Rho GTPase, Rac1, regulates Skp2 levels, vascular smooth muscle cell proliferation, and intima formation in vitro and in vivo

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Aims Vascular smooth muscle cell (VSMC) proliferation contributes to intima formation after angioplasty or venous by-pass grafting, and during atherosclerosis. VSMC proliferation requires degradation of p27Kip1 promoted by S-phase kinase-associated protein-2 (Skp2), an F-box protein component of the Skp-Cullin-F-boxSkp2 ubiquitin-ligase. We investigated the role of Rac1 in the regulation of Skp2 in rat VSMC.

Methods and results Rat carotid balloon injury increased Rac1 activity. Rho GTPase inhibition with Clostridium difficile Toxin B or specific Rac1 inhibition with adenovirus-mediated expression of dominant-negative Rac1 reduced Skp2 levels, and VSMC proliferation in vitro and intima formation in vivo following carotid balloon injury. Inhibition of Skp2 expression and proliferation by dominant-negative Rac1 was reversed by exogenous Skp2. Elevation of endogenous adenosine 3’5’-cyclic monophosphate (cAMP) with forskolin-inhibited Rac1 activity, reduced Skp2, increased p27Kip1 and inhibited VSMC proliferation, effects that were reversed by constitutively active Rac1. These effects were independent of Rac1 Cdc42/Rac interactive binding (CRIB)-domain effector proteins but associated with Rac1-dependent actin polymerization.

Conclusion Rac1 activity regulates VSMC proliferation by controlling Skp2 levels. Activation of Rac1 induced by balloon injury in vivo increases Skp2 levels, which promotes VSMC proliferation and intima formation. Inhibition of this novel pathway underlies the negative effects of cAMP on VSMC proliferation.

KEYWORDS
Skp2; Rac; Cell cycle; Neointima; Smooth muscle cell

1. Introduction
Vascular smooth muscle cell (VSMC) proliferation contributes to intima formation after balloon injury with and without stenting, venous graft failure, and atherosclerosis. This motivates efforts to understand the mechanisms regulating VSMC proliferation after vessel injury. In healthy vessels, VSMCs exist in a quiescent non-proliferative state. However, their proliferation rate is dramatically increased following vascular injury, which activates multiple pro-mitogenic signals, including release of growth factors and remodelling of the vascular extracellular matrix. Ultimately, these stimuli act together to regulate VSMC proliferation at the level of the cell cycle.1

Progression through the G1 phase of the cell cycle is regulated by phosphorylation and inactivation of retinoblastoma (Rb) proteins. This is promoted by the Cyclins (Cyclin A, D, and E), which associate with and activate the Cyclin-dependent kinases (cdk2, cdk4, and cdk6). These Cdk:Cyclin complexes are subject to negative regulation by the Cyclin-dependent kinase inhibitors (CDKIs), such as the Cip/Kip family of CDKIs (p21Cip1, p27Kip1, and p57Kip2).2,3 For example, increased levels of p27Kip1 mediated by elevated intracellular levels of adenosine 3’,5’-cyclic monophosphate (cAMP) and other growth inhibitory signals cause cell-cycle arrest.4-6 Moreover, down-regulation of CDKIs during late G1 following addition of growth factors in vitro or balloon injury in vivo is a critical step in allowing activation of cyclin:cdk complexes and subsequent G1/S phase transition.7,8

Down-regulation of p27Kip1 in late G1 phase is mediated by ubiquitin-dependent proteasomal degradation. This is promoted by S-phase kinase-associated protein-2 (Skp2), an F-box protein component of the Skp-Cullin-F-box (SCF)Skp2 ubiquitin-ligase.9 We recently demonstrated that Skp2 expression is absent in healthy uninjured rat carotid arteries but up-regulated in response to balloon injury co-ordinately with the reduction in p27Kip1 levels and increased VSMC

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proliferation. Furthermore, forced expression of Skp2 promotes p27<sup>Kip1</sup> degradation and increases proliferation, whereas siRNA-mediated silencing of Skp2 has the opposite effects. This demonstrates how increased expression of Skp2 can contribute towards increased VSMC proliferation and intima formation. However, the mechanisms that promote Skp2 expression in response to culture and vascular injury are not fully understood.

There is increasing evidence that the Rho GTPases regulate levels of p27<sup>Kip1</sup> and cell proliferation. Moreover, Rho GTPases can be activated by adhesion-dependent signal- and inhibited by intracellular cyclic nucleotides, which mirrors their effects on Skp2 levels in VSMC. We therefore hypothesized that Rho GTPase activation might increase Skp2 protein levels in VSMC and that this might be responsible for down-regulating p27<sup>Kip1</sup> and promoting VSMC proliferation.

2. Methods

Male Sprague-Dawley (SD) rats were obtained from Charles River. Culture media and additives were obtained from Invitrogen (Paisley, Scotland). Monoclonal antibody to Skp2 was obtained from Zymed (San Francisco, USA). Rabbit antibody to hyper-phosphorylated Rb was from Cell Signalling Technology (MA, USA).

Primers for quantitative RT-PCR for Skp2 (forward: 5′ AGG AGG TGG ACA GTG AGA ACA T 3′; reverse: 5′ CCT CTT GCA GAC TCC AGA GAC T 3′) were synthesized by Sigma-Genosys and used at an annealing temperature of 60°C. Quantitect-SYBR green PCR mix was obtained from Qiagen.

2.1 Balloon injury of rat common carotid artery

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Balloon injury of left common carotid artery was performed as described by Clowes et al. Briefly, male SD rats (~400 g) were anaesthetized with desflurane and subjected to a peritoneal injection of a mixture of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) right after brief inhalation of halothane. The section of left common carotid artery was surgically exposed, and a 2F arterial embolectomy balloon catheter (Actamed, Wakefield, West Yorkshire, UK) was introduced through an arteriotomy on left external carotid artery. The balloon was inflated and pulled back with rotation through common carotid artery three times. The proximal external carotid artery was ligated after withdrawal of the catheter. The injured arteries were cultured in 200 µL of 30% (w/v) pluronic gel with or without 20 ng/mL of Clostridium difficile Toxin B before closure of skin. Alternatively, the injured common carotid arteries were subjected to adenosviruses-mediated gene delivery.

2.2 Smooth muscle cell culture and bromo-deoxyuridine labelling

Isolated VSMCs were prepared using a modification of the explant technique described previously. Unless otherwise stated, all cells were cultured in the presence of 10% foetal calf serum. Where indicated, cells were rendered quiescent by serum deprivation for 72 h. VSMC proliferation was quantified by labelling cells with 10 µmol/L BrdUrd for 18 h. Cells were then fixed in ice cold 70% ethanol and analysed for BrdUrd incorporation by immuno-histochemistry using a monoclonal anti-BrdUrd antibody (ICN Biochemicals). BrdUrd-positive cells were visualized with diaminobenzidine staining.

2.3 Western blotting

Total cell lysates were prepared using SDS-lysis buffer (50 mmol/L Tris–HCl, pH 6.8, 10% glycerol, and 1% SDS). Protein content was determined (Micro BCA assay kit, Pierce), and equal amounts of reduced protein (50–100 µg) were separated by PAGE and transferred to PVDF membrane (Bio-Rad). Membranes were blocked with TBS–T (20 mmol/L Tris–HCl, pH 7.6, 137 mmol/L NaCl, 25 mmol/L KCl, and 0.25% Tween 20) containing 6% milk powder before incubation in primary antibody. Specific proteins were detected using HRP-conjugated secondary antibodies (Dako, Ely, UK). Peroxidase activity was detected using enhanced chemoluminescence (Amersham, UK). Where indicated, some westerns detecting exogenously expressed proteins were exposed to correctly detect exogenous proteins and thus endogenous expression may not be visible.

2.4 Recombinant adeno- and infection of vascular smooth muscle cell

Recombinant adenosviruses encoding wild-type Skp-2 (Ad:WT-Skp-2), dominant-negative focal activation kinase (Ad:FAK<sup>DN</sup>), or an adenovirus containing an empty expression cassette (Ad:Control) have been described previously. Dominant-negative RhoA was a kind gift from Prof. A. Ridley (UCL). Rac1 mutants (G12V—constitutively active and T17N—dominant-negative) were generated by PCR and cloned into pDC515 shuttle vector (Microbix Biosystems Inc.). Replication-deficient adenosviruses were generated by recombination of co-transfected shuttle and genomic plasmids in HEK293 cells. Virus stocks were plaque-purified, ampliﬁed, CsCl-banded, and titrated by plaque assay. Asynchronous rat VSMCs were infected with adenosviruses at multiplicity of infection of 200 for 3 h, unless otherwise stated.

For infection of rat common carotid artery, both proximal end of common carotid artery and internal carotid artery were temporarily clamped and an aliquot (100 µL) of adenosvirus (1 × 10<sup>10</sup> pfu/mL/DMEM solution was infused into the balloon-injured common carotid artery through external carotid artery. Rats were given intra-peritoneal injections of heparin (500 IU/kg) to prevent arterial thrombosis (in balloon-injured arteries). The adenosviral solution was retained in the vessel for 30 min and then withdrawn. After ligation of external carotid artery, the clamps were removed and the blood flow was restored.

2.5 Rho GTPase activation and translocation assay

Activation of Rac, and Cdc42 was analysed by GST-Pak-Cdc42/Rac interactive binding (CRIB) pull-down assays. RhoA activation was analysed by GST-Rhotekin Rho-binding domain (RBD) pull down. Briefly, cells were washed in cold PBS and lysed in 50 mmol/L Tris–HCl, pH 7.2, 1% Triton X-100, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl<sub>2</sub>, 1 mM AEBSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin. Two percent of total lysates were removed for analysis of total RAC levels. For analysis of Rac activity in vivo, three rat common carotid arteries were excised, cleaned of fat and connective tissue, and immediately lysed together in 800 µL of lysis buffer. Left common carotids were balloon inflated and compared with uninjured right common carotids. Active Rho GTPases (GTP-bound) were isolated from the remainder of the cell lysates by incubating with either GST-Pak-CRIB or GST-RBD (bound to glutathione–sepharose) at 4°C for 1 h. Following four washes in 50 mmol/L Tris–HCl, pH 7.2, 1% Triton X-100, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl<sub>2</sub>, 1 mM AEBSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin, bound proteins were eluted in SDS sample buffer and analysed by immuno-blotting for Rac<sub>1</sub>, Cdc42, or RhoA.

2.6 Statistical analysis

After calculating means and standard errors of the means, analysis was performed using a two-tailed paired t-test. Significant differences were taken when P < 0.05.
3. Results

3.1 Role of Rho GTPases in regulating Skp2 levels in vitro and in vivo

We previously demonstrated that adhesion-dependent signalling through focal adhesion kinase (FAK) increased Skp2 protein levels in cultured VSMC, whereas intracellular cAMP elevation was inhibitory. Interestingly, interruption of FAK signalling with a dominant-negative FAK-1997F mutant or treatment of VSMC with cAMP analogues or the adenylyl cyclase activator, forskolin, not only decreased Skp2 levels (Supplementary material online, Figure S1A) but also caused a dramatic change in cell morphology, including cell rounding and with forskolin, induction of a stellate-shaped morphology (Supplementary material online, Figure S1B). Although different cell morphology was induced by forskolin and DN-FAK, both resulted in a dramatic loss of F-actin structures revealed by Phalloidin staining (Supplementary material online, Figure S1B). Given that the Rho GTPase Rac1 has recently been implicated in mediating the morphological changes induced by cAMP in VSMC, we sought to determine whether Rho GTPase activity and specifically Rac1 were involved in the regulation of Skp2.

We initially used the Rho GTPase inhibitor D. C. difficile Toxin B to inhibit Rho GTPase activity, including Rac1. Treatment of rat VSMC with 1 ng/mL Toxin B for 4 h resulted in a complete inhibition of Rac1 activity (Figure S1A) without effecting cell viability (Supplementary material online, Figure S2). Treatment with 1 ng/mL also resulted in loss of actin-stress fibres (Figure 1B). Toxin B also potently suppressed expression of Skp2 protein without affecting levels of GAPDH protein (Figure 1C). We next sought to determine whether Rho GTPase activity contributes towards the induction of Skp2 protein expression, VSMC proliferation, and intima formation following balloon injury in vivo. Consistent with our previous observations, Skp2 and BrdU-positive cells were undetectable in uninjured carotids (Figure 1D). Treatment of injured vessels with Toxin B significantly decreased the number of VSMCs expressing Skp2 (from 11.8 ± 2.6% to 4.1 ± 0.8%, P = 0.0445) and those that incorporated BrdUrd (from 11.42 ± 1.67% to 2.97 ± 1.33%, n = 4, P = 0.0056) 2 days after injury (Figure 1D). Furthermore, Toxin B treatment significantly reduced the intima:media ratio 14 days after injury (from 2.02 ± 0.13 to 0.97 ± 0.18, n = 5, P = 0.0013) (Figure 1E). This data demonstrates that Toxin B-mediated Rho GTPase inhibition prevents Skp2 protein expression in vitro and in vivo following vascular injury and inhibits VSMC proliferation in vivo, suggesting a potential role of Rho GTPases.

3.2 Rho GTPase, Rac1, controls Skp2 levels by preventing proteasomal degradation

To confirm a specific role for Rac1 in the regulation of Skp2, we used adenovirus-mediated expression of a dominant-negative mutant of Rac1 (Rac1-T17N). Infection of cultured VSMC with dominant-negative Rac1 significantly inhibited Skp2 levels to 14.8 ± 1.8% (n = 6, P < 0.0001) compared with control virus (Ad:Control) infected cells (Figure 2A) without reducing cell viability (Figure 2B). Neointima formation was analysed 18 hours post infection, for a further 6 hours, as indicated. Skp2 protein was detected in uninjured carotids (Figure 2C). We next sought to determine whether Rho GTPase activity contributes towards the induction of Skp2 protein expression, VSMC proliferation, and intima formation following balloon injury in vivo. Consistent with our previous observations, Skp2 and BrdU-positive cells were undetectable in uninjured carotids (Figure 1D). Treatment of injured vessels with Toxin B significantly decreased the number of VSMCs expressing Skp2 (from 11.8 ± 2.6% to 4.1 ± 0.8%, P = 0.0445) and those that incorporated BrdUrd (from 11.42 ± 1.67% to 2.97 ± 1.33%, n = 4, P = 0.0056) 2 days after injury (Figure 1D). Furthermore, Toxin B treatment significantly reduced the intima:media ratio 14 days after injury (from 2.02 ± 0.13 to 0.97 ± 0.18, n = 5, P = 0.0013) (Figure 1E). This data demonstrates that Toxin B-mediated Rho GTPase inhibition prevents Skp2 protein expression in vitro and in vivo following vascular injury and inhibits VSMC proliferation in vivo, suggesting a potential role of Rho GTPases.

Figure 1 Rho GTPase activity controls Skp2 levels in vitro and in vivo. Vascular smooth muscle cells (VSMCs) were treated with 1 ng/mL of D. C. difficile Toxin B. Rac activity was determined 4 h later (A), and F-actin staining (B) and Skp2 protein expression (C) were analysed 18 h later. Rat left common carotid arteries were treated with 20 ng of D. C. difficile Toxin B in 100 μL of pluronic gel immediately after balloon injury (n = 5). Skp2 expression and BrdUrd incorporation were quantified 2 days after injury (D). Neointima formation was analysed 14 days post-injury (n = 5) (E). *P < 0.05.

Figure 2 Rac1-dependent regulation of Skp2 protein stability. Rat VSMC were infected with 200 pfu/cell of adenovirus expressing DN-Rac1, Skp2, Rac1, and GAPDH protein levels were analysed 18 hours later (A). Skp2 mRNA was quantified using real-time RT-PCR (B). Cells were treated with either 1 ng/mL Clostridium difficile Toxin B (C) or infected with Ad:Control or Ad:DN-Rac1 adenovirus (D) and treated with 10 μM/L MG-132, 16 hours post infection, for a further 6 hours, as indicated. Skp2 protein was detected by western blotting (C and D). Skp2 protein stability was analysed by western blotting in cells treated with 1 ng/mL Toxin B for the times indicated in the presence of 2 μg/mL Cyclohexamide (C). Arrows indicate exogenous protein. Arrow heads indicate endogenous protein.
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viability (Supplementary material online, Figure S2). Importantly, DN-Rac1 had no effect on Skp2 mRNA levels detected by quantitative RT-PCR (Figure S2B), demonstrating post-transcriptional regulation. We previously showed that adhesion-dependent signalling and cAMP influence Skp2 protein stability.6,10 We therefore investigated whether DN-Rac1-mediated loss of Skp2 protein was also due to accelerated proteolysis. Consistent with this, treatment of cells with MG-132 alone for 6 h had little effect (1.3-fold increase) on Skp2 levels (Figure 2C). However, loss of Skp2 protein after expression of DN-Rac1, or Toxin B treatment was completely blocked by co-treatment with the proteasome inhibitor, MG-132 (Figure 2C and D). MG-132 treatment of DN-Rac1-expressing cells actually resulted in a super-induction of Skp2, which may indicate a high rate of Skp2 turnover in these cells. Furthermore, treatment with Toxin B in the presence of cyclohexamide reduced the half-life of Skp2 protein from 6.35 ± 1.14 h to 2.59 ± 0.68 h (n = 5, P = 0.0084) (Figure 2E). These experiments used Toxin B only because immediate inhibition was not possible with DN-Rac1, owing to the lag time in adenovirus-driven expression.

3.3 Rac1-dependent regulation of Skp2 controls vascular smooth muscle cell proliferation

To determine whether loss of Skp2 after Rac1 inhibition impacts on cell-cycle progression in VSMC, we tested the ability of exogenously expressed Skp2 (Ad:Skp2) to rescue markers of G1–S phase progression in VSMC expressing DN-Rac1. Consistent with our previous observations,10 expression of WT-Skp2 alone promoted BrdUrd incorporation, Rb phosphorylation, and a decrease in p27\(^{\text{Kip1}}\) levels (Figure 3A). DN-Rac1 significantly inhibited S-phase entry measured by incorporation of BrdUrd (from 71.9 ± 4.3% to 18.7 ± 4% BrdUrd-positive cells, n = 3, P = 0.0007) (Figure S3A). This was accompanied by a significant increase (to 142 ± 8.1% of controls, n = 3, P = 0.0356) in p27\(^{\text{Kip1}}\) protein and a significant decrease (to 1.1 ± 0.62% of controls, n = 3, P < 0.0001) in hyper-phosphorylated Rb protein, a marker of progression through the G1 restriction point (Figure 3A and B). Co-expression of Skp2 reversed the DN-Rac1-induced increase in p27\(^{\text{Kip1}}\) to levels that were not significantly different from controls or cells expressing exogenous WT-Skp alone. Co-expression of Skp2 also partially rescued BrdUrd incorporation (from 19 ± 4% to 65 ± 12% BrdUrd-positive cells, n = 3, P = 0.0347). Rb hyper-phosphorylation was also partially restored (from 8.15 ± 0.15% to 34.6 ± 0.9%, n = 3, P < 0.05) (Figure 3A and B). This demonstrates that modulation of Skp2 protein levels underlies the Rac1-dependent regulation of p27\(^{\text{Kip1}}\) levels and contributes towards Rac1-dependent G1–S phase progression.

3.4 Rac1 activity controls Skp2 protein, VSMC proliferation, and intima formation in vivo

We next sought to determine whether Rac1 signalling up-regulates Skp2 and induces VSMC proliferation following rat carotid balloon injury. Initially, we tested whether vascular injury resulted in an activation of Rac1 in vivo using Rac1 pull-down assays to quantify the levels of GTP-bound (active) Rac and total Rac1. Basal Rac1 activity in uninjured left and right carotids was not different (Supplementary material online, Figure S3). The ratio of GTP-bound to total Rac1 was low in uninjured arteries but significantly increased

2.25-fold (P = 0.03) 2 days after injury (Figure 4A). The increased ratio of GTP-bound to total Rac1 (1.87 ± 0.3 of uninjured carotids, n = 4, P = 0.07) was just non-significant 7 days after injury. We next tested whether adenovirus-mediated expression of DN-Rac1 inhibited Skp2 expression, affected p27\(^{\text{Kip1}}\) levels, VSMC proliferation, and intima formation. Effects on Skp2, p27\(^{\text{Kip1}}\), and proliferation were measured 4 days after injury to allow sufficient time for viral infection and transgene expression. DN-Rac1 significantly reduced the number of medial VSMC expressing Skp2 (from 32.3 ± 3.2% to 15.6 ± 2.4%, n = 6, P = 0.002), increase the number of cells expressing p27\(^{\text{Kip1}}\) (from 22 ± 2.9% to 38.9 ± 6.5%, P = 0.032), and reduced the number of cells incorporating BrdUrd (from 35.66 ± 6.36% to 14 ± 2.2%, n = 5–6, P = 0.024) 4 days post-injury compared with Ad:β-Gal-infected arteries (Figure 4B). No effect of DN-Rac1 expression on apoptotic cell death was observed (Supplementary material online, Figure S4). Ad:DN-Rac1 infection also significantly reduced the intima:media ratio (from

![Figure 3](https://academic.oup.com/cardiovascres/article-abstract/80/2/290/347555/figure3)

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/80/2/290/347555/figure4)
3.5 Inhibition of Rac1-dependent Skp2 up-regulation underlies the growth inhibitory effects of cyclic-adenosine monophosphate on vascular smooth muscle cell proliferation

We previously demonstrated that elevated levels of cAMP inhibit VSMC proliferation by reducing levels of Skp2.6 Here, we sought to determine whether inhibition of Rac1 up-regulation of Skp2 mediates these effects of cAMP. Elevation of endogenous cAMP levels in cultured VSMC using forskolin, an activator of adenylate-cyclase, significantly inhibited serum-stimulated Rac1 activity (to 41.5 ± 8.2%, n = 3, P < 0.05) (Figure 5A) and significantly decreased the proportion of Rac1 at the plasma membrane (Figure 5B). The stable cyclic-AMP analogue dibutyryl-cyclic-AMP (Db-cAMP) resulted in a similar inhibition of Rac1 activity (Figure 5C). This inhibition of Rac1 activity was at least in part mediated by protein kinase A (PKA) as the effect could be partially reversed by a peptide PKA inhibitor (Figure 5C), although PKA inhibitor alone had no effect. Neither forskolin nor Db-cAMP reduced cell viability (Supplementary material online, Figure S2). Next, we used a constitutively active mutant of Rac1 (Rac1-G12V) to determine whether cAMP-mediated inhibition of Rac1 activity was responsible for the loss of Skp2 and inhibition of VSMC proliferation. Expression of Rac1-G12V alone had no effect on the levels of Skp2, p27Kip1, or Rb hyper-phosphorylation (Figure 5D). Forskolin treatment significantly decreased BrdUrd incorporation (from 83 ± 1.5% to 28.4 ± 4.6%, n = 3, P < 0.05), Skp2 protein levels (to 25.6 ± 13.4% of controls, n = 4, P < 0.05), and hyper-phosphorylated Rb (to 25.1 ± 9.1% of controls, n = 3, P < 0.01) (Figure 5E). This was associated with an increase in p27Kip1 (to 220.5 ± 15.2% of controls, n = 3, P < 0.01). Expression of active-Rac1 in the presence of forskolin rescued BrdUrd incorporation (to 77 ± 3.3%), Skp2 protein levels (to 117.1 ± 25.7%), p27Kip1 protein levels (63.5 ± 28.9%), and hyper-phosphorylated Rb (to 89.8 ± 15.9%) to levels that were not significantly different from controls. Active Rac1 also reverse forskolin-induced stellate-shaped morphology and restored F-actin-stress fibre formation (Figure 5F). A similar response was observed after treatment with Db-cAMP, which significantly inhibited Skp2 (to 13.7 ± 9.9%, n = 3, P = 0.013) and Rb phosphorylation (7.8 ± 1.4%, n = 3, P = 0.0002), and elevated p27Kip1 levels (to 216 ± 25.3% of controls, n = 3, P = 0.0443) (Supplementary material online, Figure S5). Again, expression of active-Rac1 in the presence of Db-cAMP rescued Skp2 levels (to 139.6 ± 16.3%), Rb phosphorylation (to 107.6 ± 26.7%), and p27Kip1 protein levels (96 ± 1%) to levels that were not significantly different from controls (Supplementary material online, Figure S6). Taken together, these data suggest that elevated levels of cAMP inhibit VSMC proliferation by inhibiting Rac1-dependent up-regulation of Skp2.

Figure 4. Activation of Rac, controls Skp2 protein, vascular smooth muscle cell proliferation, and intima formation in vivo. Rac activity was quantified 2 and 7 days after balloon injury to left carotids (n = 3) and compared with uninjured right carotids (A). Carotids were infected with either 100 μL of 1 × 10⁹ pfu/mL Ad:β-Gal or Ad:DN-Rac1 immediately following balloon injury. Skp2 and p27Kip1-positive cells and BrdU incorporation were quantified 4 days post-injury (n = 6) (B). Intima:media ratio was calculated 14 days post-injury (C). Data expressed as means ± SEM.

3.6 Rac1-dependent regulation of Skp2 is independent of Cdc42/Rac interactive binding-domain effectors and associated with actin polymerization

Several studies have implicated signalling via CRIB-domain effector proteins such as PAK in Rac-dependent cell-cycle progression, possibly via regulation of cyclin D.19,20 Our data indicate that Skp2 levels are associated with Rac1-dependent actin polymerization. To test which pathways downstream of Rac1 are responsible for the regulation of Skp2, we constructed
effector-loop mutants (Y40C and F37A) within Rac1, both alone and in the context of the activating mutation (G12V). Previous studies established that mutation F37A impairs Rac1-dependent actin polymerization,14,21 while mutation Y40C has no effect on actin polymerization but does block interaction with CRIB-domain containing proteins such as PAK, which controls expression of Cyclin D.19,21 Consistent with this, the Y40C mutation but not F37A mutation blocked Rac1 interaction with PAK (Figure 6A). Expression of both active-Rac1 (G12V) and Rac1-G12V-Y40C also completely prevented stellate morphology and loss of actin-stress fibres in cells treated with forskolin (Figure 6B). Although Rac1-Y40C did not affect Skp2 levels, it did inhibit the expression of Cyclin D and partially block the Rb hyperphosphorylation (Figure 6E). Taken together, these observations indicate that Skp2 levels are independent of Rac1 association with CRIB-domain effector proteins but associated with Rac1-mediated actin polymerization.

To directly test whether Rac1-mediated actin polymerization underlies the regulation of Skp2, we treated cells with Cytochalasin D to induce actin depolymerization. Treatment of Ad:Control-infected cells with Cytochalasin D resulted in a significant inhibition of Skp2 compared with controls (Figure 6D). Although Rac1-Y40C did not affect Skp2 levels, it did inhibit the expression of Cyclin D and partially block the Rb hyperphosphorylation (Figure 6E). Taken together, these observations indicate that Skp2 levels are independent of Rac1 association with CRIB-domain effector proteins but associated with Rac1-mediated actin polymerization.

Inhibition of Rac1-dependent Skp2 up-regulation underlies the growth inhibitory effects of cyclic-AMP on vascular smooth muscle cell (VSMC) proliferation. Quiescent rat aortic VSMCs were stimulated with 10% foetal calf serum in the presence of 100 μM forskolin, as indicated, and Rac1 activity quantified 6 h later (A) by Rac pull-down assays and expressed as means ± SEM and is representative of three independent experiments. Rac1 localization was quantified by cell fractionation and western blotting (B). Cells were pre-treated 40 μmol/L PKA inhibitor for 30 min prior to stimulation with 500 μmol/L Db-cAMP for a further hour. Rac1 activity was quantified by Rac pull-down assays (C) and expressed as means ± SD and is representative of two independent experiments. VSMC were infected with 50 pfu/cell Ad:Control or Ad:Rac1-G12V (D and E) and treated with 100 μmol/L forskolin (E) for 18 h. Skp2, p27Kip1, hyper-phosphorylated Rb, and GAPDH protein levels were quantified by western blotting (E) and cell morphology and F-actin-stress fibres analysed by phase contrast microscopy and phalloidin staining, respectively (F). Data expressed as means ± SEM and is representative of at least three independent experiments. *P < 0.05 compared with controls.
significant inhibition (to 25.4 ± 4.3% of controls, n = 3, P ≤ 0.05) of Skp2 protein levels (Figure 6F). Expression of active-Rac1 (G12V) completely prevented this loss of Skp2 protein, indicating that Rac1-dependent actin polymerization controls Skp2 levels.

4. Discussion

In this study, we show for the first time that activity of Rho GTPases, in particular Rac1, is required for up-regulation of Skp2, the F-box component of the SCF-Skp2 ubiquitin-ligase that promotes ubiquitin-mediated proteasomal degradation of p27\textsuperscript{kip1}. Moreover, Rac1 promotes VSMC proliferation and subsequent intima formation following balloon injury, at least in part through this mechanism. Rho GTPases increase Skp2 levels by reducing proteolysis of Skp2 protein. Interruption of Rac1-mediated Skp2 up-regulation is involved in the inhibitory effects of cAMP on VSMC proliferation.

Our experiments using the broad-spectrum Rho GTPase inhibitor, C. difficile Toxin B, and DN-Rac1 imply that Rho GTPase activity increases Skp2 protein levels by reducing the rate of proteosomal degradation. This is consistent with previous work showing that APC\textsuperscript{cdh1} and SCF-based ubiquitin-ligases can target Skp2 for proteosomal degradation.\textsuperscript{22,23} Future work (beyond the scope of the present studies) will be needed to determine whether either of these ubiquitin-ligases mediates Rac1-dependent down-regulation of Skp2.

Our data demonstrating a role for Rac1 in the regulation of Skp2 levels is consistent with previous reports that document the growth regulatory properties of Rac1 in fibroblasts. For example, active-Rac1 mutants are sufficient to induce DNA synthesis and dominant-negative mutants decrease it in fibroblasts.\textsuperscript{24,25} Furthermore, Rac1 is essential for Ras-mediated transformation of fibroblasts.\textsuperscript{26} Our observation that Rac1-dependent Skp2 protein expression decreased p27\textsuperscript{kip1} levels also accounts, at least in part, for the reported effect of Rac1 to decrease p27\textsuperscript{kip1} levels in fibroblasts.\textsuperscript{13} However, participation of Skp2 in these growth regulatory properties has not been proposed previously. Instead, workers have suggested that active-Rac1 stimulates G1–S phase transition in NIH 3T3 fibroblasts by the induction of
Cyclin D1,19,20 This cannot, however, be entirely correct, since Y40 Rac1 mutants unable to activate this pathway retain the ability to promote G1–S phase progression and transformation.21 Our observations that Rac1 activity also controls Skp2 levels and subsequent degradation of p27Kip1 may account for this discrepancy. Furthermore, our experiments using Rac1-effector-loop mutants demonstrate that, unlike Cyclin D1, Skp2 levels are independent of Rac-Y40 and association with CRIB-domain effector proteins such as PAK19,20 but dependent on Rac-F37 and associated with Rac-dependent actin polymerization. This was confirmed by the ability of active-Rac1 to reverse the loss of Skp2 induced by Cytochalasin D. This suggests that at least two distinct pathways may exist downstream of Rac (Rac-PAK-Cyclin D and Rac-actin-Skp2), both of which are required for efficient cell-cycle progression. Consistent with a role for this novel Rac-Skp2 pathway in the regulation of VSMC proliferation, forced expression of exogenous wild-type Skp2 was able to completely prevent the DN-Rac1-induced increase in p27Kip1 and at partially restore Rb hyper-phosphorylation and BrdU incorporation. The fact that exogenous Skp2 expression only partially rescued Rb hyper-phosphorylation and S-phase entry is consistent with our conclusion that the Rac-Skp2 pathway may co-operate with other Rac-dependent cell-cycle regulators such as the Rac1-Cyclin-D pathway to control CDK activity.21,27 Such a requirement for multiple Rac1-dependent cell-cycle regulators probably accounts for large but incomplete inhibition of Rb hyper-phosphorylation and the small inhibition of S-phase entry induced by Rac1 inhibition in cells expressing exogenous Skp2 (Figure 3, Ad:WT-Skp2 vs. Ad:WT-Skp2 + Ad:DN-Rac). Although the precise mechanisms underlying Rac1-dependent regulation of Skp2 remain to be determined, our data indicate a role for actin-cytoskeleton organization. This is consistent with previous work demonstrating a requirement for cytoskeleton integrity for G1 progression.28 Future experiments should address this possibility directly. Our in vivo data shows that Rac1 is activated 2 days after injury to the rat carotid artery, the earliest time that we detected up-regulation of Skp2.6 DN-Rac1, potently suppressed Skp2 expression, VSMC proliferation, and intima formation after balloon injury, which demonstrates for the first time that Rac1-dependent Skp2 up-regulation occurs in vivo. The stimulus for Rac1 activation in vivo is most likely the release of growth factors and remodelling of the vascular extracellular matrix since these occur after vascular injury and have been shown to activate Rac1 in other systems.29,30 We previously demonstrated that elevated levels of cAMP inhibit VSMC proliferation by promoting the destabilization of Skp2.6 Our new data now demonstrates that the decrease in Skp2, increase in p27Kip1, and the inhibition of VSMC proliferation are all almost completely reversed by a constitutively active Rac1 mutant. Hence, inhibition of Rac1-dependant Skp2 up-regulation is largely responsible for the inhibition of VSMC proliferation by cAMP. We have also observed inhibition of Rac1 activity by elevated levels of cGMP (data not shown), which we have also shown to negatively regulate Skp2 and SMC proliferation under low mitogen conditions,3 suggesting that a similar mechanism may also underlie the anti-proliferative properties of cGMP in VSMC. Interestingly, a recent study also reported that Rac can increase cGMP levels by enhancing guanylate cyclase activity, suggesting the existence of a negative feedback mechanism.31 Although cyclic nucleotides have long been known to be growth inhibitory to VSMC, they have also been reported to have little effect on proliferation32 or even to have pro-proliferative properties in endothelial cells 33,34 cAMP-elevating signals have also been reported to activate Rac1 activity in endothelial cells,35 whereas we and others report Rac1 inhibition in VSMC.14 Other studies have reported enhanced recruitment of endothelial progenitor cells in response to cAMP-elevating signals.36,37 Together, these observations highlight the cell specific effects of cAMP signaling and may indicate divergent regulation of the Rac-Skp2 pathway in VSMCs, endothelial cells, and endothelial progenitor cells. Further characterization of this pathway in these cells may identify valuable therapeutic targets for second-generation drug-eluting stents, allowing selective modulation of VSMC proliferation endothelial repair.

In summary, our new data indicate the activation of Rac1 in culture or after vessel injury is required for increased Skp2 levels and that this novel pathway contributes towards down-regulation of p27Kip1 and towards increased VSMC proliferation and intima formation. Furthermore, inhibition of this novel pathway, at least in part, underlies the negative effects of cAMP on VSMC proliferation. Our data suggest that activation of Rac1 is a key step in pathological intima formation. Further analysis of this pathway may identify useful therapeutic target for the treatment of vascular proliferative diseases.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

6. Wu Y, Bond M, Sala-Newby G, Newby A. Altered S-phase kinase-associated protein-2 levels are a major mediator of cyclic nucleotide-induced...


