Vasodilator-stimulated phosphoprotein (VASP) associates with cytoskeletal structures and promotes F-actin formation. RhoA, a member of the Ras superfamily of proteins, activates serum response element (SRE)-dependent transcription through changes in actin dynamics. We now show that the F-actin binding region of VASP is required for VASP stimulation of SRE-dependent transcription, and that VASP is downstream of RhoA in stimulating SRE-dependent transcription. The isolated carboxyl-terminal coiled-coil region of VASP mediates protein tetramerization and has been used as a dominant negative form of VASP; we found that it forms complexes with endogenous VASP in vivo and inhibits in a dose-dependent fashion serum-, RhoA-, and VASP-stimulated SRE-dependent transcription. Cyclic GMP-dependent protein kinase (G-kinase) inhibits RhoA activation of SRE-dependent transcription (Gudi, T., Chen, J. C., Casteel, D. E., Seasholtz, T. M., Boss, G. R., and Pilz, R. B. (2002) J. Biol. Chem. 277, 37382–37393). We now show that the G-kinase inhibition that occurs downstream of RhoA can be explained, at least in part, by G-kinase phosphorylation of VASP on Ser239 at the carboxy-terminal end of the G-kinin binding site, with some contribution by phosphorylation of Ser237, which is proximal to the profilin binding site. A phosphorylation-deficient VASP mutant can partly prevent cGMP/G-kinase inhibition of serum- and RhoA-induced SRE-dependent transcription. These studies show that VASP, an important component of the cellular microfilament system, plays a major role in regulating SRE-dependent transcription, and that G-kinase regulates VASP activity.

Serum response factor (SRF) is a ubiquitous transcription factor that regulates immediate early genes such as c-fos, mus-

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1 The abbreviations used are: SRF, serum response factor; SRE, serum response element; A-kinase, cAMP-dependent protein kinase; CPT, 8-p-salicylalylthio; CRE, cAMP-response element; DMEM, Dulbecco's modified Eagle's medium; 8-Gal, 8-galactosidase; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; G-kinase, cGMP-dependent protein kinase; Luc, luciferase; REF, rat embryonic fibroblasts; VASP, vasodilator-stimulated phosphoprotein; VSV, vesicular stomatitis virus; EVH, Ena-VASP homology; MCFB, murine cardiac fibroblast; ROK, Rho kinase; C/EBP, CCAAT-enhancer binding protein.

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Cyclic GMP-dependent protein kinase (G-kinase), like cAMP-dependent protein kinase (A-kinase), regulates transcription of multiple genes including c-fos, and is the mediator of some of the effects of nitric oxide on gene expression (23–25). We recently showed in C6 glial cells and vascular smooth muscle cells that G-kinase inhibits SRE-dependent transcription, both by inhibiting RhoA activation as well as by a mechanism downstream of RhoA (26). Because G-kinase phosphorylates VASP at three well-characterized sites (10), and VASP phosphorylation in vitro inhibits its F-actin binding and actin polymerization activity (17, 27), we asked whether the G-kinase regulation of SRE-dependent transcription that occurs distal to RhoA could be mediated by VASP. In this study, we found that VASP activates SRE-dependent transcription downstream of RhoA, and that the EVH2 domain of VASP is required for induction of transcription of VASP. A dominant negative VASP construct containing the tetramerization region interfered with VASP- and RhoA-dependent activation of gene transcription; G-kinase phosphorylation of VASP, particularly on Ser239, inhibited the transcriptional effects of VASP. Studies with phosphorylation-deficient VASP mutants suggest that VASP is important to the G-kinase regulation of SRE-dependent transcription that occurs distal to RhoA.

**EXPERIMENTAL PROCEDURES**

**Materials and DNA Constructs**—The rabbit polyclonal anti-VASP antibody M4 (affinity purified), the mouse monoclonal anti-phosphoSer239 VASP antibody 5C6, and the mouse monoclonal anti-phosphoSer239 VASP antibody 16C2 have been described previously (28, 29). The mouse monoclonal anti-vesicular stomatitis virus (VSV) antibody P5D4 was from Sigma, the anti-Myc epitope antibody was from Santa Cruz Biotechnology, and the rabbit polyclonal anti-C-terminal G-kinase I antibody was from StressGen.

The VASP constructs were derived from the human gene, and have been described previously (14, 27–29). They were generated using pcDNA3 (Invitrogen) as the expression vector, and are amino-terminal tagged with an epitope from VSV glycoprotein G recognized by antibody P5D4 (30); green fluorescent protein (GFP)-tagged VASP was generated in pEGFP-C2 (Clontech) (27). The human G-kinase I vector, the pRSV-β-Gal control vector, and the reporter constructs pERE-Luc and pCRE-Luc have been described previously (26, 31, 32). Expression vectors encoding C3 exoenzyme, mutant RhoA(63L), RhoA(14V), RhoA(19N), constitutively active Ga11 (Ga11(Q21L)), a truncated constitutively active RhoGEF (ΔN-ρ115-RhoGEF), and the Rho-kinase (ROK) catalytic domain, were from A. Hall, J. H. Brown, M. Simon, M. Hart, and K. Kaibuchi, respectively, and were used as described previously (5, 26, 33–36).

**Cell Culture and Transfections**—Rat C6 glioma cells, rat embryo fibroblast (REF) 52 cells, rat A7R5 embryonic aortic smooth muscle cells, and murine cardiac fibroblast (McFb) cells derived from VASP−/− mice (21) were routinely cultured in Dulbecco’s modified Eagle’s (DMEM) medium supplemented with 10% bovine serum. For transfection experiments, C6 and REF 52 cells were cultured in 24-well dishes to ~70% confluence, and were transfected with a total of 0.3 μg of DNA as previously described using LipofectAMINE™ Plus (Invitrogen) (32, 37). After a 1-h recovery period in full medium, cells were cultured in serum-free DMEM for 24 h, with some cultures receiving the indicated concentrations of 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) during the last 30 min (for examination of VASP phosphorylation) or 7 h (for reporter gene assays). For A7R5 and McFb cells, subconfluent cultures in 12-well cluster dishes were transfected with a total of 0.6 μg of DNA and 1.5 μl of LipofectAMINE™ (Invitrogen); the cells were then treated as described above. In the experiments shown in Fig. 2A, inset, and Fig. 6D, C6 cells were stimulated with 10% serum for the last 4 h.

**Reporter Gene Assays and Measurement of G-kinase Activity**—Firefly luciferase activity and β-galactosidase activity were measured as described previously with luciferase activity normalized to β-galactosidase activity (32). With the exception of C3 exosome, which inhibited β-galactosidase expression from pRSV-β-Gal and multiple other control reporter genes (as described under “Results”), β-galactosidase activities varied by less than 15% between different conditions within an experiment.

G-kinase activity was measured in cell extracts as previously described as the difference between phosphorylation of Kemptide in the presence and absence of 3 μM 8-Br-cGMP (33, 38). The assays were performed in the presence of the specific A-kinase inhibitor PKI, and were linear with time and protein concentration.

**Western Immunoblots and Immunoprecipitation Studies**—Western blots were generated and developed using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence as described previously (31).

For immunoprecipitation experiments, C6 cells were transfected with the indicated amounts of expression vectors for the EVH2 domain (VSV-tagged) in the absence or presence of GFP-tagged full-length VASP. Thirty-six hours later cells were lysed and proteins were immunoprecipitated using the mouse monoclonal anti-VSV antibody and protein G-agarose beads. Western blots were generated from the immunoprecipitates using the rabbit polyclonal anti-VASP antibody. Phalloidin Staining—REF 52 cells plated on glass coverslips coated with fibronectin (10 μg/ml) were starved for 48 h, and transfected as described above; 36 h later, the cells were fixed and stained as previously described using Texas Red-labeled phalloidin (1.50, Molecular Probes), and the mouse anti-VASP antibody followed by an fluorescent isothiocyanate-labeled goat anti-mouse antibody (31). Cells were visualized using a Zeiss fluorescence microscope.

**Statistical Analysis**—The data in bar graphs and G-kinase enzyme activity are presented as the mean ± S.D. of at least three independent experiments performed in duplicate. Blots or photographs represent data that were reproduced at least three times in independent experiments. Statistical analyses were performed using the Student’s t test, with p < 0.05 considered to indicate statistical significance.

**RESULTS**

**Effect of VASP and Different VASP Subdomains on SRE-dependent Transcription**—Along with other proteins and agents that deplete the G-actin pool and induce F-actin accumulation, VASP has been shown to induce SRE-dependent transcription in NIH 3T3 cells (6). We found that transfection of full-length VASP into serum-starved rat C6 glioma cells stimulated SRE-dependent transcription: as little as two to three times the amount of endogenous VASP increased SRE-dependent transcription with further increases in transcriptional rates occurring at higher amounts of transfected VASP (Fig. 1, A and B). To determine which regions in VASP were responsible for transcriptional activation, we expressed VASP subdomains in the C6 cells and found that the amino-terminal EVH1 domain (amino acids 1–133), which includes the vinculin and gxin binding site, had no effect on SRE-dependent transcription (Fig. 1A). The carboxyl-terminal EVH2 domain (amino acids 225–380), which includes the G- and F-actin binding motifs and the tetramerization region of VASP, was about 40% as effective as full-length VASP (Fig. 1A). Further truncation of the EVH2 domain to amino acids 259–380, which removes the G-actin binding region, reduced its effectiveness little (Fig. 1A); this construct can still bind F-actin, induce actin cross-linking in vitro, and localize to stress fibers in vivo (14). However, removal of the F-actin binding region at residues 259–277 yielded a construct (EVH2, amino acids 278–380) that no longer stimulated SRE-dependent transcription (Fig. 1A), and in fact inhibited transcription in a dose-dependent manner (Fig. 2, discussed below). These data suggest that the F-actin binding region of VASP is important for its induction of SRE-dependent transcription, but that other parts of VASP, in particular the proline-rich region, contribute to the effect of VASP, possibly through interaction with other proteins (12). The VASP constructs were expressed at relatively similar levels (Fig. 1C), and thus their differential effects on SRE-dependent transcription could not be attributed to variable expression; the isolated G- and F-actin binding regions consisting of amino acids 225–278 did not express sufficiently well to be used in these studies.

Similar differential effects on SRE-dependent transcription by full-length VASP, and the EVH1 and EVH2 domains were found in REF 52 cells, a fibroblastic cell line (data not shown). Compared with C6 cells, which as neuronal cells have a cortical
vasp, were measured in cell lysates as described under Experimental Procedures, and cells were harvested after 24 h of serum-starved C6 cells, we found that this construct inhibited SRE-dependent transcription in a dose-dependent manner, both in the absence and presence of exogenous VASP. In the former case, the EVH2-(278–380) construct inhibited basal SRE-luciferase activity to 44 ± 12% of control (p < 0.05), presumably by interfering with endogenous VASP (Fig. 2A, filled circles); in the latter case, the construct almost completely prevented the marked transcriptional activation caused by transfected VASP (Fig. 2A, open circles). Moreover, the EVH2-(278–380) construct had no effect on VASP expression levels in VASP-transfected cells (Fig. 2B). We excluded nonspecific effects of the EVH2-(278–380) construct by showing that it had no effect on cAMP stimulation of a cAMP-response element-containing reporter gene (pCRE-Luc, Fig. 2C, right panel), or on CCAAT-enhancer binding protein-β (C/EBP-β)-mediated stimulation of the SRE-dependent reporter gene (pSRE-Luc; Fig. 2C, left panel).

To determine whether the EVH2-(278–380) construct oligomerized with either endogenous or transfected VASP, we performed immunoprecipitation experiments using the anti-VSV antibody to pull down the VSV-tagged EVH2-(278–380) construct. To be able to identify both endogenous and transfected VASP separately, we used GFP-tagged VASP (which migrates on gel electrophoresis differently than wild type VASP). We found that both endogenous VASP and transfected VASP co-immunoprecipitated with the EVH2-(278–380) construct, whereas neither of them were precipitated in cells lacking VSV-tagged EVH2-(278–380) (Fig. 2D, compare lanes 2 and 4 with lanes 1 and 3).

Thus, the EVH2-(278–380) construct serves as a specific dominant negative inhibitor of the activation of SRE-dependent transcription by VASP. It appears to function by oligomerizing with full-length VASP, and thereby preventing normal tetramer formation. VASP oligomerization is known to be important for in vitro actin polymerization and binding of VASP to some ligands (11, 14, 17).

VASP Acts Downstream of RhoA in Activating SRE-dependent Transcription—Because Rho proteins regulate SRE-dependent transcription through alterations in actin dynamics and VASP is involved in actin polymerization, we asked where VASP acted relative to RhoA in stimulating SRE-dependent transcription. To address this question we performed the following experiments. First, we used the C3 exoyme that ADP-ribosylates and thereby inhibits RhoA. Co-transfection of the C3 exoyme with the SRE-luciferase reporter, a control β-galactosidase expression vector, and either VASP or constitutively active RhoA had no effect on VASP-induced relative

![Graph](image_url)

**Fig. 1. Effect of different VASP domains on SRE-dependent transcription and effect of VASP on stress-fiber formation.** C6 glioma cells were transiently transfected with the reporter pSRE-Luc, the control plasmid pRSV-β-Gal, and either empty vector (E.V.) or increasing amounts of expression vectors encoding VSV-epitope-tagged full-length VASP (wild type, WT), the EVH1 domain (amino acids 1–113), the full EVH2 domain (amino acids 225–380), a truncated EVH2 domain that eliminates the G-actin binding region (amino acids 259–380), or a further truncated EVH2 domain that has the coiled-coil region (amino acids 278–380). Transfections were performed in 24-well dishes as described under “Experimental Procedures,” and cells were harvested after 24 h of culture in serum-free DMEM. A, luciferase and β-galactosidase activities were measured in cell lysates as described under “Experimental Procedures,” with luciferase activity normalized to β-galactosidase activity. Relative luciferase activity in cells transfected with empty vector was assigned a value of 1. The triangles denote 50, 100, or 200 ng of the indicated expression vector. B and C, from cells transfected with the indicated amounts of VASP expression vectors, Western blots of cell lysates were generated using either a rabbit anti-VASP (B) or a mouse anti-VSV (C) antibody. D, REF 52 cells serum starved for 48 h were transfected with 100 ng of VSV-tagged wild type VASP, and 36 h later, they were fixed and stained with Texas Red-conjugated phalloidin and the anti-VSV antibody followed by an fluorescein isothiocyanate-labeled goat anti-mouse antibody.
VASP Activation Is Inhibited by G-kinase

Luciferase activity (Fig. 3A; normalized luciferase/β-galactosidase activity ratio). However, C3 exo-enzyme markedly inhibited RhoA activation of the SRE (Fig. 3A). We should note that C3 exo-enzyme did reduce basal and VASP-stimulated luciferase activity, but this occurred to a similar extent as it inhibited β-galactosidase expression from the control vector, suggesting a nonspecific inhibitory effect; further evidence for nonspecific inhibition by C3 exo-enzyme was that it inhibited several other control reporter genes including RSV-luciferase, CMV-luciferase, and a thymidine kinase promoter-driven luciferase. Because VASP-induced SRE-luciferase activity was much less sensitive to C3 exo-enzyme inhibition compared with RhoA-stimulated activity, these results suggest that the transcriptional effects induced by VASP in C6 cells are largely independent of RhoA. Consistent with this view, we found that expression of a dominant negative RhoA (RhoA(19N)) had no effect on VASP-induced SRE-luciferase activity (data not shown).

In the second set of experiments, we used the EVH2-(278–380) construct, and found that it caused a partial but significant decrease in SRE-dependent transcription stimulated by four different Rho- (or Rho effector)-mediated mechanisms: (i) a constitutively active form of the heterotrimeric G protein Go13 (Go13(QL)), a known Rho activator (41); (ii) an amino-terminal truncated p115-RhoGEF construct (ΔN-p115-RhoGEF), which we have previously shown activates Rho (26); (iii) a constitutively active RhoA (RhoA(14V)); and (iv) a constitutively active ROK, which is a downstream RhoA effector that can mediate activation of SRE-dependent transcription (26, 36) (Fig. 3B, compare filled and striped bars, transcriptional activation in the absence and presence of EVH2-(278–380), respectively). Similar results were obtained with the constitutively active RhoA(63L) (data not shown). The EVH2-(278–380) construct had no significant effect on expression levels of ΔN-p115-RhoGEF, RhoA(14V), or the ROK construct (Fig. 3C), and did not influence β-galactosidase activity from the control pRSV-β-Gal vector. These results suggest that VASP acts downstream of RhoA in activating SRE-dependent transcription. The fact that the EVH2-(278–380) construct only partially inhibited Rho-stimulated transcription could be related to incomplete inhibition of endogenous VASP, or to Rho activating transcription via both VASP-dependent and VASP-independent pathways (26, 42, 43).

RhoA and EVH2-(278–380) Effects in VASP-deficient Cells—To examine the transcriptional effect of RhoA and the effect of the EVH2-(278–380) construct in the absence of VASP, we performed experiments in VASP-deficient murine cardiac fibroblasts (MCFB cells derived from transgenic mice carrying a VASP null mutation (21)). In these VASP-deficient cells, expression of constitutively active Rho(14V) stimulated SRE-dependent transcription 10-fold, but in contrast to C6 cells, the EVH2-(278–380) construct had no effect on Rho(14V)-induced

Lanes 2-5, cells transfected with 100 ng of full-length wild type (set) VSV-VASP, and the indicated amounts of VSV-EVH2-(278–380). C, C6 cells were transfected with pRSV-β-Gal control vector and either pSRE-Luc (left panel) or pCRE-Luc (right panel), in the absence (+) or presence (−) of 150 ng of EVH2-(278–380) as indicated. In the left panel, cells were co-transfected with empty vector, or 2 or 10 ng of the CAAT-enhancer binding protein-β (C/EBP-β) to activate the SRE-dependent reporter. In the right panel, half of the cultures were treated for 5 h with 100 μM CPT-CAMP to activate the CRE-dependent reporter. D, C6 cells were transfected with empty vector (lanes 1 and 3), 100 ng of full-length GFP-tagged VASP (lanes 1, 2, 5, and 6), and/or 100 ng of VSV-tagged EVH2-(278–380) (lanes 2, 4, and 6). Forty-eight hours later cells were lysed with 5% of the lysate applied directly to the gel (lanes 5 and 6); to the remaining lysate anti-VASP antibody was added and immunoprecipitates were collected on Protein A-coated agarose beads. Washed immunoprecipitates were applied to the gel (lanes 1–4), and Western blots were generated using an anti-VASP antibody.
VASP acts downstream of RhoA in stimulating SRE-dependent transcription. Cells were transfected, serum starved, and processed as described in the legend to Fig. 1, with all cells receiving pSRE-Luc and pRSV-β-Gal. A, as indicated, C6 cells were co-transfected with 50, 100, or 200 ng of wild type VASP vector in the absence (bars with left diagonal stripes) or presence (filled bars) of 50 ng of an expression vector encoding C3 exoymse. Some cells received 50 ng of an expression vector for activated RhoA(63L) instead of VASP, in the absence (cross-hatched bar) or presence (filled bar) of 50 ng of the C3 exoymse vector. Relative luciferase activities were determined as described in the legend to Fig. 1. B, C6 cells were transfected with the indicated amounts of vectors encoding a constitutively active Gα13 (Gα13 QL), a constitutively active form of p115-RhoGEF (Myc-tagged ΔN-p115-RhoGEF), activated RhoA (EE-tagged RhoA(14V)), or the catalytic domain of Rho-kinase (Myc-tagged ROK-CD), in the absence (filled bars) or presence (right diagonal bars) of 150 ng of vector encoding VSV-tagged EVH2(278–380). Relative luciferase activity of cells transfected with empty vector in the absence of other constructs was assigned a value of 1. The difference between the absence and presence of the EVH2(278–380) construct was significant in all cases (p < 0.05). C, C6 cells were transfected with the indicated amounts of ΔN-p115-RhoGEF, RhoA(14V), or ROK-CD in the absence or presence of 150 ng of EVH2(278–380), and cell lysates were subjected to Western blot analysis using the following antibodies: anti-Myc epitope (to demonstrate expression of RhoGEF and ROK-CD); anti-VSV epitope (to demonstrate expression of EVH2(278–380)); or anti-RhoA (to demonstrate expression of mutant RhoA(14V), which migrates with a higher apparent molecular weight compared with endogenous RhoA; similar results were obtained for the RhoA(14V) construct using an anti-EE epitope antibody (not shown)). D, VASP-deficient MCFB cells were transfected in 12-well dishes with pSRE-Luc, pRSV-β-Gal, and either empty vector (E.V.), 50 ng of EE-tagged RhoA(14V), and/or 100 ng of wild type VASP; some cultures also received 300 ng of EVH2(278–380), as indicated. Relative luciferase activity was determined as described in panel B. E, cell lysates of VASP-deficient MCFB cells transfected as described in panel D were subjected to Western blotting using either an anti-VASP antibody (upper two panels; the antibody recognizes EVH2(278–380) much less efficiently than full-length VASP, therefore, the second panel was exposed longer), or an anti-EE antibody (lower panel, to demonstrate RhoA(14V) expression).
SRE-dependent transcription (Fig. 3D, compare filled and striped bars, transcriptional activation in the absence and presence of EVH2-(278–380), respectively). Transfection of exogenous VASP was titrated to induce SRE-luciferase activity similarly 10-fold, and effect of VASP was almost completely prevented by co-expression of the EVH2-(278–380) construct, clearly demonstrating the dominant negative mode of interference of this VASP fragment. Co-transfection of Rho(14V) and VASP produced an additive effect, and EVH2-(278–380) expression reduced transcription to the level observed in the presence of Rho(14V) alone. Expression levels of Rho(14V) and VASP were not influenced by EVH2-(278–380) (Fig. 3E). These results demonstrate that the dominant negative effect of the EVH2-(278–380) construct depends on the presence of VASP; they also show that RhoA can stimulate SRE-dependent transcription in the absence of VASP, i.e. through a VASP-independent pathway, but that VASP clearly enhances RhoA-induced SRF activation.

Effect of cGMP/G-kinase on RhoA- and VASP-stimulated SRE-dependent Transcription—We have shown previously that the cGMP/G-kinase signal transduction pathway inhibits RhoA activation of SRE-dependent transcription, and that cGMP/G-kinase act both upstream of RhoA, reducing RhoA activation, and at an unknown point downstream of the RhoA effectors ROK, PKN, and PRK-2 (26). Because VASP appears to act downstream of RhoA and ROK in stimulating SRE-dependent transcription and because VASP phosphorylation decreases its F-actin binding and polymerization activities in vitro (17, 27), we asked whether cGMP/G-kinase affected VASP-induced SRE-dependent transcription. We chose C6 glioma cells for these studies because they contain extremely low endogenous G-kinase activity, and they, therefore, provide the opportunity to determine whether the effects of cGMP require G-kinase, or could be attributable to other cGMP effectors, or cross-activation of A-kinase. Transfection of 50–100 ng of G-kinase Iα expression vector produced specific activities similar to those found in smooth muscle cells (0.39 ± 0.04 nmol/min/mg protein for C6 cells transfected with 100 ng of G-kinase vector (23, 38); see Fig. 7A for a direct comparison with A7R5 cells). As we reported previously (26), G-kinase activation by cGMP inhibited RhoA(63L)-induced SRE-dependent transcription by ~50% in C6 cells (Fig. 4, in left half of figure compare cross-hatched bar to left diagonally striped bar; all cells were transfected with G-kinase Iα). Similar results were obtained with a different constitutively active RhoA mutant, RhoA(14V) (Fig. 6D). When the cells were transfected with VASP, G-kinase activation reduced VASP-stimulated transcription by about 50% (Fig. 4, in right half of figure compare filled bar to right diagonally striped bar). At the amounts of DNA transfected in these experiments, the combination of RhoA(63L) and VASP on SRE-dependent transcription was approximately additive, and again, cGMP/G-kinase inhibited transcription by about 50% (Fig. 4, right half of figure; compare cross-hatched bar to left diagonally striped bar).

To study the effect of cGMP and G-kinase on VASP-stimulated transcription further, we performed dose response experiments (Fig. 5). Transfecting increasing amounts of G-kinase Iα into C6 cells caused a small decrease in VASP induction of SRE-dependent transcription that reached statistical significance at 100 ng of G-kinase vector (Fig. 5A, filled circles; all cells were transfected with 150 ng of VASP vector). These results likely reflect low intracellular cGMP concentrations in serum-starved cells. When the same experiment was performed in the presence of CPT-cGMP (100 μM), transfection of as little as 12.5 ng of G-kinase Iα vector significantly inhibited VASP-stimulated transcription, with maximal inhibition observed at 25–50 ng of G-kinase DNA (Fig. 5A, open circles). This effect was not because of interference with VASP expression (Fig. 5B, anti-VSV blot). G-kinase and A-kinase phosphorylate VASP on three sites, Ser157, Ser239, and Thr278, with Ser239 (a phosphorylation site-specific antibody for Thr 278 is available), Thr278, and Ser157 being the preferred G-kinase site (10). Using phosphorylation site-specific antibodies against VASP Ser157 and Ser239 (a phosphorylation site-specific antibody for Thr278 is not available), we found a modest increase in phosphorylation of the two serine residues on adding increasing amounts of G-kinase Iα in the absence of CPT-cGMP that became significant at 100 ng of G-kinase vector (Fig. 5B, left panels). Marked increases in phosphorylation of both sites were observed in the presence of CPT-cGMP with as little as 12.5 ng of G-kinase vector DNA, and maximal effects at 25–50 ng of vector (Fig. 5B, right panels, upper two blots; note the well documented gel shift of VASP to an apparently higher molecular weight on phosphorylation of Ser157, with the phospho-Ser157-specific antibody recognizing only the shifted upper band (10)). When we performed similar experiments but varied the concentration of CPT-cGMP while keeping the amount of G-kinase constant, we found a small but significant decrease in SRE-dependent transcription at the highest CPT-cGMP concentration (250 μM) in the absence of G-kinase (possibly because of cross-activation of A-kinase, Fig. 5C, filled circles), and marked inhibition in the presence of G-kinase (Fig. 5C, open circles; all cells were transfected with 150 ng of VASP vector). Consistent with the possibility of A-kinase cross-activation at high CPT-cGMP concentrations, we found some phosphorylation of VASP Ser157 (which is the preferred A-kinase phosphorylation site) but little phosphorylation of VASP Ser239 in the absence of G-kinase (Fig. 5D, left panels). In the presence of 25 ng of G-kinase, significant phosphorylation of both VASP Ser157 and Ser239 occurred with 10 μM CPT-cGMP, with maximal phosphorylation at 100 μM of the drug (Fig. 5D, right panels).

Taken together, these results suggest that G-kinase phosphorylation of VASP inhibits the ability of VASP to increase
SRE-dependent transcription. Of note, even in the presence of maximal amounts of G-kinase and CPT-cGMP, VASP phosphorylation at Ser\(^ {157} \) reached only about 60–70% of total VASP, as evident by the gel shift. This is similar to the maximal cGMP-induced VASP phosphorylation observed in intact platelets (10).

Analysis of VASP Phosphorylation Sites That Mediate G-kinase Inhibition of SRE-dependent Transcription—Because cGMP/G-kinase inhibition of VASP-stimulated transcription correlated with increased phosphorylation of serine residues 157 and 239, we studied the effects of cGMP/G-kinase on VASP mutants with individual and combined mutations at its three phosphorylation sites. We found that mutation of VASP Ser\(^ {157} \) or Thr\(^ {278} \) to Ala prevented to a small, statistically non-significant degree the ability of cGMP to inhibit the transcriptional effect of VASP; cGMP inhibited reporter gene activity induced by wild type VASP, VASP-Ala\(^ {157} \), and VASP-Ala\(^ {278} \) by 45 ± 8, 34 ± 6, and 40 ± 9%, respectively (Fig. 6A, all cells expressed G-kinase Ia). Mutation of VASP Ser\(^ {239} \) to Ala caused considerably more resistance to cGMP inhibition than mutation of either of the other two sites: cGMP inhibited SRE-dependent transcription induced by the VASP-Ala\(^ {239} \) mutant by only 20 ± 6% (Fig. 6A). The comparison between the effect of cGMP on wild type VASP versus mutant VASP-Ala\(^ {239} \) was statistically significant with a p value of <0.01; reporter gene activity induced by the VASP-Ala\(^ {239} \) mutant alone was still significantly (p < 0.05) inhibited by cGMP. Mutation of both Ser\(^ {157} \) and Ser\(^ {239} \) to Ala, or of all three phosphorylation sites to Ala, rendered VASP essentially unresponsive to cGMP/G-kinase inhibition (Fig. 6A; for VASP-AAT and VASP-AAA, there was no significant difference (p > 0.5) between the absence and presence of cGMP). Cyclic GMP alone had no effect on reporter gene activities induced by wild type or mutant VASP in the absence of G-kinase (data not shown). The VASP mutants were all expressed at similar levels with cGMP/G-kinase having no effect on expression levels (Fig. 6B, anti-VSV blot). Western blots with antibodies specific for phospho-Ser\(^ {157} \) and phospho-Ser\(^ {239} \) demonstrated that VASP-Ala\(^ {157} \) and VASP-Ala\(^ {239} \) mutants were lacking phosphorylation by cGMP/G-kinase at their respective mutated sites but were normally phosphorylated at the other site (Fig. 6B; the small amount of phospho-Ser\(^ {239} \) observed in cells transfected with VASP-Ala\(^ {239} \) was likely because of phosphorylation of endogenous VASP). These results suggest that the cGMP inhibition of the transcriptional effect of VASP is mediated primarily by G-kinase phosphorylation of Ser\(^ {239} \), with some contribution of Ser\(^ {157} \) phosphorylation.

To obtain further evidence for the effect of phosphorylation of VASP on its ability to activate SRE-dependent transcription, we studied a VASP mutant with all three phosphorylation sites mutated to aspartic acid residues, a change that should simulate a fully phosphorylated protein. The VASP-DDD mutant induced only a modest increase in SRE-dependent transcription, and it was refractory to the effects of cGMP/G-kinase (Fig. 6A). The DDD mutant was expressed at slightly lower levels than wild type VASP (Fig. 6C), but even when the effect of 200 ng of VASP-DDD was compared with 100 ng of wild type VASP, the DDD mutant induced reporter gene activity about half as efficiently as wild type VASP that was phosphorylated in the presence of cGMP/G-kinase (Fig. 6A). This may be explained by the fact that wild type VASP was only partially phosphorylated when G-kinase was activated in vivo (as evidenced by the incomplete gel shift in Fig. 6C indicating the presence of unphosphorylated Ser\(^ {157} \), Ser\(^ {239} \), and Thr\(^ {278} \)) (9, 10).

Because SRE-dependent transcription induced by VASP was inhibited by cGMP/G-kinase and VASP appears to mediate some transcriptional effects of RhoA, it is possible that VASP

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Fig. 5. Dose response of G-kinase and cGMP effects on VASP-stimulated SRE-dependent transcription. C6 cells were transfected with pSRE-Luc, pRSV-β-Gal, and 150 ng of VSV-epitope-tagged VASP. In panels A and B, cells were co-transfected with the indicated amounts of G-kinase Ia expression vector (PKG) and were treated with 100 μM CPT-cGMP; in panels C and D, cells were co-transfected with 25 ng of the G-kinase vector and treated with the indicated concentrations of CPT-cGMP. In panel A, open and closed circles represent cells cultured in the presence and absence of 100 μM CPT-cGMP, respectively, and in panel C, open and closed circles represent cells transfected with 25 ng of G-kinase or empty vector, respectively. Relative luciferase activity was determined as described in the legend to Fig. 1 with cells transfected with empty vector and no VASP assigned a value of 1. In panels B and D, Western blots were generated from cell lysates using antibodies specific for VASP phospho-Ser\(^ {157} \) or phospho-Ser\(^ {239} \). Blots generated with anti-VSV antibodies demonstrate the amount of transfected VASP, and blots with a G-kinase specific antibody demonstrate the amount of transfected G-kinase Ia.
cultures were left untreated (H9262), and half received 100 ng constructs: wild type VASP (WT); VASP (Ala157); VASP (Ala239); VASP (Ala278); VASP Ser157 and Ser239 mutated to Ala (AAT); VASP Ser157, Thr278 mutated to Ala (AAA); or VASP Ser157, Ser239, and Thr278 mutated to Asp (DDD). Cells were additionally transfected with pSRE-Luc, pRSV-β-Gal, and G-kinase In (25 ng), and were serum starved as described under "Experimental Procedures." A, half of the cultures were left untreated (filled bars), and half received 100 μM CPT-cGMP 7 h prior to extraction (cross-hatched bars). Relative luciferase activities were determined as described in the legend to Fig. 1 with untreated cells not receiving VASP assigned a value of 1. cGMP treatment had no effect on VASP-stimulated reporter gene activity in the absence of G-kinase (not shown). B, as indicated, cells were transfected with the VASP constructs described in panel A, with some co-transfected with 25 ng of G-kinase In, and some treated with 100 μM CPT-cGMP for 30 min prior to extraction. Lysates were subjected to Western blotting using VASP phospho-Ser157 and phospho-Ser239-specific antibodies or anti-VSV antibody. On the Western blot developed with anti-phospho-Ser239 antibody, a double band of wild type and mutant VASP was present in cells co-transfected with G-kinase and treated with cGMP, but it was not well separated. E.V., empty vector. C, cells were transfected with 25 ng of G-kinase and the indicated amount of wild type or DDD mutant VASP expression vectors as described in panel A; they were treated with 100 μM CPT-cGMP and phosphorylation by G-kinase is linked to the cGMP inhibition of serum- and RhoA-induced SRE-dependent transcription. To test this hypothesis, we examined the effect of overexpressing the phosphorylation-deficient VASP-AAA mutant on cGMP/G-kinase inhibition of serum- and RhoA-stimulated transcription. As previously described (26), cGMP inhibited serum- and RhoA(14V)-induced pSRE-luciferase activity by 45 ± 9% and 35 ± 5%, respectively, in G-kinase-expressing C6 cells (Fig. 6D, filled bars); however, when cells were co-transfected with VASP-AAA, this cGMP-mediated inhibition was reduced to 25 ± 9 and 10 ± 6%, respectively (Fig. 6D, striped bars). These results suggest that overexpression of VASP-AAA can protect serum- and RhoA-induced SRE-dependent transcription from inhibition by cGMP, and support the notion that at least some of the downstream effects of cGMP on RhoA are mediated by VASP phosphorylation.

Effect of cGMP on VASP-stimulated SRE-dependent Transcription in Vascular Smooth Muscle Cells—A7R5 cells are rat embryonic aortic smooth muscle cells. They express endogenous G-kinase at levels similar to those found in C6 cells transfected with 50–100 ng of G-kinase vector (Fig. 7A, transfection efficiency of C6 cells is about 50%), and contain similar G-kinase specific activity (0.34 ± 0.03 nmol/min/mg protein in subconfluent A7R5 cells); the specific activity is comparable with that found in other cells and tissues (26, 38). In A7R5 cells, cGMP caused a small but significant reduction in SRE-dependent transcription (Fig. 7B, cells transfected with empty vector, compare cross-hatched bars to filled bars for cells cultured in the presence or absence of 100 μM CPT-cGMP, respectively). As in C6 cells, transfection of VASP into A7R5 cells increased transcription in a dose-dependent manner, and cGMP significantly inhibited VASP-stimulated transcription (Fig. 7B, cells transfected with wild type VASP). Even at the lowest amount of transfected DNA, VASP levels were severalfold higher than endogenous VASP, and cGMP had no effect on expression levels other than than inducing the expected gel shift because of Ser157 phosphorylation (Fig. 7C, anti-VASP blot). As in C6 cells, the VASP-Ala239 mutant exhibited less inhibition by cGMP than wild type VASP, and the triple alanine mutant showed virtually no inhibition by cGMP (Fig. 7B). Phosphorylation of Ser157 and Ser 239 of endogenous VASP was detectable in cells treated with cGMP, but as one would anticipate, the signal was much stronger in cGMP-treated cells transfected with wild type VASP (Fig. 7C). The apparent phosphorylation induced by cGMP of Ser239 in cells transfected with the VASP-Ala239 mutant, and of both Ser157 and Ser239 in cells transfected with the triple alanine mutant, corresponded to phosphorylation of endogenous VASP (Fig. 7C). Thus, in cells containing endogenous G-kinase, cGMP inhibited VASP activation of SRE-dependent transcription, requiring G-kinase phosphorylation of Ser239 with phosphorylation of Ser157 and/or Thr278 contributing to the inhibition.

DISCUSSION

The transcription factor SRF is regulated by changes in actin dynamics (6) and VASP plays an important role in regulating actin dynamics in many cell types; VASP is a prominent

Western blots were developed with anti-VSV antibody. D, C6 cells were transfected with pSRE-Luc, pRSV-β-Gal, and G-kinase as described above; cells additionally received 25 ng of Rho(14V) or were serum-stimulated for 4 h to induce SRE-luciferase activity. As indicated, cells were co-transfected with 150 ng of either empty vector (filled bars) or vector encoding VASP-AAA (striped bars). G-kinase was activated by treating half of the cultures with 100 μM CPT-cGMP for 7 h, and relative luciferase activities were determined as described in the legend to Fig. 1. Data are expressed as the percent inhibition of relative luciferase activity caused by cGMP.
published a study on the role of VASP in SRE-dependent transcription, in which they also found that the F-actin binding portion of the EVH2 domain is important for activation of SRF by VASP, and they confirmed that the EVH2 domain mediates localization of VASP to F-actin structures and induction of F-actin assembly in NIH 3T3 cells (14). Similar conclusions concerning the importance of Mena’s EVH2 domain and actin binding region were reached by Loureiro et al. (46) studying Mena function during cell migration.

The distal part of the EVH2 domain contains the region responsible for tetramerization of VASP, and constructs encoding this region have been proposed to act as dominant negative proteins because they interfere with various VASP functions including F-actin assembly and formation of intercellular adhesion zippers (14, 15, 19). However, previously neither dose dependence nor specificity of the observed effects, nor direct physical interaction in vivo of these VASP fragments with wild type VASP were demonstrated. We showed that expression of EVH2-(278–380) dose dependently inhibited SRE-dependent transcription induced by exogenous VASP, serum, Rho activators, and constitutively active RhoA. In accordance, Grosse et al. (45) found inhibition of VASP- and serum-induced SRF activity by a similar construct comprised of the distal EVH2 domain. They also observed inhibition of SRF activity by a full-length VASP construct containing only a deletion in the F-actin binding region (45). We found no effect of EVH2-(278–380) on CRE-dependent transcription, nor on C/EBP-β-induced SRE-dependent reporter gene activity, excluding nonspecific effects. Moreover, Rho-induced SRE-dependent transcription was refractory to interference by EVH2-(278–380) in VASP-deficient cells, indicating that the effect of this VASP fragment depends on the presence of VASP and thus represents a specific dominant negative effect. The VASP—/ cells express normal amounts of Mena and Evl (21), suggesting that these two VASP family members either were not efficiently inhibited by the VASP-derived EVH2-(278–380) construct, and/or differ functionally from VASP and are not involved in Rho-dependent transcription. Clearly, RhoA could activate SRE-dependent transcription in VASP-deficient cells, although expression of VASP enhanced the transcriptional effect of Rho significantly. Multiple Rho effector pathways activate SRF, and importantly, Rho signaling to SRF can occur in the absence of RhoA-induced cytoskeletal changes (26, 36, 42, 47, 48). It is tempting to speculate that those Rho effector pathways that involve changes in actin dynamics may require VASP function, whereas others that do not involve cytoskeletal changes may not. We observed that ROK-induced transcription was inhibited by the dominant negative EVH2-(278–380) construct, whereas Grosse et al. (45) found that transcription induced by the diaphanosus-related protein mDia1 was inhibited by a dominant negative VASP; the Rho effectors ROK and mDia1 both induce changes in actin dynamics and cooperate to regulate SRF activity (36, 43). Thus, we conclude that VASP is downstream of RhoA, but that VASP is not absolutely required for RhoA activation of SRE-dependent transcription.

Tetramerization of VASP occurs via a coiled-coil arrangement and tetramers demonstrate extraordinary thermodynamic stability (15), suggesting that pre-formed VASP tetramers may be difficult to disrupt in intact cells. However, we showed in co-immunoprecipitation studies that the distal EVH2 domain bound in vivo to both endogenous and transfected VASP, and we obtained further evidence for the presence of mixed wild type/mutant VASP oligomers among cell extracts by nondenaturing gel electrophoresis and gel filtration
chromatography.\textsuperscript{2} Formation of wild type/mutant heteromers \textit{in vivo} may occur preferentially with newly synthesized VASP, \textit{i.e.} directly post-translational. This could explain why EVH2-(278–380) was a more potent inhibitor of the actions of cotransfected VASP compared with endogenous VASP. The fact that the EVH2-(278–380) construct only partially inhibited RhoA-induced SRE-dependent transcription could indicate persistence of intact endogenous VASP tetrammers, in addition to the existence of both VASP-dependent and VASP-independent RhoA effector pathways.

VASP oligomerization is important \textit{in vitro} binding, nucleation, and cross-linking of F-actin (14, 16, 17). VASP and Mena oligomerization is also required for proper subcellular localization, and for full function in epithelial cell-cell adhesion and fibroblast motility assays (19, 46). The exact mechanism whereby the EVH2-(278–380) construct disrupts wild type VASP function requires further studies, but our results and those of Grosse \textit{et al.} (45) suggest that the dominant negative effect is observed when mixed tetramers are formed that lack the F-actin binding region on some monomers. We speculate that the F-actin binding site in 4-fold copy in a \textit{trans}-configuration may be necessary for efficient VASP functions that require multivalent binding of interaction partners, \textit{i.e.} for enhancement of F-actin polymerization, SRE-dependent transcription, and cellular motility. Our results suggest that the transcriptional effects of VASP do not require RhoA, because C3 exonuclease, which potently inhibited RhoA(63L) activation of SRE-dependent transcription, did not specifically reduce VASP stimulation of SRE-dependent transcription. Because C3 exonuclease decreased basal and VASP-stimulated SRE-dependent transcription non-specifically to the same extent as it decreased expression of several different control reporter genes, it appears that decreasing basal Rho activity may interfere with transcription in a general, nonspecific fashion. In contrast, Grosse \textit{et al.} (45) reported that C3 exonuclease inhibited the transcriptional effects of full-length VASP in NIH 3T3 cells by almost 90\%, whereas transcription induced by the isolated VASP EVH2 domain was largely uninhibited by C3 exonuclease. Because the isolated EVH2 domain was about half as effective as full-length VASP in activating SRF, it is unclear why both constructs should be differentially sensitive to C3 exonuclease inhibition. However, cell types may differ with respect to the degree of cooperativity between VASP-dependent and VASP-independent pathways downstream of RhoA required for optimal SRF activation. Thus, it is possible that NIH 3T3 cells have a high degree of cooperativity and therefore, Rho inhibition by C3 exonuclease could decrease VASP-induced SRF activity by inhibiting the VASP-independent pathway.

VASP was initially identified as a protein rapidly phosphorylated in platelets in response to cGMP- or cAMP-elevating agents (9, 49). Phosphorylation of VASP selectively changes its interaction with some structures but not others, \textit{e.g.} VASP phosphorylation \textit{in vivo} decreases its association with focal adhesions but not with cell-cell contacts, and VASP phosphorylation by A-kinase induces its dissociation from c-Abl, but not from profilin (12, 29, 50). Phosphorylation of VASP on Ser\textsuperscript{157} and Ser\textsuperscript{239} inhibits its ability to nucleate actin polymerization and bundle actin filaments \textit{in vitro} (17, 27). We found that VASP phosphorylation on Ser\textsuperscript{157} and Ser\textsuperscript{239} inhibited its ability to induce SRE-dependent transcription, because mutation of these two sites to alanines rendered the mutant protein completely resistant to the inhibitory effects of G-kinase (or A-kinase).\textsuperscript{2} Of these two serines, Ser\textsuperscript{239}, which is nearest to the G-actin binding region of the EVH2 domain, was more critical to the effect of G-kinase than Ser\textsuperscript{157}, which is near the profilin binding site in the proline-rich domain (11, 12). Consistent with our results, phosphorylation of VASP at Ser\textsuperscript{239} only modestly reduces F-actin binding \textit{in vitro}, whereas additional phosphorylation at Ser\textsuperscript{157} reduces F-actin binding much more dramatically (27). Because expression of the phosphorylation-deficient VASP-AAA mutant largely protected cells from cGMP/G-kinase-mediated inhibition of serum- or RhoA-induced transcription, we conclude that VASP mediates, at least in part, the G-kinase inhibition of SRE-dependent transcription that occurs downstream of RhoA (26).

The inability of the phosphomimetic VASP DDD mutant to stimulate transcription is consistent with the view that phosphorylation inactivates VASP functions required for SRE-dependent transcription \textit{in vivo}. In contrast, phosphorylation of Mena on the amino-terminal protein kinase AG/G-kinase phosphorylation site corresponding to Ser\textsuperscript{157} plays a positive role in the regulation of cell motility, because an alanine mutation causes a loss-of-function phenotype, whereas an aspartic acid substitution at the same site functions similarly as wild type VASP (46).

In G-kinase-deficient C6 cells we found only minimal effects of CPT-cGMP on VASP-induced transcription, and these occurred only at high CPT-cGMP concentrations. When physiologically relevant amounts of G-kinase were transfected into the cells, low concentrations of CPT-cGMP significantly inhibited the effect of VASP on SRE-dependent transcription. We conclude that the effects of cGMP require G-kinase and do not involve other cGMP effector proteins or cross-activation of A-kinase. Studies with ATR5 smooth muscle cells confirmed that cGMP-mediated inhibition of VASP-stimulated transcription occurred in cells expressing endogenous G-kinase.

Whereas Grosse \textit{et al.} (45) reported that mutation of the three VASP phosphorylation sites to alanines had no effect on the ability of VASP to activate SRF, they did not study the consequences of phosphorylation of these sites, nor did they study the role of G-kinase in the RhoA-VASP-SRE pathway. Thus, while our study and the study of Grosse \textit{et al.} (45) reach several of the same conclusions, our work provides information about the importance of VASP phosphorylation and cGMP/G-kinase in regulating SRE-dependent transcription.

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Vasodilator-stimulated Phosphoprotein Activation of Serum-response Element-dependent Transcription Occurs Downstream of RhoA and Is Inhibited by cGMP-dependent Protein Kinase Phosphorylation
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