Altered S-Phase Kinase-Associated Protein-2 Levels Are a Major Mediator of Cyclic Nucleotide–Induced Inhibition of Vascular Smooth Muscle Cell Proliferation

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Abstract—Cyclic nucleotides inhibit vascular smooth muscle cell (VSMC) proliferation but the underlying molecular mechanisms are incompletely understood. We studied the role of S-phase kinase-associated protein-2 (Skp2), an F-box protein of SCF^Skp2 ubiquitin ligase responsible for polyubiquitylation of and subsequent proteolysis of p27Kip1, a key step leading to cell cycle progression. Skp2 mRNA and protein were upregulated in mitogen-stimulated VSMCs and after balloon injury in rat carotid arteries, where the time course and location of Skp2 expression closely paralleled that of proliferating cell nuclear antigen. Skp2 small interference RNA (siRNA) reduced Skp2 expression, increased p27Kip1 levels, and inhibited VSMC proliferation in vitro. cAMP-elevating agents prominently inhibited VSMC proliferation and Skp2 expression through inhibiting Skp2 transcription as well as decreasing Skp2 protein stability. Consistent with this, activation of protein kinase A, a downstream target of cAMP, was shown to negatively regulate focal adhesion kinase (FAK) phosphorylation and Skp2 expression. Adenovirus-mediated Skp2 expression reversed cAMP-induced p27Kip1 upregulation and rescued cAMP-related S-phase entry inhibition up to 50%. 8-Bromo-cGMP also moderately reduced Skp2 and cell proliferation when VSMCs were incubated with low serum concentration. Interestingly, we showed that 8-bromo-cGMP inhibited Skp2 expression also through activation of protein kinase G, which conversely enhanced FAK Y397 phosphorylation and Skp2 expression. After balloon injury of rat carotid arteries, local forskolin treatment significantly reduced FAK Y397 phosphorylation, Skp2 expression, VSMC proliferation, and subsequent neointimal thickening. These data demonstrate for the first time that Skp2 is an important factor in VSMC proliferation and its inhibition by cyclic nucleotides. (Circ Res. 2006;98:1141-1150.)

Key Words: Skp2 ■ cyclic nucleotides ■ smooth muscle cell ■ proliferation ■ focal adhesion kinase

Vascular smooth muscle cell (VSMC) proliferation contributes to intima formation after balloon injury with and without stenting, venous graft failure, transplant vasculopathy, and atherosclerosis. This motivates efforts to understand the regulation of VSMC proliferation and in particular to identify pathways that could mediate inhibition of proliferation. In healthy, uninjured vessels, VSMCs are quiescent and exhibit extremely low rates of proliferation, even in the presence of mitogenic stimuli. The mechanisms responsible for overcoming VSMC quiescence and initiating VSMC proliferation in response to vessel injury include release of growth factors and remodeling of the vascular extracellular matrix. This stimulates signaling pathways downstream of growth factor receptors or focal adhesions, respectively. Signaling pathways related to cell–cell adhesion through cadherins may also be involved.

Cell-type specific antiproliferative effects of cyclic nucleotides (cAMP and cGMP) are well documented. In particular, increased levels of cAMP and cGMP were shown to inhibit VSMC proliferation both in vitro in response to mitogens and in vivo after vascular injury. However, the mechanism of growth inhibition by cyclic nucleotides remains unclear. Early studies pointed to effects on mitogen-activating protein kinase (MAPK) signaling and the expression of cyclin D1. However, experiments showing that delaying addition of cyclic nucleotides for up to 16 hours after growth factors still results in inhibition, suggesting additional major effects on later events in the cell cycle. Consistent with this, several studies demonstrated that inhibition of proliferation by cAMP is associated with upregulation of the cyclin-dependent kinase inhibitor p27Kip1. Furthermore, prostacyclin mimetics, cicaprost or beraprost, which increase intracellular cAMP, also inhibit cyclin E-cyclin–dependent kinase (cdk) 2 activity via upregulation of p27Kip1. cGMP has also been demonstrated to inhibit VSMC proliferation through a transient increase of p27Kip1. Besides, VSMCs overexpress endothelial nitric oxide (NO) synthase, which increase NO production and thereby intracellular
cGMP levels, is also demonstrated to upregulate p27Kip1. However, it is not known how cyclic nucleotides regulate p27Kip1 levels and whether this mechanism is essential for their growth inhibitory effects.

S-phase kinase-associated protein 2 (Skp2), an F-box protein component of the SCF^{Skp2} ubiquitin ligase, is responsible for polyubiquitylation of many important cell-cycle regulators, including cdk inhibitor p27Kip1. Skp2 has been thought to be the major physiological target of Skp2, based on evidence that deletion of the p27Kip1 gene almost completely rescues the abnormal phenotypes of Skp2 knockout mice. Ectopic Skp2 expression in various cell types, including VSMC, has been shown to decrease p27Kip1 levels and force cells into S phase. Skp2, therefore, could play a key role in VSMC proliferation and related vascular pathologies.

The proposed role of Skp2 in regulation of p27Kip1 levels in VSMCs and the effects of cyclic nucleotides on p27Kip1 prompted us to test the hypothesis that Skp2 is an important factor in VSMC proliferation and neointimal thickening and that the growth-inhibitory effects of cyclic nucleotides in VSMCs are mediated via changes in the levels of Skp2 protein.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture
VSMCs were obtained by the explant method from thoracic aortas of male Sprague–Dawley rats as described previously. VSMC proliferation was quantified by bromodeoxyuridine (BrdUrd) labeling.

Balloon Injury of Rat Carotid Arteries and Local Administration of Forskolin
Balloon injury of left common carotid artery was performed in male Sprague–Dawley rats as described in detail in the online data supplement. Where indicated, pluronic gel containing either forskolin (200 μM) or dimethyl sulfoxide (control) was applied around the injured common carotid arteries (1 cm in length) before closure of the wound.

Gene Expression Analysis
Gene expression was quantified by RT-PCR and Western blotting, as described in detail in the online data supplement.

Results

Skp2 Regulates VSMC Proliferation In Vitro
Serum stimulation increased Skp2 protein levels 10±2-fold (n=6; P<0.05). Skp2 mRNA was also significantly upregulated by mitogen stimulation (Figure 1A) but to a lesser extent than the protein (1.8±0.4-fold; n=6; P<0.05). To determine the importance of this increase in Skp2 expression for S-phase entry and proliferation in VSMC, we designed small interfering RNA (siRNA) to specifically silence Skp2 expression. Transfection of VSMCs with siRNA targeting Skp2 but not siRNA targeting luciferase inhibited Skp2 mRNA and protein expression (by 79±4% and 93±3%, respectively; n=3; P<0.05) and increased the levels of p27Kip1 3 days post transfection. Importantly, total protein levels of GAPDH and focal adhesion kinase (FAK) were not affected by Skp2 siRNA, further demonstrating specificity. Skp2 siRNA significantly reduced VSMC proliferation measured by BrdUrd incorporation (from 24±1% to 12±2%; n=4; P<0.05) and cell counts per microscopic field (795±79 to 240±31 cells/field; n=4; P<0.05) (Figure 1B). Interestingly, VSMCs treated with Skp2 siRNA demonstrated the giant nuclei with increased size variation (Y.-J.W. and M.B., unpublished data, 2005), consistent with the phenotype of cells derived from Skp2-deficient mice.

Skp2 Is Upregulated In Vivo in the Proliferating Arterial VSMCs Induced by Balloon Injury
As a first step to investigate the role of Skp2 in VSMC proliferation in vivo, we compared the distribution of Skp2 protein and proliferating cells (using proliferating cell nuclear antigen [PCNA]) in rat carotid arteries after balloon injury. As shown in Figure 2A and 2B (n=3 for each time point), Skp2 protein and PCNA were almost undetectable in the uninjured arteries. However, Skp2 protein and PCNA increased in response to balloon injury, with medial expression of both being maximal 2 days after injury. Medial Skp2 staining waned after day 7; however, VSMCs were highly

Figure 1. Expression and silencing of Skp2 in cultured VSMC. A, Western blotting and quantitative RT-PCR analysis for Skp2 expression in quiescent VSMCs (Q) or 24 hours after adding 10% FCS (S). B, Asynchronous VSMCs were transfected with 40 nmol/L of BrdUrd for the last 8 hours. Results are expressed as mean ± SE (n=4; P<0.05 vs quiescent or luciferase treatment).
proliferative and strongly Skp2 positive in the neointima. Intimal Skp2 and PCNA expression was maximal on day 10 and then gradually returned to almost baseline levels on day 28. Quantitative results confirmed that the expression of PCNA was temporally associated with expression of Skp2 (Figure 2B). From Figure 2A, the distribution of Skp2 appears broader than that of PCNA, and this finding was confirmed by dual-labeling studies (supplemental Figure IA). This result was to be expected because Skp2 expression occurs in cells from late G1 phase onward, whereas PCNA is confined to S-phase cells. Neither rabbit immunoglobulin fraction nor mouse IgG as the substitute for primary antibody produces any staining on the sections (supplemental Figure IB). To confirm upregulation of Skp2 following injury, mRNA and protein expression were quantified after 7 days using real-time RT-PCR and Western blotting. Skp2 mRNA was upregulated 8.4±2.7-fold, whereas Skp2 protein was increased 12±3-fold, versus uninjured (3 experiments, 3 rats for each experiment) (Figure 2C).

**Regulation of Skp2 Transcription and Stability by cAMP and cGMP**

Treatment of VSMCs with dibutyryl-cAMP (db-cAMP) (0.1 to 1 mmol/L) or forskolin (adenylate cyclase activator,
10 to 100 μmol/L), or isobutylmethylxanthine (IBMX) (nonspecific phosphodiesterase inhibitor, 0.1 to 0.5 mmol/L), but not 8-bromo-cGMP (Br-cGMP, up to 1 mmol/L), resulted in a dose-dependent inhibition of Skp2 protein (supplemental Figure II). At the maximal dose used, Skp2 remaining was 16±4%, 25±11%, and 20±8% of control, respectively (Figure 3A). The same cAMP-elevating agents elevated p27kip1 levels (to 279±43%, 233±31%, and 226±34% of control, respectively) and inhibited hyperphosphorylation of retinoblastoma (Rb) protein, a marker of G1–S-phase progression (to 7.0±4.4%, 14±4%, and 18±5% of control, respectively) (Figure 3A). The same agents also similarly, but to a lesser extent, decreased Skp2 mRNA expression (to 48±8%, 53±9%, and 36±4% of control, respectively) (Figure 3B).

To further elucidate whether cAMP inhibits Skp2 mRNA expression through regulation of promoter activity, we performed luciferase reporter assays on VSMCs following transfection of reporter plasmid containing Skp2 promoter. Treatment with db-cAMP significantly decreased Skp2 promoter activity to an extent similar to that seen in mRNA expression (to 50±5% of control, P<0.05) (Figure 3C). The failure of db-cAMP to reduce the SV40 and CMV promoter activities indicates a specific effect on the Skp2 promoter rather than a global inhibition of transcriptional activity (Figure 3C). Intriguingly, Br-cGMP treatment, which did not show any effect on Skp2 protein expression, did result in a moderate inhibition of Skp2 mRNA levels (to 79±9% of control, P<0.05) (Figure 3B) and Skp2 promoter activity (to 71±3% of control, P<0.05) (Figure 3C). The disproportionate regulation between Skp2 protein and mRNA levels by cyclic nucleotides may also have their distinct posttranscriptional effects on Skp2 protein expression. We demonstrated that db-cAMP treatment but not Br-cGMP treatment results in a significant reduction in Skp2 protein stability (half-life of Skp2: db-cAMP=3.1±1.2 hours; control: 8.4±1.3 hours; Br-cGMP: 11±1 hours;
ANOVA, \( P < 0.01 \) (Figure 3D). The opposite effects of cAMP and cGMP on Skp2 stability help to explain their divergent effects on steady-state Skp2 protein and mRNA levels. Consistent with a decrease in levels of Skp2, which targets p27Kip1 for proteasomal degradation, we observed an increase in the stability of p27Kip1 after db-cAMP treatment (Figure 3D).

**Figure 4.** Serum concentration affects cGMP-mediated inhibition of VSMC proliferation and Skp2. VSMCs were incubated for 24 hours with 1 mmol/L Br-cGMP or 1 mmol/L db-cAMP (as positive control) in the presence of 0%, 2%, 5%, 10%, and 15% FCS. A, Cell proliferation was measured by BrdUrd incorporation. B, Skp2 expression was quantified by Western blotting. Data were obtained from at least 3 independent experiments. *\( P < 0.05; \) †\( P = 0.06; \) ‡\( P = 0.09 \) vs corresponding control.

**Figure 5.** Skp2 overexpression reverses forskolin-induced p27Kip1 upregulation and rescues proliferation. Asynchronous VSMCs were infected with either AdSkp2 or Ad\( \beta \)Gal at a multiplicity of infection of 150. Twenty-four hours post infection, cells were incubated with forskolin (100 \( \mu \)mol/L) for 24 hours in the presence of 15% FCS. A, Skp2, p27Kip1, and phosphorylated Rb (phospho-Rb) were determined by Western blotting. B, Cell proliferation was quantified by BrdUrd incorporation. Data were from at least 3 independent experiments. *\( P < 0.05 \) vs Ad\( \beta \)Gal, †\( P < 0.05 \) vs Ad\( \beta \)Gal + forskolin.

**Inhibition of VSMC Proliferation and Skp2 Expression by cGMP Is Observed at Low Serum Concentrations**

Although the growth inhibitory effects of cAMP were consistently observed in previous studies, the effects of cGMP were variable.\(^ {13,28,29} \) Either treatment with high concentration...
of cGMP\textsuperscript{13} or stimulation with relatively low concentrations of mitogens\textsuperscript{29,30} appears to be required for the growth inhibitory effects of cGMP. To further clarify this controversial issue and determine whether Skp2 regulation is also a common underlying mechanism in inhibition of proliferation by cGMP, we treated quiescent VSMCs with Br-cGMP (1 mmol/L) in the presence of different FCS concentrations. Unlike db-cAMP (1 mmol/L), which potently inhibited BrdUrd incorporation from 69±8% to 8.4±3.1% (P<0.05) in response to 15% serum mitogens, Br-cGMP did not show any significant effect on proliferation in the presence of 15% or even 10% of serum mitogens. However, at lower concentrations of FCS (ie, 5%, 2%, and 0%), Br-cGMP treatment resulted in a significant reduction in BrdUrd incorporation (by 30%, P<0.05; 45% and 65%, both P<0.05, respectively) (Figure 4A). Similar to the pattern of BrdUrd incorporation, Skp2 expression (Figure 4B) was potently inhibited by db-cAMP by 84% (P<0.05) in response to 15% FCS. However, Skp2 expression was only inhibited by Br-cGMP at lower FCS concentrations (ANOVA, P<0.01); the inhibition was concentration-dependent (supplemental Figure II). Taken together, these data suggest that regulation of Skp2 expression, at least in part, through FAK signaling. A, Opposite actions of cAMP and cGMP on FAK phosphorylation at 15% FCS. Quiescent VSMCs were treated as described in Figure 3A, and cell lysates were analyzed for the levels of phosphorylated FAK\textsuperscript{397} (phospho-FAK\textsuperscript{397}) and GAPDH by Western blot. Data were from 3 independent experiments. *P<0.05 vs control. B, Biphasic effects of Br-cGMP on phospho-FAK\textsuperscript{397} in the presence or absence of serum stimulation. Quiescent VSMCs were subjected to either 15% FCS or serum-free medium in the presence or absence of 1 mmol/L Br-cGMP for 4 hours. Cell lysates were analyzed as in A. Data were obtained from 4 independent experiments. *P<0.05 vs 0% FCS, †P<0.05 vs 15% FCS, ‡P<0.05 vs 15% FCS+Br-cGMP. C, Expression of constitutively active FAK (cd2-FAK) mutant reverses p27\textsuperscript{kip1} levels and partially rescues Skp2 expression and Rb hyperphosphorylation. Asynchronous VSMCs were infected with either Ad\textsubscript{cd2-FAK} or Ad\textsubscript{Gal} at a multiplicity of infection of 150. Twenty-four hours post infection, cells were incubated with forskolin (100 \mu mol/L) for 24 hours in the presence of 15% FCS. Cell lysates were subjected to phospho-FAK\textsuperscript{397}, Skp2, p27\textsuperscript{kip1}, phospho-Rb, and GAPDH analysis by Western blot. *P<0.05 vs Ad\textsubscript{Gal}; †P<0.05 and ‡P<0.05 vs Ad\textsubscript{Gal}+forskolin.
sion is a common mechanism underlying the antiproliferative effects of both cAMP and cGMP.

Adenovirus-Mediated Expression of Skp2 Reverses Forskolin-Related p27^Kip1 Upregulation and Rescues VSMC Proliferation

To investigate whether Skp2 downregulation is necessary for antiproliferative effects of cAMP in VSMCs, we attempted to rescue VSMC proliferation in cells treated with forskolin using adenovirus-mediated expression of exogenous wild-type Skp2. Infection with adenoviral vector expressing Skp2 (AdSkp2) resulted in increased expression of Skp2 protein and completely prevented p27^Kip1 upregulation by forskolin (from 166±21% to 66±6% of control, P<0.05). Furthermore, AdSkp2 infection also significantly, although partially, rescued Rb hyperphosphorylation inhibited by forskolin treatment (from 5.1±1.8% to 54±9% of control, P<0.05) (Figure 5A). More importantly, exogenous Skp2 expression reversed, at least partially, the forskolin-induced inhibition of BrdUrd incorporation (from 11±2% to 52±2% of control, P<0.05, Figure 5B).

cAMP and cGMP Have Opposite Effects on Focal Adhesion Kinase Phosphorylation at High Serum Concentration

Because we previously demonstrated that Skp2 expression is absolutely dependent on FAK activity,6 we investigated whether inhibition of Skp2 expression by cyclic nucleotides and cyclic nucleotide–elevating agents was associated with a change of FAK signaling. Treatment of cultured VSMCs with db-cAMP, forskolin or IBMX in the presence of 15% FCS significantly inhibited FAKY397 phosphorylation (Figure 6A; n=3, all P<0.05), an autophosphorylation site required for FAK activation. Levels of phospho-FAKY397 were significantly increased by Br-cGMP (to 154±7% of control; n=3; P<0.05 vs control) in the presence of 15% FCS but inhibited in serum-free conditions (Figure 6B).
overexpression can reverse the increase in p27Kip1 and partially rescue Skp2 expression, reversed the upregulation of p27Kip1 levels, and increased Rb hyperphosphorylation, a marker of G1–S-phase progression (Figure 6C), confirming that changes in FAK activity are, at least in part, responsible for regulating Skp2 expression.

**Protein Kinases A and G Have Opposite Effects on Skp2 Expression**

We investigated the role of protein kinase A (PKA) and G (PKG) signaling in the regulation of FAK phosphorylation and downregulation of Skp2 by cyclic nucleotides. Treatment of VSMC with a cell-permeable peptide inhibitor of PKA (PKAI) significantly rescued FAKY397 phosphorylation and Skp2 expression after treatment with db-cAMP (500 μmol/L) in presence of 10% serum (Figure 7A). Conversely, treatment with a PKGI resulted in a further dose-dependent inhibition of Skp2 expression and FAKY397 phosphorylation after Br-cGMP treatment in 2% serum (Figure 7B). Interestingly, PKAI treatment was also able to completely rescue Skp2 expression after Br-cGMP treatment in the presence of 2% serum (Figure 7C), which implies that the inhibitory effect of Br-cGMP on Skp2 levels is mediated by PKA.

**Forskolin Inhibits FAK Phosphorylation, Skp2 Expression, and BrdUrd Incorporation After Balloon Injury of Rat Carotid Artery**

We observed a significant increase in phospho-FAKY397–positive VSMCs in vivo 2 days after balloon injury to the rat carotid artery compared with uninjured control arteries (Figure 8A). Consistent with a role of FAK as a downstream effector of cyclic nucleotides, treatment with 30% pluronic gel containing 200 μmol/L forskolin significantly inhibited medial phospho-FAKY397 expression (from 6.6±1.2% to 1.4±0.4%; *P*<0.05) (Figure 8A). Forskolin treatment also inhibited Skp2 expression (from 7.5±2.7% to 1.0±0.2%, *P*<0.05) and BrdUrd incorporation (from 12.2% to 3.8±1.2%, *P*<0.05) (Figure 8B) 2 days after balloon injury. Consistent with previous studies, forskolin treatment significantly reduced the intima/media ratio 7 days after balloon injury, from 0.33±0.05 to 0.14±0.02 (P<0.05).

**Discussion**

In this study, we show for the first time that Skp2, an F-box component of the SCF^Skp2^ ubiquitin ligase that targets p27Kip1 for proteasome-mediated degradation,23,24 is an essential intermediate in the inhibitory effects of cAMP on VSMC proliferation in vitro. We show, moreover, that Skp2 expression closely parallels VSMC proliferation after injury to the rat carotid artery in vivo and that the cAMP-elevating agent forskolin suppresses both Skp2 expression and proliferation. Previously, work in a number of cell types demonstrated that growth inhibition by cAMP or cAMP-elevating agents is associated with increases in expression of p27Kip1.19–21 but these studies did not establish a causal relationship or define any upstream regulators. Our studies now show that Skp2 overexpression can reverse the increase in p27Kip1 and partially rescue VSMC proliferation from inhibition by cAMP. These experiments not only demonstrate that Skp2 is an essential upstream regulator of p27Kip1 degradation but that both Skp2 downregulation and p27Kip1 upregulation are essential for the full antiproliferative effects of cAMP. Interestingly, we found that the relative effects of cAMP and cGMP on Skp2 protein expression closely mirror their inhibitory effects on VSMC proliferation, which further supports our conclusion that Skp2 is a major effector of cyclic nucleotide–mediated growth arrest in VSMC. Previous data showing that 8-Br-cAMP has no effect on Skp2 expression in mouse embryonic fibroblasts20 suggest an interesting species
or cell-type difference between VSMCs and fibroblasts. Skp2 overexpression completely reversed the effect of cyclic nucleotides on p27Kip1 but only partially rescued VSMC proliferation. Hence our results complement rather than contradict previous studies showing important effects of cAMP on MAPK activation,16 cyclin D1,13,18,19 and cyclin A expression.31,32 Notwithstanding, our results demonstrate that Skp2 is a major effector of the growth inhibitory effects of cyclic nucleotides.

Consistent with our previous work,9 we demonstrated that Skp2 mRNA and protein levels are both elevated in serum-stimulated isolated VSMCs. Silencing Skp2 expression by siRNA inhibited proliferation, confirming our previous results with adenovirus-mediated overexpression of dominant negative F-box–deleted Skp2.9 Although these results firmly establish a role for Skp2 in VSMC proliferation in vitro, no previous studies have addressed its role in vivo. As a first step, we showed that Skp2 mRNA and protein levels were also upregulated after balloon injury to the rat carotid in vivo. Secondly, the time course and localization of Skp2 protein expression closely paralleled that of the proliferation marker PCNA. Interestingly, the expression pattern of Skp2 reported here appears to be the reciprocal of that previously reported for p27Kip1 by Tanner et al.33 In that study, p27Kip1 protein levels are high in uninjured vessels and decline sharply after injury. Subsequently, p27Kip1 rises to very high levels 14 to 21 days after injury, coinciding with the fall in Skp2 expression we report here, and VSMC proliferation levels returned toward baseline values. Thirdly, and most importantly, we showed that Skp2 upregulation and VSMC proliferation were inhibited in parallel by the cAMP-elevating agent forskolin, which also inhibited neointima formation.

To elucidate the mechanisms by which cyclic nucleotides regulate Skp2 expression, we first compared the effects of serum and cyclic nucleotides on steady-state Skp2 mRNA and protein levels. Skp2 mRNA levels were less responsive to serum than protein levels, which implied both transcriptional and posttranscriptional controls. This was confirmed by studies of promoter activity and protein stability, both of which were inhibited by cAMP-related interventions and only transcriptional signal. Notably, the net outcome of elevated cGMP is only able to inhibit Skp2 expression in low serum concentrations, which probably reflects the unmasking of cGMP/PKA signaling, and thereby inhibit FAK phosphorylation in these conditions. In the presence of high serum, cGMP stimulates PKG-dependent increase of FAK phosphorylation, which we previously demonstrated promotes Skp2 protein stability.9 Presumably, this mechanism counters the negative effects of cGMP/PKA signaling on Skp2 and, at least in part, explains the relatively poor effects of cGMP on VSMC proliferation compared with cAMP.

The cyclic nucleotides cAMP and cGMP are important intracellular secondary messengers for prostanoids and NO, respectively, which regulate several functions of VSMC, including contraction as well as proliferation.34,35 The ability of cells to synthesize cAMP and cGMP is high in healthy uninjured vessels,37 and this probably contributes toward the maintenance of VSMC quiescence. Furthermore, reduced synthesis of cyclic nucleotides after vessel injury37 is likely to be an important mechanism in removing the “brake” on proliferation.

Taken together, our data demonstrate that cyclic nucleotide–dependent regulation of Skp2 represents an important pathway for controlling VSMC proliferation. Hence, Skp2 may emerge as a promising target of therapy for vasculoproliferative disorders.

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Materials and Methods

Materials

All chemicals, except those stated, were obtained from Sigma-Aldrich Co.

Cell-permeable, myristoylated protein kinase A inhibitor peptide (14-22 amide) (PKAI) and protein kinase G1α inhibitor peptide (PKGI) were both from Calbiochem (EMD Biosciences Inc.). Mouse monoclonal and rabbit polyclonal antibodies to Skp2 were purchased from Zymed Laboratories and US Biological, respectively. Mouse monoclonal antibody to p27^Kip1 and rabbit polyclonal antibody to phosphorylated retinoblastoma protein (Rb) (ser807/811) were from Transduction Laboratories and Cell Signaling Technology Inc., respectively. Rabbit polyclonal focal adhesion kinase (FAK) and phospho-FAK_Y397 antibodies were both from Upstate. Rabbit polyclonal and mouse monoclonal proliferating cell nuclear antigen (PCNA) antibodies were obtained from Abcam Ltd.

Methods

Cell Culture. VSMCs were obtained by the explant method from thoracic aortas of male Sprague-Dawley rats as described previously. Cells were maintained in DMEM containing 10% FCS and were used between passages 2-6. Cells were rendered
quiescent by serum starvation for 72 hours before simultaneously treated with
mitogens, cyclic nucleotides and other chemicals.

**Small Interfering RNAs (siRNAs) and Their Transfection.** siRNA targeting rat
Skp2 (5’-AGAGCAAAGGGAGUGACAA-3’ and
5’-GCUGCAGAAUCUGAGUCUG-3’ ) and luciferase
(5’-GUGCGUUGCUAGUACCAAC-3’) were generated using the Silencer siRNA
Construction Kit 1620 (Ambion Inc.) according to manufacturer’s instruction..
VSMCs were transfected with 40 nmol/L of siRNA (20 nmol/L each Skp2 target or 40
nmol/L luciferase control) using calcium phosphate co-precipitation as previously
described by Deroanne et al.(reference and private communication). The cells were
subjected to analysis 72 hours after transfection.

**Cell Proliferation Assay.** VSMC proliferation was quantified by labelling cells with
10 μM BrdU for 8 hours. Cells were then fixed in ice-cold 70% ethanol and analysed
for BrdU incorporation by immunocytochemistry using a monoclonal anti-BrdU
antibody (MP Biomedicals). BrdU-positive cells were visualised with
diaminobenzidine (DAB) staining.

**Quantitative and Semi-quantitative RT-PCR.** Total RNA was extracted from
VSMCs and rat carotid arteries using RNeasy RNA extraction kit (Qiagen). RNA
quality was determined using an Agilent Bioanalyzer 2100 and samples with rRNA
28S/18S ratios more or equal to 1.8 were used. cDNA was synthesized using ProStar First-Strand Kit (Stratagene). Quantitative PCR was performed using a LightCycler (Roche) with QuantiTect SYBR Green PCR system (Qiagen) and primers for Skp2 (forward: 5’-CAAGGAAAAGGCTAAAGAGCA A-3’, reverse: 5’-CCTCTTGCAAAACTCCAGAGACT-3’) and 18S ribosomal RNA (forward: 5’-CGCGGTTCTATTTTGTTGGT-3’, reverse: 5’-CTTCAAACCTCCGACTTTTCG-3’). Semi-quantitative PCR was performed as previously described.3

**Western Blotting.** Immunoblot analysis was done as described elsewhere.3 Briefly, total VSMC or arterial lysates were prepared at the indicated times using SDS-lysis buffer. The equal amounts of reduced protein (50-100 µg) were separated by polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Bio-Rad). Blots were blocked in 5% non-fat milk and specific proteins detected using primary antibodies as indicated and HRP-conjugated secondary antibodies (DAKO). Bands were visualized using ECL detection (Amersham Biosciences).

**Cloning of Skp2 Promoter, Plasmid Transfection and Luciferase Assay.** The human Skp2 promoter (-1511 to +67) was cloned from human genomic DNA using PCR with the following primers (forward 5’- GCAATGCTTTCTCCTTAGCCAAAGTAAC-3’ and reverse...
5’-CCAGCAGCCAGACCCGCTAAG-3’) and subcloned into pGL-Basic luciferase reporter gene (Promega). Rat VSMCs were transfected with 1µg of reporter plasmids using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) and assayed for luciferase activity 48 hours later using Luciferase Assay System (Promega). Transfected cells were incubated in presence or absence of cyclic nucleotides for the last 4 hours. Luciferase reporter plasmids containing simian virus (SV) 40 or cytomegalovirus (CMV) promoters served as specificity controls.

**Recombinant Adenoviruses and Infection of VSMCs.** Recombinant adenoviruses encoding wild-type Skp2 (AdSkp2) or β-galactosidase (AdGal) driven by CMV promoter was generated as previously described. Briefly, the coding sequences were cloned into the adenovirus shuttle vector pDC515 (Microbix Biosystems Inc.). To generate the constitutively-active FAK adenovirus (Adcd2FAK), the membrane targeting domain of CD2 was fused to the wild-type FAK coding sequence and cloned into pDC515 shuttle vector. Replication-deficient adenoviruses 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. Asynchronized rat VSMCs were infected with adenovirus at multiplicity of infection (MOI) of 150 for 3 hours. Cells were treated with forskolin 24 hours post infection.

**Balloon Injury of Rat Carotid Arteries and Local Administration of Forskolin.**
Male Sprague-Dawley rats (350-400 g) were obtained from B&K Universal Ltd (UK).

The housing and care of the animals and all the procedures used in the study adhered
to the guidelines and regulations of Animal Scientific Procedures Act 1986. Balloon
injury of left common carotid artery was performed as described by Clowes et al.4
Briefly, rats were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg)
(i.p.) following inhalation of halothane. Left common carotid arteries were injured by
three rotating passes with a 2F Forgaty catheter (Actamed, West Yorkshire, UK)
introduced through the left external carotid artery. Proximal external carotid arteries
were ligated after withdrawal of the balloon catheter. Where indicated, 200 µl of 30%
(wt/vol) pluronic gel (F127, Sigma) containing either forskolin (200 µmol/L) or 0.1%
DMSO (control) was applied around the injured common carotid arteries (~ 1 cm in
length) before closure of the wound.

Rats were killed at the indicated time points using pentobarbital (100 mg/kg i.p.)
and vessels collected after perfusion with heparinized PBS or perfusion-fixation with
10% formalin/PBS solution. Another set of animals received 3 subcutaneous
injections of BrdU (25 mg/kg) at 17, 9 and 1 hour before euthanasia.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded arterial sections were
deparaffinized, rehydrated and subjected to antigen retrieval (sodium citrate buffer,
0.1 mol/L, pH=6.0, at 100°C for 10 minutes). Sections were incubated with specific
primary antibody, biotin- or alkaline phosphatase-conjugated secondary antibody

(Dako) followed by detection with BCIP/NBT (Vector Laboratories), NovaRed

(Vector Laboratories) or DAB. Nuclei were counterstained with hematoxylin. Mouse

IgG or rabbit immunoglobulin fraction (Dako) were used as negative controls.

**Statistical Analysis.** All data are shown as mean ± SEM. Statistical analysis between
groups was performed by analysis of variance (ANOVA) using InStat. 2-tailed $t$ test
or Mann-Whitney U test were used where appropriate. $p<0.05$ was considered
significant.
Results

Supplement Figure 1

A

Dual labeling

B

Nonimmune Ig
Supplement Figure 1

(A) The representative section of 10-day injured carotid artery was examined by dual-labeling for Skp2 (brown) and PCNA (blue) in a high-power field (1000x). (B) The negative control of dual staining using mouse IgG (brown) and rabbit immunoglobulin fraction (blue) as the substitutes for primary antibodies. Scale bar=25 \( \mu \text{m} \).
Supplement Figure 2

Dose-dependent inhibition of Skp2 expression by cyclic nucleotides. cAMP-elevating agents (A) and Br-cGMP (B) inhibit Skp2 expression dose-dependently at 15% and 2% FCS, respectively. Quiescent rat VSMCs were incubated with indicated doses of db-cAMP, forskolin, IBMX or Br-cGMP and indicated concentrations of FCS for 24 hours and then subjected to western blot analysis for Skp2 and GAPDH expression.
References


