Vasodilator-Stimulated Phosphoprotein Is Involved in Stress-Fiber and Membrane Ruffle Formation in Endothelial Cells

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Abstract—Vasodilator-stimulated phosphoprotein (VASP) is highly expressed in vascular endothelial cells, where it has been implicated in cellular reorganization during angiogenesis, as well as in endothelial retraction and changes in vessel permeability. However, the cellular functions of VASP are not known. In this study, we have expressed wild-type and mutant forms of VASP in endothelial cells to determine in what aspects of cytoskeletal behavior this protein participates. Expression of wild-type VASP induces marked membrane ruffling and formation of prominent stress fibers in bovine aortic endothelial cells. Deletion of the proline-rich domain of VASP abolishes its ability to bind profilin but does not affect ruffling or stress fiber formation. Further deletions reveal a sequence within the carboxy-terminal domain that is responsible for in vivo bundle formation. Ruffling occurs only on the expression of forms of VASP that possess bundling activity and the capacity to bind zyxin/vinculin-derived peptide. The ability of distinct subdomains within VASP to bind adhesion proteins and induce F-actin bundling in vivo suggests that this protein could function in the aggregation and tethering of actin filaments during the formation of endothelial cell–substrate and cell-cell contacts. These data provide a mechanism whereby VASP can influence endothelial migration and organization during capillary formation and modulate vascular permeability via effects on endothelial cell contractility. (Arterioscler Thromb Vasc Biol. 2000;20:2051-2056.)

Key Words: endothelium | vasodilator-stimulated phosphoprotein | actin | angiogenesis | vasodilators
VASP-deficient animals, is functional compensation by other members of the Ena/VASP family. Indeed, VASP has been shown to be able to substitute for Ena in Ena-null mutants of Drosophila. Therefore, as an alternative approach to gain insight into the functions of VASP in vascular endothelial cells, we have constructed and expressed a series of deleted forms of the protein. The effects of these constructs on the endothelial cytoskeleton were examined. We show that VASP induces membrane ruffling and stress-fiber formation in endothelial cells. Use of the deletion mutants allowed definition of specific regions within VASP participating in these activities. Our data provide the first indication of functions for VASP in vascular endothelial cells and are consistent with the involvement of this protein in endothelial movement and retraction.

Methods

Materials

Generation of full-length human VASP has been described previously. Deleted forms of VASP were generated by using existing restriction sites or introducing appropriate new restriction sites by site-directed mutagenesis with use of the Altered Sites Mutagenesis System (Promega Ltd), as per the manufacturer’s directions. For expression, VASP constructs were ligated into the pFLAG epitope-tag vector (Sigma-Aldrich). Profilin II cDNA was a kind gift from Dr Henrik Leffers, Aarhus University, Aarhus, Denmark. The sequences of all clones were confirmed by using a 373A ABI automated DNA sequencer. A monoclonal antibody recognizing VASP was obtained from Transduction Laboratories. All other reagents were from sources described previously.

Binding Studies

Radiolaabeled VASP and deleted forms of VASP were tested for their ability to bind immobilized FP, peptide or control peptide coupled to Actigel (Sterogene), glutathione-S-transferase (GST), GST-profilin, or GST full-length VASP. The FP2 peptide (839-EPDFPPPPPDPDE-850) encompassing the VASP binding motif from vinculin and the control peptide that was derived from an adjacent proline-rich region of vinculin (859-APPKPLPEGEVPPPRPPPPE-879) have been described previously. Radiolaabeled VASP and deleted forms of VASP were produced by using T7 RNA polymerase in a rabbit reticulocyte lysate in vitro transcription/translation system (Promega Ltd) in the presence of [35S]methionine and [35S]cysteine with a specific activity of 37 TBq/mmol (Trans3S-Label, ICN Biomedicals), as per the manufacturer’s instructions. Binding studies were performed as described previously. Bound proteins were eluted into Laemmli sample buffer, containing 100 mmol/L dithiothreitol, and separated by SDS-PAGE. Fluorography was as described previously.

Cell Culture and Transfection

Bovine aortic endothelial cells were obtained as described previously and cultured in DMEM containing 10% FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin under 5% CO2/95% air in a humidified incubator at 37°C. Cells were used between passages 3 and 7. For immunofluorescence studies, cells were plated onto sterile glass coverslips. For transfection, cells were plated at 40% to 50% confluence 24 hours before transfection. Cells were then washed with PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, and 1.8 mmol/L KH2PO4, pH 7.3) and transfected with 2.5 μg DNA and 10 μL Superfect (Qiagen Ltd) in 35-mm dishes, as detailed in the manufacturer’s instructions. After transfection, cells were washed in PBS and maintained for 24 hours in complete growth medium. In some experiments, cells were serum-starved for 24 hours before use.

Immunoprecipitation and Western Blotting

Cells were rinsed in PBS and lysed by the addition of ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin). Lysates were cleared by centrifugation at 12000 g for 10 minutes and then incubated for 2 hours or overnight at 4°C with anti-FLAG monoclonal antibody and protein G–agarose beads. Immune complexes were washed extensively with lysis buffer, and proteins were eluted by boiling in SDS-sample buffer in the presence of 10% glycerol and 2% SDS. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Epitope-tagged VASP was detected by probing the membranes with monoclonal antibody recognizing the FLAG epitope tag. Immunoreactive bands were detected with a peroxidase-conjugated secondary antibody and the ECL chemiluminescent detection system (Amersham International Plc).

Immunofluorescence Microscopy

Immunofluorescence was essentially as described by Symons and Mitchison. Cells were washed in PBS and fixed by incubation for 15 minutes at room temperature in 4% formaldehyde in cytoskeletal buffer (10 mmol/L MES, pH 6.1, 138 mmol/L KCl, 3 mmol/L MgCl2, 2 mmol/L EGTA, and 0.32 mol/L sucrose). Permeabilization was accomplished by incubation for 10 minutes at room temperature in cytoskeletal buffer containing 0.5% Triton X-100. Cells were washed with cytoskeletal buffer containing 0.1% Triton X-100 and blocked for 10 minutes in antibody buffer (137 mmol/L NaCl, 25 mmol/L Tris, pH 7.4, 2.7 mmol/L KCl, 0.1% Triton X-100, and 2% BSA). Coverslips were incubated at 37°C with antibody buffer and primary antibody. After 60 minutes, coverslips were washed extensively with antibody buffer before incubation with fluorescently labeled secondary antibodies at 37°C. After 45 minutes, coverslips were washed extensively. After FLAG immunodetection, filamentous actin was stained by probing with fluorescein-conjugated phalloidin at 2 μg/mL in cytoskeletal buffer for 30 minutes before extensive washing in antibody buffer and mounting in 220 mmol/mL diazobicyclo-octane in 90% (vol/vol) glycerol and 10% (vol/vol) PBS, pH 8.6. Cells were viewed by use of a Bio-Rad MRC 600 Laser Scanning Confocal Microscope.

Results

Expression of VASP Induces Ruffling and F-Actin Bundle Formation in Endothelial Cells

Indirect evidence suggests that VASP is involved in some aspect of cytoskeletal dynamics in vascular endothelial cells. To gain insight into the functions of VASP in the endothelium, bovine aortic endothelial cells were transfected with constructs encoding wild-type and mutant forms of the protein, and F-actin organization was examined. The distribution of expressed wild-type VASP, examined in cells by immunofluorescence microscopy of epitope-tagged protein, reveals a distribution similar to that of endogenous VASP, being observed in focal adhesions, along stress fibers, and in lamellipodia (Figure 1A). In all figures depicting transfected cells expressing VASP constructs, the expressing cells are revealed by anti-FLAG immunofluorescence and shown in the right panels to allow location of the relevant cells in the F-actin–stained field. Examination of the F-actin organization in endothelial cells reveals the nontransfected cells to have fine bundles of actin filaments and minimal membrane ruffling, as shown in Figure 1B. In contrast, transfected cells expressing full-length VASP show conspicuous membrane ruffles and prominent thick stress fibers (Figure 1B). These data indicate that increased expression of full-length VASP leads to the induction of marked ruffling and filament bundling in endothelial cells. On the basis of fluorescence
intensity of transfected and nontransfected cells after staining with antibodies against VASP, the expression level of VASP in transfected cells was determined to be, on average, between 4- and 6-fold that of the endogenous protein. In the experiments described in the present study, subconfluent cells were used partly to allow examination of peripheral ruffling. However, VASP-overexpressing cells can achieve confluence and continue to demonstrate prominent actin stress fibers (data not shown). As detailed earlier, VASP is known to bind profilin, FP4-containing cytoskeletal proteins, and full-length VASP. Therefore, deletion mutants of VASP were used to test which, if any, of these interactions were required for the ruffling and stress fiber–inducing effects of the full-length protein.

**Binding Interactions of Deleted Forms of VASP**

The structure of VASP and deletion mutants used in the present study are shown schematically in Figure 2. Before examination of their effects on the cytoskeleton, the ability of wild-type and deleted forms of VASP to bind to FP4 peptide, profilin, and full-length VASP was analyzed (Figure 3). Full-length VASP, the EVH-1 domain, and ΔGP5 VASP were produced by in vitro transcription/translation and tested for their capacity to bind immobilized FP4 peptide and control peptide. As shown in Figure 3A, full-length, EVH-1, and ΔGP5 VASP all bind to FP4 peptide but not appreciably to control peptide. For examination of binding to profilin and VASP, full-length, ΔGP5, and ΔC VASP were produced by in vitro transcription/translation, and binding to immobilized GST-profilin or GST-VASP was assessed; nonspecific binding was assessed by using immobilized GST (Figure 3B). Full-length VASP binds profilin II; however, ΔGP5 and ΔC

Figure 1. Actin filament reorganization is induced by expression of VASP in endothelial cells. A, Distribution of endogenous VASP and expressed epitope-tagged VASP in endothelial cells. Endogenous VASP was detected by using a monoclonal antibody against VASP antibody (VASP). For expressed VASP, endothelial cells were transfected with plasmids encoding full-length (FL) VASP, and the distribution of expressed protein was determined by detection with an antibody recognizing the FLAG epitope tag (FLAG). The optical section reveals distribution to focal adhesions, stress fibers, and cell perimeter. B, Endothelial cells transfected with plasmids expressing FL VASP, as indicated. F-actin organization was determined by staining with FITC-phalloidin (actin), and cells expressing the VASP constructs were identified by anti-FLAG immunofluorescence (FLAG). Examples of the fine-filament bundles in cells not expressing transfected VASP are indicated with arrows, and basal ruffling is indicated by an arrowhead. In the expressing cell, an example of a prominent stress fiber is indicated by a double-headed arrow, and the marked membrane ruffling is indicated by a double arrowhead. Bar=25 μm.

Figure 2. Domain structure of VASP and deletion mutants. Schematic representation of the domain structure of VASP shows EVH-1, EVH-2, and ΔGP5 domains. Deletion mutants of VASP and their designation in the present study are shown.

Figure 3. Binding of FL and deleted forms of VASP to FP4 peptide, profilin, and full-length VASP. A, Binding of FL, VASP EVH-1 domain (EVH-1), and ΔC VASP (ΔC) to FP4 peptide (FP4) and control peptide (C). Radiolabeled FL and deleted forms of VASP produced by in vitro transcription/translation (IVT/T) were incubated with agarose-coupled peptides, as indicated for each lane, for 2 hours at 4°C. B, Binding of FL, ΔGP5, VASP (ΔGP5), and ΔC VASP (ΔC) to GST, GST-profilin, and GST-VASP. Radiolabeled FL and deleted forms of VASP produced by IVT/T were incubated with glutathione-agarose bound GST, GST-profilin, or GST-VASP, as indicated for each panel, for 2 hours at 4°C. In the experiments depicted in panels A and B, bound protein was collected by centrifugation, washed extensively, eluted by boiling in electrophoresis sample buffer, and analyzed by SDS-PAGE. Bound radiolabeled VASP and deletion mutants were detected after fluorography, as described in Experimental Procedures. IVT/T lanes show the sizes of the relevant IVT/T products used in the binding assays.
VASP do not (Figure 3B). Thus, deletion of residues 175 to 196, which disrupts (GP)₃, by removing 2 of the tandem repeats but not that between residues 169 and 174 or the single GP₃ motif between residues 117 and 122, abolishes the binding of profilin. Full-length and ΔGP₃ VASP are both able to bind to full-length VASP, but ΔC VASP is not (Figure 3B).

### Effects of VASP Deletion Mutants on Endothelial Cell Ruffling and Stress Fiber Formation

Human VASP has a calculated Mr of 39.8 kDa and has an apparent molecular mass of 46 kDa under the conditions of SDS-PAGE. Phosphorylation of VASP on Ser157 causes a shift in mobility to an apparent Mr of 50 kDa.⁵ Western blots of cells expressing epitope-tagged VASP mutants are shown in Figure 4; full-length, ΔGP₃, and ΔC VASP mutants, but not EVH-1 (which lacks Ser157), are present as doublets, similar to the doublet previously observed for the endogenous wild-type protein,⁶ indicating that they exist in phosphorylated and dephosphorylated forms in the endothelial cells. The predominant form of the expressed full-length, ΔGP₃, and ΔC deletion mutants is the higher mobility form, suggesting that expressed VASP is largely unphosphorylated on Ser157.

Membrane ruffling usually reflects actin filament assembly.⁷ Binding of profilin to VASP could provide a means for the local accumulation of polymerization-competent G-actin, which, in the presence of nucleating activity, can lead to filament assembly and membrane ruffling.⁸ Therefore, we examined whether profilin binding is required for the VASP-induced ruffling observed in the present study. Expression of ΔGP₃, VASP, which is able to bind both FP₄ and VASP but not profilin, surprisingly still results in the marked membrane ruffling and stress fiber formation similar to those induced by wild-type VASP (Figure 5). This suggests an alternative mechanism whereby VASP induces endothelial ruffling. The ΔC form of VASP is unable to bind to any known partners and was therefore tested for its effects on the endothelial cytoskeleton (Figure 5). Prominent bundles of F-actin are observed in ΔC VASP–expressing endothelial cells. However, the induction of marked ruffling seen with full-length or ΔGP₃, VASP is not observed with ΔC VASP, indicating that the ability to induce stress fibers is independent of the effects of VASP on ruffling. Expression of the EVH-1 domain of VASP, VASP 1-245, and VASP 225-340 does not induce the marked membrane ruffles seen with full-length VASP (Figures 1 and 6). Ruffling is observed only with forms of VASP that can bind FP₄ and localize to the cell periphery (Figures 1, 3, and 5).

### A Subdomain Within the EVH-2 Domain Induces F-Actin Bundles in Endothelial Cells

The finding that VASP induces F-actin bundle formation in endothelial cells was unexpected and may be highly significant for endothelial organization and retraction. Therefore, it was of interest to identify the domain responsible for this activity. The EVH-1 domain of VASP is able to bind FP₄ peptide but not profilin or VASP (Figure 3). EVH-1 expression fails to induce bundle formation in vivo (Figure 6). Similarly, VASP 1-245 has no effects on cytoskeletal organization (Figure 6). Because VASP 1-325 induces bundle formation but VASP 1-245 does not, we asked whether the sequence between these residues is sufficient for in vivo bundling activity. Expression of VASP sequence 225-340 results in prominent bundle formation in vivo (Figure 6).

### Discussion

The data reported in the present study implicate VASP in membrane ruffling and stress fiber formation in vascular endothelial cells. VASP has been hypothesized to have a role in membrane ruffling⁸ by virtue of its ability to recruit profilin, a 14-kDa polypeptide that binds G-actin and stimulates the exchange of ADP for ATP on the monomer.⁹ The data in the present study are the first to demonstrate the direct actions of VASP on membrane ruffling. Rather surprisingly, however, experiments with the ΔGP₃ mutant indicate this did...
A possible mechanism for the effects of VASP on stress fiber formation is provided by identification of the subdomain responsible for this activity. Our findings that removal of residues 340-380 prevents VASP-VASP binding but not bundle formation in endothelial cells indicates that multimerization of VASP is not required for the formation of F-actin bundles in vivo. Alignment of sequences of Ena/VASP family members corresponding to the region of VASP shown in the present study to induce F-actin bundles reveals 2 highly conserved motifs corresponding to VASP 225-245 and VASP 262-277 (data not shown). The second motif includes a positive charge cluster. Such a region could act to induce filament bundling via its interaction and to charge neutralizing effects on acidic actin molecules within filaments. During preparation of the present article, it was reported that VASP 259-380 binds F-actin and forms bundles in vitro. This is consistent with our findings on the effects VASP 225-340 in vivo and the scheme outlined in the present study.

In conclusion, the present study demonstrates that VASP is intimately involved in membrane ruffling and stress fiber formation in vascular endothelial cells. This provides a rationale for the increased expression of VASP seen in endothelial organization in angiogenesis. Phase of angiogenesis requires endothelial cells to migrate, involving peripheral actin assembly, as well as to undergo shape change, a process in which actin bundles would be expected to contribute to individual cell shape changes involved in lumen formation. It has already been shown that VASP phosphorylation status is altered under conditions leading to endothelial retraction. Given the involvement of actin bundles in cell retraction, it is likely that VASP phosphorylation modulates its ability to participate in actin bundling. This possibility is under investigation. Finally, the prominence of stress fibers in endothelial cells within blood vessels and their importance in vascular function are consistent with the finding of high expression levels of VASP in endothelial cells in vivo.

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References


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