Sphingosine-1-Phosphate and Its Potentially Paradoxical Effects on Critical Parameters of Cutaneous Wound Healing

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The sphingolipid metabolite sphingosine-1-phosphate has emerged as a new bioactive molecule involved in the regulation of cell growth, differentiation, survival, and chemotaxis as well as angiogenesis and embryogenesis. These effects are mediated either via G-protein-coupled receptors or through intracellular actions. The most prominent sources of sphingosine-1-phosphate are human platelets suggesting its potential role in wound healing. In agreement with a positive function on reconstruction of wounded skin, we identified sphingosine-1-phosphate as a potent chemoattractant for keratinocytes as well as an activator of extracellular matrix production by fibroblasts. An unexpected finding is a strong cell growth arrest of keratinocytes after exposure to sphingosine-1-phosphate, as keratinocyte proliferation is critical for re-epithelialization of the wound. Most interestingly, the anti-proliferative effect of sphingosine-1-phosphate is not a result of cytotoxicity or apoptosis as sphingosine-1-phosphate even protects these cells from programmed cell death. Moreover, sphingosine-1-phosphate enhances differentiation of keratinocytes. To investigate further by which signaling pathway cell growth inhibition is mediated expression of the mRNA of all sphingosine-1-phosphate receptors ($S1P_{1-5}$) was identified. 1 (Edg 1), 2 (Edg 5), 3 (Edg 3), 4 (Edg 6), and 5 (Edg 8) mRNA in keratinocytes was identified. As demonstrated in guanosine 5-[γ-^{35}S] triphosphate-γS binding assays, these G-protein-coupled receptors are functional at nanomolar concentrations. As the anti-proliferative effect of sphingosine-1-phosphate is only partially inhibited in the presence of pertussis toxin, it was investigated if intracellular actions are also involved. Microinjections of sphingosine-1-phosphate in keratinocytes also reduce proliferation suggesting that both sphingosine-1-phosphate receptors as well as intracellular actions mediate sphingosine-1-phosphate-induced cell growth arrest. ***Key words: sphingolipids/sphingosine/1-phosphate receptors/keratinocyte proliferation/migration/extracellular matrix protein formations. J Invest Dermatol 120:693–700, 2003***

The bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) plays a prominent part as a signaling molecule to elicit a variety of physiologic and pathophysiologic responses. In particular, S1P is involved in the regulation of cell proliferation, differentiation, survival, and motility (Goetzl and An, 1998; Spiegel *et al*, 1998; Pyne and Pyne, 2000; Spiegel and Milstien, 2000). A well known source of S1P are human platelets, from which it is released upon activation by physiologic stimuli, suggesting that S1P is a significant factor involved in endothelial injury, inflammation, thrombosis, angiogenesis, and wound healing by increasing migration and proliferation of endothelial cells (Yatomi *et al*, 1997; Ruwisch *et al*, 2001).

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Abbreviations: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; BrdU, bromodesoxyuridine; Edg, endothelial differentiation gene; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; LPA, lysophosphatidic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAI-1, plasminogen activator inhibitor-1; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; S1P, sphingosine-1-phosphate.

Growing interest in S1P has been increased by the discovery of a family of distinct G-protein-coupled receptors, which originally were designated endothelial differentiation gene (Edg) receptors (Verlinden *et al*, 1998). Most recently, a nomenclature subcommittee of the International Union of Pharmacologists has renamed these receptors according to the binding ligand and the order of discovery. To date, five S1P receptors and three lysophosphatidic acid (LPA) receptors have been identified, which are named $S1P_1$ (Edg 1), $S1P_2$ (Edg 5), $S1P_3$ (Edg 3), $S1P_4$ (Edg 6), $S1P_5$ (Edg 8); and LPA_1 (Edg 2), LPA_2 (Edg 4), LPA_3 (Edg 7) (Fukushima *et al*, 2001; Chun *et al*, 2002). Intracellular signaling pathways activated by the cloned S1P receptors have been characterized in heterologous expression systems indicating an influence on cyclic adenosine monophosphate levels as well as activation of phospholipase C, Ras, mitogen-activated protein kinase, Rho, and several protein tyrosine kinases (An *et al*, 1998; Lee *et al*, 1998; Okamoto *et al*, 1998, 1999; Van Brocklyn *et al*, 1998; Zondag *et al*, 1998; Gonda *et al*, 1999).

Nevertheless, several lines of incidence indicate that S1P also acts as an intracellular second messenger. As sphingosine kinase is the crucial enzyme for the formation of S1P, numerous stimuli have been identified to increase its activity and subsequent intracellular S1P levels. These stimuli comprise platelet-derived growth factor, nerve growth factor, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), phorbol myristate acetate, activation of the formyl peptide receptor by *N*-formyl-methionyl-leucyl-

phenylalanine, and cross-linking of the FcεRI receptors by antigens (Olivera and Spiegel, 1993; Mazurek *et al*, 1994; Choi *et al*, 1996; Edsall *et al*, 1997; Kleuser *et al*, 1998; Melendez *et al*, 1998; Alemany *et al*, 1999). In agreement, inhibition of sphingosine kinase activity utilizing *N,N*-dimethylsphingosine prevents cellular proliferation induced by mitogenic stimuli and revokes the cytoprotective effects of protein kinase C, nerve growth factor, and 1,25-$(OH)_2D_3$ (Cuvillier *et al*, 1996; Edsall *et al*, 1997; Kleuser *et al*, 1998). In addition, increase of intracellular S1P levels by microinjection mobilizes calcium from internal stores and induces proliferation of Swiss 3T3 cells (Van Brocklyn *et al*, 1998). Furthermore, sphingosine kinase has been overexpressed in NIH 3T3 and HEK 293 cells leading to an increase of intracellular S1P levels and a subsequent promotion of cell growth and survival (Olivera *et al*, 1999). In these studies an export of S1P from the cytoplasm to the extracellular space was not observed. On the contrary in a further study it has been reported that transfection of HEK 293 with sphingosine kinase leads to a constitutive export of the enzyme into the extracellular environment suggesting an external formation of S1P (Ancellin *et al*, 2002).

Interestingly, S1P is stored in human platelets and released at wounded sites suggesting a positive role of this sphingolipid metabolite in the process of wound healing of the skin (Lee *et al*, 2000). Contrary to these expectations, most recently we found that S1P is increased in keratinocytes after treatment with those doses of 1,25-$(OH)_2D_3$, which inhibit keratinocyte proliferation (Manggau *et al*, 2001). This is of interest as a cell growth inhibitory effect of keratinocytes opposes re-epithelialization of the skin. Therefore, the influence of S1P on critical parameters of wound healing such as proliferation and migration of keratinocytes as well as proliferation and matrix formation of fibroblasts was investigated. In agreement with results of several fibroblast cell lines S1P induced proliferation of primary fibroblasts. Moreover, matrix protein formation by S1P was observed. In keratinocytes S1P enhanced migration but induced a significant cell growth arrest of human keratinocytes not due to toxic or apoptotic effects. The latter finding was unexpected as inhibition of cell growth of keratinocytes decelerates re-epithelialization of the cutaneous barrier. Additionally, despite expression of functional S1P receptors, the anti-proliferative effect was also visible after microinjection of S1P.

MATERIALS AND METHODS

Materials 1,25-$(OH)_2D_3$ was a generous gift from Leo Pharmaceuticals (Ballerup, Denmark). [methyl-^{3}H]Thymidine (35 Ci per mmol), [^{3}H]putrescine (80 Ci per mmol), [^{35}S]guanosine 5-[y-^{35}S] triphosphate (GTPγS) (1000 Ci per mmol), and gelatin Sepharose 4B were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). [^{35}S]Methionine (1175 Ci per mmol) was from Perkin Elmer (Boston, MA). S1P was purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). Transforming growth factor (TGF)-β was from Calbiochem (Bad Soden, Germany). Mouse monoclonal anti-human plasminogen activator inhibitor 1 (PAI-1) and mouse monoclonal anti-human fibronectin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dimethylcasein, putrescine, propidium iodide, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, deoxypyridoxine, bovine serum albumin (BSA), Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide, GIEMSA, Dulbecco's modified Eagle's medium (DMEM), aprotinin, pepstatin, and leupeptin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal bovine serum was from Seromed Biochrom (Berlin, Germany). Keratinocyte growth medium (KGM), keratinocyte basal medium (KBM), epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, gentamicin sulfate, and amphotericin B were purchased from Cell Systems (St Katharinen, Germany).

Cell culture Human keratinocytes and fibroblasts were isolated from juvenile foreskin from surgery. Skin was incubated at 4°C in a solution of 0.25% trypsin and 0.2% ethylenediamine tetraacetic acid for 20 h and trypsinization was terminated by addition of ice-cold DMEM containing 10% fetal bovine serum. Keratinocytes were scraped from the epidermis, washed with phosphate-buffered saline (PBS) and centrifuged at $250 \times g$ for 5 min. The pellet was resuspended in KGM that was prepared from KBM by the addition of 0.1 ng recombinant epidermal growth factor per ml, 5.0 μg insulin per ml, 0.5 μg hydrocortisone per ml, 0.15 mM Ca^{2+}, 30 μg bovine pituitary extract per ml, 50 μg gentamicin sulfate per ml, and 50 ng amphotericin B per ml. The remaining skin was trypsinized for another 10 min at 37°C. The enzymatic reaction was terminated by the addition of ice-cold DMEM containing 10% fetal bovine serum. Fibroblasts were scraped from the dermis, filtered, centrifuged, and resuspended in DMEM containing 10% fetal bovine serum. Both keratinocytes and fibroblasts were pooled from several donors and cultured at 37°C in 5% CO_2. For all experiments only cells of the second or third passage were used.

MTT dye-reduction assay Keratinocytes (8×10^4 cells per well), seeded into 24-well plates for 24 h, were incubated with test substances for 24 h at 37°C in 5% CO_2. After the addition of 100 μl MTT solution (5 mg per ml) per well, the plates were incubated for another 4 h. The supernatants were removed and the formazan crystals were solubilized in 1 ml of dimethyl sulfoxide. The optical density was determined at 540 nm using a scanning microplate spectrophotometer (Multiscan® Plus, Labsystems, Helsinki, Finland).

DNA synthesis Cells (4×10^4 cells per well) were grown in 24-well plates for 24 h. Then medium was replaced by fresh KBM or KGM for keratinocytes and by serum-free DMEM for fibroblasts. Cells were incubated with the indicated substances for 72 h and pulsed with 1 μCi of [methyl-^{3}H]thymidine per well. After 23 h medium was removed and cells were washed twice each with PBS and ice-cold trichloroacetic acid (5%). The precipitated material was dissolved in 0.3 M NaOH solution and incorporated [methyl-^{3}H]thymidine was determined in a scintillation counter (MicroBeta™ Plus, Wallac Oy, Turku, Finland).

Cell cycle analysis Cell cycle analysis was performed using a cycle test plus DNA reagent kit (Becton and Dickinson, Heidelberg, Germany). Keratinocytes were fixed, RNA was digested and DNA was labeled with propidium iodide according to the manufacturer's instructions. Propidium iodide staining was determined by flow cytometry using a FACScalibur™ (Becton and Dickinson).

Transglutaminase assay Transglutaminase activity was determined by the method described by Wakita *et al* (1994). Cells were cultured in KGM and incubated with the test substances for 96 h. Keratinocytes were collected with a rubber policeman in 20 mM Tris–HCl buffer containing 2 mM ethylenediamine tetraacetic acid (pH 8.0) and homogenized by freeze thawing. After centrifugation at $600 \times g$ for 10 min, 100 μl of the supernatant were mixed with 600 μl 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM $CaCl_2$, 5 mM dithiothreitol, 540 μg dimethylcasein, 1 mM putrescine, and 2.5 μCi [^{3}H]putrescine (80 Ci per mmol). The mixture was incubated for 1 h at 37°C and the enzymatic reaction was stopped by addition of 600 μl ice-cold trichloroacetic acid (10%). The protein precipitate was washed three times with ice-cold trichloroacetic acid (5%) containing 10 mM putrescine and once with ethanol (95%). The pellet was solubilized in 200 μl 1 M NaOH solution and radioactivity was determined in the scintillation counter.

Extracellular matrix protein assays Measurement of fibronectin and PAI-1 synthesis was assayed as previously described (Wrana *et al*, 1992). Briefly, fibroblasts (1×10^5 cells per well) were incubated overnight in serum-free DMEM, followed by 12 h stimulation with the indicated concentrations of S1P. For control assays cells were stimulated with TGF-β (2 ng per ml). Cultures were labeled with 50 μCi per ml [^{35}S]methionine for 4 h. For measurement of newly synthesized secreted fibronectin, an aliquot of labeled media was incubated with gelatin-Sepharose in the presence of 0.5% Triton X-100. The beads were washed once with Tris-buffered saline (50 mM Tris–HCl, pH 7.4, 150 mM NaCl), once with 0.5 M NaCl, once with 50 mM Tris–HCl (pH 7.4), and once again with Tris-buffered saline. Fibronectin was eluated by boiling in electrophoresis sample buffer containing dithiothreitol and samples were analyzed by sodium dodecyl sulfate–gel electrophoresis. Fibronectin was identified as a band of 250 kDa. For measurement of PAI-1 labeled cells were removed by washing once with PBS, three times with 10 mM Tris–HCl (pH 8.0), 0.5% sodium deoxycholate, 1 mM PMSF, once with 10 mM Tris–HCl (pH 8.0), and once again with PBS. Matrix proteins were extracted by scraping into electrophoresis sample buffer containing dithiothreitol. PAI-1 was identified as a characteristic protein band of 45 kDa.

For western blot analysis of fibronectin and PAI-1, treated or control cells were washed twice with PBS before being lyzed on ice in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediamine

tetraacetic acid, 1% Nonidet P-40, 1 mM PMSF, and 1 μg per ml each of leupeptin, pepstatin, and aprotinin). The cell lysates were centrifuged at 12,000 × *g* for 5 min at 4°C and the supernatant was collected. Samples were prepared in Laemmli reducing buffer, boiled for 10 min, and analyzed by sodium dodecyl sulfate–gel electrophoresis. Gels were blotted on to PDVF-membranes. After blocking with 5% nonfat dry milk overnight at 4°C membranes were incubated with the primary antibodies (mouse monoclonal anti-human PAI-1, mouse monoclonal anti-human fibronectin) for 2 h. The blots were washed three times in PBS-Tween (0.1%) followed by incubation with the secondary antibodies (rabbit anti-mouse IgG-horseradish peroxidase, rabbit anti-mouse IgG-AP alkaline phosphatase) for 1 h. After washing, the blots were developed according to the manufacturer's protocol.

Migration assay Chemotactic migration of cells in response to a gradient of S1P or TGF-β was measured in a modified Boyden chamber as described (Wang *et al*, 1999). Cells were added to the upper well of the chamber. The lower chamber, separated by a fibronectin-coated membrane, contained S1P in the indicated concentration. As control for the chemotactic response TGF-β (1 ng per ml) was used. Cells that had migrated through the membrane were fixed and stained by GIEMSA. The migrated cells were quantified by light microscopy at a magnification of × 150 by counting the stained cells from four randomly selected fields.

Reverse transcription and amplification by polymerase chain reaction The mRNA of human keratinocytes or intact skin, and Jurkat cells were isolated by QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. Aliquots of mRNA preparation were frozen at −80°C until use. One microgram of mRNA was reverse transcribed (Superscript reverse transcriptase, Invitrogen, Karlsruhe, Germany) in the presence of 1 pmol of a 25–30 mer oligo(dT) primer. Based upon the nucleotide sequences of the human S1P receptors in the database, oligonucleotide primer pairs were prepared:

S1P1: 5′-CCC AAG CTT ATG GGG CCC ACC AGC GTC CCG-3′ and 5′-GCT CTA GAC TAG GAA GAA GAG TTG ACG TTG CC-3′;
S1P2: 5′-CAT TGC CAA GGT CAA GCT GT-3′ and 5′-ACG ATG GTG ACC GTC TTG AG-3′;
S1P3: 5-CCC AAG CTT ATG GAC ACT GCC CTC CCG-3′ and 5′-CGG GAT CCT CAG TTG CAG AAG ATC CC-3′;
S1P4: 5′-ACG GGA GGG CCT GCT CTT CA-3′ and 5′-AAG GCC AGC AGG ATC ATC AG-3′;
S1P5: 5′-GTG GAC TTG AGC TTC AAG AC-3′ and 5′-CAC TTT GGG GAG GAT TTG GA-3′.

Polymerase chain reaction amplification was carried out in a Thermocycler (T Gradient, Whatman Biometra, Göttingen, Germany) using the Thermoprime Plus polymerase (Advanced Biotechnologies, Columbia, MD) under the following cycling conditions: (1) 94 C for 1 min; (2) 94°C for 30 s; (3) 55°C for 30 s; (4) 72°C for 1 min; (5) repeat of steps 2–4 for 30 cycles; (6) 72°C for 2 min; and (7) 4°C for 1 s. Polymerase chain reaction products were size-fractionated in a 2% agarose gel, and visualized by ethidium bromide staining.

Membrane preparation and [^{35}S]-GTPγS-binding assay Keratinocytes were washed with ice-cold PBS, scraped in buffer A containing 20 mM Tris (pH 7.4), 500 μM PMSF, 1 μg per ml each leupeptin and aprotinin, and 0.5 μg pepstatin per ml and homogenized by passing through a 28 gauge needle 10 times. The homogenate was centrifuged for 5 min at 5000 × *g* and the pellet was discarded. The supernatant was spun for 40 min at 43,000 × *g*, the resulting pellet was resuspended in buffer A and frozen at −80°C until use. Ten micrograms of protein per assay were incubated for 45 min in buffer B containing 20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′, N′*-tetraacetic acid, 100 μM PMSF, 1 μg per ml each leupeptin and aprotinin, 0.5 μg pepstatin per ml, 3 μM guanosine diphosphate (GDP), 50 pM [^{35}S]-GTPγS, and indicated concentrations of S1P. The samples were rapidly filtered on GF/B glass microfiber filters (Whatman, Kent, U.K.) presoaked in buffer C (20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM β-mercaptoethanol). The filters were washed three times with buffer C and radioactivity was determined in a scintillation counter.

Microinjection and immunostaining Keratinocytes (approximately 60% confluent) were cultured on gridded glass coverslips. Then cells were microinjected using an Eppendorf Micromanipulator 5171 and Microinjector 5242 (Eppendorf, Hamburg, Germany) at a pressure of 210 hPa for 0.3 s. Approximately 400 cells were microinjected cytoplasmatically with S1P diluted in injection buffer (27 mM K_2HPO_4, 8 mM Na_2HPO_4, 26 mM KH_2PO_4, and 1 mg per ml BSA pH 7.2). Texas red-conjugated dextran (5 mg per ml) was coinjected for identification of microinjected cells. Cells injected only with Texas red-conjugated dextran and injection buffer served as a control. After microinjection of 400 cells, they were cultured in KGM supplemented with bromodesoxyuridine (BrdU, 10 mM) for 24 h at 37°C. Then keratinocytes were fixed in acidic ethanol (70% in 50 mM glycine buffer, pH 2.0) and stained with mouse monoclonal anti-BrdU antibody according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Coverslips were mounted on slides and fixed with 20% glycerol/PBS. Cell counts of nuclear BrdU labeling were determined by a Zeiss Axiovert 100 fluorescence microscope (Zeiss, Jena, Germany) using a triple bandpass filter.

RESULTS

S1P stimulates migration but inhibits proliferation of human keratinocytes As S1P is released from degranulating platelets at wound sites, we have investigated its effects on different parameters of dermal and epidermal cells involved in wound healing of human skin. In particular these parameters include migration and proliferation of keratinocytes responsible for the reconstruction of the cutaneous barrier as well as proliferation of and matrix formation by fibroblasts.

Exogenous S1P possessed no mitogenic effect in primary human quiescent keratinocytes, isolated from skin samples and cultured in KBM. But intriguingly, S1P decreased thymidine incorporation of proliferating keratinocytes, cultured in KGM, in a concentration-dependent manner indicating an anti-proliferative action in these epidermal cells. An inhibition of DNA synthesis of more than 60% was observed at a concentration of 10 μM S1P with an IC_{50} of 1 μM (**Fig 1*A***). Direct assessment of cell growth confirmed the anti-proliferative property of 10 μM S1P with a 40% inhibition of cell growth after 3 d. In addition, DNA flow cytometry was used to characterize the distribution of cells in the different phases of the cell cycle. S1P caused a concentration-dependent accumulation of cells in the G_0/G_1 phase and, in agreement with the DNA-synthesis data, decreased the percentage of keratinocytes entering the S phase. The number of cells in the G_2 compartment was relatively unaffected by treatment with S1P (**Fig 1*B***).

As cell growth arrest is often connected with an increase of the apoptotic rate it is important to mention an opposing action of S1P, which possesses a cytoprotective effect in keratinocytes in the same concentration range as it induces cell growth arrest

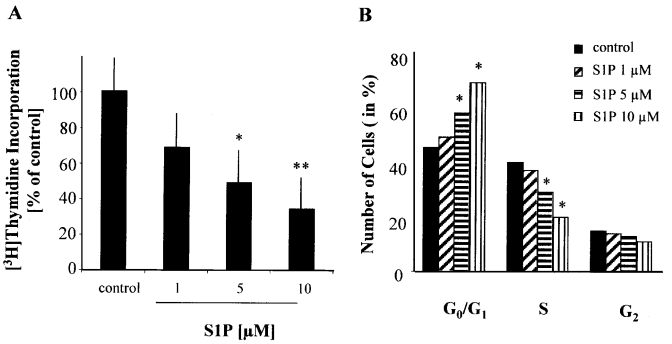

Figure 1. S1P inhibits cell proliferation of human keratinocytes. Human keratinocytes were incubated with the indicated concentrations of S1P as BSA complex (4 mg BSA per ml PBS) for 72 h and pulsed with [^{3}H]thymidine. Incorporation into DNA was determined as described under *Materials and Methods*. Data are expressed as percentage of control (unstimulated keratinocytes) and are the mean ± SEM of results from at least four experiments, each run in triplicate (*A*). For cell cycle analysis keratinocytes were fixed, RNA was digested, DNA was labeled with propidium iodide, and staining was determined by flow cytometry. The experiment was repeated independently three times obtaining similar results (*B*). $^*p < 0.05$ and $^{**}p < 0.001$ indicate a statistically significant difference from control values.

(Manggau *et al*, 2001). Only higher S1P concentrations exceeding 20 μM resulted in toxic effects, visible by propidium iodide staining, MTT dye reduction, and promoted detachment of the cells from the dishes.

Moreover, the anti-proliferative effect was accompanied by the induction of the differentiation process. Measurement of transglutaminase activity, the crucial enzyme for the formation of the cornified envelope, indicated that S1P promotes differentiation to corneocytes (**Fig 2*A***).

Previous data have shown that S1P generally has mitogenic actions and moreover is a positive regulator of human endothelial wound closure. For this reason it was surprising that S1P inhibits keratinocyte cell growth and consequently the re-epithelialization of the cutaneous barrier, which is a critical factor during this process.

As keratinocyte migration is another pivotal component of skin wound healing and S1P is a well known chemoattractant in a variety of cells, its chemotactic potency on primary keratinocytes was determined in a modified Boyden chamber with fibronectin-coated filters. Indeed, S1P was identified as a potent factor to stimulate keratinocyte cell migration. The chemotactic response was enhanced in a concentration-dependent and saturable manner. The EC_{50} value for S1P was estimated to be 100 nM. The S1P-induced migration was comparable with that of TGF-β1 (1 ng per ml), a potent chemoattractant for keratinocytes (**Fig 2*B***).

S1P induces proliferation of and matrix formation by primary fibroblasts In several cell lines such as Swiss 3T3 fibroblasts or rat fibroblasts S1P has been identified as an effective mitogenic agent. Indeed, a similar effect was measured in primary fibroblasts isolated from the same skin samples as keratinocytes. S1P significantly increased DNA synthesis in a concentration-dependent manner with an EC_{50} of 1 μM, which is in accordance to the concentration of the cell growth inhibitory effect in keratinocytes. S1P in a concentration of 10 μM induced a 4-fold increase of thymidine incorporation in nascent DNA (**Fig 3*A***). Cell cycle analysis revealed that S1P increased the proportion of cells in the S-phase, whereas the number of cells in the $G_{0/1}$ phase was reduced. These results indicate that S1P may stimulate or inhibit cell proliferation depending on whether the target is a fibroblast or a keratinocyte.

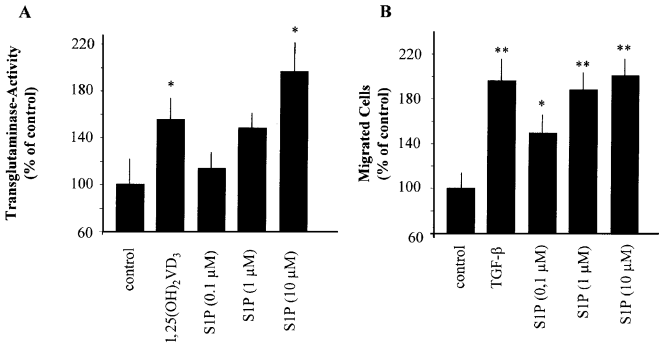

Figure 2. S1P enhances differentiation and migration of human keratinocytes. Transglutaminase activity as differentiation marker was determined as described in *Materials and Methods*. Cells were cultured for 96 h in the presence of 1,25-$(OH)_2D_3$ 100 nM as positive control or with the indicated concentrations of S1P delivered as BSA complex (4 mg BSA per ml PBS). Values are expressed as percentage of control (unstimulated cells) and are the mean ± SEM of results from at least three experiments, each run in triplicate (*A*). Chemotactic migration of keratinocytes in response to a gradient of S1P or TGF-β as positive control was measured in a modified Boyden chamber as described. Data are presented as a percentage of control and are the mean ± SEM of results from at least four experiments. Each run was done using triplicate wells (*B*). $^{*}p < 0.05$ and $^{**}p < 0.001$ indicate a statistically significant difference *vs* unstimulated control cells.

The homeostasis of different extracellular matrix proteins influences the process of wound healing. Two positive cooperative regulators are fibronectin and PAI-1, which are probably critical to the motile process and the resulting tissue plasticity. To the best of our knowledge until now an influence of S1P on matrix protein generation has not been examined. Therefore the synthesis and accumulation of fibronectin and PAI-1 in cells, respectively, in medium were measured in response to S1P using either radioactive labeling or western blot analysis. As shown in **Fig 3** (***B,C***) synthesis of both fibronectin and PAI-1 was induced following treatment with S1P. The accumulation of PAI-1 was concentration dependent, the generation of PAI-1 started at 0.1 μM S1P, a maximal formation was observed with a concentration of 10 μM of S1P. In contrast, fibronectin generation started also after exposure to 0.1 μM of S1P but could not be increased using higher S1P concentrations (**Fig 3*B,C***).

Characterization of receptors for S1P in human keratinocytes To characterize further and to examine whether the unexpected anti-proliferative action of S1P in keratinocytes is mediated through specific S1P receptors, the expression of these receptors in the epidermal cells was investigated.

Reverse transcription-polymerase chain reaction revealed that transcripts of mRNA for all known S1P receptors, namely $S1P_1$

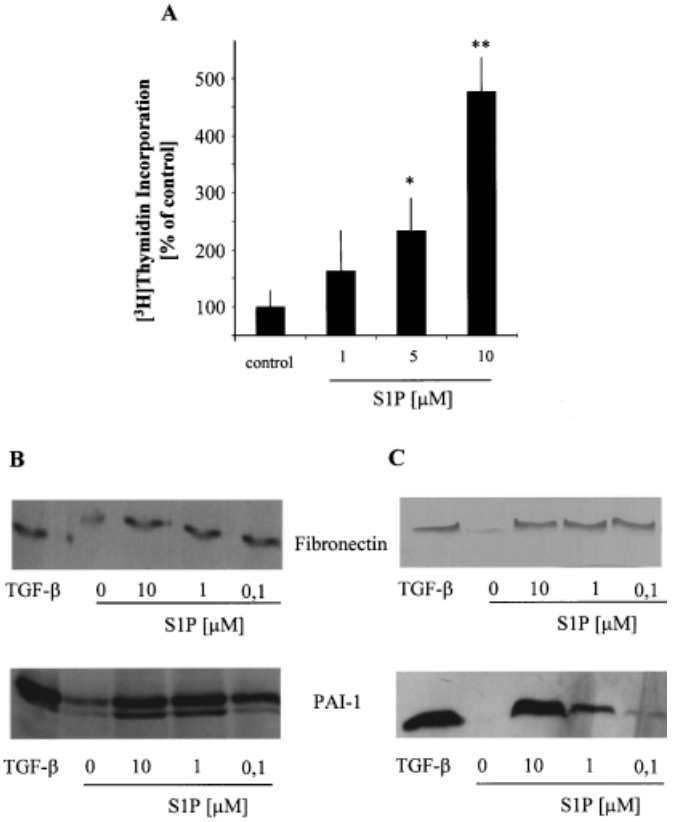

Figure 3. S1P enhances proliferation of primary fibroblasts and matrix protein formation by fibroblasts. Human fibroblasts were incubated with the indicated concentrations of S1P, pulsed with [^{3}H]thymidine and incorporation into DNA was determined as described. Values are presented as percentage of control (unstimulated fibroblasts) and are the mean ± SEM of results from at least four experiments, each run in triplicate (*A*). Measurement of the matrix proteins fibronectin and PAI-1 was assayed as described. For control assays cells were stimulated with TGF-β (2 ng per ml). Radiolabeled fibronectin was identified as a band of 250 kDa and labeled PAI-1 as a characteristic protein band of 45 kDa (*B*). Western blot analysis confirmed fibronectin and PAI-1 formation after treatment with S1P (*C*). Similar results were obtained in three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.001$.

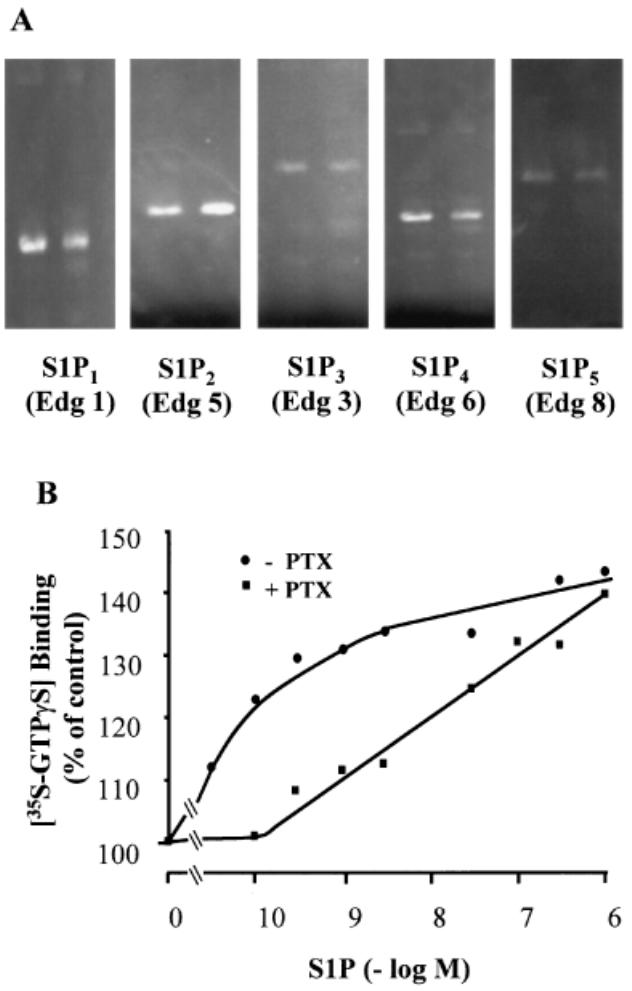

Figure 4. Expression and characterization of S1P receptors in human keratinocytes. The mRNA of cultured keratinocytes or intact skin was isolated, reverse transcribed, and amplified as described in *Materials and Methods.* Polymerase chain reaction products of cultured keratinocytes (left band) and intact skin (right band) were size-fractionated in an agarose gel, and visualized by ethidium bromide staining (*A*). Membranes from human keratinocytes were examined for [^{35}S]-GTPγS binding assays in the presence of the indicated S1P concentrations with or without PTX (3 ng per ml) preincubation for 3 h as described (*B*).

(Edg 1), $S1P_2$ (Edg 5), $S1P_3$ (Edg 3), $S1P_4$ (Edg 6), and $S1P_5$ (Edg 8) are present in cultured keratinocytes (**Fig 4*A***). To exclude that the receptor expression is an artifact of culture, the expression profile was also demonstrated in intact skin confirming the existence of $S1P_{1–5}$ (**Fig 4*A***). To evaluate coupling of S1P receptors to G proteins the GDP/GTP exchange was measured by the use of [^{35}S]-GTPγS, which measures GDP/GTP exchange of the α-subunit of the G-protein and, therefore, the initial steps of G-protein activation by a receptor ligand. Optimal conditions were defined and S1P-induced GDP/GTP exchange was determined. Indeed, S1P enhanced binding of [^{35}S]-GTPγS to G-proteins of isolated membranes in a concentration-dependent manner with an EC_{50} of 5 nM indicating that human keratinocytes express S1P receptors, which are functionally coupled to G-proteins (**Fig 4*B***). It should be mentioned that the estimated EC_{50} value is in agreement with the reported K_d of S1P for its receptors. Furthermore, we investigated the coupling of S1P receptors in the presence of pertussis toxin (PTX), which inactivates the α-subunit of G_i/G_0 maintaining them in a nondissociated form. Treatment with PTX resulted in a right shift of the binding curve indicating that the stimulatory effect of S1P on [^{35}S]-GTPγS binding is mediated by both PTX-sensitive and PTX-insensitive G-proteins.

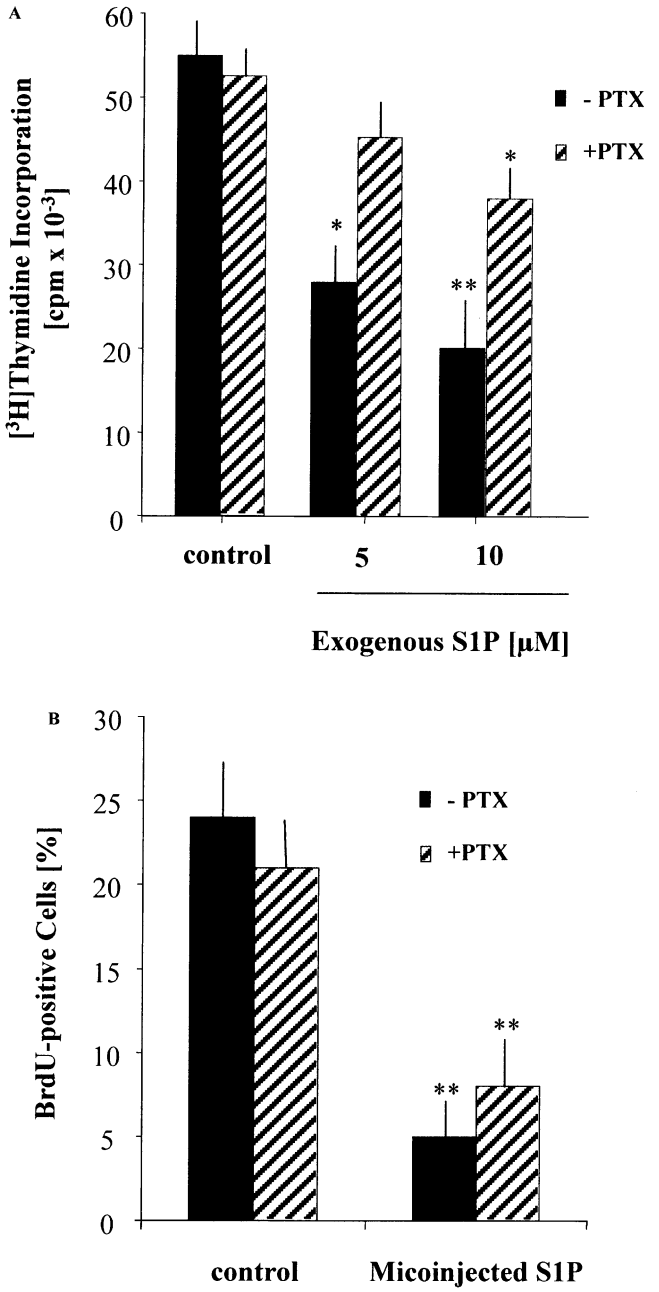

Figure 5. Exogenous S1P-treatment as well as microinjection of S1P into keratinocytes decreases DNA synthesis. Human keratinocytes were pretreated with 3 ng PTX per ml for 3 h or vehicle and incubated with the indicated concentrations of S1P for 72 h and pulsed with [^{3}H]thymidine. Incorporation into DNA was determined as described under *Materials and Methods.* Data are expressed as percentage of control (mean ± SEM) of four experiments, each run was performed in triplicate (*A*). Human keratinocytes were microinjected with vehicle or S1P. Microinjected cells were visualized by Texas red fluorescence and BrdU incorporation by green fluorescein isothiocyanate using a triple band pass filter. Microinjection was also performed after preincubation of keratinocytes with 3 ng PTX per ml for 3 h. Values (mean ± SD) are the percentage of cells positive for BrdU staining and correspond to the average of three measurements in which 100 cells were scored. Similar results were obtained in three independent experiments (*B*). $^*p < 0.05$, $^{**}p < 0.001$.

Microinjection of S1P induces cell growth arrest of human keratinocytes To examine whether the anti-proliferative property and the differentiation inducing effect of S1P in keratinocytes are mediated by the binding of S1P to its receptors, cells were treated with S1P in the presence of PTX. Indeed, preincubation of keratinocytes with PTX caused only a

partially inhibition of the anti-proliferative effect induced by S1P (**Fig 5*A***). In analogy, PTX pretreatment diminished S1P-induced transglutaminase activity by only 40%. These results suggest that either PTX-insensitive S1P receptors or intracellular targets are also involved in the anti-proliferative and differentiation inducing properties of S1P. Moreover, the fact that the IC_{50} of S1P to inhibit proliferation and to induce differentiation are almost 200 times higher than the EC_{50} to stimulate [^{35}S]-GTPγS binding leads to the assumption that these properties of S1P are due to intracellular actions rather than binding to a cell surface receptor despite their functional active expression. In view of this controversy it was of interest to examine the proliferation of keratinocytes whose intracellular levels of S1P are increased. Therefore, S1P was microinjected into the cytosol of keratinocytes together with Texas red-conjugated dextran to identify microinjected cells and the incorporation of BrdU into DNA was examined 24 h later. Using double immunofluorescence to visualize injected cells and BrdU incorporation, it is clearly demonstrated in **Fig 5** that DNA synthesis decreased after S1P microinjection, as only 7% of S1P-injected cells were positive for BrdU incorporation compared with 25% of control cells in the same field (**Fig 5**). Microinjection of Texas red-conjugated dextran alone had no significant effect on DNA synthesis. As uninjected cells adjacent to S1P-microinjected cells showed no significant decrease in BrdU incorporation it can be excluded that S1P is leaking from microinjected cells acting in an autocrine mechanism. To exclude further an autocrine mechanism of S1P, microinjection was performed in the presence of PTX. The anti-proliferative effect of S1P was insensitive to PTX treatment confirming that reduction of DNA synthesis by microinjected S1P does not require at least interaction with a PTX-sensitive G-protein-coupled S1P receptor. Collectively, these results strongly suggest that there is a complex interplay between cell surface receptor signaling and intracellular targets for S1P to mediate cell growth arrest of keratinocytes.

DISCUSSION

Cutaneous wound healing, a dynamic process that combines tissue regeneration events with local activation of immune functions, involves diverse cell types. These cells include keratinocytes for the regeneration of the cutaneous barrier, fibroblasts for the production of extracellular matrix proteins, and monocytes for the defeat of infections. Proliferation and migration of human keratinocytes is crucial for the re-epithelialization of the cutaneous barrier. Here we report, that the effects of S1P are paradoxical concerning these parameters: keratinocyte migration is potently enhanced, whereas keratinocyte proliferation is strongly inhibited. These findings were unexpected in some respects: first, the anti-proliferative observation is in contrast to the generally known growth promoting effect of S1P, and further on, in contrast to the suggested role of S1P in wound healing as inhibition of cell growth of keratinocytes prevents efficient closure of the wound. But it has to be considered that a variety of mediators are released from platelets at the wounded site. Growth factors such as epidermal growth factor, platelet-derived growth factor, TGF-β, and fibroblast growth factor are stored or formed in thrombocytes, many of them affect keratinocyte cell function allowing a complex interplay (Piazza *et al*, 1995). Moreover, our data indicate that the migration response of keratinocytes is more sensitive to S1P than the cell growth inhibitory effect. Thus, it depends on the S1P levels present in a cutaneous wound, whether cell growth inhibition and differentiation will be influenced by S1P. But it should be mentioned that differentiation of keratinocytes is also a crucial event to reconstruct the cutaneous barrier. It could also be possible that S1P may have disparate effects on different keratinocyte populations. For instance, perhaps S1P enhances migration of stem and transit amplifying cells, but stimulates differentiation and inhibits proliferation of late stage transit amplifying cells.

The stimulation of cell growth is a well established biologic response to S1P in a variety of fibroblast cell lines (Zhang *et al*, 1991; Olivera and Spiegel, 1993; Gomez-Munoz *et al*, 1994; Bornfeldt *et al*, 1995; Olivera *et al*, 1999). Consistent with these studies we found a profound proliferative action of S1P in primary human fibroblasts isolated from the same origin as keratinocytes indicating a bimodal effect of S1P on cell proliferation depending on the cell type. Until now an anti-proliferative effect of S1P has only been described in human myofibroblasts (Davaille *et al*, 2000). In these cells, S1P rapidly increased prostaglandin E_2 production and in turn cyclic adenosine monophosphate, both have been recognized as growth inhibitory messengers for human myofibroblasts.

Moreover, the presented results show significant differences of S1P on crucial parameters of endothelial and cutaneous wound healing. S1P at low concentrations enhances migration of both endothelial cells and keratinocytes. If the concentration is increased, S1P influences also proliferative events in endothelial and epidermal cells. But as S1P increases the proliferation rate of endothelial cells, there is an opposing action in keratinocytes (Lee *et al*, 2000).

The cell growth inhibitory effect in keratinocytes is consistent with our preliminary studies indicating that 1,25-$(OH)_2D_3$, at least in concentrations higher than 1 nM, in analogy to S1P is an anti-proliferative agent and enhances S1P levels in these epidermal cells (Manggau *et al*, 2001). Moreover, both S1P and 1,25-$(OH)_2D_3$ possess cytoprotective actions in keratinocytes indicating that their anti-proliferative properties are not due to cytotoxicity (Cuvillier *et al*, 1996; Manggau *et al*, 2001).

Although it is unusual for an effector to both stimulate and inhibit cell growth it is not unprecedented. TGF-β is in analogy to S1P a strong inhibitor of keratinocyte proliferation, but enhances proliferation of fibroblasts as well as activation of extracellular matrix production by fibroblasts (Alexandrow and Moses, 1995; O'Kane and Ferguson, 1997). Moreover it is a very potent chemoattractant and an inducer of its own production in monocytes (Massague, 1998). Interestingly, whereas the effects of TGF-β on fibroblast proliferation and its effect to induce chemotaxis are consistent with a positive role in wound healing, the growth inhibitory effect on keratinocytes is not. Indeed, studies of wound healing in mice, in which the TGF-β gene has been deleted by homologous recombination, revealed that the release of the growth factor from degranulating platelets or secretion by infiltrating macrophages and fibroblasts is not critical to initiation or progression of tissue repair. Surprisingly, no impairment in wound healing was seen in TGF-β1 null mice. Instead wounds showed an overall reduction in the amount of granulation tissue and an increased rate of epithelialization compared with littermate controls suggesting that endogenous TGF-β may actually retard wound closure (Koch *et al*, 2000). In analogy to S1P, it has also been shown that 1,25-$(OH)_2D_3$ promotes TGF-β synthesis in human keratinocytes and originates an autocrine/paracrine loop, which is partly responsible for the anti-proliferative action of 1,25-$(OH)_2D$ (Heberden *et al*, 1998).

A major point of this study was to identify whether the unexpected anti-proliferative effect in keratinocytes is linked to an intracellular effect or to its plasma membrane receptors. It seems likely that many, but not all events, are the result of S1P interaction with its G-protein-coupled receptors. In Swiss 3T3 fibroblasts, however, S1P was first proposed as a second messenger involved in the proliferative effect of growth factors such as platelet-derived growth factor or serum, which enhance sphingosine kinase activity and increase intracellular S1P levels (Olivera and Spiegel, 1993). Moreover, competitive inhibitors of sphingosine kinase block some of the proliferative signals elicited by those growth factors (Edsall *et al*, 1997; Kleuser *et al*, 1998; Alemany *et al*, 1999). But it should be mentioned that in Swiss 3T3 fibroblasts the proliferative response to exogenously added S1P is partially sensitive to PTX, suggesting at least the potential

involvement of G-protein-coupled receptors (Goodemote *et al*, 1995). Thus, it seems possible that there is a complex interplay between cell surface receptor signaling and intracellular S1P formation. Indeed, recently in HEK293 cells it has been shown that stimulation of S1P receptors results in the activation of sphingosine kinase leading to an intracellular increase in S1P (Meyer zu Heringdorf *et al*, 2001).

Although we have identified functional acting S1P receptors in keratinocytes several lines of evidence suggest that the anti-proliferative effect of S1P in human keratinocytes is not only mediated by binding of S1P to its receptors. Cell growth arrest of keratinocytes requires micromolar concentrations of S1P, whereas putative receptor-mediated effects of S1P have been shown to occur in the nanomolar range. This is consistent with the proliferative action of S1P in NIH 3T3 fibroblasts and HEK 293 cells. Moreover, when S1P was microinjected in keratinocytes BrdU incorporation revealed that S1P microinjection induces a decrease of the proliferation rate of keratinocytes in the same extent as exogenously added S1P. This result was even confirmed in the presence of PTX. Moreover, as uninjected cells adjacent to S1P microinjected cells showed no significant decrease in BrdU incorporation, it is unlikely that S1P is leaking from the pipette. These data demonstrate that besides S1P-induced receptor stimulation intracellular targets of S1P may contribute to the anti-proliferative property of S1P in keratinocytes. But, although microinjection bypasses cell surface receptors, it cannot be excluded that S1P is transported into the extracellular medium acting in an autocrine fashion via PTX-insensitive coupled S1P receptors.

In summary this study indicates a dual effect of S1P on parameters in wound healing. In fact, S1P is brought to the wound site by the massive degranulation of platelets and therefore has been implicated to reinforce re-epithelialization. Our findings, in particular cell growth arrest of keratinocytes, interfere with a rapid wound repair. Moreover, the anti-mitogenic effect mediated by S1P may be important to prevent excessive proliferation of the skin. Owing to this property, it would be of interest to determine whether S1P is a promising candidate in the therapy of hyperproliferative skin diseases such as psoriasis vulgaris. As we recently identified (Manggau *et al*, 2001) that 1,25-$(OH)_2D_3$, which is used for the treatment of psoriasis vulgaris, enhances S1P levels in keratinocytes, it seems likely that some actions of secosteroid is via the formation of this sphingolipid metabolite.

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