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Transcriptional regulation of connective tissue growth factor by sphingosine 1-phosphate in rat cultured mesangial cells

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Abstract Connective tissue growth factor (CTGF) is induced by transforming growth factor-β (TGF-β) via Smad activation in mesangial cells. We recently reported that sphingosine 1-phosphate (S1P) induces CTGF expression in rat cultured mesangial cells. However, the mechanism by which S1P induces CTGF expression is unknown. The present study revealed that S1P-induced CTGF expression is mediated via pertussis toxin-insensitive pathways, which are involved in the activation of small GTPases of the Rho family and protein kinase C. We also showed by luciferase reporter assays and chromatin immunoprecipitation that S1P induces CTGF expression via Smad activation as TGF-β does.

Keywords: Sphingosine 1-phosphate; Connective tissue growth factor; Mesangial cell; Smad

1. Introduction

Transforming growth factor-β (TGF-β) not only directly induces extracellular matrix (ECM) production, but also induces expression of other biologically active profibrotic mediators, such as connective tissue growth factor (CTGF)[1,2]. CTGF promotes fibronectin synthesis in mesangial cells [3,4] and mediates at least part of TGF-β-induced collagen and fibronectin synthesis in renal fibroblasts [5]. CTGF is overexpressed in a variety of fibrotic disorders such as renal fibrosis and expression levels of CTGF is well correlated with the severity and progression of renal fibrosis [6–10]. Therefore CTGF is a useful molecular marker of the fibrotic response [11] and might play an important role in the progression of renal fibrotic disorders [6,8].

Sphingosine 1-phosphate (S1P) is a polar sphingolipid metabolite which has been considered to act as an extracellular mediator and as an intracellular second messenger [12–15]. Extracellular effects of S1P are mediated through G protein-coupled receptors (GPCRs). To date, five closely related GPCRs, $S1P_{1\text{-}5}$, have been identified as high-affinity S1P receptors [16]. Each S1P receptor subtype couples to different G-proteins, thereby differentially regulating intracellular signal transduction that results in diverse biological functions. Previously, we reported that S1P-induced mesangial cell proliferation is mediated by pertussis toxin (PT)-sensitive G-proteins and extracellular signal-regulated kinase (ERK) [17].

By cDNA microarray analysis, we have recently shown that S1P induces CTGF expression in rat cultured mesangial cells [18]. However, the mechanism by which S1P promotes CTGF expression in mesangial cells is unknown. In this study, we examined the signaling pathway involving S1P-induced CTGF expression in rat cultured mesangial cells, and found that S1P induces CTGF expression by Smad binding to the CTGF promoter as TGF-β does.

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Abbreviations: ChIP, chromatin immunoprecipitation; CTGF, connective tissue growth factor; GPCR, G protein-coupled receptor; PT, pertussis toxin; S1P, sphingosine 1-phosphate; TGF-β, transforming growth factor-β

2. Materials and methods

2.1. Materials

Fatty acid-free bovine serum albumin (FAF-BSA) was obtained from Sigma. U0126, PD98059, SB203580, staurosporin, genistein, bisindolylmaleimide I (BIS), Ro-31-8220, wortmannin, cytochalasin D and latrunculin B were purchased form Calbiochem. S1P and SP600125 were from Biomol. PT was obtained from Seikagaku Corporation. Recombinant TGF-β1 was from PeproTech. Antibodies against phospho-protein kinase C (PKC) (pan) and PKCδ were obtained from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Phospho-PKC (pan) antibody detects endogenous levels of PKCα, β, δ, ε, and η isoforms only when phosphorylated at a carboxyl-terminal residue homologous to serine 660 of $PKC\beta_{II}$.

2.2. Cell culture, transfection, and plasmids

Rat cultured mesangial cell line immortalized with pSV3-Neo (American Type Culture Collection: ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum and 0.4 mg/ml G418. Mesangial cells were serum-starved for 24 h and treated with or without S1P or TGF-β1 in serum-free DMEM containing 0.4% FAF-BSA. Plasmids were transfected into mesangial cells using Lipofectamine PLUS (Invitrogen) as described previously [17]. pCMV-$G\alpha_{i2}$, pCMV-$G\alpha_{i2}$Q205L, pCMV-$G\alpha_{q}$, pCMV-$G\alpha_{q}$Q209L, pCMV-$G\alpha_{12}$Q229L, pCMV-$G\alpha_{12}$, pCMV-$G\alpha_{13}$Q226L, pCMV-$G\alpha_{13}$, pCMV-βARK1nt and pCMV-βARK1ct were kindly provided by J. Yamauchi (Nara Institute of Science and Technology).

2.3. Real-time PCR analysis

Total RNA was isolated from mesangial cells using Isogen (Nippon Gene), and subjected to polymerase chain reaction with reverse transcription (RT-PCR). Total RNA was reverse transcribed and used

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for real-time PCR analysis by using DNA Engine Opticon2 System (MJ Research) according to the manufacture's instructions [19]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control. PCR primers were as follows:
for rat CTGF:
rCTGF-F2, 5′-ccgactggaagacacatttg-3′
rCTGF-R1, 5′-ccagcctgcagaaggtattg-3′
for rat S1P1:
rS1P1-F1, 5′- agctaacctgctgttgtctg-3′
rS1P1-R2, 5′- actgatcagcagaaaggagc-3′
for rat S1P2:
rS1P2-F2, 5′- actcagccatgtacctgttc-3′
rS1P2-R1, 5′- actgcaagggagttaaggac-3′
for rat S1P3:
rS1P3-F2, 5′- tgtctccaacagtgtggttc-3′
rS1P3-R2, 5′- cagcacatcccaatcagaag-3′
for rat GAPDH:
rGAPDH-F1, 5′-tccgttgtggatctgacatg-3′
rGAPDH-R2, 5′-ggagttgctgttgaagtcac-3′

2.4. Transfection of mesangial cells with short hairpin RNA (shRNA) expression vector

DNA oligonucleotides targeting $S1P_1$, $S1P_2$, and $S1P_3$ were synthesized and inserted into the siRNA expression vector pSilencer4.0 (Ambion). The sequences for two complementary oligonucleotides with single-stranded overhangs encoding restriction enzyme sites were as follows:
S1P1 sense:
5′-GATCCCCAGAGACCATTATGTCTTTTCAAGAGAAAGACATAATGGTCTCTGGATA-3′
S1P1 antisense:
5′-AGCTTATCCAGAGACCATTATGTCTTTCTCTTGAAAAGACATAATGGTCTCTGGG-3′
S1P2 sense:
5′-GATCCGCTCTACGGCAGTGACAAATTCAAGAGATTTGTCACTGCCGTAGAGCTTA-3′
S1P2 antisense:
5′-AGCTTAAGCTCTACGGCAGTGACAAATCTCTTGAATTTGTCACTGCCGTAGAGCG-3′
S1P3 sense:
5′-GATCCGGCACCTGACCATGATCAATTCAAGAGATTGATCATGGTCAGGTGCCGCA-3′
S1P3 antisense:
5′-AGCTTGCGGCACCTGACCATGATCAATCTCTTGAATTGATCATGGTCAGGTGCCG-3′

Plasmids were transfected into mesangial cells with Lipofectamine 2000 (Invitrogen). Transfected cells were examined for $S1P_1$, $S1P_2$, and $S1P_3$ mRNA levels by real-time RT-PCR.

2.5. Western blotting

Mesangial cells were serum-starved for 24 h, and treated with S1P. After stimulation, total cell extracts were prepared and subjected to Western blotting as described previously [17].

2.6. Rho activity assay

Mesangial cells were serum-starved for 18 h and stimulated with S1P for the indicated time points. Rho activity was examined by Rho Activation Assay Kit (Upstate). Total and activated Rho in the original cell lysate was detected by Western blotting as described above.

2.7. Luciferase reporter assays

All constructs used in this study were shown in Fig. 6A. DNA fragments of rat CTGF promoter lying between −807 and +16 (designated as P1), −240 to +16 (P2), and −143 to +16 (P3) were amplified by PCR using rat genomic DNA, and were subcloned into pGL3-basic vector (Promega). A construct in which the Smad binding element was mutated to *Eco*RI site in the context of the −807 to +16 construct (M1) was also generated. Rat Smad3 cDNA was cloned from rat mesangial cells, and ligated into pIRES-neo expression vector (Clontech). Reporter plasmids were cotransfected with a Smad3 expression plasmid and pRL-TK (Renilla luciferase expression plasmid, Promega) into mesangial cells using Lipofectamine PLUS (Invitrogen) as described above. Twenty four hours after transfection, cells were serum-starved, treated with or without S1P, and luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega).

2.8. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out by ChIP Assay Kit (Upstate). In brief, mesangial cells were serum-starved for 24 h and treated with or without S1P for 0.5 h. After crosslinking by adding formaldehyde, cells were washed with phosphate-buffered saline, resuspended in SDS lysis buffer, and sonicated to shear genomic DNA. Immunoprecipitation was done by adding an antibody against Smad3 or normal rabbit IgG (Santa Cruz). After adding protein A agarose, immune complex was washed and co-precipitated DNA fragment was eluted. Before adding antibodies, we kept a portion of the diluted cell supernatant as 'input' to estimate the amount of DNA present in different samples. Recovered DNA was purified by QIAquick PCR purification kit (Qiagen) and used as a PCR template. PCR primers for ChIP assays were as follows:
rCTGFChIP1, 5′- ctcacaccggattgatcctg-3′
rCTGFChIP2, 5′- ggtgcgaagaggatacagag-3′

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate treatment effects. If the ANOVA value was significant, comparisons between the control and treatment group were performed using ANOVA followed by Dunnett's test to localize the significant difference. A P value of less than 0.05 was considered significant. All statistics were run with InStat 2.00 (GraphPad Software).

3. Results and discussion

3.1. S1P induces CTGF expression in rat cultured mesangial cells

TGF-β has been characterized as a potent inducer of CTGF in mesangial cells [1,2]. This induction of CTGF was confirmed when rat cultured mesangial cells were treated with TGF-β (5 ng/ml) (Fig. 1A). S1P showed a similar but more transient response. Maximal induction of CTGF expression by S1P was observed after 2 h stimulation (Fig. 1A). S1P-induced CTGF expression in a dose-dependent manner up to a concentration of 5 μM (Fig. 1B).

3.2. S1P-induced CTGF expression is mediated through $S1P_2$ and $S1P_3$ and PT-insensitive G proteins

In rat cultured mesangial cells, several S1P receptors (S1PRs) are expressed on the mRNA level, including $S1P_1$, $S1P_2$, and $S1P_3$ [17]. We previously showed that S1P-promoted mesangial cell proliferation via PT-sensitive G protein ($G_{i/o}$) [17]. Also, it was reported that $S1P_1$ couples to G_{13}, and both $S1P_2$ and $S1P_3$ can signal through $G_{12/13}$ and G_q [20,21]. We first examined which S1PRs are involved in S1P-induced CTGF expression. We transfected shRNA vectors targeting S1PRs into mesangial cells, and examined mRNA levels of S1PRs by RT-PCR. As shown in Fig. 2A, $S1P_1$, $S1P_2$, or $S1P_3$ mRNA expression was significantly reduced in cells transfected with each shRNA vector compared with that of cells transfected with the control vector. Then, we measured S1P-induced CTGF expression in these cells. Quantitative RT-PCR analysis indicated that reduced expression of $S1P_2$ and $S1P_3$, but not $S1P_1$, decreased the level of S1P-induced CTGF expression (Fig. 2B), suggesting that S1P induces CTGF expression mainly via $S1P_2$ and $S1P_3$ in rat cultured mesangial cells.

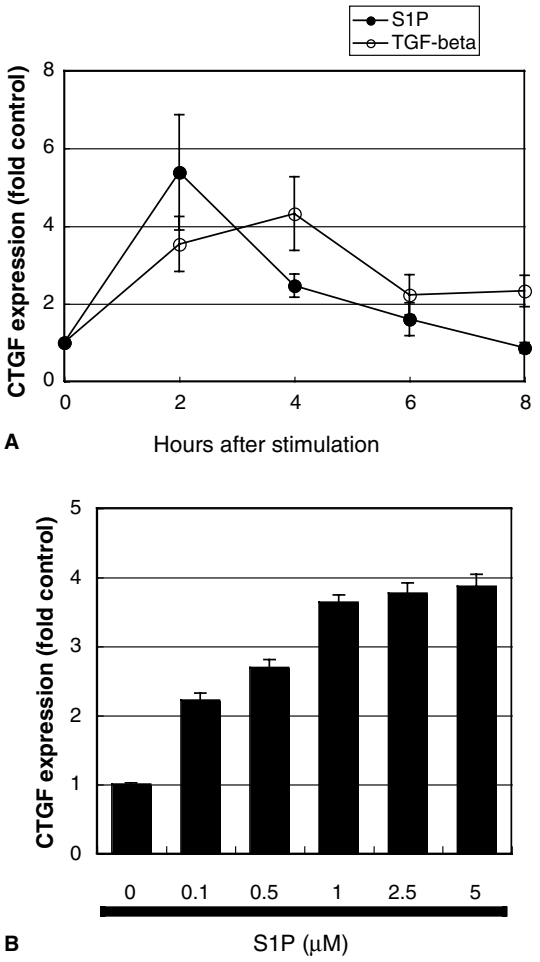

Fig. 1. Induction of CTGF expression by S1P. (A) Mesangial cells were stimulated with S1P (5 μM) and TGF-β (5 ng/ml) for the times indicated. CTGF mRNA expression was measured by real-time RT-PCR. Data show means ± S.E. of three independent experiments. (B) Mesangial cells were treated with different concentrations of S1P for 2 h. CTGF mRNA expression was measured by real-time RT-PCR. Data show means ± S.E. of three independent experiments.

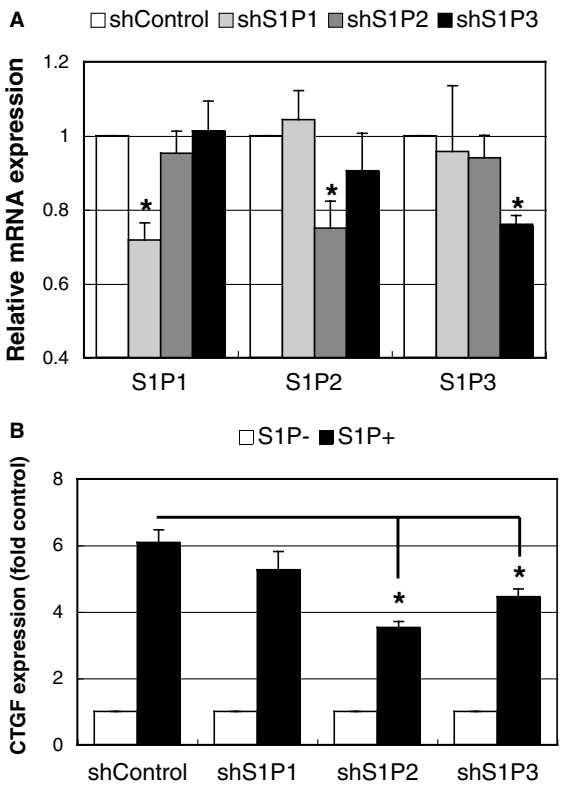

Fig. 2. S1P-induced CTGF expression is mediated through $S1P_2$ and $S1P_3$. (A) shRNA-mediated knockdown of S1PRs in mesangial cells. Plasmids were transfected into mesangial cells, and transfected cells were examined for the levels of S1PR mRNA by real-time RT-PCR. mRNA levels were expressed as a relative mRNA level compared with those of control vector-transfected cells. Data show means ± S.E. of three independent experiments. $^*P < 0.05$. (B) Mesangial cells transfected with shRNA vectors were treated with S1P (0.5 μM) for 2 h. CTGF mRNA expression was measured by real-time RT-PCR. Data show means ± S.E. of three independent experiments. $^*P < 0.05$.

We next investigated which G proteins are involved in S1P-induced CTGF expression. Treatment of rat cultured mesangial cells with PT (200 ng/ml, 24 h) did not inhibit S1P-promoted CTGF expression (Fig. 3A), although this treatment completely inhibited S1P-promoted cell proliferation (data not shown). This suggests that S1P-induced CTGF expression through PT-insensitive G proteins. At the concentrations of 1, 2.5, and 5 μM, PT treatment slightly enhanced S1P-induced CTGF expression, suggesting that PT-sensitive pathway may be negatively involved in the S1P-induced CTGF expression. We also examined whether S1P-induced CTGF expression is mediated through the $G_{q/11}$ pathway. We measured CTGF expression levels in rat cultured mesangial cells transfected with a plasmid encoding the regulator of G-protein signaling (RGS) domain of β-adrenergic receptor kinase 1 (βARK1nt), which is known to bind to $G_{q/11}$ and inhibit those cellular functions [22,23]. As shown in Fig. 3B, transfection of βARK1nt did not inhibit S1P-promoted CTGF expression in rat cultured mesangial cells. However, transfection of the C-terminus of βARK1 (βARK1ct), which is known to bind to Gβγ and inhibit those cellular functions [24,25], partially inhibited S1P-promoted CTGF expression in rat cultured mesangial cells (Fig. 3B). Furthermore, we measured CTGF expression levels in mesangial cells transfected with plasmids expressing wild-type or constitutive active forms of Gα cDNAs. As shown in Fig. 3C, transfection of $G\alpha_{12}$Q229L, a constitutive active form of $G\alpha_{12}$, significantly induced CTGF expression. Taken together, these results suggest that S1P induces CTGF expression partially through $G\alpha_{12}$ and Gβγ, but not $G\alpha_{i/o}$, $G\alpha_{q/11}$, or $G\alpha_{13}$.

3.3. Small GTPases of the Rho family and the actin cytoskeleton were critical factors for S1P-induced CTGF expression in mesangial cells

Previously it was reported that S1P mediates activation of small GTPases of the Rho family via $G_{12/13}$ [21]. In addition, the Rho family proteins were shown to be critical determinants of CTGF expression induced by lysophosphatidic acid, serotonin, and TGF-β in mesangial cells [26]. As shown in Fig. 4A, pretreatment of mesangial cells with toxin B, an inhibitor of small GTPases of the Rho family, led to significant inhibition of S1P-induced CTGF expression. In addition, we measured

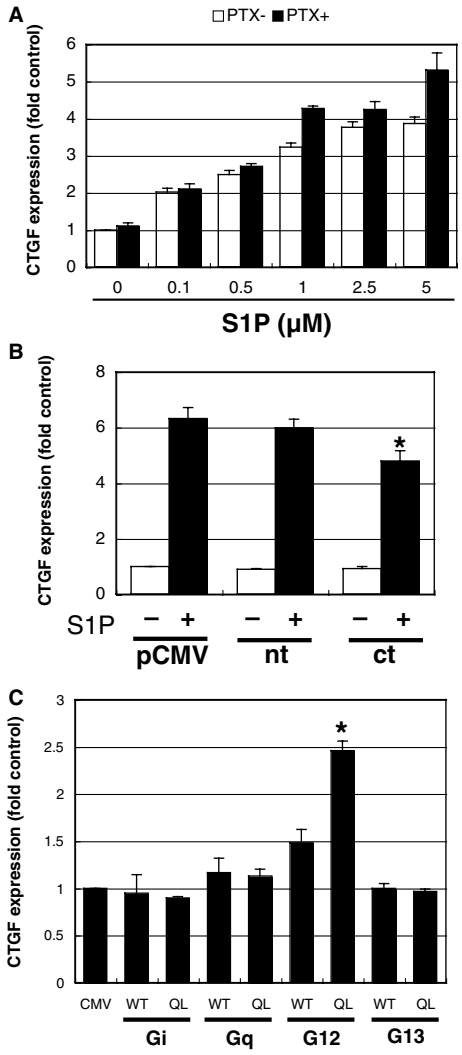

Fig. 3. S1P-induced CTGF expression is mediated through the PT-insensitive G protein pathway. (A) Effect of PT on S1P-induced CTGF expression. Cells were treated with PT (200 ng/ml) for 24 h, and treated with S1P. After 2 h of stimulation, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$. (B) Involvement of Gβγ on S1P-induced CTGF expression in mesangial cells. Mesangial cells were transfected with pCMV-βARK1nt, pCMV-βARK1ct or empty vector pCMV. After 24 h, cells were serum-starved for 24 h and treated with or without S1P (5 μM). After 2 h of stimulation, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$. (C) Effects of overexpression of Gα subunits on CTGF expression in mesangial cells. Mesangial cells were transfected with wild type (WT) or constitutive active forms (QL) of GαcDNAs or empty vector pCMV. After 24 h, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$.

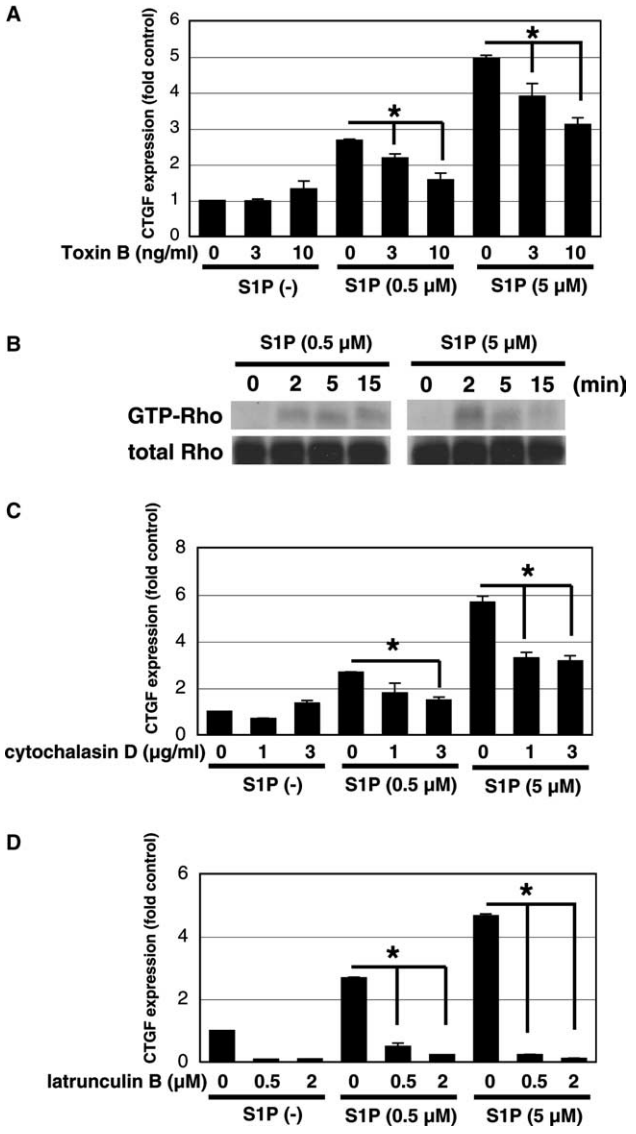

Fig. 4. Small GTPases of the Rho family and an intact cytoskeleton are required for S1P-induced CTGF expression. (A) Effect of toxin B on S1P-induced CTGF expression. Cells were treated with toxin B (3 or 10 ng/ml) for 3 h, and treated with 0.5 or 5 μM of S1P. After 2 h of stimulation, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$. (B) Rho activity assay. Mesangial cells were serum-starved for 18 h and stimulated with 0.5 or 5 μM of S1P for the indicated time points. Rho activity was examined by Rho Activation Assay Kit (Upstate). Total and activated Rho in the original cell lysate was detected by Western blotting. (C) Effect of cytochalasin D on S1P-induced CTGF expression. Cells were treated with cytochalasin D (1 or 3 μg/ml) for 3 h, and treated with 0.5 or 5 μM of S1P. After 2 h of stimulation, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$. (D) Effect of latrunculin B on S1P-induced CTGF expression. Cells were treated with lactrunculin B (0.5 or 2 μM) for 3 h, and treated with 0.5 or 5 μM of S1P. After 2 h of stimulation, total RNA were prepared and subjected to real-time RT-PCR analysis. Data show means ± S.E. of three independent experiments. $^{*}P < 0.05$.

S1P-induced activation of Rho in mesangial cells using a GST-rhotekin pull-down assay. Following stimulation with S1P, a rapid increase in the GTP-bound form of Rho was detected in mesangial cells (Fig. 4B), suggesting that S1P-induced CTGF expression is mediated via small GTPases of the Rho family. Inhibition of Rho family proteins affects the actin cytoskeleton [26]. To assess the role of the actin cytoskeleton in CTGF induction, we examined the effects of cytochalasin D and latrunculin B, toxins that disrupt the actin cytoskeleton [26,27]. As shown in Fig. 4B and C, pretreatment of the cells with these toxins dramatically decreased S1P-induced CTGF expression, suggesting that an intact actin cytoskeleton is a

critical factor for S1P-induced CTGF expression in mesangial cells. Taken together, small GTPases of the Rho family and the actin cytoskeleton play important roles in S1P-induced CTGF expression in mesangial cells.

3.4. Protein kinase C involved S1P-promoted CTGF induction

To delineate the signaling pathways involved in S1P-induced CTGF expression in more detail, we examined the effects of specific inhibitors for various protein kinases: U0126 and PD98059, ERK kinase inhibitors; SB203580, a p38 mitogen-activated protein kinase inhibitor; SP600125, a c-Jun NH_2-terminal kinase inhibitor; bisindolylmaleimide I, staurosporine, and Ro-31-8220, PKC inhibitors; genistein, a tyrosine kinase inhibitor; wortmannin, a phosphatidylinositol 3-kinase inhibitor. We measured mRNA expression levels of CTGF in S1P-stimulated mesangial cells with or without pretreatment of these inhibitors. As shown in Fig. 5A, pretreatment with three PKC inhibitors, bisindolylmaleimide I, staurosporine, and Ro-31-8220, significantly reduced S1P-induced CTGF expression. In addition, Western blot analysis using phospho-PKC (pan) antibody showed that S1P rapidly induced PKC phosphorylation in mesangial cells (Fig. 5B). These results suggest that PKC involves S1P-induced CTGF induction in mesangial cells.

3.5. A Smad binding element in the CTGF promoter region is required for S1P-promoted CTGF induction

To determine if the ability of S1P to induce CTGF expression in mesangial cells was due to elevated CTGF promoter activity, mesangial cells were transfected with luciferase reporter plasmids containing CTGF promoter elements (Fig. 6A). The fragment containing a Smad binding element had previously been shown to be responsive to TGF-β in fibroblasts and mesangial cells [1,28]. As shown in Fig. 6B, S1P-stimulation resulted in an induction of CTGF promoter activity. To further elucidate the elements in the CTGF promoter necessary for S1P-induced CTGF expression, various deleted and mutated constructs of the CTGF promoter (Fig. 6A) were trasnfected into mesangial cells and assayed for reporter gene expression (Fig. 6B). Either the deletion or mutation of the Smad binding element in the CTGF promoter completely abolished the ability of S1P to induce CTGF promoter activity in mesangial cells (Fig. 6B). This suggests that the Smad recognition sequence in the CTGF promoter is necessary for S1P-induced CTGF expression (Fig. 6B).

Also, we examined by ChIP assay whether Smads bind to the CTGF promoter region. An antibody against Smad3 was used in ChIP experiments because CTGF induction by TGF-β does not occur in Smad3 knockout fibroblasts [28] and S1P stimulation promotes Smad3 activation (phosphorylation) in mesangial cells [29]. As shown in Fig. 6C, Smad3 actually bound to a putative Smad binding element in the CTGF promoter when mesangial cells were stimulated with S1P. These results strongly suggest that S1P-induced CTGF expression in mesangial cells is mediated by Smad3 binding to the CTGF promoter region.

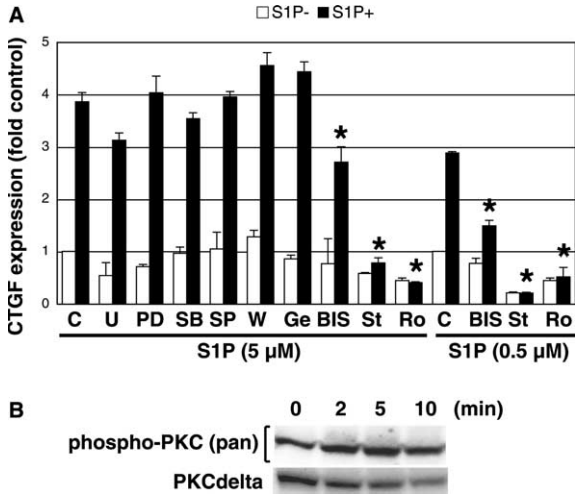

Fig. 5. Effects of protein kinase inhibitors on S1P-induced CTGF expression. (A) Effects of protein kinase inhibitors on S1P-induced CTGF expression. Serum-starved mesangial cells were preincubated for 0.5 h with the following inhibitors: U0126 (U: 10 μM), PD98059 (PD: 30 μM), SB203580 (SB: 10 μM), SP600125 (SP: 10 μM), wortmannin (W: 500 nM), genistatin (Ge: 10 μM), bisindolylmaleimide I (BIS: 10 μM), staurosporine (St: 1 μM), and Ro-31-8220 (Ro: 10 μM). Cells were then stimulated with or without S1P (0.5 or 5 μM) for 2 h, and CTGF expression was measured by quantitative RT-PCR. Data show means ± S.E. of three independent experiments. $^*P < 0.05$. (B) PKC activation. Mesangial cells were starved for 18 h and stimulated with 0.5 μM of S1P for the indicated time points. Total (PKCδ) and phosphorylated PKC (pan) in the cell lysate was detected by Western blotting.

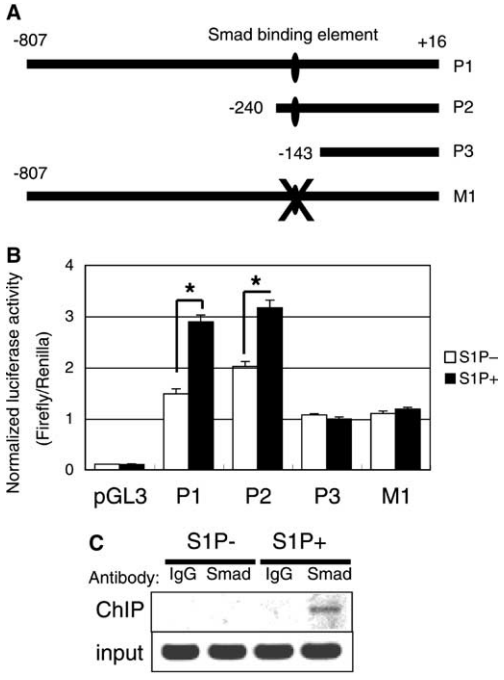

Fig. 6. Involvement of Smads on S1P-induced CTGF expression (A) Schematic diagram of promoter constructs used. (B) *Reporter assay*. After 24 h of transfection with plasmids, cells were serum-starved, and treated with or without S1P (5 μM). After 5 h, luciferase activity was measured. $^*P < 0.05$. (C) *ChIP assay*. Mesangial cells were serum-starved for 24 h and treated with or without S1P for 0.5 h. ChIP assay was carried out as described in Section 2. The lower panel indicates PCR reactions with aliquots of samples before immunoprecipitation (inputs).

4. Conclusion

We previously showed that S1P induces mesangial cell proliferation through cell-surface S1P receptors coupled with PT-sensitive G protein(s) [17]. Also, we showed by cDNA microarray analysis that S1P promotes CTGF expression in rat cultured mesangial cells [18]. The present study revealed that S1P-induced CTGF expression is mediated via PT-insensitive pathways, which are involved in the activation of small GTPases of the Rho family, PKC, and Smad. We previously reported that S1P receptors were markedly upregulated in the kidney of mice with immunoglobulin A nephropathy (IgAN) [30]. Taken together with the observations that CTGF is often overexpressed in a variety of fibrotic disorders such as renal fibrosis and enhances ECM production in mesangial cells [6–10], it is tempting to speculate that an enhanced activation of S1P–S1P receptor signaling pathways may promote not only mesangial cell proliferation but also CTGF-mediated ECM production, which results in the enhanced progression of renal fibrotic disorders. Very recently, Xin et al. [29] reported that S1P trans-activates the TGF-β receptor and triggers activation of Smads in mesangial cells. It is well known that TGF-β is a potent inducer of CTGF in mesangial cells and this induction is mediated through the Smad pathways [1,28]. These suggest that S1P and TGF-β may cooperate in the enhanced CTGF expression via Smad pathways, leading to the progression of fibrotic renal disorders, such as IgAN [30].

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