Sphingosine Kinase 1 (SPHK1) Is Induced by Transforming Growth Factor-β and Mediates TIMP-1 Up-regulation*

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transforming growth factor-β (TGF-β) signaling plays a pivotal role in extracellular matrix deposition by stimulating collagen production and other extracellular matrix proteins and by inhibiting matrix degradation. The present study was undertaken to define the role of sphingosine kinase (SphK) in TGF-β signaling. TGF-β markedly up-regulated SphK1 mRNA and protein amounts and caused a prolonged increase in SphK activity in dermal fibroblasts. Concomitantly, TGF-β reduced sphingosine-1-phosphate phosphatase activity. Consistent with the changes in enzyme activity, corresponding changes in sphingolipid levels were observed such that sphingosine 1-phosphate (S1P) was increased (~2-fold), whereas sphingosine and ceramide were reduced after 24 h of TGF-β treatment. Given the relatively early induction of SphK gene expression in response to TGF-β, we examined whether SphK1 may be involved in the regulation of TGF-β-inducible genes that exhibit compatible kinetics, e.g. tissue inhibitor of metalloproteinase-1 (TIMP-1). We demonstrate that decreasing SphK1 expression by small interfering RNA (siRNA) blocked TGF-β-mediated up-regulation of TIMP-1 protein suggesting that up-regulation of SphK1 contributes to the induction of TIMP-1 in response to TGF-β. The role of SphK1 as a positive regulator of TIMP-1 gene expression was further corroborated by using ectopically expressed SphK1 in the absence of TGF-β. Adenovirally expressed SphK1 led to a 2-fold increase of endogenous S1P and to increased TIMP-1 mRNA and protein production. In addition, ectopic SphK1 and TGF-β cooperated in TIMP-1 up-regulation. Mechanistically, experiments utilizing TIMP-1 promoter constructs demonstrated that the action of SphK1 on the TIMP-1 promoter is through the AP1 response element, consistent with the SphK1-mediated up-regulation of phospho-c-Jun levels, a key component of AP1. Together, these experiments demonstrate that SphK/SIP are important components of the TGF-β signaling pathway involved in up-regulation of the TIMP-1 gene.

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Transforming growth factor-β (TGF-β) is a member of a large growth factor family with diverse functions in embryonic and adult tissues (1, 2). TGF-β signaling plays a pivotal role in extracellular matrix (ECM) deposition in fibroblasts by stimulating the production of collagens and other ECM proteins and inhibiting matrix degradation (3). The negative effects of TGF-β on ECM degradation are mediated via direct inhibition of the principal matrix-degrading proteinase, metalloproteinase-1 (MMP-1) (4), and stimulation of endogenous inhibitors of MMP-1, including TIMP-1 (5). In healthy tissues, a balance between matrix synthesis and degradation precisely controls ECM homeostasis, and shifting this balance can lead to pathological ECM turnover (6). Excessive matrix degradation occurs in rheumatoid arthritis, osteoarthritis, periodontitis, and tumor invasion (6). An imbalance between the tissue inhibitors of matrix metalloproteinases and matrix metalloproteinases is believed to be one of the mechanisms contributing to these pathologies. Recent observations on the role of TIMPs in tumorigenesis suggest that these factors have additional MMP-independent functions, including growth stimulatory and anti-apoptotic effects (7). Thus, TIMPs may enhance tumor growth during the early stages of tumorigenesis, although inhibiting tumor progression through their inhibitory effects on MMPs.

Sphingosine kinase (SphK) is a ubiquitously expressed, evolutionary conserved enzyme that catalyzes the phosphorylation of sphingosine to sphingosine 1-phosphate (SIP) (8). To date, two mammalian isomers of SphK (types 1 and 2), including their splice variants, have been identified. In addition, sphingosine kinase activity that was not attributable to SphK1 or SphK2 was detected in selected mouse tissues (9); however, because the search of the human genome did not reveal any additional proteins with the SphK homology (10), this activity may be due to the presence of proteins with overlapping functions not related to SphK. SphK1 and SphK2 differ in their relative tissue distribution, subcellular localization, and biochemical activities, consistent with distinct biologic functions for these two enzymes (11). SphK1 is primarily localized in the cytosol and has been shown to translocate to the membrane upon activation with selected agonists (12), whereas SphK2 has a predominantly nuclear localization (13). SphK1 is slightly more efficient than SphK2 in phosphorylating their primary intracellular substrate, sphingosine, whereas SphK2

1 The abbreviations used are: TGF-β, transforming growth factor-β; ECM, extracellular matrix; SIP, sphingosine 1-phosphate; siRNA, small interfering RNA; TIMP-1, tissue inhibitor of metalloproteinase-1; SphK, sphingosine kinase; RT, reverse transcription; CAT, chloramphenicol acetyltransferase; DME, Dulbecco’s modified Eagle’s medium; PCS, fetal calf serum; m.o.i., multiplicity of infection; MMP, metalloproteinase; GFP, green fluorescent protein; IS, internal standards; SPPase, sphingosine-1-phosphate phosphatase.

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is significantly more efficient toward unnatural substrates such as the immunomodulatory drug FTY720 (10). SphK1 has been shown to regulate a wide variety of cellular processes, including promotion of cell proliferation, survival, and motility (8). In contrast, SphK2 has been shown to inhibit DNA synthesis and also to induce apoptosis in a variety of cell types (13, 14). Significantly, nuclear localization was essential for the antiproliferative effects of SphK2.

SphK appears to have a dual role in regulating cellular function. At the basal catalytic level, SphK is involved in sphingomyelin and glycosphingolipid turnover and is responsible for clearing the cell of sphingosine and ceramide (11). Upon activation with agonists, SphK functions as a component of the signal transduction cascades. For example, tumor necrosis factor-α-induced activation of SphK1 is required for the TRAF2-dependent activation of NF-κB (15). Furthermore, activation of SphK1 and its translocation to the membrane is mediated by the ERK1/2-induced phosphorylation at Ser-225 (16). Phosphorylation of SphK1 by ERKs was dependent on its association with TRAF2. SphK1 is not only an effector of ERK1/2, but it is also involved in signaling cascades leading to ERK1/2 activation. It has been shown that activation of ERK1/2 by tumor necrosis factor-α (16), vascular endothelial growth factor (17), and 17β-estradiol (18) requires SphK1. Because intracellular receptors for SIP have not been described, it is unclear at this point how SphK1 couples to intracellular signaling cascades.

Our previous study (19) in dermal fibroblasts revealed a cross-talk between the sphingolipids and the TGF-β signaling pathways. We have shown that TGF-β treatment leads to a rapid and transient induction of ceramide. Furthermore, changes in the intracellular levels of ceramide due to the rapid and transient induction of ceramide. Furthermore, cross-talk between the sphingolipids and the TGF-β signal transduction cascades. For example, tumor necrosis factor-α-induced activation of SphK1 is required for the TRAF2-dependent activation of NF-κB (15). Furthermore, activation of SphK1 and its translocation to the membrane is mediated by the ERK1/2-induced phosphorylation at Ser-225 (16). Phosphorylation of SphK1 by ERKs was dependent on its association with TRAF2. SphK1 is not only an effector of ERK1/2, but it is also involved in signaling cascades leading to ERK1/2 activation. It has been shown that activation of ERK1/2 by tumor necrosis factor-α (16), vascular endothelial growth factor (17), and 17β-estradiol (18) requires SphK1. Because intracellular receptors for SIP have not been described, it is unclear at this point how SphK1 couples to intracellular signaling cascades.

Our previous study (19) in dermal fibroblasts revealed a cross-talk between the sphingolipids and the TGF-β signaling pathways. We have shown that TGF-β treatment leads to a rapid and transient induction of ceramide. Furthermore, changes in the intracellular levels of ceramide due to the overexpression of the yeast sphingosine-1-phosphate phosphatase (YSR2) resulted in potentiation of TGF-β signaling, including Smad2/3 phosphorylation. Recent studies (20) have also demonstrated cooperation between TGF-β and SIP signaling. Stimulation of mesangial cells with SIP led to phosphorylation of Smads1–3 and mimicked some of the TGF-β downstream effects. It was also shown that SIP effects on Smad phosphorylation were dependent on the presence of TGF-β receptor type II and the SIP3 receptor subtype, suggesting transactivation of TGF-β receptors by SIP. Thus, it appears that interactions between sphingolipid and TGF-β pathways occur at many levels with both exogenous and endogenous sphingolipids capable of modulating TGF-β signaling. In the present study, we describe an additional mode of interaction between these two pathways. We demonstrate for the first time that TGF-β induces SphK synthesis. Furthermore, we show that SphK mediates TIMP-1 up-regulation by TGF-β via an AP1-dependent mechanism.

EXPERIMENTAL PROCEDURES

Materials—Sphingosine 1-phosphate and PD98059 were obtained from Biomol (Plymouth Meeting, PA). TGF-β1 was from R & D Systems (Minneapolis, MN). PCR reagents were bought from Applied Biosystems (Foster City, CA). Radionucleotides, [γ-32P]ATP, and [γ-32P]ATP were purchased from Enzyme Fundamentals, Life Sciences. DNA 1 and QuanturmRNA™ Classic 18 S rRNA standards were obtained from Ambion (Austin, TX). Tissue culture reagents, Dulbecco’s modified Eagle’s medium (DMEM), and fetal calf serum (FCS) were obtained from Invitrogen. Primers were purchased from Sigma-Genosys (The Woodlands, TX). Nylon membranes were from StrataGene (La Jolla, CA). Dimethyl sulfoxide (Me2SO) and monoclonal β-actin antibody (clone AC-150) were obtained from Sigma. Protease inhibitor mixture set III was from Calbiochem. ECL reagent and anti-rabbit horseradish peroxidase-linked whole antibody (from donkey) were obtained from Amer sham Biosciences. D.C. protein assay reagent was from Bio-Rad. Bicinchoninic acid protein assay reagent was from Pierce. Mouse monoclonal TIMP-1 antibody (Ab4) was from Oncogene. Rabbit polyclonal antibodies for phospho-c-Jun and c-Jun were from Santa Cruz Biotechnology.

Cell Culture—Human fibroblasts were obtained from the foreskins of healthy newborns from the Medical University of South Carolina Hospital. Dermal fibroblasts were established from biopsy specimens obtained from the dorsal forearm of healthy volunteers after informed consent. Tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma), and 0.05% DNase (Sigma) in Dulbecco’s modified Eagle’s medium (Invitrogen) with 20% fetal bovine serum (Invitrogen) overnight at 37 °C. Primary fibroblast cultures were established in 25-cm² culture flasks in DMEM supplemented with 20% FCS (Invitrogen) and 2 mM glutamine. Monolayer cultures were maintained at 37 °C in 10% CO2 until confluence. Cells were then passaged and used for experiments.

Measurement of Sphingosine Kinase Activity—SphK activity was determined as described previously by Olivera et al. (21). Cells were harvested and lysed in a buffer containing Tris-HCl (20 mM, pH 7.4), EDTA (1 mM), 20% glycerol, deoxyribozyme (0.5 mM), NaF (15 mM), β-glycerophosphate (40 mM), β-mercaptoethanol (1 mM), sodium orthovanadate (1 mM), phenylmethyl sulfonyl fluoride (1 mM), dithiothreitol, aprotinin (10 μg/ml), leupeptin (10 μg/ml). Sphingosine kinase assay was determined in the presence of sphingosine (50 μM) complexed with 0.3% bovine serum albumin and [γ-32P]ATP (10 μCi, 1 mM) containing MgCl2 (10 mM). The labeled SIP was separated by thin layer chromatography on Silica Gel G60 with 1-butanol/methanol/acetic acid/water (80:20:10: 20, v/v) and visualized by autoradiography. The labeled SIP was quantitated by phosphorimaging. Bands corresponding to SIP and S1P were also scraped from the plates and counted in a scintillation counter.

Measurements of Phosphatase Activity—Phosphatase activity was performed as described previously (22). Cells were harvested and lysed on ice in Buffer A containing Heps (100 mM, pH 7.5), EDTA (10 mM), 1 mM dithiothreitol, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and soybean trypsin inhibitor. Cells were freeze-thawed seven times and spun for 10 min at 3000 g and the supernatant was collected and spun at 100,000g for 1 h. Pellet (membrane fraction) was dissolved in Buffer A (32P-Labeled SIP, 10,000 cpm) was incubated with 2 μg of membrane fraction in Buffer A for 30 min at 37 °C followed by chloroform/methanol extraction and TLC analysis. The TLC plate was autoradiographed, and sphingosine was scraped and counted in a scintillation counter. S1Pase activity was determined by subtracting background blank counts.

Measurements of S1P/Sphingosines/Ceramides by Multiple Reaction Monitoring Method—Electrospray ionization/mass spectrometry/mass spectrometry analysis of endogenous sphingoid bases, sphingoid base 1-phosphates, and ceramide species was performed on a Thermo Finnn gan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction monitoring positive ionization mode using a modified version of a published protocol (23). Briefly, cell pellets corresponding to ~3 × 10⁶ cells were fortified with the internal standards (IS) (C₁₇ base t-erythrosphingosine: 17CSphe, C₁₇ sphingosine 1-phosphate: 17CSphe-1P, N-palmitoyl-t-erythro-C₁₃ sphingosine: 13C/16-Cer and N-heptadecanoyl-d-erythro-C₁₇ sphingosine: 17C/17-Cer) and extracted into a one-phase solvent system with ethyl acetate/isopropl alcohol/water (60:30:10 v/v). After evaporation and reconstitution in 1 μl methanol, samples were injected on to the 7000 series liquid chromatography/mass spectrometry system, and the gradient was eluted from the BDS Hypersil CS, 150 × 3.2 mm, 3 μm particle size column, with 1.0 mm methanolic ammonium formate, 2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and IS were collected and processed using the Xcalibur software system.

Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the IS. The target analyte peak areas ratios from the samples were similarly normalized to their respective IS and compared with the calibration curves using a linear regression model.

Adenoviral Constructs—Adenoviral vectors expressing human SphK1 were generated using the method described by He et al. (24). Briefly, the cDNA encoding full-length human SphK1 (12) was cloned into the shuttle vector, pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate cytomegalovirus promoter. The shuttle vector containing SphK1 was cotransformed into Escherichia coli BJ5163 cells with the AdEasy-1 adenoviral backbone plasmid, which lacks the E3 and E1 regions of the adenoviral genome. Linearized recombinant plasmid DNA was then transfected into 293 cells, an adenoviral packaging cell line, using the FuGENE 6 transfection reagent (Roche Applied Science) to generate the recombinant adenovirus expressing SphK1 and GFP (AdSphK1).
containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), 1% Nonidet P-40, 15 mM NaF, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Cell lysates were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with a rabbit polyclonal antibody against human SphK1 (12). Polyconal phospho-Jun, c-Jun, phospho-Smad2, and Smad2 antibodies were purchased from Cell Signaling. Bands were detected using ECL (Amersham Biosciences).

RNA Isolation and RT-PCR for SphK1 and Sphk2 Expression—Confluent cultures of foreskin fibroblasts were serum starved for 24 h, followed by stimulation with TGF-β (2.5 ng/ml) for the indicated time. Total RNA was isolated using the guanidinium thiocyanate/phenol/chloroform method (25). Total RNA (1 μg) was converted to cDNA using a random hexamer primer. The following primers were used for SphK1: forward, 5′-CGG CCC AGG GAA TGA CAC C-3′, and reverse, 5′-GCC TGT CCC CCC AAA GCA TAA C-3′, and the PCR product size was 1492 bp; for SphK2: forward, 5′-CCA AGG CAG CTC TAC ACT CAC-3′, and reverse, 5′-CCA CAC CCC CTA GCT CAA G-3′, and the product size was 654 bp. Control products (18 S rRNA) were amplified using the QuantumRNA Classic 18 S Standards (Ambion, Austin, TX) to yield a 488-bp product.

Northern Blot Analysis—Fibroblasts were grown to confluence in 10-cm² dishes in DMEM supplemented with 10% FCS. Confluent fibroblasts were serum-starved in DMEM containing 0.1% bovine serum albumin for 24 h followed by transduction with AdGFP or AdSphK1 at a multiplicity of infection (m.o.i.) of 100. The cells were incubated for 30 min or for 24 h with TGF-β (2.5 ng/ml) for an additional 24 h. Total RNA was extracted, and 3.5 μg was analyzed by Northern blotting as described previously (26). Nylon membranes were hybridized with 32P-labeled cDNA probes for TIMP-1 (as described in Ref. 27) and Mediates TIMP-1 Up-regulation

Results SphK Activity Is Up-regulated by TGF-β in Human Fibroblasts—Previous reports have shown that SphK can be activated acutely by a number of agonists including growth factor receptor tyrosine kinases and G-protein-coupled receptors (32), but whether TGF-β regulates SphK has not been investigated. We examined the effects of TGF-β on activity and expression levels of SphK in human fibroblasts. A pronounced increase in SphK activity was consistently observed in fibroblasts after 24 h of TGF-β stimulation, with the earliest effects observed starting at 3 h (Fig. 1, A and B). Thus, unlike growth factor signaling, TGF-β results in sustained induction of SphK activity.

To determine whether TGF-β stimulation resulted in corresponding increases of S1P, sphingolipid amounts were quantitated by mass spectrometry in confluent serum-starved cells treated for 30 min or for 24 h with TGF-β. Two independently isolated foreskin fibroblast and one adult dermal fibroblast lines were used in these experiments. Both foreskin and adult cell lines responded similarly to TGF-β treatment. Intracellular amounts of S1P were very low in dermal fibroblasts under our experimental conditions and varied from 0.17 to 0.29 pmol/10⁶ cells. In agreement with our previously published data using the diaicylglycerol kinase assay (19), after 30 min of stimulation with TGF-β we observed an increase in ceramide, whereas the amounts of S1P were further decreased below detection levels (data not shown). In contrast, after 24 h stimulation with TGF-β, S1P amounts were consistently increased (0.26–0.95 pmol/10⁶ cells) (Fig. 1C). We did not detect any measurable changes in the secreted S1P amounts after TGF-β stimulation. Concomitant with S1P increases, the amounts of sphingosine were decreased from 8 to 3.7 pmol/10⁶ cells (Fig. 1C). The basal level of C₁₅₆-ceramide was higher in adult fibroblasts (264 pmol/10⁶ cells) than in foreskin fibroblasts lines (101 pmol/10⁶ cells), but in all cell lines ceramide decreased about 15% after 24 h of TGF-β treatment. Other ceramide subtypes, including C₁₄, C₁₈, and C₂₁ showed inconsistent changes in different primary cell lines (Fig. 1C and data not shown). Together, these results demonstrate that 24-h TGF-β
TGF-β stimulation leads to the generation of S1P and sustained changes in sphingolipid content, consistent with other prolonged effects of TGF-β on ECM synthesis in dermal fibroblasts.

**SPHK1 Is Induced by TGF-β and Mediates TIMP-1 Up-regulation**

**SPHK mRNA and Protein Levels Are Induced by TGF-β in Human Fibroblasts**—The TGF-β-mediated increase in SPHK activity may be a result of SPHK protein up-regulation or a direct effect on its activity. To examine these possibilities, human SPHK1 protein was measured by Western blot. As shown in Fig. 2A, TGF-β (24 h) treatment markedly up-regulated SPHK1 protein amounts (2.13 ± 0.39-fold increase, n = 3). We next examined the effects of TGF-β on SPHK1 mRNA synthesis by using semi-quantitative RT-PCR. As shown in Fig. 2, B and C, TGF-β rapidly stimulated SPHK1 mRNA steady-state levels. Stimulation was observed at 20 min (data not shown), increasing after 1 h (3.7 ± 0.67-fold increase, n = 3), with a maximal increase after 3 h (14 ± 0.55-fold increase, n = 3). Elevated levels persisted for at least 24 h (11.4 ± 0.40-fold increase, n = 3). Together, these data demonstrate that SPHK1 is a novel TGF-β-inducible gene in fibroblasts. SPHK2 expression levels are low in quiescent dermal fibroblasts as compared with MOLT4 cells and were not affected by TGF-β treatment. In contrast to TGF-β, connective tissue growth factor had no appreciable effect on SPHK activity (data not shown), suggesting divergent pathways for these two pro-fibrotic cytokines on sphingolipid metabolism in dermal fibroblasts.

**TGF-β Down-regulates Sphingosine-1-Phosphate Phosphatase (SPPase)**—Our findings of SPHK1 up-regulation by TGF-β are in agreement with a recent comprehensive TGF-β array study, which identified 360 TGF-β target genes in fibroblasts (33). Among the genes that were up-regulated by the TGF-β/Smad3 cascade was SPHK1, whereas among the small number of genes that were down-regulated by TGF-β was SPPase. We have measured sphingosine phosphatase activity by using an enzymatic assay. A marked down-regulation of SPPase activity in dermal fibroblasts was observed in response to TGF-β (Fig. 3), as shown in Fig. 3A, addition of cycloheximide in combination with TGF-β strongly inhibited the TGF-β-mediated induction of TIMP-1 mRNA. To investigate the possible role of SPHK1 in TIMP-1 induction by TGF-β, we utilized siRNA to inhibit SPHK1 expression. Fig. 4B demonstrates that sphingosine kinase activity was markedly decreased up to 96 h after treatment with siRNAs directed against SPHK1. The intracellular levels of sphingolipids were measured 72 h after treatment with the SPHK1-specific siRNA or the control, nonsilencing RNA. As expected, cells treated with SPHK1-specific siRNA showed an approximate 2-fold decrease of S1P, more, some of the TGF-β early response genes such as connective tissue growth factor contribute to the up-regulation of genes induced with delayed kinetics such as collagen (35). Given the relatively rapid induction of SPHK1 mRNA in response to TGF-β, and its possible role as a signaling mediator, we proceeded to examine whether SPHK1 may be involved in the regulation of TGF-β-inducible genes that exhibit a delayed response, such as TIMP-1. Previous studies have demonstrated that maximal stimulation of TIMP-1 transcription by TGF-β required 24 h and was dependent on new protein synthesis (36, 37). We confirmed this observation in our experimental system using foreskin fibroblasts. As shown in Fig. 4A, addition of cycloheximide in combination with TGF-β strongly inhibited the TGF-β-mediated induction of TIMP-1 mRNA. To investigate the possible role of SPHK1 in TIMP-1 induction by TGF-β, we utilized siRNA to inhibit SPHK1 expression. Fig. 4B demonstrates that sphingosine kinase activity was markedly decreased up to 96 h after treatment with siRNAs directed against SPHK1. The intracellular levels of sphingolipids were measured 72 h after treatment with the SPHK1-specific siRNA or the control, nonsilencing RNA. As expected, cells treated with SPHK1-specific siRNA showed an approximate 2-fold decrease of S1P,
whereas the amounts of several ceramide subspecies including C18, C20, C24, and C24:1 consistently increased (Fig. 4B and data not shown). C24:1-ceramide showed the most pronounced increase (1.7-fold, n = 2) from 42 to 86 pmol/10^6 cells, whereas C24-ceramide increased about 10% from 85 to 94 pmol/10^6 cells. Under this experimental condition, TIMP-1 up-regulation by TGF-β was abrogated (Fig. 4, C and D). Together these observations strongly suggest that endogenous SphK1 contributes to the TGF-β-induced up-regulation of the TIMP-1 gene.

Endogenous S1P Is Increased in Fibroblasts Overexpressing SphK1—Elevated expression levels of SphK have been found in various tumors (38), but the biological consequences of SphK overexpression, especially in stromal cells, are presently unknown. To study the effects of elevated amounts of SphK1 in human fibroblasts, we utilized cells overexpressing SphK1. Because of the limited life span of dermal fibroblasts, the use of stable transfectants is impractical; therefore, an adenoviral vector encoding full-length SphK1 was generated for these stable transfectants. Cells were transduced at an increasing m.o.i. to establish optimal conditions, such that the levels of C14-, C24-, and C24:1-ceramides were consistently decreased, although the levels of C16-ceramide did not display a significant change (Fig. 5C). Thus, both TGF-β treatment and ectopic expression of SphK1 led to similar increases in intracellular amounts of S1P, demonstrating that expression of SphK1 may be employed to mimic the effects of TGF-β on endogenous S1P. We have not detected secreted S1P under the experimental conditions used in this study.

Ectopic SphK1 Up-regulates TIMP-1 mRNA and Protein Levels in the Absence of TGF-β Signaling—We first asked whether SphK1 is able to modulate TIMP-1 expression independently of TGF-β. The effects of adenoviral expression of SphK1 on TIMP-1 production were examined in dermal fibroblasts under serum-free conditions. SphK1 on its own up-regulated the levels of TIMP-1 protein (1.78 ± 0.18-fold increase, n = 3) (Fig. 6A). In addition, SphK1 overexpression enhanced the effects of TGF-β in the induction of TIMP-1. Likewise, SphK1 increased TIMP-1 mRNA expression alone and further potentiated the induction of TIMP-1 mRNA by TGF-β (Fig. 6, B and C), suggesting that the activation of SphK1 and the generation of intracellular S1P are part of the signaling cascade involved in the induction of the TIMP-1 gene.

SphK1 Stimulates TIMP-1 Promoter Activity via an AP1 Site—It has been reported recently (5) that TGF-β up-regulates the TIMP-1 promoter through an AP1-response element in a Smad2/3-independent manner. Therefore, we investigated whether the AP1-binding site is involved in TIMP-1 promoter regulation by SphK. By using “big” (−736 to +95) TIMP-1 promoter CAT reporter constructs, we observed similar levels of stimulation of CAT activity by either TGF-β or SphK alone (Fig. 7A). Consistent with the protein and mRNA data, SphK1 cooperated with TGF-β in the stimulation of the TIMP-1 promoter. As reported previously, TGF-β stimulation was also observed with the minimal TIMP-1 promoter (−102 to +95), whereas this promoter construct was less responsive to SphK1
Most interestingly, the cooperation between SphK1 and TGF-β/H9252 was maintained with the minimal promoter. Therefore, these data demonstrate that the actions of SphK1 on the TIMP-1 promoter involve two distinct mechanisms, such that the actions of SphK1 on its own are distinct from its cooperative actions with TGF-β.

The TGF-β-independent action of SphK1 is located in a currently uncharacterized promoter region of TIMP-1 between −736 and −102. Therefore, we focused on characterizing the response element that mediates the cooperation between TGF-β and SphK1. We utilized a TIMP-1 minimal promoter.
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A)  

**Fig. 8. SphK1 induces c-Jun phosphorylation.** A, human fibroblasts were transduced with AdSphK1 and empty control virus (AdGFP) at a m.o.i. of 100 and incubated for 24 h. Cell layers were sequentially analyzed by Western blot for phospho- and total c-Jun. A representative blot from two independent experiments is shown. B, human fibroblasts were transduced with AdSphK1 or empty control virus (AdGFP) at a 100 m.o.i. for 24 h (top panel) or transfected with SphK-siRNA (250 nM) for 24 h (bottom panel), followed by TGF-β addition (2.5 ng/ml) where indicated. Western blots were sequentially performed with anti-phospho-SMA2 and anti-SMA2.

We have shown previously that overexpression of sphingosine 1-phosphate phosphatase-1 (YSR2) stimulated basal and TGF-β-dependent collagen α2(I) promoter activity via phosphorylation of Smad2/3, whereas overexpression of SphK had an inhibitory effect on collagen promoter activity (19). However, the effects of SphK on Smad2/3 phosphorylation have not been examined. Therefore, we examined whether SphK1 affects Smad2 phosphorylation levels. As shown in Fig. 8B, neither forced expression with SphK1 nor reducing SphK1 amounts with siRNA had an effect on Smad2 phosphorylation. This result is consistent with the previously shown Smad2/3 independent regulation of the TIMP-1 promoter by TGF-β (5).

**DISCUSSION**

TGF-β is a primary regulator of fibroblast function through its multiple effects on extracellular matrix remodeling, cell adhesion, and migration (2, 3). The molecular mechanisms by which TGF-β exerts its pleiotropic effects are not fully defined, but a recent extensive gene profile analyses suggested a hierarchical model of gene regulation in which initial TGF-β/Smad3 signaling induces expression of a number of secondary regulatory factors, including transcription factors and signaling molecules, in turn may initiate additional signaling cascades (33). Here we demonstrate that TGF-β induced a prolonged activation of sphingosine kinase via an increase in SphK1 mRNA and protein levels. Concomitant with the increased SPHK1 levels, TGF-β treatment resulted in a significant reduction of SPPase activity. These data are in agreement with the gene array study that demonstrated TGF-β/Smad3-dependent down-regulation of SPPase mRNA levels. Thus, in dermal fibroblasts the prolonged effects of TGF-β on the levels of sphingolipid enzymes are consistent with the enhanced generation of S1P.

Because SphK, and its metabolite S1P, play important roles in regulating various cell functions, including survival and proliferation, we asked whether endogenous SphK1 mediates some of the effects of TGF-β in fibroblasts. This study focuses on TIMP-1, which is induced by TGF-β with delayed kinetics. We provide evidence that endogenous SphK1 and its product S1P are the critical mediators of TIMP-1 up-regulation by TGF-β. Accordingly, an siRNA that targeted SphK1 and reduced the amount of intracellular S1P blocked TIMP-1 up-regulation by TGF-β. Conversely, ectopically expressed SphK1 cooperated with TGF-β in TIMP-1 induction. The cooperation between TGF-β and SphK1 was mediated via an AP1-response element in the TIMP-1 promoter. These data are consistent with a recently published study (5), which demonstrated that the up-regulation of TIMP-1 promoter activity by phorbol 12-myristate 13-acetate and TGF-β also required the AP1-response element. Phorbol 12-myristate 13-acetate is a potent activator of SphK1 (12), and it is likely that the stimulatory effects of phorbol 12-myristate 13-acetate on TIMP-1 promoter may be mediated in part via the activation of endogenous SphK1.

The role of SphK1 in TIMP1 gene regulation was further investigated using ectopically expressed SphK1. Elevated SphK1/S1P expression was sufficient to induce TIMP-1 protein, mRNA, and promoter levels independent of TGF-β stimulation. In addition, a marked increase in the amounts of phospho-c-Jun were observed in response to the increased amounts of SphK1. Whether c-Jun is involved in the SphK1-dependent TIMP-1 up-regulation is currently not known, and answering this question would require further analyses of the TIMP-1 promoter. On the other hand, current findings may explain our previous results demonstrating the inhibitory effect of SphK1 on the COL1A2 promoter (19). It was shown recently that the ability of inflammatory cytokines to antagonize the up-regulation of collagen type I and III by TGF-β is mediated by the JNK-dependent c-Jun and JunB phosphorylation (39). Thus, the previously observed inhibitory effect of SphK1 on the collagen promoter may be mediated via phosphorylation of c-Jun. In our experimental system, SphK1 had no direct effect on Smad2 phosphorylation. This contrasts recent observations from Xin et al. (20) that linked S1P to Smad phosphorylation in mesangial cells. Different cell types, mesangial cells versus fibroblasts, and different modes of action, exogenous versus endogenous, may account for the different results.

Our study reveals time-dependent effects of TGF-β on sphingolipid content in dermal fibroblasts. At an early time point (30 min) TGF-β treatment resulted in a general increase in ceramide amounts, whereas sphingosine and S1P amounts were reduced. These data corroborate our earlier observation show-
SphK1 and SPPase expression levels by TGF-β. Changes in sphingolipid content result from the modulation of levels were decreased. As shown in this study, prolonged in vivo expression of SphK1 in fibroblasts also led to increased intracellular levels of S1P, but no evidence of S1P secretion either after TGF-β treatment or in cells with high ectopic expression of SphK1 was found (data not shown). This observation is consistent with a report by Olivera et al. (40) that shows no secretion of S1P from NIH3T3 fibroblasts. Whereas some variations were observed between individual fibroblast cell lines, in general endogenous S1P levels were below 0.5 pmol/10^6 cells in quiescent fibroblasts. Only a 2-fold increase in S1P levels was observed after TGF-β stimulation and in cells with forced expression of SphK1. These data suggest that the intracellular levels of S1P are tightly controlled in dermal fibroblasts. S1P levels were more readily detectable in other cell types, e.g., in lung epithelial cells, and the intracellular levels of S1P were >5 pmol. Because no secretion of S1P was detected, it suggests that the elevation of the intracellular SphK1/S1P is responsible for the observed biologic effects.

How SphK1/S1P couples to the intracellular signaling pathways in dermal fibroblasts remains to be elucidated. In other experimental models, activation of SphK1 and generation of intracellular S1P has been linked to activation of the Ras/Raf/ERK cascade (11). However, in dermal fibroblasts, neither TGF-β (41) nor ectopic SphK1 (data not shown) led to appreciable ERK activation. At present, the intracellular function of SphK1 is poorly understood, and it is possible that different experimental models will operate in different cell types and under different experimental conditions. Isolation and characterization of the SphK1-interacting proteins, such as TRAF2 (15), AKAP (42), RPK118 (43), and δ-catenin/NRAP (44) would be helpful in elucidating the intracellular function of SphK1 and its mediator S1P.

Increasing evidence suggest that sphingolipid metabolism plays an important role in regulating cell fate. In general, SphK1 and S1P have been associated with the promotion of cell growth and survival, whereas ceramide has been associated with anti-proliferative and pro-apoptotic pathways (8). Because TIP-1 has been shown previously to stimulate cell growth (7), SphK1/S1P may contribute to the mitogenic effects of TGF-β via up-regulation of TIP-1 production. However, because TGF-β is a weak mitogen for fibroblasts, and its main effects are related to extracellular matrix modulation, cell adhesion, and migration, SphK1/S1P may rather function as a mediator of these cellular processes. In support of this conclusion, it has been shown that similarly to TGF-β, SphK1 promotes growth of NIH3T3 fibroblasts in soft agar, as well as formation of tumors in vivo (45). A recent study has also demonstrated that SphK1 specifically interacts with the δ-catenin/NRAP, and mediates δ-catenin/NRAP-induced cell migration (44). The biological consequences related to activation of the SphK1/S1P pathway in the context of TGF-β signaling remain to be fully elucidated.
Sphingosine Kinase 1 (SPHK1) Is Induced by Transforming Growth Factor-β and Mediates TIMP-1 Up-regulation
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