Development 122, 3557-3566 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV1102

BMP signaling during bone pattern determination in the developing limb

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SUMMARY

To examine the role of BMP signaling during limb pattern formation, we isolated chicken cDNAs encoding type I (BRK-1 and BRK-2) and type II (BRK-3) receptors for bone morphogenetic proteins. BRK-2 and BRK-3, which constitute dual-affinity signaling receptor complexes for BMPs, are co-expressed in condensing precartilaginous cells, while BRK-1 is weakly expressed in the limb mesenchyme. BRK-3 is also expressed in the apical ectodermal ridge and interdigital limb mesenchyme. BRK-2 is intensely expressed in the posterior-distal region of the limb bud. During digit duplication by implanting Sonic hedgehog-producing cells, BRK-2 expression is induced anteriorly in the new digit forming region as observed for BMP-2 and BMP-7 expression in the limb bud. Dominant-negative effects on BMP signaling were obtained by over-expressing kinase domain-deficient forms of the receptors. Chondrogenesis of limb mesenchymal cells is markedly inhibited by dominant-negative BRK-2 and BRK-3, but not by BRK-1. Although the bone pattern was not disturbed by expressing individual dominant-negative BRK independently, preferential distal and posterior limb truncations resulted from co-expressing the dominant-negative forms of BRK-2 and BRK-3 in the whole limb bud, thus providing evidence that BMPs are essential morphogenetic signals for limb bone patterning.

Key words: limb, BMP, BMP receptor, pattern formation, chondrogenesis, chick

INTRODUCTION

Bone pattern determination during vertebrate limb morphogenesis provides an experimental paradigm to study fundamental developmental mechanisms, including tissue interaction, induction and morphogenetic field determination (Tickle and Eichele, 1994; Johnson et al., 1994). The limb bone pattern is determined in a proximal-to-distal sequence within the distal limb mesenchyme called the progress zone (Summerbell et al., 1973), but chondrogenesis occurs after the mesenchymal cells leave the progress zone. This three-dimensional patterning of the limb skeleton is determined along three axes: anterior-posterior, proximal-distal and dorsal-ventral axes dominated by signaling centers called the polarizing region, apical ectodermal ridge and dorsal ectoderm, respectively. Sonic hedgehog, FGF-4, FGF-8 and Wnt-7a have been identified as the key instructive signals produced by these signaling centers (Riddle et al., 1993; Niswander et al., 1993; Yang and Niswander, 1995; Parr and McMahon, 1995; Crossley et al., 1996). These secretory signals cooperate to determine the limb pattern: a positive feedback loop between Sonic hedgehog and FGF-4 maintains their expression in the posterior-distal limb bud (Niswander et al., 1994; Laufer et al., 1994), Wnt-7a produced by the dorsal ectoderm has positive influence on *Sonic hedgehog* expression (Yang and Niswander, 1995; Parr and McMahon, 1995), and FGF-4 and FGF-8 also can function in the induction of the limb field (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996).

The most intensely studied one of the three signaling centers is the polarizing region, which influences the anterior-posterior axis by secretory Sonic hedgehog. Grafting of Sonic hedgehog-producing cells or, most notably, a source of an amino terminal peptide of Sonic hedgehog (Shh-N), into anterior margin of the limb bud at stages 19 to 21 (Hamburger and Hamilton, 1951) results in mirror-image duplication of the digit pattern (Riddle et al., 1993; Lopez-Martinez et al., 1995). The pattern duplication has been ascribed to the gradient of morphogenetic field established by Shh-N that being a putative morphogen. However, distribution of the secreted Shh-N protein is limited to the narrow region nearby within the limb bud (Marti et al., 1995; Lopez-Martinez et al., 1995), suggesting that Shh-N is unlikely to form a long-range gradient that accounts for concentration-dependent digit pattern formation, although diffusion of low level of protein remains possible. Conserved signaling mechanisms between the vertebrate limb and the *Drosophila* appendage (Perrimon, 1995) suggest that BMP-2 and BMP-4 may be a downstream signal of Sonic hedgehog, as their *Drosophila* homolog *decapentaplegic* (*dpp*) is down-

stream of *hedgehog* in anterior-posterior patterning of the *Drosophila* appendage primordium (Ingham and Fietz, 1995). Consistent with this, the expression of BMP-2 and BMP-7 overlaps with that of *Sonic hedgehog* in the limb bud (Francis et al., 1994; Francis-West et al., 1995). However, BMP-2 protein does not affect the digit patterning when implanted into the limb bud (Francis et al., 1994). To test whether BMPs are indispensable for pattern formation in the limb bud, we have taken advantages of the ability to generate a specific signal blockade. Specific inhibition of BMP signals can be obtained by overexpressing the dominant-negative forms of BMP receptor kinases (BRKs) lacking intracellular kinase domain (Suzuki et al., 1994).

BMP receptors, like the receptors for other members of the TGF-β superfamily, consist of heterodimers of inducible and constitutively active kinases of type I and type II receptors, respectively. Both type I and type II receptors for BMPs have been identified in vertebrates (Koenig et al., 1994; ten Dijke et al., 1994; Liu et al., 1995; Nohno et al., 1995a; Rosenzweig et al., 1995). Both contain intracellular serine/threonine kinase domains and form heteromeric high-affinity complexes for BMPs to transduce external signals to an intracellular phosphorylation cascade. We have identified type I and type II receptors for BMP from the chicken, and found that one of the type I receptors, BRK-2, is predominantly expressed in chondrocytes of the limb bud. Kinase domain-deficient form of BRK-2 (DN-BRK-2) inhibits endogenous and BMP-induced chondrogenesis of the limb mesenchyme in culture. Furthermore, the limb bone pattern in the posterior-distal region is disturbed by DN-BRK-2, when expressed in the chick limb bud in combination with DN-BRK-3. Position-dependent bone defects with these DN-BRKs suggest that their ligands, the BMPs, are essential signaling factors, involved in determining bone pattern as well as chondrogenesis in the developing limb bud.

MATERIALS AND METHODS

Cloning of chicken BMP receptors

Chicken BRK-1 was obtained by PCR amplification of the library cDNA, based on the nucleotide sequence of partial cDNA clone CST2-2 (Sumitomo et al., 1993). Chicken BRK-2 was already characterized as RPK-1 (Sumitomo et al., 1993). To obtain chicken BRK-3, the following degenerate primers were synthesized based on the human BRK-3 sequence (Nohno et al., 1995a): 5′-CGNTAYGGNGCNGTNTAYAA-3′ and 5′-CANCKCATRAADATYTCCCA-3′. PCR was carried out using these primers and chick embryo cDNA at annealing temperature at 63 to 53˚C for 20 cycles decreasing 0.5˚C per cycle followed by 15 additional cycles at 53˚C. The PCR products were identified by nucleotide sequencing. Full coding sequence was determined using an ABI Sequencer after isolation of the chicken BRK-3 by hybridization screening of a cDNA library (Sumitomo et al., 1993) and also a chicken genomic library (Clontech).

In situ hybridization

Chick embryos were staged according to Hamburger and Hamilton (1951). Whole-mount in situ hybridization was carried out as described (Wilkinson, 1992) using the following chicken cDNAs. Antisense cRNA probes were synthesized with 1.0 kb *Hin*dIII-*Bam*HI fragment encoding an amino-terminal half of BRK-1, 1.4 kb *Pst*I-3′-end fragment encoding BRK-2 (Sumitomo et al., 1993), and 1.6 kb *Bam*HI-3′-end fragment encoding a carboxy-terminal tail of BRK-3. *HoxD12*, *Msx-1* and *Sonic hedgehog* expression was determined using the cDNAs described previously (Nohno et al., 1991, 1992, 1995b). Partial cDNAs encoding chicken BMP-2, BMP-4 and BMP-7 were obtained by PCR based on the published nucleotide sequences (Francis et al., 1994; Houston et al., 1994), and used as templates for cRNA synthesis. Sense probes were used to estimate signal specificity and showed no significant hybridization signal.

Expression of kinase domain-deficient BMP receptors

Amino-terminal coding sequences of chicken BRK-1 and BRK-2 were subcloned into modified pBluescript that contains *myc* epitope (MEQKLISEEDLN) and stop codon. DN-BRK-1 and DN-BRK-2 contain amino-terminal 203 and 178 amino acids, respectively. The DN-BRK-3 construct was derived from the human BRK-3 (Ishikawa et al., 1995). These DN-BRK cDNAs were finally subcloned into the RCAS(A) retroviral vector (Hughes et al., 1987) that had been modified to contain the polylinker sequence recognized by *Cla*I, *Nsi*I, *Pml*I, *Spe*I, *Bst*BI and *Not*I. Primary culture of chicken embryonic fibroblasts was obtained from the torso of virus-free White Leghorn embryos (line M, obtained from Nisseiken) at stage 24. Chicken fibroblasts were transfected with DN-BRKs using Lipofectin (GIBCO-BRL). The recombinant virus in the medium was harvested daily and concentrated by ultracentrifugation (Fekete and Cepko, 1993). Virus titer was determined using anti-gag monoclonal antibody AMV-3C2 (Potts et al., 1987) after infecting chicken fibroblasts with the diluted virus stock. Mesenchymal cells were obtained by trypsin digestion of the limb bud from line M embryos at stages 22-24, cultured in Ham's F12 containing 1% FBS (Sasse et al., 1984). Mesenchymal cells (about 5×10^5 cells/ml) were infected with the recombinant virus bearing DN-BRK or the human placental alkaline phosphatase (PLAP) gene (Fekete and Cepko, 1993). After 5-6 days of cultivation, the infected cells were stained with Alcian blue (pH 1) to visualize chondrogenetic nodule formation, or cultured for additional 24 hours in the presence of 1 μCi sodium [^{35}S]sulfate to determine the incorporation into cetylpyridinium chloride-precipitable proteoglycan fraction as described previously (Iwamoto et al., 1993).

Ectopic Shh-N expression in the limb bud

The chicken *Sonic hedgehog* cDNA encoding amino-terminal peptides (Shh-N) was subcloned into RCAS by inserting a stop codon at residue 200. Virus-free chicken embryonic fibroblasts were transfected with Shh-N-RCAS. The cells were grown for 2 days in α-MEM containing 5% chicken serum and confluent cells were scraped and cut into small pieces. Wild-type chicken embryos were used as hosts for implantation at stages 18-21. A cell pellet was grafted to the anterior region of limb buds as described previously for implantation of the polarizing region (Noji et al., 1991). The embryos were fixed 24-72 hours after implantation to carry out whole-mount in situ hybridization.

Effect of dominant-negative BMP receptors on limb patterning

Soluble form of DN-BRK-3 was constructed by subcloning amino-terminal 125 amino acid-coding sequence of the chicken BRK-3 cDNA. This construct is called DN-BRK-3S to distinguish from DN-BRK-3 that contains a transmembrane helix. Concentrated virus stocks of DN-BRK-2 and DN-BRK-3S were co-injected into the right wing field at stages 11-13. After incubation for 12-16 hours, the embryos received subsequent injection of the same viruses to the right wing bud at stages 16-18. For implantation of virus-producing cells, chicken fibroblasts transfected with DN-BRKs were grafted to the right wing bud at stages 16-17. To observe the effect on bone pattern formation, the embryos were fixed in 10% (v/v) formalin in phosphate-buffered saline, stained with Alcian green and finally clarified in methyl salicylate (Tickle et al., 1985). Efficiency of RCAS-mediated gene expression in the limb bud was monitored by staining with the alkaline phosphatase substrate as described (Fields-Berry et al., 1992).

RESULTS

Expression of BMP receptors in the limb bud

Chicken cDNAs encoding the type I receptors, BRK-1 and BRK-2, and the type II receptor, BRK-3, were isolated by screening a cDNA library with PCR-amplified probes. The deduced amino acid sequence of BRK-1 is 90-91% identical to corresponding human, rat and mouse cognates (Fig. 1). Chicken BRK-2, formerly called RPK-1 (Sumitomo et al., 1993), is also conserved. The type II receptor BRK-3, which has long carboxy-terminal tail following the kinase domain, is also conserved between human and chicken (Fig. 1).

A complex between the type I and type II receptors showed high binding affinity for BMP-4, and cells acquired the capa-

A

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BRK-1 (GG)       MTRLRVCERLLGAYLLIILHVQGQNLDSMLHGTGMKTNPDQKKQGNGVTLAPEDTLPFLKCYCSGHCPDDAINNTCITNGHCFAIIEEDE   90
BRK-1/TFR11 (MM) ..Q.YTYI.....C.F..S.................SDL....PE.........................................D   90
BRK-2 (GG)                                 .PLLSSS.LSMESR.EDSEG.-..APPQKK.S.Q.HH...E.SV.S..S.D.Y..T.....D   61
ALK-6 (MM)                                 ..LRSSG.L.VGT..EDGES.-..TPRPKI.R.K.HH...E.SV..I.S.D.Y..TM....D   61

BRK-1 (GG)       HGEPTLASGCMKYEGSDFQCKDSPKAQLRRTIECC-RTDFCNQDLQPTLPPLDSTDGLFDGSIRWMAVLISMAVCIIVMIILFSCFCYKH  179
BRK-1/TFR11 (MM) Q..T..T..........................-..NL...Y.......V-VIGPF......LV.........VA...FS.......  178
BRK-2 (GG)       S.GHLVTK..LGL.......R.T.IPHQ..S....TGQ.Y..KH.H......-KNRDFAE.N.HHK.L...VT..-SILLV.III...FR  149
ALK-6 (MM)       S.M.VVT...LGL.......R.T.IPHQ..S....TERNE..K..H......-KDRDFV..P.HHK.L...VT..-SLLLV.IIL...FR  149
                                                                                  Transmembrane
                                                                    ► Kinase domain
BRK-1 (GG)       YCKSMAKRHCYNRDLEQDEAFIPAGESLKDLIDQSQSSGSGSGLPLLVQRTIAKQIQMVRQVGKGRYGEVWMGKWRGEKVAVKLFFTTEE  269
BRK-1/TFR11 (MM) ....ISS.GR.............V.....................................................V.......  268
BRK-2 (GG)       .-.RQEA.PR.SIG.....TY..P........E.........................K.I.....................V.......  238
ALK-6 (MM)       .-.RQEA.PR.SIG.....TY..P....R...E.........................K.I.....................V.......  238

BRK-1 (GG)       ASWFRETEIYQTVLMRHENILGFIAADIKGTGSWTQLYLITDYHENGSLYDFLKCTTLDNRALLKLAYSAACGLCHLHTEIYGTQGKPAI  359
BRK-1/TFR11 (MM) ..................................................A...T.............................  358
BRK-2 (GG)       ..................................................Y..S....TKGM......SVS.......G.FS.......  328
ALK-6 (MM)       ..................................................Y..S....AKSM......SVS.........FS.......  328

BRK-1 (GG)       AHRDLKSKNILIKKNGTCCIADLGLAVKFNSDTNEVDVPLNTRVGTKRYMAPEVLDESLNKNHFQPYIMADIYSFGLIIWEMARRCVTGG  449
BRK-1/TFR11 (MM) ..............S.....................I.....................................................I...  448
BRK-2 (GG)       ...........V.................I.......I.P..........P.........R....S.....M......L..I.....S..  418
ALK-6 (MM)       ...........V.................I.......I.P..........P.........R....S.....M......L..I.....S..  418
                                                                                  Kinase domain ◄
BRK-1 (GG)       IVEEYQLPYYDMVPNDPSYEDMREVVCVKRLRPVVSNRWNSDECLRAILKLMSECWAHNPASRLTALRIKKTLAKMVESQDVKI         533
BRK-1/TFR11 (MM) ..........N...S.................I.............V.....................................        532
BRK-2 (GG)       .........H.L..S.........I..I.....SFP...S......QMG...M..............V.......S....I.L       502
ALK-6 (MM)       .........H.L..S.........I..M.K...SFP...S......QMG...T....Q..........V.......S....I.L       502
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B

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BRK-3 (GG)                  MKSVTKNI-FHSLSKAAQSEERLCAFKDPYQQDHGISESRISQENGTILCMKGSTCYGLWEKTREGDIHLVKQGCWSHIG   79
BRK-3 (HS)       MTSSLQRPWRVPWLPWT.LLV.TAA.S.NQ.............L..G.....H.......S...........SK-...N...........   89

BRK-3 (GG)       DPQECHFEECIVTTTPSLIQNGTYRFCCCSTDLCNVNFTENFPPPDPTDTTPYNSSHSFHRDETIVIVLASVSVLAVLIAALFFGYRMLA  169
BRK-3 (HS)       ......Y...V.....PS.....................-..-.-....LSPP...N....I.A...........V..C....T...T  176
                                                                                  Transmembrane
                                                                    ► Kinase domain
BRK-3 (GG)       GDRKQGLHSMNMMEAAASEPSLDLDNLKLLELIGRGRYGAVYKGSLDERPVAVKVFSFANRQNFVNERNIYRIPLMEHDNIARFIVGDER  259
BRK-3 (HS)       ..................................................I..K....V.................  266

BRK-3 (GG)       FTADGRMEYLLVMEYYPNGSLCKYLSLHTSDWVSSCRLAHSITRGLAYLHTELPRGDHYKPAISHRDLNSRNVLVKNDGTCVISDFGLSM  349
BRK-3 (HS)       V.....................................V.....................................................  356

BRK-3 (GG)       KLTGNRLVRPGEEDNAAISEVGTIRYMAPEVLEGAVNLRDCESALKQVDMYALGLIYWEIFMRCTDLFPGESVPEYQMAFQTEVGNHPTF  439
BRK-3 (HS)       R.....................................................................................  446
                                                                    Kinase domain ◄
BRK-3 (GG)       EDMQVLVSREKQRPKFPEAWKENSLAVRSLKETIEDCWDQDAEARLTAQCAEERMAELMMIWERNKSVSPTVNPMSTAMQNERNLSHHRR  529
BRK-3 (HS)       .....................................................................................N..  536

BRK-3 (GG)       VSKIRPYPDYSSSSYIEDSIHHTDSIVKNISSEHSMSTTPLTSGEKNRNSINYERQQAQARIPSPETSVTSLSTNTTTTNTTGLTPSTGM  619
BRK-3 (HS)       .P..G.............................S....I.....................................................  626

BRK-3 (GG)       TTISEIPYSDETNLHTTNVMQPGGPTPVCLQLTEEDLETNKLDPKEVDKNLKESSDENLMEHSLKQFSGPDPLSSTSSSLLYPLIKLAVE  709
BRK-3 (HS)       .....M..P.........A.SI.....................................................................  716

BRK-3 (GG)       VTGQQDFTQASNGQACLIPDVPSAQVYPLPKQQNLPKRPTSLPLNTKNSTKEPRLKFGGKHKSNLKQVETGVAKMNTINAAEPHVVTVTM  799
BRK-3 (HS)       A........TA..........LPT.I.............................S.............................  806

BRK-3 (GG)       NGVAGRTHSINSHAGTTQYANGAVPSGQTASSVAQRAQEMLQNQFSGEDSRLNINSSPDEHEPLLRREQQVGHDEGVLDRLVDRRERQLD  889
BRK-3 (HS)       ......N..V....A.......T.L....TNI.TH..........I...T.....................A..............P.E  896

BRK-3 (GG)       NGRTNVNNNNSNPCPVQDTATLSIQNVARNPGQTQTRRAQRPNSLDLSATNSLDSSSMQLGDSSQDGKSGSGEKIKKRVKTPYSLKRWRP  979
BRK-3 (HS)       G....S........SE..VLAQGVPST.AD..PSKP..............V..G..I.I.E.T.....................  986

BRK-3 (GG)       STWVISTEPLHCEVNNNGGDRAVHSKSSTAVYLAEGGTATTMVSKDAGVNCL                                1031
BRK-3 (HS)       ........S.D.......SN.........................I.M...                                  1038
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Fig. 1. Comparison of the deduced amino acid sequences of BRK-1, BRK-2 and BRK-3 between chicken and mouse or human. A gap is introduced to improve alignment and the identical residues are indicated by dots. A transmembrane helix is underlined and intracellular serine/threonine kinase domain is indicated by arrowheads. (A) Chicken BRK-1 and BRK-2 are 91% and 92% identical to the mouse cognates, respectively, although chicken BRK-1 and BRK-2 are 69% identical. (B) Chicken BRK-3 is 88% identical to human BRK-3. GSDB/DDBJ/EMBL/GenBank accession number of chicken BRK-3 is L77660.

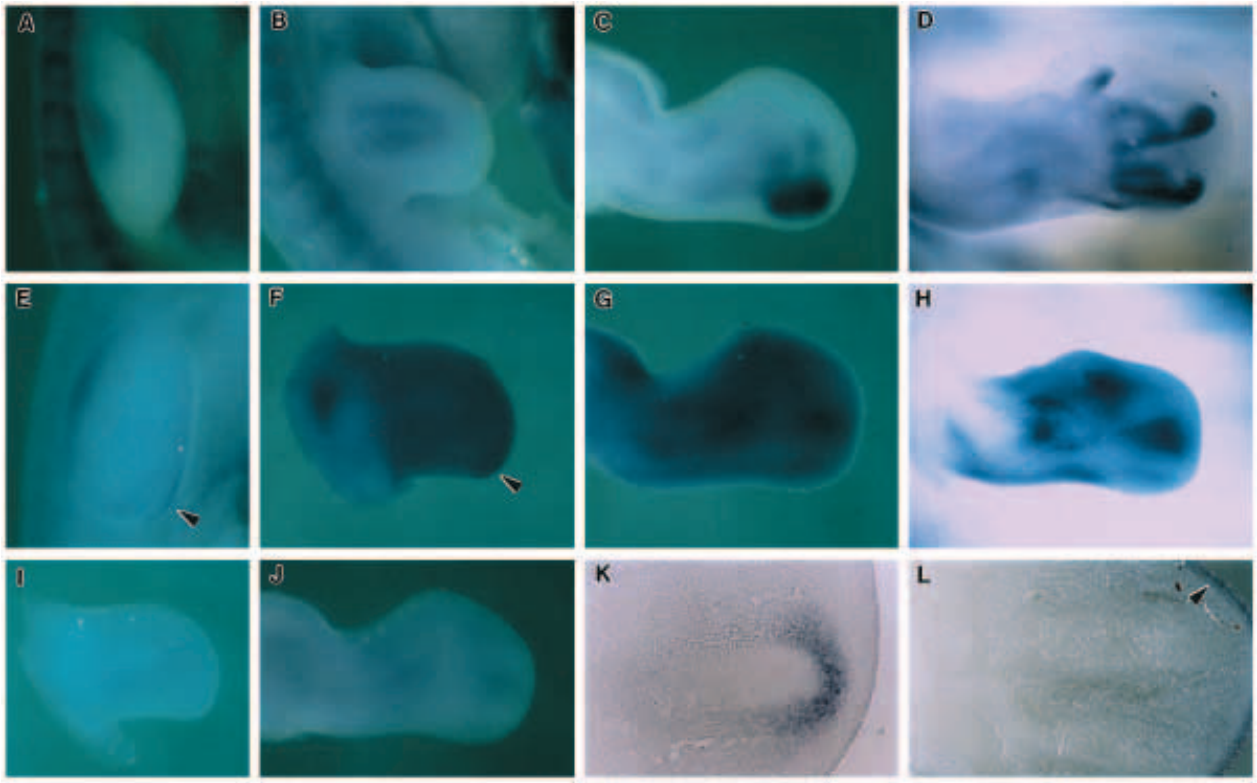

Fig. 2. Differential expression of three BMP receptors in the developing chick limb. (A-D,K) BRK-2; E-H,L, BRK-3; I-J, BRK-1. BRK-2 expression is weak in the limb bud at stages 19 (A), gradually confined to precartilaginous cells at stage 24 (B), and intense in the posterior-distal region at stages 28 (C) and 30 (D,K). BRK-3 transcripts are relatively abundant in the developing limb bud at stages 21 (E), 24 (F), 28 (G), and 30 (H,L), and intense signals are detectable in the apical ectodermal ridge throughout (arrowheads). BRK-1 signals are not detectable in the limb bud at stages 24 (I) and weak in the limb mesenchyme at stage 28 (J).

bility to respond to BMP-7 signaling when BRK-3 was co-expressed with BRK-2 (Nohno et al., 1995a; Rosenzweig et al., 1995). However, high-affinity binding sites for BMP-4 could not be detected when type I receptor was expressed alone. These results suggest co-expression of the type I and type II BMP receptors during embryogenesis will be indicative of tissues responsive to physiological levels of BMP signals.

Although BRK-1 and BRK-2, also known as ALK-3 and ALK-6, respectively, are structurally related type I receptors for BMPs (ten Dijke et al., 1994), their expression patterns differ significantly during chick embryogenesis. The BRK-1 gene is only weakly expressed in the limb bud at stages 17-32, whereas the BRK-2 gene is intensely expressed in prechondrogenic cells of the skeletal elements in the limb bud (Fig. 2A-D,I,J). Expression of BRK-1 is not detected in the precartilaginous condensation, while BRK-2 signals are detectable in this region. At stage 28, BRK-2 signals were confined to chondrogenic cells, intensifying within the posterior-distal region of the limb bud (Fig. 2C). Signaling through BRK-2, but not BRK-1, is therefore potentially involved in the cartilage differentiation predominantly in the posterior-distal region of the developing limb. At stages 28-32, intense BRK-2 signals are detected in the distal end of the phalangeal primordium (Fig. 2C,D,K). Weak BRK-1 signals are detectable in the interdigital limb mesenchyme at stages 28-30.

The type II receptor BRK-3 is intensely expressed in the apical ectodermal ridge at stages 17-24 and continues to be expressed in this region until at least stage 30. BRK-3 is also expressed in the prechondrogenic region at stages 21-24 (Fig. 2E,F). The BRK-3 signal is relatively abundant, detectable in the whole limb mesenchyme at stages 24-28 and intensified in the interdigital mesenchyme at stages 28-30 (Fig. 2G,H,L). Distinct but overlapping expression of the BRK-2 and BRK-3 genes in the limb chondrocyte suggests an important role for BMPs in chondrogenesis by forming differential heteromeric receptor complex.

The expression of BRK-2 in the posterior region of a stage 28 limb bud (Fig. 2C) suggests that it might be regulated, in part, by Sonic hedgehog. To test this, polarizing region cells and *Shh-N*-expressing cells were grafted into the anterior of a host limb bud. Both operations resulted in the induction of BRK-2 in the anterior region (Fig. 3), but BRK-2 expression in the new-digit forming region was only detectable 48 hours after grafting. BMP-2 is also known be induced in the new-digit forming region after grafting polarizing region (Francis et al., 1994), or infection with a *Sonic hedgehog*-expressing virus in the anterior margin (Laufer et al., 1994). Similar results were also obtained by using Shh-N producing fibroblasts, and the onset of BMP-2 and BMP-7 induction in the anterior region was about 12-24 hours earlier than BRK-2 (Fig. 3). Since the BMP-4, BRK-1 and BRK-3 genes were not expressed in the posterior mesenchyne of the limb bud, we could not detect the induction of these genes in the anterior wing bud by Shh-N grafting (data not shown). Thus, BMP-2 and BMP-7 as well as

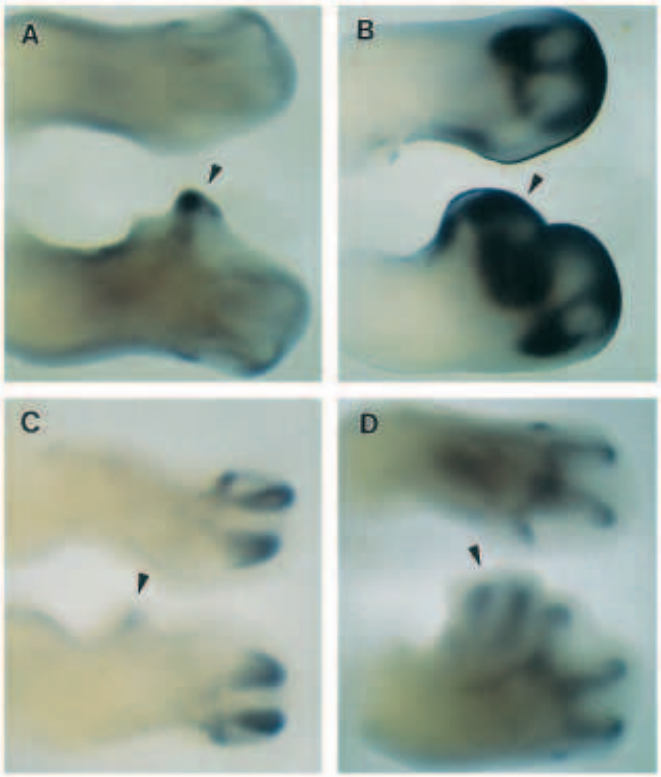

Fig. 3. BMP-2, BMP-7 and BRK-2 expressions after implantation of Shh-N-producing cells into anterior margin of the right wing bud of virus-resistant embryos at stages 18-21. (A,B) Dorsal views of BMP-2 and BMP-7 expressions, respectively, 48 hours after grafting. BMP-2 and BMP-7 expressions are induced in the new-digit forming region (arrowheads). (C,D) Dorsal views of BRK-2 expression 48 and 72 hours after implantation, respectively, of Shh-N-producing cells. BRK-2 expression is induced in the anterior region (arrowheads) of the treated wing bud, as compared to the contralateral untreated wing bud. At 24-36 hours after implantation, expression of BMP-2 and BMP-7, but not BRK-2, is slightly induced in the anterior limb bud. Control left limb buds are placed on top of each panel with the reversed anterior-posterior orientation.

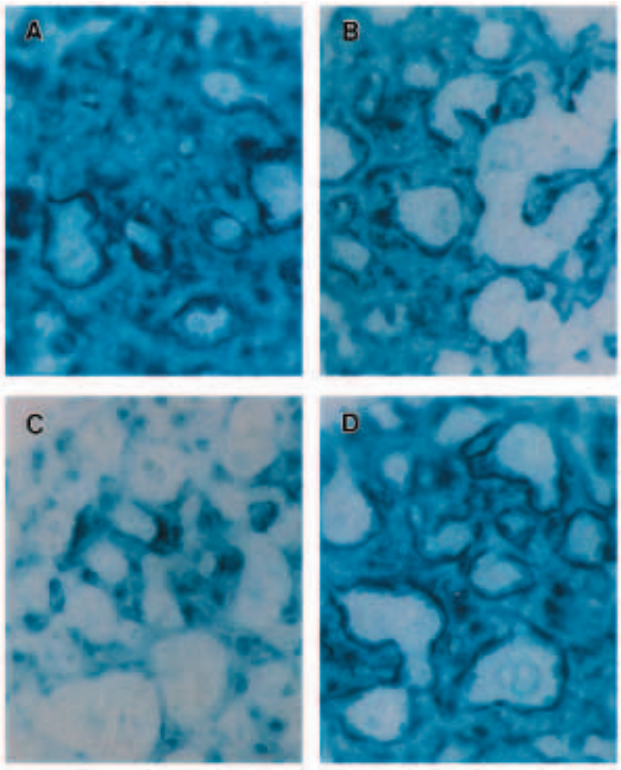

Fig. 4. Alcian blue staining of limb mesenchymal cells from stage 22 embryos after cultivation for 6 days in the presence of the dominant-negative BRK viruses. (A) RCAS vector alone; (B) DN-BRK-1; (C) DN-BRK-2; (D) DN-BRK-3.

BRK-2 are positively regulated by Shh-N, although the receptor gene is induced late as compared to the BMP ligands. Sequential induction of the BMP and receptor genes by Sonic hedgehog is correlated well with the normal expression pattern in the posterior region of the wing bud.

Dominant-negative effect elicited by modified BMP receptors

To examine the roles of the BMP receptors, we constructed a dominant-negative version of BRK-2 (DN-BRK-2) and BRK-3 (DN-BRK-3), and inserted them into a retroviral vector (RCAS) for introducing the gene into chick cells. Chondrogenesis of cultured limb mesenchymal cells from stage 22 embryo could be inhibited by infecting with the RCAS bearing DN-BRK-2 (Figs 4, 5). Limb mesenchymal cells spontaneously form cartilaginous nodules in culture and nodule formation was enhanced by adding BMP-2 protein. DN-BRK-2 significantly inhibited chondrogenesis of cultured limb mesenchyme as observed by the decrease in the number and size of Alcian blue-stained nodules (Fig. 4). Sulfate incorporation into proteoglycan was also reduced by DN-BRK-2 as compared to the control vector, or vector bearing the alkaline phosphatase gene (Fig. 5). In contrast, DN-BRK-3 had a weak activity to inhibit proteoglycan synthesis, while DN-BRK-1 was not inhibitory. However, when we used limb bud mesenchymal cells from stage 24 embryo, proteoglycan synthesis and chondrogenic nodule formation were profoundly inhibited by both DN-BRK-3 and DN-BRK-2, accompanying extensive cell proliferation (data not shown). Again, DN-BRK-1 had no inhibitory effect on the stage 24 mesenchyme. Therefore, BMP signals recognized and transduced by BRK-1 seem to differ from those of BRK-2, and may represent differential signaling capability of the BMP receptor system. It is worth noting that BRK-2 and BRK-3 form the heteromeric type I/type II receptor complex with dual-affinity binding to BMP-4, whereas BRK-1 and BRK-3 form a low-affinity receptor complex (Nohno et al., 1995a). We therefore used DN-BRK-2 and DN-BRK-3 for ectopic expression by injecting the recombinant virus or implanting virus-producing fibroblasts.

Limb bone pattern induced by dominant-negative BMP receptors

The total skeletal pattern was not changed by independently expressing DN-BRK-1, DN-BRK-2 or DN-BRK-3 in ovo. However, when DN-BRK-2 was expressed in the limb bud with a modified form of DN-BRK-3, cartilage formation in the limb bud was inhibited in a position-dependent manner. Ideally, it would have been desirable to infect each cell with both dominant-negative receptors. Unfortunately, the

Table 1. The number of embryos showing bone defects after ectopic DN-BRKs expression in the wing bud

Treatment	Sites in the wing bud	Treated embryos	Survived	Normal pattern	Bone defects					
					Humerus	Radius	Ulna	Digit II	Digit III	Digit IV
DN-BRK-2	Whole	49	36	36	0	0	0	0	0	0
DN-BRK-2	Whole	43	30	22	1	4	7	1	6	6
+	Anterior	12	10	8	0	2	0	1	0	0
DN-BRK-3S	Distal	34	29	21	0	4	2	2	2	2
	Posterior	6	4	2	0	0	1	0	0	2

RCAS(A) viral vector used here prevents superinfection by a second virus of the same subgroup once the viral genome is established in a cell. We therefore used a different construct of the dominant-negative receptor, DN-BRK-3S, which lacks a transmembrane helix and is therefore expected to distribute extensively from the site of expression. In theory, this approach can lead to wide-spread expression of DN-BRK-3S superimposed on focal infection of DN-BRK-2, since DN-BRK-2 virus will be unable to infect cells already producing DN-BRK-3. In some percentage of the limbs, this will lead to the majority of the cells expressing DN-BRK-2 and effectively receiving secreted DN-BRK-3S. By repetitive injections of the mixture of DN-BRK-2 and DN-BRK-3S at stages 12 and 18, skeletal defects were observed in the posterior and distal region. In the most severe cases, the ulna and autopods were absent (Fig. 6). Most of the infected embryos developed with normal bone pattern (Table 1). Besides the virological limitations, the low frequency of limb bone defects may be due to decrease in the viability upon increasing doses of the DN-BRK recombinant virus, or to low efficiency of the DN-BRKs in overcoming endogenous signaling through the receptor complex. Similar results were obtained by implanting virus-producing fibroblasts into the limb bud at stage 17 (Fig. 6). Autopods of posterior digit IV in the wing were missing when DN-BRKs were expressed predominantly in the posterior wing bud, whereas anterior digit II and radius were absent when DN-BRKs-expressing fibroblasts were implanted to the anterior wing bud (Table 1). We could not exactly control the site of ectopic expression because of extensive distribution of the recombinant retrovirus owing to self-replication in proliferating cells in the developing limb bud and therefore obtained variable phenotype, especially when DN-BRK-expressing cells were implanted to the distal wing bud. Nevertheless, the bone defects were consistent with the region where the endogenous BRK-2 gene was intensely expressed in the wing bud.

We examined whether co-injection of DN-BRK-2 with DN-BRK-3S into the wing-forming region had an inhibitory effect on the *Sonic hedgehog* and *HoxD12* expression.We used more than 12 embryos in each experiments, since bone defects were observed with low frequency. *Sonic hedgehog* expression in the treated wing was not significantly different from that in the contralateral wing bud at 48-72 hours after injection (data not shown), consistent with previous reports that BMP signaling was downstream of Sonic hedgehog (Laufer et al., 1994). *HoxD12* expression in the posterior-distal region was also not affected by DN-BRK-2 and DN-BRK-3 in combination. Therefore, it is possible that BMP regulation of posterior limb bone formation may be downstream of *HoxD12* and not influence *HoxD12* expression. Alternatively, *HoxD12* may not be required for specification of the posterior limb elements. We also examined several additional marker genes, including BMP-2, BMP-7, FGF-4 and *Msx-1*. However, major differences in the expression patterns of these genes between DN-BRKs-treated and control wing buds were not detectable, although it was difficult to determine subtle differences due to the low efficiency of signal blockade that resulted in bone defects (data not shown).

DISCUSSION

Role for BMP in bone pattern formation

The present studies implicate that BMP signaling is in the process of chondrogenesis and also in pattern determination during limb development. The limb pattern is initially laid down as mesenchymal condensations which then undergo cartilage differentiation. BMP family members have been suggested as candidates involved in this process (Johnson et al., 1994). Role for BMPs in limb pattern formation has been suggested by the several lines of evidence. The BMP-2 and BMP-7 genes are expressed in specific regions of the limb bud, including posterior-distal mesenchymal region and the apical ectodermal ridge (Francis et al., 1994; Francis-West et al.,

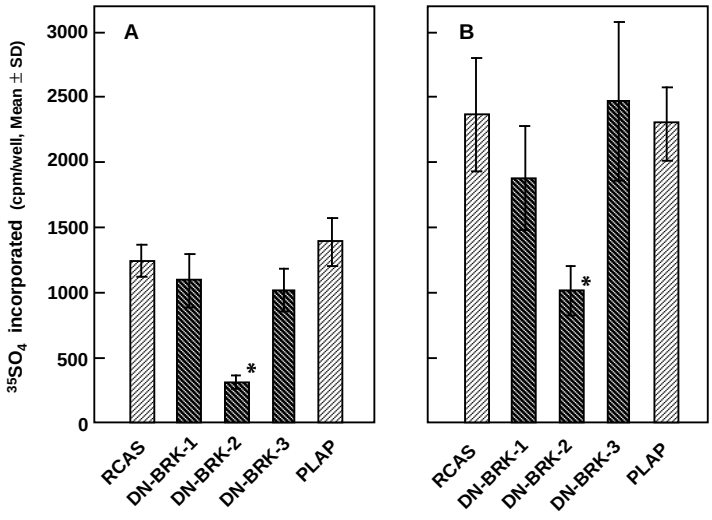

Fig. 5. Effect of dominant-negative BRKs on chondrogenesis of the cultured limb mesenchyme from stage 22 embryos. Mean ± s.d. of proteoglycan synthesis determined by [^{35}S]sulfate incorporation into proteoglycan fraction in the presence (B) or absence (A) of added BMP-2 (100 ng/ml). *Significantly different from controls received the RCAS vector virus at P<0.01.

1995). BMP-2 is induced by Sonic hedgehog during the process of establishing anterior-posterior limb axis (Laufer et al., 1994). We showed here that the type I receptor BRK-2, which is part of functional receptor system for BMP-4 and BMP-7, is expressed in the prechondrogenic and distal regions of limb cartilage, most intensely in the posterior-distal region of the limb bud. The spatial and temporal expression patterns of several BMPs and their receptors implicate them in anterior-posterior pattern formation.

We observed dominant-negative effects on chondrogenesis of limb mesenchymal cells in culture, when BRK-2, but not BRK-1 and BRK-3, was used to generate a kinase domain-deficient form of the receptors. Furthermore, cartilage and bone formation is prevented in the distal region when DN-BRK-2 and DN-BRK-3S are co-expressed in the whole limb bud. Chondrogenic condensation that leads to bone formation is stimulated by BMPs, mediated through high-affinity binding to BRK-2 and BRK-3 heteromer, since these receptor genes are co-expressed in the chondrogenic condensation and perichondrial region of the limb bud. These results suggest that BMPs are essential factors for cartilage and bone pattern formation in the limb bud.

Position-dependent effects of BMP signaling through receptor system

Both BMP ligands and their receptors are expressed in the limb bud with specific patterns that account for their differential signaling capabilities. BMPs, as members of the TGF-β superfamily, are known to function as dimers. BMP-2 and BMP-7 are expressed in an overlapping region of the limb bud, and therefore both homodimers and heterodimers are presumably formed in the posterior-distal limb bud. The BMP heterodimers are much more active in the induction of ectopic bone formation than are homodimers (Aono et al., 1995). In addition, there are myriad combinations of BMP-related proteins produced in the limb bud including GDFs (Storm et al., 1994; Chang et al., 1994; Lyons et al., 1995). The differential activities of the various BMP-related peptides to induce cartilage differentiation may be responsible for determining aspects of the limb bone pattern.

The receptor BRK-2 shows graded expression, intensifying distally, in the chondrogenic region. Consistent with this, all of the cartilaginous cells making up limb bones are not affected equally by co-introduction of DN-BRK-2 and DN-BRK-3S in the whole wing bud. Our results show that bone formation in the distal limb bud is most susceptible to the BMP signal blockade through BRK-2/BRK-3 receptor system. Most severely affected parts of limb bones were derived from the limb mesenchyme where BRK-2 is intensely expressed. Therefore, position-dependent defects of the limb bones by these DN-BRKs represent graded and differential competence of limb mesenchyme to BMP signaling.

Differential signaling by BMPs may provide a partial explanation for positional information along the anterior-posterior and proximal-distal axes of the limb bud. Because BRK-2 and BRK-3 constitute a heteromeric receptor complex that has dual-affinity for BMP-4 (Nohno et al., 1995a) and signaling capability to BMP-7 (Rosenzweig et al., 1995), overlapping expression of BRK-2 and BRK-3 is important in signal recognition at extremely lower level of BMPs. High affinity binding of BMPs is only detected by co-expressing BRK-3 with BRK-2, but not with BRK-1, suggesting the formation of differential receptor complexes (Nohno et al., 1995a). Since BRK-2 and BRK-3 are co-expressed predominantly in the cartilage-forming region of the developing limb, high affinity binding may be implicated in the chondrogenic differentiation whereas low affinity sites elicited either with type I receptor alone or with BRK-3 and BRK-1 combination are presumed to be involved in other developmental processes, such as regulation of cell death in the interdigital region, as discussed below.

A *Drosophila* type II receptor that binds on its own to activin, called AtrII/punt, has been shown to bind BMP-2 in the presence of *thick vein* and *saxophone* type I receptors for *dpp*, the BMP-2 homologue (Letsou et al., 1995). Similarly, vertebrate type II receptors for activins are involved in the formation of signaling receptor complexes responsive to BMPs (Yamashita et al., 1995). The type II receptors for activin, called ActRIIA and ActRIIB, are intensely expressed in chick limb chondrocytes (Ohuchi et al., 1992; Nohno et al., 1993), and activin has an activity to induce limb chondrogenesis (Jiang et al., 1993). Overlapping expression of these activin receptors with BRK-2 in the prechondrogenic region of the limb bud suggests signal cross-talk between activin receptor system and BMP receptor system during chondrogenesis. Since activins are only weakly expressed in the limb bud, compared to BMPs, the type II receptors for activins may participate in the BMP signaling as a distinct type II receptor. It remains to be determined, however, whether heterodimers of BMP-2/BMP-7 and BMP-4/BMP-7 bind to these type I/type II receptor heteromers with high affinity and which combinations of the receptors are most effective at inducing cartilage differentiation in the limb bud.

Roles for BRK-1 and BRK-3 heteromer

Although the specific blockade of BMP signaling through ectopic expression of the DN-BRK-2 profoundly inhibits chondrogenesis of limb mesenchyme, DN-BRK-1 and DN-BRK-3 have weak ability to inhibit chondrogenesis on their own. This suggests that BMP signaling through BRK-1 entirely differs from BRK-2. Since BRK-1 is weakly expressed in the interdigital limb mesenchyme where BRK-3 is expressed, BRK-1 and BRK-3 complex may be involved in the programmed cell death in this region.

After submitting our paper, Zou and Niswander (1996) reported that interdigital cell death of the chicken leg could be prevented by the dominant-negative form of BMPR-IB, that is equivalent, but not identical to, DN-BRK-2 used in this study. Unfortunately, we did not examine the effect of DN-BRK-2 on the leg bud, and therefore we could not compare these results in details. Since BRK-1 and BRK-3 in combination produce only a single low-affinity site for BMP-4, DN-BRK-2 alone may not interfere with high affinity binding site elicited by BRK-2 and BRK-3 heteromer. Therefore, inhibition of cell death by DN-BRK-2 alone is likely to result from BMP signaling blockade at low affinity site produced by BRK-1 and BRK-3 complex. Limb bone defects observed here using DN-BRK-2 and DN-BRK-3S in combination probably result from BMP signaling blockade at both high-affinity and low-affinity sites. Efficient blockade of the high-affinity site is only achieved by using DN-BRK-3, and this may partially explain the different phenotypes obtained by using either type I receptor alone (Zou and Niswander, 1996) or type I and type II receptors in combination (present study).

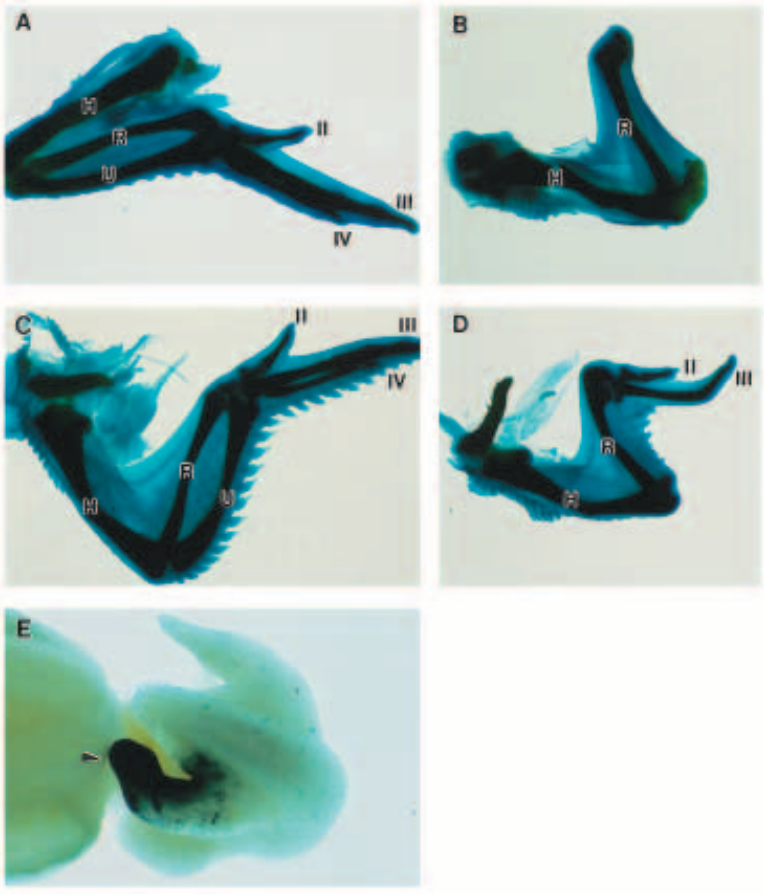

Fig. 6. Effect of dominant-negative BRKs on the wing bone pattern. (A,B) Skeletal pattern on the 6th day after final injection of both DN-BRK-2 and DN-BRK-3 viruses in the whole wing bud at stages 12 and 17. The non-injected left wing (A) is shown as a control to the treated right wing (B). Ulna, carpal, metacarpal and phalanx were absent in the right wing. (C,D) Skeletal pattern on the 7th day after implantation of DN-BRK-2 and DN-BRK-3 virus-producing cells to the distal region of the right wing bud at stage 16. The non-treated left wing (C) serves as a control to the treated right wing (D). Bone formation of ulna and autopods of digit IV was inhibited here. The phenotypes were somewhat variable probably due to uneven distribution of the virus in the wing bud, as summarized in Table 1. (E) Alkaline phosphatase staining of the limb bud at 60 hours after implantation of RCAS-PLAP virus-producing cells to the whole right leg bud. The virus spread and expressed the alkaline phosphatase gene as visualized by blue color in the treated right leg (arrowhead). Outgrowth of the right leg is slightly inhibited, but bone pattern was not changed by RCAS-PLAP. Abbreviations: H, humerus; R, radius; U, ulna; II, digit II; III, digit III; IV, digit IV.

BMPs as downstream signaling factors of Sonic hedgehog

Shh-N is likely to exert at least part of its patterning activity through BMP-2 and BMP-7 as ligands and BRK-2 and BRK-3 as receptors. Several lines of evidence suggest that BMP-2 and BMP-7 are essential downstream signals of Sonic hedgehog. There is a correlation between Sonic hedgehog and BMP for their temporal and spatial expression during embryogenesis (Bitgood and McMahon, 1995; Roberts et al., 1995). Both the ligand BMP-2/-7 and the receptor BRK-2 are positively regulated by Shh-N as shown here by the increased expression in the anterior limb mesenchyme following implantation of Shh-N-producing cells, and previously for BMP-2 by ZPA implantation (Francis et al., 1994) and Sonic hedgehog infection (Laufer et al., 1994). A similar signaling cascade is conserved in insects during appendicular development, where the posterior signal *hedgehog* patterns the developing wings and legs, acting exclusively via the induction of *dpp* in adjacent cells which, in turn, acts in a concentration-dependent manner (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Zecca et al., 1995; Nellen et al., 1996).

Signaling factor interaction

Inhibition of Wnt-7a signaling either by genetic knockout or by removing dorsal ectoderm results in defects of posterior limb bones (Parr and McMahon, 1995; Yang and Niswander, 1995). The posterior bone defects have been explained as down regulation of Sonic hedgehog that depends on Wnt-7a expressed predominantly in the dorsal ectoderm (Gavin et al., 1990; Parr et al., 1993; Dealy et al., 1993). Our results using DN-BRKs in the developing limb are somewhat similar to those obtained by Wnt-7a knockout. Because BMP-2 and BMP-7 are positively regulated by Sonic hedgehog, inhibition of Wnt-7a signaling resulted in reduction of BMP signaling through down regulation of Sonic hedgehog. We showed here that Sonic hedgehog expression in the posterior limb bud is not inhibited by DN-BRK-2 and DN-BRK-3S. Therefore, BMP is presumed to be involved in limb pattern formation as a downstream signal of Sonic hedgehog.

We thank Clifford J. Tabin, Harvard Medical School, for his critical reading of the manuscript, and Hideyo Ohuchi, University of Tokushima, for carrying out preliminary experiments with DN-BRKs in replication-incompetent retrovirus. The AMV-3C2 antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, and the Department of Biological Sciences, University of Iowa, under contract NO1-HD-2-3144 from the NICHD. This work was supported in part by Grants-in-Aids from the Japanese Ministry of Education, Science and Culture, and by a Research Project Grant from Kawasaki Medical School.

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(Accepted 4 August 1996)