Induction of Interleukin-6 Expression by Bone Morphogenetic Protein-6 in Macrophages Requires Both SMAD and p38 Signaling Pathways*\[5\]

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Unlike the prototype transforming growth factor-β (TGF-β), bone morphogenetic protein-6 (BMP-6) activates macrophages. Here, we report that BMP-6 induces the expression of IL-6 in macrophages. Using overexpression and knockdown experiments, we demonstrate that BMP receptor type II and activin-like kinase-2 are necessary for IL-6 induction by BMP-6. At the intracellular level, both Smad and p38 signaling pathways are required for the induction of IL-6. The cross-talk between the two pathways occurs at the level of transcription factor GATA4 and Smad 1/4. These results, taken together, demonstrate a novel BMP-6 signaling mechanism in which both the Smad and non-Smad pathways directly interact to activate the transcription of a target gene.

Bone morphogenetic proteins (BMPs) have been initially identified as factors critical for bone and cartilage formation (1). Subsequent investigations have revealed that BMPs are members of the TGF-β superfamily that regulate embryonic development as well as normal tissue homeostasis (2). To date, more than 20 subtypes of BMPs have been identified. Results of knock-out studies in mice suggest that these ligands have different functions (2). BMPs signal through a heteromeric, most likely heterotetrameric, complex of type I and type II receptors (3, 4). There are three type I (ALK-2/Act-RIA, ALK-3/BMPRIA, and ALK-6/BMP-RIB) and three type II receptors (ActRIIA, Act-RIIB, and BMP-RII) (3). The ligand binds to type I and II receptors, which then phosphorylate receptor-activated Smads (R-Smads) (Smads 1, 5, and 8) (5, 6). Subsequently, the phosphorylated R-Smads interact with the common mediator Smad (Co-Smad) (Smad 4) and translocate into the nucleus and regulate specific gene expressions. Currently, the precise role of each BMP receptor and R-Smad remains unclear.

In addition to the canonical Smad signaling pathway, BMPs can signal independent of Smads (7). For example, BMP-4 has been reported to stimulate vascular endothelial growth factor synthesis in osteoblasts via p38 (8). Similarly, BMP-7 stimulates renal epithelial cell morphogenesis through the activation of p38 that is negatively regulated by Smad 1 (9), whereas BMP-2 activates both p38 and c-Jun-NH$_2$-terminal kinase (JNK) (10). Thus, upon activation of the receptors by BMPs, both Smad and non-Smad signaling pathways can be activated simultaneously. At the present time, the precise mechanism as well as the biological consequences of the concurrent activation of Smad and non-Smad signal transduction cascades by BMPs remain largely unknown.

In the context of immune regulation, BMP-6 has been reported to suppress both B and T cells (11, 12). More recently, we have reported that BMP-6 activates macrophages (13, 14). Consistent with our previous publications, we demonstrate here that BMP-6 induces the expression of interleukin-6 (IL-6). In investigating the mechanism of IL-6 induction by BMP-6 in macrophages, we have unexpectedly uncovered a novel mechanism in which a cross-talk between the canonical Smad pathway and the noncanonical p38 pathway is required for IL-6 expression.

EXPERIMENTAL PROCEDURES

Cell Culture—RAW 264.7 and THP-1, cells were purchased from American Type Tissue Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. BMPs were purchased from R&D Systems.

Peritoneal macrophages were obtained from C57BL/6 and IL-6 KO mice (Jackson Laboratory) and cultured in 10% FBS/20% L929 culture medium supplemented DMEM. Cycloheximide and actinomycin D were used at 50 μg/ml and 1 μg/ml,
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FIGURE 1. Induction of IL-6 expression by BMP-6 in macrophages. A, murine macrophage (Mc) cell line RAW 264.7 and murine peritoneal macrophages were treated with increasing concentrations of BMP-6. RT-PCR (left panel) and immunoblot (right panel) analysis demonstrated that BMP-6 induced expression of IL-6 in macrophages in a concentration-dependent manner. B, RT-PCR (left panel) demonstrated that BMP-6 at 100 ng/ml induced expression of IL-6 mRNA within 1 h after treatment in RAW 264.7 and murine peritoneal macrophages. Immunoblot analysis (right panel) supported the results of RT-PCR. C, RAW 264.7 and murine peritoneal macrophages were treated with BMP-2, -4, -6, and -7 at 100 ng/ml, and IL-6 expression was measured by RT-PCR (top panel) and immunoblot analysis (bottom panel). Only BMP-6 induced IL-6 expression. D, RAW 264.7 and murine peritoneal macrophages were treated with cycloheximide (Cyclo) and actinomycin D (ActD) along with BMP-6 and IL-6 expression level was measured by RT-PCR. IL-6 mRNA induction by BMP-6 was blocked by actinomycin D but not by cycloheximide, suggesting that BMP-6 directly activates IL-6 promoter in macrophages. Con, control.

RESULTS

BMP-6 Induces IL-6 Expression in Macrophages—We initially treated the murine macrophage cell line RAW 264.7 with BMP-6 and performed a multiplex RT-PCR for cytokines. This revealed that BMP-6 altered the expression of multiple cytokines in RAW 264.7 cells (data not shown).
Among the cytokines induced by BMP-6, we focused on IL-6. BMP-6 induced IL-6 expression in a concentration-dependent manner in both RAW 264.7 and murine peritoneal macrophages at the RNA and protein level (Fig. 1A). A similar degree of IL-6 mRNA induction by BMP-6 was observed in the human monocytic cell line THP-1 (supplemental Fig. 1). The consistent induction of IL-6 by BMP-6 in RAW 264.7, murine peritoneal macrophages, and THP-1 demonstrates that RAW 264.7 is a reasonable model for investigating the mechanism of BMP-6-induced IL-6 expression in macrophages. Kinetic studies using 100 ng/ml BMP-6 showed IL-6 induction at the mRNA level within 1 h, whereas protein induction was seen in 1–6 h (Fig. 1B). Among BMP-2, -4, -6, and -7, only BMP-6 induced the expression of IL-6 in RAW 264.7 and murine peritoneal macrophages (Fig. 1C). This induction of IL-6 was blocked by actinomycin D but not by cycloheximide, demonstrating that BMP-6 induces IL-6 expression directly at the transcription level (Fig. 1D).

**Smad 1 Transduces BMP-6 Signaling for IL-6 Expression**—The canonical BMP signaling pathway requires R-Smads (Smad 1, 5, and 8) and Co-Smad (Smad4). When RAW 264.7 cells were treated with BMP-6, confocal immunofluorescence

A plasmid IL-6-Luc containing IL-6 promoter and luciferase reporter was transfected into RAW 264.7 cells and treated with 100 ng/ml BMP-6. Twenty-four hours after adding BMP-6, IL-6 promoter activity increased more than 3-fold. B, constitutively active ALK-2 and -3 were co-transfected with IL-6-Luc reporter. Although both ALK-2 and -3 induced IL-6 promoter activity to a statistically significant level, ALK-2 increased IL-6 promoter activity more than 3-fold. C, lentiviruses containing shRNA sequences targeting ALK-2 and -3 were infected into RAW 264.7 cells. Statistically significant knockdown of target gene expression was confirmed previously and published (13). When transfected with IL-6-Luc and treated with BMP-6, only the knockdown of ALK-2 blocked the induction of luciferase activity. D, macrophages express all three known BMP type II receptors (BMP-RII, Act-RIIA, and Act-RIIB). Thus, each of the three type II BMP receptors was co-transfected with IL-6-Luc into RAW 264.7 cells and treated with BMP-6. Only in cells transfected with BMP-RII was a significant increase in IL-6 promoter activity observed. Interestingly, overexpression of Act-RIIB suppressed IL-6 promoter activity consistently. E, lentiviruses containing shRNA sequences targeting each of the three type II BMP receptors were infected into RAW 264.7 cells. We have previously confirmed the knockdown of the target gene expression (13). Among the three type II BMP receptors, knockdown of BMP-RII reversed the induction of IL-6 promoter.*, statistically significant.

Among the type II BMP receptors, we have reported that macrophages express only ALK-2 (Act-RIA) and -3 (BMP-RIA) (13). Thus, only ALK-2 and -3 were studied. The results demonstrated that 18 h after the co-transfection of constitutively active ALK-2, luciferase activity increased more than 3.5-fold (Fig. 2B). Co-transfection of constitutively active ALK-3 also increased luciferase activity but to a more modest level. When the expression of ALK-2 and -3 was knocked down using the lentivirus-based shRNA approach (13), BMP-6-induced IL-6 expression was blocked only in cells infected with the ALK-2 shRNA lentivirus (Fig. 2C).

In contrast to type I receptors, type II BMP receptors are constitutively active serine/threonine kinases. Thus, RAW 264.7 cells were co-transfected with each of the three known BMP type II receptors along with IL-6-Luc plasmid and treated with BMP-6. As shown in Fig. 2D, cells transfected with BMP-RII exhibited the highest level of luciferase activity following treatment with BMP-6. Interestingly, co-transfection with ActRIIB repeatedly decreased the luciferase activity, suggesting that receptor stoichiometry may play a role in BMP-6 signaling. As a complementary approach, cells were infected with lentivirus containing shRNA sequence targeting each of the type II BMP receptors (13). Only BMP-RII shRNA significantly blocked the induction of luciferase activity in RAW 264.7 cells (Fig. 2E). These results suggest that ALK-2 and BMP-RII are the optimal and functional BMP-6 receptors in the context of IL-6 expression in macrophages.

**ALK-2 and BMP-RII Are the Functional BMP-6 Receptors in the Context of IL-6 Induction**—Because there are three each of type I and II BMP receptors, we next investigated the functional BMP-6 receptors in the context of IL-6 in macrophages. To this end, we obtained the previously reported luciferase reporter construct containing the 1.2-kb human IL-6 promoter (IL-6-Luc) (15). When this construct was transfected into RAW 264.7 cells, BMP-6 increased luciferase activity more than 3.5-fold in 24 h (Fig. 2A). Next, constitutively active type I BMP receptors were co-transfected with the IL-6-Luc plasmid. Among the three known type I BMP receptors, we have reported that macrophages express only ALK-2 (Act-RIA) and -3 (BMP-RIA) (13). Thus, only ALK-2 and -3 were studied. The results demonstrated that 18 h after the co-transfection of constitutively active ALK-2, luciferase activity increased more than 3.5-fold (Fig. 2B). Co-transfection of constitutively active ALK-3 also increased luciferase activity but to a more modest level. When the expression of ALK-2 and -3 was knocked down using the lentivirus-based shRNA approach (13), BMP-6-induced IL-6 expression was blocked only in cells infected with the ALK-2 shRNA lentivirus (Fig. 2C).
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To identify the BMP-6-response element within the IL-6 promoter, serial deletion constructs were made using PCR and subcloned. The results demonstrated that the BMP-6-response element is located between -50 and -150 bp 5’ to the transcription initiation site (Fig. 3E). Using the ChIP assay, the -50 to -150 bp region was amplified readily using PCR when immunoprecipitated with either Smad 1 or 4 antibodies (Fig. 3F). As a control, -150 to -300 bp region was targeted.

Activation of p38 Is Required for IL-6 Induction by BMP-6—When a software-based analysis of the 100-bp region containing the BMP-6-response element was carried out for potential transcription factor binding sites, surprisingly no consensus Smad-binding element was identified. This suggested that the non-Smad pathway may be involved in BMP-6-mediated IL-6 induction. Thus, the effect of BMP-6 on p38 was investigated. Immunoblot analysis demonstrated that BMP-6 induced the phosphorylation of p38 within 15 min in both RAW 264.7 and murine peritoneal macrophages (Fig. 4A). Immunofluorescence microscopy showed a consistent localization of p38 in the cytosol of RAW 264.7 cells following treatment with BMP-6 (Fig. 4B).

When RAW 264.7 was pretreated with the p38 inhibitor SB 203580 (10 μM) for 1 h, IL-6 mRNA expression was no longer induced by BMP-6 (Fig. 4C). Control experiments showed that SB 203580 has no significant effect of Smad phosphorylation up to 10 μM (supplemental Fig. 3). Consistent with this observation, the transfection of dominant negative p38 (p38DN) into RAW 264.7 cells also blocked IL-6 mRNA induction following BMP-6 exposure (Fig. 4D). These results suggest that p38 activation, in addition to the Smad 1, is required for IL-6 induction by BMP-6.

Because NF-κB is the classic activator of IL-6 promoter, we next studied the potential relationship between BMP-6 signaling and NF-κB in murine peritoneal macrophages using the IKK inhibitor, BAY11-7082. The results demonstrated that the inactivation of NF-κB pathway did not significantly alter IL-6 mRNA expression following exposure to BMP-6 (Fig. 4E). One potential mechanism for the observed requirement of Smad-dependent and p38 pathways is that p38 regulates the nuclear translocation of Smads following BMP-6 treatment. To test this concept, RAW 264.7 cells were again treated with the p38 inhibitor SB 203580, and confocal immunofluorescence mi-
GATA4 binds to the BMP-6-response element in IL-6 promoter (Fig. 5B). This interaction between GATA4 and the BMP-6-response element was disrupted by the p38 inhibitor SB 203580 (Fig. 5C), demonstrating that GATA4 signals downstream of p38. Next, confocal immunofluorescence microscopy demonstrated a simultaneous nuclear translocation of GATA4 and Smad 1 following BMP-6 treatment (Fig. 5D).

Based on the observation that Smad 1 is required for IL-6 promoter activation by BMP-6 (Fig. 3D), we hypothesized that GATA4 and Smad 1 may complex to activate the transcription of IL-6 in the context of BMP-6 in macrophages. To test this concept, RAW 264.7 cells were transfected with varying combinations of Smads and GATA4 along with IL-6-Luc. The results showed that the overexpression of GATA4 augmented the capacity of Smad 1/4 to induce IL-6 promoter activity (Fig. 5E). However, GATA4 alone did not increase IL-6 promoter activity. To characterize the interaction between Smad 1 and GATA4 further, myc-tagged Smad 1, 4, 5, and 8 along with FLAG-tagged GATA4 were expressed in RAW 264.7 cells. Following the transfection, immunoprecipitation against the FLAG-tagged GATA4 followed by immunoblotting for the myc-tagged Smads demonstrated that Smad 1 interacted with GATA4 (Fig. 5F). In addition, Smad 4 and 8 also complexed with GATA4. Because the observed binding among Smads and GATA4 may be an artifact of overexpression, we next wanted to assay for the interaction of endogenous molecules. However, antibodies that reliably distinguish each of the R-Smads do not exist. Thus, myc-tagged Smads were overexpressed in RAW 264.7 cells and immunoprecipitation against myc-epitope followed by immunoblotting for the endogenous GATA4 was performed. Following treatment with BMP-6, higher levels of GATA4 were immunoprecipitated out in lysates obtained from cells overexpressing Smad 1 and 4 (Fig. 5G). Interestingly, binding of Smad 8 to endogenous GATA4 did not change with the addition of BMP-6. These results along with the observation that overexpression of Smad 1/4 (but not Smad 8) led to an induction of IL-6 expression by BMP-6 (Fig. 3C) demonstrate that GATA4 and Smad 1/4 are part of the transcription machinery that drives the expression of IL-6 in response to BMP-6. To investigate interaction between endogenous Smad 1 and GATA4 further, shRNA knockdown experiments were performed. When Smad1 was knocked down, immunoprecipitation for GATA4...
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A. BMP-6 and IL-6 in Macrophages

B. BMP-6 siRNA

C. BMP-6 (100ng/ml) IP Ab

D. BMP-6 (100ng/ml) WB Ab

E. BMP-6 (100ng/ml) Con vs. GATA4

F. BMP-6 (100ng/ml) Con vs. Smad

G. BMP-6 (100ng/ml) Smad1, Smad4, Smad5, Smad8

H. IB: Smad1, IP: GATA4, IB: β-actin

I. BMP-6 (100ng/ml) shRNA

J. Macrophones

K. Type II Receptor

L. Type I Receptor

M. Non-Smad pathway

N. Smad pathway

O. IL-6
followed by immunoblotting against Smad 1 demonstrated no protein band (Fig. 5H, top panel). Conversely, when GATA4 was knocked down, immunoprecipitation for Smad 1 followed by immunoblotting against GATA4 revealed no positive protein band (Fig. 5H, bottom panel).

Based on these results, we propose a new mechanism of BMP-6 signaling in macrophages (Fig. 5I). In this model, BMP-6 induces macrophages to produce IL-6 expression via a cross-talk between the canonical Smad-dependent and the noncanonical p38 pathways. The actual interaction between the two pathways occurs through GATA4 and Smad 1/4 in the context of IL-6 expression. This view of the stoichiometry of the receptors indicates the optimal ligand-receptors interaction is consistent with the report demonstrating that knocking out the expression of BMP-RII attenuated BMP-2 and -4 signaling and augmented BMP-6 and -7 signaling (19). Additional work is necessary to verify this concept.

Despite the varying effects of each BMP subtype, the common theme in BMP signal transduction is the activation of the Smads. Specifically, the canonical BMP signaling pathway involves the R-Smads (Smad 1, 5, and 8) and the Co-Smad (Smad 4). Upon receptor activation by the ligand, the R-Smads are phosphorylated, form heteromeric complexes with Co-Smad, and translocate into the nucleus to regulate gene expression. In addition to this Smad-dependent pathway, the existence of non-Smad pathways has been reported (20). Now, the observation that both p38 and Smad pathways are required for BMP-6 signaling has revealed a novel mechanism of BMP signal transduction in which two signaling pathways interact at the transcriptional level to activate the target gene expression. In this work, we have demonstrated that GATA4, a transcription factor implicated in development and heart formation (16), and Smad 1 cooperate to activate IL-6 promoter. Because the BMP-6-responsive region in IL-6 promoter contains the consensus GATA but not the Smad-binding element, it is likely that GATA4 interacts directly with the promoter whereas Smad 1 is part of the transcription complex that drives IL-6 expression. We cannot rule out though, the possibility of direct binding between Smad 1/4 and IL-6 promoter at nonconsensus Smad-binding element sites. Regardless, this cross-talk between the Smad and p38 pathways adds another point of

This observation suggests that for a given BMP function, there is an optimal receptor. But in the absence of the optimal receptor, the remaining receptor subtype(s) may substitute partly for the missing receptor. This concept requires further investigations for confirmation.

Interestingly, the overexpression of BMP-RII increased whereas that of Act-RIIB decreased the expression of IL-6 upon stimulation with BMP-6. Because different BMP ligands have different binding affinity for each of the type I and type II receptors, it is plausible that the overexpression of Act-RIIB may, in essence, function as a competitive inhibitor of BMP-RII in the context of IL-6 expression. This view of the stoichiometry of the receptors dictates the optimal ligand-receptors interaction is consistent with the report demonstrating that knocking out the expression of BMP-RII attenuated BMP-2 and -4 signaling and augmented BMP-6 and -7 signaling (19). Additional work is necessary to verify this concept.

FIGURE 5. GATA 4 and IL-6 expression in macrophages. A, RAW 264.7 was transfected with GATA4 siRNA (siGATA4), and the effect on IL-6 expression was measured using RT-PCR. When treated with BMP-6, induction of IL-6 was no longer observed when GATA4 was knocked down. B, ChIP was carried out to determine the interaction between GATA4 and the IL-6 promoter. GATA3 was used as a control. Following treatment with BMP-6, the −50 to −150 bp region was amplified in samples immunoprecipitated with GATA4 but not GATA3 antibody. C, when RAW 264.7 cells were treated with SB 203580 and BMP-6, ChIP assay using GATA4 antibody no longer amplified the −50 to −150 bp region. This observation demonstrates that GATA4 signals downstream of p38. D, confocal immunofluorescence microscopy was carried out using antibodies against Smad 1 and GATA4. BMP-6 treatment induced nuclear translocation of Smad 1 and GATA4 simultaneously. E, RAW 264.7 was transfected with indicated combination of Smad 1, 4, 5, and 8, and BMP-6 and IL-6 in Macrophages
regulation in BMP signaling in which the host regulates the function of BMPs through the non-Smad pathway.

The observation that a cross-talk between the canonical and the noncanonical pathways is required for BMP-6 signaling may explain, in part, the multifunctional effect of BMPs. Since the initial identification of BMPs, it has been reported that these growth factors have varying effects depending on the cellular context (2). The precise mechanism for these variable effects has been difficult to explain because the activation of Smad-dependent pathway is nearly universal in cells that are responsive to BMPs. Results of the present study revealed the critical role of the noncanonical non-Smad pathway in regulating the expression of certain target genes. Because there are multiple different non-Smad signaling pathways and BMPs do not uniformly activate all the non-Smad pathways (20), the effect of BMPs may be determined by the presence/absence of the noncanonical pathways. In short, the ultimate effect of BMPs may be determined by the non-Smad pathway where the canonical Smad-dependent pathway plays a permissive role.

It should be pointed out that both Smad 4 and 8 also complexed with GATA4. The implications of this interaction are yet to be established. Overexpression of dominant negative Smad 4 completely blocked IL-6 expression. Smad 4 is the lone Co-Smad and likely plays a role in translocation of the R-Smads (Smad 1 in the case of the IL-6 promoter). More recently, nuclear translocation of R-Smads has been reported to occur independently of Co-Smad (21, 22). Once in the nucleus, Smad 4 has been reported to activate gene transcriptional activity (23). Because the nuclear translocation activity and the transcriptional function of Smad 4 cannot be uncoupled in our experimental setting, the precise biological effect of the GATA4-Smad 4 interaction is uncertain at the present time. Nevertheless, ChIP demonstrated increased binding of Smad 4 to the BMP-6-response element in the IL-6 promoter upon addition of BMP-6. Similarly, increased endogenous GATA4 was seen when immunoprecipitated against Smad 4 following stimulation with BMP-6. These results suggest that Smad 4 is also part of the transcription complex that drives IL-6 expression. With respect to Smad 8, the overexpression of Smad 8 quite surprisingly resulted in consistent suppression of IL-6 expression. In essence, Smad 8 appears to act opposite of Smad 1 with respect to the regulation of the IL-6 promoter. The precise reason for the negative effect of Smad 8 is unclear at the present time. However, ChIP assay against Smad 8 demonstrated that Smad 8 does not bind to the BMP-6-response element within the IL-6 promoter (Fig. 4F). Thus, one potential explanation is that Smad 8 may compete against Smad 1 and prevent the binding of the transcription complex to the target promoter. However, because Smad 8 did not demonstrate increased binding to GATA4 upon stimulation with BMP-6 (Fig. 5G), the possibility that the interaction between Smad 8 and GATA4 may be an artifact of the overexpression study cannot be ruled out. Currently, our laboratory is actively evaluating the interaction between GATA4 and Smad 4/8.

In conclusion, the current study demonstrates that BMP-6 induces IL-6 expression in macrophages. This induction involves a novel mechanism in which a cross-talk between p38 and Smad-dependent signaling pathways via the interaction between GATA4 and Smad 1/4 is necessary for the target gene expression. In the future, the nature of interaction between Smad 1, 4, and 8 and GATA4 as well as the upstream activator(s) of p38 in the context of BMP-6 signaling will be investigated in macrophages.

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