Sphingosylphosphorylcholine (SPC), a potent mitogen for Swiss 3T3 cells, rapidly induced tyrosine phosphorylation of multiple substrates including bands of M, 110,000–130,000 and M, 70,000–80,000 in Swiss 3T3 cells. Focal adhesion kinase (p125FAK) and paxillin were identified as prominent substrates for SPC-stimulated tyrosine phosphorylation. An increase in tyrosine phosphorylation of p125FAK was detected as soon as 30 s after SPC stimulation, reaching a maximum after 2.5 min. SPC induced tyrosine phosphorylation of p125FAK in a concentration-dependent fashion; a half-maximum effect occurred at 250 nM. Tyrosine phosphorylation of p125FAK induced by SPC could be dissociated from both protein kinase C activation and Ca2+ mobilization from intracellular stores. SPC induced a unique pattern of reorganization of the actin cytoskeleton with a rapid appearance of actin microspikes at the plasma membrane that was followed by the formation of actin stress fibers. This pattern of cytoskeletal changes was clearly distinguishable from that induced by bombesin and 1-oleoyl-lysophosphatidic acid. Formation of microspikes and actin stress fibers were accompanied by striking assembly of focal adhesion plaques. Cytochalasin D, which disrupts the network of actin microfilaments, completely prevented SPC-induced tyrosine phosphorylation of p125FAK. In addition, tyrosine phosphorylation of p125FAK was markedly inhibited in the presence of platelet-derived growth factor at a concentration (30 ng/ml) that disrupts actin stress fibers. Finally, microinjection of Clostridium botulinum C3 exoenzyme, which inactivates p21rho, prevented SPC-induced formation of actin stress fibers, focal adhesion assembly, and tyrosine phosphorylation. Thus, p21rho is upstream of both cytoskeletal reorganization and tyrosine phosphorylation in SPC-treated cells.

Sphingosylphosphorylcholine (SPC) acts as a potent mitogen for Swiss 3T3 cells in the absence of any other exogenously added growth factor (1, 2), but the molecular basis for this effect is largely unknown. The best characterized signaling event in response to SPC is the release of Ca2+ from intracellular stores in a variety of cell lines (2–5). However, it appeared unlikely that all cellular responses elicited by SPC are mediated by Ca2+ mobilization. In the accompanying paper (6), we demonstrated that SPC induces transient activation of MAPK and p90SK by a pathway dependent on the activity of PKC and a pertussis toxin-sensitive G protein. In this paper, we identify further cellular and molecular responses to exogenously added SPC to elucidate the mechanisms by which lysosphingolipids modulate cell function.

Tyrosine phosphorylation of the cytosolic protein kinase p125SK and of the cytoskeletal-associated protein paxillin has recently been identified as an early event in the action of diverse signaling molecules that mediate cell growth and differentiation, including mitogenic neurotransmitters (7–9), growth factors such as PDGF (10), the bioactive lipid LPA (11–13), sphingosine (14), extracellular matrix proteins (15–19), and transforming variants of p60src (16, 20). The increases in p125SK and paxillin tyrosine phosphorylation are accompanied by profound alterations in the organization of the actin cytoskeleton and in the assembly of focal adhesions (10, 13, 14, 21, 22), the distinct areas of the plasma membrane where p125SK and paxillin are localized (23–26). Recently, the small G protein p21rho, a member of the Ras superfamily of small GTP binding proteins, has been implicated in mitogen-stimulated formation of focal adhesions and stress fibers as well as tyrosine phosphorylation of p125SK and paxillin (21, 27–29). The effects, if any, of exogenously added SPC on tyrosine phosphorylation, the organization of the actin cytoskeleton and focal adhesion assembly, and the potential involvement of small G proteins such as p21rho in this signaling pathway remain unknown.

In this paper, we demonstrate that SPC rapidly stimulates tyrosine phosphorylation of multiple proteins including p125SK and paxillin in Swiss 3T3 cells and induces a unique pattern of actin organization, which was accompanied by the formation of focal contacts. Microinjection of C. botulinum C3 exoenzyme, which ADP-ribosylates and inactivates p21rho, blocked the changes in the organization of the actin cytoskeleton, the assembly of focal contacts, and tyrosine phosphorylation in response to SPC.

**Experimental Procedures**

**Cell Culture and Down-regulation of PKC**—Cell culture of Swiss 3T3 cells and down-regulation of PKC by prolonged pretreatment with PDB were performed as described in the accompanying paper (6).

**Immunoprecipitation**—Quiescent cultures of Swiss 3T3 cells (1–2 × 106) were washed twice with DMEM, treated with SPC or other factors in 10 ml of DMEM for the times indicated, and lysed at 4 °C in 1 ml of a solution containing 10 mm Tris-HCl, pH 7.6, 5 mm EDTA, 50 mm NaCl,
SPC-Induced Tyrosine Phosphorylation of p125\textsuperscript{FAK}  

30 mM sodium pyrophosphate, 50 mM NaF, 100 \mu M Na\textsubscript{2}VO\textsubscript{4}, and 1% Triton X-100 (lysis buffer). Proteins were immunoprecipitated at 4°C for 14 h with anti-mouse I\textgamma G-agarose-linked mAbs directed against phosphotyrosine, p125\textsuperscript{FAK} as indicated. Immunoprecipitates were washed three times with lysis buffer and extracted for 10 min at 95°C in 2 x SDS-PAGE sample buffer (200 \mu M Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and analyzed by SDS-PAGE.

Western Blotting—Treatment of quiescent cultures of cells with factors, cell lysis, and immunoprecipitations were performed as described above. After SDS-PAGE, proteins were transferred to Immobilon membranes. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.4, and incubated for 2 h at 22°C with a mixture of anti-Tyr(P) mAbs (1 \mu g/ml of each). Immunoreactive bands were visualized by autoradiography using \textsuperscript{125}I-labeled sheep anti-mouse IgG (1:1000) followed by autoradiography. Autoradiograms were scanned using an LKB Ultrascan XL densitometer, and labeled bands were quantified using the Ultrascan XL internal integrator. The values expressed represent percentages of the maximum increase in tyrosine phosphorylation above control values.

Measurement of [Ca\textsuperscript{2+}i].—Confluent and quiescent Swiss 3T3 cells in 100-mm dishes were washed twice with DMEM and incubated for 10 min in DMEM containing 1 \mu M fura-2-tetraacetoxymethyl ester. After this time, the cells were washed twice with PBS at 37°C and once with electrolyte solution, which contained 120 mM NaCl, 5 mM KCl, 1.8 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.4, 10 mM glucose, 16 mM HEPEs, 6 mM Tris/HCl, and a mixture of amino acids at the same concentrations as are present in DMEM, pH 7.2. The cells were then suspended in 2 ml of electrolyte solution by gentle scraping and transferred to a 1-cm\textsuperscript{2} quartz cuvette, which was placed in a Perkin-Elmer LS-5 luminescence spectrophotometer. The cell suspension was stirred continuously at 37°C, and fluorescence was monitored as indicated. [Ca\textsuperscript{2+}i] was determined as described previously (30).

Immunostaining of Cells—Quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for the indicated time at 37°C with the indicated factor or mixture of SPC or other factors. Thereafter, for staining of actin, cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min at 4°C, and permeabilized with PBS containing 0.2% Triton X-100 for 8 min at room temperature. The cells were then incubated with TRITC-conjugated phalloidin (0.25 \mu g/ml) in PBS for 10 min at room temperature and visualized utilizing a Zeiss Axiopt fluorescence microscope. In experiments in which quiescent Swiss 3T3 cells were labeled with TRITC-conjugated phalloidin and anti-vinculin or anti-Tyr(P) antibody, after fixing and permeabilizing the cells as described above, TRITC-conjugated phalloidin (0.25 \mu g/ml) and anti-vinculin antibody (dilution 1:100) or anti-Tyr(P) mAb 4G10 (dilution 1:300) were added together to the cells for 30 min at room temperature. Cells were subsequently washed three times in PBS at 4°C, incubated with FITC-labeled anti-mouse IgG for 30 min at 4°C, and washed three times with PBS at 4°C. The cells were then fixed in 4% paraformaldehyde in PBS and permeabilized with a mixture of anti-Tyr(P) mAb 4G10 and FITC-labeled anti-mouse IgG for 45 min at 4°C, mounted on a glass slide, and analyzed using a Bio-Rad MRC-1000 laser scanning head fitted onto a Diaphot 200 microscope. A 60 x N.A.1.4 planapochromato oil immersion lens (Nikon) and the dicroic filter blocks T1 and E2 were used. Images were collected sequentially using a krypton/argon mixed gas laser (Bio-Rad) with excitation filters at 488, 568, and 647 nm for FITC, TRITC, and Cy5 fluorescent dyes, respectively, and emission filters at 510, 560, and 680 nm, respectively. Correction of images for bleed through and other processing was carried out using the COMOS program (Bio-Rad) run on a QE 486 33C computer. Optical sections were recorded at 0.5 \mu M. Final images were photographed directly from the VDU screen or processed on a Silicon Graphics Indigo XS 24 workstation using a Sony digital color printer.

Microinjection—For microinjection experiments, Swiss 3T3 cells were plated in 35-mm Nunc Petri dishes at 10\textsuperscript{4} cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent. To facilitate localization of microinjected cells, a circle was scored on the bottom of the dishes, and in each experiment approximately 60 cells in the circle were microinjected. The efficiency of injection was determined by co-injecting rabbit IgG at 0.5 mg/ml followed by staining of the cells with FITC-linked anti-rabbit IgG. The same batch of C3 exoenzyme was used in all experiments described.

Materials—SPC, LPA, PDB, bombesin, cytchalasin D, TRITC-conjugated phalloidin, monoclonal anti-vinculin antibody, and FITC-linked anti-rabbit IgG were obtained from Sigma. Cy5-conjugated affinity pure sheep anti-mouse IgG and goat anti-rabbit IgG were from jackson ImmunoResearch Laboratories Inc. The specific PKC inhibitor GF 109203X and thapsigargin were obtained from Calbiochem-Novabiochem Ltd. (Nottingham, UK). Anti-mouse IgG-agarose-linked anti-Tyr(P) mAb done Py72 was obtained from the hydridoma development unit (Imperial Cancer Research Fund, London, UK). Py20 anti-Tyr(P) mAb and the mAb directed against paxillin (mAb 165) were from ICN (High Wycombe, UK). 4G10 anti-Tyr(P) mAb and mAb 2A7 directed against p125\textsuperscript{FAK} were from TCS Biologicals Ltd. (Buckingham, UK). Anti-p125\textsuperscript{FAK} mAb for Western blotting was obtained from AFFINITI Research Products Ltd. (Nottingham, UK). 125I-Sheep anti-mouse IgG (15 mCi/mg) and recombinant BB homodimer PDGF were from Amersham. Recombinant C. botulinum exoenzyme C3 was the kind gift of Prof. Shuh Narumiya (Dept. of Pharmacology, Faculty of Medicine, Kyoto University, Japan). All other reagents used were of the purest grade available.

RESULTS

SPC Induces Tyrosine Phosphorylation of Several Proteins

In including p125\textsuperscript{FAK} and Paxillin—To examine the effect of SPC on protein tyrosine phosphorylation, quiescent Swiss 3T3 cells were incubated with 5 \mu M SPC for 5 min and lysed. Lysates of the treated cells were incubated with anti-Tyr(P) mAb, and the immunoprecipitates were further analyzed by Western blotting with a mixture of anti-Tyr(P) mAbs. As shown in Fig. 1, upper panel, left, SPC stimulated tyrosine phosphorylation of multiple components including bands migrating with an apparent M\textsubscript{r} of 110,000–130,000 and 70,000–80,000. The pattern of tyrosine-phosphorylated proteins induced by SPC was comparable to that obtained with 10 nm bombesin (Fig. 1, upper panel, right). The increase in the tyrosine phosphorylation of the M\textsubscript{r} 110,000–130,000 and the M\textsubscript{r} 70,000–80,000 bands in response to 5 \mu M SPC occurred rapidly, reaching half-maximum levels after 45 s and 2 min and maximum levels after 2.5 and 10 min, respectively (Fig. 1, lower panel).

The tyrosine kinase inhibitor genistein (10 \mu M, 24) and the cytoskeleton-associated protein paxillin (25, 26) have been identified as prominent tyrosine-phosphorylated proteins in bombesin-treated Swiss 3T3 cells (7–9). As SPC elicited a pattern of tyrosine-phosphorylated bands similar to that induced by bombesin (Fig. 1, upper panel, left), we determined whether p125\textsuperscript{FAK} and paxillin were also substrates for SPC-mediated tyrosine phosphorylation. Lysates of quiescent Swiss 3T3 cells incubated with 5 \mu M SPC for 5 min were immunoprecipitated with mAb 2A7 directed against p125\textsuperscript{FAK} or mAb 165 directed against paxillin and further analyzed by immunoblotting with a mixture of anti-Tyr(P) mAbs. As Fig. 2, top panel, left and right, shows that SPC markedly induced tyrosine phosphorylation of p125\textsuperscript{FAK} and paxillin, respectively. Thus, p125\textsuperscript{FAK} is a component of the broad M\textsubscript{r} 110,000–130,000 band, and paxillin is a component of the diffuse M\textsubscript{r} 70,000–80,000 band of tyrosine-phosphorylated proteins in SPC-treated Swiss 3T3 cells. The degree of tyrosine phosphorylation of these specific substrates in response to 5 \mu M SPC was comparable to that induced by 10 nm bombesin in parallel cultures (Fig. 2, top panel, left and right). As shown in Fig. 2, middle panel, SPC induced tyrosine phosphorylation of p125\textsuperscript{FAK} in a concentration-dependent manner.
tyrosine phosphorylation of p125 FAK is shown in Fig. 2, in 0.25 and 1 nM; half-maximum and maximum effects were achieved at 70,000–80,000 band was virtually superimposable to that of the 110,000–130,000 band (error bar is not shown). To examine whether the extracellular Ca2+ has been implicated in tyrosine phosphorylation above unstimulated control values in each experiment. The dose response of SPC-induced tyrosine phosphorylation of the M, 70,000–80,000 band was virtually superimposable to that of the M, 110,000–130,000 band, which is shown in the upper right panel. Where an error bar is not shown, it lies within the dimensions of the symbol.

Fig. 1. Dose response and time course of SPC-stimulated tyrosine phosphorylation. Left upper panel, lysates of quiescent Swiss 3T3 cells were treated with 5 μM SPC or 10 nM bombesin for 10 min were immunoprecipitated with anti-Tyr(P) mAb and further analyzed by autoradiography. Right upper panel, quiescent Swiss 3T3 cells were treated with various concentrations of SPC for 5 min, and cell lysates were further analyzed as described above. The increase in tyrosine phosphorylation of the M, 110,000–130,000 band (closed circles) and the M, 70,000–80,000 band (open circles) was quantified by scanning densitometry. Values shown are the mean ± S.E. of at least five independent experiments and are expressed as percentages of the maximum increase in tyrosine phosphorylation above unstimulated control values in each experiment. The dose response of SPC-induced tyrosine phosphorylation of the M, 70,000–80,000 band is shown in the upper panel and the results expressed as percentages of the maximum increase in tyrosine phosphorylation in response to SPC (data not shown).

Fig. 2. SPC induces tyrosine phosphorylation of p125 FAK and paxillin in Swiss 3T3 cells. Upper panel, quiescent Swiss 3T3 cells were treated with 5 μM SPC or 10 nM bombesin for 5 min and subsequently lysed. Tyrosine phosphorylation was analyzed by immunoprecipitation using mAb ZD against p125 FAK (left panel) or mAb 165 directed against paxillin (right panel) followed by Western blotting with anti-Tyr(P) mAbs. The positions of p125 FAK and paxillin are indicated by arrows. Middle panel, quiescent Swiss 3T3 cells were treated with various concentrations of SPC for 5 min and lysed, and the lysates were immunoprecipitated with anti-Tyr(P) mAb followed by Western blotting with anti-p125 FAK mAbs. Lower panel, quiescent Swiss 3T3 cells were treated with 5 μM SPC for various times and lysed, and the lysates were immunoprecipitated with mAb ZD against p125 FAK followed by Western blotting with anti-Tyr(P) mAb. Experiments shown are representative of at least two independent experiments.

SPC induces activation of MAPK by a pertussis toxin-sensitive mechanism, suggesting the involvement of a G protein of the G12/13 subfamily in the signaling pathway (6). To examine whether SPC stimulates p125 FAK tyrosine phosphorylation via G1, quiescent Swiss 3T3 cells were pretreated with various concentrations of pertussis toxin for 3 h and subsequently challenged with 5 μM SPC for 5 min. Pertussis toxin at concentrations between 0.1 and 100 ng/ml did not affect tyrosine phosphorylation of p125 FAK in response to SPC (data not shown).

In the accompanying paper (6), we show that SPC stimulates activation of PKC in Swiss 3T3 cells. As activation of PKC is also a potential pathway leading to tyrosine phosphorylation of p125 FAK and paxillin (8), we examined the role of PKC in SPC-induced p125 FAK and paxillin tyrosine phosphorylation. PKC was either selectively inhibited by pretreatment of quiescent Swiss 3T3 cells for 1 h with the bisindolylmaleimide GF 109203X at 3.5 μM (33) or down-regulated by prolonged exposure (48 h) to 800 nM PDB (34). As shown in Fig. 3, middle panel, both treatments completely blocked stimulation of p125 FAK tyrosine phosphorylation by 200 nM PDB. In contrast, inhibition or down-regulation of PKC only slightly reduced p125 FAK tyrosine phosphorylation in response to 5 μM SPC (Fig. 3, middle panel). Thus, SPC induces p125 FAK tyrosine phosphorylation largely through a PKC-independent pathway.

Paxillin contains several potential target sites for phosphorylation by PKC (35). Tyrosine phosphorylation of paxillin in response to bombesin, LPA, or phorbol esters is accompanied by a mobility shift that results in the appearance of slower migrating forms of this protein, a process mediated by PKC (9).
SPC-induced Tyrosine Phosphorylation of p125<sup>FAK</sup>

In conclusion, the results depicted in Fig. 3 indicate that SPC induces tyrosine phosphorylation of p125<sup>FAK</sup> and paxillin through a pathway largely independent of Ca<sup>2+</sup> mobilization, a G protein, or PKC activation.

SPC Induces a Unique Pattern of Organization of the Actin Cytoskeleton—As p125<sup>FAK</sup> and paxillin are localized at focal adhesion plaques, the distinct sites on the plasma membrane where actin stress fibers emanate, we examined whether SPC modulates actin cytoskeletal organization and focal adhesion assembly. As shown in Fig. 4A, quiescent Swiss 3T3 cells contain only very few actin stress fibers. Incubation of cells with SPC for various times induced dramatic changes in the organization of the actin cytoskeleton. Within 1 min, SPC induced actin reorganization with loss of punctate actin and an increase in polymerized actin as cell surface protrusions. These protrusions formed microspikes. The reorganization of actin into microspikes peaked within 3–5 min of addition of SPC. Interestingly, 7–10 min after addition of SPC the pattern of actin organization changed, and a diffuse increase in actin fibers throughout the cytoplasm was visible. By 20 min, the cells contained numerous densely packed stress fibers, whereas the microspikes were markedly reduced (Fig. 4A). All of these effects were independent of an increase in [Ca<sup>2+</sup>], as treatment of Swiss 3T3 cells with 30 nM thapsigargin for 30 min did not prevent the subsequent changes in the actin cytoskeleton in response to SPC (data not shown).

Both the neuropeptide bombesin and the bioactive lipid LPA stimulate the reorganization of actin with a time course comparable to that of SPC. However, these compounds primarily induce organization of actin into stress fibers visible as early as 1 min after addition of the factors but not into microspikes (21). Therefore, the pattern of the changes in actin organization induced by SPC seemed to be unique. To further substantiate this observation, we compared the early effects of 5 μM SPC, 10 nM bombesin, and 2 μM LPA on the actin cytoskeleton in parallel cultures. As shown in Fig. 4B, SPC induced the typical microspike pattern of actin arrangement. In contrast, bombesin induced actin stress fiber formation and membrane ruffling, whereas LPA predominantly induced actin stress fiber formation in accordance with previous observations (13, 21). Therefore, the pattern of actin rearrangements in response to SPC showing first microspikes and later stress fibers is clearly distinguishable from that induced by other factors that cause rapid changes in the organization of the actin cytoskeleton in Swiss 3T3 cells.

SPC-induced Assembly of Focal Contacts Is Related to the Formation of Both Actin Microspikes and Actin Stress Fibers—Focal adhesions are subcellular structures that are formed at regions of close contacts between cells and their underlying substratum. Several proteins are specifically localized in focal adhesions including vinculin, paxillin, talin, and α-actinin (36). To assess the effect of SPC on the assembly of focal adhesions, vinculin, a major focal adhesion-associated protein, was localized by immunofluorescence with monoclonal anti-vinculin antibody. Surprisingly, both the reorganization of actin into microspikes and the later occurrence of actin stress fibers were accompanied by the formation of focal contacts as seen by the time-dependent aggregation of vinculin into focal adhesions starting as early as 1 min after addition of SPC to the cells (Fig. 5A).

To corroborate these findings and clarify the spatial relationship between microspikes and focal contacts in response to SPC, we used double labeling to visualize actin and vinculin in the same cells. Addition of 5 μM SPC to quiescent Swiss 3T3

2 T. Seufferlein and E. Rozenzurt, unpublished observations.
cells for 5 min led to recruitment of actin into microspikes at the plasma membrane, giving the cells a "hedgehog"-like appearance (Fig. 5B, left picture). Vinculin staining of focal contacts was clearly visible at the ends of these microspikes (Fig. 5B, right picture). Thus, in SPC-stimulated cells, focal adhesion assembly with localization of vinculin to focal contacts clearly occurred before formation of actin stress fibers and was also related to a different form of actin organization, namely the formation of actin microspikes.

The Integrity of the Actin Cytoskeleton Is Necessary for SPC-stimulated Tyrosine Phosphorylation—The effects of SPC on actin stress fiber formation and focal adhesion assembly depicted in Figs. 4 and 5 and the strong correlation of the kinetics of cytoskeletal changes and tyrosine phosphorylation in response to SPC prompted us to examine whether the integrity of the actin cytoskeleton was necessary for SPC-induced tyrosine phosphorylation. As shown in Fig. 6A, cytochalasin D inhibited SPC-induced tyrosine phosphorylation in a concentration-dependent manner, completely preventing tyrosine phosphorylation of p125FAK at a concentration of 1.2 \( \mu M \).

The inhibitory effect of cytochalasin D on SPC-induced tyrosine phosphorylation was specific, as cytochalasin D at the concentrations used profoundly disrupted the network of actin filaments and focal contacts in Swiss 3T3 cells (data not shown) but did not interfere with SPC-induced Ca\(^{2+}\) mobilization (Fig. 6B) or with SPC-stimulated MAPK activation as shown in the accompanying paper (6). This suggests that SPC induces tyrosine phosphorylation of p125FAK and MAPK activation by clearly distinct mechanisms.

**Effect of High Concentrations of PDGF on SPC-induced Actin Stress Fiber Formation and p125FAK Tyrosine Phosphorylation**—Recent data from our laboratory have shown that PDGF, at a high concentration (30 ng/ml), completely abolishes bombesin-, LPA-, and sphingosine-induced actin stress fiber formation (10, 13, 14). As SPC induced a unique pattern of cytoskeletal changes (Figs. 4 and 5), we examined the effect of low (5 ng/ml) and high (30 ng/ml) concentrations of PDGF on actin microspikes and stress fibers induced by SPC. As shown in Fig. 7A, the marked increase in actin stress fiber formation induced by 5 \( \mu M \) SPC was unaffected by PDGF at 5 ng/ml, but it was completely prevented when cells were incubated with both 5 \( \mu M \) SPC and 30 ng/ml PDGF for 20 min. Assembly of focal contacts in response to SPC was also reduced in the presence of PDGF at 30 ng/ml (data not shown). However, organization of actin into microspikes was still visible in these cells, resembling the pattern of actin organization at earlier times (e.g. 3 min) of incubation with SPC.

The results shown in Fig. 6 demonstrated that SPC stimulated p125FAK tyrosine phosphorylation by a mechanism dependent on the integrity of the actin cytoskeleton. In view of...

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**Fig. 4. Effect of SPC on the actin cytoskeleton.** Swiss 3T3 cells were washed twice with DMEM and incubated with 5 \( \mu M \) SPC for various times as indicated (panel A) or with 5 \( \mu M \) SPC, 10 nM bombesin (BOM), or 2 \( \mu M \) LPA for 3 min (panel B). Cells were subsequently fixed in 4% paraformaldehyde and stained for actin using TRITC-conjugated phalloidin. Changes in the actin cytoskeleton were visualized using a Zeiss Axiophot immunofluorescence microscope.
the results depicted in Fig. 7A, we examined whether SPC-stimulated tyrosine phosphorylation of p125FAK could also be affected by high concentrations of PDGF. As shown in Fig. 7B, PDGF at 30 ng/ml markedly reduced tyrosine phosphorylation of p125FAK induced by 5 μM SPC in agreement with its effects on SPC-induced actin reorganization.

Effects of C3 Exoenzyme on SPC-induced Actin Stress Fiber Formation, Focal Contact Assembly, and Protein Tyrosine Phosphorylation—Recently, the small G protein p21rho has been implicated in the formation of actin stress fibers and focal contacts in response to bombesin and LPA in Swiss 3T3 cells (21). The function of p21rho is specifically impaired by treatment with C. botulinum C3 exoenzyme, which ADP-ribosylates the Asn41 of p21rho and thereby prevents its interaction with downstream targets (37). To examine whether SPC-induced changes in actin cytoskeleton and focal contact assembly were dependent on p21rho, confluent and quiescent Swiss 3T3 cells were microinjected with 100 μg/ml C3 exoenzyme and subsequently incubated with 5 μM SPC for various times. The efficiency of injection was determined by coinjection of rabbit IgG at 0.5 mg/ml together with C3 exoenzyme followed by immunostaining with FITC-linked anti-rabbit IgG. Fig. 8, C and F, shows the cells injected with C3 exoenzyme and rabbit IgG. The formation of actin stress fibers after 20 min of incubation with 5 μM SPC was completely prevented in cells microinjected with C3 exoenzyme. The injected cells appeared contracted, suggesting a complete breakdown of the actin cytoskeleton as compared to the surrounding cells not injected (Fig. 8, A and D). C3 exoenzyme also prevented the formation of focal adhesions in response to SPC as shown by additional staining of the cells with anti-vinculin mAb (Fig. 8B). C3 exoenzyme was equally efficient at concentrations between 20 and 400 μg/ml. Thus, p21rho is required for the organization of the actin stress fibers and focal adhesion assembly induced by SPC. Swiss 3T3 cells injected with rabbit IgG alone exhibited the typical pattern of actin organization and focal contact assembly in response to SPC, demonstrating that microinjection itself did not attenuate SPC-induced changes in the actin cytoskeleton and focal adhesion formation (data not shown).

To examine whether C3 exoenzyme could also prevent tyrosine phosphorylation in response to SPC, microinjected cells were stained for actin, Tyr(P), and rabbit IgG as described under “Experimental Procedures.” In control cells stimulated with 5 μM SPC for 20 min, anti-Tyr(P) staining was visible at the ends of actin stress fibers in focal contacts (Fig. 8, D and E). In microinjected cells, this pattern of staining was not detect-
SPC-induced intracellular Ca\textsuperscript{2+} coordinate increase in tyrosine phosphorylation of p125\textsuperscript{FAK} and paxillin revealed that this protein contains multiple domains but associates with other proteins including v-Src and tyrosine residues in response to SPC, p125\textsuperscript{FAK}, and paxillin.

The recent molecular cloning and sequencing of domains but associates with other proteins including v-Src and tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells. We identified two substrates that were phosphorylated on tyrosine phosphorylation of p125\textsuperscript{FAK} is completely independent of intracellular Ca\textsuperscript{2+} release or Ca\textsuperscript{2+} influx. As shown in the accompanying paper (6), SPC rapidly activates PKC. Since direct activation of PKC has been shown to stimulate tyrosine phosphorylation of p125\textsuperscript{FAK}, SPC could activate tyrosine phosphorylation of p125\textsuperscript{FAK} through a PKC-dependent mechanism. However, neither down-regulation of PKC with PDB nor the PKC inhibitor GF 109203X blocked SPC-stimulated tyrosine phosphorylation of p125\textsuperscript{FAK}.
of p125FAK. Thus, SPC induces tyrosine phosphorylation of p125FAK by a pathway largely independent of both Ca\textsuperscript{2+} mobilization and PKC activation.

The increases in tyrosine phosphorylation of p125FAK and paxillin in response to SPC were accompanied by a novel and dramatic reorganization of the actin cytoskeleton. Specifically, SPC evoked a striking formation of peripheral actin microspikes followed by the development of actin stress fibers. Both events were associated with the assembly of focal adhesion plaques, which were localized at early times at the ends of actin microspikes and later at the ends of actin stress fibers. Thus, focal adhesion assembly in response to SPC occurs prior to actin stress fiber formation and is also linked to the formation of microspikes at the plasma membrane.

Tyrosine phosphorylation of p125FAK and paxillin in response to bombesin and other agents is critically dependent on the integrity of the actin cytoskeleton (8). Given the similar kinetics of SPC-stimulated tyrosine phosphorylation and cytoskeletal changes, it was of interest to establish whether these events were also linked in SPC-treated Swiss 3T3 cells. Pretreatment of quiescent Swiss 3T3 cells with cytochalasin D led to a complete disruption of the actin cytoskeleton and abolished tyrosine phosphorylation of p125FAK stimulated by SPC. Thus, the integrity of the actin cytoskeleton is essential for SPC-induced tyrosine phosphorylation. This conclusion was substantiated by experiments using PDGF at a high concentration (30 ng/ml), which disrupted actin stress fibers in response to SPC. At this concentration, PDGF also profoundly decreased SPC-induced tyrosine phosphorylation of p125FAK, revealing a novel cross-talk between SPC and PDGF.

Recent findings indicate that both cytoskeletal changes and tyrosine phosphorylation of p125FAK induced by bombesin require functional p21\textsuperscript{rho} protein (21, 27). These findings suggested the existence of a pathway activated by seven transmembrane domain receptors in which p21\textsuperscript{rho} is upstream of both cytoskeletal responses and tyrosine phosphorylation of specific proteins. In view of the results described above, it was plausible that the small G protein p21\textsuperscript{rho} could also be involved in SPC-induced cytoskeletal changes. Microinjection of C. botulinum C3 exoenzyme, which ADP-ribosylates and inactivates of p125FAK. Thus, SPC induces tyrosine phosphorylation of p125FAK by a pathway largely independent of both Ca\textsuperscript{2+} mobilization and PKC activation.

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Tyrosine phosphorylation of p125FAK and paxillin in response to bombesin and other agents is critically dependent on the integrity of the actin cytoskeleton (8). Given the similar kinetics of SPC-stimulated tyrosine phosphorylation and cytoskeletal changes, it was of interest to establish whether these events were also linked in SPC-treated Swiss 3T3 cells. Pretreatment of quiescent Swiss 3T3 cells with cytochalasin D led to a complete disruption of the actin cytoskeleton and abolished tyrosine phosphorylation of p125FAK stimulated by SPC. Thus, the integrity of the actin cytoskeleton is essential for SPC-induced tyrosine phosphorylation. This conclusion was substantiated by experiments using PDGF at a high concentration (30 ng/ml), which disrupted actin stress fibers in response to SPC. At this concentration, PDGF also profoundly decreased SPC-induced tyrosine phosphorylation of p125FAK, revealing a novel cross-talk between SPC and PDGF.

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p21^{tho} function, prevented actin stress fiber formation, focal contact assembly, and tyrosine phosphorylation in response to SPC. Thus, p21^{tho} is upstream of both cytoskeletal changes and tyrosine phosphorylation in response to SPC.

Recently, a possible link between p125^{FAK} tyrosine phosphorylation and the MAPK pathway has been suggested. Several groups demonstrated that integrin-mediated cell adhesion activates MAPK by a pathway critically dependent on the integrity of the actin cytoskeleton (40, 41). Schlaepfer et al. (42) showed that integrin engagement stimulates an interaction between p125^{FAK} and the adapter protein Grb2, suggesting a possibility of linking between p125^{FAK} and the p21^{tho} MAPK activation. In view of the data provided in the accompanying paper (6) on SPC-induced MAPK activation and here on p125^{FAK} tyrosine phosphorylation, it was important to define whether these events are causally related. Several lines of evidence demonstrate a dissociation of p125^{FAK} tyrosine phosphorylation from MAPK activation in response to SPC in Swiss 3T3 cells. SPC-induced MAPK activation is dependent on the activity of PKC and a pertussis toxin-sensitive Gi protein. In contrast, tyrosine phosphorylation of p125^{FAK} was largely independent of PKC and not affected by treatment with pertussis toxin. Crucially, tyrosine phosphorylation of p125^{FAK} in response to SPC was completely prevented by disruption of the actin cytoskeleton D, whereas MAPK activation by SPC was unaffected by an identical treatment with cytochalasin D. Thus, p125^{FAK} tyrosine phosphorylation and MAPK activation are independently regulated in SPC-treated cells.

In conclusion, our results demonstrate, for the first time, that SPC stimulates tyrosine phosphorylation of p125^{FAK} and paxillin. Furthermore, SPC induces a unique pattern of reorganization of the actin cytoskeleton and focal adhesion assembly in Swiss 3T3 cells. The integrity of the polymerized actin network and functional p21^{tho} are essential for SPC-induced tyrosine phosphorylation.

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