Modulation of transforming growth factor-β (TGF-β) signaling by endogenous sphingolipid mediators

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Abbreviations: TGF-β, transforming growth factor-β; S1P, sphingosine 1-phosphate; SPHK1, sphingosine kinase; TβRI, TGF-β type I receptor; ERK, extracellular signal-regulated kinase; COL1A1, collagen α1(I); COL1A2, collagen α2(I)
ABSTRACT

Transforming growth factor-β (TGF-β) is a multifunctional growth factor that plays a critical role in tissue repair and fibrosis. Sphingolipid signaling has been shown to regulate a variety of cellular processes and has been implicated in collagen gene regulation. The present study was undertaken to determine whether endogenous sphingolipids are involved in the TGF-β signaling pathway. TGF-β treatment induced endogenous ceramide levels in a time-dependant manner within 5-15 min of cell stimulation. Using human fibroblasts transfected with a α2(I) collagen promoter/reporter gene construct (COL1A2), C₆-ceramide (10 µM) exerted a stimulatory effect on basal and TGF-β-induced activity of this promoter. Next, we employed ectopic expression of enzymes involved in sphingolipid metabolism in order to define the effects of endogenous sphingolipids on TGF-β signaling. Sphingosine 1-phosphate phosphatase (YSR2) stimulated basal COL1A2 promoter activity and cooperated with TGF-β in activation of this promoter. Furthermore, overexpression of YSR2 resulted in the pronounced increase of COL1A1 and COL1A2 mRNA levels. Conversely, overexpression of sphingosine kinase (SPHK1) inhibited basal and TGF-β-stimulated COL1A2 promoter activity. These results suggest that endogenous ceramide, but not sphingosine or sphingosine 1-phosphate, is a positive regulator of collagen gene expression. Mechanistically, we demonstrate that Smad3 is a target of YSR2. TGF-β-induced Smad3 phosphorylation was elevated in the presence of YSR2. Cotransfection of YSR2 with wild-type Smad3, but not with the phosphorylation-deficient mutant of Smad3 (Smad3A) resulted in a dramatic increase of the COL1A2 promoter activity. In conclusion, this study demonstrates a direct role for the endogenous sphingolipid mediators in regulating the TGF-β signaling pathway.
INTRODUCTION

Transforming growth factor-β (TGF-β) is a member of a large family of growth factors with diverse functions in embryonic and adult tissues (1). TGF-β plays a critical role in regulating immune cell function, epithelial cell growth, and extracellular matrix deposition (2). Abnormal TGF-β signaling has been implicated in the pathogenesis of a number of diseases including cancer and fibrosis (3). TGF-β signals through a heteromeric receptor complex of type I and type II receptor serine-threonine kinases. Intracellular signal transducers termed Smads transmit signal from the receptor to the nucleus. The members of the Smad family have been divided into three functional subgroups: receptor-regulated Smads (R-Smads), the common mediator Smads (co-Smads), and inhibitory Smads (I-Smads). R-Smads (Smad2 or 3) are phosphorylated on a conserved SSXS motif by the activated type I receptor leading to their dissociation from the receptor. Subsequently, they form heterocomplexes with co-Smads, and upon translocation into the nucleus contribute to transcriptional regulation of the target genes (1). A Smad interacting membrane-bound protein SARA (Smad anchor for receptor activation) facilitates binding of the Smad2/3 to the activated type I receptor (4). Recent studies suggest that interaction of SARA with Smads occurs in early endosomes (5,6). Another endosomal FYVE domain protein, Hgs, has also been shown to cooperate with SARA in recruiting Smad2 and Smad3 to the activin receptor complex (7). Interestingly, translocation of Smads to the nucleus can be blocked by phosphorylation of the linker region of R-Smads by Ras/extracellular signal-regulated kinases (ERKs) (8), suggesting that Smads can be directly targeted by other signaling pathways. There is also increasing evidence for cross-talk between TGF-β and other signaling pathways (9).

Sphingolipids, in addition to their role as structural molecules of the plasma membrane, are now recognized as important bioactive mediators of a variety of cellular processes (10-12). In particular, biologic functions of ceramide, sphingosine, and their phosphates have been extensively studied. Intracellular ceramide is primarily generated via hydrolysis of sphingomyelin by a family of sphingomyelinases (SMases). In addition, ceramide can be formed from sphingosine by ceramide synthase. Sphingosine can be further converted to sphingosine 1-phosphate (S1P) by the action of sphingosine kinase (SPHK1). On the other hand, S1P phosphatase dephosphorylates S1P back to sphingosine (Fig. 1). The yeast S1P phosphatase, YSR2 (yeast sphingosine resistance gene), which has a high degree of specificity, has recently been cloned and characterized (13). It has been shown that overexpression of YSR2 in yeast increased ceramide formation. Conversely, deletion of YSR2 decreased ceramide levels (14). The biosynthetic pathways of sphingoid bases, their phosphates, and ceramide are conserved for the most part from yeast to mammals. It was
shown that mammalian S1P phosphatase was able to substitute for YSR2 when expressed in mutant yeast lacking YSR2 function (15). Thus, it has been proposed that S1P phosphatase may play an important role in altering the balance between ceramide and intracellular S1P. Significantly, ceramide and S1P appear to have antagonistic functions particularly in the regulation of cell growth. Ceramide, which is induced by inflammatory cytokines, various damaging agents, and stress signals, seems to be mainly associated with cell growth arrest and apoptosis, while intracellular S1P has been shown to promote cell proliferation (10,11). It is important to note that the effects of ceramide and S1P on cell growth and other cellular functions are cell type specific.

Previous studies have suggested that ceramide may also be involved in regulation of collagen metabolism. It was shown that C₂-ceramide and sphingomyelinase inhibit collagen gene expression in hepatic stellate cells and dermal fibroblasts (16,17). Furthermore, C₂-ceramide suppressed α2(I) collagen (COL1A2) promoter activity (16). Because elevated collagen production is associated with fibrotic diseases and TGF-β is a primary inducer of collagen, this study was undertaken to examine the possible role of sphingolipids in TGF-β regulation of collagen production. Using exogenous S1P and C₆-ceramide and enzymatic manipulation of endogenous sphingolipids, we demonstrate that intracellular sphingolipids modulate the TGF-β signaling pathway. Furthermore, we provide evidence that the effects of sphingolipids are Smad3-dependent. This study establishes for the first time direct functional interaction between TGF-β and sphingolipid signaling pathways.

MATERIAL AND METHODS

**Cell Cultures**

Foreskin fibroblast cultures were established from newborn foreskins obtained from delivery suites of local hospitals. Tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma, Saint Louis, MO) and 0.05% DNase (Sigma) in DMEM with 10% fetal bovine serum (FBS). COS, Mv1Lu, and HEK 293 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

**Plasmid Constructs**

The COL1A2-luciferase construct contains sequences from -353 to +58 bp of the human COL1A2 promoter (18) fused to the luciferase reporter gene (pGL2 basic, Promega, Madison, WI). Cloning of YSR2 has been described previously (13,14). The ORF of YSR2
was recloned into KpnI and EcoR1 sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). The EGFP coding sequence was amplified from pEGFP-N1 (Clontech, Palo Alto, CA) and inserted into HindIII and KpnI sites upstream of YSR2. p3TP-Lux was a gift from J. Massagué (Memorial Sloan-Kettering Cancer Center, NY). The expression construct for the constitutively active form of TGF-β type I receptor (HA-TβRI(TD)) was provided by K. Miyazono (University of Tokyo). Expression vectors for Smad3 and Smad3A, which carries three carboxy-terminal serine-to-alanine substitutions, were gifts from H. Lodish (Whitehead Institute, MA). SPHK1a was subcloned as described by Olivera et al. (19) into pcDNA3.1 vector.

**Adenoviral constructs**

Adenoviral vectors expressing YSR2 and green fluorescent protein (GFP) were generated using a published protocol (20). Briefly, the cDNA encoding YSR2 was cloned into the shuttle vector pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate CMV promoter. The shuttle vector containing YSR2 was cotransformed into E. coli BJ5183 cells with the AdEasy-1 adenoviral backbone plasmid, which lacks the E1 and E3 regions of the adenoviral genome. Linearized recombinant plasmid DNA was transfected into 293 cells, an adenoviral packaging cell line, using the FuGENE 6 transfection reagent (Roche applied Science, Indianapolis, IN) to generate the recombinant adenovirus expressing YSR2 and GFP from separate CMV promoters (AdYSR2). An adenovirus expressing GFP alone (AdGFP) was generated via the same method for use as a control vector.

**Transient Transfection and Luciferase Assay**

For transient transfection, 60-80% confluent cells in 6-well plates were transfected using FuGENE 6 transfection reagents (Roche, Indianapolis, IN) following manufacturer's recommendations. After transfection, cells were incubated for 20 h and stimulated where indicated for additional 24 h with TGF-β (R&D, Minneapolis, MN), C₆-ceramide, S1P, or sphingosine (synthesized as described by (21)). Luciferase activities in the cell lysates were measured with a dual luciferase reporter assay system (Promega, Madison, WI).

**RNA preparation and northern blot analysis**

Human fibroblasts (5x10⁴) were plated in 10%FCS/DMEM in 6-well plates. Next day, cells were transfected using FuGENE 6 reagent with either empty vector or YSR2. 20 h after transfection, the number of GFP-positive cells was visually determined using a Zeiss Axiovert 35 microscope. Efficiency of transfection in dermal fibroblasts varied from 5 to 15%. Experiments in which efficiency of transfection was below 10% were not continued.
Medium was changed to 1%FBS/DMEM and TGF-β1 (5 ng/ml) was added for additional 24 h. Total RNA was extracted, and analyzed by Northern blot as described previously (22). Filters were sequentially hybridized with radioactive probes for COL1A1, COL1A2, and 18S ribosomal RNA. The filters were scanned with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation and Immunoblotting**

For determination of phosphorylation levels of ectopically expressed Smad3, cell lysates were subjected to immunoprecipitation with the anti-Flag antibody followed by adsorption to protein G sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). Precipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting with the anti-phosphoserine antibody (Zymed, South San Francisco, CA), the membranes were subjected to re-blotting with anti-Flag (M2, Sigma) antibody to confirm levels of expression of the Flag-tagged Smad3 protein. To determine levels of expression of the receptors, aliquots of cell lysates were subjected to SDS-PAGE and immunoblotting with anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA). To determine the kinetics of phosphorylation of endogenous Smad3, Mv1Lu cells or dermal fibroblasts were treated with TGF-β1 (2.5 ng/ml) for various time intervals. Cell lysates were subjected to immunoprecipitation with the anti-Smad2/3 antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) followed by adsorption to protein G sepharose. Western blotting was performed using an anti-phosphoserine antibody. As loading control, membranes were subjected to re-blotting using an antibody against Smad3 (FL-425, Santa Cruz Biotechnology).

**Assay of Sphingosine Kinase Activity**

Foreskin fibroblasts were transiently transfected with either SPHK1a or empty vector. After a 24 h incubation in DMEM/1%FBS, cells were harvested and lysed in buffer containing 20 mM Tris, pH 7.4, 20% glycerol, 1mM β-mercaptoethanol, 1mM EDTA, 1mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 µg/ml leupeptin, aprotinin and soybean trypsin inhibitor. Sphingosine kinase activity was determined in the presence of 50 µM sphingosine, dissolved in 5% Triton X-100 (final concentration 0.25%), and [32P]ATP (10 µCi, 1 mM) containing MgCl2 (10 mM) as described (23). The labeled S1P was identified by thin layer chromatography with 1-butanol/ethanol/acetic acid/water (80:20:10:20) and visualized by autoradiography.

**Assay of S1P phosphatase activity**
Phosphatase activity was measured as previously described (15). Briefly, HEK 293 cells were transfected with either YSR2 or empty vector. Forty-eight hours after transfection, cells were lysed in buffer A [100 mM Hepes (pH 7.5) containing 10 mM EDTA, 1 mM DTT, and 10 µg/ml each leupeptin, aprotinin, and soybean trypsin inhibitor], and cells were scraped on ice. Cells were subsequently freeze-thawed seven times, then centrifuged at 100,000 g for 1 h. Supernatants were removed, and the membrane fractions were resuspended in 200 µl of buffer A and homogenized. \(^{32}\)P-labeled S1P was prepared by \textit{in vitro} phosphorylation of sphingosine using cell lysates obtained from 293 cells overexpressing SPHK1. Alternatively, D-erythro-[4,5-\textsuperscript{3}H]dihydrosphingosine-1-phosphate (ARC, Inc.) was used as a substrate. Similar results were obtained with both substrates. S1P phosphatase activity was measured by adding \(^{32}\)P-labeled S1P to 20 µg of membrane preparations in 200 µl of buffer A and incubated for 30 min at 30°C. The assay was terminated by adding 20 µl of 1N HCl, and lipids extracted with 0.8 ml of chloroform/methanol/HCl (100:200:1). To separate phases, 0.24 ml of chloroform and 0.24 ml of 2M KCl were added. After centrifugation, 100 µl of the aqueous layer was counted by liquid scintillation. Remaining \(^{32}\)P-labeled S1P was extracted and analyzed by TLC.

\textit{Measurement of cellular ceramide}

Mv1Lu cells or dermal fibroblasts were grown to near confluence, and culture medium was changed to serum-free DMEM for 24h, followed by the stimulation with TGF-\(\beta\)1 (2.5 ng/ml) for various time intervals. Lipids were then extracted with chloroform/methanol/water and processed for determination of ceramide as described (24).

\textbf{RESULTS}

\textit{TGF-\(\beta\) stimulates endogenous ceramide levels}

Modulation of ceramide levels by a variety of stimuli have been observed in many cell types; however, only limited information is currently available regarding TGF-\(\beta\) regulation of ceramide (25). To examine whether ceramide is involved in TGF-\(\beta\) signaling, we measured ceramide levels in cells treated with TGF-\(\beta\) for various time intervals. Ceramide measurements were performed in human dermal fibroblasts and in Mv1Lu cells. Both cell types responded in a similar manner with a rapid increase of the ceramide levels in response to TGF-\(\beta\). As shown in Fig. 2A, ceramide increased 5 minutes after TGF-\(\beta\) addition picked by 10 min, and very slowly declined over the next 2 hours. We also examined phosphorylation of Smad3 in cells treated with TGF-\(\beta\) for the same time intervals (Fig. 2B). Detectable phosphorylation of Smad3 occurred at 10 min after TGF-\(\beta\) addition, with the peak
phosphorylation between 20-60 min and a decline at 120 min. These results indicate that TGF-β treatment leads to a relatively rapid induction of endogenous ceramide. Furthermore, generation of ceramide preceded Smad3 phosphorylation, raising the possibility that ceramide may be involved in TGF-β signaling.

**Exogenous S1P and C₆-Ceramide modulate TGF-β dependent COL1A2 promoter activity**

Since our laboratory is particularly interested in defining TGF-β signaling pathways relevant to fibrosis, we utilized a well characterized COL1A2 promoter reporter construct to delineate the role of ceramide in TGF-β regulation of matrix genes (18,22,26). It has been previously shown that TGF-β stimulation of this promoter is Smad3-dependent (27,28). We first examined the effects of exogenously added sphingolipids on the basal and TGF-β-dependent COL1A2 promoter activity. Dermal fibroblasts were transfected with the COL1A2 promoter and treated with S1P (10-30 µM), C₆-ceramide (10-50 µM), and sphingosine (1 µM) in the presence or absence of TGF-β. S1P at micromolar concentrations slightly stimulated basal and TGF-β-induced COL1A2 promoter activity (Fig. 3A) whereas nanomolar doses of S1P had no effect on the COL1A2 promoter (data not shown). C₆-ceramide (10 µM) stimulated COL1A2 promoter activity with an effect comparable to that of TGF-β, a known inducer of collagen gene transcription (Fig. 3B). Simultaneous addition of C₆-ceramide (10 µM) and TGF-β resulted in a further increase of the COL1A2 promoter activity. On the other hand, C₆-ceramide at a higher concentration (50 µM) did not affect basal promoter levels and decreased TGF-β induced COL1A2 promoter activity (Fig. 3B). Sphingosine (1 µM) had no effect on the COL1A2 promoter activity in the presence or absence of TGF-β (Fig. 3C). Higher concentrations of sphingosine were toxic. These experiments suggest that sphingolipids maybe modulators of the basal and TGF-β-dependent COL1A2 promoter activity in dermal fibroblasts.

Interestingly, ceramide had both stimulatory (at lower doses) and inhibitory (at higher doses) effects. One possible interpretation of these results is based on the current view of the dynamic balance between intracellular levels of ceramide, sphingosine and S1P (10). As the relative ratio of endogenous ceramide and S1P is tightly controlled, the stimulatory effect of high concentrations of S1P may be due to its conversion to ceramide. Also, the failure of S1P to act in nanomolar range argues against its effects being mediated through the edg (SIP) receptors, and further supporting intracellular action which would include metabolic interconversion, as a distinct possibility. Conversely, inhibitory effects of high doses of ceramide may reflect its metabolism to S1P or other inhibitory intermediates. Together, the experiments using exogenous sphingolipids suggest that balance between components of
sphingolipid pathway may be important for collagen gene regulation.

**YSR2 and TGF-β synergize to induce COL1A2 promoter**

To gain more understanding into the nature of endogenous sphingolipids involved in TGF-β signaling, we took advantage of the availability of recombinant enzymes involved in regulation of the balance between S1P and ceramide (see Fig.1). First, we utilized YSR2 to generate endogenous ceramide. YSR2 specifically dephosphorylates S1P to sphingosine, which is then converted to ceramide. Control experiments indicated that cell lysates prepared from cells ectopically expressing YSR2 contained higher levels of S1P phosphatase activity as compared to cell lysates obtained from cells expressing empty vector (Fig. 4A). We also determined that ectopic expression of YSR2 elevates endogenous ceramide levels (Fig.7D). Overexpression of YSR2 in dermal fibroblasts led to a modest but consistent induction of the COL1A2 promoter activity. Significantly, TGF-β stimulation of this promoter was greatly potentiated by the presence of YSR2 (Fig. 4B). To determine whether activation by YSR2 is specific for the COL1A2 promoter or whether YSR2 is a general coactivator of the TGF-β pathway, we used a TGF-β-responsive artificial reporter system (p3TP-Lux promoter). YSR2 induced basal activity of the p3TP-Lux promoter and synergized with TGF-β in activation of this promoter, suggesting a general effect of YSR2 on the TGF-β signaling pathway (Fig. 4C).

We next examined the effects of overexpressing SPHK1 on COL1A2 promoter activity. This enzyme generates S1P by phosphorylating sphingosine. Control experiments indicated that cell lysates prepared from cells ectopically expressing SHPK1 contained higher levels of SPHK1 activity (Fig. 5A). Overexpression of SHPK1 in dermal fibroblasts slightly decreased both basal and TGF-β stimulated COL1A2 promoter activity (Fig. 5B). Together, these experiments indicate that perturbation of the intracellular sphingolipid-ceramide pathway has both positive and negative effects on TGF-β signaling, such that tilting the balance from S1P to ceramide exerts a pronounced stimulatory effect whereas tilting the balance from ceramide S1P induces a modest inhibitory effect.

**YSR2 induces endogenous collagen type I gene expression**

Next, we examined the effects of the ectopically expressed YSR2 on expression levels of COL1A1 and COL1A2 mRNAs in human dermal fibroblasts transiently transfected with YSR2. Both, COL1A1 and COL1A2 mRNAs, were significantly increased in cells transfected with YSR2 as compared to cells transfected with empty vector (Fig. 6). Depending on the individual cell line, stimulation of COL1A1 by YSR2 varied from 1.6 to
9.9 folds, whereas stimulation by TFG-β varied from 1.7 to 8.8 folds. When both stimuli were present, an additional modest increase was observed (2.2-11.3 folds). It should be noted that in human dermal fibroblasts autocrine TGF-β signaling pathway is operational and contributes to basal collagen gene expression (29). Thus, increased COL1A1 and COL1A2 mRNA levels in cells transfected with YSR2 may reflect cooperation between YSR2 and low levels of endogenous TGF-β signaling pathway. Presently, we cannot exclude that other signaling pathways that are involved in collagen gene regulation are also affected by YSR2.

YSR2 regulates Smad3

Smad2 and Smad3 are mediators of TGF-β signaling. Furthermore, we have previously showed that Smad3 mediates TGF-β stimulation of the COL1A2 promoter in dermal fibroblasts (28). Therefore, we next examined the effects of YSR2 on Smad3 stimulation of the COL1A2 promoter. Transfection with Smad3 or YSR2 alone resulted in induction of the promoter activity. However, cotransfection of the COL1A2 promoter with Smad3 and YSR2 led to a dramatic increase of the collagen promoter activity (Fig. 7A). Interestingly, cotransfection of Smad3A (a mutant of Smad3 that carries three carboxy-terminal serine-to-alanine substitutions) (30) also produced a modest stimulatory effect. This may be due to activation of endogenous Smad3 or perhaps YSR2 can enhance TGF-β signaling through an additional yet unidentified mechanism. Since recent studies have shown that nuclear import of Smad can occur without phosphorylation on the SSXS motif (31), it is possible that the action of a phosphatase facilitates dissociation of Smad3 from SARA. In any case, our data suggest that Smad3 is a major effector of YSR2.

Since phosphorylation of C-terminal serine residues of Smad3 is necessary for its activation, we investigated the effect of YSR2 on Smad3 phosphorylation. Using COS cells, Smad3 was coexpressed with the constitutively active form of the TGFβ receptor [TβRI(TD)] in the absence or presence of YSR2. Phosphorylation of Smad3 was detected by immunoblotting using antibodies to phosphorylated serine residues. As previously shown, in the presence of TβRI(TD), Smad3 phosphorylation was observed. YSR2 significantly increased Smad3 phosphorylation (Fig. 7B). This result indicates that YSR2 has an additive, or synergistic effect on the intensity of the TGF-β signaling cascade acting at the post-receptor level.

To further investigate the role of YSR2 in Smad3 activation, we ectopically expressed YSR2 in human fibroblasts using adenoviral vector. As shown in Fig. 7C, Smad2/3 was not phosphorylated in unstimulated cells but became rapidly phosphorylated in response to TGF-β stimulation. In contrast, in cells transduced with YSR2, Smad3 became fully
phosphorylated even in the absence of TGF-β with no additional increases in phosphorylation after addition of TGF-β. Thus, the endogenous metabolic products of YSR2 (e.g. ceramide) are capable of inducing phosphorylation of Smad3.

Previous studies have shown that overexpression of S1P phosphatase results in generation of ceramide (13-15). The endogenous levels of ceramide were measured in cells transduced either with control-GFP or with YSR2 adenoviruses and compared to cells stimulated with TGF-β. As shown in Fig. 7D, ceramide levels were similarly induced by YSR2 overexpression or TGF-β treatment.

DISCUSSION

The present study establishes for the first time that endogenous sphingolipids are co-regulators of the TGF-β signaling pathway. The following observations support this conclusion. First, exogenously added C₆-ceramide stimulated basal and TGF-β-induced collagen promoter activity. Second, overexpression of the yeast S1P phosphatase had a synergistic effect with TGF-β in activation of the collagen promoter and stimulated endogenous collagen gene. Both the yeast and the murine enzymes have been previously shown to decrease endogenous S1P levels and to increase endogenous ceramide levels (14,15). On the other hand, TGF-β induction of this promoter was inhibited by overexpression of SPHK1, an enzyme that promotes formation of S1P. More importantly, TGF-β treatment induced endogenous ceramide levels, which correlated with TGF-β induced Smad3 phosphorylation. These results strongly suggest that ceramide is a coactivator of the TGF-β signaling pathway and that S1P is a possible endogenous inhibitor. We have also begun to unravel the specific regulatory mechanisms involved in the interaction of sphingolipids and TGF-β signaling pathways. We demonstrate by functional assays that the effects of YSR2 are mediated via Smad3. Consistent with these results, Smad3 phosphorylation is increased in the presence of ectopically expressed YSR2.

Our results also clarify previous observations indicating an inhibitory role of C₂-ceramide in collagen production (16,17). In our study the inhibitory effects of exogenous ceramides on COL1A2 transcription were also observed with higher doses of C₆-ceramide. These observations suggest that sphingolipids may have a dual role in collagen gene regulation and that other metabolites with inhibitory functions may also be formed. S1P may be one of the metabolites with the inhibitory function. Our recent studies have shown that exogenously added S1P and lysophosphatidic acid inhibit TGF-β stimulation of collagen mRNA stability. These effects were mediated via MEK/ERK (Sato and Trojanowska,
unpublished observations). Induction of the ERK pathway was also responsible for collagen inhibition by C2-ceramide (16). Although induction of ERK pathway in response to exogenously added ceramide or SMase has been observed in several experimental systems (11), the specific intracellular mediators of this induction are presently unknown. C2-ceramide has also been shown to activate p38 pathway (32). In contrast to ERKs, p38 has been shown to cooperate with TGF-β signaling in collagen gene stimulation (33). However, p38 does not appear to be involved in the effects observed in our study. First, Smad3 is not a direct target of p38 (34,35). Second, stimulation of the COL1A2 promoter by YSR2 was insensitive to SB203580, a specific inhibitor of p38 (data not shown). We could also exclude stress-activated protein kinase (SAPK)/JNK pathway as it was shown that induction of this pathway downregulates COL1A2 promoter in dermal fibroblasts (36). Thus, it appears that the members of MAPK superfamily, which have been previously shown to mediate many of the ceramide effects, are not involved in the process described herein.

The question remains about the nature of the interaction between the sphingolipid and TGF-β signaling pathways. It has been demonstrated that both TGF-β receptor type I and type II, which form homodimers in the endoplasmic reticulum, are clustered in the cell surface into discrete cellular subdomains (37,38). Furthermore, a TGF-β-dependent interaction between TβRI and caveolin-1 has recently been described (39). Interestingly, caveolin-1 was shown to suppress TGF-β mediated Smad2 phosphorylation and subsequent downstream events. It is possible that ceramide may interfere with this interaction resulting in increased Smad3 phosphorylation. This possibility will be tested in our future studies. Recent studies suggest that that assembly of the TGF-β signaling pathway occurs in early endosomes (5,6). Consistent with this possibility, an endosomal protein SARA has been shown to colocalize with TGF-β receptors and recruit Smad to the receptor complex (4,31,40,41). Other endosomal proteins including Hgs (Hrs) may also contribute to TGF-β signaling (7). It has been recently shown that ceramide binds to and activates the endosomal protease, cathepsin D (42). Thus, endosomal ceramide may facilitate interaction of the TGF-β receptor complex with SARA and Smad3. Recent studies suggest that the lipid environment may be critical for the proper interactions of the cell surface receptors with downstream signaling molecules (43). Our data demonstrating increased Smad3 phosphorylation in cells overexpressing YSR2 are consistent with this possibility. Whereas the specific subcellular localization of the mammalian S1P phosphatase has not been determined, yeast S1P phosphatases have been localized to the endoplasmic reticulum (13), consistent with the possibility of interaction of this enzyme with TGF-β receptors or components of the TGF-β signaling pathway. In conclusion, this study demonstrates for the
first time a role for the endogenous sphingolipid mediators in regulating the TGF-β pathway. Further work is needed to delineate the specific mechanism of this interaction.

**FOOTNOTES**

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FIGURE LEGENDS

Figure 1. Schematic view of sphingolipid metabolism. Abbreviations: SM, sphingomyelin; SMase, sphingomyelinase; S1P, sphingosine 1-phosphate; GlcCer, glucosylceramide.

Figure 2. TGF-β induces endogenous ceramide levels and Smad3 phosphorylation with similar kinetics. Mv1Lu cells were treated with TGF-β (2.5 ng/ml) for the indicated time intervals. The measurements of ceramide levels and detection of phosphorylated Smad3 were performed as described in Methods section. Values represent the mean ± SD of three independent experiments.

Figure 3. S1P and C6-Ceramide stimulate COL1A2 promoter activity in fibroblasts, while sphingosine has no effect. Foreskin fibroblasts were transiently transfected with the COL1A2 promoter construct (1 µg) for 20 h. Cells were subsequently treated with S1P (A), C6-ceramide (B), or 1 µM sphingosine (C) alone or in combination with TGF-β (5 ng/ml) for 24 h. Values represent the mean ± SEM of five independent experiments performed in duplicate (A) or three independent experiments in duplicate (B, C).

Figure 4. YSR2 cooperates with TGF-β to activate COL1A2 promoter. (A) Phosphatase activity was measured in cell lysates obtained from 293 cells transiently transfected with YSR2 (see Methods section for details). Values represent the mean ± SD of three independent experiments. Values from control/untransfected cells (1) were arbitrarily set at 100%; values from vector transfected cells (2) and YSR2 transfected cells (3) indicate increases over control. (B) Foreskin fibroblasts were transiently transfected with 0.1 µg of YSR2 expression vector along with the COL1A2 promoter construct (0.9 µg) or (C) the p3TP-Lux construct (0.9 µg). Luciferase activity was determined following treatment with TGF-β (5 ng/ml) for 24h. Values represent the mean ± SEM of three independent experiments done in duplicate.

Figure 5. SPHK inhibits basal and TGF-β stimulated COL1A2 promoter activity. (A) Foreskin fibroblasts were transiently transfected with either 0.1 µg of SPHK1a (SPHK) or empty vector (control). Twenty four hours post-transfection, sphingosine kinase activity in cell lysates was determined using 50 µM sphingosine and [32P]ATP (10 µCi, 1 mM). Analysis of [32P]S1P was performed by thin layer chromatography. (B) Foreskin fibroblasts were transiently transfected with 0.1 µg of SPHK1 expression vector along with the COL1A2 promoter construct (0.9 µg). Luciferase activity was determined following treatment with TGF-β (5 ng/ml) for 24 h. Values represent the mean ± SEM of three independent experiments done in duplicate.

Figure 6. YSR2 stimulates endogenous type I collagen gene expression. Dermal fibroblasts were transiently transfected with YSR2 or empty vector and analyzed by northern blot as described in the Methods section. Where indicated, TGF-β (5 ng/ml) was added for 24 h. Efficiency of transfection varied between 10 - 15%. The experiment was performed nine times, representative three experiments using independently isolated human fibroblast cell lines are shown.
Figure 7. Smad3 is a target of YSR2 action. (A) Foreskin fibroblasts were transiently transfected with 0.1 µg of the wild-type Smad3 (WT) or phosphorylation-deficient Smad3, Smad3A(3A), or the empty vector along with the COL1A2 promoter construct (0.9 µg). Luciferase activity was determined following treatment of fibroblasts with TGF-β (5 ng/ml) for 24 h. Values represent the mean ± SEM of three independent experiments done in duplicate. (B) TGF-β-induced phosphorylation of Smad3 is enhanced by YSR2. COS cells were transfected with Flag-Smad3 in the presence or absence of HA-TβRI(TD) and YSR2. Cell lysates were subjected to immunoblotting with anti-phosphoserine (P-ser) antibody. The membrane was re-blotted with anti-Flag antibody. Aliquots of cell lysates were also subjected to immunoblotting with anti-HA antibody to detect HA-TβRI(TD). (C) Adenovirally-expressed YSR2 enhances endogenous Smad2/3 phosphorylation level. Fibroblasts were transduced with control GFP-adenovirus or YSR2-adenovirus (MOI=70) and next day stimulated with TGF-β. Whole cell extracts were immunoprecipitated with goat anti-Smad2/3 antibody and immunoblotted with either anti-phosphoserine antibody (upper panel) or rabbit anti-Smad3 antibody (lower panel). (D) YSR2 induces endogenous ceramide levels. Ceramide levels were measured as described in the Methods section in cells transduced with control GFP-adenovirus and treated with TGF-β or in cells transduced with YSR2-adenovirus. Values represent the mean ± SEM of two independent experiments done in duplicate.
Figure 1
Figure 2
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