

JunB Is Involved in the Inhibition of Myogenic Differentiation by Bone Morphogenetic Protein-2*

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Bone morphogenetic proteins (BMPs) constitute a family of multifunctional growth and differentiation factors structurally related to transforming growth factor- β . BMPs were first identified by their osteoinductive effects, inducing ectopic bone formation when implanted in skeletal muscle, and have an important role as regulators of skeletal development *in vivo*. *In vitro*, BMP-2 is able to transdifferentiate myogenic C2C12 cells into the osteoblastic phenotype. In this report, we show that the osteoinductive effects of BMP-2 in C2C12 cells are mediated by bone morphogenetic protein receptor type-IA in combination with both activin receptor type II and bone morphogenetic protein receptor type II. We also analyzed the expression levels of nuclear protooncogenes to understand early transcriptional events induced by BMP-2. We show that *junB* is an immediate early gene induced by BMP-2 and transforming growth factor- β . BMP-2 induces transcriptional activation of JunB expression as early as 30 min after ligand addition, reaching maximal levels after 90 min. Increase of JunB mRNA correlates with a higher AP-1 binding activity. Furthermore, ectopic overexpression of JunB is sufficient to inhibit expression of myoblast differentiation markers in C2C12 cells. These data, taken together, show the involvement of JunB in the early steps of inhibition of myogenic differentiation induced by transforming growth factor- β family members.

During mammalian development, pluripotent mesenchymal cells give rise to several specialized cell types, including cells with myogenic, osteogenic, or adipogenic potential. This differentiation process consists of at least two stages. In the first step or commitment, upon certain stimuli, undifferentiated cells become committed to a particular lineage. Later, during terminal differentiation, cells acquire specific phenotypes by expressing genes encoding a set of proteins unique to a certain type of cells. The best known example of master genes governing cell fate are the family of myogenic basic helix-loop-helix (bHLH)¹ transcription factors (1). In muscle, the bHLH factors

MyoD and Myf-5 play a role in lineage determination, whereas myogenin and MRF4 appear soon thereafter to execute the differentiation program (1–2). Other examples of such factors are the peroxisome proliferator-activated receptor γ and CCAAT/enhancer-binding protein family members, which control adipocyte differentiation or the transcription factor Cbfa1, recently found as an activator of osteoblast differentiation (3–5). The fact that ectopic expression of each these factors is sufficient to induce a unique differentiation program or even to transdifferentiate cells from other lineages emphasizes their key function (6–8).

Each specific cell commitment and differentiation program is ultimately controlled by signals and regulatory pathways that converge to activate a small number of transcription factors. These mechanisms involve cell-cell and cell-matrix interactions, as well as extracellular diffusible factors. Among the latter, bone morphogenetic proteins (BMPs) have been shown to be regulators of skeletal development (9). They were originally identified by their osteoinductive effects, inducing ectopic bone formation when exogenously implanted in rat or mouse muscle (10). Furthermore, knock-out mice showed marked skeletal defects (11–14), and they are expressed in the embryo at sites of cartilage and bone formation (15). BMP-2/4 also play a role in inductive interactions during early development, regulating dorso-ventral patterning and mesoderm induction (16). In addition to their function during morphogenesis and determination of the body axis, BMPs are also involved in later stages of development. BMPs not only stimulate maturation of osteoblast precursor cells, they also convert the differentiation pathway of myoblastic cells into the osteoblast lineage (17).

BMPs belong to the transforming growth factor- β (TGF- β) superfamily encompassing TGF- β s, activins, and Mullerian inhibiting substance. BMPs and other members of the TGF- β superfamily exert their biological function by interacting with two types of related transmembrane serine/threonine kinase receptors, known as type I and type II (18). For both TGF- β and activin, receptor activation requires binding of ligand to the type II receptor, which then recruits and phosphorylates the type I receptor (19). Phosphorylation on the GS region activates the kinase activity of the type I receptor, which propagates the signal to downstream substrates (19–20). The BMP receptor system is somewhat different. They also require heteromeric complex formation for signaling, but, unlike TGF- β or activin, BMP type I receptors are able to bind the factor without coexpression of type II receptors; however, both together achieve high affinity binding (21–23). Downstream signaling by these receptors is mediated by the recently identified family of Smad proteins (18, 24). In response to receptor activation, Smad

ase chain reaction; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium.

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¹ The abbreviations used are: bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; TGF- β , transforming growth factor- β ; BMPR-I, bone morphogenetic protein receptor type I; T β R-I, transforming growth factor- β receptor type I; ActR-II, activin receptor type II; BMPR-II, bone morphogenetic protein receptor type II; PCR, polymer-

proteins become phosphorylated at their carboxyl termini and translocate into the nucleus (25–27). Recent results support the notion that distinct Smad family members transduce signaling from specific receptor combinations. Smad1 is a BMP signal transducer, and the closely related Smad2 and Smad3 are TGF- β /activin signal transducers. Smad4/DPC4, originally found as a gene mutated in pancreatic carcinomas (28), functions as a shared partner, which hetero-oligomerizes with all other Smads upon phosphorylation (29). Once into the nucleus, Smad complexes probably exert a direct role in transcriptional control. Consistent with this function, transcriptional activity of various Smad proteins and association of Smad2 with the DNA-binding protein FAST1 have recently been shown (30).

Although the molecular mechanisms involved in BMP signaling are beginning to emerge, the precise BMP receptor combinations as well as early transcriptional events that mediate the osteogenic differentiation effects of BMP in muscular tissues have remained elusive. Here, we report that the osteoinductive response to BMP-2 in C2C12 cells is mediated by the type I receptor BMPR-IA in combination with both type II receptors BMPR-II and ActR-II. Additionally, to understand early events induced by BMP-2, we analyzed the expression level of nuclear protooncogenes. Results presented here indicate that *junB* is an immediate early gene transcriptionally induced by BMP-2. Moreover, ectopic overexpression of JunB is sufficient to inhibit expression of myogenic markers on C2C12 cells. Therefore, these data suggest the involvement of JunB in the inhibition of myogenic differentiation induced by BMP-2.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Transfections— α -Actin and α -acetylcholine receptor promoter regions fused to a CAT reporter were kindly provided by Dr. Kocieczny (Purdue University; West Lafayette, IN) (31). T β R-I^{HA} and ActR-I^{HA} vectors have been described previously (32). BMPR-IA^{HA} construct containing the hemagglutinin (HA) epitope at the carboxyl terminus was generated by PCR. A full-length JunB cDNA was subcloned into pCDNA3 for mammalian expression. A 3-kilobase mouse JunB promoter fused in a CAT reporter (JB3000CAT) was kindly provided by Dr. Nakajima (Osaka University; Osaka, Japan). C2C12 were cultured in DMEM supplemented with 20% fetal bovine serum and transiently transfected with the indicated vectors by the calcium phosphate method (33). Cell lines COS-1 and R1B/L17 were cultured and transiently transfected with the indicated vectors as described previously (32).

Immunofluorescence—C2C12 cells were grown on coverslips and treated with BMP-2 (Genetics Institute, Cambridge, MA) or TGF- β 1 (Sigma) for 3 days in DMEM supplemented with 2% inactivated horse serum. Cells were fixed in 4% paraformaldehyde and processed as described in Ref. 34, using rabbit anti-myosin antiserum as primary antibody (1/100 dilution) (Sigma) and Texas red-labeled anti-rabbit antibody as secondary antibody (Amersham Life Science Inc.).

Alkaline Phosphatase and CAT Assays—Alkaline phosphatase was assayed as follows. Cells were washed with phosphate-buffered saline and lysed in 50 mM Tris-HCl, 0.5% Nonidet P-40, pH 7.5. Cell lysates were incubated in a buffer containing 0.1 M glycylglycine, 5 mM MgCl₂, and 10 mM *p*-nitrophenyl phosphate, pH 9.5. The reaction was stopped with 0.5 M NaOH, and absorbance was measured at 405 nm. CAT activity from transiently transfected cells was measured as described in Ref. 33.

RNA Preparation and Northern Blots—Total RNA was extracted from C2C12 cells according to the Chomczynski method (33). Northern analysis was done as described in Ref. 33.

Affinity Labeling in Vivo and in Vitro—Receptor affinity labeling assays *in vivo* were performed as described in Ref. 32. For *in vitro* affinity labeling, cell extracts from COS-1 cells expressing epitope-tagged receptors were immunoprecipitated using anti-HA antibody (12CA5 Babco) and protein A-Sepharose (Pharmacia Biotech Inc.). Immunoprecipitates were washed five times in 50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, pH 7.5. After washing twice in Krebs-Ringer Hepes buffer, bound receptors were incubated with ¹²⁵I-BMP-2 for 3 h at 4 °C, followed by four washes in the same buffer. Receptors were cross-linked to bound ligand with disuccinimidyl suberate (Pierce) for

15 min, washed twice, and subjected to SDS-PAGE and autoradiography.

Immunoprecipitations—Antibodies against ActR-I, BMPR-IA, and BMPR-IB were kindly provided by Dr. P. ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden) (35). Antibodies against ActR-II were kindly provided by Dr. W. Vale (The Salk Institute, La Jolla, CA) (36). Affinity-labeled receptors from C2C12 cells were solubilized in 50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, pH 7.5. Cell extracts, clarified by centrifugation, were incubated with each antibody for 1 h at 4 °C, followed by incubation with protein A-Sepharose for another 1 h. Bound receptors were then washed five times in solubilization buffer, and subjected to SDS-PAGE and autoradiography.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared by the method of Andrews and Faller (33). Pelleted cells were resuspended in 400 μ l of cold Buffer A (10 mM Hepes-KOH, pH 7.9 at 4 °C, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) by flicking the tube. After a 10-min incubation on ice, cells were vortexed for 10 s and then centrifuged. The pellet was resuspended in 20–100 μ l of cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min, and the supernatant fraction was stored at –70 °C. The protein content was determined using the Bradford protein concentration assay (Bio-Rad) with bovine serum albumin as standard. The AP-1 oligonucleotide 5'-CGCTTGATGAGTCAGCCGAA-3' (Promega) was labeled with [γ -³²P]ATP and T4 polynucleotide kinase.

Four micrograms of the nuclear protein were diluted to a final volume of 20 μ l in a reaction mixture containing 20 mM Tris, pH 7.9, 50 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, and 1.25 μ g of poly(dI-dC); 0.2 pmol of labeled probe (4×10^4 cpm) was added after 15 min. After an additional 30-min incubation at room temperature, the reaction mixture was loaded onto a 6% polyacrylamide gel, 0.25 \times TBE, and 4% glycerol, and resolved at 20 mA for 2.5 h. Gels were dried and autoradiographed at –80 °C.

RESULTS

C2C12 myoblasts constitute an *in vitro* model system to study the ability of BMPs to alter cell lineage from the myogenic to the osteogenic phenotype. C2C12 cells differentiate into multinucleated myotubes when concentration of serum is reduced from 20% to 2%. BMP-2 inhibits myotube formation, inducing osteoblastic markers such as alkaline phosphatase activity or osteocalcin production (17). Treatment with 1 nM BMP-2 or 1 nM TGF- β completely inhibited myotube formation and myosin expression on C2C12 cells exposed to differentiation media (Fig. 1A). This BMP2-dependent inhibition of myogenic differentiation could be also seen using reporter constructs containing muscle-specific promoter regions of myogenic markers such as α -actin or α -acetylcholine receptor. Addition of 1 nM BMP-2 to cells treated with differentiation media inhibited 50–70% of their CAT reporter activity (Fig. 1B). Inhibition of reporter expression is not a general transcriptional effect of BMP-2 since expression from constitutive promoters did not show any change (data not shown). C2C12 cells express extremely low levels of alkaline phosphatase and other osteoblastic markers when cultured both in proliferative or differentiation media. Time-course studies show that, of all the TGF- β superfamily members tested, only BMP-2 is able to stimulate alkaline phosphatase expression, beginning to appear within 2 days, and increasing until day 4 (Fig. 1C). These data indicate that different members of the TGF- β superfamily have distinct biological effects on selection of C2C12 terminal differentiation.

We analyzed the expression and binding affinity of distinct activin and BMP receptors in C2C12 cells to determine which receptor combinations are involved in osteogenic BMP responses. Northern analysis indicates that C2C12 cells express BMPR-II and ActR-II as activin/BMP type II receptors and ActR-I and BMPR-IA as type I receptors (Fig. 2A). In contrast, both ActR-IIB and BMPR-IB were expressed below detection limits of either Northern or reverse transcription-PCR analysis

FIG. 1. Effects of BMP-2 and TGF- β in C2C12 differentiation. A, immunofluorescence analysis using anti-myosin antibodies of C2C12 cells cultured for 3 days in differentiation media in the absence (*left panel*), with 1 nM BMP-2 (*middle panel*), or with 1 nM TGF- β (*right panel*). B, BMP-2 blocks expression of myogenic markers. C2C12 cells were transiently transfected with α -actin CAT or α -acetylcholine receptor (*Ach R*)-CAT. 24 h after transfection, cells were incubated with differentiation media in the absence (*open bars*) or the presence of 1 nM BMP-2 (*solid bars*), and CAT activity was measured 2 days later. Results are the mean \pm S.E. of three separate experiments. C, time-course assay of alkaline phosphatase activity of C2C12 cells treated as in A. Results are the mean \pm S.E. of three experiments.

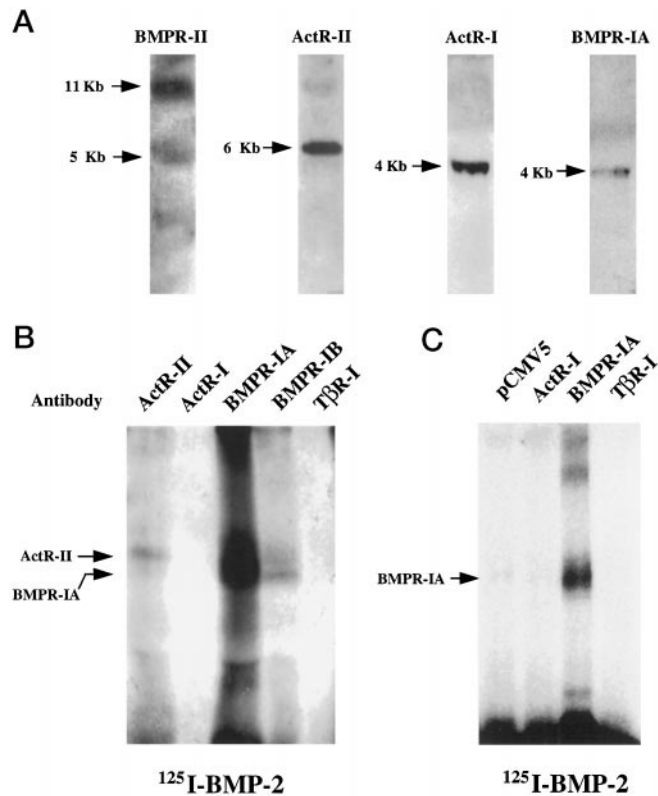
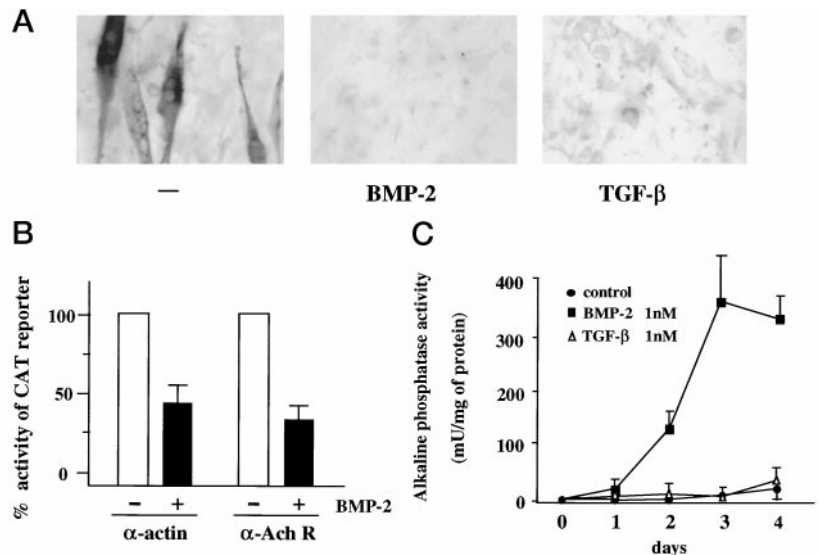


FIG. 2. Expression and binding abilities of receptors for BMP-2 in C2C12 cells. A, Northern blots of RNA (20 μ g) obtained from undifferentiated C2C12 cells were sequentially hybridized with cDNA probes for known receptors for BMPs. RNA size for each receptor is indicated by arrows. B, affinity labeling of different BMP-2 receptors expressed in C2C12. C2C12 cells were affinity-labeled *in vivo* as described under "Experimental Procedures." Equal amounts of cell lysates were immunoprecipitated with the indicated antibodies, subjected to SDS-PAGE and autoradiography. C, receptors expressed in COS-1 cells transfected with different type I receptors or with vector alone were immunopurified by sequential incubation with anti-HA antibody and protein A-Sepharose. Purified receptors were affinity-labeled *in vitro* with 125 I-BMP-2, and binding was visualized by SDS-PAGE and autoradiography.

(data not shown).

We also tested binding of BMP-2 to the endogenous receptors expressed in C2C12 cells. Immunoprecipitation of the cross-linked receptor complexes using antiserum against BMPR-IA gave a strong signal of about 75 kDa (Fig. 2B). A labeled band

could be seen in immunoprecipitates using an antiserum against ActR-II. In addition, a weak signal was detected when antiserum against BMPR-IB was used. This signal could be generated by a small number of BMPR-IB receptor mRNA that could not be detected by reverse transcription-PCR. Alternatively, since BMPR-IB antibody was raised against peptides derived from its juxtamembrane region (35), and BMPR-IA and -IB have closely related juxtamembrane regions, it is likely that the antibody against BMPR-IB cross-reacts with small amounts of BMPR-IA. Although it has been reported that ActR-I is able to bind BMP-2/4 when overexpressed in COS-1 cells (23), using the same immunoprecipitation approach, we could not detect any BMP-2 binding to ActR-I at physiological receptor levels.

Some cross-linking studies showed that when transiently transfected, type I receptors for BMP are capable of binding to BMP2/4 without coexpression of the type II receptor (21, 23). To confirm these data and exclude the possibility that endogenous type II receptor expressed in these cells could allow binding to type I receptors, we analyzed the ability of distinct purified type I receptors to bind BMP-2 *in vitro*. Epitope-tagged BMP-2 receptors expressed in COS cells were immunopurified and subjected sequentially to binding and cross-linking to 125 I-BMP-2. This binding assay showed specific binding of the ligand to BMPR-IA compared with control lanes derived from mock-transfected cells or cells transfected with transforming growth factor- β receptor type I (T β R-I), which is unable to bind BMP-2. In agreement with the *in vivo* assays, purified ActR-I was also unable to bind BMP-2 by itself (Fig. 2C). Altogether, these results suggest that the major receptor complexes involved in osteogenic responses of BMP-2 are those including BMPR-IA in combination with ActR-II and probably BMPR-II.

TGF- β has been shown to modulate expression of several nuclear protooncogenes in epithelial and fibroblastic cells (37–38). In addition, knock-out mice for transcription factors involved in AP-1 function, such as c-Fos or ATF2, exhibited profound defects in bone formation (39–40). These two lines of evidence led us to investigate the expression levels of nuclear protooncogenes to understand early transcriptional events in myogenic/osteogenic transdifferentiation processes induced by BMP-2. RNAs from C2C12 cells cultured with or without 1 nM BMP-2 in differentiation media were analyzed by Northern blotting. As shown in Fig. 3, expression of *c-jun*, *junD*, or *c-myc* was not significantly modified at any time point, either with or without ligand addition. However, 1 nM BMP-2 transiently increased JunB mRNA. Induction of JunB is detected as early

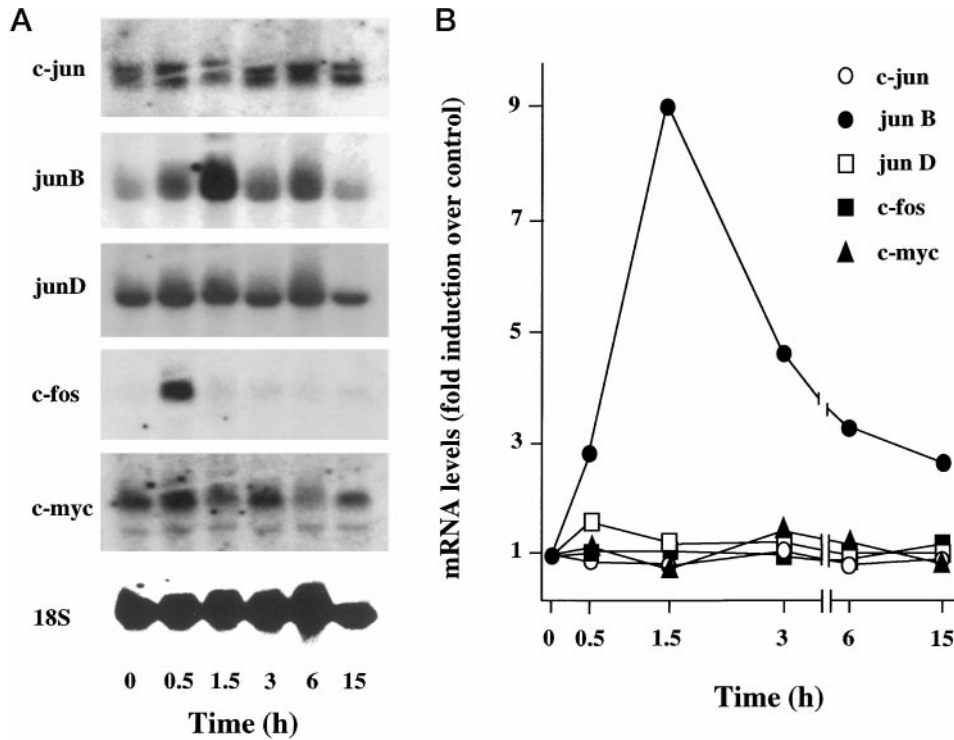


FIG. 3. Effect of BMP-2 on gene expression of nuclear protooncogenes. Cultured C2C12 cells were induced to differentiate in the presence or absence of BMP-2. Total RNA was extracted at different time points, analyzed by Northern blotting, and hybridized with the indicated cDNA probes. *A*, analysis of expression of cells treated with BMP-2 for different times. *B*, densitometric analysis of signals detected on Northern blots of cells treated with BMP-2 relative to cells treated only with differentiation media. Signals were normalized using expression of 18 S rRNA as control. To indicate the specific effects of BMP-2 on gene expression, results are expressed as -fold induction relative to cells treated for the same period of time with only differentiation media. Results are the mean of two separate experiments.

as 30 min after BMP-2 addition, reaching maximal (9-fold over control levels) levels after 90 min and decreasing thereafter. Interestingly, we also detected a sharp induction of *c-fos* transcription at 30 min, followed by a rapid decline to control levels. Nevertheless, this induction was observed both in BMP-2-treated and untreated cells, suggesting this effect was likely due to media replacement and is not a consequence of BMP-2 addition.

We next assessed whether induction of JunB is dependent on new protein synthesis. C2C12 cells were treated with BMP-2 for 90 min, together with the protein synthesis inhibitor, cycloheximide (10 μg/ml), or the RNA synthesis inhibitor, actinomycin D (1 μg/ml). Northern analysis revealed an even higher induction of JunB mRNA in the presence of cycloheximide and a complete block of induction when actinomycin D was added (Fig. 4). We also tested the effects of other members of the TGF-β superfamily on JunB mRNA levels. TGF-β at 1 nM strongly induced JunB mRNA, whereas no significant induction was detected in RNA from C2C12 cells treated with 1 nM activin A (Fig. 4).

Transient cell transfections with a JunB promoter/CAT reporter gene construct were performed to confirm whether BMP-2 and TGF-β regulate JunB mRNA steady state levels through modulation of its transcription rate. R1B/L17 clone, which is unresponsive to TGF-β due to mutations at its receptor type I (32), and C2C12 cells were transfected with a CAT construct containing a 3-kilobase proximal region of mouse JunB promoter and treated with BMP-2 or TGF-β both at 1 nM. Assay of CAT activity after 16 h of incubation indicated that both factors induced promoter activity 2–3-fold as compared with control cultures in C2C12 cells, whereas only BMP-2 was able to stimulate CAT activity in L17 cells (Fig. 5). Thus, these results indicate that the enhancement of *junB* gene expression in myogenic cells does not require protein synthesis and is

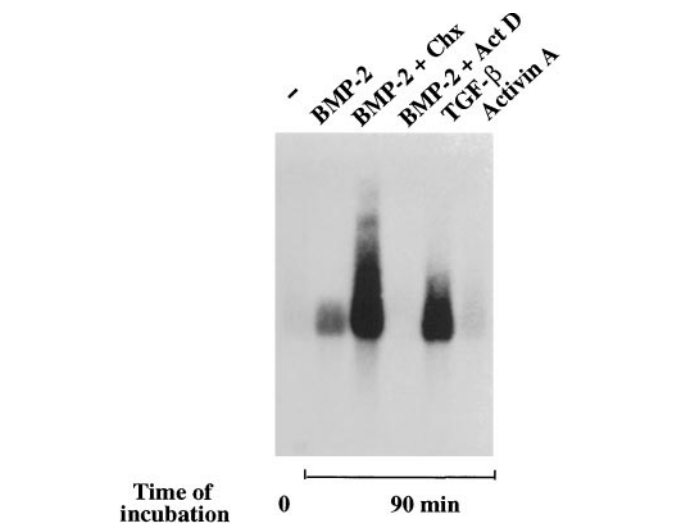


FIG. 4. Effect of different TGF-β family members on JunB transcriptional induction. C2C12 cells were treated for 90 min with 1 nM of the indicated factors, and some cultures also received cycloheximide (*Chx*) (10 μg/ml) or actinomycin-D (*Act D*) (1 μg/ml). Total RNA was extracted, and JunB expression was measured by Northern blot analysis.

mediated, at least in part, by modulation of its promoter activity.

AP-1 is a collective term referring to homo- and heterodimeric transcription factors composed of members of the Jun, Fos, or ATF families that bind to a common DNA site known as the AP-1 binding site. Distinct combinations of AP-1 factors regulate different target genes and thus execute distinct biological functions (41). We tested if higher expression levels of JunB mRNA induced by BMP-2 correlate with higher

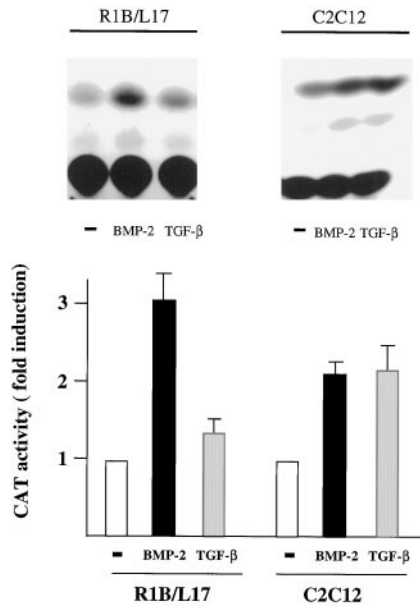


FIG. 5. Effect of BMP-2 and TGF- β on JunB promoter activity. R1B/L17 cells, deficient in TGF- β receptor type I, and C2C12 cells were transfected with JunB promoter-CAT (JB3000CAT) as described under "Experimental Procedures." Two days later, cells were incubated with the indicated factors at 1 nM in DMEM supplemented with 0.1% serum. CAT activity was assayed 16–20 h later. The upper panel shows representative results; in the lower panel, results, presented as relative promoter activity, are the means \pm S.E. of three separate transfections.

AP-1 function. Gel mobility shifts were performed with a radiolabeled AP-1 consensus oligonucleotide incubated in the presence of nuclear extracts from cells treated with or without 1 nM BMP-2 at different times. As shown in Fig. 6, we obtained a single shifted band. This band was completely competed with a 50-fold molar excess of unlabeled probe (data not shown). There was a binding increase in extracts from cells treated or not with factor for 1 h. This BMP-independent increase is likely to be due to the strong activation of c-Fos transcription, seen at 30 min in Fig. 3, since it has been described that c-Fos can be rate-limiting in the formation of heterodimers with the other AP-1 members (41). At later times, the intensity of the shifted band progressively increased in extracts from cells treated with BMP-2 in respect to their control counterparts. Preincubation of the extracts with antibody against JunB partly abrogates the binding, further suggesting the involvement of JunB in these AP-1 complexes (Fig. 6). These data suggest that induction of JunB results in formation of transcriptionally active AP-1 complexes specific for JunB-regulated genes.

Whereas both BMP-2 and TGF- β induce JunB transcription and inhibit myogenic differentiation of C2C12 cells, only BMP-2 is able to transdifferentiate these cells to the osteoblastic lineage. Thus, we analyzed the relationship between JunB expression and myogenic differentiation. We used regulatory sequences of α -actin and α -acetylcholine receptor genes fused to a CAT reporter gene. These enhancers contain binding sites for myogenin and MyoD that are strictly muscle-specific (31). Fig. 7 shows that CAT activities were induced 2–4-fold in C2C12 cells transfected with those reporters when shifted to differentiation media for 3 days. These transcriptional activations were efficiently blocked by addition of both BMP-2 or TGF- β . More interestingly, when cotransfection assays were performed with a JunB expression vector, transactivation of both reporters by differentiation media was completely suppressed. No effects were seen using SV40- β -galactosidase as reporter or when other unrelated genes were cotransfected with the CAT reporters (data not shown). Thus, we conclude

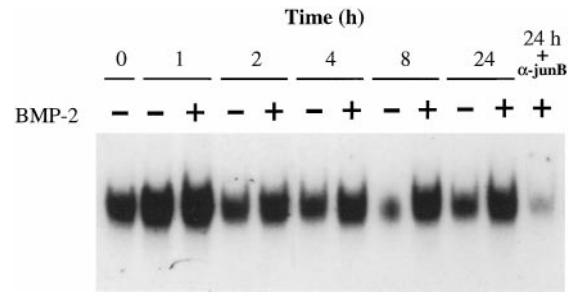


FIG. 6. AP-1 binding activity is increased in C2C12 cells treated with BMP-2. Nuclear extracts were obtained from C2C12 cells treated with or without BMP-2 in differentiation media for different times. Extracts were incubated with 32 P-labeled AP-1 consensus oligonucleotide, and, when indicated, JunB antibody (α -junB) was added. Binding mixtures were separated by nondenaturing PAGE and subjected to autoradiography.

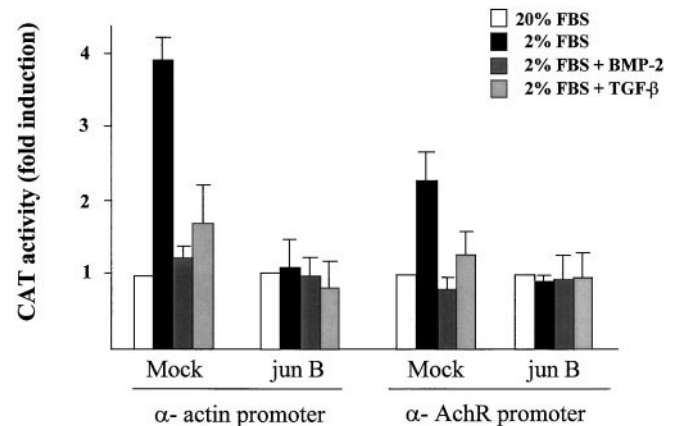


FIG. 7. JunB is sufficient to inhibit myogenic differentiation of C2C12 cells. C2C12 cells were cotransfected with myogenic reporters in combination with JunB expression vector (*jun B*) or vector alone (*Mock*). The day after transfection, medium was changed to DMEM plus 20% serum (*open bars*), DMEM plus 2% serum (*solid bars*), DMEM plus 2% serum with 1 nM BMP-2 (*dashed bars*), or DMEM plus 2% serum with 1 nM TGF- β (*gray bars*). Two days later, cells were harvested and CAT activity assayed. All values are reported as -fold induction relative to the CAT level of cells grown in proliferative media, referred as 1. Results are expressed as mean \pm S.E. of four separate transfections. FBS, fetal bovine serum; AchR, acetylcholine receptor.

that induction of JunB is sufficient and could be, at least partly, responsible of the inhibition of myogenic differentiation of C2C12 cells by BMP-2 and TGF- β .

DISCUSSION

Our results show that the ability of BMP-2 to transdifferentiate myogenic cells to the osteoblastic lineage is mediated by pathways activated by BMPR-IA. We also demonstrate that activation of these pathways lead to early transcriptional activation of *junB* gene expression with a concomitant increase in JunB-dependent AP-1 binding activity. TGF- β , another member of the TGF- β superfamily, shares with BMP-2 both the ability to inhibit myogenesis and to activate transcription of JunB. On the basis of this evidence and the observation that ectopic expression of JunB in myogenic precursor cells is sufficient to inhibit myogenic differentiation, we propose that the induction of JunB has an important role in the control of myogenic differentiation by BMP-2 and TGF- β .

During the process of ectopic bone formation, after bone-inducing factors are implanted into muscular tissues, these factors appear to alter the differentiation of muscle cells around the implantation sites (9). BMPs have been shown to be potent bone inducers *in vivo* (9–10). Although related factors failed to induce ectopic bone formation, some of them are able

to act as negative regulators of muscle cell development. In this regard, it has recently been shown that knock-out mice for GDF-8, a member of the TGF- β superfamily, have 2–3-fold increased body mass (42). In the present study, we show that the TGF- β -related factors studied differ in their transdifferentiation effects in C2C12 cells. Whereas, in agreement with Ref. 17, BMP-2 both inhibits myogenesis and activates osteogenesis, TGF- β is only capable of inhibit myogenesis (Fig. 1). The above data suggest that osteogenic effects are elicited by a unique signaling pathway initiated by BMP-2.

BMPR-IA Is the Main Receptor for BMP-2 in C2C12 Cells—BMP signaling at the plasma membrane is mediated by a subset of transmembrane serine/threonine kinase receptors (18). However, a certain redundancy in the ligand binding of the type I receptors has been reported (18, 21–23). For this reason, the assignment of precise receptor combinations that mediate each activin or BMP response remains tentative. For example, BMP-4 binds to BMPR-IA and BMPR-IB, whereas BMP-7 binds to BMPR-IB and less efficiently to BMPR-IA (21). Both BMP-2 and BMP-7 can also bind to one of the activin type I receptors, ActR-I, in the presence of a suitable receptor type II (23). In respect to type II receptors, BMPR-II has been shown to bind BMP-2, -4, and -7 and is required for signaling in combination with certain type I receptors (22–23). Additionally, BMP-7 has been shown to bind to ActR-II/ActR-I complexes, signaling certain activin-like effects (43). The present identification of ActR-II as a BMP-2 binding receptor at physiological expression levels further extends the notion of versatility in this receptor system. Our conclusion that the osteogenic responses induced by BMP-2 in C2C12 cells arise from activation of BMPR-IA rests on several lines of evidence. First, immunoprecipitation assays show that BMPR-IA is the expressed receptor on C2C12 cells (BMPR-IB is not expressed or expressed at very low levels) with higher binding affinity for BMP-2 (Fig. 2). Second, although required for signaling, its ability to bind BMP-2 is independent of the presence of a type II receptor, suggesting that in the cellular context it could combine with both BMPR-II and ActR-II acting as activating receptors. Furthermore, constitutively active BMPR-IA is sufficient to mediate osteoinductive responses in chicken wing development (44), suggesting that, as with other TGF- β superfamily receptors, signaling depends on the specificity of receptor type I kinase.

***junB* Is an Immediate Early Gene Induced by BMP-2**—Developmental studies performed *in vitro* and *in vivo* implicate the Fos and Jun family of transcription factors in the regulation of bone tissue formation (39–41). Here we show that, from all AP-1 transcription factors studied, only JunB is highly induced at early times after BMP-2 or TGF- β addition (Fig. 3). Regulation of the steady-state levels of JunB is likely to be due to regulation of its transcription rate since addition of actinomycin D completely blocks induction of its mRNA by BMP-2. Furthermore, BMP-2 and TGF- β are able to induce expression from an heterologous reporter construct. R1B/L17 cells lack functional type I receptors for TGF- β , but they have receptors for BMPs (21–22). Thus, the fact that, in these cells, only BMP-2 activated reporter expression indicates that the signal transduction elicited by BMP-2 and TGF- β are initiated by distinct subsets of receptors at the cell membrane, that at some later point, would converge in this specific JunB transcriptional response.

Since induction of the *junB* gene is not affected by the protein synthesis inhibitor cycloheximide, it would appear that transacting factors required for transcription preexist in the cell. The Smad family of proteins are the only known mediators of the TGF- β superfamily signaling. In response to receptor activation, Smad proteins become phosphorylated and translocate

into the nucleus where is likely they activate transcription by interacting FAST-like DNA-binding proteins (18, 24–27, 29–30). Time-course assays showed that BMPR-IA-dependent Smad1 phosphorylation and nuclear translocation begins 10–20 min after BMP-2 addition and remains elevated for up to 2 h (25), which is in agreement with the profile of JunB mRNA induction by BMP-2. Smad1 is expressed in C2C12 cells, and its mRNA levels do not change after BMP-2 addition (data not shown). Taken together, these data support the attractive possibility that the Smad family may be the direct mediators of this JunB transcriptional activation. If this is true, two alternative possibilities could be envisaged. One is that the JunB promoter could contain distinct response elements for BMP-2 and TGF- β ; alternatively, since phosphorylation of Smad family members by different receptors has been described to be specific (26–27), downstream signaling components could have overlapping specificities.

Ectopic Expression of *JunB* Is Sufficient to Inhibit Myogenic Differentiation—Several studies reported differences in the pattern of expression and response to stimuli between Jun family members (41). In addition, considerable differences in their transactivation and transforming activities have been reported (38, 41, 45–46); c-Jun is an effective activator of human collagenase and synthetic promoters containing a single 12-*O*-tetradecanoylphorbol-13-acetate response element, whereas JunB transactivates promoters with multiple 12-*O*-tetradecanoylphorbol-13-acetate response elements (46). In addition, c-Jun^{-/-} mouse showed embryonic lethality, further suggesting Jun family members have non-equivalent transcriptional functions (39–40). In addition, overexpression of JunB has been shown to counteract activation of c-Jun-responsive genes, suggesting a role for JunB as a negative regulator of c-Jun (45, 46). Protein interaction assays showed, in the absence of c-Fos, preferential formation of c-Jun/JunB heterodimers that have decreased DNA binding activity in respect to their homodimeric counterparts, whereas, in the presence of c-Fos, preferential formation of JunB/c-Fos heterodimers should contribute to AP-1 activity (45). Therefore, modification in the relative levels of Fos and Jun family members by extracellular factors, such as BMP-2 and TGF- β , would result in the presence of a specific subset of AP-1 dimers with altered transcriptional activity. This hypothesis has been demonstrated in the differential regulation of collagenase transcription by cell-specific induction of JunB or c-Jun and could also be the case in the C2C12 model presented here since ectopic overexpression of JunB, altering the c-Jun/JunB ratio within the cell, is sufficient to mimic inhibition of myogenic differentiation by BMP-2 and TGF- β .

Evidence points to inhibition of myogenic bHLH transcriptional activity as the key event in inhibition of myogenic differentiation by TGF- β family members. Not only do BMP-2 and TGF- β inhibit expression of myogenic factors in myoblasts (47), they also inhibit terminal differentiation of cells overexpressing myogenin without altering their DNA binding ability (47, 48). These results suggest that the myogenic activity of these transcription factors is also inhibited at posttranscriptional levels. Id-1 antagonizes myogenic factor binding to the E-boxes in muscle-specific promoters (49), and its expression is increased by BMP-2 (50). In addition, besides its own transcriptional activity, increased levels of JunB also interferes with bHLH function (51). It has been shown that MyoD directly interacts with Jun family members (52). Additionally, both the MyoD and Jun family of proteins require coactivation by p300/CBP (41, 53) and, since availability of these coactivators could be rate-limiting, a competition between distinct transcription factors could take place within the cell.

In conclusion, the present results show that *junB* is an immediate early gene induced by BMP-2 through a pathway initiated by BMPR-IA, and they also implicate JunB as a factor involved in some of the mechanisms by which TGF- β family members inhibit myogenic bHLH transcriptional activity. Further research is needed to clarify the molecular mechanisms of JunB-mediated inhibition of myogenesis and to analyze the pathways that lead to transcriptional activation of JunB expression induced by BMP-2.

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