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# Differential effect of simvastatin on various signal transduction intermediates in cultured human smooth muscle cells

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**Abstract**

The underlying mechanism of the antiproliferative effect of S (simvastatin), a HMG-CoA reductase inhibitor, in vascular smooth muscle cells (SMC) is still poorly understood. In the present study, we used synchronized human SMC, isolated from left interior mammary artery, as an *in vitro* model to test the effects of S on platelet-derived growth factor (PDGF)-induced DNA synthesis, extracellular-regulated kinase 1/2 (ERK1/2), p38/stress-activated protein kinase 2 (SAPK2), RhoA and Rac1 activation. ERK1/2 phosphorylation was triggered within 2 min of PDGF stimulation (early G1 phase) and was blocked by PD98059, a specific inhibitor of the ERK1/2 pathway, which also strongly inhibited PDGF-induced DNA synthesis ($IC_{50}$ = 10 $\mu$mol/L). PDGF quickly induced p38 phosphorylation (early G1 phase) and SB203580, a specific inhibitor of the p38/SAPK2 pathway, also blocked PDGF-induced DNA synthesis ($IC_{50}$ = 0.3 $\mu$mol/L). Translocation to the plasma membrane of small GTPases, such as RhoA and Rac1, could not be detected within 15 min of stimulation with PDGF or lysophosphatidic acid (LPA) (early G1 phase), but occurred after 24 hr of PDGF stimulation (late G1/S phase). S inhibited PDGF-induced DNA synthesis ($IC_{50}$ = 3.5 $\mu$mol/L), and this effect was dependent on intracellular mevalonate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate availability. The critical time period for the reversal of the S effect by mevalonate comprised both the early and late G1 phase of the SMC cycle. PDGF-induced ERK1/2 phosphorylation and PDGF-induced p38 phosphorylation were not markedly affected by S during the whole G1 phase. However, S treatment blocked the PDGF- and LPA-induced membrane translocation of RhoA that occurred during the late G1/S phase. In the case of Rac1, the same process was also inhibited by S treatment. We concluded from these results that, in SMC, the early events associated with ERK1/2 and p38 signal transduction pathways, recruited for PDGF-mediated DNA synthesis, were insensitive to S action, whereas the mevalonate-dependent, posttranslational modification of RhoA and Rac1 molecules, required for PDGF-induced membrane translocation, was blocked by this drug. These results suggest that the antiproliferative effect of S can be explained not only by the blockage of RhoA-mediated signaling events but also by Rac1-mediated signaling events. 

*Keywords:* Human smooth muscle cells; Simvastatin, ERK1/2; p38/SAPK2, DNA synthesis; RhoA; Rac1.

## 1. Introduction

SMC are normally present in a relatively quiescent state in the blood vessel wall. However, a proliferative response is observed in the case of arterial injury following percutaneous transluminal coronary angioplasty or during the process of atherosclerosis. Among the various mitogens involved in SMC growth, PDGF interacts with specific SMC receptors, which ultimately leads to DNA synthesis and cell proliferation involving signal transduction proteins such as PKC [1], Ras, Rac, and the MAPK family [2]. Ten members of this family have thus far been identified in mammalian cells [3]. Two of these, MAPK1/ERK1 and MAPK2/ERK2, are known to be activated by growth factors with a tyrosine kinase receptor such as PDGF. In contrast, the other MAPK family members are strongly activated by stress signals and proinflammatory cytokines and, thus, are frequently referred to as SAPKs. The occurrence of an inflammatory response

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*Abbreviations:* SMC, smooth muscle cells; S, simvastatin; ERK1/2, extracellular-regulated kinase 1/2; SAPK, stress-activated protein kinase; PDGF, platelet-derived growth factor; PMA, phorbol myristil acetate; POD, peroxydase; MAPK, mitogen-activated protein kinase; TBS, Tris-buffered saline; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; Mev, mevalonic acid lactone, LPA, lysophosphatidic acid; PKC, protein kinase C; GST, glutathione *S*-transferase; and PAK-CD, p21-activated kinase CRIB domain.

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in atherosclerotic lesions involving cellular immune processes is now well documented [4]. The precise role of SMC proliferation in atherosclerosis and the question as to whether intimal SMC contribute to the pathogenesis and progression of vascular lesions are today under debate [5]. Since the stability of the plaque seems to be directly related to its SMC content and inversely related to its macrophage content [4], inhibition of SMC proliferation may not be desirable in the context of plaque stabilization.

Statins, cholesterol-lowering drugs, are extensively used for the prevention and treatment of atherosclerosis [6,7]. The antiproliferative effect of these drugs *in vitro* has been associated with the lowering of the cellular content of mevalonate, an intermediate of the cholesterol biosynthesis pathway [8]. This compound is the precursor of FPP and GGPP, isoprenoid intermediates that are important for the posttranslational modification of a variety of proteins [9], including Ras, Rho, and Rac. We previously observed, in cultured human SMC, that the antiproliferative effect of S was accompanied by a decrease in the farnesylation of newly synthesized Ras [10]. This fact, together with the observation that S could inhibit PDGF-induced DNA synthesis in synchronized SMC [10,11], led us to analyze Ras-mediated ERK1/2 activation in these cells. Moreover, very little information on the activation of stress/inflammatory pathways by PDGF has been available until now. Stimulation of quiescent cells with growth factors has been shown in many cases to induce rapid organization of the actin cytoskeleton, the regulation of this process by Rac and Rho being dependent on the cell type [12]. In Swiss 3T3 fibroblasts, Rho activation was found to be responsible for stress fibre assembly, whereas membrane ruffling was mediated through Rac activation [12]. Recently, isoprenylation of RhoA and its membrane translocation was shown to coincide with cell cycle progression in rat thyroid cells [13] and in human SMC [15]. These facts led us to investigate the effect of PDGF on DNA synthesis, ERK1/2 phosphorylation, p38/SAPK2 phosphorylation, and RhoA and Rac1 activation in synchronized, quiescent human SMC. The effect of S on all these processes was analyzed during early and late G1 phase so as to gain more insight into the mechanism of action of S in blocking SMC proliferation.

## 2. Materials and methods

### 2.1. Materials

S was kindly provided by Sankyo Co. The drug was in the open sodium salt form and kept at −20° as a 10 mM stock solution (in 100% ethanol) for up to 2 weeks. SB203580 was from Alexis Corp. PD98059 and SB203580 were kept at −20° as 100 mM (in 100% DMSO) and 100 μM (in 100% ethanol) stock solutions, respectively. [$^3$H]Thymidine (specific radioactivity of 70–86 Ci/mmol) was purchased from Amersham. GGPP, FPP, Mev, phosphatidylcholine, and LPA were purchased from Sigma. Recombinant, human PDGF B/B, polyclonal peroxydase-conjugated, anti-rabbit immunoglobulin G (anti-rabbit IgG–POD), and protease inhibitor cocktail tablets (Complete™) were from Boehringer Mannheim B.V. Rabbit polyclonal immunoglobulin G anti-ERK1/2 (anti-ERK1/2), rabbit polyclonal immunoglobulin G anti-RhoA (anti-RhoA), and polyclonal peroxydase-conjugated, anti-mouse immunoglobulin G (anti-mouse IgG–POD) were from Santa Cruz Biotechnology. Rabbit polyclonal anti-phosphorylated p38 (anti-phospho p38) and PD98059 were from New England Biolabs Inc. Mouse polyclonal immunoglobulin G anti-Rac1 (anti-Rac1) was from Transduction Lab. The GST–PAK-CD fusion protein was generated and used as previously described [14].

### 2.2. SMC cultures

Human SMC were isolated from left interior mammary artery explants obtained from patients undergoing bypass surgery. Vascular specimens, discarded after the operation, were kindly provided by the Department of Thorax Surgery of the Leiden University Medical Center according to the rules of the local ethical committee. The cells were cultured as previously described [10]. SMC cultures were used within 6 passages in this study. The average doubling time was 2.5 days.

### 2.3. Preparation of liposomes of isoprenoids

Liposomes containing a mixture of dipalmitoylphosphatidylcholine (3.6 mg) and either GGPP or FPP (200 μg) were prepared according to Hirai *et al*. [13]. They were kept at 4° until use.

### 2.4. DNA synthesis

After trypsinization, SMC were seeded (25,000 cells/2.5 cm$^2$ well) in serum-poor Dulbecco's modified Eagle's medium (DMEM), containing 0.4% fetal bovine serum, for 65–70 hr to obtain quiescent, synchronized cells. After this "growth arrest" period, cells were preincubated with S, Mev, isoprenoids, PD98059, SB203580, or control medium and stimulated, as indicated in the figure legends. Cells were further processed for determination of DNA synthesis activity by a [$^3$H]thymidine incorporation assay, as previously described [10].

### 2.5. Measurement of membrane translocation by subcellular fractionation

The detailed protocol of this technique is described in Hirai *et al*. [13]. Cells (200,000 cells/10 cm$^2$ well; 2 wells/sample) were made quiescent, as described above, incubated further, and stimulated as described in the figure legends. They were then washed in ice-cold PBS, disrupted

by incubation (30 min, 4°) in hypotonic buffer (5 mM Tris–HCl, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM $MgCl_2$, 2 mM dithiothreitol; pH 7.0) containing a mixture of protease inhibitors, and separated into membrane-associated and cytosolic fractions by centrifugation (100,000 *g*, 1 hr at 4°). The volume of each fraction was adjusted to comparable values, diluted in SDS–PAGE buffer, and specific proteins contained in the membrane-associated fractions were detected by Western blotting.

### 2.6. Western blot analysis

After trypsinization, SMC (200,000 cells/10 $cm^2$ well) were made quiescent, as described above, further incubated, and stimulated as described in the figure legends. Cells were then lysed in SDS–PAGE buffer and processed for SDS–PAGE analysis. Only in the case of ERK1/2 detection, were phosphorylated and non-phosphorylated forms of the proteins separated by electrophoretic migration on an 8.4% polyacrylamide gel. All other proteins were resolved on pre-cast 10% polyacrylamide mini-gels (Gradipore Ltd.). Proteins were transferred onto polyvinylidene fluoride membranes (0.45 $\mu$m; Millipore Corp.). The membranes were first shaken for 1 hr in TBS (20 mmol/L of Tris, 136 mmol/L of NaCl, pH 7.6) containing 0.25% Tween 20 and 5% non-fat, protein-enriched milk (Protifar™), then incubated with anti-ERK1/2 (1/3000 dilution in TBS/Tween, overnight at 4°), anti-phospho p38 (1/5000 dilution in TBS/Tween/milk, overnight at 4°), anti-RhoA (1/500 dilution in TBS/Tween, overnight at 4°), or anti-Rac1 (1/500 dilution in TBS/Tween, overnight at 4°), followed by an incubation with an anti-rabbit IgG–POD (1/1000 dilution in TBS/Tween, 1 hr at room temperature) or with an anti-mouse IgG–POD (1/2000 dilution in TBS/Tween, 1 hr at room temperature). When the anti-phospho p38 antibody was used, 0.5 mmol/L of vanadate was present in all incubations to prevent phosphatase activity. The immunoreactive proteins were visualized by chemiluminescence using a peroxidase enzymatic reaction (enhanced chemoluminescence kit, Amersham). In some cases, the intensity of each specific band was quantified by densitometry after scanning of the exposed film. Unless otherwise specified, the results of 1 experiment, representative of a series of 3–4 experiments, are shown.

### 2.7. Rac activation assay

The detailed protocol of this technique is described in Sander *et al*. [14]. Cells (200,000 cells/10 $cm^2$ well; 3 wells/sample) were made quiescent, as described above, further incubated, and stimulated as described in the figure legends. They were then washed in ice-cold PBS, lysed in the presence of a mixture of protease inhibitors (Complete™), and then centrifuged for 5 min at 13,000 *g* at 4°. Aliquots were taken from the supernatant to compare the amounts of total Rac1 among the samples. The supernatant was incubated with bacterially produced GST–PAK-CD fusion protein bound to glutathione-coupled Sepharose beads at 4°, for 1 hr. The protein/fusion protein/bead complex was washed 3 times in an excess of lysis buffer, eluted in SDS-containing Laemmli buffer, and then analyzed for bound Rac1 molecules (GTP-bound Rac1) by Western blotting.

### 2.8. Statistical analysis

Quantitative results are given as means ± SD. For statistical evaluation, the results were analyzed by means of a Student's *t*-test. Differences were considered statistically significant at values $P > 0.05$.

## 3. Results

### 3.1. Inhibition of PDGF-induced DNA synthesis in synchronized HSMC by simvastatin and specific inhibitors of MAPK and SAPK pathways

In synchronized SMC, PDGF-induced DNA synthesis was inhibited by S in a concentration-dependent manner ($IC_{50}$ = 3 $\mu$mol/L; Figs. 1A,B and 2C). This inhibitory effect was fully overcome by the presence of Mev during the whole period of S incubation, during the first 10 hr, and during the time interval of 10–20 hr after PDGF addition (values not statistically different from control value after PDGF stimulation, $P > 0.05$) (Fig. 1A). When Mev was present only before PDGF addition or during the last 6 hr of PDGF addition, no overcoming effect was observed (Fig. 1A). There is, therefore, a critical time period, comprising both early and late G1 phase, when PDGF-stimulated cells require Mev for DNA synthesis to proceed. Incubation of FPP- or GGPP-containing liposomes during the whole period of S treatment prevented its inhibitory effect on PDGF-induced DNA synthesis (values not statistically different from control value after PDGF stimulation, $P > 0.05$) (Fig. 1B), suggesting that the mode of action of S takes place through inhibition of both farnesylation and geranylgeranylation of signal transduction protein(s), probably at various steps of the G1 phase. When FPP or GGPP alone was used, FPP did not prevent the S effect (Fig. 1B), probably because this compound is less hydrophobic than GGPP and is not as well taken up by the cell in such a state.

In order to block the ERK1/2 pathway, we used PD98059, a specific inhibitor of MAPK kinase 1 and (to a lesser extent) MAPK kinase 2 activation. This compound inhibited PDGF-induced DNA synthesis in a concentration-dependent manner ($IC_{50}$ = 10 $\mu$mol/L) (Fig. 2A). Similarly, SB203580, a specific inhibitor of p38/SAPK2 substrate activation, even more strongly inhibited PDGF-induced DNA synthesis in a concentration-dependent manner ($IC_{50}$ = 0.3 $\mu$mol/L) (Fig. 2B). Moreover, up-regulation of PKC by PMA, which induced a partial phosphorylation of ERK1/2

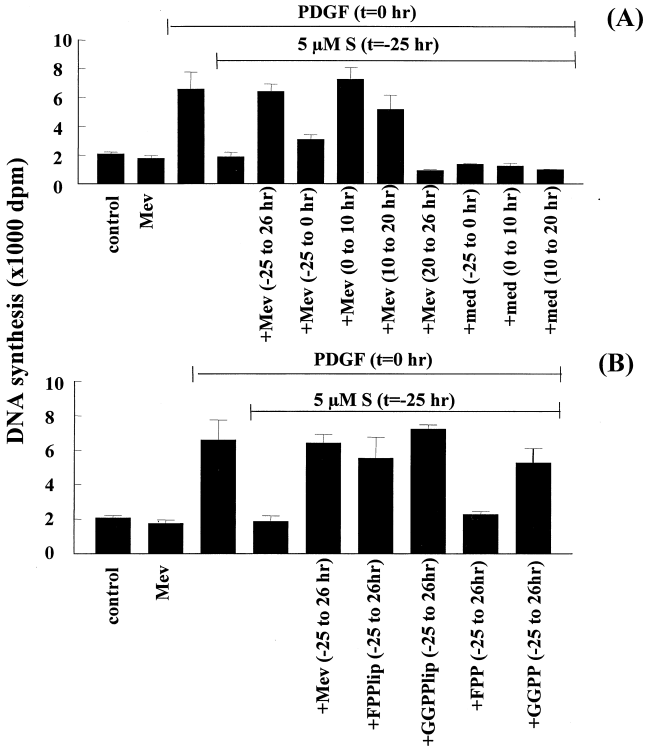


Fig. 1. Inhibition of PDGF-induced DNA synthesis by simvastatin in synchronized SMC. Influence of mevalonate (A) and of isoprenoid compounds (B) on the simvastatin inhibitory effect. Cells were preincubated with S for 25 hr, PDGF (20 ng/mL) added, and DNA synthesis measured 26 hr later. Incubations were performed in the absence or presence of Mev (100 $\mu$M), FPP- or GGPP-containing liposomes (+FPPlip, +GGPPlip, 5 $\mu$M), or medium (+med) for the indicated lengths of time. Each assay was performed in triplicate. Bars depict SD of a typical experiment.

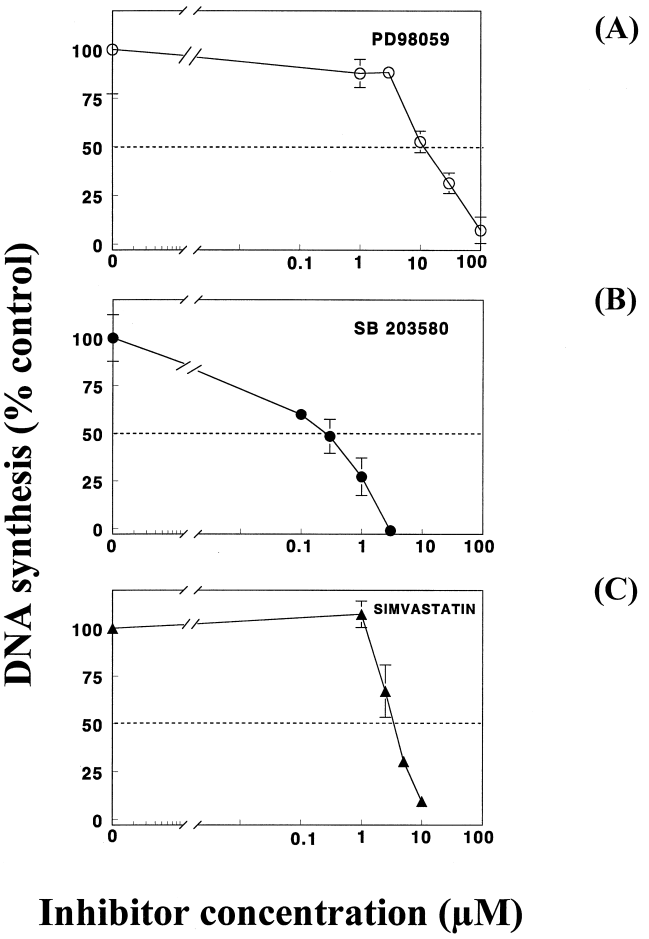


Fig. 2. Comparison of the inhibitory effect of PD98059 (A), SB203580 (B), and simvastatin (C) on PDGF-induced DNA synthesis, in synchronized SMC. Cells were preincubated with PD98059 or SB203580 for 1 hr or with S for 25 hr, at the indicated concentrations. PDGF (20 ng/mL) was then added and DNA synthesis measured 26 hr later. The data were expressed in % of control [control value, PDGF added (A): 29,261 ± 6,654 dpm/well; control value, PDGF added (B): 14,077 ± 1,360 dpm/well; control value, PDGF added (C): 8,243 ± 230 dpm/well]. Each assay was performed in triplicate. Bars depict SD of a typical experiment.

(Fig. 3A) but no phosphorylation of p38 (Fig. 3B), was not able to trigger DNA synthesis (Fig. 3C). These data taken together indicate that both the ERK1/2 and p38/SAPK2 pathways must be recruited for DNA synthesis to proceed.

### *3.2. Absence of effect of simvastatin on the activation of ERK1/2 by PDGF*

In order to further investigate whether the inhibitory effect of S on PDGF-induced DNA synthesis was mediated through its interference on the ERK1/2 pathway, we analyzed ERK1/2 phosphorylation patterns in PDGF-stimulated SMC. As shown in Fig. 4A, a 10-min incubation with PDGF induced a strong activation of ERK1/2. This process was already attenuated after 2 hr and very moderately sustained up to 24 hr after PDGF addition (non-phosphorylated and phosphorylated forms both present). S treatment inhibited neither the early induced nor long-lasting ERK1/2 phosphorylation (Fig. 4A).

Since the PDGF concentration used in our experiments was quite high (20 ng/mL) and could stimulate DNA synthesis up to 20-fold in quiescent HSMC, we investigated whether ERK1/2 stimulation could occur at much lower PDGF concentrations and whether, under these less extreme conditions, S could block this process. PDGF-induced ERK1/2 phosphorylation was total and complete at concentrations as low as 2 ng/mL (Fig. 4B), whereas the induction of DNA synthesis by PDGF followed a typical concentration-dependent pattern with a maximal effect at concentrations higher than 20 ng/mL (Fig. 4C). S failed to inhibit ERK1/2 phosphorylation at all PDGF concentrations tested. In contrast, the amplitude of the effect of PD98059, a specific inhibitor of the ERK1/2 pathway, was inversely proportional to PDGF concentration (Fig. 4B).

### *3.3. Absence of effect of simvastatin on the activation of p38/SAPK2 by PDGF*

Since PDGF-induced DNA synthesis was inhibitable by SB203580, a specific inhibitor of p38/SAPK2 substrate ac-

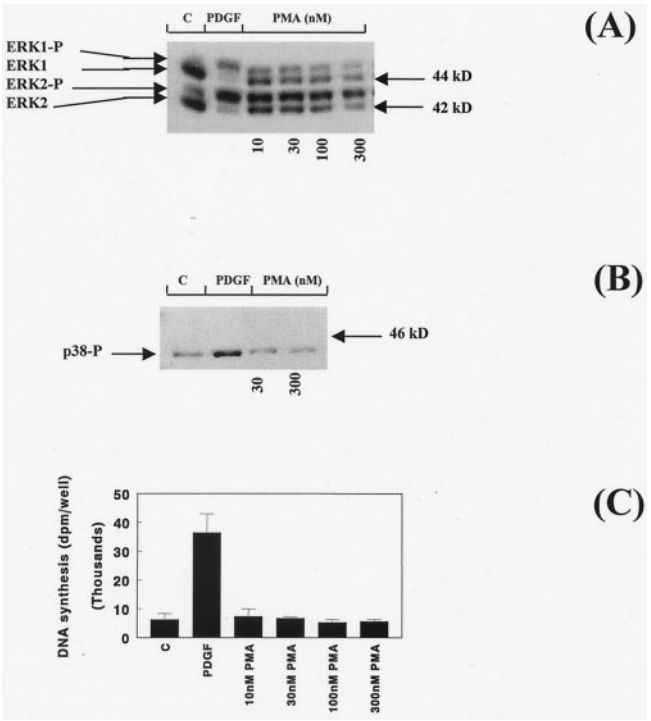


Fig. 3. Influence of PMA on ERK1/2 phosphorylation (A), p38 phosphorylation (B), and DNA synthesis (C). Quiescent cells were preincubated with PDGF (20 ng/mL) or PMA (at the indicated concentrations) for 10 min. The reaction was then terminated and the samples processed for Western blotting, as described in Figs. 4A and 5B, or for measurement of DNA synthesis, as described in Fig. 1.

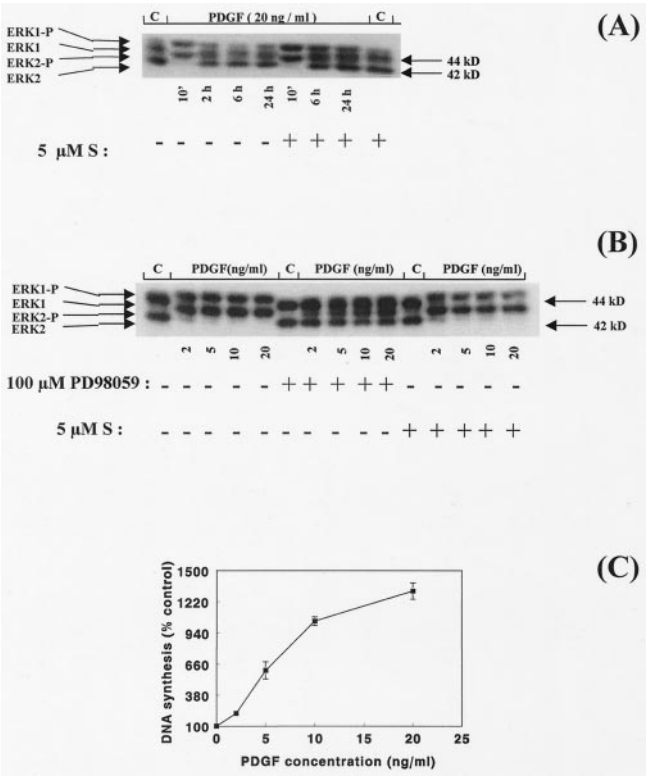


Fig. 4. Time-course of PDGF-induced ERK1/2 phosphorylation (A); influence of PDGF concentration on ERK1/2 phosphorylation (B) and on DNA synthesis (C), in synchronized SMC. Effect of simvastatin and PD98059 on these processes. (A and B) Quiescent cells were preincubated in the absence or presence of S for 25 hr, at the indicated concentrations. PDGF was then added for 10 min, or the indicated times, and at 20 ng/mL, or the concentrations indicated. The reaction was terminated by lysing the samples in SDS–sample buffer, and the proteins were separated by SDS–PAGE and immunolabeled by Western blotting using anti-ERK1/2. (C) Cells were incubated with PDGF at the indicated concentrations and DNA synthesis was measured 26 hr later. Each assay was performed in triplicate. Bars depict SD. ERK1, ERK2: ERK1/2 non-phosphorylated forms; ERK1-P, ERK2-P: ERK1/2 phosphorylated forms.

tivation (see Fig. 2B), we investigated the effect of this growth factor on the activation of p38 by detecting the phosphorylated form of the enzyme with a specific antibody. P38 phosphorylation was strongly induced by PDGF at concentrations as low as 2 ng/mL (Fig. 5C). As shown in Fig. 5, A and B, this process was triggered during the early G1 phase (2–10 min after PDGF addition), followed by inactivation and re-activation during the late G1 phase. S (2-hr pretreatment, 10 $\mu$M) failed to inhibit p38 phosphorylation during the early G1 phase (Fig. 5, A and B) and even seemed to sustain it during the late G1/S phase of the cell cycle. The same results were obtained with a 24-hr pretreatment with S (not shown). Measurement of total p38 levels by Western blotting using an anti-p38 antibody indicated that the total amount of this protein was not affected by S treatment (not shown).

### *3.4. Inhibition of PDGF-induced membrane translocation of RhoA and Rac1 by simvastatin*

Protein prenylation is essential for the attachment of small GTPases to the plasma membrane and for their subsequent membrane translocation, a crucial step for the activation of their respective signal transduction pathways. The membrane translocation of RhoA and Rac1 was assessed by measuring the amount of these proteins in the membrane-associated fraction obtained after subcellular fractionation. In quiescent cells (3 days in serum-poor medium), the level of RhoA translocated to the membrane was quite high and was not increased by LPA or PDGF, after 15 min of stimulation (Fig. 6A). A 2-hr pretreatment with 10 $\mu$M S did not remarkably affect the levels of translocated RhoA during this time period (early G1 phase; Fig. 6A). When the amounts of membrane-associated RhoA were measured 24 hr later (4 days in serum-poor medium), a much lower background signal was observed (Fig. 6A). After 24 hr of incubation with LPA or PDGF, a high level of membrane translocation was observed and S treatment entirely blocked this process (Fig. 6A).

The high level of membrane-associated Rac1 detected in quiescent cells (3 days in serum-poor medium) was not noticeably increased by LPA or PDGF after 15 min of stimulation (Fig. 6B). However, S reduced the levels of Rac1 translocation in cells stimulated with LPA for 15 min (Fig. 6B). As observed with RhoA, a much lower background

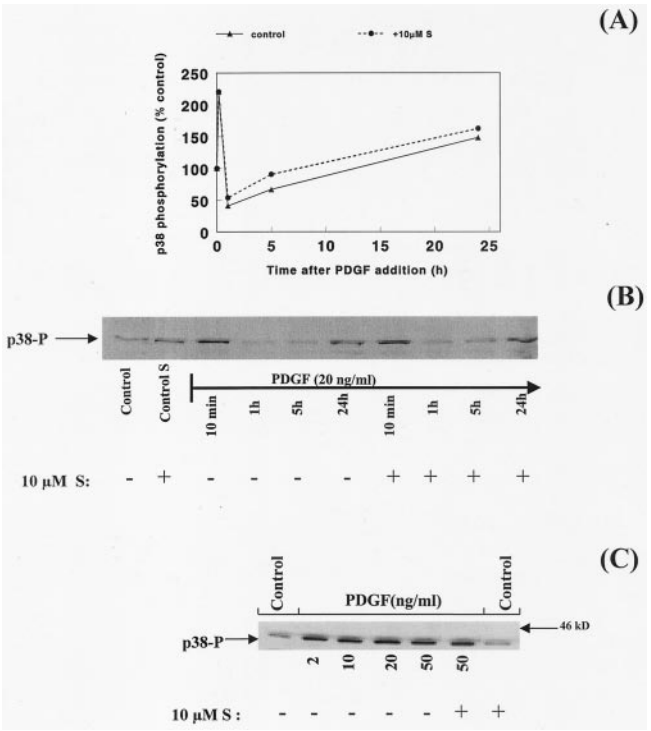


Fig. 5. Time-course of PDGF-induced p38 phosphorylation (A and B); influence of PDGF concentration on p38 phosphorylation (C), in synchronized SMC. Effect of simvastatin on these processes. Quiescent cells were preincubated in the absence or presence of S (10 $\mu$M) for 2 hr. PDGF was then added for 10 min, or the indicated times, and at 20 ng/mL, or the concentrations indicated. The samples were then processed as described in Fig. 4, except that anti-phospho p38 was used. (A) Average curve from 2 independent experiments after quantification by densitometry of specific bands obtained in Western blots (as shown in B). p38-P: p38 phosphorylated form.

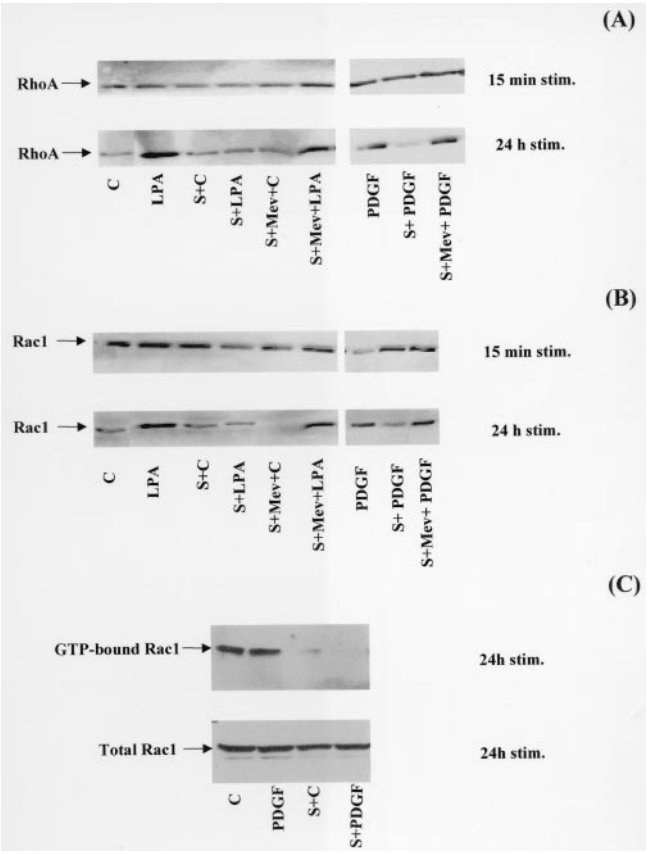


Fig. 6. Influence of PDGF and LPA on the membrane translocation of RhoA (A) and Rac1 (B) in synchronized HSMC. Comparison with the influence of PDGF on the levels of GTP-bound Rac1 (C). Effect of simvastatin on these processes. (A and B) Quiescent cells were pretreated for 2 hr with medium, S (10 $\mu$M), or S (10 $\mu$M) and Mev (200 $\mu$M). Cells were then incubated with medium, LPA (100 $\mu$M), or PDGF (20 ng/mL) for 15 min or 24 hr. The reaction was stopped by lysing the cells in hypotonic buffer, and the total cell lysate was separated in membrane-associated and cytosolic fractions by high-speed centrifugation. The volume of each fraction was adjusted to comparable values, and proteins from the membrane-associated fractions were analyzed by Western blotting with anti-RhoA (A) or anti-Rac1 (B). (C) Quiescent cells were pretreated for 24 hr with medium or S (10 $\mu$M). Cells were then incubated with medium or PDGF (20 ng/mL) for 24 hr. After stopping the reaction, cells were processed for the Rac1 activation assay and GTP-bound Rac molecules were analyzed by Western blotting with anti-Rac1.

signal was observed in cells incubated for 4 days in serum-poor medium. A 24-hr incubation with LPA or PDGF strongly induced Rac1 translocation to the membrane, a process blocked by S treatment. All the observed inhibitory effects of S were counteracted by the presence of Mev in the incubation medium, indicating that they are related to Mev depletion and posttranslational modification of the proteins analyzed.

### *3.5. Influence of simvastatin on the levels of GTP-bound Rac1 after PDGF stimulation*

Since Rac1 is a known activator of p38 and no inhibitory effect of S was detected on p38 activation (Fig. 5A), we tested the possibility that soluble, GTP-bound Rac1 could account for p38 activation in the presence of S. We measured the levels of GTP-bound Rac1 in synchronized SMC using a GST–PAK fusion protein with a binding domain (CRIB domain:CD) for GTP-bound Rac1 (Fig. 6C). No specific effect of PDGF was detected on Rac1 activation, probably due to high background levels of GTP-bound Rac1, possibly in part cytosolic, in quiescent cells. However, when quiescent cells were preincubated for 24 hr with S and further incubated, in the presence of S, with PDGF or medium, the levels of GTP-bound Rac1 decreased dramatically, indicating that p38 activation occurring in the late G1 phase is independent of this parameter.

## 4. Discussion

We observed in synchronized, cultured human SMC that S is able to inhibit PDGF-induced DNA synthesis in a concentration-dependent manner ($IC_{50}$ = 3 $\mu$mol/L). Although it was previously reported that the growth of S-treated cells can be restored by Mev, geranylgeraniol, and farnesol [11,15], the direct effect of geranylgeraniol on various cellular mechanisms [16–18] makes it difficult to

rely on the use of this compound to analyze isoprenylation pathways specifically. By using FPP and GPP, which are directly used for farnesylation or geranylgeranylation of cellular proteins, we demonstrated that both compounds, when incorporated into liposomes, prevented the inhibitory effect of S on PDGF-induced DNA synthesis, suggesting that in human SMC, both farnesylation and geranylgeranylation of transduction protein(s) are necessary for G1/S cell cycle transition. In contrast, in rat thyroid cells, Hirai *et al*. observed that only GGPP-, and not FPP-, containing liposomes could rescue statin-induced G1 arrest [13]. Moreover, we observed that the critical time period during which PDGF-stimulated HSMC require Mev for DNA synthesis to proceed encompasse the early G1 as well as the late G1 phase of their cell cycle. In contrast, in the case of human fibroblasts [19], this critical time period is present only during the late G1 phase of the cell cycle, implying that the involvement of various isoprenylated proteins in signal transduction pathways leading to cell cycle progression might be of varying importance depending on the cell type.

We showed in human SMC that both stimulation of the ERK1/2 pathway (blocked by PD98059) and the p38/SAPK2 pathway (blocked by SB203580) are required for PDGF-induced DNA synthesis. Indeed, PKC activation by PMA, which stimulates ERK1/2 but not p38 phosphorylation, does not trigger DNA synthesis in quiescent SMC. However, we cannot exclude the possibility that PMA alone failed to induce ERK1/2-sustained phosphorylation, a necessary step for progression into G1 phase and DNA synthesis. By expressing a dominant-negative ras gene in vascular smooth muscle cells, Irani *et al*. [20] showed that Ras activation is essential for SMC proliferation. We have previously shown that treatment with S results in a decrease in farnesylation of the newly synthesized Ras in exponentially growing human SMC [10]. However, we observed in the present study that this treatment did not inhibit PDGF-induced ERK1/2 phosphorylation at any PDGF concentration (Fig. 4B). Moreover, neither immediate (10 min after stimulation) nor sustained (up to 24 hr after stimulation) phosphorylation of ERK1/2 was blocked by S (Fig. 4A), indicating that its inhibitory effect on PDGF-induced DNA synthesis was not the result of a concomitant blockage of ERK1/2 activation at any time during the G1 phase. In addition, a 2-hr preincubation with S was sufficient to block PDGF-induced DNA synthesis in quiescent human SMC (results not shown), suggesting that depletion of the pool of the *de novo* synthesized, farnesylated Ras is probably not sufficient to suppress the signal-transducing activity of stable proteins such as Ras. It is generally admitted that p38/SAPK2 is strongly activated by proinflammatory cytokines and environmental stress [21,22]. In our study, we observed in human SMC that p38 phosphorylation was not only stimulated by stress factors such as LPS or hyperosmolarity (not shown), but also by mitogenic agents such as PDGF (Fig. 5). Accordingly, it was recently reported that growth factors could induce p38/SAPK2 activation in airway SMC [23] and endothelial cells [24]. We demonstrated in human SMC that the recruitment of the p38/SAPK2 pathway by PDGF was necessary for its mitogenic effect, since it was blocked by SB203580, a specific inhibitor of p38/SAPK2 substrate activation. However, S treatment did not markedly affect p38 activation at any time during the G1 phase of the PDGF-induced cell cycle (Fig. 5A).

During reorganization of actin cytoskeleton and/or cell migration, the recruitment of the p38/SAPK2 pathway was demonstrated in canine SMC [25] and human endothelial cells [24]. The Rho GTPase family, which includes RhoA, RhoB, Rac1, and Cdc42, plays a key role in these processes [12]. In particular, the involvement of RhoA in growth factor-induced cell cycle progression was described recently in rat [26] and human SMC [15]. Moreover, Rac1 was shown to be required in PDGF-stimulated migration [27,28] and cyclic strain stress-stimulated proliferation [28] of vascular SMC. These proteins are isoprenylated, and geranylgeranylation in particular is important for cellular localization while targeting these Rho GTPases to the cellular membrane [29,30]. In our model system, the membrane translocation of Rho A occurred during the late G1 phase of the cell cycle and was strongly inhibited by S treatment, probably due to the blockage of its posttranslational modification. In contrast with a previous observation in Swiss 3T3 fibroblasts [31], we observed that Rac1 translocation was also induced by LPA in human SMC and, more interestingly, by PDGF. S strongly inhibited membrane translocation and GTP binding of Rac1 during the late G1 phase. Consequently, the antiproliferative effect of S can be related not only to the inhibition of RhoA-mediated signaling events, but also to the inhibition of Rac1-mediated signaling events occurring during the late G1 phase. This is the first time, to our knowledge, that the inhibitory effect of S on PDGF-induced membrane translocation of Rac1 has been described in vascular human SMC. Rac1 is a known activator of p38 and, paradoxically, blockage of its membrane translocation had no influence on p38 activation in our model system. However, it is important to note that p38 activation is a very early event (peak 2–10 min after PDGF addition) and that Rac1 translocation is a long-lasting event (high levels 24 hr after PDGF addition). These results support the concept that early membrane translocation of Rac1, insensitive to S, leads to strong p38 activation, and that long-lasting membrane translocation of Rac1, blocked by S, does not influence p38 phosphorylation but rather other unknown pathways leading to cell proliferation. In human SMC, the effectors of Rac1 involved in the PDGF-mediated signaling pathway are not yet known. However, Rho is a good candidate, and, whether growth factors act through Rac to stimulate a Rho-dependent response (i.e. formation of actin stress fibers) [32], or Rac signaling is able to antagonize Rho activity directly at the GTPase level [33], is still controversial. Moreover, the type of interaction might be strongly dependent on the cell type analyzed [12]. It will be of interest to investigate this matter in more detail in

human SMC. In view of these results, it is crucial to underline that the net effect of PDGF on SMC is the result of a complex interaction of various signal transduction pathways involved in actin reorganization, migration, and proliferation. The study of the molecular cross-talk of Rho and Rac activation by growth factors will be of great importance in light of the inhibitory effect of S on SMC proliferation.

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## References

[1] Lee MW, Severson DL. Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. Am J Physiol 1994;267:659–78.
[2] Denhardt DT. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochem J 1996;318:729–47.
[3] Cohen P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends in Cell Biology 1997;7:353–61.
[4] van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994;89:36–43.
[5] Weissberg PL, Clesham GJ, Bennett MR. Is vascular smooth muscle cell proliferation beneficial? Lancet 1996;347:305–7.
[6] Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 1994;344:1383–9.
[7] Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med 1995;333:1301–7.
[8] Jakóbisiak M, Bruno S, Skierski JS, Darzynkiewicz Z. Cell cycle-specific effects of lovastatin. Proc Natl Acad Sci USA 1991;88:3628–32.
[9] Fu HW, Casey PJ. Enzymology and biology of CAAX protein prenylation. Recent Prog Horm Res 1999;54:315–42.
[10] Nègre-Aminou P, van Vliet AK, van Erck M, van Thiel GC, van Leeuwen RE, Cohen LH. Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors; comparison with other human cell types. Biochem Biophys Acta 1997;1345:259–68.
[11] Corsini A, Mazzotti M, Raiteri M, Soma MR, Gabbiani G, Fumagalli R, Paoletti R. Relationship between mevalonate pathway and arterial myocyte proliferation: *in vitro* studies with inhibitors of HMG-CoA reductase. Atherosclerosis 1993;101:117–25.
[12] Ridley AJ. Rho-related proteins: actin cytoskeleton and cell cycle. Curr Opin Genet Dev 1995;5:24–30.
[13] Hirai A, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Tatsuno I, Oeda T, Tahara K, Terano T, Narumiya, Kohn LD, Saito Y. Geranylgeranylated Rho small GTPase(s) are essential for the degradation of $p27^{Kip1}$ and facilitate the progression from $G_1$ to S phase in growth-stimulated rat FRTL-5 cells. J Biol Chem 1997;272:13–6.
[14] Sander EE, van Delft S, ten Klooster JP, Reid T, van der Kammen RA, Michiels F, Collard JG. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J Cell Biol 1998; 143:1385–98.
[15] Laufs U, Marra D, Node K, Liao JK. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing Rho GTPase-induced down-regulation of $p27^{Kip1}$. J Biol Chem 1999;274:21926–31.
[16] Crick DC, Andres SA, Waechter CJ. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. Biochem Biophys Res Commun 1997;237:483–7.
[17] Forman BM, Ruan B, Chen J, Schroepfer GJ Jr, Evans RM. The orphan nuclear receptor LXR$\alpha$ is positively and negatively regulated by distinct products of mevalonate metabolism. Proc Natl Acad Sci USA 1997;94:10588–93.
[18] McGuire T, Sebti SM. Geranylgeraniol potentiates lovastatin inhibition of oncogenic H-Ras processing and signaling while preventing cytotoxicity. Oncogene 1997;14:305–12.
[19] Fairbanks KP, Witte LD, Goodman DW. Relationship between mevalonate and mitogenesis in human fibroblasts stimulated with platelet-derived growth factor. J Biol Chem 1984;259:1546–51.
[20] Irani K, Herzlinger S, Finkel T. Ras proteins regulate multiple mitogenic pathways in A10 vascular smooth muscle cells. Biochem Biophys Res Commun 1994;202:1252–8.
[21] Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420–6.
[22] Geng Y, Valbracht J, Lotz M. Selective activation of the mitogen-activated protein kinase subgroups c-Jun NH2 terminal kinase and p38 by IL-1 and TNF in human articular chondrocytes. J Clin Invest 1996;98:2425–30.
[23] Pyne NJ, Pyne S. Platelet-derived growth factor activates a mammalian Ste20 coupled mitogen-activated protein kinase in airway smooth muscle. Cell Signal 1997;9:311–7.
[24] Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene 1997;15:2169–77.
[25] Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA, Gerthoffer WT. A role for p38MAPK/HSP27 pathway in smooth muscle cell migration. J Biol Chem 1999;274:24211–9.
[26] Seasholtz TM, Majumdar M, Kaplan DD, Brown JH. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ Res 1999;84:1186–93.
[27] Doanes AM, Irani K, Goldschmidt-Clermont PJ, Finkel T. A requirement for Rac1 in the PDGF-stimulated migration of fibroblasts and vascular smooth muscle cells. Biochem Mol Biol Int 1998;45:279–87.
[28] Li C, Hu Y, Sturm G, Wick G, Xu Q. Ras/Rac-dependent activation of p38 mitogen-activated protein kinases in smooth muscle cells stimulated by cyclic strain stress. Arterioscler Thromb Vasc Biol 2000;20:E1–E9.
[29] Didsbury JR, Uhing RJ, Snyderman R. Isoprenylation of the low molecular mass GTP-binding proteins rac 1 and rac 2: possible role in membrane localization. Biochem Biophys Res Commun 1990;171: 804–12.
[30] Yamane HK, Farnsworth CC, Xie H, Evans T, Howald W, Gelb MH, Glomset JA, Clarke S, Fung BK. Membrane-binding domain of the small G protein G25K contains an *S*-(all-*trans* geranylgeranyl)cysteine methyl ester at its carboxyl terminus. Proc Natl Acad Sci USA 1991;88:286–90.
[31] Fleming IN, Elliot CM, Exton JH. Differential translocation of Rho family GTPases by lysophosphatidic acid, endothelin-1, and platelet-derived growth factor. J Biol Chem 1996;271:33067–73.
[32] Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. Cell 1992;70:401–10.
[33] Sander EE, ten Klooster JP, van Delft S, van der Kammen RA, Collard J. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. J Cell Biol 1999;147:1–13.