terminus) showed high similarity to all known SMADs including the pathway-specific SMADs. The highest homology, however, resided with hSmad6, mSmad6, and xSmad6. The putative cSmad6 lacked the SSXS motif found at the end of the MH2 domain of the pathway-specific SMADs. This motif serves as a receptor phosphorylation site and its absence is a hallmark of inhibitory SMADs (Kretzschmar and Massague, 1998). Taken together, sequence homology and the presence or absence of certain features strongly suggests that the isolated chicken cDNA encodes the chicken Smad6 protein, one of several known inhibitory SMADs.

Temporal and Spatial Expression Pattern of cSmad6 in the Developing Heart

In order to determine the spatiotemporal expression of cSmad6, whole-mount in situ hybridization was performed on Hamburger–Hamilton stage 4 to 30 chick embryos. Expression of cSmad6 was first detected at stage 4 in the posteriormost part of the primitive streak and at the margin of the embryo (Fig. 2A) in a region of prospective extraembryonic tissue (Garcia-Martinez and Schoenwolf, 1993). Expression in these domains increased by stage 5 (Fig. 2B), a
stage at which cSmad6 mRNA also began to appear in an anterior lateral region in a crescent-shaped pattern, previously described as a cardiogenic region or a cardiac crescent (Rosenquist and DeHaan, 1966; Ehrman and Yutzey, 1999). Expression in the crescent was identical to that of Nkx2-5 (compare Figs. 2B and 2G), the earliest known marker gene for cardiogenic tissue (Schultheiss et al., 1995). Unlike Nkx2-5, cSmad6 expression at this stage was found not only in cardiogenic mesoderm but also in the underlying endoderm (data not shown). At stage 7, the expression pattern of cSmad6 was similar to that seen at earlier stages, but transcript levels were further increased in the cardiac region, while expression in the posterior part of embryo became more diffuse (Fig. 2C). At stage 8, cSmad6 expression was very pronounced in the nascent bilateral heart tubes (Fig. 2H) and in the posteriormost tip of the primitive streak (data not shown). Cross-sections at this stage showed cSmad6 transcripts in anterolateral splanchnic mesoderm and the underlying endoderm (Fig. 2O). After stage 8, bilateral heart tubes begin to fuse at the midline, and a single primitive straight heart tube is formed. During formation of the single heart tube and later, cSmad6 was strongly expressed throughout the entire heart (Figs. 2I and 2J), both in the myocardium and in the endocardium (Figs. 2P and 2Q).

SMAD proteins are known to mediate BMP signal trans-
duction (reviewed in Whitman, 1998). Therefore, we compared the expression of cSmad6 with that of several BMPs expressed in the cardiac region (Schultheiss et al., 1997). The transcripts of bmp-2, -4, and -7 were detected in the cardiogenic region at stage 5 to 6 (Figs. 2D–2F), and expression resembled that of cSmad6 (Fig. 2B). bmp-2 is endoderm specific, whereas bmp-4 and -7 are expressed only in ectoderm (Schultheiss et al., 1997). cSmad6 transcripts were found in endoderm and mesoderm (Fig. 2O), but since BMPs are secreted factors any of the three BMPs could enter tissue that expressed cSmad6. Until stage 6, the location of cSmad6 expression overlapped with those of bmp-2, -4, and -7. Thereafter, the patterns of expression diverged. By stage 10, when the formation of a primitive straight heart tube was completed, bmp-2 expression in the heart had disappeared except in the vitelline veins (Fig. 2K). Likewise, expression of bmp-4 was no longer detectable in the heart (Fig. 2L), bmp-7 (Fig. 2M) and cSmad6 (Fig. 2I) transcripts, however, were present throughout the heart.

At stage 11 when the heart started looping to the right, cSmad6 was strongly expressed in the entire heart (Fig. 2J) and in the dorsal aorta and the dorsal wall of the vitelline veins, both of which were connected to the heart (Figs. 2J and 2Q and data not shown). cSmad6 expression in the developing heart persisted at least to stage 28, in the myocardium and in the endocardium.

To investigate cSmad6 expression at later stages of heart development, in situ hybridization was conducted on tissue sections. We were particularly interested in the overlapping pattern of expression of cSmad6 and BMPs, since BMPs were considered to be involved in the process of septation at this stage (reviewed in Eisenberg and Markwald, 1995; Yamagishi et al., 1998). After the formation of the straight heart tube, the looping process follows, and subsequently each chamber of the heart starts to be separated by septum formation to eventually form a complete four-chamber heart (Bellairs and Osmond, 1998; Colvin, 1990). At stage 17, a subpopulation of endocardial cells located at the junction of the primitive atrium and ventricle undergoes a transformation to mesenchymal cells (Bolender and Markwald, 1979). Three cells then invade the underlying extra-cellular matrix and therein form endocardial cushion tissue. This cushion tissue consists of two components; the atrioventricular cushion (AV cushion), which contributes to the formation of atrioventricular septum and valves, and the outflow tract cushion (OT cushion), which forms aorticopulmonary septum (Keyes and Sanders, 1999). These cushions are essential elements for septation and valve formation accomplished by stage 34. Mesenchymal transformation that generates the cushion tissue is thought to be regulated by signaling molecules secreted by the underlying myocardium (Krug et al., 1987; Markwald et al., 1990). Several BMPs and other TGF-β family members are expressed in these valve- and septum-forming regions (Lyons et al., 1990; Jones et al., 1991; Nakajima et al., 1994, 1998; Ramsdell and Markwald, 1997) and may mediate the above-described processes.

At stage 20, when the formation of endocardial cushion had begun, cSmad6 was expressed throughout the entire heart (Fig. 3A). Expression was particularly strong in the myocardium at the atrioventricular junction, where highly localized bmp-2 expression was also observed (Fig. 3B). bmp-4 was not expressed at this stage (data not shown), but bmp-7 was expressed in the myocardium throughout the entire heart (Fig. 3C), which was also the case for Nkx2-5 (Fig. 3D).

At stage 26–28, when the septation process was close to completion, bmp-2 expression was highly restricted to the myocardium at the nascent interatrial septum, the atrioventricular grooves, the tip of interventricular septum (Fig. 3F), and the aorticopulmonary septum (Fig. 3K). cSmad6 expression was seen throughout the heart, not only in the myocardium and endocardium, but also in the endocardial AV cushion (Fig. 3E). Expression was high in the regions forming a ventricular septum and the aorticopulmonary septum (Figs. 3I and 3J). Interestingly, the OT cushion, unlike the AV cushion, never expressed cSmad6 (Fig. 3I). Nkx2-5 was expressed only in the myocardium, but not in the endocardial cushion (Fig. 3H). In comparison, the expression of bmp-4 reappeared at this stage and was even more restricted than bmp-2, being found only in the myocardium surrounding the OT cushion (data not shown). Another BMP member, bmp-7, showed ubiquitous expression in myocardium, with clear border between conus muscle and truncal muscle (Figs. 3G and 3L). The expression of bmp-2, -4, and -7 covered all areas of cSmad6 expression, except for the endocardial cushion. Figure 3N summarizes the overlapping patterns of expression of cSmad6 and bmp-2, -4, and -7 at stage 26–28.

**Temporal and Spatial Expression Pattern of cSmad6 during Eye and Limb Development**

cSmad6 expression was also observed in developing eyes, limbs, somites, and lungs. Because BMP-2, -4, and -7 are implicated in eye and limb development (Dudley et al., 1995; Luo et al., 1995; Solursh et al., 1996; Furuta and Hogan, 1998; Wawersik et al., 1999; Fransis et al., 1994; Hogan, 1996), we briefly describe cSmad6 expression patterns in these tissues. The expression in the prospective eye was first seen at stage 10 (Fig. 4A) in the neuroepithelium of optic vesicles (Fig. 4D). As the surface ectoderm contacting the optic vesicle started to form lens placode, cSmad6 mRNA began to be expressed in the placode (Fig. 4E). Expression in the optic cup decreased during stage 13 (Fig. 4B) and cSmad6 was no longer detectable in the optic cup by stage 17 (Figs. 4C and 4F) but only in the dorsal portion of the lens vesicle (Fig. 4F). cSmad6 was also expressed in developing limbs. At stage 14, expression was seen uniformly along the entire flank in the lateral plate mesoderm (data not shown). By stage 18, expression disappeared in the interlimb flank but increased in the nascent limb buds (Fig. 4G). As the limb buds extended distally, the signal became concentrated to the mesenchyme underneath the apical...
FIG. 3. Comparison of the expression pattern of cSmad6, bmp-2, bmp-7, and Nkx2-5 in the developing heart at stage 20 and stage 26-28 by in situ hybridization on tissue sections. The levels of sectioning are indicated in the diagrams of the heart on the left. (A) At stage 20, when septation of the heart had begun, cSmad6 was expressed throughout the heart, both in the myocardium and in the endocardium. High
ectodermal ridge. cSmad6 transcript levels were elevated at the anterior and posterior limb bud margins, and low expression was also found in the apical ectodermal ridge (Fig. 4H). At the time when digits started to form, cSmad6 expression was restricted to the interdigital zones (Figs. 4I and 4J).

Expression of BMP-Pathway-Specific cSmad1 and cSmad5 in the Cardiogenic Region

As described above, the spatiotemporal patterns of cSmad6 expression resembled those of bmp-2, -4, and -7 in the developing heart. Since several in vitro studies have suggested that Smad6 was a negative regulator of BMP signaling (Imamura et al., 1997; Topper et al., 1997; Hata et al., 1998), we expected that positive mediators of BMP signaling, such as Smad1 and Smad5 (Kretzchmar and Massagué, 1998), might also be expressed in the cardiogenic region. This prompted us to survey the expression pattern of these two genes, during the early stages of cardiac development.

Partial cDNAs of cSmad1 and cSmad5 were isolated using the RT-PCR strategy described here in detail for cSmad6. Corresponding riboprobes were used for whole-mount in situ hybridization analysis. cSmad1 and -5 were expressed along the primitive streak and in the anterior region of stages 6–7 embryos (Figs. 5A and 5B). The cSmad1 expression pattern (Fig. 5A) was similar to that of the BMPs (Figs. 2D–2F) and of cSmad6 (Fig. 2B), but expression was more diffuse including anterior lateral and medial areas and weaker (note riboprobes of similar length were used for cSmad1 and cSmad5). Of note, cSmad1 was expressed in the cardiogenic region (Fig. 5A) and continued to be expressed in the cardiac tissue to at least stage 7 (Fig. 5E), although expression levels were low when compared with that of cSmad6 (Fig. 2H). cSmad5 was expressed in neuroectoderm and anterior medial area of the embryo at stages 6–7, but transcripts were not found in the anterior lateral cardiogenic region, suggesting that this gene was not involved in heart formation at this stage. Cardiac cells exhibited weak cSmad5 expression at stage 8 (Fig. 5F). Although cSmad1, a positive regulator of BMP signaling, is expressed levels of cSmad6 are observed in the myocardium at the atrioventricular junction. (B) bmp-2 was expressed only in the myocardium at the atrioventricular junction. bmp-7 (C) and Nkx2-5 (D) were expressed in entire myocardium, similar to cSmad6. (E) At stage 28, cSmad6 expression was detected in the myocardium, the endocardium, and the atrioventricular endocardial cushion. Expression of cSmad6 was accentuated in the forming interventricular septum (I) and aortico-pulmonary septum (J). (H, M) Nkx2-5 mRNA was found only in the myocardium and not in the endocardial cushion and the aortico-pulmonary septum. (G, L) bmp-7 expression was seen in the myocardium, from atria, through ventricles until conus muscles. (F, K) bmp-2 expression was limited to the portions of the myocardium contributing to septum formation, and there was no bmp-2 expression in the endocardial cushion. (N) Diagrams showing the overlapping patterns of expression of cSmad6 and bmp-2, -4, and -7 at stage 26–28. The four-chamber view of the heart representing the level of E–H is shown on the left, and the section of outflow tract representing the level of J–M is on the right. Abbreviations: a, anterior; aps, aortico-pulmonary septum; av, atrioventricular endocardial cushion; avg, atrioventricular groove; avj, atrioventricular junction; cns, conus; d, dorsal; ecc, endocardial cushion; end, endocardium; ias, interatrial septum; ivs, intraventricular septum; la, left atrium; lv, left ventricle; myo, myocardium; otc, outflow tract endocardial cushion; p, posterior; pv, primitive ventricle; ra, right atrium; rv, right ventricle; tr, truncus; v, ventral.

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in the cardiogenic region, expression is clearly less cardiac-specific than that seen for cSmad6, indicating that BMP specificity in the heart is primarily affected by the inhibitory SMADs, cSmad6 in this case.

Regulation of cSmad6 by BMPs

The strong expression of cSmad6 in the cardiogenic region as well as the expression of BMPs and Nkx2-5 suggested that cSmad6 might have a specific active function in the signaling pathway between BMPs and Nkx2-5 during the period of cardiac induction. Schultheiss et al. (1997) showed that BMP-2 could induce Nkx2-5 expression when BMP-2-soaked beads were implanted to the anterior medial area of stage 5 embryos, which was normally non-cardiogenic but competent to form cardiac tissues. We tested whether cSmad6 could be induced by ectopic application of BMP to this noncardiogenic tissue. Heparin–acrylamide beads or rat bone matrix particles soaked in 30 μg/ml BMP-2 were implanted into the anterior medial area of the stage 5 embryos. The embryos were incubated for 4 to 9 h after implantation and were subsequently processed for whole-mount in situ hybridization to evaluate the expression of cSmad1, -5, and -6 and Nkx2-5. BMP-2 induced intense expression of cSmad6 in cells surrounding the beads in a domain medial to the endogenous cSmad6 expression in 75% of embryos implanted (n = 36) (Fig. 5C, Table 1). A cross-section at the level of the implant showed the strong induction of cSmad6 around the bead, not only in the mesoderm but also in surrounding ectoderm and even a part of a neural tube (Fig. 5G). A similar induction was found for Nkx2-5 (Fig. 5D and Schultheiss et al., 1997).

We observed that release of BMP-2 from either carrier was able to induce cSmad6. Moreover, induction was dose dependent, in that the lower dose evoked cSmad6 expression in about half of the cases, while the higher dose was 100% effective (Table 1). Longer incubation with BMP-2 decreased the percentage of cSmad6 induction by BMP-2 (Table 1), while Nkx2-5 induction was still observed in all cases (data not shown). Neither cSmad1 (Fig. 5E) nor cSmad5 (Fig. 5F) was induced by BMP-2, even when the concentration of BMP-2 was increased to 90 μg/ml.

We then asked whether cSmad6 mediated the effect of BMP on Nkx2-5 expression or acted in some other fashion in cardiac development. Tissue explants obtained from the anterior medial region from stage 5 to 6 embryos were cultured in either presence or absence of BMP-2 and were analyzed for expression of cSmad6 and Nkx2-5 by RT-PCR (Fig. 6A). Transcripts of Nkx2-5 were not detected in anterior medial explants prior to incubation and 1 h after incubation with BMP-2. By 3 h of BMP-2 treatment, how-

### Table 1

<table>
<thead>
<tr>
<th>Carrier type</th>
<th>Concentration of BMP-2</th>
<th>Duration of treatment</th>
<th>Percentage of Embryos Which Showed cSmad6 Induction</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>4–5 h</td>
<td>8–9 h</td>
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<tr>
<td>Bone particle</td>
<td>5 μg/ml</td>
<td>43% (n = 7)</td>
<td>—</td>
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<tr>
<td></td>
<td>30 μg/ml</td>
<td>100% (n = 12)</td>
<td>57% (n = 7)</td>
</tr>
<tr>
<td>Heparin bead</td>
<td>30 μg/ml</td>
<td>60% (n = 5)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>90 μg/ml</td>
<td>100% (n = 5)</td>
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Note. n, number of cases. Induction was assessed by whole-mount in situ hybridization.
FIG. 6. (A) Induction of cSmad6 and Nkx2-5 by BMP-2 in chick explant cultures. Tissues from anterior medial (AM) region of stage 5–6 embryos were cultured in either presence (+) or absence (−) of BMP-2. Cardiogenic tissue explants (anterior lateral tissue, AL) were used as a positive control. Ethidium bromide staining of RT-PCR products showed induction of both cSmad6 and Nkx2-5 within 3 h of BMP-2 treatment. After 6 h of BMP-2 treatment, Nkx2-5 was still present, but cSmad6 induction was no longer obvious. A low level of cSmad6 expression was always detected in these anterior medial explants without the presence of BMP-2, probably because of the greater sensitivity of RT-PCR. GAPDH was used as an internal standard. (B) Reduction of cSmad6 by the BMP antagonist Noggin. Cardiogenic mesoendoderm of stage 4–5 embryos were incubated with (+) or without Noggin (−) for 48 h and analyzed for the expression of cSmad6 and GAPDH by RT-PCR. Ethidium bromide staining (right) and 32P labeling (left) both showed a significant reduction of cSmad6 expression by Noggin.

However, Nkx2-5 expression was induced and it continued for at least 6 h. Control tissues incubated without addition of BMP-2 did not express Nkx2-5 (Fig. 6A). cSmad6 was detectable at low levels in nontreated anterior medial explants, even though in situ hybridization did not show any expression of cSmad6 in this region, probably because of the greater sensitivity of RT-PCR. cSmad6 expression was not changed by a 1-h long treatment with BMP-2, but the level of cSmad6 had clearly increased after 3 h of BMP-2 treatment. Interestingly, after 6 h of BMP-2 treatment, induction of cSmad6 expression was no longer obvious (Fig. 6A). This might be the same outcome that we have seen in the BMP-2 beads experiment, as the reduction of percentage cSmad6 induction after 8–9 h of BMP-2 treatment (Table 1). It has been suggested that once it is turned on, Nkx2-5 expression persists through autoregulatory pathway (Reecy et al., 1999); therefore, in the case of Nkx2-5, expression observed at 6 h of BMP-2 treatment may not be a direct effect of BMP-2, but reflect autoregulation. These data suggest that induction of cSmad6 and Nkx2-5 occurs with a similar time course and in a parallel fashion, yet cSmad6 induction is transient while that of Nkx2-5 persists. This result makes it less likely that cSmad6 is upstream of Nkx2-5, but still supports the specific requirement of cSmad6 for the induction of Nkx2-5 by BMPs.

Explants taken from anterior lateral cardiogenic region of stage 4 chick embryos fail to differentiate into myocardial tissue when cultured in the presence of Noggin, a secreted protein acting as a BMP antagonist (Schultheiss et al., 1997). Because cardiac-specific marker genes such as Nkx2-5, GATA4, or MHC are not expressed in Noggin-treated tissue, it is proposed that BMP signaling is required for the differentiation of cardiogenic mesoendoderm into cardiac tissue (Schultheiss et al., 1997). We asked whether the level of cSmad6 was altered by the presence of Noggin. Anterior lateral mesoendoderm from stage 4 to 5 embryos was incubated with conditioned medium from CHO cells stably transfected with Noggin (Lamb et al., 1993). After 48 h of incubation, RNAs were harvested and subjected to RT-PCR to analyze expression of cSmad6, Nkx2-5, and GAPDH. Explants incubated with Noggin-free medium (i.e., supernatant from nontransfected CHO cells) showed expression of Nkx2-5 as well as of cSmad6 as would be expected (Fig. 6B; data not shown). In contrast, tissues incubated with Noggin-containing CHO cell supernatant exhibited a marked reduction of cSmad6 and Nkx2-5 expression. GAPDH, a marker gene, was not affected by the treatment.

In summary, the expression of cSmad6 is rapidly induced by ectopic application of BMP-2 and, moreover, endogenous cSmad6 expression is effectively blocked by Noggin, an inhibitor of BMP signaling. These findings thus support the hypothesis that cSmad6 expression is regulated by BMP in the developing heart.

DISCUSSION

We have studied the pattern of expression of cSmad6 and the regulation of this gene by BMP in the developing heart. Pathway-specific SMAD family transduces growth factor receptor signals to the nucleus, while others known as inhibitory SMADs serve as negative regulators of this process. Smad1 is an example for the first type of protein, whereas Smad6 is a negatively acting SMAD protein. We found that in the developing chick embryo the expression pattern of cSmad6 resembled the expression pattern of bmp-2, -4, and -7 (Schultheiss et al., 1997; Andréé et al., 1998; Fransis et al., 1994; Lyons et al., 1995; Solursh et al., 1996; Luo et al., 1995). This was especially evident in the developing heart where cSmad6 was expressed in the cardiogenic region in a manner identical to that seen with bmp-2, -4, and -7. This suggests that cSmad6 is involved in BMP-dependent aspects of cardiogenesis. In addition to cSmad6, the cardiogenic region also expressed cSmad1, which is known to transduce the BMP signal (Kretzschmar and Massagué, 1998). The link between BMP signaling and...
cSmad6 expression in heart development is bolstered by two additional observations. First, ectopically applied BMP-2 protein induced cSmad6 expression as well as cardiac fate markers in anterior medial explants. Second, blockage of BMP signaling using Noggin protein downregulated cSmad6 in the cardiogenic tissue (Schultheiss et al., 1997). These findings, together with the overlapping patterns of expression of bmps and cSmad6, suggest that BMPs are necessary and sufficient for cSmad6 expression in the cardiogenic region of the chick embryos.

The fact that inhibitory SMADs can be induced by the ligands whose signal transduction they then inhibit has been demonstrated in several in vitro systems (Takase et al., 1998; Afrakhte et al., 1998; Nakao et al., 1997). Smad6 and Smad7 can be induced by TGF-β, activin, and BMP-7 after 30–90 min of stimulation in certain cell lines that are responsive to these ligands. This occurs even in the presence of cycloheximide, a protein synthesis inhibitor (Nakao et al., 1997; Afrakhte et al., 1998), and has led to the suggestion that Smad6 and Smad7 could be direct target genes of this signaling pathway. The induction of inhibitory SMADs by a ligand results in establishment of a negative feedback that dampens the positive signaling activities of ligand. In the case of cardiac induction, the BMP signal could be transduced by the BMP-pathway-specific cSmad1 that we found to be expressed in the cardiogenic region and which is not ligand inducible. As a result of such a positive signaling molecule, cardiac genes such as Nkx2-5, GATA4, and MHC are induced. In parallel, BMP would also induce cSmad6, which would then counteract the activating role of cSmad1. We found that the induction of Nkx2-5 and cSmad6 occurred on a similar time scale, indicating that the balance between a constitutively expressed positive factor (cSmad1) and a ligand-inducible negative factor (cSmad6) could be important in orchestrating cardiac induction as manifested by Nkx2-5 expression.

Several studies have explored the issue of BMP-dependent regulation of other inhibitory SMADs (Tsuneizumi et al., 1997; Caselas and Hemmati-Brivanlou, 1998; Nakayama et al., 1998b; Bhushan et al., 1998). The spatiotemporal expression pattern of Xenopus Smad7 is similar to that of bmp-4. Ectopic BMP signaling significantly increases the level of xSmad7 expression, whereas blocking BMP with a dominant-negative BMP receptor decreases xSmad7 expression (Nakayama et al., 1998b). These authors suggest that Smad7 functions directly within BMP-responsive cells in which it modulates the amplitude or duration of BMP signaling, similar to what we have proposed above for cSmad6 in cardiac development. Drosophila Dad is another inhibitor SMAD whose MH2 domain shares the highest homology with Smad6 (Tsuneizumi et al., 1997). Dad expression in the wing disc overlaps with the expression of Dpp, a homolog of bmp-2 and -4, and ectopic expression of Dpp induces the expression of Dad. Again, the response of cells to Dpp may depend on the balance between the transduction of Dpp signals by the positive regulator Mad and antagonism by Dpp-inducible inhibitory Dad protein.

Taken together, the mechanism of action of cSmad6 in cardiac induction might be to keep the response to the BMP signal at a certain level, by countering the Smad1-mediated positive regulation. Conceivably, if cSmad6 failed to be induced in the presence of BMPs, then upregulation of BMP signaling may cause the appearance of inappropriate cell lineages, predictably osteogenic rather than cardiogenic. On the other hand, if cSmad6 was expressed at greater levels than normally seen, BMP signaling might be downregulated and as a result, all lineages induced by BMPs, including cardiogenic lineage, may fail to appear.

The cardiogenic region at stage 5 to 6 consists of ectoderm, mesoderm, and endodermal layers, and cardiac induction is thought to involve signaling between these layers (Jacobson, 1960; Jacobson and Sater, 1988; Sugi and Lough, 1994; Naasone and Mercola, 1995). Expression of Nkx2-5, a marker for cardiac fate, is restricted to mesoderm. By contrast, bmp-2 is expressed in endoderm (Schultheiss et al., 1997). cSmad1 and cSmad6 transcripts are found in endoderm and mesoderm. Assuming that these two factors mediate the BMP-2 signal, two pathways could be envisaged. In an autocrine mechanism, BMP-2 could signal within the endoderm and activate the expression of a yet unknown factor that subsequently induces Nkx2-5 in the overlying mesoderm via cSmad1 and cSmad6. The existence of such a hypothetical factor has previously been postulated (Jacobson and Sater, 1988; Schultheiss et al., 1997). Alternatively, BMP-2 could act in a paracrine fashion, i.e., be secreted by endoderm and diffuse into mesoderm and therein induce Nkx2-5 via cSmad1 and cSmad6.

CSmad6 is also expressed later during cardiac development. Strong expression is seen in the atrioventricular endocardial cushion and the myocardium at the site of septum formation. We found, however, that the endocardial tissue was devoid of bmp-2, -4, and -7 mRNA. Possibly BMP-2 or BMP-7 synthesized in the myocardium adjacent to the endocardial cushion could diffuse into the cushion tissue that expresses cSmad6. This would, however, require diffusion of these BMPs over many cell diameters. A more likely mechanism is that in the cushion, cSmad6 modulates the signal of another TGF-β family member. In the chick embryo, TGF-β1, TGF-β2, and TGF-β3 are expressed in the endocardial cushion tissue (Choy et al., 1991; Barnett et al., 1994; Ramsdell and Markwald, 1997; Nakajima et al., 1998). Therefore, in this tissue cSmad6 may be involved in modulating TGF-β signaling. It has been shown that Smad6 can associate not only with BMP type I receptor, but also with TGF-β type I receptor (Imamura et al., 1997). Thus it is possible that cSmad6 mediates both TGF-β- and BMP-signaling pathways during cardiogenesis.

The expression of cSmad6 is remarkably restricted in the embryo. In addition to the heart, the developing limb is a region where this gene is prominently expressed. In the limb bud cSmad6 transcripts are seen in the apical ectodermal ridge (AER) and in underlying mesenchyme. Moreover, there is an elevated expression of cSmad6 along the posterior limb bud margin, a region that also contains the zone of...
polarizing activity (ZPA). Both, the AER and ZPA express bmp-2 and bmp-4 (Fransis et al., 1994) and it seems likely that cSmad6 regulates the action of these growth factors in the developing limb. Later in limb development, programmed cell death occurs in the interdigital region. Studies with dominant-negative BMP-receptor constructs have shown that such programmed cell death depends on BMP signaling (Zou and Niswander, 1996). The expression pattern of cSmad6 suggests that the response of cells destined to undergo apoptosis in response to BMP may also involve cSmad6.

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