View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Lysophosphatidic Acid Interacts with Transforming Growth Factor-β Signaling to Mediate Keratinocyte Growth Arrest and Chemotaxis

Bettina Sauer,* Rüdiger Vogler,* Karsten Zimmermann,* Makiko Fujii,† Mario B. Anzano,† Monika Schäfer-Korting,* Anita B. Roberts,† and Burkhard Kleuser*

*Institute of Pharmacy, Pharmacology and Toxicology, Free University Berlin, Berlin, Germany; †Laboratory of Cell Regulation and Carcinogenesis, NCI, National Institutes of Health, Bethesda, Maryland, USA

Lysophosphatidic acid (LPA, 1-acyl-glycerol-3-phosphate) plays an important role in diverse biological responses including cell proliferation, differentiation, survival, migration, and tumor cell invasion. The most prominent source of LPA is platelets from which it is released after thrombin activation and is assumed to be an essential function of this lysophospholipid in cutaneous wound closure. Therefore, we examined the role of LPA on biological responses of keratinocytes. Although LPA potently enhances keratinocyte migration, it strongly induces growth arrest of proliferating epidermal cells. Thus, LPA possesses analogous actions to transforming growth factor-β (TGF-β), which is also released from degranulating platelets at wounded sites. In contrast to LPA, the intracellular signaling events of TGF-β have been clearly identified and indicate that Smad3 is involved in chemotaxis and cell growth arrest of keratinocytes induced by this cytokine. Here we show that LPA, although it does not alter TGF-β release is capable to activate Smad3 and results in a heteromerization with Smad4 and binding of the complex to its specific DNA-promoter elements. LPA completely fails to induce chemotaxis in Smad3-deficient cells, whereas growth inhibition is at least in part reduced. These findings indicate an essential role of Smad3 in diverse biological properties of LPA-stimulated keratinocytes.

Key words: lipid mediators/migration/proliferation/smad3 protein
J Invest Dermatol 123:840–849, 2004

Lysophosphatidic acid (LPA, 1-acyl-glycerol-3-phosphate), one of the simplest phospholipids, has been identified as a pluripotent biological mediator that can be produced by a variety of cells including platelets, fibroblasts, adipocytes, or cancer cells (Tigyi *et al*, 1994; Gaits *et al*, 1997; Pages *et al*, 2000; Shida *et al*, 2003). The most prominent responses induced by LPA include cell proliferation, differentiation, survival, chemotaxis, platelet aggregation, and tumor cell invasion (Moolenaar, 1999, 2000). Moreover, dysregulation of LPA production has also been suggested in pathophysiological processes such as cardiovascular and neoplastic diseases (Mills *et al*, 2002; Osborne and Stainier, 2003). LPA evokes its multiple effects through G-protein-coupled receptors that belong to the Edg (endothelial differentiation gene) family, namely Edg2/LPA_1, Edg4/LPA_2, and Edg7/LPA_3 (Contos *et al*, 2000). Depending on the expression of LPA receptors and the involvement of different G-proteins, LPA may influence a wide array of biological responses in diverse cell types; however, control of these complex processes is difficult to reconcile with only these three receptors suggesting further unidentified targets of LPA. Indeed, the transcription factor peroxisome proliferator-activated receptor γ has been recognized as an intracellular receptor for LPA (McIntyre *et al*, 2003).

The leading source of LPA are platelets from which it is generated and released after thrombin activation (Sano *et al*, 2002). Thus, serum contains micromolar concentrations of the lysophospholipid, whereas levels in the plasma are much lower (Aoki *et al*, 2002). For that reason, it has been proposed that LPA is a key mediator in the regulation of wound healing. Indeed, it has been shown that LPA promotes endothelial as well as intestinal epithelial wound closure, a dynamic process that combines tissue regeneration events with local activation of immune functions (Sturm *et al*, 1999; Lee *et al*, 2000). Diverse cell types are involved in these processes and modified by LPA. But there exists a fundamental divergence between epithelial and endothelial wound closure, although LPA strongly enhances migration of both cell types, there is an opposing action on cell proliferation. In analogy to fibroblasts and vascular smooth muscle cells, LPA stimulates endothelial cell proliferation (Tigyi *et al*, 1994; Tokumura *et al*, 1994; Lee *et al*, 2002), whereas a cell growth arrest in intestinal epithelial cells is observed (Sturm *et al*, 1999). Although a cell growth inhibitory effect may counteract a sufficient wound closure, it has to be considered that LPA ameliorates further aspects of

Abbreviations: Edg, endothelial differentiation gene; EGF, epidermal growth factor; LPA, lysophosphatidic acid (1-acyl-glycerol-3-phosphate); PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; PTX, pertussis toxin; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor β

Copyright © 2004 by The Society for Investigative Dermatology, Inc.

healing like cell surface fibronectin assembly and infiltration of immune cells (Panetti *et al*, 2001; Huang *et al*, 2002).

Among the numerous mediators that modulate epithelial and endothelial restitution, transforming growth factor β (TGF-β) plays a central role as it is released in response to many other cytokines promoting wound closure (O'Kane and Ferguson, 1997; Sturm *et al*, 1999). Like LPA, TGF-β is a strong inhibitor of epithelial cell growth, but enhances proliferation of fibroblasts as well as endothelial cells (Suemori *et al*, 1991; Roberts and Sporn, 1993; Dignass and Podolsky, 1993). It should be noted that TGF-β influences epidermal cells, responsible for the reconstruction of the cutaneous barrier, in a similar manner as epithelial cells since migration of keratinocytes is enhanced, whereas cell growth is inhibited (Alexandrow and Moses, 1995; Ashcroft *et al*, 1999; Ashcroft and Roberts, 2000).

A disarmingly simple system has been elucidated that mediates many diverse TGF-β responses. This system includes a family of TGF-β receptor substrates, the Smad proteins, which translocate into the nucleus where they act as transcription factors. Binding of TGF-β links its receptors TβRII and TβRI activating the serine/threonine kinase function of TβRII, which then phosphorylates TβRI. This process is followed by the anchoring of receptor-activated Smads (R-Smads) to the TβRI kinase domain (Massague, 1998; Tsukazaki *et al*, 1998; Massague, 2000). Phosphorylation on the C-terminal SSVS motif of Smad causes a conformational change that results in the heteromerization with Smad4 (Co-Smad) and correlates with the movement of the complex from the cytoplasm to the nucleus. Here they directly or indirectly regulate the transcription of target genes (Massague and Chen, 2000; Massague and Wotton, 2000). In human keratinocytes Smad3 was clearly identified as an essential receptor-activated Smad protein associated with TGF-β-induced biological responses. Knocking out Smad3 completely diminished TGF-β-induced chemotaxis and partially cell growth arrest of keratinocytes (Ashcroft *et al*, 1999; Ashcroft and Roberts, 2000).

The aim of this study was to characterize the effects of LPA on critical functions of keratinocytes. Most interestingly, LPA affects biological responses of kerationcytes like migration and proliferation in a similar manner as TGF-β. Therefore, we aimed to analyze whether TGF-β signaling is essential for LPA-mediated biological responses. Our results clearly indicate that LPA, like TGF-β, leads to a functional activation of the TFG-β signal transducer Smad3 and that both LPA and TGF-β receptors are involved in this process.

Results

Modulation of keratinocyte growth and migration by LPA Although several studies indicate that LPA is a strong chemoattractant for various cells (Sturm *et al*, 1999; Lee *et al*, 2000; Panetti *et al*, 2001), the chemotactic activity in keratinocytes in response to LPA has not been defined. Therefore, the effect of LPA on migration of primary human keratinocytes was assessed in a modified Boyden chamber using fibronectin-coated filters. As presented in Fig 1*A*, keratinocytes migrated toward LPA in a concentration-dependent and saturable manner showing a maximal effect at 5 μM. For control purposes, the migratory effect of TGF-β was also evaluated as this cytokine has been identified as a potent chemoattractant for keratinocytes. Both, TGF-β and LPA led to a comparable enhancement of migrated cells (Fig 1*A*). Moreover, when keratinocytes were co-stimulated with TGF-β and LPA in those concentrations, which did not lead to a maximal effect, an additive migration rate was visible (Fig 1*A*).

LPA has also been reported as a potent mitogen in several cell lines and primary cell cultures. In contrast, LPA impaired proliferation of keratinocytes in the logarithmic growth phase in a concentration-dependent manner. A maximal effect, which was not due to cytotoxicity, appeared at a concentration of 10 μM leading to a decrease of [^{3}H]thymidine incorporation of almost 60%. Cell cycle analysis revealed an increased cell number in the G_0/G_1 phase after exposure to LPA, whereas keratinocytes entering the S phase were significantly reduced (Fig 1*C*). The antiproliferative effect was comparable with the action of the well known growth inhibitory factor TGF-β (Ashcroft *et al*, 1999), which served as positive control (Fig 1*B*). In analogy to the migration experiments, there was an increase of cell growth arrest, when keratinocytes were treated with both TGF-β and LPA (Fig 1*B*).

Smad3 activation in response to LPA The presented results revealed analogous effects of LPA and TGF-β in human epidermal cells. Therefore, we measured whether treatment of keratinocytes with LPA leads to a secretion of the cytokine TGF-β. Indeed, LPA neither enhanced peptide levels of TGF-β nor increased latent complexes of TGF-β over a time period of 24 h. As positive control 1α,25-dihydroxyvitamin D_3 was used, which significantly augmented TGF-β release after a stimulation period of 24 h (Fig 2*A*). To further substantiate that LPA-induced keratinocyte migration and cell growth arrest are independent of TGF-β release, both effects were measured under immunoneutralized conditions. As presented in Fig 2*B* and *C*, LPA enhanced migration and induced cell growth arrest of keratinocytes in the presence of anti-TGF-β antibodies, whereas the effects in response to TGF-β were significantly diminished.

In contrast to LPA, the signaling cascade of TGF-β is well characterized, suggesting that intracellular Smad3 protein mediates the migratory response toward TGF-β and in part its antiproliferative properties (Ashcroft *et al*, 1999). To reveal whether LPA provokes its effects in analogy to TGF-β via Smad3 signaling, we first determined whether Smad3 is phosphorylated in response to LPA. Therefore, lysates from LPA-stimulated keratinocytes were immunoprecipitated with anti-Smad3 antibodies, and western blot analysis was performed with anti-phosphoserine antibodies. As shown in Fig 3*A*, a pronounced phosphorylation of Smad3 occurred in response to LPA, whereas its protein levels were unaffected. The phosphorylation was visible after 10 min and the most effective dose was 1 μM of LPA (Fig 3*B*). This rapid phosphorylation of Smad3 in response to LPA confirms a mechanism that is independent of a LPA-mediated TGF-β release.

Next, it was of interest to determine whether phosphorylation of Smad3 by LPA leads to the heterodimerization

with Smad4, as this process is crucial for the translocation into the nucleus. To address this, LPA-stimulated cells were immunoprecipitated with anti-Smad3 antibodies and immunoblotted with anti-Smad4 antibodies. Indeed, Smad4 was co-immunoprecipitated after treatment with LPA (Fig 3*C*). To substantiate a translocation of the complex we measured the occurrence of both Smad proteins in the nucleus. In non-stimulated nuclei Smad4 as well as Smad3 was not detectable, but both appeared in the nuclear fraction, when cells were treated with LPA (Fig 3*D*). But it should be mentioned that changes of the level of Smad3 and Smad4 in the cytosol were not visible, indicating that only a minor part of Smad proteins is translocated.

To prove the DNA-binding of the active complex, a DNA-affinity precipitation assay was performed. Therefore, lysates of stimulated keratinocytes were incubated with a biotinylated oligonucleotide probe representing a tandemly repeated CAGA Smad3/Smad4 binding element (Nishihara *et al*, 1999). Treatment with LPA resulted in an increased binding of the Smad3/Smad4 complex to the oligonucleotide (Fig 3*E*). The DNA binding was specific as a biotinylated scrambled fragment did not precipitate Smad3 protein. Moreover, competition experiments using biotinylated and non-biotinylated CAGA sequences together resulted in a slight decrease of the Smad3 band (Fig 3*E*). Consistent with the LPA-dependent Smad3 activation, TGF-β induced Smad3/Smad4 complex formation, nuclear translocation of the complex, and promoter binding (Fig 3*C–E*).

Involvement of Smad3 in LPA-induced migration and cell growth arrest These results clearly demonstrate the activation of Smad3 by LPA, but do not indicate an essential role of this signaling pathway for biological responses. Therefore, we examined whether keratinocyte migration and proliferation were modified by Smad3 disruption. Keratinocytes were isolated from newborn wild-type or Smad3$^{(ex8/ex8)}$-deficient mice. In analogy to cells of human origin, wild-type keratinocytes strongly migrated to both LPA and TGF-β. Most interestingly, Smad3$^{(-/-)}$ keratinocytes, which showed a normal migration rate toward serum, were not responsive toward LPA and TGF-β (Fig 4*A*). These results confirm the essential migratory role of this signal transducer for both molecules.

Proliferation measurements also revealed an inhibitory effect of LPA and TGF-β on growth of wild-type keratinocytes. The antiproliferative property of LPA but not of TGF-β, however, was less pronounced than in human cells (Fig 4*B*). Thus, a maximal effect appeared at a concentration of 5 μM LPA leading to a significant decrease ($p < 0.05$) of

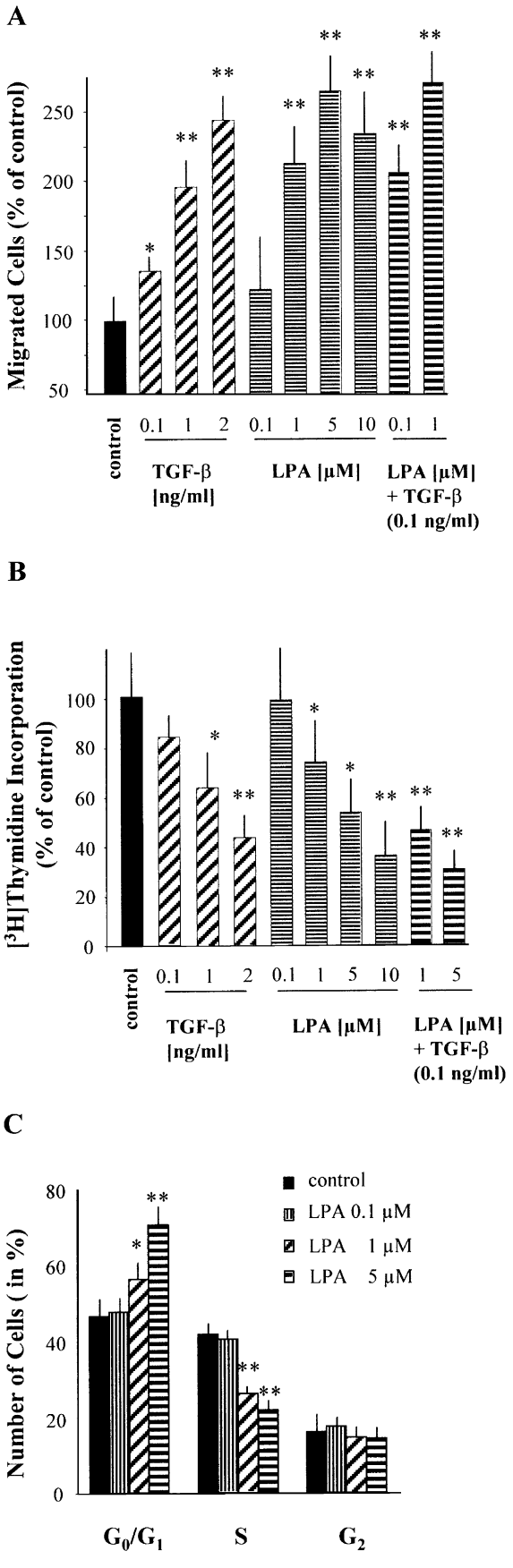

Figure 1
LPA increases migration but inhibits proliferation of human keratinocytes. Chemotactic migration of keratinocytes in response to a gradient of LPA, TGF-β, or LPA/TGF-β was measured in a modified Boyden chamber as described. Values are expressed as percentage of control and are the mean ± SEM from at least three experiments, each run in triplicate (*A*). Cells were incubated with the indicated concentrations of LPA, TGF-β, or LPA/TGF-β 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 and are the mean ± SEM of results of at least four experiments (*B*). For cell cycle analysis keratinocytes were incubated for 24 h with the indicated concentrations of LPA. Then, RNA was digested and DNA was labeled with PI and staining was determined by flow cytometry. Data are expressed as mean ± SEM from three independent experiments (*C*).*$p < 0.05$ and **$p < 0.001$ indicate a statistically significant difference *versus* unstimulated control cells.

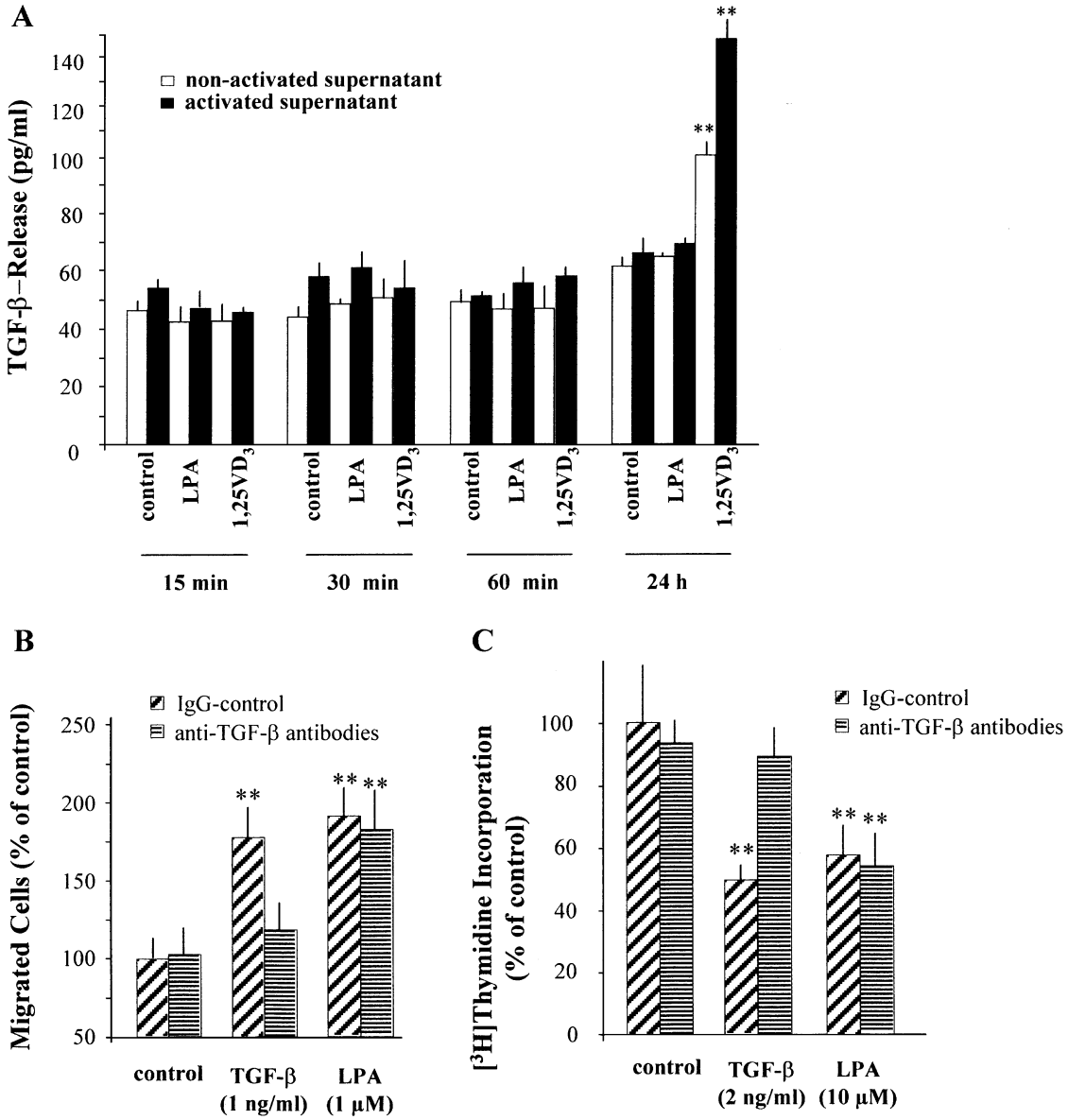

Figure 2
LPA induces migration and cell growth arrest of keratinocytes independent from TGF-β secretion. Cells were stimulated with LPA (1 μM) or 1α,25-dihydroxyvitamin D_3 (1,25VD_3, 100 nM) for the indicated time periods. Then latent and active TGF-β was measured in the supernatant as described under "Materials and Methods". Values are expressed as total amount of TGF-β ± SEM from three experiments (*A*). Chemotactic migration and [^{3}H]thymidine incorporation in response to LPA or TGF-β was measured in the presence of anti-TGF-β- or IgG-control antibodies (25 μg per mL). Values are expressed as percentage of control and are the mean ± SEM from at least three experiments (*B*, *C*).

[^{3}H]thymidine incorporation of only 30%, whereas TGF-β (2 ng per mL) reduced proliferation rates by more than 70%. Nonetheless, a reduced sensitivity to growth inhibition occurred in keratinocytes from Smad3-deficient mice, when stimulated with TGF-β or LPA (Fig 4*B*). These data clearly indicate that Smad3 is at least in part responsible for the antiproliferative property of not only TGF-β but also of LPA.

LPA induces growth inhibition and migration as well as Smad-activation through $G_{i/o}$-coupled receptors Most of the effects of LPA are mediated through G-protein-coupled receptors, namely LPA_{1-3} (Moolenaar *et al*, 1997; Contos *et al*, 2000). But the expression of these receptors in epidermal cells until now has not been characterized. RT-PCR indicated that mRNA transcripts for all three LPA receptors, LPA_1, LPA_2, and LPA_3, exist in human primary keratinocytes as well as in intact skin (Fig 5*C*). To evaluate coupling of LPA receptors to G-proteins, the GDP/GTP exchange was measured by the use of [^{35}S]GTPγS, and indeed, LPA enhanced binding of [^{35}S]GTPγS to G-proteins of isolated membranes indicating that human keratinocytes express functionally active LPA receptors (data not shown).

To address, whether LPA receptors are essential for both biological responses of LPA and Smad3 activation, we assessed these actions after pre-treatment with pertussis toxin (PTX). Our findings indicate that PTX prevented cell growth arrest and caused a strong inhibition of keratinocyte migration (Fig 5*A, B*). In similar, treatment of keratinocytes with PTX also completely diminished Smad3 phosphorylation indicating that the phosphorylation event is a consequence of LPA-receptor stimulation (Fig 5*D*). To address whether also TGF-β receptors are involved in LPA-induced activation of Smad signaling, cells were treated with SB431542, which specifically blocks the ATP-binding site of the TβRI (Laping *et al*, 2002), followed by the measurement of Smad3 phosphorylation. As expected, Smad3 activation by TGF-β was completely abolished in the presence of SB431542 (Fig 5*E*). Accordingly, LPA was not able to phosphorylate Smad3, when cells were pre-treated with the inhibitor, indicating the involvement of TGF-β receptors to mediate LPA-induced Smad signaling (Fig 5*E*).

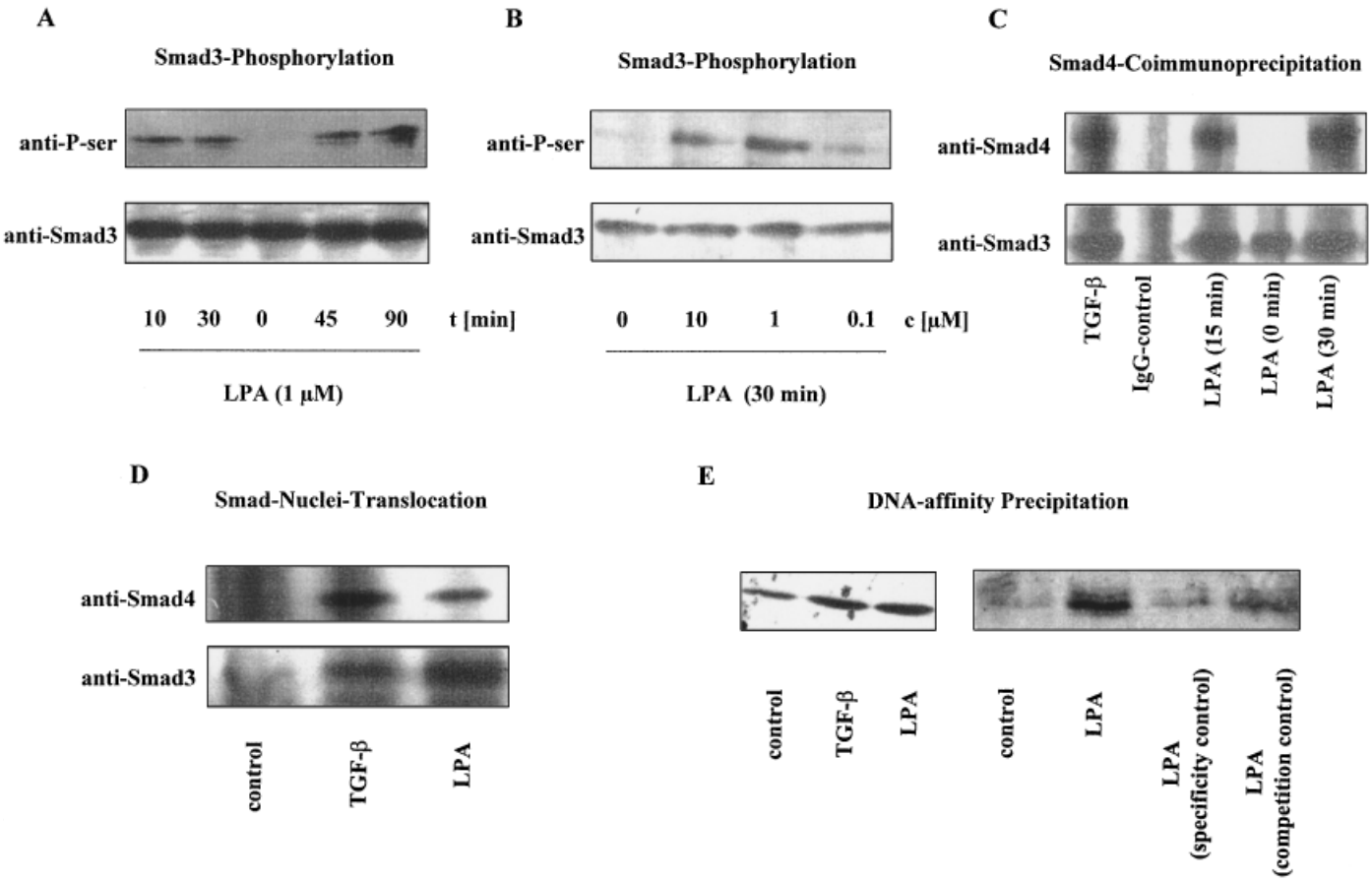

Figure 3
LPA induces Smad3 activation in human keratinocytes. Cells were incubated with 1 μM LPA for different time periods (*A*) or with the indicated concentrations of LPA for 30 min (*B*). After cell lysis, Smad3 protein was immunoprecipitated with anti-Smad3-antbodies and western blot analysis was performed with anti-Smad3- (*lower panel*) or anti-phosphoserine (anti-P-ser) antibodies (*upper panel*) as described under "Materials and Methods" (*A*, *B*). Cells were treated with control vehicle, TGF-β (2 ng per mL) or LPA (1 μM) for the indicated periods. After immunoprecipitation with normal goat IgG (IgG-control) or anti-Smad3 antibodies, western blot analysis was performed. The top blot was developed using anti-Smad4-, and the bottom blot with anti-Smad3 antibodies (*C*). Keratinocytes were stimulated with control vehicle, TGF-β (2 ng per mL) or LPA (1 μM) for 45 min followed by the isolation of nuclei as described under "Materials and Methods". After protein determination, lysates of nuclei (200 μg protein per sample) were immunoprecipitated with anti-Smad3 antibodies, followed by immunoblotting. The top blot was developed using anti-Smad4- and the bottom blot with anti-Smad3 antibodies (*D*). Lysates of TGF-β (2 ng per mL, 30 min), LPA (1 μM, 30 min) ore control vehicle stimulated cells were incubated with a biotinylated double-stranded DNA (30 pM) composed of three tandemly repeated CAGA sequences. To control the specificity of the oligonucleotides, stimulated lysates were incubated with biotinylated scramled DNA fragments (30 pM). For competition control, lysates were incubated with 30 pM of biotinylated and 30 pM of non-biotinylated three tandemly repeated CAGA sequences. DNA-bound proteins were precipitated with streptavidin–agarose and western blot analysis using anti-Smad3 antibodies was performed (*E*). Smad3 was detected at 56 kDa and Smad4 at 66 kDa. All results were confirmed in three independent experiments.

Discussion

These data indicate that LPA enhances migration and inhibits cell growth of keratinocytes. Since intracellular targets of LPA have not been well defined, we clearly provide evidence that Smad-signaling is essential for LPA-mediated biological responses. Thus, LPA not only induces an activation of Smad3 but also fails to mediate migration and in part growth inhibition, when Smad3 is eliminated.

Rapid migration and proliferation of keratinocytes are essential to improve wound healing after various forms of cutaneous injury. A multitude of biological mediators and growth factors are formed and stored in human platelets, which in response to an injury are released at the wounded site (Lee, 2000). One of these factors is LPA suggesting a potential relevance for the wound-healing process (Sturm *et al*, 1999; Lee *et al*, 2000; Moolenaar, 2000). Indeed, here we show that migration and proliferation events of keratinocytes are modulated by this lysophospholipid. Exogenously added LPA promotes cell migration and inhibits proliferation of human keratinocytes. These data are consistent with intestinal wound healing as similar effects of LPA on intestinal epithelial cells have been observed (Sturm *et al*, 1999). Our data, however, are unexpected as it has been suggested that LPA possesses proliferative properties in primary keratinocytes (Piazza *et al*, 1995). Therefore, it should be considered that these studies were performed with quiescent keratinocytes and moreover a slight increase only occurred in the millimolar range. Our results clearly indicate, that lower micromolar levels of LPA potently inhibit growth of proliferating keratinocytes. The concentration, in which chemotaxis and cell growth inhibition by LPA takes place, is in agreement with serum levels, which have been estimated in the range of 2–20 μM of LPA (Aoki *et al*, 2002).

Interestingly, LPA possesses analogous actions on cell proliferation and migration in epithelial and epidermal cells

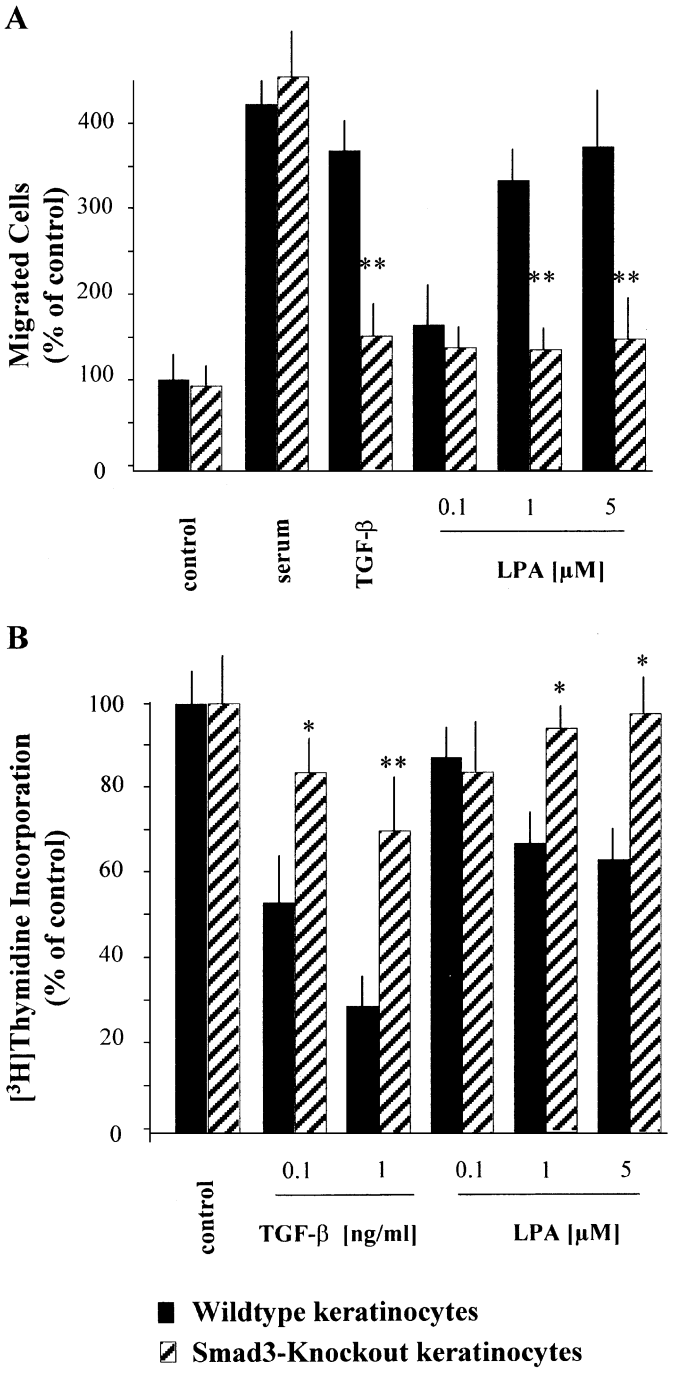

Figure 4
LPA-mediated migration and cell growth arrest is inhibited by the abrogation of Smad3 signaling. Chemotactic migration of keratinocytes isolated from wild-type and Smad3$^{(-/-)}$ mice was measured in response to a gradient of LPA (concentrations as indicated), TGF-β (2 ng per mL) or serum (10%) in a chemotaxis chamber as described (*A*). Wild-type and Smad3$^{(-/-)}$ keratinocytes were treated with TGF-β or LPA in the indicated concentrations for 24 h and pulsed with [^{3}H]thymidine. Then incorporation into DNA was determined as described in "Materials and Methods" (*B*). Values are percentage of control ± SEM from at least three experiments. $^*p<0.05$ and $^{**}p<0.001$ indicate a statistically significant difference *versus* unstimulated control cells.

as the related lysophospholipid sphingosine 1-phosphate and the cytokine TGF-β, which are derived from blood platelets (Dignass and Podolsky, 1993; Roberts *et al*, 1990; Vogler *et al*, 2003). Measurement of peptide levels elucidated that LPA does not modulate epidermal cell functions through the secretion of TGF-β. This corresponds with studies by Sturm *et al* (1999), which show that proliferation and migration of epithelial cells by LPA are independent of a release of TGF-β. TGF-β is known to mediate its chemotactic response and its cell growth inhibition in keratinocytes by the activation of the TGF-β receptor substrate Smad3 (Ashcroft *et al*, 1999; Ashcroft and Roberts, 2000). Most recently several studies indicate that Smad proteins can be activated in a TGF-β-independent way. Such an activation of Smad proteins has been shown for insulin-like growth factor binding protein-3 as well as for epidermal growth factor (EGF) and hepatocyte growth factor (de Caestecker *et al*, 1998; Fanayan *et al*, 2000; Fanayan *et al*, 2002; Firth and Baxter, 2002). These molecules are capable to stimulate Smad2 phosphorylation in breast cancer cells even in the absence of endogenous or exogenous TGF-β. Thus, it was of interest to figure out, whether LPA may also modulate Smad protein activity in a ligand-independent fashion. Indeed, here we clearly present evidence that LPA phosphorylates the Smad3 protein, which results in a heteromerization with Smad4, translocation into the nucleus, and the binding to target genes. The crucial role of Smad3 for biological actions of LPA becomes visible after abrogation of this transducing molecule as both the migratory response and the cell growth arrest are completely lost and reduced, respectively.

Next it was of interest to further elucidate the mechanism underlying the Smad activation by the lysophospholipid. Although intracellular targets of LPA have been discussed, most biological responses are mediated through the activation of the G-protein-coupled LPA receptors (Mills *et al*, 2002; Takuwa *et al*, 2002). But to our knowledge the expression of LPA receptors in primary keratinocytes has not been examined. Here, we show that not only all LPA receptors are present in human keratinocytes but also contribute to the activation of TGF-β signaling. Thus, Smad phosphorylation is completely abolished in the presence of PTX. In accordance also LPA-induced migration and cell growth arrest are sensitive to this toxin. Moreover, our results clearly indicate also an involvement of TβRI in LPA-induced Smad3 activation. These data suggest a cross-communication between LPA- and TGF-β receptors. Indeed, such a receptor interaction has also been described to contribute to Smad activation by insulin-like growth factor binding protein-3 as it needs the existence of type I and II TGF-β receptors. In contrast, Smad activation by EGF also occurs in TGF-β receptor deficient cells (de Caestecker *et al*, 1998; Fanayan *et al*, 2000, 2002; Firth and Baxter, 2002).

Indeed, several lines of evidence also support the finding that LPA receptors build functional heterodimers with other plasma membrane receptors. Initially, it was found that in Rat-1 fibroblasts LPA induces a rapid phosphorylation of the EGF receptor suggesting that LPA effects are executed through the stimulation of receptor tyrosine kinase activity (Hordijk *et al*, 1994). Actually, in analogy to LPA, the EGF receptor plays a central role in regulating cell proliferation, differentiation, and migration (Brown, 1995). Internalization of the EGF-receptor represents an important facet for the control of mitogenic responses (Kim *et al*, 2003). Thus, it has

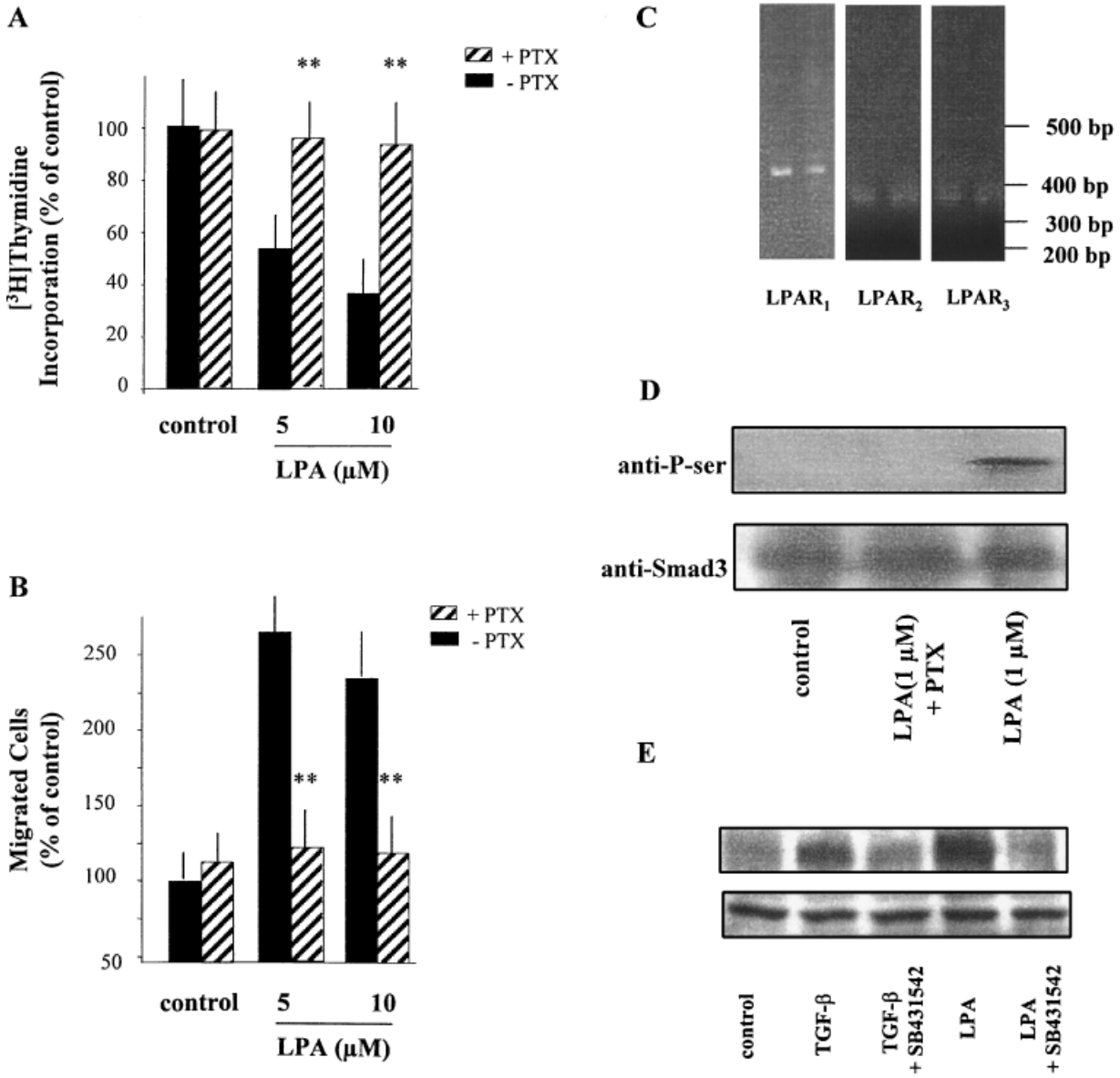

Figure 5
Expression of LPA receptors in human keratinocytes and intact skin and their involvement in LPA-mediated functions. The mRNA of cultured keratinocytes or intact skin was isolated, reverse transcribed and amplified as described in "Materials and Methods". PCR products of cultured keratinocytes (left band) and intact skin (right band) were size-fractionated in an agarose gel, and visualized by ethidium bromide staining. PCR results were confirmed by two more independent experiments (*C*). Human keratinocytes were pre-treated with control vehicle or 200 ng per mL PTX for 3 h. Afterwards, growth inhibition (*A*), chemotaxis (*B*) and Smad3 phosphorylation (*D*) in response to the indicated concentrations of LPA was determined. Keratinocytes were pre-incubated with SB431542 (10 μM) for 30 min. After treatment with LPA (1 μM) or TGF-β (2 ng per mL), Smad3 phosphorylation was detected (*E*). Values of migration and proliferation assays are percentage of control ± SEM from at least three experiments. $^{*}p<0.05$ and $^{**}p< 0.001$ indicate a statistically significant difference *versus* unstimulated control cells. Similar results of Smad3 phosphorylation were obtained in two further experiments.

been shown that the EGF-receptor is removed from the cell surface not only following stimulation with EGF, but also in response to stimulation of LPA receptors (Kim *et al*, 2003). Several mechanisms have been discussed how LPA receptors induce an activation of the EGF receptor. Thus, stimulation of the EGF receptor involves activation of membrane-bound metalloproteinases, which, in response, induce the release of heparin-EGF from its latent membrane-spanning precursors (Yan *et al*, 2002). But EGF-receptor transactivation by LPA receptors may also occur independently of metalloproteinases. Accumulating evidence suggests that c-Src tyrosine kinase activity mediates EGF receptor activation downstream of LPA receptors as this kinase is activated in reponse to LPA, and moreover, has been shown to directly phosphorylate and activate the receptor tyrosine kinase (Biscardi *et al*, 1999; Kim *et al*, 2003). Additionally, it has been suggested that transactivation of receptor tyrosine kinases by LPA receptors is a more general phenomenon, which is not restricted to only EGF receptors. Thus, ligand-independent activation of platelet-derived growth factor receptor is essential to mediate the mitogenic activity of LPA (Herrlich *et al*, 1998).

In summary, we present data that show a novel cross-talk between LPA receptors and TGF-β signaling and further

on indicate the essential role of this pathway for biological responses of LPA. It is of interest that TGF-β signaling is involved in physiological and pathophysiological processes such as wound healing or hyperproliferative skin diseases. Owing to their transactivation effects, drug development of LPA agonists or antagonists might present a novel method to regulate such biological responses.

Materials and Methods

Chemicals Specific primers (LPA_{1-3}) were synthesized at Tib Molbiol (Berlin, Germany). [*methyl*-^{3}H]Thymidine (35 Ci/mmol), and [^{35}S]-GTPγS (1000 Ci per mmol) were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). TGF-β, PTX, protein G plus agarose, molecular weight markers, sodium dodecyl sulfate (SDS)-sample buffer, and dithiothreitol were from Calbiochem (Bad Soden, Germany), poly dI-dC from Roche Diagnostics (Mannheim, Germany). Goat polyclonal anti-Smad3-, goat polyclonal anti-Smad4- antibodies, normal goat IgG, anti-goat and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit polyclonal anti-phosphoserine antibody and goat anti-TGF-β_1 antibodies were obtained from Zymed Laboratories (San Francisco, California), LumiGlo reagent and peroxide from New England Biolabs (Beverly, Massachusetts). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, aprotinin, bovine serum albumin, collagen-1, deoxycholic acid, Dulbecco's Modified Eagle's Medium, Eagle's Minimal Essential Medium, eosin, EDTA, fetal bovine serum, fibronectin, GIEMSA, hematoxilen, HEPES, β-glycerophosphate, LPA, leupeptin, nonidet P-40, penicillin/streptomycin, pepstatin, phenylmethylsulfonylfluoride (PMSF), potassium glycine, SB431542, SDS, sodium fluoride, sodium orthovanadate, streptavidin sepharose, sucrose, trichloroacetic acid, Tris, Triton X-100, and trypsin were purchased from Sigma-Aldrich (Taufkirchen, Germany). HEPES buffer and calcium- and magnesium-free phosphate-buffered saline (PBS) were purchased from Invitrogen (Karlsruhe, Germany). 1α,25-Dihydroxyvitamin D_3 was a gift from Leo Pharmaceuticals (Ballerup, Denmark). Keratinocyte basal medium, epidermal growth factor (EGF), insulin, hydrocortisone, bovine pituitary extract, gentamicin sulfate, and amphotericin B were from Cell Systems (St Katharinen, Germany).

Keratinocyte culture To isolate human keratinocytes, juvenile foreskin was incubated at 4°C in a solution of 0.25% trypsin and 0.2% EDTA for 20 h and trypsinization was terminated by addition of ice-cold Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. Keratinocytes were separated, washed with PBS and centrifuged. The pellet was resuspended in keratinocyte growth medium that was prepared from keratinocyte basal medium by the addition of 0.1 ng per mL recombinant EGF, 5.0 μg per mL insulin, 0.5 μg per mL hydrocortisone, 0.15 mM Ca^{2+}, 30 μg per mL bovine pituitary extract, 50 μg per mL gentamicin sulfate, and 50 ng per mL amphotericin B. Keratinocytes were pooled from several donors and only used in the second or third passage.

Murine keratinocytes were isolated from PCR-genotyped Smad3 wild-type and knockout newborn mice as recently reported (Yang *et al*, 1999). Skin of sacrified mice was numbered and disinfected by iodine solution. Mice were washed three times in 70% ethanol and tails from each mouse were removed for RT-PCR. 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 RT-PCR, the epidermal layers of homozygote Smad3-knockout- and wild-type mice were pooled separately and homogenized. The cell suspensions were centrifuged, washed with PBS and resuspended in growth medium that was prepared from Eagle's Minimal Essential Medium by the addition of 8% of fetal bovine serum, 0.2 mM Ca^{2+} and 1% penicillin/streptomycin. The next day, medium was switched to Eagle's Minimal Essential Medium/serum containing 0.05 mM Ca^{2+}. The protocol used in this study was approved by the National Cancer Institute Animal Care and Use Committee and was in accordance with the guidelines of the Institute of Laboratory Animal Resources, National Research Council.

Abrogation of Smad3 Smad3$^{(ex8/ex8)}$ mice were generated by targeted disruption of the Smad3 gene by homologous recombination using a pLoxpneo cassette (Yang *et al*, 1999). Targeted embryonic stem-cell clones were microinjected into C57BL/6 blastocysts to obtain germline transmission. Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring. Keratinocytes were isolated from wild-type or Smad3-deficient homozygous mice. Genotypes were determined by PCR. For detection of wild-type and Smad3$^{ex8/+}$ mice the primer sequences 5′-CCACTTCATTGCCATATGCCCTG-3′ (located 5′ to the deletion) and 5′-CCCGAACAGTTGGATTCACACA-3′ (located within the deletion) were used. Smad3$^{(-/-)}$ mice were identified using the primer located to the 5′deletion and a primer specific for the pLoxpneo cassette (5′-CCAGACTGCCTTGGGAAAAGC-3′).

Reverse-transcription and amplification by PCR The mRNAs of human keratinocytes or intact skin were isolated by QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. One microgramm of mRNA was reverse transcribed (Superscript reverse transcriptase, Invitrogen) in the presence of 1 pmol of a 25–30 mer oligo(dT) primer. Based upon the nucleotide sequences of the human LPLR family, oligonucleotide primer pairs were prepared (LPA_1: 5′-CCC AAC CAA CAG GAC TGA CT-3′, 5′-GGT CCA GAA CTA TGC CGA GA-3′, LPA_2: 5′-CCC AAC CAA CAG GAC TGA CT-3′, 5′-GAG CCC TTA TCT CTC CCC AC-3′; LPA_3: 5′-GGA CAC CCA TGA AGC TAA TG-3′, 5′-TCT GGG TTC TCC TGA GAG AA-3′). PCR amplification was carried out in a Thermocycler (T Gradient, Whatman Biometra, Göttingen, Germany) using the Thermoprime Plus polymerase (Advanced Biotechnologies, Columbia, Maryland) under 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. PCR 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 a buffer containing 20 mM Tris (pH 7.4), 500 μM PMSF, 1 μg per mL each leupeptin, aprotinin, and 0.5 μg per mL pepstatin and homogenized by passing through a 28-gauge needle for 10 times. The homogenate was centrifuged for 5 min at 5000 × *g* and the supernatant was spun for 40 min at 43 000 × *g*. Ten micrograms protein/assay were incubated for 45 min in a buffer containing 20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 100 μM PMSF, 1 μg per mL each leupeptin, aprotinin, 0.5 μg per mL pepstatin, 3 μM GDP, 50 pM [^{35}S]-GTPγS, and different concentrations of LPA. The samples were rapidly filtered on GF/B glass microfiber filters (Whatman, Kent, UK) presoaked in a buffer [20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM β-mercaptoethanol]. The filters were washed three times and radioactivity was determined in a scintillation counter.

Measurement of TGF-β secretion Keratinocytes (4×10^4 cells per mL) were stimulated with LPA over a time period of 24 h. As positive control, 1α,25-dihydroxyvitamin D_3 was used. Then levels of TGF-β in the supernatant were quantified by selective ELISA kits following the instructions of the manufacturer (Amersham Pharmacia Biotech). For measurement of latent complexes of TGF-β, activation was accomplished by acid treatment. Therefore, 0.5 mL of cell culture supernatants were treated with 0.1 mL of 1 M HCl, incubated for 10 min, and then neutralized with 0.1 mL of 1.2 M NaOH/0.5 M HEPES. Samples were analyzed by an ELISA reader from Merlin (Bornheim, Germany). The detection limit was 4 pg per mL.

Immunoprecipitation of Smad proteins and western blot analysis Keratinocytes were seeded in six-well plates and cultured for 24 h. After a 2 h medium switch to HEPES buffer (1 M), cells were treated with TGF-β or LPA in different concentrations and time periods. To finish the stimulation, cells 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 mM PMSF, 1mM EDTA, 1 μg per mL leupeptin, 1 μg per mL aprotinin and 1 μg per 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 and 100 μg of lysate protein was immunoprecipitated overnight at 4°C with 0.2 μg anti-Smad3 antibodies, followed by a precipitation with 10 μL 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 SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and separated by SDS/PAGE. Gels were blotted overnight onto PVDF membranes. After blocking with 5% non-fat dry milk (for anti-Smad3 and anti-Smad4) or 3% bovine serum albumin (for anti-phosphoserine) in Tris-buffered saline/Tween-20 (0.1%) overnight at 4°C. Membranes were incubated with anti-Smad3-, anti-Smad4-, or anti-phosphoserine antibodies (each 0.2 μg per 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- and anti-rabbit IgG-horseradish peroxidase) for 1 h. After washing, the blots were developed according to the manufacturer's protocol.

Isolation of nuclei Nuclei were isolated as described by Bunce *et al* (1988). Briefly, keratinocytes were washed with PBS, scraped into PBS and then centrifuged at 5000 × *g* for 5 min. Cells were resuspended in 400 μL of the following buffer (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 15 mM KCl, 1 mM PMSF) at 5×10^6 cells per mL, frozen in liquid nitrogen, thawed rapidly at 37°C, and then passed 15 times through a 25-gauge needle. 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 3 min. Only intact nuclei pelleted through the cushion, as confirmed by light microscopy. Nuclei were lysed in 100 μL of complete RIPA buffer by seven freeze–thawing cycles. To determine the purity of the nuclei lysate, the activity of a specific marker enzyme for endoplasmic reticulum, α-glucosidase II, was measured as described by Fujimoto *et al* (1976). After protein determination, lysates of nuclei (200 μg protein/sample) were immunoprecipitated with anti-Smad3 antibodies. The immunoprecipitates were eluted and separated by SDS/PAGE. Then western blot analysis was performed by the use of anti-Smad3- and anti-Smad4 antibodies.

Binding of the Smad3/Smad4 complex to biotinylated DNA Human keratinocytes were plated into 100 mm dishes (1×10^6 cells per dish). Cells of 30%–40% confluence were stimulated, 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 PMSF and 1 μg per mL aprotinin, leupeptin and pepstatin. Lysate protein (1 mg) was incubated at 4°C for 1 h with a biotinylated double-stranded DNA (30 pM) composed of three-tandemly repeated CAGA sequences in the presence of 12 μg of poly dI-dC (5′Bio-TCGAGAGCCAGACAAGG AGCCAGACAAGGAGCCAGACACTCCAG-3′ 5′-CTCGAGTGTCTG GCTCCTTGTCTGGCTCCTTGTCTGGCTCTCGA-3′). For control experiments, lysates were incubated with a non-specific biotinylated double-stranded DNA fragment of scrambled nucleotides (5-Bio-TCG AGA GCT AGA TAA GGA GCT AGA TAA GGA GCT AGA TAC TCG AG, 5′-GAG TAT CTA GCT CCT TAT CTA GCT CCT TAT CTA GCT CTC GA). For competition control, lysates were incubated with 30 pM of biotinylated and 30 pM of non-biotinylated three-tandemly repeated CAGA sequences. 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 SDS sample buffer for electrophoresis and western blot analysis using anti-Smad3 antibodies (Nishihara *et al*, 1999).

DNA synthesis, cell cycle and cytotoxicity analysis Keratinocytes (4×10^4 cells per well) were grown in 24-well plates for 24 h. Then medium was replaced by fresh keratinocyte growth medium and cells were incubated with TGF-β (2 ng per mL) or the indicated concentrations of LPA for 72 h and pulsed with 1 μCi of [*methyl*-^{3}H]thymidine per well. After 23 h, the 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 N NaOH-solution and incorporated [*methyl*-^{3}H]thymidine was determined in a scintillation counter (MicroBeta Plus, Wallac Oy, Turku, Finland).

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

For the measurement of cytotoxicity, keratinocytes (8×10^4 cells per well) were seeded into 24-well plates for 24 h and incubated with LPA for 24 h at 37°C in 5% CO_2. After the addition of 100 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg per mL) per well for 4 h at 37°C the supernatants were removed and the formazan crystals were solubilized in 1 mL of dimethyl sulfoxide. The optical density was measured at 540 nm using a scanning microplate spectrophotometer (Multiscan Plus, Labsystems, Helsinki, Finland).

Migration assay Chemotaxis of human keratinocytes in response to a gradient of serum, LPA or TGF-β was measured in a modified Boyden chamber with fibronectin-coated filters (Wang *et al*, 1999). Cells were added to the upper well of the chamber. The lower chamber contained LPA in the indicated concentration. As control for the chemotactic response TGF-β (2 ng per mL) was used. Cells that had migrated through the membrane were fixed, stained by GIEMSA and counted.

Murine keratinocytes were cultured until a confluence of approximately 30%–40%, trypsinized, washed and resuspended in serum-free Eagle's Minimal Essential Medium. They were added to the upper well of a chemotaxis chamber which was separated from the serum-, TGF-β-, or LPA-supplemented test medium by a fibronectin/collagen-I-coated membrane. Cells that had migrated through the membrane after 5 h at 37°C were stained with ethanol for 1 min, hematoxylene and eosin each for 1 min and counted. Each value represents the average number of cells migrated from triplicate wells.

Statistical analysis Data are expressed as the mean ± SEM of results from at least three experiments, each run in triplicate. Statistics were performed using Student's *t* test. $^*p < 0.05$ and $^{**}p < 0.001$ indicate a statistically significant difference *versus* control experiments.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to B.K. (FG 463).

DOI: 10.1111/j.0022-202X.2004.23458.x

Manuscript received December 18, 2003; revised June 9, 2004; accepted for publication June 24, 2004

Address correspondence to: Burkhard Kleuser, Institute of Pharmacy, Pharmacology and Toxicology, Free University Berlin, Königin-Luise-Str. 2 + 4, D-14195 Berlin, Germany. Email: kleuser@zedat.fu-berlin.de

References

Alexandrow MG, Moses HL: Transforming growth factor beta 1 inhibits mouse keratinocytes late in G1 independent of effects on gene transcription. Cancer Res 55:3928–3932, 1995

Aoki J, Taira A, Takanezawa Y, *et al*: Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J Biol Chem 277:48737–48744, 2002

Ashcroft GS, Roberts AB: Loss of Smad3 modulates wound healing. Cytokine Growth Factor Rev 11:125–131, 2000

Ashcroft GS, Yang X, Glick AB, *et al*: Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1:260–266, 1999

Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, Parsons SJ: c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. J Biol Chem 274:8335–8343, 1999

Brown KD: The epidermal growth factor/transforming growth factor-alpha family and their receptors. Eur J Gastroenterol Hepatol 7:914–922, 1995

Bunce CM, Thick JA, Lord JM, Mills D, Brown G: A rapid procedure for isolating hemopoietic cell nuclei. Anal Biochem 175:67–73, 1988

Contos JJ, Ishii I, Chun J: Lysophosphatidic acid receptors. Mol Pharmacol 58:1188–1196, 2000

de Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ: Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. Genes Dev 12:1587–1592, 1998

Dignass AU, Podolsky DK: Cytokine modulation of intestinal epithelial cell restitution: Central role of transforming growth factor beta. Gastroenterology 105:1323–1332, 1993

Fanayan S, Firth SM, Baxter RC: Signaling through the Smad pathway by insulin-like growth factor-binding protein-3 in breast cancer cells. Relationship to transforming growth factor-beta 1 signaling. J Biol Chem 277:7255–7261, 2002

Fanayan S, Firth SM, Butt AJ, Baxter RC: Growth inhibition by insulin-like growth factor-binding protein-3 in T47D breast cancer cells requires transforming growth factor-beta (TGF-beta) and the type II TGF-beta receptor. J Biol Chem 275:39146–39151, 2000

Firth SM, Baxter RC: Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824–854, 2002

Fujimoto A, Fluharty AL, Stevens RL, Kihara H, Wilson MG: Two alpha-glucosidases in cultured amniotic fluid cells and their differentiation in the prenatal diagnosis of Pompe's disease. Clin Chim Acta 68:177–186, 1976

Gaits F, Fourcade O, Le Balle F, *et al*: Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. FEBS Lett 410:54–58, 1997

Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G, Gudermann T: Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. Proc Natl Acad Sci USA 95:8985–8990, 1998

Hordijk PL, Verlaan I, van Corven EJ, Moolenaar WH: Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of map kinase is mediated by the Gi-p21ras pathway. J Biol Chem 269:645–651, 1994

Huang MC, Graeler M, Shankar G, Spencer J, Goetzl EJ: Lysophospholipid mediators of immunity and neoplasia. Biochim Biophys Acta 1582:161–167, 2002

Kim J, Ahn S, Guo R, Daaka Y: Regulation of epidermal growth factor receptor internalization by G protein-coupled receptors. Biochemistry 42:2887–2894, 2003

Laping NJ, Grygielko E, Mathur A, *et al*: Inhibition of Transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity: SB431542. Mol Pharmacol 62:58–64, 2002

Lee H, Goetzl EJ, An S: Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. Am J Physiol Cell Physiol 278: C612–C618, 2002

Lee SJ: Cytokine delivery and tissue engineering. Yonsei Med J 41:704–719, 2002

Massague J: TGF-beta signal transduction. Annu Rev Biochem 67:753–791, 1998

Massague J: How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1:169–178, 2000

Massague J, Chen YG: Controlling TGF-beta signaling. Genes Dev 14:627–644, 2000

Massague J, Wotton D: Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 19:1745–1754, 2000

McIntyre TM, Pontsler AV, Silva AR, *et al*: Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. Proc Natl Acad Sci USA 100:131–136, 2003

Mills GB, Eder A, Fang X, *et al*: Critical role of lysophospholipids in the pathophysiology, diagnosis, and management of ovarian cancer. Cancer Treat Res 107:259–283, 2002

Moolenaar WH: Bioactive lysophospholipids and their G protein-coupled receptors. Exp Cell Res 253:230–238, 1999

Moolenaar WH: Development of our current understanding of bioactive lysophospholipids. Ann NY Acad Sci 905:1–10, 2000

Moolenaar WH, Kranenburg O, Postma FR, Zondag GC: Lysophosphatidic acid: G-protein signalling and cellular responses. Curr Opin Cell Biol 9:168–173, 1997

Nishihara A, Hanai J, Imamura T, Miyazono K, Kawabata M: E1A inhibits transforming growth factor-beta signaling through binding to Smad proteins. J Biol Chem 274:28716–28723, 1999

O'Kane S, Ferguson MW: Transforming growth factor beta s and wound healing. Int J Biochem Cell Biol 29:63–78, 1997

Osborne N, Stainier DY: Lipid receptors in cardiovascular development. Annu Rev Physiol 65:23–43, 2003

Pages G, Girard A, Jeanneton O, *et al*: LPA as a paracrine mediator of adipocyte growth and function. Ann NY Acad Sci 905:159–164, 2000

Panetti TS, Magnusson MK, Peyruchaud O, Zhang Q, Cooke ME, Sakai T, Mosher DF: Modulation of cell interactions with extracellular matrix by lysophosphatidic acid and sphingosine 1-phosphate. Prostaglandins 64: 93–106, 2001

Piazza GA, Ritter JL, Baracka CA: Lysophosphatidic acid induction of transforming growth factors alpha and beta: Modulation of proliferation and differentiation in cultured human keratinocytes and mouse skin. Exp Cell Res 216:51–64, 1995

Roberts AB, Flanders KC, Heine UI, *et al*: Transforming growth factor-beta: Multifunctional regulator of differentiation and development. Philos Trans R Soc Lond B Biol Sci 327:145–154, 1990

Roberts AB, Sporn MB: Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors 8:1–9, 1993

Sano T, Baker D, Virag T, *et al*: Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. J Biol Chem 277:21197–21206, 2002

Shida D, Kitayama J, Yamaguchi H, *et al*: Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. Cancer Res 63:1706–1711, 2003

Sturm A, Sudermann T, Schulte KM, Goebell H, Dignass AU: Modulation of intestinal epithelial wound healing *in vitro* and *in vivo* by lysophosphatidic acid. Gastroenterology 117:368–377, 1999

Suemori S, Ciacci C, Podolsky DK: Regulation of transforming growth factor expression in rat intestinal epithelial cell lines. J Clin Invest 87:2216–2221, 1991

Takuwa Y, Takuwa N, Sugimoto N: The Edg family G protein-coupled receptors for lysophospholipids: Their signaling properties and biological activities. J Biochem (Tokyo) 131:767–771, 2002

Tigyi G, Dyer DL, Miledi R: Lysophosphatidic acid possesses dual action in cell proliferation. Proc Natl Acad Sci USA 91:1908–1912, 1994

Tokumura A, Iimori M, Nishioka Y, Kitahara M, Sakashita M, Tanaka S: Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. Am J Physiol 267:204–210, 1994

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL: SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell 95:779–791, 1998

Vogler R, Sauer B, Kim DS, Schäfer-Korting M, Kleuser B.: Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing. J Invest Dermatol 120:693–700, 2003

Wang F, Nohara K, Olivera A, Thompson EW, Spiegel S: Involvement of focal adhesion kinase in inhibition of motility of human breast cancer cells by sphingosine 1-phosphate. Exp Cell Res 247:17–28, 1999

Yan Y, Shirakabe K, Werb Z: The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. J Cell Biol 158:221–226, 2002

Yang X, Letterio JJ, Lechleider RJ, *et al*: Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J 18:1280–1291, 1999