Involvement of Smad Signaling in Sphingosine 1-Phosphate-mediated Biological Responses of Keratinocytes*

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The lysophospholipid sphingosine 1-phosphate and the cytokine-transforming growth factor β are both released from degranulating platelets at wound sites, suggesting a broad spectrum of effects involved in wound healing. Interestingly, both of these molecules have been previously shown to induce chemotaxis but to strongly inhibit the growth of keratinocytes, while stimulating the proliferation of fibroblasts. In contrast to sphingosine 1-phosphate, the signaling cascade of the growth factor has been extensively examined. Specifically, Smad3 has been shown to be an essential mediator of transforming growth factor β-dependent chemotaxis of keratinocytes and mediates, in part, its growth-inhibitory effect. Here we show that sphingosine 1-phosphate, independently of transforming growth factor β secretion, induces a rapid phosphorylation of Smad3 on its C-terminal serine motif and induces its partnering with Smad4 and the translocation of the complex into the nucleus. Moreover, sphingosine 1-phosphate fails to induce chemotaxis or inhibit the growth of Smad3-deficient keratinocytes, suggesting that Smad3 plays an unexpected functional role as a new target in sphingosine 1-phosphate signaling. Both sphingosine 1-phosphate receptors and the transforming growth factor β-type I receptor serine/threonine kinase are essential for activation of Smad3 by this lysophospholipid and the dependent biological responses, indicating a novel cross-talk between serine/threonine kinase receptors and G-protein-coupled receptors.

Sphingosine 1-phosphate (S1P) has been shown to act as a critical signaling molecule to elicit a variety of physiological and pathophysiological responses (1–3). A well known source of S1P are human platelets, which release it upon activation by physiological stimuli, suggesting that S1P may play a role in endothelial injury, inflammation, thrombosis, angiogenesis, and wound healing (4, 5).

Like other crucial phospholipid mediators, S1P has been suggested to exert its actions either intracellularly as a second messenger following its formation from sphingosine by the activation of sphingosine kinase or extracellularly as a ligand for G-protein-coupled receptors (GPCR) that were originally known as the endothelial differentiation gene-1 (Edg1) family of proteins but were recently renamed S1P receptors (6). To date, five members of the S1P receptor family have been cloned, namely S1P1 (Edg1), S1P2 (Edg5), S1P3 (Edg3), S1P4 (Edg6), and S1P5 (Edg8) (7). Depending on the expression of S1P1–5 and the involvement of different G-proteins, S1P is able to influence a wide array of signal transduction pathways in diverse cell types (8). It is noteworthy, that all S1P receptors have been shown to be involved in the regulation of cell migration (8). Thus, activation of S1P1 and S1P3 by S1P results in an enhanced migratory response of a variety of cells, whereas S1P2 stimulation conversely inhibits chemotaxis of these cells (9).

Among the cell types responsive to S1P, it has been shown to be a potent chemoattractant for keratinocytes (10). Indeed, migration of this cell type is crucial for reepithelialization suggesting a positive role of S1P on cutaneous wound closure. However, whereas S1P is generally known as an important mitogen stimulus for many cell types, it strongly inhibits the proliferation and induces differentiation of human epidermal cells. Growth inhibition of keratinocytes forestalls wound closure and implies a more complex regulatory role of S1P in preventing excessive epidermal proliferation (10).

Although it is remarkable for an effector to both stimulate and inhibit cell growth, it is not unprecedented. To our knowledge, the only other factor that mimics the action of S1P on migration and proliferation of dermal and epidermal cells is transforming growth factor β (TGF-β) (11–13). TGF-β is secreted by many cells including platelets and mediates a multiplicity of biological effects depending on the cell type and its differentiation state (14). TGF-β signaling from the surface to the nucleus requires a series of interdependent events. It is initiated by the association between TGF-β and the TGF-β type II receptor, TβRII, resulting in recruitment of the TGF-β type I receptor (TβRI) into a heteromeric complex, thereby enabling activation of TβRI by the TβRII receptor serine/threonine kinase (15). The anchor protein SARA (Smad anchor for receptor activation) recruits the cytoplasmic signal transducers Smad2 and Smad3, classified as receptor-activated Smads (R-Smads) to the TβRI kinase domain, resulting in their phosphorylation on serine residues in the C-terminal SSXS motif (16). Activated R-Smads dissociate from the receptor complex and associate, caused by conformational changes at the phosphorylation sites,
with the common partner Smad4. The resulting heteromeric Smad complexes are translocated into the nucleus, where they directly or indirectly regulate the transcription of target genes (17, 18). It has been demonstrated that the effects of TGF-β on keratinocyte migration and proliferation are due to activation of a specific member of the Smad family, namely Smad3. Keratinocytes prepared from Smad3 null mice show little or no TGF-β-induced chemotaxis and partly reduced inhibition of growth following treatment with TGF-β (19, 20).

Interestingly, agents not belonging to the TGF-β superfamily can also activate Smad2 and Smad3. Thus, it has been reported that the insulin-like growth factor binding protein-3 (IGFBP-3) can stimulate Smad2 and Smad3 phosphorylation. Although this activation occurs in the absence of endogenous or exogenous TGF-β, it requires the TpRII, implying an interaction between IGFBP receptors and TGF-β receptors (21–23).

SIP receptors are also known to cross-communicate with other GPCRs as well as other plasma membrane receptors, including receptor-tyrosine kinases (24–26). For example, SIP and platelet-derived growth factor (PDGF) have been shown to act via PDGF-β- and SIP-1-receptor complexes in airway smooth muscle cells (27).

Given the strong functional overlap of the actions of TGF-β and SIP on keratinocytes, we have examined possible cross-talks between the TGF-β signaling pathway and that of SIP. We now report that SIP can stimulate phosphorylation of Smad3 in human as well as mouse keratinocytes requiring both SIP receptors and TGF-β receptors. Moreover, we have used both antisense technology and Smad3 null mouse keratinocytes to show that this activation is essential for the stimulation of migration and the inhibition of proliferation of the epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**SB431542 was a gift from Dr. Laping (GlaxoSmithKline, King of Prussia, PA), and hemagglutinin (HA)-tagged TpRII plasmids (backbone pCMV5, which served as control vector) were constructed by Dr. Attisano and Dr. Wrana (Memorial Sloan-Kettering Cancer Center, New York). Oligonucleotides were synthesized at Tib Molbiol (Berlin, Germany). SIP, sphingosine, and N-acetylphosphosine (C2-ceramide) were purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). TGF-β(C2-ceramide) were purchased from Bioventura (Berlin, Germany). Rabbit polyclonal antiphosphoserine antibodies, followed by a precipitation with 10 mg/g of anti-Smad3 or anti-Smad4 antibodies and normal goat IgG, anti-goat, anti-mouse, and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antiphosphoserine antibodies were obtained from Zymed Laboratories (San Francisco, CA). LumiGlo reagent and peroxide were obtained from New England Biolabs (Beverly, MA). Aprotinin, collagen-1, deoxycholic acid, Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Keratinocytes were scraped from the epidermis, washed with PBS, and cultured for 5 min at 25°C. The pellets were resuspended in keratinocyte growth medium that was prepared from keratinocyte basal medium by the addition of 0.1 ng/ml recombinant EGF, 5.0 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.15 mM CaCl2, 30 μg/ml bovine pituitary extract, 50 μg/ml gentamicin sulfate, and 50 ng/ml amphotericin B. Keratinocytes were pooled from several donors and cultured at 37°C in 5% CO2. For all experiments, only cells of the second or third passage were used.

Murine keratinocytes were isolated from polymerase chain reaction-genotyped Smad3 wild-type and knockout newborn mice as described (28). Briefly, skin of sacrificed mice was numbered and disinfected by iodine solution. Mice were washed three times in 70% ethanol, and tails from each mouse were removed for reverse transcriptase-PCR. The entire skin was mechanically separated and incubated at 4°C in a solution of 0.25% trypsin and 0.2% EDTA for 20 h. According to the results of the reverse transcriptase-PCR, the epidermal layers of homozygous wild-type and Smad3 knockout mice were pooled separately and homogenized. The cell suspensions were centrifuged at 250 × g for 5 min, washed with PBS, and resuspended in growth medium that was prepared from Eagle’s minimal essential medium by the addition of 8% fetal bovine serum, 0.2 mM CaCl2, and 1% penicillin/streptomycin. The next day, the medium was switched to Eagle’s minimal essential medium/serum containing 0.05 mM CaCl2.

**Deletion of Smad3 and SIP—**Using Antisense—Smad3 and SIP, antisense oligonucleotides were designed to surround the transcellular activation site, a place empirically known to be most effective for inhibition of gene expression. Smad3 antisense oligonucleotides 5′-GAGTtggACgACAT-3′ were synthesized based on the mammalian Smad3 cDNAs. Same-length control oligonucleotides (with the same nucleotides but randomly scrambled sequence) to Smad3 were also synthesized: 5′−TggACgTAgTAgACgC-3′. To reduce SIP, protein expression, the following oligonucleotide was used: 5′-GAGTGGtggCCCCAT-3′. The following oligonucleotide served as control: 5′-ATgggCACCACAgtgC-3′.

**Immunoprecipitation of Smad Proteins—**Smad immunoprecipitation and blotting were performed as described recently (30). Briefly, keratinocytes seeded in 6-well plates were cultured for 24 h, and then medium was replaced by RIPA buffer (1 ml) for 2 h. Cells were treated with TGF-β or SIP in different concentrations and time periods. Keratinocytes were rinsed twice with ice-cold PBS and harvested in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS), containing protease inhibitors (1 mg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotonin, and 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM NaF, and 40 mM β-glycerophosphate). Lysates were centrifuged at 14,000 × g for 30 min. 100 μg of lysate protein was immunoprecipitated overnight at 4°C with 0.2 μg of anti-Smad3 or anti-Smad2 antibodies, followed by a precipitation with 10 μl of protein G plus agarose at 4°C for 90 min. After four washes with complete RIPA buffer, the immunoprecipitates were eluted by boiling for 5 min in 60 μl of SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue, 20% glycerol, 200 mM dithiothreitol).

**Western Blot Analysis—**For Western blot analysis, immunoprecipitates (20 μl) were separated by SDS-PAGE. Gels were blotted overnight onto polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk (for anti-Smad3, anti-Smad4, and anti-phospho-Smad3-antibodies) or 3% bovine serum albumin (for anti-phospho-serine) in Tris-buffered saline-Tween (0.1%) overnight at 4°C, the blots were incubated with anti-Smad3, anti-Smad4, anti-phospho-Smad3 (each 0.2 μg/ml), or antiphosphoserine antibodies (1 μg/ml) for 2 h at room temperature. The blots were washed three times in Tris-buffered saline-Tween followed by incubation with the secondary antibodies (anti-goat IgG-horseradish peroxidase, anti-rabbit IgG-horseradish peroxidase, and anti-mouse IgG-horseradish peroxidase). After washing, the blots were developed according to the manufacturer’s protocol.

**Isolation of Nuclei—**Nuclei were isolated as described (31). Briefly, keratinocytes were washed with PBS, scraped into PBS, and then...
centrifuged at 5,000 × g for 5 min. Cells were resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.5, 5 mM MgCl₂, 15 mM KCl, 1 mM phenylmethylsulfonyl fluoride) at 5 × 10⁶ cells/ml, frozen in liquid nitrogen, thawed rapidly at 37 °C, and then passed through a 25-gauge needle 15 times. The homogenate was layered on top of 200 μl of a sucrose gradient (50% sucrose in buffer A) and centrifuged at 15,000 × g for 5 min. Intact nuclei, pelleted through the cushion, were suspended in RIPA buffer and lysed by freezethawing. For determination of the nuclei purity, activities of marker enzymes for cytosol and endoplasmic reticulum, lactate dehydrogenase, and α-glucoisidase II were measured. Lactate dehydrogenase activity was examined according to the manufacturer’s protocol, and α-glucoisidase II activity was measured as described (32). Briefly, 4-methylumbelliferyl-α-D-glucoside was used as substrate in pH 4.5 buffer (pH 7.0, containing 0.1% Triton X-100), and assays were carried out for 15 min at 37 °C. Reactions were terminated by the addition of ice-cold 250 mM potassium glycine (pH 10.3), and the release of 4-methylumbellifereone was measured (365 nm excitation, 450 nm emission).

**Binding of the Smad3-Smad4 Complex to Biotinylated DNA—Human keratinocytes were plated into 100-mm dishes (1 × 10⁶ cells/dish) and cultured until a confluence of 30–40% was reached. Cells were stimulated as described above, rinsed twice with ice-cold PBS, and harvested into lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA), which was supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin, leupeptin, and pepstatin. After centrifugation at 14,000 × g for 30 min by protein detection, 1 mg of lysate protein was incubated at 4 °C for 1 h with a biotinylated double-stranded DNA (30 μM) composed of three tandemly repeated CAGA sequences in the presence of 12 μg of poly(dI-dC). DNA-bound proteins were precipitated with 80 μl of streptavidin-agarose at 4 °C for 30 min, washed several times with lysis buffer, and eluted by boiling for 5 min in 50 μl of SDS sample buffer for electrophoresis and Western blot analysis using anti-Smad3 antibodies.

**Cross-communication of TGF-β and S1P—Receptors—**Human keratinocytes (5 × 10⁵/10-cm dish) were cultured for 12 h in keratinocyte growth medium and then transfected with HA-tagged TβRI receptor plasmids (1 μg of DNA/ml, 1:2 complex with Fugene). Transfection control was performed by the use of a control plasmid (1:2 complex with Fugene). After 24 h, medium was replaced again by fresh keratinocyte growth medium following by a stimulation with control vehicle or 10 μM S1P. Keratinocytes were rinsed twice with ice-cold PBS and harvested in complete RIPA buffer. After centrifugation and protein determination, 10 μg of each lysate was prepared for electrophoresis and Western blot analysis with anti-HA antibodies to reveal an equally high transfection efficacy. 1 mg of lysate protein was immunoprecipitated overnight at 4 °C with 2 μg of anti-HA antibodies, followed by a precipitation with 40 μl of protein G plus agarose at 4 °C for 90 min. After four washes with complete RIPA buffer, the immunoprecipitates were eluted by boiling for 5 min in 80 μl of SDS sample buffer and electrophoresed in 10% SDS-gels. Western blot analysis was performed with anti-S1P, antibodies as primary antibodies.

**Measurement of TGF-β Secretion—**Cells were stimulated with S1P (10 μM) over a time period of 1 h. Then TGF-β was quantified by selective enzyme-linked immunosorbent assay kits following the instructions of the manufacturer. Samples were analyzed by an enzyme-linked immunosorbent assay reader from Merlon (Bornheim, Germany). The detection limit was 4 pg/ml.

**DNA Synthesis—**Keratinocytes (4 × 10⁶ cells/well) were grown in 24-well plates for 24 h. Then medium was replaced by fresh keratinocyte growth medium, and cells were incubated with the indicated substances for 72 h and pulsed with 1 μCi of [methy]-H-thymidine per well. At the end of incubation, medium was removed and each cell was washed twice with each with PBS and ice-cold trichloroacetic acid (5%). The precipitated material was dissolved in 0.3 N NaOH solution, and incorporated [methy]-H-thymidine was determined in a scintillation counter (MicroBeta™ Plus, Wallac Oy, Turku, Finland).

**Migration Assays—**Migration of human keratinocytes was measured using chemotaxis chambers as recently described (10). Therefore, the lower chamber, separated by a fibronectin-coated membrane, was supplemented with S1P, serum, or TGF-β in the indicated concentrations. Cells that had migrated through the membrane were fixed, stained by Giemsa, and counted. Each value represents the average number of cells migrated from triplicate wells.

**RESULTS**

**SIP Induces Smad3 Activation in Human Native Keratinocytes—**Based on the similarity between the actions of S1P and TGF-β on keratinocytes (10, 11) and the requirement for Smad3 to mediate the chemotactic as well as the antiproliferative properties of TGF-β (19), we investigated whether Smad3 might also be a direct or indirect mediator of S1P on keratinocyte actions. To eliminate possible artifacts based on overexpression systems using transformed cells, we examined effects of TGF-β and S1P on Smad3 activation in primary human keratinocytes. In agreement with previous studies using transformed cells, TGF-β induced a transient phosphorylation of Smad3, in the absence of effects on its level of expression. A phosphorylation was detectable 10 min after exposure to TGF-β, which returned to basal levels after 90 min (Fig. 1A). Most interestingly, treatment of keratinocytes with S1P also resulted in phosphorylation of Smad3 on its C terminus (Fig. 1B). The kinetics of S1P phosphorylation of Smad3 were slightly more rapid than that seen for activation by TGF-β. Keratinocytes responded to S1P in a rapid activation of Smad3 after 10 min, which declined to baseline after 90 min. The phosphorylation process induced by S1P was also dose-dependent. A significant increase was detected at a concentration of 1 μM S1P, whereas a maximal effect occurred at 10 μM S1P, inducing a phosphorylation similar to that of the most effective dose of TGF-β. It should be mentioned that also a transient Smad2 phosphorylation occurred in response to TGF-β and S1P (Fig. 1D).

**SIP Induces the Dimerization of Smad3 and Smad4, the Translocation of the Complex into the Nucleus, and Its Binding to DNA Target Promoters—**To verify that Smad3 phosphorylation is specific for S1P and not a consequence of S1P metabolites, we also examined the effects of exogenously added sphingosine and ceramide on Smad3 activation. As presented in Fig. 2A, neither sphingosine nor the short chain C2-ceramide at micromolar concentrations induced a phosphorylation of Smad3 after 10 min. A similar result was obtained in response to a stimulation period of 30 and 45 min. To further substantiate the particular role of S1P, we also measured downstream events after phosphorylation of Smad3. Phosphorylation on the C-terminal SSXS motif of Smad3 enables it to dimerize with Smad4 and correlates with translocation of the complex from the cytoplasm to the nucleus and binding to promoters of Smad3-responsive genes. Based on this, we examined whether phosphorylation of Smad3 by S1P is accompanied by the formation of a functional Smad3-Smad4 complex. Immunoprecipitation of keratinocyte lysates with anti-Smad3 antibodies followed by Western blotting for Smad3 or Smad4 showed that Smad3 expression levels were not influenced by the treatment with TGF-β or S1P. As expected, no Smad4 co-immunoprecipitated under control conditions. However, Smad3-Smad4 complexes were detected not only after stimulation with TGF-β but also after exposure to S1P (Fig. 2B). Similar to the phosphorylation experiments, both TGF-β and S1P led to a Smad3-Smad4 complex formation after 10 min and peaked at 30 min (data not shown).

We also investigated whether S1P would induce nuclear translocation of the Smad3-Smad4 complex as reported for TGF-β. Cells were stimulated with TGF-β or S1P, lysed, and separated into nuclear and cytosolic fractions using a sucrose gradient. Measurement of marker enzymes specifically localized in the cytosol (lactate dehydrogenase) or in the endoplasmic reticulum (α-glucoisidase II) confirmed the purity of the
nuclei. Thus, less than 4% of lactate dehydrogenase activity and 8% of α-glucosidase II was found in the purified nuclei (not shown). Indeed, Western blotting of Smad3 and Smad4 in the lysates of nuclei revealed the absence of both Smad proteins under control conditions. However, when cells were stimulated either with TGF-β or S1P for 30 min, both Smad3 and Smad4 appeared in the nuclear fraction, indicating their translocation from the cytosol (Fig. 2).

As final confirmation of the formation of a functional DNA-binding complex, we used a DNA affinity assay with a biotinylated oligonucleotide probe representing a tandemly repeated CAGA Smad3-Smad4 binding element (33). The DNA-Smad complex was precipitated with streptavidin-agarose followed by Western blot analysis for Smad3. Again, a significant increase in Smad3/DNA binding occurred, when cells were exposed to either TGF-β or S1P, consistent with the S1P-dependent activation of Smad3 (Fig. 2D).

**Smad3 Is Essential for S1P-mediated Proliferation and Migration of Keratinocytes**—Since these results clearly demonstrate activation of Smad3 by S1P, a further aspect of interest was to examine whether Smad3 is necessary to mediate effects of S1P on chemotaxis and growth inhibition of keratinocytes, as shown previously for TGF-β (19). Incubation of keratinocytes with Smad3-antisense phosphothioate oligonucleotides diminished Smad3-protein expression, whereas scrambled oligonucleotides had no effect (Fig. 3A). In the presence of scrambled oligonucleotides, both TGF-β and S1P potently induced native human keratinocyte migration in a similar extension (Fig. 3B). However, in the presence of Smad3 antisense oligonucleotides, the chemotactic response toward TGF-β was almost completely lost, whereas a normal motility to serum-containing media was maintained. This result demonstrates that the chemotactic activity of S1P on human keratinocytes, similar to that of TGF-β, is dependent on Smad3 expression (Fig. 3B).

As a measure of effects of Smad3-antisense oligonucleotides on growth inhibition by TGF-β and S1P, DNA synthesis was determined by thymidine incorporation (Fig. 3C). Both TGF-β (1 ng/ml) and S1P (10 μM) decreased thymidine incorporation by greater than 60%, and this was partially, but significantly, reversed by the addition of Smad3 antisense but not of scrambled control oligonucleotides (Fig. 3C). To more rigorously confirm that these effects of S1P were dependent on Smad3, we utilized primary keratinocytes isolated from newborn wild-type or Smad3<sup>−/−</sup> mice (29). Similar to our results in human keratinocytes, a strong migratory response toward S1P and TGF-β was visible in control keratinocytes derived from wild-type mice (Fig. 4A). On the contrary, Smad3-deficient keratinocytes did not migrate toward either stimulus. Again, stimulation with serum served as a positive control, with Smad3-deficient keratinocytes showing a normal chemotactic activity toward serum-containing medium (Fig. 4A).

Smad3-deficient and wild-type keratinocytes are also distinguished by their growth inhibitory responses to TGF-β and S1P (Fig. 4B). Thus, the antiproliferative effect of either TGF-β or S1P was reduced in Smad3-deficient keratinocytes compared with wild-type cells (Fig. 4B). Taken together, these results clearly indicate the critical role of Smad3 in both S1P- and TGF-β-mediated chemotaxis and proliferation of keratinocytes.

**Cross-talk of S1P Receptors and TGF-β Signaling**—It has been suggested that S1P mediates its action either extracellularly via GPCR (S1P<sub>1-3</sub>) or intracellularly after being formed by sphingosine kinase (5, 7). To address whether S1P receptors are required for the migratory response of the lysosphospholipid, we assessed the effects of pretreatment with PTX on chemotaxis and Smad3 phosphorylation induced by S1P. The migratory response to exogenously added S1P was completely sensitive to PTX, indicating the involvement of G<sub>αi</sub>-coupled S1P receptors (Fig. 5A). As expected, PTX had no effect on chemotaxis induced by TGF-β (Fig. 5A). In parallel, preincuba-
**Fig. 2. Smad3 phosphorylation is a specific response to S1P, which also induces dimerization of Smad3-Smad4, translocation into the nucleus, and binding of the dimer to DNA target promoters.** Keratinocytes were stimulated with the indicated concentrations of S1P, C2-ceramide (C2-Cer), or sphingosine (Sph) for 10 min. Lysates were immunoprecipitated with anti-Smad3 antibodies or with normal goat IgG (IgG-control), and Western blot analysis was performed with anti-Smad3 (lower panel) and anti-P-Smad3 antibodies (upper panel) (A). Cells were treated with control vehicle, TGF-β (2 ng/ml), or S1P (10 μM) for 30 min. After immunoprecipitation with normal goat IgG (IgG-control) or anti-Smad3 antibodies, the complexes were electrophoresed followed by immunoblotting. The top blot was developed using anti-Smad4 antibodies, and the bottom blot was developed with anti-Smad3 antibodies (B). Keratinocytes were stimulated with control vehicle, TGF-β (2 ng/ml), or S1P (10 μM) for 45 min, followed by the isolation of nuclei as described under “Experimental Procedures.” Lysates of nuclei were immunoprecipitated with anti-Smad3 antibodies, followed by electrophoresis and immunoblotting. The top blot was developed using anti-Smad4 antibodies, and the bottom blot was developed with anti-Smad3 antibodies (C). Lysates of TGF-β (2 ng/ml)- or S1P (10 μM)-stimulated cells were incubated with a biotinylated double-stranded DNA (30 nM) composed of three tandemly repeated CAGA sequences. DNA-bound proteins were precipitated with streptavidin-agarose, and electrophoresis and Western blot analysis using anti-Smad3 antibodies were performed (D). All results were confirmed in three independent experiments.

**DISCUSSION**

These data are, to our knowledge, the first to demonstrate a complex interplay between both S1P and TGF-β receptors in S1P-mediated actions, we also measured migration in the presence of SB 431542. Consistent with the phosphorylation experiments, migration of keratinocytes toward S1P was inhibited, whereas serum-induced chemotaxis was not influenced (Fig. 6B). To more rigorously prove a cross-communication between S1P receptors and TGF-β receptors, we transfected human keratinocytes with an HA-conjugated TβRII plasmid. Then keratinocytes were stimulated with S1P followed by an immunoprecipitation of TβRII receptors with anti-HA antibodies and protein G plus agarose. After electrophoresis of precipitates, Western blot analysis was performed with anti-HA antibodies. As presented in Fig. 6C (lower panel), precipitated lysates of transfected keratinocytes showed equal amounts of HA-coupled TβRII protein. Most interestingly, development with anti-S1P1 antibodies revealed that a complex of the HA-conjugated TβRI receptors and the S1P1, receptors was only formed when keratinocytes were stimulated with the lysophospholipid (Fig. 6C, upper panel). These experiments suggest a complex interplay between both S1P and TGF-β receptors to affect S1P-mediated Smad3 activation.

**A** Smad3 phosphorylation. **B** Smad3/Smad4 co-immunoprecipitation. **C** Nuclear Smad3 and Smad4. **D** DNA-affinity precipitation.
Chemotaxis and growth inhibition of wild-type and Smad3-deficient keratinocytes in response to S1P and TGF-\(\beta\). For Smad3 expression and migration assays, cells were seeded in 10-cm diameter dishes and incubated with Smad3-antisense or scrambled oligonucleotides (ODN; each 20 \(\mu\)M) for 24 h. To reveal the down-regulation of Smad3 by the antisense treatment, 10 \(\mu\)g of lysate protein was electrophoresed and immunoblotted with anti-Smad3 antibodies (A, upper panel). As loading control, immunoblotting was performed using anti-actin antibodies (A, lower panel). The chemotactic migration of keratinocytes in response to a gradient of S1P (1 \(\mu\)M) or TGF-\(\beta\) (2 ng/ml) was measured in a modified Boyden chamber as described. Each value represents the average number of cells \(\pm\) S.D. migrated from triplicate wells (B). For growth inhibition experiments, cells were seeded in 24-well plates and incubated with Smad3-antisense or scrambled oligonucleotides as indicated. Medium was renewed and supplemented for 24 h with oligonucleotides, TGF-\(\beta\) (2 ng/ml), or S1P (10 \(\mu\)M) followed by a treatment with \(^{3}\text{H}\)-thymidine for another 24 h. Incorporation of radioactivity into cells was determined by scintillation counting. Each condition was analyzed in triplicate (C). Similar results were obtained in three independent experiments. *, \(p < 0.05\); **, \(p < 0.001\).
of S1P receptors have not been well defined. Here we provide evidence that activation of the TGF-β cascade is a crucial event for biological responses of S1P, at least in keratinocytes, since it fails to induce chemotaxis and is a less potent growth inhibitor when Smad3 signaling is disrupted.

Our data reveal a phosphorylation of Smad3 and Smad2 in response to S1P. Normally, C-terminal phosphorylation by the TβRI is the key event in Smad2 and Smad3 activation (18). However, other kinase pathways further regulate Smad signaling as suggested by the complex phosphorylation patterns of endogenous Smads. It has been reported that mitogen-activated protein kinase activation also causes phosphorylation of Smad3 at specific sequences, which are separate from the SSXS motif, leading to an inhibition of nuclear translocation (40). Since S1P can stimulate mitogen-activated protein kinase activity in various cells (41), it was important to show that S1P-dependent phosphorylation of the C-terminal SSXS motif leads to a functional Smad3-Smad4 complex. Indeed, S1P, like TGF-β, resulted in an increased hetero-oligomerization and nuclear translocation of Smad3 and Smad4, demonstrating that Smad3 is not only C-terminally phosphorylated but also functionally activated by S1P. The conclusive proof that Smad3 protein is not only activated but also plays a pivotal role in biological responses to S1P is the complete loss of chemotaxis and a partial reduction of the cell growth-inhibitory effect in Smad3-deficient keratinocytes stimulated with S1P.

The query remains about the nature of the interaction between the S1P and TGF-β signaling. S1P has been shown to act both intracellularly as a second messenger and extracellularly through binding to GPCR (8, 42–45). Indeed, in human fibroblasts, it has been shown that endogenous sphingolipid metabolites are involved as coregulators of the TGF-β-induced signaling cascade (46). Thus, TGF-β increases levels of intracellular ceramide, which is generated via degradation of sphingomyelin by a family of sphingomyelinases. Both TGF-β and exogenously added cell-permeable ceramides activate an α2(I) collagen promoter/reporter gene construct transfected into fibroblasts. It has also been suggested that ceramide may facilitate the interaction of the endosomal anchor protein SARA, which colocalizes with the TGF-β receptors and facilitates the recruitment of Smad2 and Smad3 to the receptor complex. Most interestingly, intracellular S1P has also been proposed to influence TGF-β signaling. However, in contrast to ceramides, it has been argued that S1P may act as an endogenous inhibitor of TGF-β signaling, since overexpression of sphingosine kinase, the crucial enzyme in the formation of S1P, interferes with induction of the α2(I) collagen promoter by TGF-β (46).

In contrast to an endogenous role of S1P, here we clearly provide evidence, at least in keratinocytes, that S1P influences TGF-β signaling through activation of GPCR, since Smad3 phosphorylation is completely abolished in the presence of PTX. Moreover, keratinocytes treated with PTX showed no migratory response toward S1P. Consistently, many studies revealed the participation of S1P receptors to induce chemotaxis (47–52). An involvement of GPCR in the S1P-induced growth inhibition has also been described, although intracellular actions may additionally contribute to the antiproliferative properties (10).

Furthermore, our data suggest that also the TβRI is essential for Smad3 phosphorylation by S1P, since inhibition of the ATP binding of the kinase domain by SB 431542 (53) blocks not only Smad3 activation but also the S1P-induced migration. This is not surprising, since TβRI is, to our knowledge, the only kinase with the specificity for the C-terminal phosphorylation of Smad2 and Smad3. However, since S1P induces Smad signaling in the absence of exogenously added TGF-β, our findings reveal for the first time a cross-communication of GPCR with receptor serine/threonine kinases.

Indeed, similar interactions have also been suggested between GPCR and receptor tyrosine kinases, since there exists a transactivation of S1P1 with the PDGF receptor (26, 27, 34, 54). In these reports, it has been shown that cell migration toward PDGF is dependent on expression of S1P1, and moreover PDGF is capable of activating this S1P receptor. However, it should be mentioned that controversial studies indicate that PDGF-in-
duced chemotaxis does not require the S1P1 receptor (55).

A more detailed mechanism has been described for lysophosphatidic acid, which is also an agonist for the Edg family of lysophospholipid receptors. Daub et al. (56) found that in fibroblasts, lysophosphatidic acid induces a rapid phosphorylation of the epidermal growth factor receptor (EGFR). Several mechanisms are discussed that connect plasma membrane-receptor interactions. GPCR transactivation of EGFR occurs through generation of cognate ligand, the heparin-binding EGF, which activates the EGFR signal transduction. Heparin-binding EGF is generated through extracellular proteolytic cleavage of proheparin-binding EGF, a membrane-spanning latent form of this growth factor, that is mediated by the action of a GPCR-stimulated metalloproteinase (57–59). A more direct mechanism involves the GPCR-triggered recruitment of receptor tyrosine kinases in a complex with cytoplasmic tyrosine kinases and the subsequent activation of the EGFR. This might also occur in higher order complexes that involve scaffolds or membrane microdomains (60–62).

In this context, it is of interest that the EGF cascade is also connected to TGF-β signaling. Thus, EGF can mediate Smad-dependent reporter gene activation and induces a phosphorylation of endogenous Smad proteins (63). Most interestingly, these studies suggest that the TβRI is not responsible for the EGF-induced Smad phosphorylation, since it is evident in R1B cells, which lack the type I receptor. Additionally, IGFBP-3 is a further biological mediator, which is capable to stimulate Smad2 and Smad3 phosphorylation and moreover to potentiate TGF-β-induced Smad activation (21, 23). However, in contrast to the EGF-induced Smad activation, these studies indicate that IGFBP-3 signaling in T47D and MCF-7 breast cancer cells activates TβRII and that this requires the presence of TβRII. It is of interest that this stimulation even occurs in the absence of endogenous or exogenous TGF-β. However, it is not yet known whether IGFBP-3 is a direct ligand for TβRII or initiates signaling in an indirect manner. Thus, it has been considered that specific cell surface receptors for IGFBP-3 exist that may cross-talk to TGF-β receptors. But even an intracellular interaction of IGFBP-3 with TβRI cannot be excluded (22).

Thus, an attractive area of future research is to further elucidate the mechanism of how S1P receptors may interact with receptor serine/threonine kinases. A tantalizing notion is that a variety of biological actions of TGF-β are mediated by the activation of other Smad family members (29, 39, 64, 65). Some of these biological responses like matrix protein formation, embryogenesis, and angiogenesis are mimicked by S1P. Thus, it is of great interest to investigate the involvement of Smad protein family members in further S1P-mediated effects, since this will uncover new insights into related diseases and might result in new treatment opportunities.

**FIG. 6. Involvement of TβRI receptors in S1P-mediated migration and Smad3 phosphorylation.** Human keratinocytes were pretreated with SB431542 (10 μM) for 30 min (A and B). Then chemotactic migration (A) or Smad3 phosphorylation (B) in response to a gradient of S1P (1 μM), TGF-β (2 ng/ml), or serum (10%), where indicated, was measured as described (*, p < 0.05; **, p < 0.001). Human keratinocytes were transfected with HA-tagged TβRI or control plasmids. Transfected cells were treated with S1P in the indicated concentrations, followed by lysing and immunoprecipitation using anti-HA antibodies and protein G plus agarose. Western blot analysis using anti-HA antibodies confirmed equal amounts of HA-TbRI protein (C, lower panel). Co-immunoprecipitation of HA-tagged TβRI with S1P receptors was revealed by Western blot analysis using anti-S1P1 antibodies (C, upper panel). Similar results were obtained in three independent experiments.
Involvement of Smad Signaling in Sphingosine 1-Phosphate-mediated Biological Responses of Keratinocytes

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