Expression and Regulation of Type I BMP Receptors during Early Avian Sympathetic Ganglion Development

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We have investigated the expression and regulation of the mRNAs for the type I BMP receptors, BMPR-IA and BMPR-IB, in quail embryos in vivo and in neural crest cultures in vitro. BMPR-IB mRNA was expressed in the primordial sympathetic ganglia at stage 17, soon after the first expression of Cash-1 mRNA, the avian homolog of the Drosophila transcription factor achaete-scute. BMP-4 mRNA was detected in the dorsal aorta at stage 17, coincident with BMPR-IB mRNA expression in the sympathetic ganglia. BMPR-IA mRNA was first expressed in the sympathetic ganglia at stage 18. Moreover, BMP-4 ligand mRNA was detected in the sympathetic ganglia starting at stage 18. BMPR-IA and BMPR-IB were differentially regulated in cultured neural crest cells. BMPR-IB was expressed in primary outgrowths of neural crest cells but was downregulated after primary outgrowths were harvested and replated in secondary cultures. In secondary cultures of neural crest cells, exogenous BMP-2 and BMP-4 increased the expression of BMPR-IA but decreased the expression of BMPR-IB. The expression of both type I BMP receptors was inhibited by exogenous TGF-β1. Our results suggest distinct roles for BMPR-IA and BMPR-IB in the development of the sympathoadrenal phenotype from cells of the neural crest.

Key Words: neural crest; BMP; BMP receptor; development; neurogenesis; sympathetic ganglia; dorsal aorta.

INTRODUCTION

Extracellular signals play an important role in influencing the developmental decisions of progenitor cells as they mature into adult cell types. Neural crest cells are an excellent model system in which to study this process. The cells of the neural crest originate from the dorsal neural tube and migrate to many other sites in the embryo, giving rise to numerous cell types in the developing vertebrate embryo (Weston, 1970; Noden, 1978; LeDouarin, 1982; Hall and Horstadius, 1988; Anderson, 1993; Bronner-Fraser, 1993; Marusich and Weston, 1991; Selleck et al., 1993). Several studies have shown some neural crest cells to be multipotent and that the molecular environment in the developing embryo is important in influencing their eventual fate (Bronner-Fraser and Fraser, 1988; Bariffio et al., 1988; Bariffio et al., 1988; Sieber-Blum and Cohen, 1980; Sieber-Blum, 1991).

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lesser extent BM-7 increase the number of adrenergic cells which develop in primary cultures of neural crest cells, as indicated by an increase in the number of cells that express tyrosine hydroxylase (TH) and catecholamines (Varley et al., 1995; Varley and Maxwell, 1996; Reissmann et al., 1996). BM-2 appears to play an important role in sympathetic neurogenesis in mammals as well (Shah et al., 1996). In vivo, BMP-4 is expressed in the avian dorsal aorta (Reissmann et al., 1996), immediately adjacent to the location where neural crest cells coalesce to form the primordial sympathetic ganglia. Moreover, we have previously shown that the expression of a constitutively active form of the BMP type I receptor (BM-PR-IA) mimics the effects of BMP-4 on cultured neural crest cells (Varley et al., 1998). Thus, BMP-4 and its receptor(s) play critical roles in the differentiation of the developing sympathetic ganglia.

An important question is when do neural crest cells first express differentiated markers in the sympathoadrenal lineage. Migrating neural crest cells express cell surface antigens which react with the monoclonal antibody HNK-1 as early as stage 13 (Bronner-Fraser, 1986). The expression of the HNK-1 epitope persists in neural crest cells that differentiate along the adrenergic pathway, but HNK-1 does not uniquely define adrenergic progenitors (Maxwell et al., 1988; Maxwell and Forbes, 1990, 1991; Henning and Maxwell, 1995). Cash-1, the chick homolog of the Drosophila transcription factorachaete-scute, is first expressed in the developing sympathetic ganglia in stage 15 (Hamburger and Hamilton, 1951) chick embryos (Groves et al., 1995; Ernsberger et al., 1995). Mash-1, the mammalian homolog of achaete-scute, is expressed in the developing sympathetic ganglia before other adrenergic phenotypes are observed, and mice lacking Mash-1 fail to develop sympathetic neurons (Johnson et al., 1990; Lo et al., 1991; Guillemot et al., 1993). BMPs are first detected in the chick dorsal aorta as early as stage 16, prior to the expression of TH (Reissmann et al., 1996), and BMP-2 and BMP-4 increase the expression of Mash-1 in neural crest cells adjacent to the dorsal aorta in mice (Shah et al., 1996). Mash-1 then induces the expression of the transcription factor Phox2a, which binds to the TH promoter and activates its expression (Lo et al., 1998; Hirsh et al., 1998; Goridis and Brunet, 1999). Transcripts encoding cPhox-2, cGATA-2, and TH are first detected in chick sympathetic ganglia at stage 18 (Ernsberger et al., 1995; Groves et al., 1995). Evidence indicates that Cash-1 expression can be unlinked from the BMP signaling pathway (Groves et al., 1995; Ernsberger et al., 1995; Schneider et al., 1999). Taken together, these results establish a partial chronology of the molecular events in the differentiation of the sympathoadrenal phenotype and demonstrate a crucial role for BMPs in this process. However, the temporal expression of the BMP receptors and what factors regulate their expression in the developing sympathetic ganglia are less well understood. In order to fully understand how BMPs affect the development of the sympathetic ganglia, it is important to understand the regulation of BMP receptor expression.

BMPs transduce their signal by binding a heterodimer composed of a type I and a type II receptor (Massague, 1998). There are three type I BMP receptors, BM-PR-IA, BM-PR-IB, and the activin type I receptor. BM-2 and BM-4 ligands act primarily through BM-PR-IA and BM-PR-IB, while BM-7 acts through BM-PR-IB (Massague, 1998). There appears to be a single type II BMP receptor (BM-PR-II) (Massague, 1998); thus the ligand specificity is determined by the type I BMP receptor. Ligand binding brings the type I and type II BMP receptors together, after which the type II receptor phosphorylates the type I receptor (Massague, 1998). Activated type I BMP receptors phosphorylate Smad proteins, which then enter the cell nucleus and affect gene transcription. Single amino acid changes within the kinase domain of type I BMP receptors result in an increased kinase activity and the ability to signal in the absence of BMP ligand or type II receptors (Weiser et al., 1995; Massague, 1998; Varley et al., 1998). In the mouse, a knockout mutation of the BM-PR-IA gene results in early embryonic lethality (Mishina et al., 1995). These results indicate that the type I BMP receptors are key signal transduction molecules for BMPs.

Limited information is available about the tissue distribution of mRNAs encoding the BMP receptors during development. In the mouse, BM-PR-II is ubiquitously expressed in the developing embryo until at least midgestation (Roelen et al., 1997). The expression of BM-PR-IA and BM-PR-IB has been examined in the developing mouse and zebrafish embryos (Dewulf et al., 1995; Zhang et al., 1998; Roelen et al., 1997; Nikaido et al., 1999). One or both type I BMP receptors are expressed in most tissues in the developing mouse embryo (Dewulf et al., 1995), and BM-PR-IA and BM-PR-IB are differentially expressed in the developing mouse brain (Zhang et al., 1998). Moreover, BM-PR-IA and BM-PR-IB have distinct functional roles in the differentiation of cartilage, osteoblasts, and adipocytes (Zou et al., 1997; Chen et al., 1998). BM-PR-IA expression has been reported in the sympathetic ganglia of the mouse at E16 (Zhang et al., 1998). However, little detailed information is known about the temporal pattern of expression of BM-PR-IA and BM-PR-IB in the developing sympathetic ganglia. For the reasons outlined above, we have chosen to focus our attention on the expression and regulation of BM-PR-IA and BM-PR-IB.

We have previously shown that neural crest cells express BM-PR-IA, but not BM-PR-IB, mRNA after 18 h in culture and that the activity of this receptor is related to adrenergic differentiation (Varley et al., 1998). It is not known when cells of the developing sympathetic ganglia in vivo first express BMP receptors. Based on our previous in vitro results (Varley et al., 1998), we hypothesized that BM-PR-IA mRNA would be expressed in the sympathetic ganglia in vivo. In order to examine the expression of the BMP type I receptors during quail embryogenesis, we have isolated cDNA clones corresponding to the quail homologs of the two BMP type I receptors, BM-PR-IA and BM-PR-IB. We have investigated the expression of mRNA encoding the type IA and IB receptors, as well as the expression of mRNAs...
encoding BMP-4, Cash-1, and TH, in vivo by in situ hybridization. Our results revealed that Cash-1 mRNA was expressed in the developing sympathetic ganglia before the expression of either type I BMP receptor mRNA. Surprisingly, we also found that the expression of BMP-IB mRNA in the developing sympathetic ganglia precedes that of BMP-IA mRNA and coincides with the expression of BMP-4 mRNA in the dorsal aorta. Moreover, we found that BMP-4 mRNA is expressed in the cells of the developing sympathetic ganglia coincident with the expression of BMPR-IA and TH mRNA. Because of these surprising results concerning the expression of BMP type I receptor mRNAs in vivo, we further investigated the regulation of their expression in vitro in cultured neural crest cells. We found that BMPR-IA and BMPR-IB mRNAs are differentially regulated by culture conditions and by members of the transforming growth factor-β (TGF-β) superfamily of growth factors.

**MATERIALS AND METHODS**

**Cloning of quail BMPR-IA and BMPR-IB cDNA.** For the quail BMPR-IA clone, gene-specific first-strand cDNA was primed from RNA isolated from neural crest cultures grown in the presence of BMP-4 by an oligonucleotide complementary to the 3′ end of the chick BMPR-IA gene using Superscript II reverse transcriptase ( Gibco BRL). This cDNA was then amplified by PCR using oligonucleotides based on the chicken BMPR-IA sequence to generate full-length, double-stranded cDNA fragments. The PCR consisted of 1 cycle of 95°C for 3 min, 48°C for 2 min, and 72°C for 5 min, followed by 29 cycles of 92°C for 2 min, 48°C for 2 min, and 72°C for 5 min. The oligonucleotides, which contained Clal restriction endonuclease sites at their 5′ ends, were BMPR-IA top strand, 5′-ACATATATCGATACGCTAGAGT-3′, and BMPR-IA bottom strand, 5′-ACATATATCGATCTCAGAGT-3′, and BMPR-IB top strand, 5′-ACATATATCGATACGCTAGAGT-3′, and BMPR-IB bottom strand, 5′-ACATATATCGATCTCAGAGT-3′. For the quail BMPR-IB clone, first-strand cDNA synthesis was primed from 4-day embryo RNA by 0.5 mg of oligo(dT) using Superscript II reverse transcriptase. This cDNA was then amplified by PCR using oligonucleotides derived from the chicken BMPR-IA and BMPR-IB sequences and containing a Clal restriction endonuclease site at their 5′ ends. The PCR consisted of 2 cycles of 94°C for 3 min, 40°C for 2 min, and 72°C for 5 min, followed by 28 cycles of 92°C for 2 min, 44°C for 2 min, and 72°C for 5 min. The oligonucleotides sequences, including Clal restriction endonuclease sites, were BMPR-IB top strand, 5′-ACATATATCGATACGCTAGAGT-3′, and BMPR-IB bottom strand, 5′-ACATATATCGATACGCTAGAGT-3′. The quail BMPR-IA and BMPR-IB cDNA fragments were cleaved with Clal, gel purified, and ligated into the Clal site of Bluescript II SK(+). Ligations were used to transform MAX Efficiency DH5α competent cells ( Gibco BRL). Colonies were screened for inserts by miniprep analysis, and clones containing inserts were further analyzed by restriction endonuclease cleavage. One clone each of BMPR-IA and BMPR-IB was selected and sequenced in an automated sequencer. The GenBank accession numbers are AF189777 and AF189778 for BMPR-IA and BMPR-IB, respectively.

**Probe preparation.** Riboprobes containing digoxigenin-labeled uracil were generated with the Genius System (Boehringer Mannheim Biochemicals). Full-length probes were transcribed from the appropriate cDNA clones. Probes greater than 1 kb in length were hydrolyzed to reduce probe size to fewer than 700 nucleotides. For BMPR-IA, BMPR-IB, and TH, probes were generated from quail cDNAs; for Cash-1 and BMP-4, probes were generated from chick cDNAs.

**In situ hybridization of tissue sections.** Quail embryos of the appropriate age were dissected free of extraembryonic membrane, the stage was determined ( Hamburger and Hamilton, 1951), and their heads were removed. After being washed in 4% paraformaldehyde, embryos were incubated overnight at 4°C in 4% paraformaldehyde. Embryos were washed in PBS and incubated overnight in PBS containing 30% sucrose at 4°C. The embryos were then embedded in mounting medium and sectioned onto silane-treated microscope slides ( Sigma ). Slides were stored at −70°C for up to 1 week until needed.

In situ hybridizations were conducted essentially as described by Groves et al. ( 1995 ). Slides containing embryo sections were warmed to room temperature and dried in a 50°C oven. After fixation for 20 min in 4% paraformaldehyde, slides were washed twice in PBS. Slides were then treated with 10 μg/ml proteinase K at room temperature for 10 min, washed in PBS, and fixed again in 4% paraformaldehyde for 15 min. After a wash in H2O, slides were incubated in 0.1 M triethanolamine, pH 8.0, 0.25% acetic anhydride for 10 min and washed in PBS. After successive washes of 25, 50, 75, and 100% methanol in PBS, slides were prehybridized at the appropriate temperature for 3–4 h in hybridization buffer (50 μg/ml salmon sperm DNA, 1× SSC, pH 5.3, 50 μg/ml tRNA, 100 μg/ml heparin, 0.2% Tween 20, 0.5% CHAPS, 5 mM EDTA). The hybridization buffer was then replaced with fresh hybridization buffer containing 1 μg/ml of the appropriate riboprobe and incubated overnight at the appropriate temperature (determined empirically for each probe; typically 45–55°C). Slides were washed free of probes in two washes of 2× SSC, 0.1% CHAPS at the hybridization temperature for 30 min, followed by two washes of 0.2× SSC, 0.1% CHAPS at the hybridization temperature for 30 min. Slides were then washed twice in PBS containing 0.1% Tween 20, followed by one wash in PBT (1× PBS, 2% BSA, 0.1% Triton X-100). After being blocked with PBT containing 20% heat-inactivated sheep serum ( Sigma ), slides were incubated overnight at 4°C in alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim Biochemicals) diluted 1:500 in PBT plus 20% sheep serum. After three washes with PBT and two washes in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, 5 mM Levamisole) probes were detected with NBT/BCIP as directed by the manufacturer (Boehringer Mannheim Biochemicals). After the alkaline phosphatase reaction product was evident, the slides were washed in PBS and coverslips were mounted with Aquamount.

In order to increase the sensitivity of the in situ hybridizations, relatively low hybridization temperatures were used (45–55°C). This resulted in background signals for some probes. Hybridizations carried out with sense strand probes or no probe resulted in very low background signals. Labeling above background was determined based on the overall level of background hybridization seen in each section.

**Neural crest cultures.** Primary and secondary cultures of quail trunk neural crest cells were prepared and grown as described (Varley et al., 1998). Primary cultures were prepared by dissecting neural tubes from stage 14–15 ( Hamburger and Hamilton, 1951) Japanese quail embryos ( Coturnix coturnix ) and cultured at 37.5°C on a thin film of Vitrogen 100 ( Collagen Corp. ) in 60-mm culture dishes. Secondary cultures were prepared by harvesting the neural crest outgrowths from the primary cultures after 42 h in culture.
Neural tubes were removed, and the neural crest cells were harvested by trypsinization and replated in 1-cm diameter-culture wells. Twenty-five thousand cells were plated in each 1-cm culture well. Growth factors were added to secondary cultures after cell attachment to the culture dishes. The RT-PCR analysis of RNA. Total cellular RNA was isolated from quail trunk neural crest cultures using the Ultraspec RNA isolation system. Within a given experiment, RNA was isolated from cells in the same number of 1-cm culture wells for each condition. First-strand cDNA was primed with 0.5 mg oligo(dT) and synthesized with Superscript II reverse transcriptase (Gibco BRL). For each condition examined, identical aliquots of first-strand cDNA were used as templates for PCR using oligonucleotide primers specific for BMPR-IA, BMPR-IB, and β-actin. PCR conditions consisted of denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min, and extension at 72°C for 3 min. Cycle numbers varied between different primer sets, and at least two different numbers of cycles were performed for each primer set to determine the optimal number of cycles. For actin, a series of cycle numbers was performed to determine the linear range of amplification. Changes in actin levels in RT-PCR experiments reflect the change in cell number or slight variations in RNA concentration. In each RT-PCR experiment, 10 μl of each reaction product was separated on agarose gels. The sequences of the oligonucleotide primers were BMPR-IA 5'-CCAGCAGTCTTCTGTGAT-3'; BMPR-IB 3'-TCAAA-TCTTTTACATCTTGTGAT-3'; BMPR-IA 5'-CTCAAA-TCTTTTACATCTTGTGAT-3'; BMPR-IB 5'-TGCTACTTCCAGG-TATAAGCGGC-3'; BMPR-IB 5'-TCTTTTACATCTTGTGAT-3'; β-actin 5'-5'-TGGTCTCCCAGGCT-3'; β-actin 3'-5'-CCGGATTCTACGTACTC-3'.

PCR data were quantitated using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on a Power Macintosh computer. Because different cycles numbers were used for each primer set, absolute levels of expression for BMPR-IA, BMPR-IB, and β-actin could not be compared. The relative differences in BMPR-IA or BMPR-IB expression under different conditions were compared with the relative differences in the expression of β-actin mRNA within a given RT-PCR experiment.

RESULTS

Cloning of the Quail Type I BMP Receptors

To determine the degree of similarity between the chick and the quail sequences, we cloned the quail homologs of BMPR-IA and BMPR-IB. Since the chick sequences were known, we made oligonucleotide primers corresponding to the 5’ and 3’ ends of the chick BMPR-IA and BMPR-IB coding sequences (Kawakami et al., 1996). A single, abundant band corresponding to each gene was obtained after RT-PCR using as a template RNA isolated from either quail trunk neural crest cultures or intact quail embryos. After cloning and screening, single colonies containing cDNA clones of either quail BMPR-IA or BMPR-IB were selected, and the plasmids were sequenced. Each clone contained a single open reading frame. The deduced amino acid sequences for both cDNAs are shown in Fig. 1. The quail BMPR-IA and BMPR-IB cDNAs are greater than 98% identical to their chick homologs at the amino acid level. The chick BMPR-IA- and BMPR-IB-derived amino acid sequences are 91 and 92% identical to the mouse sequences, respectively (Kawakami et al., 1996). The quail cDNA clones were used as templates for the synthesis of antisense riboprobes for in situ hybridization.

Expression of BMPR-IA, BMPR-IB, and BMP-4 mRNA in Stage 23 Quail Embryos

To determine which type I BMP receptor mRNAs were expressed in neural crest cells in vivo, we initially performed in situ hybridization on frozen sections of stage 23 (Hamburger and Hamilton) quail embryos. At this stage, neural crest cells which form the developing sympathetic ganglia have ceased migration and coalesced adjacent to the dorsal aorta. In more rostral sections at stage 23, the cells of the developing sympathetic ganglia no longer lie adjacent to the dorsal aorta but are located in a more dorsal position. The expression of TH mRNA, a marker for adrenergic cells, was detected in the developing sympathetic ganglia adjacent to the dorsal aorta (Fig. 2). We observed the expression of both BMPR-IA and BMPR-IB mRNA in the developing sympathetic ganglia (Fig. 2). Expression of mRNAs for both type I BMP receptors was also detected in the dorsal aorta in stage 23 embryos (Fig. 2). As previously reported, BMP-4 mRNA is expressed in the dorsal aorta, and Cash-1 mRNA is expressed in the developing sympathetic ganglia (Reissmann et al., 1996; Groves et al., 1995; Ernsberger et al., 1996). Interestingly, we also detect BMP-4 mRNA in the developing sympathetic ganglia (Fig. 2). Thus, mRNAs for the BMP-4 ligand and both BMP type I receptors are expressed in the developing sympathetic ganglia, cells that are responding to the BMP-4 signal. BMPR-IA, BMPR-IB, BMP-4, Cash-1, and TH mRNAs are all expressed in the developing sympathetic ganglia as late as stage 26, the latest stage we examined (data not shown).

Our observation of BMP-4 mRNA expression in both the dorsal aorta and the developing sympathetic ganglia of the quail contrasts the observations of Reissmann et al. (1996) who report BMP-4 mRNA expression in the chick dorsal aorta but not in the sympathetic ganglia. To further investigate this discrepancy, we performed in situ hybridizations on frozen sections from both quail and chick stage 24 embryos to compare BMP-4 mRNA expression. Our results reveal BMP-4 mRNA expression in the dorsal aorta and the developing sympathetic ganglia of both the quail and the chick at stage 24 (Fig. 3).

Expression of BMPR-IA, BMPR-IB, and BMP-4 mRNA in Stage 16, 17, and 18 Quail Embryos

Both BMPR-IA and BMPR-IB mRNA are expressed in the developing sympathetic ganglia in stage 23 embryos, but only BMPR-IA mRNA is present in neural crest cells after 18 h in culture. We therefore wished to determine whether only one or the other type I BMP receptor was expressed at earlier stages.
of development in vivo. We examined the expression of BMP-4, BMP-IA, BMP-IB, Cash-1, and TH mRNA by in situ hybridization on frozen sections from stage 16, 17, and 18 embryos. At stage 16, some migrating neural crest cells have begun to coalesce adjacent to the dorsal aorta. We observed expression of Cash-1 mRNA in the developing sympathetic ganglia in stage 16 embryos, but neither type I BMP receptor mRNA was expressed at this time (Figs. 4A, 4C, and 4D). In some sections, a low level of BMP-4 mRNA was detected in the dorsal aorta at stage 16 (Fig. 4B). TH mRNA expression

**FIG. 1.** Deduced amino acid sequence of quail BMPR-IA and BMPR-IB. The sequences of the quail BMP type I receptors are greater than 98% identical to their chick counterparts at the amino acid level. The quail and chick amino acid sequences are identical, except where indicated. The GenBank accession numbers are AF189777 and AF189778 for BMPR-IA and BMPR-IB, respectively. To determine the specificity of riboprobes generated from the quail BMPR-IA and BMPR-IB clones, they were used to probe a Northern blot of total RNA isolated from 5-day quail embryos. The BMPR-IA and BMPR-IB riboprobes hybridized to distinct bands of sufficient size to encode the receptors; no cross-hybridization between the two probes was evident (data not shown).
was not detected in the developing sympathetic ganglia at stage 16 (data not shown). We found weak expression of BMPR-IB mRNA in the cells of the developing sympathetic ganglia at stage 17, but BMPR-IA mRNA was not detected (Figs. 4G and 4H). We also continued to observe expression of Cash-1 mRNA in the developing sympathetic ganglia in stage 17 embryos (Fig. 4E). At stage 17, BMP-4 mRNA was expressed by some cells in the wall of the dorsal aorta. This expression appeared to be more prevalent in the ventral and lateral portions of the dorsal aorta (Fig. 4F). BMP-4 mRNA expression was not detected in the cells of the developing sympathetic ganglia in stage 17 embryos. As observed in stage 16 embryos, TH mRNA expression was not detected in the developing sympathetic ganglia (data not shown). Thus, Cash-1 mRNA was detected in the sympathetic ganglia prior to the uniform expression of BMP-4 mRNA in the dorsal aorta and prior to the detection of either type I BMP receptor mRNA in the sympathetic ganglia. Interestingly, BMPR-IB mRNA was expressed in the developing sympathetic ganglia prior to the expression of mRNAs encoding either BMP-4 or BMPR-IA.

Both BMPR-IA and BMPR-IB mRNAs were observed in the developing sympathetic ganglia of stage 18 embryos (Figs. 4K and 4L). In addition, BMPR-IA mRNA was expressed in the dorsal aorta at this stage (Fig. 4K). A higher magnification view of BMPR-IA and BMPR-IB mRNA expression in the developing sympathetic ganglia and dorsal aorta is shown in Fig. 5. Moreover, we observed expression of BMP-4 mRNA in both the dorsal aorta and the developing sympathetic ganglia at stage 18 (Fig. 4J). We also detected both Cash-1 (Fig. 4I) and TH (data not shown) mRNA in the developing sympathetic ganglia in stage 18 embryos. Thus, Cash-1 mRNA is expressed in the developing sympathetic ganglia as early as stage 16. BMPR-IB mRNA is first expressed in the sympathetic ganglia at stage 17, and BMPR-IA and TH mRNA are first expressed at stage 18. BMP-4 mRNA is detected in the dorsal aorta in stage 17 embryos, followed by expression in the sympathetic ganglia at stage 18. The expression of all of these genes in the developing sympathetic ganglia persists at least until stage 26, the latest stage examined.

BMPR-IA and BMPR-IB Expression in Cultured Neural Crest Cells

We previously showed that BMPR-IA, but not BMPR-IB, is expressed in neural crest cells after 18 h in secondary cultures (Varley et al., 1998). In contrast, both receptors are expressed in the developing sympathetic ganglia at stage 16 (data not shown).

FIG. 2. Cash-1, BMP-4, BMPR-IA, BMPR-IB, and TH mRNAs are expressed in the sympathetic ganglia of stage 23 quail embryos. In situ hybridizations were performed on serial sections from the trunk region of stage 23 quail embryos. Arrows indicate hybridization signal in the developing sympathetic ganglia. Results show expression of mRNAs encoding Cash-1, BMP-4, BMPR-IA, BMPR-IB, and TH in the developing sympathetic ganglia. BMP-4, BMPR-IA, and BMPR-IB mRNA expression is also observed in the dorsal aorta (da). The section labeled sense shows the lack of a specific signal obtained using a sense-strand probe for BMPR-IA in a section from a stage 24 quail embryo. The notochord is indicated by "n".

FIG. 3. BMP-4 mRNA is expressed in both the developing sympathetic ganglia and the dorsal aorta of quail and chick embryos. In situ hybridizations were performed on sections from the trunk region of stage 24 chick and quail embryos. Results show the expression of BMP-4 mRNA in the developing sympathetic ganglia of the chick, as we observe in quail embryos. Arrows indicate the sympathetic ganglia, and the dorsal aorta is labeled da.

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expressed in vivo, with BMPR-IB detected earlier than BMPR-IA. We therefore wished to explore the regulation of the expression of the type I receptors in greater detail. RNA was isolated from neural crest cultures grown in the presence or absence of exogenous BMP-4 ligand after 18, 48, or 96 h in culture and was assayed by RT-PCR. As shown in Fig. 6, BMPR-IA expression increased with time, both in the absence and in the presence of BMP-4. The level of expression of β-actin also increases over time, indicating that the increased BMPR-IA expression we observe may be due to the increase in cell number (Fig. 6). As previously shown, significant BMPR-IB expression was not detected after 18 h in culture in either the presence or the absence of BMP-4 (Fig. 6). However, BMPR-IB expression was detected after 96 h in culture, but only in the absence of exogenous BMP-4 (Fig. 6). Only a low level of BMPR-IB expression was observed at any time point in cells grown in the presence of exogenous BMP-4 (Fig. 6). These results indicate that BMPR-IA and BMPR-IB are differentially regulated both in vivo and in vitro.

**BMPR-IA and BMPR-IB Expression in Primary Outgrowths of Neural Crest Cells**

To generate neural crest cultures for the experiment described above, neural tubes were excised from stage 14–15 quail embryos and cultured for 42 h. During this time in primary culture, neural crest cells migrate out of

**FIG. 4.** Expression of Cash-1, BMP-4, BMPR-IA, and BMPR-IB mRNA in stage 16, 17, and 18 quail embryos. Serial sections from the trunk region of stage 16 (A–D), stage 17 (E–H), and stage 18 (I–L) quail embryos were analyzed by in situ hybridization. Cash-1 mRNA is expressed in the sympathetic ganglia (arrows) starting at stage 16 (A, E, I). BMP-4 mRNA is expressed in the dorsal aorta (da), indicated by asterisks, at stage 17, although BMP-4 mRNA is detected in some sections at stage 16 (B, F, J). BMPR-IB mRNA is expressed in the sympathetic ganglia (arrows) starting at stage 17 (H, L). BMPR-IA and BMP-4 mRNAs are expressed in the sympathetic ganglia (arrows) starting at stage 18, and BMPR-IA mRNA is expressed in the dorsal aorta at stage 18 (K, J). Some hybridization signal was observed in the dermamyotome with all probes.
the neural tubes. The neural tubes were then removed, and the neural crest cell outgrowths were harvested by trypsinization, counted, and replated in secondary cultures. We hypothesized that the lack of BMPR-IB in the 18-h secondary cultures could be due to either the act of replating the cells or the culture conditions used. To address this question, we isolated RNA from primary outgrowths of neural crest cells immediately after harvesting, from cells allowed to "heal" in suspension in growth medium for 4 h, and from cells 4, 24, and 96 h after replating in secondary cultures in the absence of exogenous BMP-4. BMP receptor expression was assayed by RT-PCR, and the results were analyzed using NIH Image software. We found that BMPR-IB was expressed in primary outgrowths and at a slightly lower level in healed cells (Fig. 7, lanes 1 and 2). After replating in secondary culture, the level of expression of BMPR-IB decreased at 4 h and was undetectable after 24 h (Fig. 7, lanes 3 and 4). By 96 h, BMPR-IB was expressed at a relatively high level, as observed above (Fig. 7, lane 5). In contrast, β-actin expression did not decrease in healed cells or after replating and increased over time in culture (Fig. 7). BMPR-IA was expressed in primary outgrowths and at a slightly lower level in healed cells (Fig. 7, lanes 1 and 2). BMPR-IA expression then increased over time in culture (Fig. 7, lanes 3-5). These results provide a possible explanation for the difference between our in vivo data and our earlier in vitro data.

**Effects of TGF-β Superfamily Members on BMPR-IA and BMPR-IB Expression**

BMP-2, BMP-4, and BMP-7, but not BMP-6, increase the number of cells that develop the adrenergic phenotype in quail trunk secondary neural crest cultures (Varley et al., 1995; Varley and Maxwell, 1996; Reissmann et al., 1996). TGF-β1 reduces the number of adrenergic cells in avian neural crest cultures (Varley et al., 1995) and promotes smooth muscle differentiation in cultures of mammalian neural crest stem cells (Shah et al., 1996). To further explore the regulation of the type I BMP receptors, we examined their expression in neural crest cultures treated with various BMPs and TGF-β1. Secondary neural crest cultures were grown for 96 h in the presence of exogenous BMP-2, BMP-4, BMP-6, BMP-7, or TGF-β1, and RNA was isolated and analyzed by RT-PCR. The resulting data were quantitated with NIH Image software. BMPR-IA was expressed in control cultures with no exogenous growth factors, and its expression was increased by both BMP-2 and BMP-4 (Fig. 8, lanes 1-3). BMP-7, as well as the combination of BMP-2 and 7, had no appreciable effect on BMPR-IA expression (Fig. 8, lanes 5 and 6). BMPR-IA expression was also increased in cultures treated with a combination of BMP-4 and 7 (Fig. 8, lane 7). In contrast, addition of BMP-6 or TGF-β1 resulted in a marked decrease in BMPR-IA expression (Fig. 8, lanes 4-6).
DISCUSSION

In the present study, we have investigated the expression and regulation of the type I BMP receptors in the developing quail embryo and in an in vitro culture system. We have previously shown the importance of both BMP-4 and BMP-IA for the development of the adrenergic phenotype in cultured neural crest cells in vitro (Varley and Maxwell, 1996; Varley et al., 1998). We find that Cash-1 mRNA is expressed in the sympathetic ganglia at stage 16. We also show that BMP-RIB mRNA is expressed at stage 17 in the developing sympathetic ganglia, prior to the expression of BMP-RIA mRNA. The expression of BMP-RIB mRNA at stage 17 coincides with the expression of BMP-4 mRNA in the dorsal aorta but is after the first expression of the transcription factor Cash-1. At stage 18, 4 h later, we detected expression of BMP-4, BMP-RIA, and TH mRNA in the developing sympathetic ganglia. We also show that BMP-RIA and BMP-RIB are differentially regulated both in vivo and in vitro and that BMP-RIB expression is regulated by members of the TGF-β superfamily of growth factors.

The expression of BMP receptors has been investigated in the developing mouse embryo (Dewulf et al., 1995; Roelen et al., 1997; Zhang et al., 1998), and BMP-RIB expression has been examined in the developing zebrafish (Nikaido et al., 1999). Dewulf et al. (1995) found that BMP-RIA (ALK3) is almost ubiquitously expressed throughout the mouse embryo from 6.5 to 15.5 days postcoitum, with a notable lack of expression in the liver. BMP-RIB (ALK6) was found to have a more restricted distribution but was still expressed in numerous tissues including the central nervous system (Dewulf et al., 1995). Zhang et al. (1998) investigated the expression of BMP receptors in the developing nervous system of the mouse, primarily focusing on the brain, and they reported a more restricted pattern of expression for BMP-RIB than for BMP-RIA. However, they did report that BMP-RIA is expressed in the sympathetic ganglia at E16, and this expression persists until adulthood in the sympathetic cervical ganglia (Zhang et al., 1998). BMP-RIB is also expressed in a restricted manner in the developing zebrafish embryo (Nikaido et al., 1999). There is little information concerning when the type I BMP receptors are first expressed in the sympathetic ganglia and how such expression correlates with other phenotypic markers of adrenergic development.

BMP-4 mRNA is detected in the dorsal aorta as early as stage 16 (Reissmann et al., 1996), and TH is first detected in the sympathetic ganglia at stage 18 (Embsberger et al., 1995). Our results are in general agreement with these findings, although we detected only limited BMP-4 expression in the dorsal aorta before stage 17, perhaps because of the axial level examined. In addition, we detected the expression of BMP-4 mRNA in the sympathetic ganglia beginning at...
stage 18, the same time as the first expression of TH. This novel observation of BMP-4 ligand mRNA expression in the developing sympathetic ganglia contrasts the observations of Reissmann et al. (1996), who do not detect such expression in chick embryos. This raised the possibility of a species-specific difference between chick and quail in the expression of BMP-4 mRNA in the sympathetic ganglia.

However, we observed BMP-4 mRNA expression in the sympathetic ganglia of both the chick and the quail embryos at stage 24, suggesting that our in situ hybridization protocol is more sensitive. This observation raises the possibility that BMP-4 ligand from the dorsal aorta may induce the expression of BMP-4 in the developing sympathetic ganglia. The expression of BMP-4 mRNA in the sympathetic ganglia persists at least until stage 26. This finding is significant, since BMP-4 expression in the sympathetic ganglia could provide a local source of BMP ligand at later stages of development after the cells of the sympathetic ganglia are no longer in contact with the dorsal aorta. Autocrine stimulation of the ganglion by BMP-4 may be important for later events in the differentiation of the sympathoadrenal phenotype, beyond the initial appearance of the adrenergic phenotype.

The timing of the expression of Cash-1 relative to BMP ligand and receptor expression may provide clues to its role in the differentiation of the sympathetic ganglia. Several studies have investigated the regulation and expression of Cash-1 and its mammalian counterpart, Mash-1. Cash-1 mRNA has been detected in the developing sympathetic ganglia in stage 15 embryos (Ernsberger et al., 1995; Groves et al., 1995). Consistent with these previous results, we detected Cash-1 mRNA in the developing sympathetic ganglia in stage 16 embryos, the earliest stage we examined. Cash-1 mRNA is expressed in the sympathetic ganglia prior to the detection of BMP-4 mRNA in the dorsal aorta, and we observe Cash-1 mRNA expression prior to the expression of BMP type I receptor mRNAs in the sympathetic ganglia. However, it is important to note that Mash-1 expression is induced in cultured rat neural crest stem cells at a much lower level of BMP-2 than is required for the induction of the neuronal phenotype (Shah and Anderson, 1997). Thus, there may be a lower level expression of BMP-4 in the dorsal aorta and BMP type I receptors in the sympathetic ganglia at stage 16 sufficient to induce Cash-1 mRNA expression but too low for detection by in situ hybridization. These results are consistent with a role for Cash-1 in the initiation of sympathoadrenal differentiation. Although BMPs increase the expression of Mash-1 (Shah et al., 1996;
Lo et al., 1997), studies of Cash-1 suggest that it is regulated in part by an alternate, non-BMP-dependent, pathway (Embersberger et al., 1995; Groves et al., 1995). This conclusion is supported by the observation that the BMP antagonist noggin inhibits the induction of TH by BMPs, but the expression of Cash-1 is unaffected (Schneider et al., 1999). However, mice lacking Mash-1 fail to develop sympathetic neurons (Johnson et al., 1990; Lo et al., 1991; Guillemot et al., 1993). Persistent expression of Mash-1 maintains the responsiveness to BMPs, which then increase the expression of Mash-1 (Lo et al., 1997). Thus, while Cash-1, or Mash-1 in mammals, is required for the development of sympathetic neurons, it appears to be regulated at least in part outside of the BMP pathway (Stern et al., 1991). BMPS increase, but may not initiate, the expression of Cash-1 in the sympathetic ganglia. This conclusion is supported by our finding that Cash-1 mRNA is expressed in the sympathetic ganglia before BMP receptor mRNA is detected. One possibility is that Cash-1 acts to induce the expression of BM PR-IB, initiating BMP responsiveness in the cells of the developing sympathetic ganglia.

We also found that BM PR-IA expression in secondary cultures was increased in the presence of exogenous BMP-2 or BMP-4 and decreased by exogenous TGF-β1. BMP-2 and BMP-4 increase the number of adrenergic cells which develop in neural crest cultures, while TGF-β1 decreases the number of adrenergic cells and promotes smooth muscle development (Varley et al., 1995; Varley and Maxwell, 1996; Shah et al., 1996; Shah and Anderson, 1997). In addition, neural crest cells grown in the presence of TGF-β fail to express Mash-1 (Shah et al., 1996). Thus, there is a good correlation between BM PR-IA expression and adrenergic differentiation. In contrast, BMP-2, BMP-4, and TGF-β1 all decrease BM PR-IB expression, indicating that BM PR-IB is correlated with traits other than neurotransmitter specification.

Given that BM PR-IB mRNA is expressed in vivo in the developing sympathetic ganglia where the cells are exposed to BMP-4, the lack of expression in cultures grown in the presence of exogenous BMP-4 is surprising. BM PR-IB expression is not necessary for adrenergic development in neural crest cultures after replating, as these cells undergo a striking increase in adrenergic cell differentiation in the presence of exogenous BMP-4 (Varley et al., 1996; Reissmann et al., 1996). The presence of exogenous BMP-4 in the cultures apparently prevents the induction of BM PR-IB expression that occurs in the absence of exogenous BMP-4 ligand. In addition to the difference in expression of BM PR-IB, the expression of neurofilament proteins also differs between neural crest cells in vitro and in vivo. In vivo cells in the developing sympathetic ganglia express neurofilament proteins prior to the expression of adrenergic markers (Lallier and Bronner-Fraser, 1988; Gani and Luckenbill-Edds, 1991). These cells also express neuron-specific tubulin prior to the expression of TH (Shirley et al., 1996). However, in vitro under our culture conditions, the TH-positive and catecholamine-positive cells which develop are not neurofilament positive (Christie et al., 1987; Maxwell and Forbes, 1990). Thus, there has been an apparent dissociation of the adrenergic and panneuronal phenotypic traits in vitro (Anderson and Jan, 1997; Lo et al., 1999). One possibility is that BM PR-IB may play a role in the regulation of neurofilament expression, and its absence may result in a lack of neurofilament expression in vitro.

Another possible function for BM PR-IB in the development of sympathoadrenal cells could be to play a role in regulating cell adhesion molecule expression. During harvesting from primary culture, cells are disaggregated, and BM PR-IB is downregulated after harvesting from primary culture. By 96 h in secondary culture, when the cells express BM PR-IB again, cells are more dense and aggregated together. During embryogenesis, neural crest cells express neuronal cell adhesion molecule (N-CAM) and N-cadherin prior to migration from the neural tube. Expression of these cell adhesion molecules declines sharply upon the onset of cell migration. After these cells coalesce adjacent to the dorsal aorta, expression of N-CAM and N-cadherin returns (Thiery et al., 1982; Hatta et al., 1987; Duband, 1990; Akitaya and Bronner-Fraser, 1992). N-cadherin protein is first detected in cells of the sympathetic ganglia at stage 19 (Akitaya and Bronner-Fraser, 1992). BM PR-IB mRNA is expressed at stage 17, soon after neural crest cells coalesce adjacent to the dorsal aorta and prior to the expression of N-cadherin. Moreover, BMPS have been shown to induce the expression of N-CAM and L1 and to induce neuronal aggregation in neuroblastoma cells (Perides et al., 1992, 1993, 1994). Interestingly, BMP-2 and BMP-4 are more potent inducers of N-CAM than is BMP-7 (Perides et al., 1994), and BMP-2 and BMP-4 are more potent inducers of the adrenergic phenotype than is BMP-7 (Varley et al., 1995; Varley and Maxwell, 1996). Thus, BM PR-IB may serve to regulate cell adhesion molecules in neural crest cells after they arrive at the dorsal aorta, while BM PR-IA may regulate transmitter-specific traits such as TH expression.

Our results, taken together with other published reports, lead to a model of the activation of gene expression during sympathoadrenal differentiation. In this model, some migrating neural crest cells coalesce adjacent to the dorsal aorta, and these cells begin to express Cash-1 at stage 15. These cells then express BM PR-IB at stage 17, when BMP-4 is detected in the dorsal aorta. This leads to the induction of BM PR-IA, BMP-4, Phox2, GATA-2, and TH in the developing sympathetic ganglia at stage 18. Cell adhesion molecules are also induced at this time. After exposure to BMP-4, Cash-1 expression is increased to a higher level.

BMPS have distinct effects on neural crest cells at different times during development. For example, BMP-4 is required for the migration of cells out of the dorsal neural tube, and the expression of cell adhesion molecules declines at this time. Later, as neural crest cells coalesce adjacent to the dorsal aorta, BMPS participate in sympathoadrenal differentiation, and expression of cell adhesion molecules is induced. The distinct effects of BMPS on neural crest cells, as well as other cell types, at different times during deve-
opment may be mediated in part by the population of BMP receptors present on the cell surface at a given time. In addition, the BMP receptors may be linked to different intracellular signal transduction pathways as development proceeds. The modulation of BMP receptor expression at various times during development may be one element that allows neural crest cells to respond in distinct ways to the same ligand.

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