

EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway

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Summary

Interactions between the Eph receptor tyrosine kinase and ephrin ligands transduce short-range signals regulating axon pathfinding, development of the cardiovascular system, as well as migration and spreading of neuronal and non-neuronal cells. Some of these effects are believed to be mediated by alterations in actin dynamics. The members of the small Rho GTPase family elicit various effects on actin structures and are probably involved in Eph receptor-induced actin modulation. EphrinA1 is proposed to contribute to angiogenesis as it is strongly expressed at sites of neovascularization. Moreover, angiogenic factors induce the expression of ephrinA1 in endothelial cells. In this study, using rat vascular smooth muscle cells (VSMCs), we investigated the contribution of the small Rho GTPases in ephrinA1-induced integrin inactivation. EphrinA1 did not significantly affect early adhesion of VSMCs on purified laminin or fibronectin, but strongly impaired cell spreading. The Rho kinase inhibitor Y-27632 partly reversed the ephrinA1 effect, suggesting involvement of

Rho in this model. However, inhibition of RhoA synthesis with short interfering (si)RNA had a modest effect, suggesting that RhoA plays a limited role in ephrinA1-mediated inhibition of spreading in VSMCs. The ephrinA1-mediated morphological alterations correlated with inhibition of Rac1 and p21-activated kinase 1 (PAK1) activity, and were antagonized by the expression of a constitutively active Rac mutant. Moreover, repression of Rac1 synthesis with siRNA amplifies the ephrinA1-induced inhibition of spreading. Finally, sphingosine-1-phosphate (S1P), a lipid mediator known to inhibit Rac activation in VSMCs amplifies the ephrinA1 effect. In conclusion, our results emphasize the role of the Rac/PAK pathway in ephrinA1-mediated inhibition of spreading. In this way, ephrinA1, alone or in synergy with S1P, can participate in blood vessel destabilization, a prerequisite for angiogenesis.

Key words: EphrinA1, RhoGTPases, siRNA, Lamellipodia, Integrin, Laminin

Introduction

The Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins, are key regulators of cell organization (Holder and Klein, 1999). During development, Eph and their ephrin ligands are expressed at the limit of cellular compartments (Flenniken et al., 1996; Gale et al., 1996; Wang et al., 1998). Their interaction generates bidirectional signaling that is proposed to limit cell migration through embryonic boundaries (Mellitzer et al., 1999). Various studies on Eph/ephrin function during embryonic development suggest that Eph receptors and ephrins act through modulation of cell adhesion and motility (Bruckner and Klein, 1998; Xu et al., 2000). Both receptor- and ligand-mediated signaling can modulate adhesion, spreading or migration of various cell types, and accumulating evidence suggests that this effect is mediated through modulations of integrin function (Davy et al., 1999; Davy and Robbins, 2000; Huynh-Do et al., 1999; Miao et al., 2000). Upon activation, signaling molecules implicated in cytoskeletal organization are recruited to both Eph and ephrin, supporting the concept of direct communication with

the intracellular machinery driving cytoskeletal plasticity (Mellitzer et al., 2000). The small GTPases of the Rho family are key intermediates in cellular signaling that originates from clustered cell adhesion receptors and elicit distinct effects on the actin cytoskeleton (Hall, 1998). They have been implicated in some biological effects of ephrins in neuronal cells (Shamah et al., 2001; Wahl et al., 2000), and a recent report suggests that EphA3 activation by ephrinA5 in melanoma cells induces de-adhesion through the activation of RhoA (Lawrenson et al., 2002).

While integrin activation is required in various biological processes, such as cell-cycle progression, growth and cell survival, a dynamic in the adhesion process is associated with either physiological or pathological situations. Decreased adhesion is important in the destabilization of blood vessel structures during angiogenesis (Yancopoulos et al., 2000). EphrinA1 and its cognate receptor, EphA2, are strongly expressed at sites of neovascularization, and vascular endothelial growth factor (VEGF), one of the most-potent angiogenic factors, induces the expression of ephrinA1 in

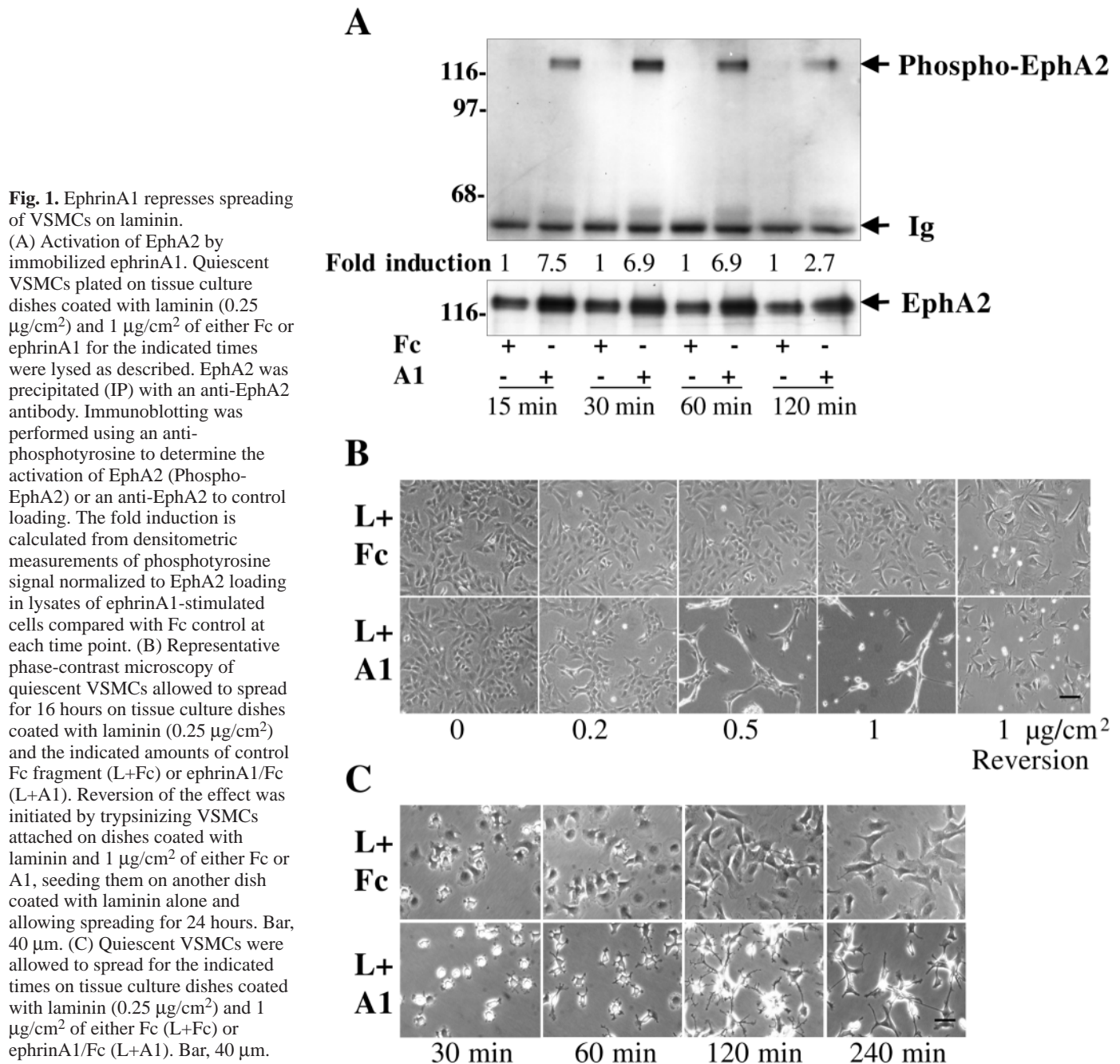


Fig. 1. EphrinA1 represses spreading of VSMCs on laminin.

(A) Activation of EphA2 by immobilized ephrinA1. Quiescent VSMCs plated on tissue culture dishes coated with laminin ($0.25 \mu\text{g}/\text{cm}^2$) and $1 \mu\text{g}/\text{cm}^2$ of either Fc or ephrinA1 for the indicated times were lysed as described. EphA2 was precipitated (IP) with an anti-EphA2 antibody. Immunoblotting was performed using an anti-phosphotyrosine to determine the activation of EphA2 (Phospho-EphA2) or an anti-EphA2 to control loading. The fold induction is calculated from densitometric measurements of phosphotyrosine signal normalized to EphA2 loading in lysates of ephrinA1-stimulated cells compared with Fc control at each time point. (B) Representative phase-contrast microscopy of quiescent VSMCs allowed to spread for 16 hours on tissue culture dishes coated with laminin ($0.25 \mu\text{g}/\text{cm}^2$) and the indicated amounts of control Fc fragment (L+Fc) or ephrinA1/Fc (L+A1). Reversion of the effect was initiated by trypsinizing VSMCs attached on dishes coated with laminin and $1 \mu\text{g}/\text{cm}^2$ of either Fc or A1, seeding them on another dish coated with laminin alone and allowing spreading for 24 hours. Bar, $40 \mu\text{m}$. (C) Quiescent VSMCs were allowed to spread for the indicated times on tissue culture dishes coated with laminin ($0.25 \mu\text{g}/\text{cm}^2$) and $1 \mu\text{g}/\text{cm}^2$ of either Fc (L+Fc) or ephrinA1/Fc (L+A1). Bar, $40 \mu\text{m}$.

endothelial cells (Cheng et al., 2002; Ogawa et al., 2000). Therefore, it is possible that the pro-angiogenic effects of Eph/ephrin are mediated not only through local modulation of cell adhesion and motility of endothelial cells but also of perivascular cells. To test this hypothesis, we analyzed the impact of EphA activation by ephrinA1 on VSMC adhesion and morphology.

Investigation of the signaling pathways involved in ephrinA1-induced morphological alterations, in particular spreading inhibition, underlined the key role played by the Rac/PAK pathway. While specific ablation of RhoA using the double-stranded siRNA approach had a limited effect, decreased levels of Rac1 using siRNA or decreased activation of Rac1 using S1P led to a decrease in cell spreading and

amplification of the action of ephrinA1. We therefore conclude that the Rac activity in VSMCs is essential in mediating the action of ephrinA1, a control point that could be reinforced in vivo by the lipid mediator S1P (Ryu et al., 2002).

Materials and Methods

Reagents and cells

EphrinA1/Fc was produced as described (Miao et al., 2000). EphrinB1/Fc and EphB2/Fc were purchased from R&D Systems. Antibodies and reagents were purchased from the following manufacturers: mouse anti-phosphotyrosine PY99, mouse anti-RhoA (26C4) and rabbit anti-EphA2 antibodies were from Santa Cruz Biotechnology; mouse anti-Rac1 was from Upstate Biotechnology; mouse anti-Cdc42 was from Transduction Laboratories; rabbit anti-

GFP was from Clontech and protein-A-Sepharose was from Amersham Pharmacia Biotech. Rabbit anti-p42MAPK kinase (E1B4) was developed in our laboratory (Le Gall et al., 1998) and rabbit anti-phospho-PAK1 antibody was a kind gift of M. E. Greenberg (Children's Hospital, Harvard Medical School, Boston, MA). Cell dissociation solution (CDS), phalloidin-TRITC, fibronectin, laminin and S1P were from Sigma. The Fc fragment of human IgG was from Jackson ImmunoResearch. VSMCs were isolated from rat thoracic aortas by enzymatic digestion (Richard et al., 2000). VSMCs were cultured in DMEM supplemented with 8% FCS, non-essential amino acids, penicillin (50 U/ml) and streptomycin (50 µg/ml). 1G11 endothelial cells were cultured as previously described (Vinals and Pouyssegur, 1999). Cells were starved by total deprivation of serum for 16-20 hours.

Cell adhesion assay

To analyze cell adhesion and spreading on immobilized proteins, tissue culture dishes were coated overnight with laminin (0.25 µg/cm²) and Fc (1 µg/cm²), or with laminin (0.25 µg/cm²) and ephrin A1/Fc (1 µg/cm²) in PBS. In some experiments, laminin was replaced by fibronectin (0.125 µg/cm²). Nonspecific sites were blocked with 1% BSA for 30 minutes. Starved VSMCs were detached non-enzymatically with CDS, washed twice in DMEM+0.1% BSA and seeded on coated dishes in the same medium. As already published by others, we observed that coating with only ephrin A1/Fc only permits cell adhesion but does not allow cell spreading.

Immunoprecipitation and immunoblotting

Subconfluent VSMCs were chilled on ice, rinsed twice with ice-cold PBS and lysed in buffer containing 1% Triton X-100, 50mM Tris pH 7.4, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM Na₃VO₄, 0.1 mM AEBSF, 4 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin. Immunoprecipitations were carried out using 1 µg of rabbit anti-EphA2. After 1 hour of incubation at 4°C, 40 µl of protein-A-Sepharose beads were added for 1 hour to capture immune complexes. Beads were washed four times with lysis buffer and boiled in 60 µl SDS-PAGE lysis buffer. For immunoblotting, samples were separated on a 6% gel and transferred to Immobilon-P PVDF membrane. EphA2 and phosphotyrosines were immunodetected with the corresponding antibodies. The bands were visualized with the electrochemiluminescence (ECL) system.

siRNA transfection

21-nucleotide RNAs were chemically synthesized and purified by reverse-phase HPLC (Eurogentec). To inhibit Rac1 synthesis, we used the 5'-GUUCUUAAUUUGCUUUUCCTT-3' and 5'-GGAAAAGCAAUUAAGAAC-3' oligoribonucleotides and, to inhibit RhoA, we used the 5'-GAAGUCAAGCAUUUCUGUCTT-3' and 5'-GACAGAAAUGCUUGACUUCTT-3' oligoribonucleotides. Control siRNA primers corresponded to 21-nucleotide RNAs from *Drosophila* hypoxia inducible factor 1α. Each pair of oligoribonucleotides was annealed at a concentration of 20 µM in 100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-KOH pH 7.4. Calcium phosphate-mediated transfection was performed in 6-well plates with a final concentration of 20 nM of siRNA. VSMCs were used in the adhesion assay 48 hours post-transfection.

GTPase assay

After seeding on coated dishes, VSMCs suspended in DMEM+0.1% BSA were harvested every 30 minutes, until 2

hours. Cells were chilled on ice and lysed in ice-cold buffer containing 1% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 0.1 mM AEBSF, 4 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin. Lysates were centrifuged for 8 minutes at 13,000 g. Supernatants were immediately frozen in liquid nitrogen and stored at -80° until further use. An aliquot of each supernatant was denatured in SDS-PAGE lysis buffer before freezing to measure the total rhoGTPase content. For pull-down assays, supernatants were incubated with 30 µg of GST-PBD protein (containing the Cdc42- and

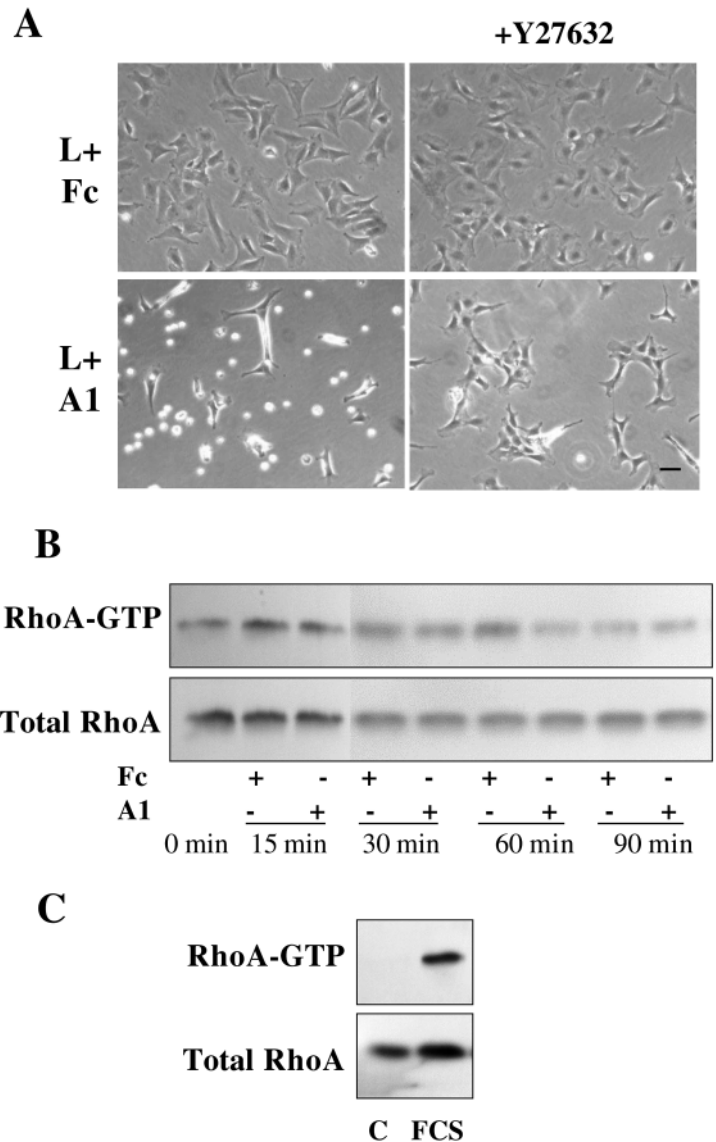


Fig. 2. ROCK inhibitor Y27632 antagonizes the ephrinA1-mediated repression of VSMC spreading whereas ephrinA1 does not modulate RhoA activity. (A) Representative phase-contrast microscopy of quiescent VSMCs allowed to spread on tissue culture dishes coated with laminin (0.25 µg/cm²) and 1 µg/cm² of either Fc (L+Fc) or ephrinA1/Fc (L+A1), in the presence of 7 µM Y27632 or vehicle alone. Bar, 20 µm. (B) Quiescent VSMCs were seeded on tissue culture dishes coated with laminin and either Fc or ephrinA1/Fc (A1). Cells were harvested at the indicated times and processed for pull-down experiments. RhoA activity is determined by the amount of GST-RBD-bound RhoA (RhoA-GTP) normalized to total RhoA in whole cell lysates. (C) Quiescent VSMCs were stimulated (FCS) or not (C) with 10% FCS. After 5 minutes, cells were harvested and processed for pull-down experiments. RhoA activity is determined as described above (B).

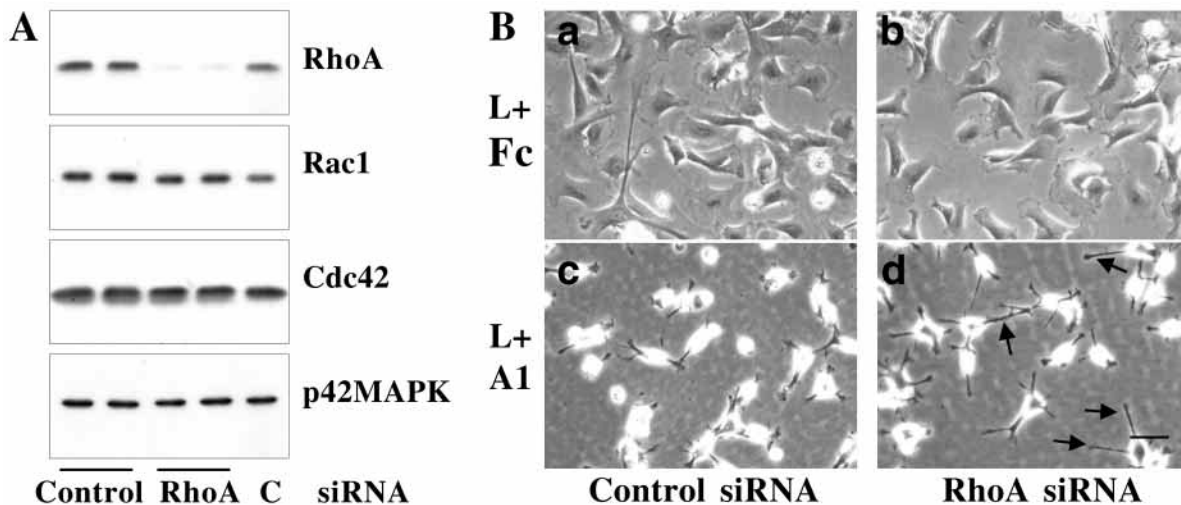


Fig. 3. Effect of RhoA silencing on cell spreading. (A) An siRNA targeting RhoA strongly reduced the RhoA protein level without a significant effect on Rac1 and Cdc42 protein levels. Western blot analysis of whole cell lysates of VSMCs transfected with 20 nM of a control siRNA (Control) or an siRNA targeting RhoA or left untransfected (C). 48 hours post-transfection, cells were lysed in SDS-PAGE loading buffer and analyzed by immunoblotting with specific antibodies to RhoA, Rac1, Cdc42 and p42 MAP kinase. (B) Inhibition of RhoA with an siRNA stabilizes process formation. Representative phase-contrast microscopy of quiescent VSMCs allowed to spread for 4 hours (a-d) on tissue culture dishes coated with laminin and either Fc (a,b) or A1 (c,d). VSMCs transfected with control siRNA (a,c) or an siRNA targeting RhoA (b,d) were used 48 hours post-transfection. Bar, 20 μ m.

Rac-binding region of PAK-1B) or GST-RBD protein (containing the Rho-binding region of rhotekin) affinity linked to glutathione Sepharose beads for 30 minutes (Ren et al., 1999; Sander et al., 1999). The beads were washed four times in lysis buffer and boiled in 60 μ l SDS-PAGE lysis buffer.

Retroviral infection

Retroviral supernatants were generated by transient transfection of Phoenix cells with pBabeGFPRacN17 or pBabeGFPRacV12 and were used to infect VSMCs. Two days post-infection, VSMCs were starved and tested on tissue-culture-coated dishes.

Immunofluorescence labeling

VSMCs cultured for 4 hours on tissue-culture-coated dishes were fixed with 3% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.2% Triton X-100 in PBS for 2 minutes. The dishes were blocked with 1% BSA in PBS for 30 minutes and incubated with phalloidin-TRITC (60 ng/ml) for 30 minutes. Fluorescence was analyzed with a Leica DM-R microscope equipped with a DC-200 digital camera.

Results

Inhibition of integrin-mediated cell spreading by ephrinA1/Fc

Coated ephrinA1/Fc induced a sustained phosphorylation of the EphA2 receptor in VSMCs, suggesting that it was able to activate, in this model, an intracellular signaling cascade (Fig. 1A). We next investigated the molecular mechanisms controlling ephrinA1 activity by analyzing its impact on cell attachment and spreading on tissue culture dishes coated with purified components of extracellular matrix (ECM). VSMCs were cultivated in dishes coated with purified ECM components (laminin or fibronectin) and either the Fc fragment

of human IgG or recombinant ephrinA1/Fc. As observed by phase-contrast microscopy, spreading of VSMCs on laminin progressively decreased as the ephrinA1/Fc coating concentration increased whereas the control Fc fragment, used at the same concentrations, did not modulate the VSMC morphology (Fig. 1B). In the presence of the highest concentration of ephrinA1/Fc (1 μ g/ml), the cells were not in the process of dying as they were able, after detachment, to re-spread as efficiently as control cells on a laminin coat (Fig. 1B, right panels). The observed smaller size of the VSMCs first attached to ephrinA1/Fc for 16 hours could be due to the widely documented repression of protein synthesis, and namely of cytoskeletal proteins, in non- or scarcely spread cells (Deroanne et al., 1996; Lambert et al., 1992; Mooney et al., 1994). A similar effect of ephrinA1/Fc was also observed with endothelial cells (1G11) (not shown). The same inhibition of laminin-mediated cell spreading was observed with ephrinB1/Fc used at the same concentrations, while EphB2/Fc had no effect (not shown). EphrinA1/Fc did not significantly alter early adhesion to laminin as nearly all the cells adhere to the coated dishes within 30 minutes in both tested conditions, but strongly impair VSMC spreading (Fig. 1C). This effect was already visible shortly after seeding the VSMCs. On control dishes, 30 minutes after seeding, all the cells attached and started to spread. Cell spreading gradually increased to reach a maximum 2 hours thereafter. Cells attached as efficiently to ephrinA1/Fc coated dishes as to control dishes, but were unable to form lamellipodia and instead projected structures that are likely to be buds of the lamellipodia. The length of these structures reached a maximum 2 hours after seeding, to progressively retract and disappear within 4 hours (Fig. 1C). Replacement of laminin by fibronectin in this cellular model gave the same results (not shown), suggesting that it was not restricted to a specific ECM component and/or integrin.

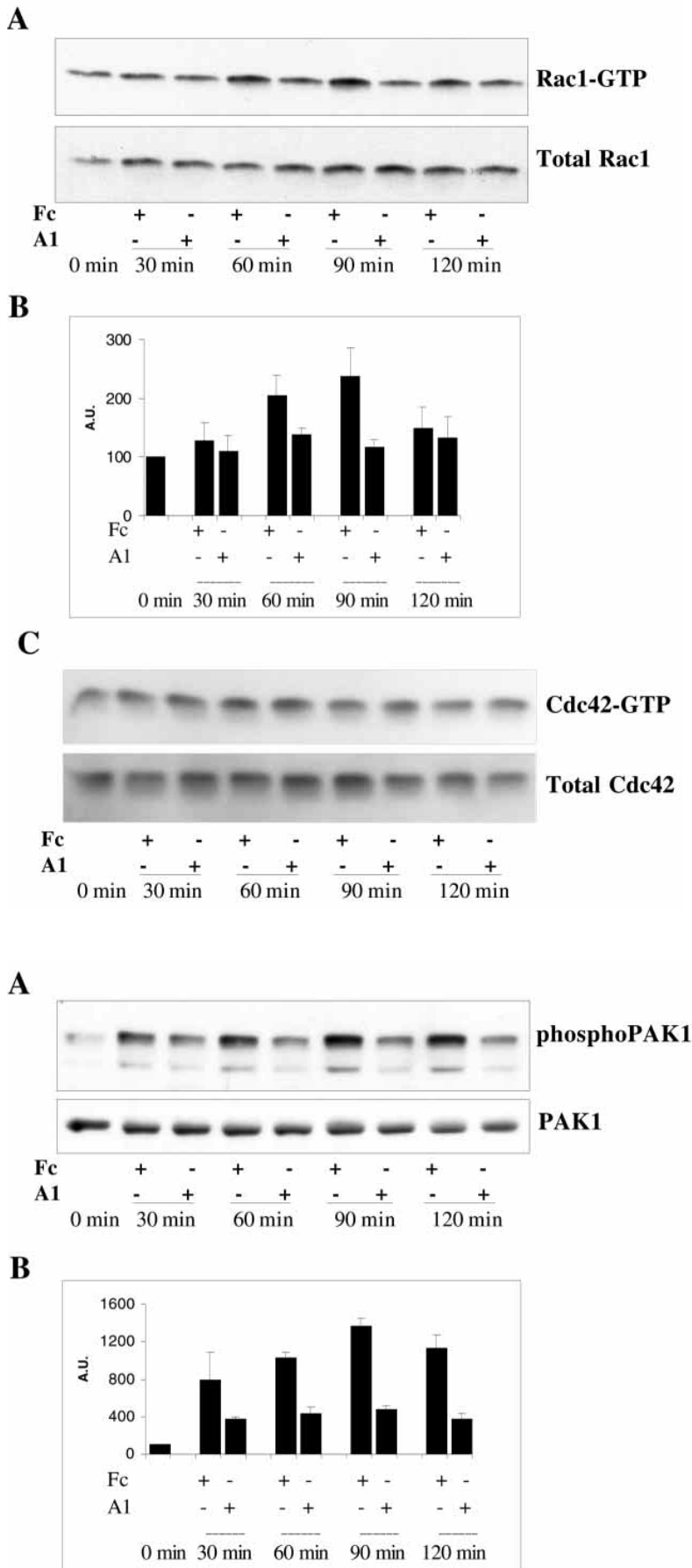


Fig. 4. EphrinA1 inhibits the activation of Rac1 but does not affect the activation of Cdc42. (A) Quiescent VSMCs were seeded on tissue culture dishes coated with laminin and either Fc or ephrinA1/Fc (A1). Cells were harvested at the indicated times and processed for pull-down experiments. Rac1 activity is determined by the amount of GST-PBD-bound Rac1 (Rac1-GTP) normalized to total Rac1 in whole cell lysates. (B) Densitometric analysis of (A). Results are the mean \pm s.d. of three independent experiments. (C) Quiescent VSMCs were seeded on tissue culture dishes coated with laminin and either Fc or ephrinA1/Fc (A1). Cells were harvested at the indicated times and processed for pull-down experiments. Cdc42 activity is determined by the amount of GST-PBD-bound Cdc42 (Cdc42-GTP) normalized to total Cdc42 in whole cell lysates.

Implication of the small GTPase RhoA

It was reported that some biological effects of ephrinA5, a member of the ephrin family, are mediated through the Rho-Rho kinase pathway (Wahl et al., 2000). We therefore wanted to determine if such pathways are implicated in ephrinA1-induced integrin inactivation. First, VSMCs were cultured in the absence or presence of the Rho kinase (ROCK) inhibitor Y-27632. As seen in Fig. 2A, the cells attached and spread as efficiently with or without Y-27632 under control conditions. By contrast, the ROCK inhibitor partially reversed the ephrinA1/Fc effect on VSMC spreading, suggesting the involvement of the Rho-ROCK pathway. Similar results were obtained when laminin was replaced by fibronectin (not shown). However, we did not observe any activation of RhoA by ephrinA1/Fc (Fig. 2B), whereas our pull-down assay revealed a strong increase in RhoA activation with 10% FCS (Fig. 2C). In an attempt to define further the implication of Rho in our model, we tried to transfect by various means an expression vector for RhoN19, a dominant-inactive form of Rho, carrying a GFP-tag, but its expression was nearly undetectable in our VSMCs (not shown). Hence, we used siRNA (Elbashir et al., 2001) to ablate specifically RhoA in VSMCs. As seen in Fig. 3, a 90% reduction in the RhoA protein level was observed 48 hours post-transfection, without significant modulation of the Rac1, Cdc42 and p42MAPK levels. These cells were seeded on dishes coated with laminin and either control Fc or ephrinA1/Fc. Interestingly, repression of RhoA synthesis markedly increases the length of processes (Fig. 3B). However, a lack of RhoA expression does not reverse the effect of

Fig. 5. EphrinA1 inhibits the activation of PAK1, an effector of Rac1 signaling. (A) Quiescent VSMCs were seeded on tissue culture dishes coated with laminin and either Fc or ephrinA1/Fc (A1). Cells were lysed in SDS-PAGE loading buffer at the indicated times. PAK1 activity is indicated by the amount of phosphorylated PAK1 (phosphoPAK1) normalized to the total PAK1 in each cell lysate. (B) Densitometric analysis of (A). Results are the mean \pm s.d. of three independent experiments.

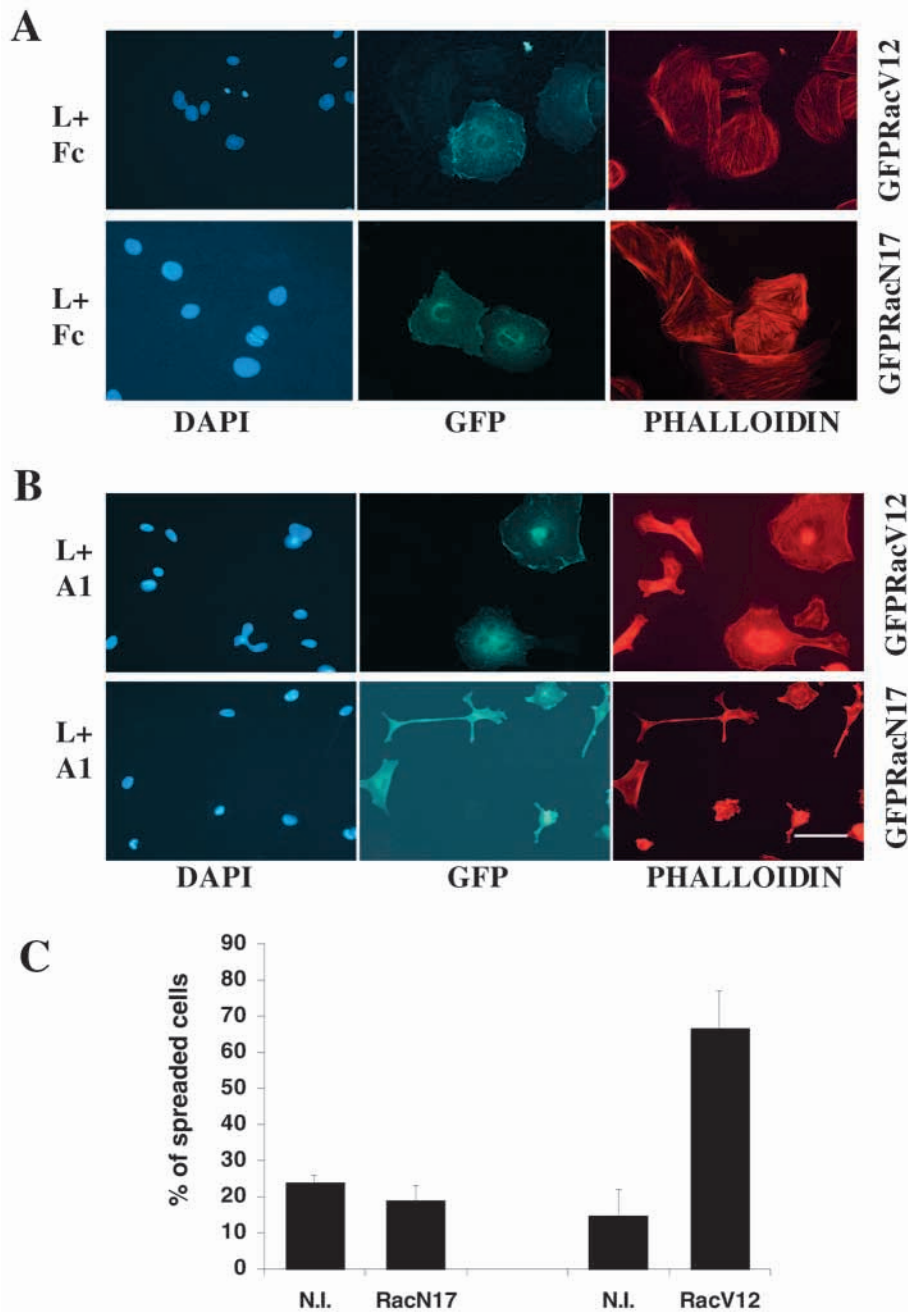


Fig. 6. Constitutively active Rac antagonizes the ephrinA1-mediated repression of VSMC spreading. (A–B) VSMCs expressing either GFP-RacN17 or GFP-RacV12 cultured on tissue culture dishes coated with laminin and either Fc (A) or ephrinA1/Fc (B) were fixed 4 hours after seeding in the adhesion assay and analyzed by immunofluorescence labeling with DAPI and phalloidin-TRITC. Bar, 20 μ m. (C) The morphology of GFP-RacN17 (RacN17)- and GFP-RacV12 (RacV12)-expressing cells cultured on tissue culture dishes coated with laminin and ephrinA1/Fc from ten randomly chosen fields were scored (spread \pm s.d. of three independent experiments). Non-infected cells (N.I.) in the same fields were also scored and used as a control for the ephrinA1 activity.

prevented this Rac1 activation mediated by either spreading on laminin (Fig. 4A) or fibronectin (not shown). As shown in Fig. 4C, we were unable to detect any significant modulation of Cdc42 activity by ephrin A1/Fc even though we were able to detect strong activation of Cdc42 in VSMCs stimulated with bradykinin (not shown). However, it is interesting to note that a basal activation of Cdc42 is detected in all tested conditions.

To evaluate further the changes in the Rac1 signaling pathway, we measured the activation level of PAK1, the immediate effector of Rac1, with an anti-phospho-PAK1 antibody. As shown in Fig. 5, ephrinA1/Fc strongly impaired the phosphorylation of PAK1, suggesting that EphA activation represses VSMC spreading through inhibition of the Rac/PAK pathway. To evaluate the importance of the inactivation of this pathway, VSMCs were infected with retroviral vectors encoding the GFP-tagged constitutive active and inactive forms of Rac, respectively RacV12 and RacN17.

ephrinA1 on cell spreading. These results suggest that RhoA has a limited involvement in this cellular model.

Implication of the Rac and Cdc42 pathways

The morphological effects of ephrinA1/Fc, mainly characterized by a lack of lamellipodia formation and a transient extension of processes (Fig. 1C), suggested an impairment of Rac1 and/or an increase in Cdc42 signaling (Hall, 1998). Hence, we measured the activation level of these small RhoGTPases with a pull-down assay. VSMCs were harvested every 30 minutes until 120 minutes after seeding. In control dishes, the level of activated Rac1 increased progressively to reach a maximum 90 minutes after seeding before returning to basal level. By contrast, ephrinA1

The constitutive activity of RacV12 was assessed in the pull-down assay used previously (not shown). 48 hours post-infection, the cells were used in the adhesion assay. More than 95% of infected or non-infected cells spread on a control coat. As seen in Fig. 6A, VSMCs expressing RacV12 or RacN17 spread as efficiently as non-infected cells on tissue culture dishes coated with laminin and control Fc. On tissue culture dishes coated with laminin and ephrinA1/Fc, 65% of the RacV12-expressing cells spread, whereas most of the RacN17-expressing cells and non-infected cells were unable to spread (Fig. 6B,C).

S1P or a decreased level of Rac (via siRNA) amplify the action of ephrinA1/Fc

To analyze further the involvement of Rac1 in the ephrinA1

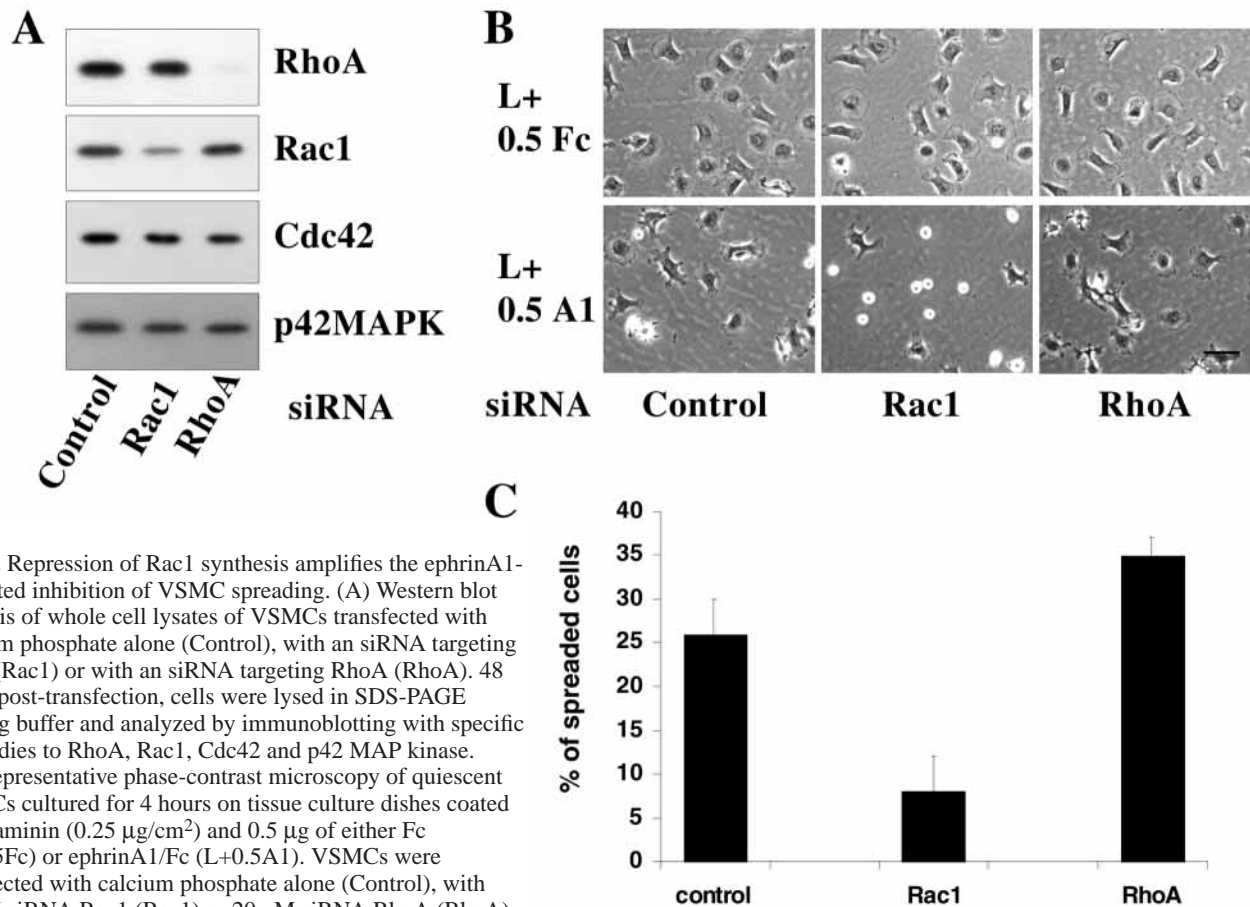


Fig. 7. Repression of Rac1 synthesis amplifies the ephrinA1-mediated inhibition of VSMC spreading. (A) Western blot analysis of whole cell lysates of VSMCs transfected with calcium phosphate alone (Control), with an siRNA targeting Rac1 (Rac1) or with an siRNA targeting RhoA (RhoA). 48 hours post-transfection, cells were lysed in SDS-PAGE loading buffer and analyzed by immunoblotting with specific antibodies to RhoA, Rac1, Cdc42 and p42 MAPK. (B) Representative phase-contrast microscopy of quiescent VSMCs cultured for 4 hours on tissue culture dishes coated with laminin (0.25 $\mu\text{g}/\text{cm}^2$) and 0.5 μg of either Fc (L+0.5Fc) or ephrinA1/Fc (L+0.5A1). VSMCs were transfected with calcium phosphate alone (Control), with 20 nM siRNA Rac1 (Rac1) or 20 nM siRNA RhoA (RhoA). Bar, 20 μm . (C) The morphology of control (Control), siRNA Rac1 (Rac1) or siRNA RhoA (RhoA)-treated cells from eight randomly chosen fields were scored (spread or retracted). Data represent the mean \pm s.d. of three independent experiments.

signaling pathway, we took advantage of interfering RNA technology. An siRNA targeting Rac1 specifically was designed and transfected into VSMCs. 48 hours post-transfection, a 60-70% decrease in the Rac1 protein level was observed, without significant modulation of the RhoA and Cdc42 levels, in VSMCs (Fig. 7A). The siRNA of RhoA was used as a positive control (Fig. 7A, lane 3). These cells were tested in the adhesion assay in the presence of a suboptimal concentration of ephrinA1/Fc. As illustrated in Fig. 7B and 7C, the repression of Rac1 synthesis significantly enhanced ephrinA1-mediated inhibition of spreading, further supporting the involvement of Rac1 in this phenomenon.

S1P is a lipid mediator exerting various effects on cytoskeletal organization depending on the cell type. In VSMCs, S1P is known to inhibit Rac and to increase Rho activity (Ryu et al., 2002). 0.5 μM S1P did not modulate the ability of VSMCs to spread on a control tissue culture dish coated with laminin and Fc (Fig. 8a,e). However, S1P significantly reduced the spreading of VSMCs with a suboptimal concentration of ephrinA1/Fc whereas, in the presence of the highest concentration of ephrinA1/Fc, all the cells were round (Fig. 8, compare b-d with f-h). These observations suggest that S1P could amplify the effect of ephrinA1/Fc on VSMCs through modulation of Rac, and perhaps Rho, activity.

Discussion

Interaction between Eph receptor tyrosine kinase and ephrin ligands transduce short-range signals regulating axon pathfinding, development of the cardiovascular system, and migration and spreading of neuronal and non-neuronal cells (Boyd and Lackmann, 2001; Cheng et al., 2002; Knoll and Drescher, 2002). Several Eph receptors and ephrins are overexpressed in tumors especially during the more invasive stages of tumor progression (Dodelet and Pasquale, 2000). They are proposed to contribute to tumor progression by modulating the adhesive properties of the cells (Lawrenson et al., 2002). EphrinA1 and EphA2 are expressed in the tumor vasculature and could contribute to the permanent remodeling of these vessels (Ogawa et al., 2000). EphrinA1 was reported to contribute to angiogenesis *in vitro* as well as in *in vivo* models, and its expression is induced by several cytokines, especially angiogenic factors such as VEGF, in endothelial cells (Cheng et al., 2002; Daniel et al., 1996; Myers et al., 2000; Pandey et al., 1995; Stein et al., 1998). We hypothesize that one function of ephrinA1 expressed in endothelial cells is to initiate a repulsive movement that will contribute to the destabilization of blood vessels, a prerequisite for angiogenesis (Yancopoulos et al., 2000). This concept encouraged us to investigate the effect of ephrinA1 on VSMC adhesive properties. It was reported that EphA activation inhibits

integrin function in PC-3 epithelial cells through SHP2-dependent FAK inactivation (Miao et al., 2000). However, this phosphatase was also reported to control the activation level of RhoA (Schoenwaelder et al., 2000). Some biological effects of the ephrins are dependent on RhoGTPases (Wahl et al., 2000). Recently, it was reported that EphA is constitutively associated with a novel RhoGTPase exchange factor, named ephexin (Shamah et al., 2001). Finally, a recent report suggested that de-adhesion of EphA3-expressing melanoma cells by ephrinA5 is mediated through a Rho and crkII-dependent pathway (Lawrenson et al., 2002).

In the present study, we have shown that the morphological effects of EphA activation in VSMCs are associated with inhibition of the Rac/PAK pathway and can be rescued by constitutive activation of Rac. Moreover, repression of Rac1 synthesis by means of an siRNA strategy amplifies the ephrinA1-mediated spreading inhibition, further supporting the key role played by Rac1 in this process. These observations are in agreement with the lack of lamellipodia formation in the presence of ephrinA1/Fc as the establishment of this cell structure is dependent on Rac activity (Hall, 1998). In this way, ephrins can compete with integrin-mediated signaling, but could also interfere with the optimal localization of activated integrins, which is mediated in some cases by activated Rac (Kiosses et al., 2001). EphrinA1 can also mediate its effect partly through decreased phosphorylation of the myosin light chain (C.D. and J.P., unpublished), a protein activated by PAK1 (Sells et al., 1999). This molecule is localized to lamellipodia and proposed to contribute to membrane extensions at the leading edge (Sells et al., 1999; Sells et al., 2000). Cdc42 is also able to activate PAK1. However, this GTPase is probably not involved in the morphological effect of ephrinA1 as we did not observe any modulation of its activity by ephrinA1/Fc, whereas our pull-down assay allowed the detection of a strong increase of Cdc42 activity by bradykinin treatment. As recently reported by Carter et al. (Carter et al., 2002), spreading of NIH3T3 on ephrinA1/Fc was also observed in our culture model while VSMCs only bind to immobilized ephrinA1/Fc and do not spread. Differences between NIH3T3 and other cell types have been previously reported. In a recent review, Coleman and Marshall discussed the discrepancies between NIH3T3 and endothelial cells in Rac signaling for cyclin D1 expression (Coleman and Marshall, 2001). Among several proposals, they suggested: "NIH3T3 can have undergone genetic changes that liberate them from Rac signaling". Differences in sensitivity to Rac signaling between the cells used in Carter's study and our VSMCs could explain our antagonistic observations. In agreement with recent reports proposing that some biological effects of ephrins are due to a Rho-mediated increase of acto-myosin contraction, we observed an enhancement of VSMC spreading on ephrinA1/Fc by the ROCK inhibitor Y-27632 (Lawrenson et al., 2002; Wahl et al., 2000). However, repression of RhoA synthesis using an siRNA strategy antagonizes this phenomenon only partly, suggesting that RhoA has a limited role in this cellular model. Nonetheless, the stabilization of

process formation in the absence of RhoA expression observed in VSMCs and in HeLa cells (not shown) cultured on a laminin+ephrinA1/Fc coat suggested that other biological effects of ephrinA1, like outgrowth of neurites or regression of melanoma cell processes, could be antagonized by an inhibition of RhoA synthesis. Alternatively, Y-27632 could also mediate its effect in our model through Rac, as it was proposed that ROCK inhibition can enhance Rac activity (Hall et al., 2001) and a recent study reported the activation of Rac by Y27632 in VSMCs (Katsumi et al., 2002). Moreover, in VSMCs cultured on laminin+ephrinA1/Fc, we observed that Y-27632 induces a modest but reproducible increase of PAK1 phosphorylation level (C.D. and J.P., unpublished). By contrast, it was also reported that Y-27632 did not exclusively inhibit ROCK. At micromolar concentration, as it is commonly used, Y-27632 can inhibit other kinases (Davies et al., 2000). These side effects can also account for the partial reversion of the ephrinA1-induced phenotype independently of its action on the Rho pathway. Finally, the cooperativity between S1P and ephrinA1/Fc reported here could represent a supplementary control level of the ephrinA1 action *in vivo*, not only by

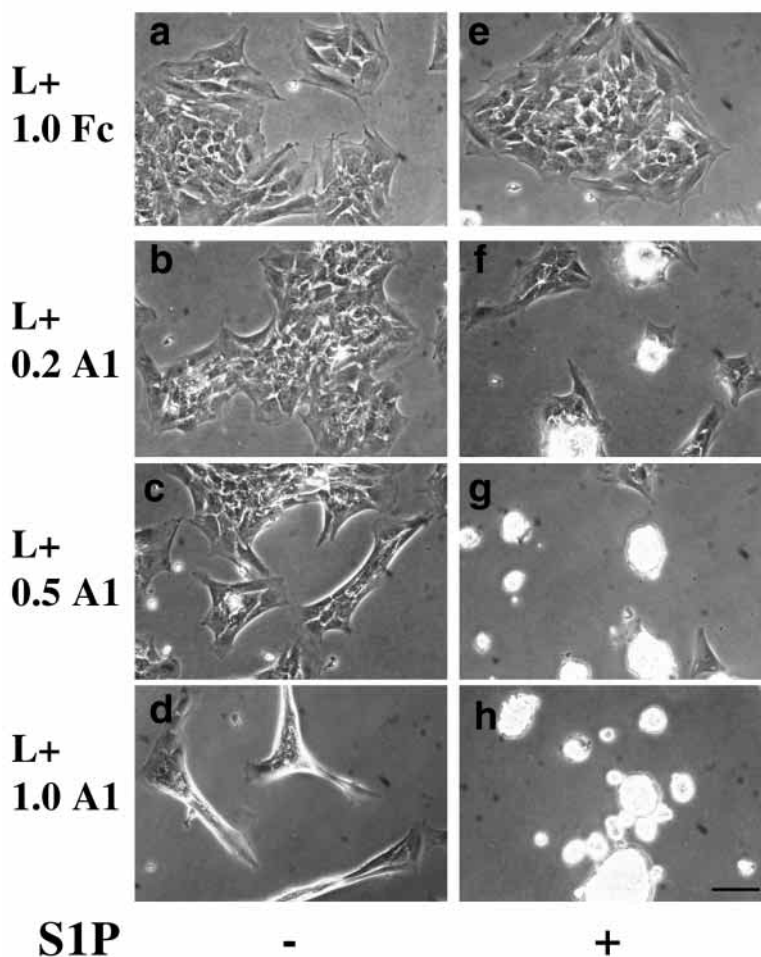


Fig. 8. S1P amplifies the ephrinA1-mediated repression of VSMC spreading. Representative phase-contrast microscopy of quiescent VSMCs cultured for 16 hours in the adhesion assay with vehicle (a-d) or with 0.5 μM S1P (e-h). VSMCs were seeded on tissue culture dishes coated with laminin (0.25 $\mu\text{g}/\text{cm}^2$) and either 1 $\mu\text{g}/\text{cm}^2$ of Fc (a,e) or 0.2 (b,f), 0.5 (c,g), 1.0 (d,h) $\mu\text{g}/\text{cm}^2$ of ephrinA1/Fc. Bar, 20 μm .

amplification of the effect but also by differential regulation on endothelial and smooth muscle cells through opposite regulation of RhoGTPases (Ryu et al., 2002).

In conclusion, our study underlines the implication of the Rac/PAK pathway in ephrinA1-mediated integrin inactivation in VSMCs whereas RhoA has a limited effect. These mechanisms are potentially regulated by SIP, which could participate in the early steps of angiogenesis. EphrinA1 can operate through various proteins regulating the activity of Rac1. Among them, GAPs like chimaerin and the p190RhoGAP, or the recently identified GEF ephexin, are potentially interesting candidates. Investigations are now in progress to analyze the implications of some of these proteins in our model.

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