Bone morphogenetic proteins (BMPs) play an important role in nephrogenesis. The biologic effect and mechanism of action of these proteins in the adult kidney has not yet been studied. We investigated the effect of BMP2, a member of these growth and differentiation factors, on mitogenic signal transduction pathways induced by platelet-derived growth factor (PDGF) in glomerular mesangial cells. PDGF is a growth and survival factor for these cells in vitro and in vivo. Incubation of mesangial cells with increasing concentrations of BMP2 inhibited PDGF-induced DNA synthesis in a dose-dependent manner with maximum inhibition at 250 ng/ml. Immune complex tyrosine kinase assay of PDGF receptor β immunoprecipitates from lysates of mesangial cells treated with PDGF showed no inhibitory effect of BMP2 on PDGF receptor tyrosine phosphorylation. This indicates that the inhibition of DNA synthesis is likely due to postreceptor events. However, BMP2 significantly inhibited PDGF-stimulated mitogen-activated protein kinase (MAPK) activity that phosphorylates the Elk-1 transcription factor, a component of the ternary complex factor. Using a fusion protein-based reporter assay, we also show that BMP2 blocks PDGF-induced Elk-1-mediated transcription. Furthermore, we demonstrate that BMP2 inhibits PDGF-induced transcription of c-fos gene, a natural target of Elk-1 that normally forms a ternary complex that activates the serum response element of the c-fos gene. These data provide the first evidence that in mesangial cells, BMP2 signaling cross-talks with MAPK-based transcriptional events to inhibit PDGF-induced DNA synthesis. One target for this inhibition is the early response gene c-fos.

Bone morphogenetic proteins are a group of growth and differentiation factors that were originally identified by their ability to form ectopic bone (1, 2). Recently several members of this family have been cloned. These proteins contain seven highly conserved cysteines in the C terminus that are characteristic of the TGF-β family of proteins and thus fall into that broad superfamily (3–6). BMPs play an important role in the development of many organs, including lung, heart, teeth, gut, skin, and particularly the kidney (4). BMP7, a member of this large family, has been shown to be abundantly expressed in the ureteric bud epithelium before condensation of mesenchyme during nephrogenesis. BMP7 is also expressed during glomerulogenesis (7). BMP7 null mice die after birth because of early cessation of kidney development (8, 9). The rudimentary kidneys at birth lack glomeruli, the filtering units, likely due to lack of differentiation of metanephric mesenchymal cells (8).

High affinity binding of BMP2, another member of this family, has been identified in a large variety of tissues and cells, including the kidney (10). Similar to TGF-β, BMPs exert their effect via type I and type II transmembrane serine-threonine kinase receptors (4–6). Binding of BMPs to the receptor induces phosphorylation of the type I receptor in the intracellular GS domain (6). Smad1 and Smad5 have been identified recently as downstream targets of the BMP receptor. Upon BMP receptor activation, these cytoplasmic proteins undergo serine phosphorylation that leads to its translocation to the nucleus (11, 12). Furthermore the C-terminal region of Smad1 contains a transcriptional activation domain (11, 13).

BMPs initiate signaling that involves synergistic and antagonistic interplay of different pathways. It has been reported recently that activation of epidermal growth factor receptor, a tyrosine kinase, leads to phosphorylation of Smad1 in serine residues in a MAPK-dependent manner. This phosphorylation inhibits localization of Smad1 in the nucleus thus inhibiting its transcriptional function (14). It is known that activators of tyrosine kinase receptors, e.g. fibroblast growth factor receptor, oppose the effect of BMP2 during limb bud outgrowth (15). Similarly, epidermal growth factor receptor can block expression of osteogenic differentiation markers induced by BMP2 (16). On the contrary, fibroblast growth factor-induced genes essential for tooth development can be blocked by BMP2 (17). These data indicate that there is cross-talk between BMP receptor serine threonine kinase and its components and the receptor tyrosine kinase cascades.

Bone morphogenetic protein 2 inhibits platelet-derived growth factor-induced c-fos gene transcription and DNA synthesis in mesangial cells

IN Volvement of Mitogen-Activated Protein Kinase*

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Platelet-derived growth factor (PDGF) is a potent mitogen for many mesenchymal cells, including mesangial cells, vascular pericytes that occupy the glomerular microvascular bed (18, 19). During embryogenesis activation of PDGF receptor β (PDGFR) in multipotent mesenchymal cells is necessary for development, growth, and survival of glomerular mesangial cells (20, 21). In the adult kidney, overexpression and activation of PDGFR is associated with proliferative and fibrotic responses (22). One of the major signal transduction pathways initiated by PDGF in target cells is the activation of MAPK (23, 24). Tyrosine phosphorylation of PDGFR creates binding sites for a number of proteins characterized by the presence of about 100-amino acid residue sequence motifs known as Src homology 2 domain (19, 25). Binding of one such Src homology 2 domain-containing protein, SHP-2, to the tyrosine-phosphorylated receptor stimulates the guanine nucleotide exchange factor Son of sevenless to activate Ras, an upstream regulator of MAPK cascade (19, 24, 26). We have shown recently that PDGF stimulates PI 3-kinase which in turn regulates MAPK activity in mesangial cells. Thus there is cross-talk between these two pathways that lead to MAPK activation and cell growth (27). Inhibition of either pathway blocks PDGF-induced mesangial cell proliferation, an important biological response of these cells during glomerular pathology (22).

Here we show that recombinant BMP2 inhibits PDGF-induced DNA synthesis in mesangial cells in a dose-dependent manner. The mechanism involves BMP2 inhibition of PDGF-induced MAPK activity resulting in inhibition of Elk-1-dependent transcription of an appropriate reporter gene. In support of this mechanism, we show that BMP2 inhibits PDGF-mediated c-fos gene transcription in mesangial cells.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture materials and LipofectAMINE were obtained from Life Technologies, Inc. Nonidet P-40, phenylmethylsulfonyl fluoride and Na3VO4 were purchased from Sigma. GALA-Elik-1 fusion plasmid and the GAL-4-luciferase reporter construct were obtained from Stratagene. The dual luciferase kit was purchased from Promega. Recombinant PDGF BB was obtained from Amgen. TGF-β1 was purchased from R&D Systems. BMP2 was from the Genentech-Institute. PDGFRβ antibody was purchased from Upstate Biotechnology Inc. MAPK antibody was from Santa Cruz. [3H]Thymidine and [γ-32P]ATP were from Amersham Pharmacia Biotech.

Cell Culture—Rat glomerular mesangial cells (kindly provided by Dr. Jeff Kreisberg) or human mesangial cells were isolated and characterized as described (27, 28). The human and rat cells were used between 6th and 10th passages and 10th and 20th passages, respectively. Cells were maintained in RPMI 1640 tissue culture medium supplemented with antibiotic/antifungal solution and 17% fetal bovine serum. Cells were serum-deprived for 48 h and treated with BMP2 for 30 min before addition of PDGF. For ease of transfection of rat mesangial cells, bio-chemical studies and transfections were carried out in these cells during glomerular pathology (22).

We and others (18, 19, 34) have shown previously that PDGF is a potent mitogen for mesangial cells in culture and that TGF-β inhibits PDGF-induced DNA synthesis. BMP2 falls in the TGF-β superfamily of proteins (3, 6). To test the effect of BMP2 in mesangial cells, we first identified the receptor in these cells with total RNA as template in reverse transcriptase polymerase chain reaction using specific primers for BMP receptor type IA (data not shown). We then studied the effect of different concentrations of BMP2 on PDGF-induced DNA synthesis. As shown in Fig. 1, PDGF stimulates DNA synthesis in mesangial cells. Significant inhibition of DNA synthesis by BMP2 was observed at doses higher than 50 ng/ml. 50% attenuation of PDGF-mediated DNA synthesis was obtained with 250 ng/ml BMP2. No further inhibition was obtained even at a dose as high as 500 ng/ml of BMP2.

RESULTS

BMP2 Modulates PDGF-induced DNA Synthesis—We and others (18, 19, 34) have shown previously that PDGF is a potent mitogen for mesangial cells in culture and that TGF-β inhibits PDGF-induced DNA synthesis. BMP2 falls in the TGF-β superfamily of proteins (3, 6). To test the effect of BMP2 in mesangial cells, we first identified the receptor in these cells with total RNA as template in reverse transcriptase polymerase chain reaction using specific primers for BMP receptor type IA (data not shown). We then studied the effect of different concentrations of BMP2 on PDGF-induced DNA synthesis. As shown in Fig. 1, PDGF stimulates DNA synthesis in mesangial cells. Significant inhibition of DNA synthesis by BMP2 was observed at doses higher than 50 ng/ml. 50% attenuation of PDGF-mediated DNA synthesis was obtained with 250 ng/ml BMP2. No further inhibition was obtained even at a dose as high as 500 ng/ml of BMP2.

Effect of BMP2 on PDGFR and MAPK Activation—PDGF exerts its biological effects by stimulating the tyrosine kinase activity of its receptor. We have shown recently that inhibition of PDGFR tyrosine kinase activity blocks PDGF-induced DNA synthesis in mesangial cells (31). Thus the inhibitory effect of BMP2 on PDGF-induced DNA synthesis (Fig. 1) may result from inhibition of tyrosine kinase activity of PDGFR. To test the hypothesis, we determined MAPK activity, which is activated downstream of PDGFR. The cell lysate was immunoprecipitated with PDGFR antibody and the receptor-associated tyrosine kinase activity was measured by immune-complex kinase assay. The results show that PDGF stimulates PDGFR-associated tyrosine kinase activity (Fig. 2, compare lane 2 with lane 1), and incubation of mesangial cells with BMP2 and PDGF does not
inhibit PDGF-induced intrinsic tyrosine kinase activity of the PDGFR (Fig. 2, compare lane 4 with lane 2). These data indicate that BMP2 does not modulate PDGF function at the level of receptor autophosphorylation but may alter downstream signaling molecule(s) of the PDGFR kinase cascade.

We have shown recently that PDGF increases MAPK activity in mesangial cells. Activation of this kinase cascade is essential for many mitogens, including PDGF (23, 27). To test if the inhibitory effect of BMP2 on PDGF-induced DNA synthesis is via modulation of MAPK activity, we measured the activity of MAPK in MAPK immunoprecipitates after BMP2 and PDGF treatment of mesangial cells. BMP2 significantly inhibited MAPK activity stimulated by PDGF (Fig. 3A). Quantitation of these data revealed that BMP2 caused approximately 60% inhibition of PDGF-induced MAPK activity (Fig. 3B). These data suggest that BMP2-mediated inhibition of PDGF-induced DNA synthesis may also result from attenuation of MAPK activity.

TGF-β, another member of this family, is known to inhibit DNA synthesis in a variety of cells, including mesangial cells (34). Since we demonstrated in Fig. 3 that one of the mechanisms of BMP2-induced inhibition of DNA synthesis may involve inhibition of MAPK, we studied the effect of TGF-β on PDGF-induced MAPK activity. Mesangial cells were treated with TGF-β and PDGF. MAPK was immunoprecipitated from the cell lysate, and its activity was assayed. As shown in Fig. 4, similar to BMP2, TGF-β also inhibited PDGF-induced MAPK activity in mesangial cells. Therefore, TGF-β intercepts an early signal transduction pathway to elicit its inhibitory effect on DNA synthesis.

BMP2 Inhibits MAPK-mediated Transcription—Activation of MAPK stimulates transcription of a variety of target genes that are necessary for the mitogenic effect of growth factors (23, 24). Part of this transcriptional activation is mediated by direct phosphorylation of certain substrate transcription factor(s) by MAPK. One target protein of MAPK is the ternary complex factor Elk-1. Elk-1 belongs to a subgroup of ETS domain family of transcription factors (35). Addition of mitogens could stimulate serine phosphorylation of the C-terminal domain of Elk-1 by MAPK. This phosphorylation of Elk-1 is necessary for its ability to transactivate target genes (36, 37). To test whether the regulation of MAPK by BMP2 in mesangial cells results in Elk-1-mediated transcriptional activation, we performed cotransfection assays with an expression vector encod-
mass markers are shown in kilodaltons in the left margin. The bottom panel shows the immunoblot analysis of cell lysates with MAPK antibody.

ing the Elk-1 C-terminal transactivation domain fused to the GAL4 DNA binding domain and a reporter plasmid under the control of GAL4 DNA element. This is a sensitive assay for Elk-1 phosphorylation and activation. As expected, PDGF increases GAL4-dependent reporter gene expression in mesangial cells transfected with GAL4-Elk-1 fusion plasmid, suggesting activation of Elk-1 transcription factor (Fig. 5A). Prior exposure of mesangial cells to BMP2 inhibited PDGF-induced reporter gene expression in the Elk-1-dependent promoter. Many mitogens, including PDGF, are potent activators of c-fos mRNA expression (38, 39). Elk-1 forms a ternary complex with serum response factor and the serum response element (SRE) that is present in many genes (35). Thus SRE present in the c-fos gene promoter is a target of Elk-1 in mitogen-stimulated cells (39). Regulation of Elk-1 activity modulates the transcription of the c-fos gene. We performed transient transfection assays in mesangial cells with a reporter gene under the control of c-fos promoter that includes SRE. PDGF stimulates c-fos gene transcription (Fig. 5B). Exposure of mesangial cells to BMP2 inhibits both basal as well as PDGF-induced c-fos transcription (Fig. 5B). Collectively these data indicate that BMP2 blocks transcriptional events that result from activation of MAPK by PDGF.

Effects of BMP2 and TGF-β on Fibronectin mRNA Expression in Mesangial Cells—Increased extracellular matrix production is one of the features of mesangial proliferative glomerulonephritis that includes fibrosis (40). One major biological activity of TGF-β, besides inhibition of DNA synthesis, is stimulation of matrix production in many cells, including mesangial cells (41). In these cells, TGF-β increases the formation of collagen type I and fibronectin (42). The increase in fibronectin production in response to TGF-β is mediated at least partially at the transcriptional level (43). However, the effect of BMP2, a member of the TGF-β family, on matrix gene expression has not been studied in mesangial cells. Mesangial cells were treated with either TGF-β or BMP2 for different periods of time. Total RNAs were analyzed by Northern blotting using a cellular fibronectin cDNA probe. The data show that after both 8 and 16 h of stimulation, TGF-β increases fibronectin mRNA expression (Fig. 6, lanes 3 and 6). Unlike TGF-β, BMP2 does not increase fibronectin mRNA expression in mesangial cells. This finding may have broader implication to the potential use of BMP2 as an antimitotic agent in proliferative and fibrotic disorders.

**DISCUSSION**

These studies demonstrate that BMP2 inhibits PDGF-induced DNA synthesis in mesangial cells by blocking PDGF-stimulated MAPK activity that leads to inhibition of Elk-1-dependent transcriptional events and c-fos gene expression. BMP2 had no inhibitory effect on PDGFR activation. These data provide the first evidence that BMP2 blocks DNA synthe-
sis in mammalian cells by blocking MAPK-mediated signal transduction.

We have shown previously that PDGF-mediated signal transduction in mesangial cells is initiated in the plasma membrane by tyrosine phosphorylation of the dimerized PDGF receptor (29, 31, 32). Three major signal transduction pathways are activated by PDGF. These are phospholipase Cγ, phosphatidylinositol 3-kinase and MAPK (19, 24, 25). Present evidence indicates that each of these signaling molecules contributes to mitogenic effect of PDGF. There is evidence of cross-talk among these signaling pathways. For example, we have recently shown that inhibition of PI 3-kinase blocks PDGF-induced DNA synthesis and that PI 3-kinase is an upstream regulator of MAPK in mesangial cells (27).

BMP2 is a member of the TGF-β superfamily of proteins, although they differ from the TGF-β family in several features. They contain seven conserved cysteines instead of nine present in TGF-β. Unlike TGF-β, each BMP family member is glycosylated (3). Both TGF-β and BMPs function through distinct type I and type II receptors (5, 6). TGF-β binds to type II receptor and then recruits the type I protein to undergo oligomerization (5). In the absence of type II receptor TGF-β cannot interact with type I receptor alone. BMPs can interact with both receptors independently with low affinity; however, in the presence of both receptors, BMPs bind with a very high affinity and can signal to induce appropriate genes. We have shown previously that TGF-β inhibits PDGF-induced mesangial cell proliferation (34). It is known that TGF-β exerts its inhibitory effect by increasing the expression of p27, leading to the inhibition of cyclin-dependent kinases, a point of convergence of many mitogenic signaling pathways (44). Thus inhibition of cyclin-dependent kinases causes cells to arrest in G1 and prevents their entry into S phase. Whether BMP2 regulates any cyclin-dependent kinase inhibitors is not known. It should be emphasized that the concentration of PDGF that was used in our studies elicits maximum stimulation of DNA synthesis in mesangial cells similar to that observed with serum (data not shown). In this report, we show that 100 ng/ml BMP2, which is rather a high concentration, significantly inhibits PDGF-induced DNA synthesis in mesangial cells (Fig. 1). There is a wide variability among various cell types in threshold doses of BMP2 that result in inhibition of DNA synthesis. In a recent report, Ide et al. (45) demonstrated that BMP2 at a dose of 100 ng/ml significantly inhibited the growth of human prostate cancer cells stimulated by serum and androgen. It remains to be determined if such requirement of high concentration of BMP2 reflects number or affinity of BMP2 receptors on the target cells. Moreover, the pathophysiological relevance of these concentrations of BMP2 to concentrations achieved locally in cell or tissue microenvironment remains to be determined.

Receptor tyrosine kinases are known to be phosphorylated at serine and threonine residues by downstream signaling kinases, and this phosphorylation inactivates receptor function (46, 47). PDGF-R may also be negatively modulated by serine phosphorylation directly via BMP receptor signaling upon exposure of mesangial cells to BMP2. However, our results argue against this possibility, since BMP2 does not inhibit ligand-induced PDGF-R-associated tyrosine kinase activity (Fig. 2). As discussed earlier, attenuation of PI 3-kinase and MAPK results in inhibition of PDGF-induced DNA synthesis (27). However the inhibitory effect of BMP2 on PDGF-mediated DNA synthesis was only 50% (Fig. 1), suggesting that BMP2 does not completely block all the parallel early mitogenic signal transduction pathways. Indeed little inhibitory effect of BMP2 was observed on PDGF-induced PI 3-kinase activity (data not shown). Although BMP2 inhibits PDGF-stimulated MAPK activity significantly (Fig. 3), it also was not sufficient to completely block PDGF-induced DNA synthesis. In fact, BMP2 alone stimulates MAPK activity very weakly (e.g. Fig. 3A, compare lane 3 with lane 1 and Fig. 3B). These data indicate that MAPK is regulated in a positive manner by BMP2 alone, while in the presence of PDGF stimulation, it exerts a negative regulatory signal. The signal initiated by BMP2 in the presence of PDGF is not sufficient to completely inhibit MAPK activity induced by PDGF (Fig. 3). It should be emphasized that TGF-β also inhibits PDGF-induced MAPK activity in mesangial cells (Fig. 4). This action of TGF-β demonstrates that along with its late effects on cell cycle proteins, it also blocks early mitogenic signal transduction pathways. MAPK is primarily regulated by activation of Ras, Raf1, and MEK (23, 24). Also protein kinase Ca can stimulate MAPK activity in cells via direct phosphorylation of Raf1 (48). PDGF is known to activate all these enzymes (27, 29, 32). Which of these proteins is targeted by BMP2 or TGF-β to exert their inhibitory effect is not yet known.

Early PDGF growth signals are amplified and eventually integrate into the nucleus to initiate de novo transcription of genes and synthesis of proteins that are necessary for their biological function. There is evidence that MAPK can positively regulate activities of several transcription factors. MAPK activity is also involved in the post-transcriptional control of gene expression (23). In Drosophila, the ETS domain containing transcription factor, PointedP2, is phosphorylated by Rolled/ERKα at a single conserved threonine-proline residue that is essential for R7 photoreceptor cell differentiation (49, 50). In mammalian cells, MAPK directly phosphorylates the ETS domain transcription factors Elk-1 in at least three serine residues in the C-terminal C-box (23, 36). Mutation of the MAPK phosphorylation sites in Elk-1 reduced serum-induced transcriptional activation by a GAL4-Elk-1 fusion protein-GAL4 reporter assay (35, 51). We now provide evidence that inhibition of PDGF-induced MAPK activity by BMP2 blocks GAL4-Elk-1-mediated transcription (Fig. 5A). These data indicate that Elk-1-mediated transcription is a necessary component of the mitogenic effect of PDGF.

c-fos expression is regulated by SRE present in its promoter. The SRE has been shown to be sufficient for rapid transcriptional induction of this gene in response to many growth factors (38, 39). The serum response factor is a 64-kDa protein with DNA binding and transactivation properties. After binding to its cognate core sequence in the SRE, it recruits Elk-1 to the 5’ end of the core sequence. The recruitment of Elk-1 in the ternary complex involving SRF and DNA depends upon the phosphorylation of its C-terminal domain by MAPK (23, 35, 36). Mutation of these phosphorylation sites inhibits c-fos gene expression (51). Our data show a linear relation of MAPK activation and c-fos transcription by PDGF (Fig. 5B). Furthermore, inhibition of PDGF-induced MAPK by BMP2 inhibits c-fos gene expression (Fig. 5B). We should emphasize that treatment of mesangial cells with BMP2 also inhibits the basal transcription of the c-fos gene (Fig. 5B). These data indicate that apart from the effect of BMP2 on MAPK that leads to inhibition of PDGF-induced c-fos expression, BMP2 modulates other pathways that regulate basal transcription of this gene.

The role of BMP2 in the biology of mesangial and other kidney cells remains to be determined. TGF-β plays a major role in fibrotic disorders, including glomerulosclerosis (41). This deleterious action of TGF-β is due to its effect on matrix accumulation in glomerular cells, including mesangial cells (41). However, unlike TGF-β, BMP2 at doses that inhibit DNA synthesis does not stimulate fibronectin gene expression in mesangial cells (Fig. 6). Nakaoka et al. (52) have demonstrated recently that in vascular smooth muscle cells, TGF-β, but not...
BMP2, stimulates collagen synthesis. These findings may have important therapeutic implication in proliferative and fibrotic disorders. Mesangial cell proliferation and matrix synthesis are prominent features of inflammatory glomerular diseases. BMP2 may prove to be an effective antiproliferative agent for mesangioproliferative disorders without matrix expansion.

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