# Sphingosine 1-phosphate stimulates Rho-mediated tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 fibroblasts

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Sphingosine 1-phosphate (SPP), a sphingolipid second messenger implicated in the mitogenic action of platelet-derived growth factor [Olivera, A. and Spiegel, S. (1993) Nature (London) **365**, 557–560], induced rapid reorganization of the actin cytoskeleton resulting in stress-fibre formation. SPP also induced transient tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$), a cytosolic tyrosine kinase that localizes in focal adhesions, and of the cytoskeleton-associated protein paxillin. Exoenzyme C3 transferase, which ADP-ribosylates Rho (a Ras-related small GTP binding protein) on asparagine-41 and renders it biologically inactive, inhibited both stress-fibre formation and protein tyrosine phosphorylation induced by SPP. Thus Rho may be an upstream regulator of both stress-fibre formation and tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Pretreatment with PMA, an activator of protein kinase C (PKC), inhibited the stimulation of stress-fibre formation induced by 1-oleoyl-lysophosphatidic acid (LPA) but not that by SPP. Similarly, PMA also decreased LPA-induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin without abrogating the response to SPP. Thus PKC is involved in LPA- but not SPP-dependent signalling. The polyanionic drug suramin, a broad-specificity inhibitor of ligand–receptor interactions, did not inhibit either the mitogenic effect of SPP or its stimulation of tyrosine phosphorylation of $p125^{FAK}$. However, suramin markedly inhibited these responses induced by LPA. These results suggest that in contrast with LPA, SPP may be acting intracellularly in Swiss 3T3 fibroblasts to stimulate tyrosine phosphorylation of $p125^{FAK}$ and paxillin and cell growth.

## INTRODUCTION

There is increased interest in physiological functions and mechanisms of action of sphingolipid metabolites, ceramide, sphingosine and sphingosine 1-phosphate (SPP), members of a new class of lipid second messengers [1–4]. Much of the interest in sphingolipid second messengers stemmed from the seminal studies of Hannun and Bell that suggested that sphingosine can act as a negative regulator of protein kinase C (PKC) [5]. Further studies revealed an important role for sphingosine as a positive regulator of cell growth acting in a PKC-independent pathway [6]. In contrast with the lack of stereospecificity in the inhibition of PKC, the mitogenic effect of sphingosine was stereospecific, since only the natural D-*erythro*-isomer of sphingosine stimulated DNA synthesis [7]. The carcinogenicity of Fumonisin B1, a fungal toxin that has a structural resemblance to long-chain sphingoid bases, has been related to its ability to increase the levels of sphinganine and sphingosine in cells by inhibition of sphinganine *N*-acyltransferase [8]. Sphingosine is rapidly taken up by cells and phosphorylated on its primary hydroxy group by the action of a specific sphingosine kinase to form SPP [9–11]. Subsequent studies have suggested that part of the mitogenic effect of exogenous sphingosine may be mediated via its conversion to SPP [10]. SPP, more potently than sphingosine, mobilizes calcium from internal sources in many cells [4], acting through both $Ins P_3$-dependent and -independent pathways [12,13]. Recently, it has been shown that SPP rapidly stimulates extracellular-signal-regulated kinase in airway smooth muscle cells [14] and the Raf/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinase signalling pathway in Swiss 3T3 fibroblasts [15], and also stimulates the transcription factor activator protein-1 (AP-1) [16], linking signal transduction by sphingolipid metabolites to gene expression.

The recent observation that intracellular levels of sphingosine and SPP and sphingosine kinase activity are rapidly and transiently increased by certain growth factors, such as platelet derived growth factor (PDGF), in quiescent fibroblasts, suggests that these breakdown products of cellular sphingolipids may be important mediators of the mitogenic action of PDGF [17]. PDGF also increased levels of sphingosine in vascular smooth muscle cells [18] and SPP in arterial smooth muscle cells [19] and airway smooth muscle cells [14]. In agreement, PDGF, but not endothelin-1, mediates proliferation of glomerular mesangial cells, in part through ceramidase-regulated sphingosine formation [20]. Furthermore, inhibitors of sphingosine and SPP production specifically and selectively reduce PDGF-induced cellular proliferation [17,20].

One of the earliest responses to many growth factors is the rapid reorganization of the actin cytoskeleton. PDGF induces actin reorganization to initially form ruffles, and after 5–10 min, actin stress fibres are formed that are linked to the plasma membrane at focal adhesion sites [21]. Rho, a Ras-related small GTP binding protein, is essential for the co-ordinated assembly of focal adhesions and stress fibres induced by PDGF. Similar to PDGF, sphingosine also induces actin stress-fibre formation and focal contact assembly in Swiss 3T3 fibroblasts [22]. However, in contrast with PDGF, sphingosine-induced tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$), a cytosolic tyrosine kinase that is located in focal adhesions, and of the cytoskeletal-associated protein paxillin, occurs more slowly and reaches a maximum after only 60 min [22]. Moreover, the effect of sphingosine was mimicked by cell-permeant ceramide analogues,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; LPA, 1-oleoyl-lysophosphatidic acid; mAb, monoclonal antibody; PDGF, platelet derived growth factor; $p125^{FAK}$, focal adhesion kinase p125; PKC, protein kinase C; SPC, sphingosylphosphocholine; SPP, sphingosine 1-phosphate; TRITC, tetramethyl rhodamine isothiocyanate.

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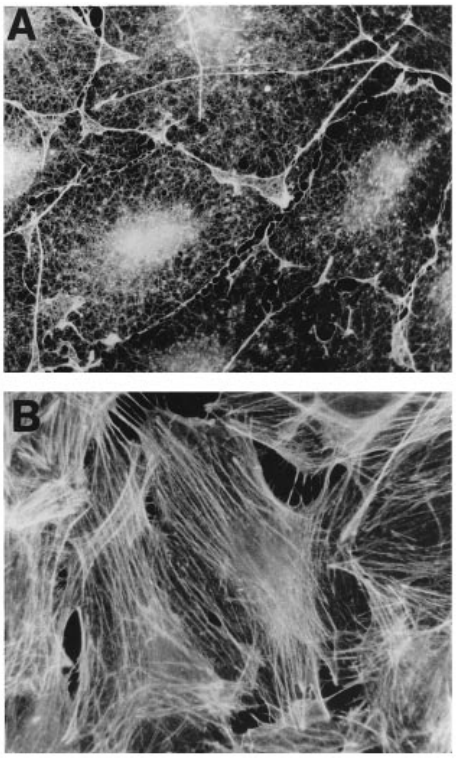


**Figure 1 Actin stress-fibre formation induced by SPP**

Serum-starved Swiss 3T3 cells treated without (**A**) or with (**B**) 5 $\mu$M SPP for 30 min were fixed and stained with TRITC-labelled phalloidin as described in the Experimental procedures section.

leading to the conclusion that the sphingosine effect was mediated via its intracellular metabolism to ceramide [22]. In agreement, treatment of Swiss 3T3 fibroblasts with sphingomyelinase also led to phosphorylation of p125$^{FAK}$ and paxillin [23]. Because sphingosine is also rapidly converted to SPP in cells, reaching a maximum within 60 min [10], and SPP has been implicated in microfilament reorganization [19], it was of interest to examine the effects of SPP on actin stress-fibre formation and phosphorylation of p125$^{FAK}$ and paxillin in Swiss 3T3 fibroblasts. In this study, we found that mitogenic concentrations of SPP induced rapid reorganization of the actin cytoskeleton resulting in stress-fibre formation and also induced transient tyrosine phosphorylation of p125$^{FAK}$ and paxillin that were dependent on activation by Rho. Moreover, suramin, an inhibitor of ligand–receptor interactions, did not inhibit either the mitogenic effect of SPP or the tyrosine phosphorylation of p125$^{FAK}$ and paxillin, suggesting that SPP acts in a distinctly different manner to 1-oleoyl-lysophosphatidic acid (LPA).

## EXPERIMENTAL PROCEDURES

### Materials

Insulin and transferrin were from Collaborative Research (Lexington, MA, U.S.A.). Calf serum was from Colorado Serum Co. (Denver, CO, U.S.A.). [*Me*-$^3$H]Thymidine (55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). LPA was from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). SPP was obtained from Biomol Research Laboratory Inc. (Plymouth Meeting, PA, U.S.A.). Suramin and PMA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-p125$^{FAK}$ and anti-phosphotyrosine (4G10) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Anti-phosphotyrosine (PY20) and anti-paxillin antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.).

### Cell culture

Swiss 3T3 cells were cultured as previously described [21]. For measurement of DNA synthesis, cells were seeded and grown on multicluster plastic tissue culture dishes (24 mm $\times$ 16 mm wells, Costar, Cambridge, MA, U.S.A.). For immunofluorescence studies, cells were seeded in dishes containing 13 mm circular glass cover slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum, and used 5–7 days later when cells were confluent and quiescent.

### Microinjection

Recombinant C3 exoenzyme was obtained from an *Escherichia coli* expression system [24] and microinjected together with fluorescein isothiocyanate (FITC)-lysinated dextran (2 mg/ml) into the cytoplasm of cells 30 min before fixation. Between 50–100 cells were microinjected in each experiment, and in all cases, cells showing a response to C3 also contained FITC-dextran.

### Immunofluorescence

For actin localization, cells were fixed in 4 % (w/v) paraformaldehyde for 10 min, permeabilized with 0.2 % (v/v) Triton X-100 for 5 min, and actin filaments were detected as previously described by staining with 0.1 $\mu$g/ml tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin [21]. Cells were viewed on a Zeiss Axiophot microscope and photographed with Kodak TMY-400 film.

### Immunoprecipitation

Serum-starved confluent Swiss 3T3 fibroblasts were treated with SPP, washed twice with PBS and lysed at 4 °C in 500 $\mu$l of lysis buffer consisting of 1 % Triton X-100 in 50 mM Hepes (pH 7.4)/150 mM NaCl/1 mM EDTA, and a cocktail of phosphatase and protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride/50 mM NaF/2 mM sodium orthovanadate/4 mM sodium pyrophosphate/1 mM PMSF/aprotinin (and leupeptin each 2.5 $\mu$g/ml)]. Lysed cells were harvested by scraping and centrifuging at 14500 ***g*** for 10 min at 4 °C. Aliquots of supernatants (350–500 $\mu$g) were incubated with 3–4 $\mu$g of monoclonal antibodies (mAbs) directed against p125$^{FAK}$ or with paxillin antibodies for 4 h at 4 °C, followed by the addition of 20 $\mu$l of Protein A/G–agarose. After overnight incubation at 4 °C, the immune complexes bound to agarose were recovered by centrifugation, washed twice with lysis buffer and heated for 10 min at 95 °C in 2 $\times$ SDS/PAGE sample buffer [200 mM Tris/2 mM EDTA/6 % (w/v) SDS/4 % (v/v) 2-mercaptoethanol/10 % (v/v) glycerol (pH 6.8)] and then analysed by SDS/PAGE.

### Western blotting

Following SDS/PAGE, proteins were transferred to PVDF membranes using a Bio-Rad transblot apparatus. Membranes were blocked using 3 % (w/v) BSA in PBS and incubated with a mixture of anti-phosphotyrosine mAbs (PY20 and 4G10, 1 : 1) or mAb directed against p125$^{FAK}$ or paxillin in PBS containing 0.1 % (v/v) Tween-20 and 1 % BSA. Immunoreactive bands were detected by chemiluminescence using horseradish peroxidase-conjugated anti-mouse IgG.

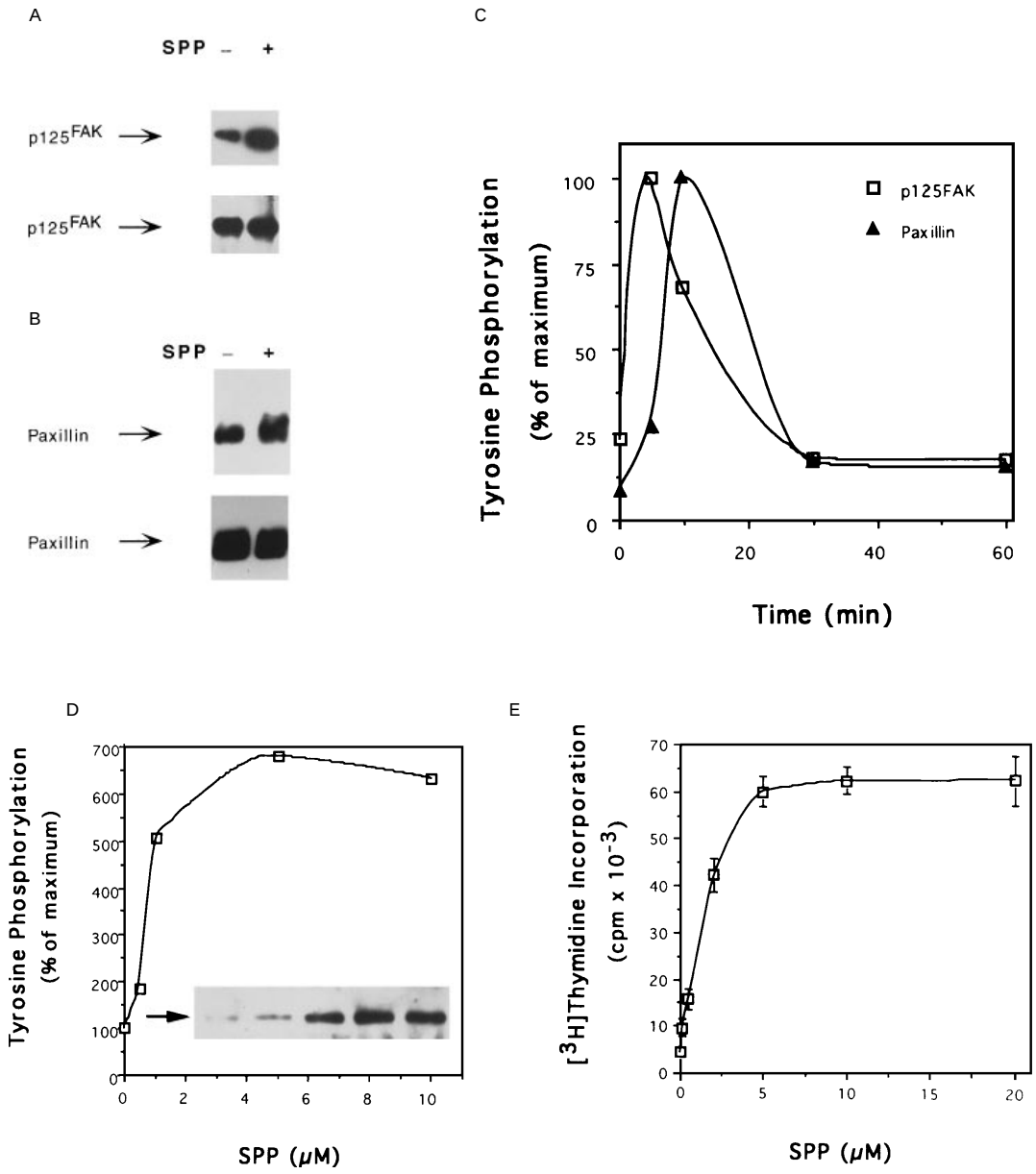


**Figure 2 SPP stimulates tyrosine phosphorylation of p125$^{FAK}$ and paxillin in Swiss 3T3 fibroblasts**

(**A**) Quiescent Swiss 3T3 fibroblasts were treated with 10 $\mu$M SPP for 10 min and lysed, and cell lysates were immunoprecipitated with anti-p125$^{FAK}$ mAb and analysed by Western blotting with anti-Tyr(P) antibody (top) or anti-p125$^{FAK}$ antibody (bottom) as described in the Experimental procedures section. (**B**) Duplicate cultures were immunoprecipitated with anti-paxillin mAb and analysed by Western blotting with anti-Tyr(P) antibody (top) or anti-paxillin antibody (bottom). The data are representative of at least three independent experiments. (**C**) Time course for SPP-induced tyrosine phosphorylation of p125$^{FAK}$ and paxillin. (**D**) Dose–response for SPP-induced tyrosine phosphorylation of p125$^{FAK}$. Phosphorylation was quantified by scanning densitometry of autoradiographs. Values are the means of duplicates and are expressed as the percentage of the maximal increase above unstimulated control values. Similar results were obtained in an additional experiment. Inset shows immunoprecipitation of lysates from cells treated with 0, 0.5, 1, 5 and 10 $\mu$M SPP respectively and analysed by Western blotting with anti-Tyr(P) antibody. The arrow indicates the migration of p125$^{FAK}$. (**E**) Dose–response for SPP-induced DNA synthesis. [$^3$H]Thymidine incorporation was measured as described in the Experimental procedures section. Value are means $\pm$ S.D. of triplicate determinations.

### Assay of DNA synthesis

Quiescent cultures were washed with DMEM to remove residual serum and subsequently 1 ml of DMEM/Waymouth medium (1:1, v/v), supplemented with 20 $\mu$g/ml BSA and 5 $\mu$g/ml transferrin, was added [25]. Cells were then treated as indicated in the Figure legends, and after 18 h pulsed with 1 $\mu$Ci/ml [$^3$H]thymidine for 6 h. Incorporation of [$^3$H]thymidine into trichloroacetic acid-insoluble material was measured as described [26]. Values are means of triplicate determinations and S.D.s were routinely less than 10 % of the mean.

## RESULTS

### SPP induces actin stress-fibre formation

Many growth factors and growth promoting agents, such as LPA [21] and sphingosine [22], have previously been shown to

induce stress-fibre formation. Because SPP is a potent mitogen for Swiss 3T3 fibroblasts [10,16], it was of interest to determine whether SPP also affects stress-fibre formation. In agreement with previous reports [21], serum-starved Swiss 3T3 fibroblasts contain only a few stress fibres (Figure 1A). Treatment with a mitogenic concentration of SPP (5 $\mu$M) induced dramatic reorganization of the actin cytoskeleton to form stress fibres (Figure 1B). The response was much weaker with 1 $\mu$M SPP.

### SPP stimulates tyrosine phosphorylation of p125$^{FAK}$ and paxillin

p125$^{FAK}$ may play a critical role in transmission of signals between growth factor receptors and the actin cytoskeleton organization. To determine whether SPP stimulates tyrosine phosphorylation of p125$^{FAK}$, confluent and quiescent Swiss 3T3 fibroblasts were treated with optimal mitogenic concentrations of SPP, and tyrosine phosphorylation of p125$^{FAK}$ was determined in cell lysates by anti-p125$^{FAK}$ immunoprecipitation followed by Western blotting with anti-phosphotyrosine antibodies (Figure 2A). In accordance with previous results [22,27], tyrosine phosphorylation of p125$^{FAK}$ in unstimulated cells was very low. SPP induced a rapid and marked increase in tyrosine phosphorylation of p125$^{FAK}$, reaching a maximum after 5 min of treatment, and declining thereafter (Figure 2C). It should be mentioned that this response to SPP was much more rapid and transient than the previously reported responses to sphingosine, cell-permeant ceramide analogues [22] or sphingomyelinase treatment [23] in these cells.

In fibroblasts, growth-promoting agents also induce tyrosine phosphorylation of the 68 kDa focal adhesion-associated protein paxillin, which may be a substrate for p125$^{FAK}$ [28]. To examine whether tyrosine phosphorylation of paxillin is stimulated in response to SPP, anti-paxillin immunoprecipitates were immunoblotted with specific anti-phosphotyrosine antibodies. Figure 2(B) shows that SPP induced an increase in tyrosine phosphorylation of paxillin. SPP did not alter the protein levels of paxillin in cells, demonstrated by reblotting with anti-paxillin antibodies. In agreement with previous findings [22,29], paxillin migrated in SDS/PAGE as a broad, diffuse band at 66–70 kDa, and the SPP-induced increase in phosphotyrosine content was associated with a shift in mobility to a more slowly migrating form. The increase in paxillin tyrosine phosphorylation followed a similar time course as described for p125$^{FAK}$ (Figure 2C), although it appears as though p125$^{FAK}$ phosphorylation somewhat precedes paxillin phosphorylation. Treatment of Swiss 3T3 fibroblasts with SPP increased the tyrosine phosphorylation of p125$^{FAK}$ and paxillin in a dose-dependent manner (Figure 2D; results not shown). A significant effect was observed at a concentration of SPP as low as 0.5 $\mu$M and maximum stimulation was achieved at 5–10 $\mu$M. This dose–response correlated closely with the dose–response for SPP-induced DNA synthesis as measured by [$^3$H]thymidine uptake (cf. Figures 2D and 2E).

### Functional Rho is required for SPP-induced stress-fibre formation and tyrosine phosphorylation of p125$^{FAK}$ and paxillin

Rho is a small regulatory GTP-binding protein that appears to integrate signals induced by integrins and growth factors with stress-fibre assembly [21,24,30]. To determine whether the stimulation of new actin stress-fibre formation was dependent on endogenous Rho protein, we used exoenzyme C3 ADP-ribosyltransferase from *Clostridium botulinum*, which specifically ADP-ribosylates Rho on asparagine-41 and renders it biologically inactive. Microinjection of 100 $\mu$g/ml C3 exoenzyme has previously been shown to interfere with Rho protein function without impairing the functions of Rac, Cdc42 or Ras [21,24]. C3

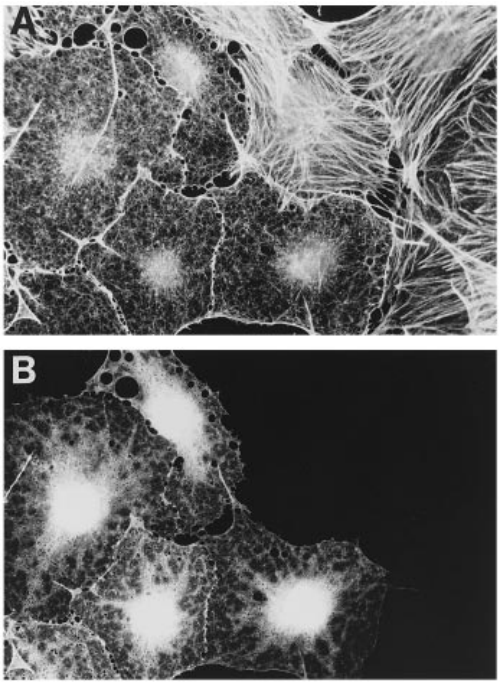


**Figure 3 *Botulinum* C3 transferase exoenzyme inhibits SPP-induced actin stress-fibre formation**

Serum-starved cells were microinjected with a mixture of C3 exoenzyme (100 $\mu$g/ml) and FITC–dextran and stimulated with 5 $\mu$M SPP for 30 min. Cells were then fixed and stained for actin filaments with TRITC-labelled phalloidin. In (**A**), cells were visualized by TRITC fluorescence and in (**B**) FITC–dextran-microinjected cells were visualized by FITC fluorescence.

exoenzyme completely inhibited stress-fibre formation induced by SPP in Swiss 3T3 fibroblasts (Figure 3). From double immunofluorescence, to visualize injected cells and stress fibres, it is evident that only cells that were microinjected with C3 exoenzyme were resistant to induction of stress-fibre formation by SPP. In the same field, adjacent cells that were not microinjected still exhibited a remarkable induction of stress fibres. To examine whether C3 exoenzyme could also prevent tyrosine phosphorylation of p125$^{FAK}$ and paxillin in response to SPP, cells were treated with 5 $\mu$g/ml C3 exoenzyme, first in complete medium for 48 h and then in serum-free medium for 12 h. Previously, it was demonstrated that such treatment of Swiss 3T3 fibroblasts led to ADP-ribosylation of Rho and consequently inactivation of Rho A protein *in situ* [27]. C3 exoenzyme pretreatment suppressed both basal (results not shown) and SPP-induced phosphorylation of p125$^{FAK}$ and paxillin (Figure 4). Neither C3 exoenzyme nor SPP treatment had any effects on levels of p125$^{FAK}$ and paxillin in cell lysates, demonstrated by reblotting with anti-p125$^{FAK}$ and anti-paxillin antibodies (results not shown). Our results indicate that Rho may be an upstream regulator of both stress-fibre formation and tyrosine phosphorylation of p125$^{FAK}$ and paxillin induced by SPP.

### Differential effects of phorbol ester PMA on stress-fibre formation and tyrosine phosphorylation of p125$^{FAK}$ and paxillin induced by SPP or LPA

Stress-fibre formation induced by many growth promoting agents, including LPA and bombesin, is blocked by the phorbol ester PMA which is known to activate PKC [30]. In contrast, formation of stress fibres following PDGF stimulation or micro-

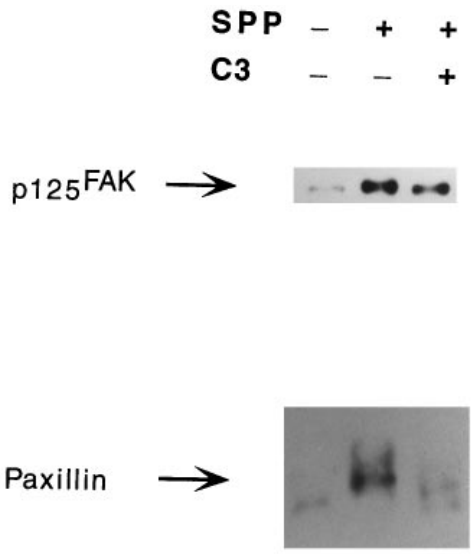


**Figure 4 Effect of *Botulinum* C3 transferase on tyrosine phosphorylation of p125[FAK] and paxillin induced by SPP**

Quiescent Swiss 3T3 cells were pretreated without or with 5 $\mu$g/ml recombinant C3 exoenzyme for 48 h in complete medium followed by 12 h in serum-free DMEM/Waymouth medium (1:1, v/v) supplemented with 20 $\mu$g/ml BSA and 5 $\mu$g/ml transferrin and then treated with vehicle or SPP (10 $\mu$M) for 10 min. Cell lysates were immunoprecipitated with anti-p125[FAK] antibody (**A**) or anti-paxillin antibody (**B**) and analysed by Western blotting with anti-Tyr(P).

injection of recombinant, constitutively active Rho protein is not affected by PMA [30]. Consistent with previous studies [30], brief treatment with PMA inhibited LPA-induced stress-fibre formation (Figures 5A and 5B). In contrast, stress-fibre formation induced by SPP was unaffected by PMA (Figures 5C and 5D). Thus despite structural similarity between LPA and SPP, both lipids appeared to stimulate stress-fibre formation in different manners. These results also suggest that activation of PKC inhibits the formation of stress fibres at a target upstream of Rho in response to LPA, but not in response to SPP or PDGF. Moreover, short-term pretreatment with PMA also reduced LPA-stimulated tyrosine phosphorylation of p125[FAK] and paxillin (Figure 6). However, in contrast, such brief treatment of the cells with PMA did not attenuate SPP-stimulated tyrosine phosphorylation of p125[FAK] and paxillin (Figure 6).

### Differential effects of suramin on DNA synthesis and tyrosine phosphorylation of p125[FAK] and paxillin induced by SPP or LPA

It has been shown recently that SPP rapidly induces Rho-dependent neurite retractions in N1E-115 neuroblastoma cells mediated through specific cell-surface receptors [31]. Thus it was of interest to examine whether the mitogenic effects of SPP as well as its effects on tyrosine phosphorylations in Swiss 3T3 fibroblasts were mediated through cell-surface receptors. To this end, we examined the effect of suramin, a polyanionic compound with broad specificity, for inhibition of ligand–receptor interactions. In agreement with previous results, suramin decreased LPA-induced DNA synthesis from 6.7-fold stimulation to 1.4-fold after 24 h treatment [32], without affecting the mitogenic effect of SPP. However, during the course of these experiments, it was observed that suramin alone was toxic to Swiss 3T3 fibroblasts and decreased DNA synthesis in unstimulated cells. To avoid complications in the interpretation of the results of treatments with suramin, we took advantage of the reversibility of the suramin effects and our previous observation that the

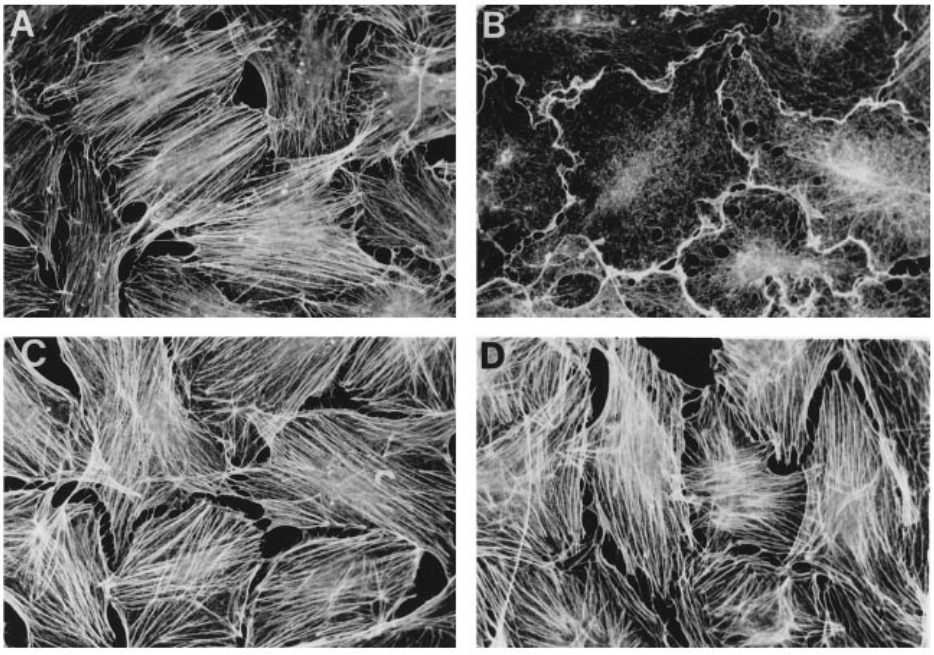


**Figure 5 PMA inhibits LPA- but not SPP-induced stress-fibre formation**

Serum-starved Swiss 3T3 cells untreated (**A**) and (**C**), or pretreated with PMA (160 nM) (**B**) and (**D**), for 5 min, were then stimulated with 100 ng/ml LPA (**A**) and (**B**), or 5 $\mu$M SPP (**C**) and (**D**), for 30 min. Cells were fixed and actin filaments visualized with TRITC-labelled phalloidin.

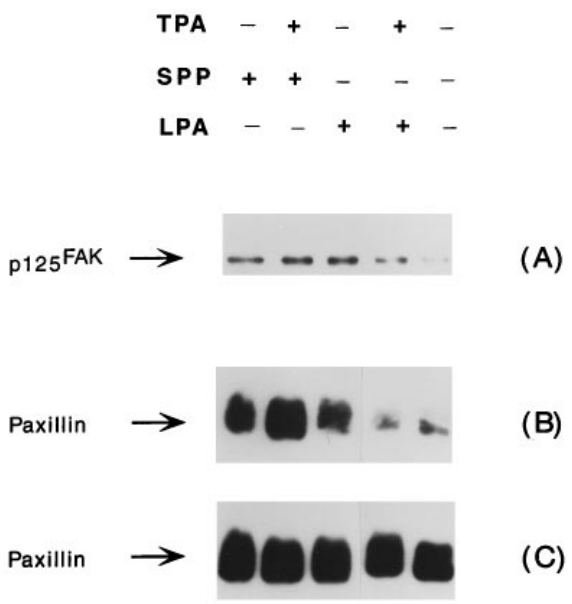


**Figure 6 Different effects of PMA on p125$^{FAK}$ and paxillin phosphorylation induced by SPP and LPA**

Quiescent Swiss 3T3 cells were pretreated without or with PMA ('TPA'; 160 ng/ml) for 5 min and then treated with vehicle, SPP (10 $\mu$M) or LPA (100 ng/ml) for 10 min as indicated. Cell lysates were immunoprecipitated with anti-p125$^{FAK}$ antibody (**A**) or anti-paxillin antibody (**B**) and (**C**) and analysed by Western blotting with anti-Tyr(P) (**A**) and (**B**) or anti-paxillin antibody (**C**).

**Table 1 Effects of suramin on DNA synthesis induced by lipid mitogens**

Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated in DMEM/Waymouth (1:1, v/v) supplemented with 20 $\mu$g/ml BSA and 5 $\mu$g/ml transferrin and treated with the indicated lipid or vehicle in the absence or presence of suramin (0.2 mg/ml) for 1 h. Cells were then washed and incubated in the same media but in the absence of lipids and suramin for a total incubation time of 24 h. Cells were pulsed for the last 6 h with [$^3$H]thymidine (1 $\mu$Ci/ml), and [$^3$H]thymidine incorporation was measured as described in the Experimental procedures section. Values are means of triplicate determinations from a representative experiment. Similar results were obtained in at least three additional experiments. $^*P \leqslant 0.002$; $\dagger P \geqslant 0.05$.

| Lipid added | $10^{-4} \times$ [$^3$H]Thymidine incorporation (c.p.m./mg of protein) | |
|---|---|---|
| Suramin ... | Minus | Plus |
| None | 18.4 ± 1.8 | 13.7 ± 2.6† |
| SPP (10 $\mu$M) | 102.3 ± 1.6 | 94.1 ± 0.6† |
| SPC (10 $\mu$M) | 129.5 ± 12 | 71.1 ± 8.2* |
| LPA (40 $\mu$M) | 35.0 ± 2.9 | 20.8 ± 0.3* |

mitogenic action of another sphingolipid mitogen, SPC, only required a short exposure time [33]. Indeed treatment of Swiss 3T3 fibroblasts with SPP or SPC, for 1 h, followed by washing and replacement of the media, resulted in 5- and 7-fold stimulation of DNA synthesis respectively. When cells were also treated with suramin in this short-treatment protocol, DNA synthesis induced by sphingosylphosphocholine (SPC) and LPA was markedly decreased by 45 % and 41 % respectively, but the response to SPP was not significantly altered (Table 1). We also found that prior addition of suramin to Swiss 3T3 fibroblasts

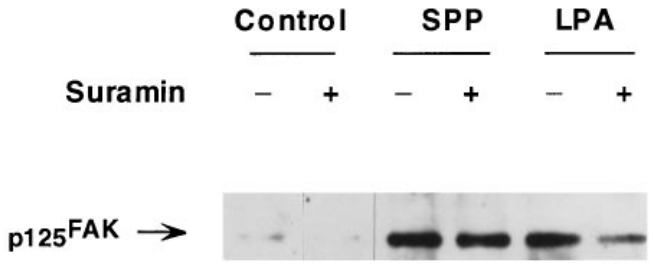


**Figure 7 Suramin has no effect on SPP-stimulated tyrosine phosphorylation of p125$^{FAK}$**

Quiescent Swiss 3T3 cells were pretreated without or with suramin (1.8 mg/ml) for 20 min and then treated with vehicle, SPP (10 $\mu$M) or LPA (20 $\mu$M) for 5 min. Cell lysates were immunoprecipitated with anti-p125$^{FAK}$ antibody and analysed by Western blotting with anti-Tyr(P) antibody.

inhibited LPA-stimulated, but not SPP-stimulated, tyrosine phosphorylation of p125$^{FAK}$ (Figure 7).

## DISCUSSION

In this study, we have demonstrated that SPP, a sphingolipid metabolite, which has been implicated in cell growth regulation [10,17], induces stress-fibre formation and tyrosine phosphorylation of p125$^{FAK}$ and paxillin in quiescent Swiss 3T3 fibroblasts. p125$^{FAK}$ and paxillin are concentrated in focal adhesions, regions of dense cellular structures that link integrin receptors attached to the extracellular matrix to actin filament bundles. Although the precise cellular function of increased tyrosine phosphorylation of p125$^{FAK}$ is still not completely understood, its subcellular localization and regulation by growth factors and integrins suggest that it may participate in the organization of focal adhesions, actin stress fibres and cell motility. In agreement, reduced cell motility and enhanced focal contact adhesion were found in cells from p125$^{FAK}$-deficient mice [34]. p125$^{FAK}$, in turn, can bind and phosphorylate other cytoskeletal components, including paxillin and tensin [28], that subsequently will establish contacts with talin, vinculin, $\alpha$-actinin and the actin polymers, essential components of stress fibres. Moreover, tyrosine kinase inhibitors, which block tyrosine phosphorylation of p125$^{FAK}$, also inhibit focal adhesion formation [30]. Paxillin shows increased tyrosine phosphorylation after treatment of cells with SPP and the time course of paxillin phosphorylation is consistent with it being a substrate of p125$^{FAK}$ *in vivo*. Furthermore, SPP treatment also increased association of phosphorylated paxillin with p125$^{FAK}$, as demonstrated by Western blotting with anti-paxillin or anti-phosphotyrosine antibodies after immunoprecipitating cell lysates with anti-p125$^{FAK}$ antibodies (F. Wang, C. D. Nobes, A. Hall and S. Spiegel, unpublished work). Sphingosine has previously been shown to induce p125$^{FAK}$ and paxillin tyrosine phosphorylation, actin stress-fibre formation and focal contact assembly in Swiss 3T3 fibroblasts [22]. This effect has been attributed to ceramide formed by acylation of sphingosine [22]. In agreement, treatment of Swiss 3T3 fibroblasts with sphingomyelinase also stimulates phosphorylation of p125$^{FAK}$ and paxillin [23]. It is interesting to note that SPC or lysosphingomyelin [35] and SPP have similar effects, suggesting that the sphingoid backbone may be sufficient to induce these responses.

In contrast with our results, in human arterial smooth muscle cells, SPP induces actin filament disassembly, resulting in in-

hibition of actin nucleation, actin filament assembly and formation of focal adhesion sites [19]. Although the reason for this discrepancy is not clear, the effects of sphingolipid metabolites may be different in different cell types. In human arterial smooth muscle cells, increased intracellular levels of calcium induced by SPP led to increased accumulation of cAMP and activation of protein kinase, A which results in actin filament disassembly [19]. In Swiss 3T3 fibroblasts, however, SPP has been shown to decrease cAMP levels [36]. Moreover, differential effects of PDGF on p125$^{FAK}$ and paxillin tyrosine phosphorylation and cell migration were observed in rabbit aortic vascular smooth muscle cells and in Swiss 3T3 fibroblasts [37]. PDGF itself has biphasic effects on phosphorylation of p125$^{FAK}$ and on stress-fibre formation in Swiss 3T3 fibroblasts, stimulating at low concentrations and inhibiting at optimal mitogenic concentrations [37,38].

The small GTP binding protein Rho appears to play a key role in integrating signals induced by integrin and growth factors [21,24,28,30]. Rho regulates both stress fibres and focal adhesion formation [21], and more recently it has been shown that Rho regulates increased tyrosine phosphorylation of several cytoskeletal proteins including p125$^{FAK}$ [27]. Interestingly, we found that inactivation of Rho with C3 transferase exoenzyme not only inhibited SPP-induced stress-fibre formation, but also inhibited SPP-stimulated tyrosine phosphorylation, indicating that Rho might act upstream of p125$^{FAK}$. Previous studies have demonstrated that, similarly, stress-fibre formation and p125$^{FAK}$ phosphorylation induced by LPA are also Rho-dependent [21,27]. The structural similarity between SPP and LPA suggests the possibility that SPP might be acting by binding to the LPA receptor [39,40]. However, there is good evidence that this is not the case. The PKC activator, PMA, inhibited stress-fibre formation and p125$^{FAK}$ phosphorylation induced by LPA. In contrast, brief treatment with PMA does not have any effect on SPP-induced stress-fibre formation or p125$^{FAK}$ and paxillin tyrosine phosphorylation. These results support the previous conclusion that PKC acts as a negative regulator upstream of Rho in the signal transduction pathway initiated by binding of LPA to its receptor and subsequent formation of stress fibres [30]. The site of PKC-mediated down-regulation is not known but it is likely to be the LPA receptor itself. Other lines of evidence also support the notion that SPP does not act through the LPA receptor. First, although SPP increased Ins$P_3$ levels, complete inhibition of inositol phosphate formation by pretreatment of cells with PMA did not inhibit SPP-mediated calcium responses, indicating that formation of Ins$P_3$ is not required for release of calcium by SPP [13]. However, PMA pretreatment suppressed not only Ins$P_3$ formation but also calcium responses elicited by LPA [13,40]. Secondly, LPA, in contrast with SPP, has been shown to release arachidonic acid [13,40]. Thirdly, SPP, in contrast with many other analogues of LPA, does not displace LPA from its receptor [41]. Finally, it has been recently demonstrated that Rho-dependent neurite retractions induced by SPP and LPA do not show cross-desensitization and probably act through distinct receptors [31]. In this regard, it should be mentioned that although SPP functions as an intracellular second messenger in calcium mobilization [12,13] and cellular proliferation [17], some of the biological responses to exogenous SPP, such as inhibition of motility [42], platelet aggregation [43] and induction of neurite retraction [31], may be due to extracellular effects on cell-surface receptors. Moreover, in guinea-pig atrial myocytes, SPP activated $G_i$-protein-regulated inwardly rectifying potassium channels only when SPP was applied to the extracellular face [44]. These results suggest that SPP might interact with a high-affinity cell-surface receptor. However, in contrast with the biological effects of SPP, which occur in the nanomolar concentration range, the responses to SPP reported here require micromolar concentrations. Moreover, we utilized suramin that has previously been reported to inhibit ligand–cell-surface receptor interactions, especially those of PDGF and LPA [32,45], but not to affect cell growth induced by calcium in human fibroblasts [45]. Suramin did not abrogate cell growth, stress-fibre formation or tyrosine phosphorylation of p125$^{FAK}$ induced by SPP. However, suramin markedly inhibited the mitogenic effects of LPA and SPC. Thus although SPC and LPA seem to act through cell-surface receptors, it is unlikely that the effects of SPP on cellular proliferation and phosphorylation of p125$^{FAK}$ and paxillin in Swiss 3T3 fibroblasts are mediated through a cell-surface receptor. It is interesting to note that SPC, in the nanomolar concentration range, activates potassium conductivity in guinea-pig atrial myocytes via a cell-surface receptor [46].

In addition to regulation of the assembly of actin stress fibres and focal adhesion complexes, it has been demonstrated recently that Rho plays an essential role in the progression of cells through the G1-phase of the cell cycle [47]. Moreover, functional Rho is required for activation of transcription via serum response factor, and acts synergistically at the serum responsive element with signals that activate ternary complex factor [48]. Because treatment of cells with SPP leads to activation of Rho, it is possible that Rho might play a role in cellular proliferation induced by SPP. However, Rho stimulation of tyrosine phosphorylation of p125$^{FAK}$ and paxillin in focal adhesions leading to formation of stress fibres, and the regulation of progression of cells through the G1-phase of the cell cycle, may be two distinct downstream effects of Rho. Understanding how the sphingolipid metabolite SPP regulates Rho, which then influences diverse cellular events occurring in different cellular compartments, is an important area of future research.

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