Vasodilator-Stimulated Phosphoprotein Regulates Proliferation and Growth Inhibition by Nitric Oxide in Vascular Smooth Muscle Cells


Objective—Vasodilator-stimulated phosphoprotein (VASP) was identified as a substrate for cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA). It is preferentially phosphorylated at serine239 by PKG, whereas serine157 is a preferred phosphorylation site for PKA. In addition, serine157 is phosphorylated by PKC in response to serum. We have investigated the effects of VASP and VASP phosphorylation at serine157 and serine239 on smooth muscle cell (SMC) proliferation and nitric oxide (NO)-mediated growth inhibition.

Methods and Results—Aortic SMCs derived from VASP-deficient mice were transduced with retroviral vectors encoding either wild-type VASP or VASP mutants (S157A-VASP and S239A-VASP), in which serine157 and serine239, respectively, were replaced by a nonphosphorylatable amino acid, alanine. Expression of wt-VASP and S239A-VASP significantly increased proliferation, whereas expression of S157A-VASP was inhibitory. Expression of S239A-VASP rendered SMCs less sensitive to growth inhibition by the NO donor, S-nitroso-n-acetylpenicillamine, when compared with cells expressing wt-VASP. Similar effects were observed in cultured rat SMCs in which wt-VASP, S157A-VASP, and S239A-VASP were expressed.

Conclusions—Our data suggest that VASP phosphorylation at serine157 is required for the growth-stimulatory effect of VASP in SMCs, whereas VASP phosphorylation at serine239 is involved in the growth inhibitory effects of NO on SMCs. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: vasodilator-stimulated phosphoprotein ♦ smooth muscle cell growth ♦ nitric oxide ♦ phosphorylation ♦ cell signaling

vasodilator-stimulated phosphoprotein (VASP) belongs to the Ena/VASP family, which plays an important role in regulating cytoskeletal dynamics and cell migration.

VASP was originally identified as a substrate for both cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA). Three phosphorylation sites on VASP have been identified, serine157, serine239, and threonine278. Serine239 is the preferential phosphorylation site for PKG, whereas serine157 is the preferential phosphorylation site for PKA. We have recently shown that serine157 is also phosphorylated in response to growth factors through activity of PKC.

Phosphorylation at serine157 leads to a mobility shift of VASP in SDS-PAGE from an apparent molecular weight of 46 kDa to 50 kDa. In platelets and various cell types, including vascular smooth muscle cells (SMCs), endothelial cells, and fibroblasts, VASP is associated with actin filaments, focal adhesions, and cell–cell contacts. VASP promotes the motility of the bacteria Listeria in host cells. VASP-deficient (VASP−/−) mice are viable with minor platelet abnormalities including an impaired inhibition of aggregation by cGMP and cAMP when compared with wild-type platelets. In addition, VASP−/− mice are characterized by enhanced in vivo platelet adhesion under physiological and pathophysiological conditions. Recent studies showed that fibroblasts derived from VASP−/− mice exhibit increased cell motility, suggesting VASP inhibits cell migration. The observation that increased or decreased VASP expression is associated with increased colony formation of NIH3T3 cells in soft agar and tumor formation in nude mice suggests that VASP may play a role in tumorigenesis. Recent studies show that the overexpression of VASP potentiates the activity of serum-response factor (SRF) in NIH3T3 fibroblasts, possibly by increasing F-actin assembly and depleting the cellular pool of G-actin.

Although VASP phosphorylation is used as a biochemical marker for activation of PKG and PKA, its precise cellular and molecular functions remain to be determined. In particular, the functional consequences of VASP phosphorylation, including its effects on protein–protein interactions, need to be determined.
be elucidated in intact cells. One study reported that VASP phosphorylation at serine157 increases its binding to F-actin, whereas another study demonstrated that VASP phosphorylation at both serine157 and serine239 reduces its binding to F-actin as well as F-actin bundling.

In human platelets VASP phosphorylation is closely correlated with fibrinogen receptor (glycoprotein a2b3) inhibition by cAMP-elevating and cGMP-elevating agents such as nitric oxide (NO) and prostaglandins, which has been observed under in vivo conditions.

We tested the hypothesis that VASP phosphorylation at serine239 regulates the inhibitory effects of NO on SMC proliferation. To study the effects of VASP and VASP phosphorylation, we used retroviral-mediated gene transfer to introduce wild-type VASP (wt-VASP) and selectively non-phosphorylatable VASP mutants (S157A-VASP and S239A-VASP) into cultured vascular SMCs from VASP−/− mice. We also used a tetracycline (Tet)-inducible gene expression system to express wt-VASP and VASP mutants (S157A-VASP and S239A-VASP) in cultured rat vascular SMCs.

### Methods

Please see online Methods section at http://atvb.ahajournals.org for further details.

### Materials

Cell culture medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen-Gibco Life Technologies. Fetal bovine serum (FBS) was purchased from Atlantic Biological. A rabbit polyclonal anti-VASP antibody was purchased from Alexis. Mouse anti-VASP phosphoserine157 antibody and anti-VASP phosphoserine239 antibodies were previously developed and characterized. A mouse anti-VSV-G antibody and protein G-agarose were purchased from Roche-Boehringer Mannheim. G418 (50 mg/mL) was purchased from Calbiochem. 3H-thymidine was purchased from ICN-Biochemicals.

### Cell Culture

Aortic Fischer rat vascular SMCs were prepared and maintained in 15% FBS as described. One-year-old VASP−/− mice were obtained in growth experiments over 7 days, in which VASP was inhibitory, whereas expression of wt-VASP and S157A-VASP (all tagged with VSV-G epitope) as well as the reporter β-galactosidase under the control of the Tet operator/promoter was suppressed. In the presence of Tet (1 μg/mL), gene expression is suppressed. Withdrawal of Tet for 48 hours induces expression of the transgene. Transduced SMCs were maintained in the presence of 1 μg/mL Tet.

### DNA Synthesis

DNA synthesis assayed by the incorporation of 3H-thymidine was measured as previously described. Assays were performed in triplicate.

### Cell Growth

Mouse vascular SMCs were seeded in 6-well plates (1×10⁶/well) and grown in DMEM containing 15% FBS. On days 1, 3, 5, and 7, cells were trypsinized and counted. The medium was changed on day 1, 3, 5, and 7. Assays were performed in triplicate.

### Protein Extraction and Western Blotting

Cells were extracted and protein samples were analyzed by SDS-PAGE and Western blotting as described.

### Transfection of Vascular SMCs From VASP−/− Mice With Retroviral Vectors Encoding wt-VASP and VASP Mutants

A retroviral construct containing either human wild-type VASP (wt-VASP) or VASP mutants (S157A-VASP and S239A-VASP) was generated by insertion of the gene into the parental retroviral vector LXSN, provided by A. D. Miller. S157A-VASP and S239A-VASP, in which serine157 and serine239, respectively, were replaced by alanine were constructed. All the constructs, wt-VASP, S157A-VASP, and S239A-VASP, were fused with the peptide epitope from the vesicular stomatitis virus glycoprotein (VSV-G) via a proline to the second amino acid of VASP. The packaging cells were transfected with the constructs and selected. Mouse SMCs were infected with LXSN, LXSN-wt-VASP, LXSN-S157A-VASP, or LXSN-S239A-VASP virus. Multiple clones were selected, propagated, and maintained in the presence of G418 (0.6 mg/mL).

### Expression of wt-VASP and VASP Mutants in Rat SMC Lines Using a Tetracycline-Inducible System

Aortic SMCs from Fischer 344 rats were sequentially transfected with 2 expression vectors: one contained the Tet transactivator protein (Tet) under the control of the Tet operator/promoter, and the other contained wt-VASP or S157A-VASP or S239A-VASP (all tagged with VSV-G epitope) as well as the reporter β-galactosidase under the control of the Tet operator/promoter. In the presence of Tet (1 μg/mL), gene expression is suppressed. Withdrawal of Tet for 48 hours induces expression of the transgene. Transduced SMCs were maintained in the presence of 1 μg/mL Tet.
SMCs expressing wt-VASP or S239A-VASP, but not S157A-VASP, displayed a significantly increased cell number at days 3 to 7 compared with vector control SMCs (Figure 1B). These results indicate that VASP expression plays a positive role in SMC proliferation only if it can be phosphorylated at serine157. Phosphorylation at serine239 is not involved in serum-stimulated SMC growth.

Phosphorylation of Serine239 on VASP Mediates Growth Inhibition by NO
To test the hypothesis that VASP phosphorylation at serine239 plays a positive role in SMC proliferation only if it can be phosphorylated at serine157. Phosphorylation at serine239 is not involved in serum-stimulated SMC growth.

SMCs expressing wt-VASP or S239A-VASP, but not S157A-VASP, displayed a significantly increased cell number at days 3 to 7 compared with vector control SMCs (Figure 1B).

These results indicate that VASP expression plays a positive role in SMC proliferation only if it can be phosphorylated at serine157. Phosphorylation at serine239 is not involved in serum-stimulated SMC growth.

The Effects of wt-VASP, S157A-VASP, and S239A-VASP in Rat SMCs
We also investigated the effects of VASP in rat SMCs by expressing wt-VASP, S157A-VASP, and S239A-VASP using a tetracycline (Tet)-inducible gene expression system. In the presence of Tet, no expression of the transgene was detected (Figure 3A). In the absence of Tet, the cells increased expression of VASP to 5- to 10-fold over endogenous VASP level (Figure 3A).

Expression of either wt-VASP or S239A-VASP significantly increased 3H-thymidine incorporation in response to 10% FBS, whereas expression of S157A-VASP was inhibitory (Figure 3B). Comparable results were obtained in cell counting experiments (data not shown).

Because of a low response to serum stimulation (Figure 1), we could not determine the IC50 value for SNAP in SMCs expressing S157A-VASP (data not shown).

To determine whether cGMP and SNAP have similar effects, we used 2 nondegradable cGMP analogues, db-cGMP (dB-cGMP) and 8-bromo-cGMP. Both inhibited DNA synthesis in response to 15% FBS in SMCs expressing wt-VASP with similar dose dependency (Figure 2B). Similar to SNAP, SMCs expressing S239A-VASP were less affected by cGMP analogues (Figure 2B).

Together, these results suggest that NO inhibits serum-induced cell proliferation by a mechanism that involves cGMP and PKG-mediated VASP phosphorylation at serine239.
Next, we investigated the effects of expression of S239A-VASP on growth inhibition by SNAP. SNAP inhibited wt-VASP expressing SMC proliferation in a dose-dependent manner, with IC$_{50}$ of 15 μmol/L (Figure 3C). SMCs expressing S239A-VASP were less affected by SNAP (IC$_{50}$ of 50 μmol/L; Figure 3C). Cyclic GMP analogues had a similar effect (data not shown).

Taken together, expression of VASP and nonphosphorylatable VASP mutants in rat SMCs produced the similar effects compared with expression of these VASP proteins in VASP$^{-/-}$ mouse SMCs.

**Discussion**

**VASP Promotes Vascular SMC Proliferation Induced by Serum Only When It Can Be Phosphorylated at Serine157**

Our data obtained with SMCs from 2 species (mouse and rat) and 2 different expression systems (retroviral-mediated gene expression and Tet-inducible expression system) support the conclusion that expression of wt-VASP enhances serum-induced cell growth (Figures 1 and 3B). This is the first evidence to our knowledge that VASP can regulate cell proliferation in nontransformed cells. As has been shown in NIH3T3 cells, however, VASP-deficiency and VASP overexpression induce neoplastic transformation and promote tumor growth. Whether similar mechanisms underlie these observations needs to be investigated.

VASP is localized at focal adhesion sites and plays a role in facilitating locally constrained actin polymerization. Its potential role in regulating SMC growth may lie at the level of regulating cytoskeletal organization. Cell spreading, adhesion, and cytoskeletal integrity are involved in growth factor-induced cell growth of all adherent cells. Actin disruption in human capillary endothelial cells suppresses cell spreading and prevents progression through G1 and S phases in the presence of growth factors. This finding suggests that tension-dependent changes in cell shape and cytoskeletal structure are required for the G1/S transition of the cell cycle. VASP is able to bind F-actin and G-actin and has been shown to regulate actin polymerization in migrating fibroblasts and Listeria movement in the host cells. VASP also interacts with other focal adhesion proteins such as vinculin, zyxin, and profilins. It is possible that VASP is involved in serum-induced cytoskeletal reorganization and/or changes in cell shape necessary for cell cycle progression.

VASP can bind to c-Abl, a pro-oncogenic tyrosine kinase. c-Abl has been implicated in cell growth, reorganization of cytoskeleton, cell death, and stress responses. Increased and deregulated c-Abl kinase activity is believed to activate signal transduction pathways, which ultimately lead to uncontrolled cell growth. VASP binding to c-Abl might be involved in regulation of c-Abl activity, which may then regulate cell growth.

Regulation of actin dynamics is necessary for serum-mediated induction of expression of a subset of SRF target genes. A possible mechanism for how VASP increases SRF activity is to promote F-actin assembly, thereby decreasing cellular G-actin, which is an inhibitor of SRF activity. However, whether serum-induced activation of SRF activity leads to cell proliferation remains to be determined.

Our data clearly show that VASP phosphorylation at serine157 is required for its growth promoting effects, because mutation of serine157 on VASP to alanine inhibits both mouse and rat SMC growth induced by serum (Figures 1 and 3B). As discussed, the mechanisms mediating the growth-stimulatory effects of VASP need to be clarified. It is possible that VASP phosphorylation at serine157 in response to serum modulates its binding to other focal adhesion proteins and cytoskeletal dynamics that are necessary for cell cycle progress.
VASP (Figures 1 and 3B). The phosphorylation of serine157 and/or serine239 of VASP is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function.142.

With respect to the kinase phosphorylating serine157 in response to serum, we have recently shown that serum induces phosphorylation of serine157 in a PKC-dependent manner.6,45 Consistent with the observation that serum does not induce VASP phosphorylation at serine239,6 expression of S239A-VASP has the same effect as expression of wt-VASP (Figures 1 and 3B).

**VASP Phosphorylation at serine239 Mediates the Inhibition of SMC Proliferation by NO/cGMP**

Our observation that SMCs expressing S239A-VASP are less sensitive to growth inhibition by NO and cGMP analogs suggests that phosphorylation of serine239 by PKG promotes detachment of VASP from focal adhesions and is associated with inhibition of migration. It is possible that PKG inhibits SMC growth by disassembly of focal adhesions and that expression of S239A-VASP would block disassembly of focal adhesions induced by NO, cGMP, and PKG. Another possibility is that PKG interferes with serum-induced gene transcription. Interestingly, it has recently been demonstrated that the potential of SRE-dependent transcription by VASP is inhibited by PKG, probably through phosphorylation of serine239.20

Because mutation of serine239 to alanine prevents phosphorylation of serine157 in response to SNAP (Figure I), we cannot rule out that serine157 phosphorylation also plays a role in NO-induced growth inhibition. However, serine239 phosphorylation is still required to mediate inhibitory effect on cell growth by NO.

Although PKA also phosphorylates VASP at serine239, it is unlikely that this event contributes to the growth inhibitory effects by forskolin, because SMCs expressing S239A-VASP is similarly affected by forskolin when compared with controls (data not shown).

In conclusion, our work identifies 2 novel functions of VASP (Figure 4). First, VASP promotes SMC growth, which requires phosphorylation of serine157. Second, VASP phosphorylation of serine239 by PKG mediates, at least in part, the inhibitory effects of NO on SMC growth. These distinct functional effects of VASP phosphorylation are most likely mediated by a distinct pattern of protein–protein interaction of serine157 and/or serine239 phosphorylated VASP that also may be cell-type specific. Although VASP-deficient mice do not exhibit an obvious phenotype in SMC function, our data predict that VASP plays a role in the response to arterial injury.

**Acknowledgments**

The study was supported by grants (HL-52549 and HL-03174) from the National Institute of Health (to A.W.C. and D.F.B.-P., respectively) and by grants from the Deutsche Forschungsgemeinschaft (SFB 355) (to M.E. and U.W.). We thank Dr Richard Kenagy for his critical discussion.

**References**


Vasodilator-Stimulated Phosphoprotein Regulates Proliferation and Growth Inhibition by Nitric Oxide in Vascular Smooth Muscle Cells

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2004/06/03/01.ATV.0000134705.39654.53.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2004/08/16/01.ATV.0000134705.39654.53.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/