Phosphorylation of the Vasodilator-stimulated Phosphoprotein Regulates Its Interaction with Actin*

Received for publication, June 12, 2000
Published, JBC Papers in Press, July 5, 2000, DOI 10.1074/jbc.M005066200

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The vasodilator-stimulated phosphoprotein (VASP) is a major substrate for cyclic nucleotide-dependent kinases in platelets and other cardiovascular cells. It promotes actin nucleation and binds to actin filaments in vitro and associates with stress fibers in cells. The VASP-actin interaction is salt-sensitive, arguing for electrostatic interactions. Hence, phosphorylation may significantly alter the actin binding properties of VASP. This hypothesis was investigated by analyzing complex formation of recombinant murine VASP with actin after phosphorylation with cAMP-dependent kinase in different assays. cAMP-dependent kinase phosphorylation had a negative effect on both actin nucleation and VASP interaction with actin filaments, with the actin nucleating capacity being more affected than actin filament binding and bundling. Replacing VASP residues known to be phosphorylated in vitro by acidic residues to mimic phosphorylation had similar although less dramatic effects on VASP-actin interactions. In contrast, phosphorylation had no significant effect on VASP oligomerization or its interaction with its known ligands profilin, vinculin, and zyxin. When overexpressing VASP mutants in eukaryotic cells, they all showed targeting to focal contacts and stress fibers. Our results imply that VASP phosphorylation may act as an immediate negative regulator of actin dynamics.

Cell morphology and motility critically depend on the remodeling of the cytoskeletal architecture in response to external stimuli. Directional locomotion requires locally confined membrane protrusion driven by actin polymerization, resulting in the formation of a leading edge. Adhesion to the extracellular matrix is mediated by distinct multi-protein complexes. The formation of these focal adhesions is initiated by the activation of integrin heterodimers, which then recruit a variety of cytoskeletal and signaling molecules (1). Most of the cytoskeletal components involved, e.g. talin, α-actinin, and vinculin, are multi-ligand proteins. They may function as structural scaffolds for other cytoskeletal and signaling proteins or interact directly with the actin cytoskeleton. Given the complexity of focal adhesions, actin dynamics at these sites is not completely understood, and the final integration of integrin-mediated signaling with de novo actin polymerization remains to be elucidated.

Several lines of evidence have implicated the vasodilator-stimulated phosphoprotein (VASP) to be involved in the regulation of filament assembly and organization. VASP was originally purified from human platelets (2). It belongs to a protein family including the Drosophila protein Enabled (Ena), its mammalian homologue Mena, and the Ena-VASP-like protein (Evl) (3). They all share a common domain structure comprising a central proline-rich core flanked by two highly conserved Ena-VASP homology domains (EVH1 and EVH2; Fig. 1 and Ref. 3). Ena/VASP proteins target to the leading edge and focal adhesions in fibroblasts (3, 4), which is mediated by the EVH1 domain recognizing the consensus motif (D/E)F PPP PX D (5, 6).

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This motif is present in several VASP ligands, including zyxin and vinculin. The central region of Ena/VASP proteins harbors proline-rich stretches that are recognized by the G-actin-binding protein profilin (3, 7). VASP oligomerization and F-actin binding are confined to the C-terminal EVH2 domain (3, 8, 9). VASP may thus control the actin cytoskeleton by three different mechanisms: (i) it may recruit G-actin via its binding to profilin, (ii) it may stabilize and possibly organize newly formed filaments by direct binding to F-actin, and (iii) oligomerization may potentiate both effects.

VASP is highly enriched in platelets (10), and it is phosphorylated in response to vasodilators and platelet inhibitors, substances that raise intracellular cAMP and cGMP levels. VASP has been shown to be an immediate target for PKA and PKG in vitro and in vivo (11), and its phosphorylation correlates with the inhibition of platelet aggregation (12). These data are further supported by genetic analyses from VASP knockout mice that display enhanced agonist-induced platelet aggregation (13, 14). How platelet inhibition is mediated by VASP is currently unknown, but phosphorylation seems to be a key factor. VASP is phosphorylated in vitro and in intact human platelets at three residues by both PKA and PKG (11), corresponding to residues Ser153, Ser235, and Thr274 in murine VASP (8). All three phosphorylation sites are positioned close to ligand-binding modules (Fig. 1): Ser153 is located N-terminal to the G-protein (Gp)3 motif in the proline-rich region that has been shown to bind to profilin (15). In the EVH2 domain, Ser235 and Thr274 neighbor basic stretches that seem to mediate VASP-actin interactions (8, 9). Hence the ligand binding properties of VASP may significantly be altered by phosphorylation.

The abbreviations used are: VASP, vasodilator-stimulated phosphoprotein; PKA, cAMP-dependent kinase; PKG, cGMP-dependent kinase; EVH, Ena-VASP homology domain; VASPwt, wild type VASP; BiProtag, birch profilin sequence tag; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay.
VASP Phosphorylation and Actin Binding

So far, VASP-ligand interactions and VASP phosphorylation have mainly been investigated separately, yielding little information about how these two are related. The aim of the present study was to directly analyze the influence of phosphorylation on VASP-ligand complex formation. Recombinant VASP was phosphorylated by PKA in vitro and tested in different assays for actin binding, oligomerization, and the interaction with its known ligands profilin, vinculin, and zyxin. These experiments reveal that VASP phosphorylation by PKA diminishes binding to F-actin and even suppresses actin nucleation, whereas oligomerization and ligand binding remain unaffected. Our data lead to a model in which phosphorylation serves as a direct regulatory switch for VASP-mediated actin polymerization at adhesion sites.

EXPERIMENTAL PROCEDURES

Cloning of VASP Constructs—Cloning of murine VASP and its EVH1 and EVH2 domains has already been reported (8). The constructs comprising either EVH domain and the proline-rich domain (EVH1-P; EVH2-P) were generated accordingly by polymerase chain reaction comprising either EVH domain and the proline-rich domain (EVH1-P; EVH2-P) were generated accordingly by polymerase chain reaction using full-length VASP as a template. Amplification primers introduced EcoRI and XhoI restriction sites for further cloning into the following vectors: pQE30 (Qiagen, Hilden, Germany) for the generation of recombinant His-tagged proteins in bacteria, pEGFP-C2 (CLONTECH, Palo Alto, CA) for expression of EGFP fusion proteins in eukaryotic cells, and a derivative of pcdNA3 (CLONTECH) bearing a sequence tag derived from birch profilin (BiPro-tag) (16) to yield sequence-tagged proteins for its derivatives were expressed in the Escherichia coli strain M15(pREP4). Bacteria were transformed with VASP expression vectors (pQE30) and were grown in 2× YT medium at 30 °C. Protein expression was induced in late log phase with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were harvested after 3 h post-induction. Recombinant proteins were purified as essentially as described in the manufacturer’s protocol (Qiagen). Protein elution was achieved by a stepwise histidine gradient (20, 30, 40, 50, and 150 mM) in VASP elution buffer (50 mM sodium phosphate, pH 7.0, 100 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 20 mM β-mercaptoethanol, 5 mM benzamidine, 20 μM leupeptin, 50 μM Pefabloc SC, 1 μM pepstatin A, and 20 μl/ml apronin). All fractions were analyzed by SDS-PAGE. VASP containing fractions with 70% purity as judged by densitometric analysis (E.A.S.Y. Image, E.A.S.Y. Imaging Systems, Heidelberg, Germany) were transferred into 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM KCl, 2.5 mM EGTA, 0.75 mM dithioerythritol, 0.1% Triton X-100, and protease inhibitors (see above). 2–3 mg of VASP were purified from 1 liter of bacterial culture by this method. VASP proteins were stored in sodium phosphate buffer with 20% glycerol added at 80 °C for up to 2 months.

Recombinant mouse profilin I and II (17) were purified by poly-l-proline affinity chromatography as described previously (18) with slight modifications: profilin I and profilin II were eluted in 6 and 8× urea, respectively. Proteins were dialyzed against 10 mM Tris-HCl, pH 7.2, 0.2 mM CaCl₂, and 1.25 mM dithiothreitol. Rabbit skeletal muscle actin was prepared from acetone powder (19) with an additional gel filtration step as described (20).

In Vitro Phosphorylation of Recombinant Murine VASP—Purified VASP (3.75 μM) was incubated at 30 °C in buffer A (50 mM KCl, 5 mM MgCl₂, 0.2 mM ATP, 1 mM dithiothreitol, 0.2 mM EGTA, 10 mM HEPES, pH 7.4, 20 units/ml apronin, and 1 μM peptatin A). For radioactive assays, buffer A was supplemented with [γ-32P]ATP yielding a specific activity of 0.5 μCi/ml. Phosphorylation was initiated by the addition of 0.5 μM catalytic subunit of PKA (Promega, Madison, WI) and stopped at times indicated with 25 μM PKA inhibitor (Promega). Radiolabeled VASP was separated on 10% polyacrylamide gel, and phosphate incorporation was visualized by autoradiography using BioMax film (Eastman Kodak Co.). For quantitative analysis, gel pieces containing VASP were excised from the gel and measured by Cerenkov counting in a scintillation analyzer (Wallac 1410 liquid scintillation counter, EG&G Berthold, Isernhagen, Germany).

Phosphoamino Acid Analysis—One-dimensional phosphoamino acid analysis on thin layer cellulose plates (Macherey and Nagel, Düren, Germany) was performed in pH 1.9 buffer (2.2% formic acid and 7.8% glacial acetic acid) essentially as described in Ref. 21. Phospho-serine and phospho-threonine (Sigma) were used as internal standards and stained with ninhydrin. Radiolabeled phosphoamino acids were detected by autoradiography.

Actin Polymerization Assay—The influence of VASP or profilin I or II on actin polymerization was determined by fluorescence with 10% pyrene-labeled actin (22) added to unlabeled actin. Actin polymerization assays were performed essentially as described (8). 1 μM actin was polymerized in the presence of 1 μM profilin I or II respectively, at 25 °C in buffer B (25 mM HEPES, pH 7.0, 0.2 mM CaCl₂, 0.5 mM dithioerythritol, and 1 mM ATP) for 30 min. Polymerization was initiated by adjusting the solution to 25 mM NaCl, 2 mM MgCl₂, and 15 mM KC1 and adding 0.25 μM VASP protein (wild type, VASP phosphorylated by PKA, or VASP phospho-mutants). Fluorescence was monitored for 1 h at 966 nm excitation (slit width, 10 nm) and 384 nm emission (slit width, 10 nm) using a 150-slitted cuvette in an LS50B fluorimeter (Perkin-Elmer, Langen, Germany).

Co- sedimentation and in Vitro Filament Assay—Co-sedimentation assays were performed essentially as described in Ref. 8. 10 μM actin was polymerized in buffer C (25 mM HEPES, pH 7.0, 0.2 mM CaCl₂, 0.5 mM dithioerythritol, 1 mM ATP, 25 mM NaCl, and 2 mM MgCl₂, KC1 (15 mM or 50 mM) for 1 h at 37 °C. 2 μM F-actin was incubated with 2 μM VASP in buffer C for 1 h at room temperature. After high speed centrifugation (100,000 × g, 60 min in an Airfuge; Beckman, Munich, Germany) pellets and supernatants were analyzed by SDS-PAGE. Coomassie Blue-stained gels were analyzed densitometrically as described above. The percentage of VASP remaining in the supernatant compared with the total amount of VASP used in the experiment was calculated. In sedimentation assays to test for ternary VASP-profilin-actin complexes, prepolymization was omitted, and actin filaments were polymerized in the presence of VASP and profilin.

2 μM unlabeled actin was polymerized in buffer C in the presence of 0.5 μM wild type VASP, the triple mutant, and VASP phosphorylated by PKA, respectively, at 37 °C for 1 h. Filaments were stained with rhodamine-labeled phalloidin (Sigma) and directly analyzed by fluorescence microscopy (Axioskop, Jenaphot, Germany) using a cooled CCD camera (Roper Scientific, Tucson, AZ) and the MetaMorph Software package (Visitron Systems, Puchheim, Germany).

Yeast Two-hybrid Analysis—Yeast two-hybrid analysis was performed with a GAL4-based MATCHMAKER System 3 (CLONTECH) with yeast strains HF7C and Y187 according to manufacturer’s instructions. VASP constructs were cloned into the “bait” vector pGAK7T as...
well as the "prey" vector pGADT7 by use of EcoRI and XhoISalI restriction sites in the multiple cloning sites of either vector. A mouse cDNA library (embryonic day 17.5, CLONTECH) was screened using either VASP or the VASP triple mutant as bait. DNA from positive clones was prepared from yeast and transformed into competent E. coli (XL1 blue/Iron Agarose) according to standard protocols. DNA sequencing was performed on an ABI PRISM™ 310 genetic analyzer (Perkin-Elmer).

Cell Culture and Immunofluorescence—C2C12 cells (mouse myogenic cell line) were grown in Dulbecco's minimum essential medium supplemented with 10% calf serum at 10% CO2. 16 h prior to transfection, the cells were seeded on collagen coverslips. Transfection of VASP constructs was achieved by calcium phosphate precipitation according to standard protocols. 48 h after transfection, cells were fixed with 4% formaldehyde followed by a 30-min permeabilization with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Actin filaments were stained with coumarin-labeled phalloidin, and vinculin was detected with a monoclonal anti-vinculin antibody (Sigma). Samples were analyzed with a Zeiss Axiopt microscope (Zeiss) equipped for triple immunofluorescence. Images were taken with a cooled CCD camera (Roper Scientific) using the MetaMorph Software package (Visitrion Systems).

Immunoprecipitation of Protein Complexes after In Situ Cross-linking—Immunoprecipitations from HeLa cells using the membrane-permeant cross-linker dithiobis(succinimidyl propionate) (Pierce) were performed as described (8) using a monoclonal antibody against the BiPro sequence (16). Endogenous proteins were detected after Western blotting with the following antibodies: anti-profilin (2H11) (23), anti-vinculin (hVIN-1; Sigma), and antibodies against human VASP and zyxin, which were a kind gift of J. Wehland, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. Horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany) were used for detection by enhanced chemoluminescence (Amersham Pharmacia Biotech).

Solid Phase Binding Assay—The interaction between VASP and both profilin isoforms was monitored by an ELISA assay. Microcolon ELISA plates (Greiner, Frickenhausen, Germany) were coated with 50 pmol of profilin I or II/well, washed three times with 0.1% Tween-20 in PBS, and blocked with 1% bovine serum albumin in PBS for 2 h at room temperature. After an additional wash with PBS, increasing amounts of recombinant Bipro-tagged VASP were added (0.1–100 pmol in 100 μl of PBS with 0.05% Tween-20 and 0.5 mM dithiothreitol) and incubated with either profilin I or profilin II for 2 h at room temperature. Unbound VASP was removed by three washing steps (PBS, 0.1% Tween-20). Bound VASP was detected with a monoclonal antibody (4A6) specific for the BiPro-tag derived from birch profilin (16, 24). After incubation with a peroxidase-conjugated polyclonal anti-mouse secondary antibody, enzymatic activity was measured using 2,2'-azino-bis(3-ethylbenzthiazolone-6-sulfonic acid) as a substrate at 410 nm using an ELISA reader (Dynatech Laboratories, Billingshurst, UK).

Surface Plasmon Resonance Studies—To determine the stoichiometry and dissociation constants (Kd) of VASP-profilin complexes, surface plasmon resonance studies were performed on a BIACORE 2000 analyzer (Biacore, Uppella, Sweden). VASP (the ligand) was immobilized in 10 mM sodium acetate, pH 6.0, on a CM5 sensor chip by covalent coupling with the following antibodies: anti-profilin (2H11) (23), anti-vinculin (hVIN-1; Sigma), and antibodies against human VASP and zyxin, which were a kind gift of J. Wehland, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. Horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany) were used for detection by enhanced chemoluminescence (Amersham Pharmacia Biotech).

RESULTS

VASP Becomes Mainly Phosphorylated at Serine Residues by PKA—To analyze VASP phosphorylation by PKA under the experimental conditions chosen (see “Experimental Procedures”), 1 μg of recombinant murine VASP was phosphorylated by the catalytic subunit of PKA in the presence of [γ-32P]ATP. Phosphorylation was monitored over a period of 60 min. At the time points indicated, phosphorylation reactions were terminated by the addition of excess of the PKA inhibitory peptide, A, Coomassie Blue staining of a 10% SDS gel (upper panel) and the corresponding autoradiograph (lower panel). Although the shift from 50 kDa (His-tagged VASP, 0 min) to a higher apparent molecular mass of approximately 54 kDa (His-tagged p-VASP) is already observed after 0.5 min, phosphate incorporation constantly increases over time. B, quantitation of phosphate incorporation as measured by Cerenkov counting after excision of protein bands from the gel. C, phosphaoino acid analysis reveals that PKA phosphorylates VASP mainly at serine residues.

M_p*RU_max,exp*N, where M_p = molecular mass profilin (15 kDa), M_v = molecular mass VASP (40 kDa), RU_max,exp = response units of VASP coupled to the sensor chip surface (1045 and 1280 for studies with profilin I and profilin II, respectively), and n = the number of binding sites of VASP for profilin, which is 2 according to data from Ref. 15.

Fig. 2. Time-dependent in vitro phosphorylation of recombinant murine VASP by PKA. 1 μg of purified His-tagged recombinant murine VASP was phosphorylated with the catalytic subunit of PKA in the presence of [γ-32P]ATP. Phosphorylation was monitored over a period of 60 min. At the time points indicated, phosphorylation reactions were terminated by the addition of excess of the PKA inhibitory peptide. A, Coomassie Blue staining of a 10% SDS gel (upper panel) and the corresponding autoradiograph (lower panel). Although the shift from 50 kDa (His-tagged VASP, 0 min) to a higher apparent molecular mass of approximately 54 kDa (His-tagged p-VASP) is already observed after 0.5 min, phosphate incorporation constantly increases over time. B, quantitation of phosphate incorporation as measured by Cerenkov counting after excision of protein bands from the gel. C, phosphaoino acid analysis reveals that PKA phosphorylates VASP mainly at serine residues.
VASP Phosphorylation and Actin Binding

VASP Phosphorylation and Binding of VASP to pre-polymerized F-actin. A, fluorescence spectroscopy monitoring the polymerization of pyrenelabeled actin in the presence of VASP phosphorylated for different periods of time as indicated by numbers: 1, 0 min; 2, 0.5 min; 3, 1.5 min; 4, 3 min; 5, 10 min; 6, 60 min; 7, actin control. B, sedimentation analysis of 2 μM prepolymerized actin with equimolar amounts of unphosphorylated VASP (left panel) phospho-VASP (P-VASP) that had been phosphorylated by PKA for similar amounts of time as in A as well as controls (right panels). The positions of PKA, actin (A), VASP (V), and phospho-VASP (P-V) are indicated. C, densitometric analysis of VASP binding. The ratio of VASP remaining in the supernatant (S) versus the total amount of VASP (S+P) with respect to phosphorylation time is given in percentages. (Fig. 2A, lower panel). Protein bands were excised from the gel and phosphate incorporation was analyzed by Cerenkov counting. After 60 min 1.8 mol phosphate/mol VASP had been incorporated (Fig. 2B). Phosphoamino acid analysis (Fig. 2C) revealed that VASP was mainly phosphorylated at serine residues even though some phospho-threonine was detectable. These results are in good agreement with a previous study on human VASP (11) showing that VASP becomes phosphorylated at three residues, both in human platelets and in vitro: Ser157, Ser235, and Thr274, corresponding to Ser153, Ser235, and Thr274 in murine VASP (8). In analogy to the data obtained by Butt and co-workers (11), we conclude that PKA first phosphorylates murine VASP at Ser153 in the proline-rich domain and subsequently at Ser235 and, to a much lesser extent, at Thr274.

VASP Phosphorylation by PKA Diminishes the Actin Nucleating Activity of VASP as Well as Its Binding to Actin Filaments—To analyze the influence of phosphorylation by PKA on VASP interaction with actin, we used unlabeled samples that had been prepared in parallel with the samples for the phosphate incorporation analysis. In a previous study we demonstrated that actin nucleation by VASP as well as its binding to actin filaments is salt-sensitive (8), indicating that the complex formation is based on electrostatic interactions. Hence all experiments were performed under low salt conditions (15 or 50 mM KCl).

Actin nucleation was monitored in a standard actin polymerization assay where 10% of the G-actin used is labeled with pyrene (Fig. 3A). Actin filament formation causes an increase in fluorescence intensity giving a direct measurement of actin polymerization. When 1 μM G-actin was transferred into a buffer promoting actin polymerization, only negligible filament formation was observed. In the presence of 0.25 μM VASP, PKA, and PKA inhibitory peptide a significant increase in fluorescence intensity was observed. Similar analysis of 0.25 μM VASP that had been phosphorylated by PKA for 0.5, 1.5, 3, 10, and 60 min, respectively, showed that actin polymerization was negatively influenced as detected by the continuous decrease in signal intensity with increasing phosphorylation. After 3 min of incubation time, hardly any actin polymerization was observable, and samples taken after 10 and 60 min were indistinguishable from the actin control. The phosphorylation analysis (Fig. 2) had already shown that most of the phosphate is incorporated during the first 10 min of incubation to more than 1 mol phosphate/mol VASP, meaning that Ser153 as well as Ser235 are phosphorylated. Although Ser153 is located in the proline-rich domain, Ser235 lies within the EVH2 domain that has been shown to mediate actin binding (8, 9). Even though it is tempting to speculate that the decrease in actin nucleating activity is mainly due to phosphorylation of Ser235, we cannot rule out that there is an additive effect of both phosphorylation sites.

Co-sedimentation assays revealed that binding of VASP to actin filaments is also affected by VASP phosphorylation. 2 μM G-actin was pre-polymerized in actin polymerization buffer. Wild type VASP (VASPwt) and phospho-VASP, respectively, were added in equimolar amounts. After centrifugation, pellets and supernatants were analyzed by SDS-PAGE (Fig. 3B). H*s-tagged VASP had a slightly higher apparent molecular mass of approximately 50 kDa and almost quantitatively co-sedimented with actin filaments. Phosphorylation decreased the amount of VASP that bound to actin filaments (Fig. 3, B and C). After 10 min of phosphorylation approximately 57% were recovered in the pellet, and after 60 min most of the protein (84%) remained in the supernatant. Phosphorylation in the proline-rich domain only (Ser153) as demonstrated by the shift to 54 kDa seems to have little effect on F-actin binding, whereas additional phosphorylation at Ser235 in the EVH2 domain significantly reduces binding of VASP to actin filaments. However, again we cannot exclude the possibility that the mere accumulation of negative charges is responsible for the decrease in actin binding.

VASP Phosphorylation Can Only Partially Be Mimicked by Introducing Acidic Amino Acids—Because one cannot obtain a homogeneously phosphorylated population of VASP by in vitro phosphorylation, we sought to circumvent this problem by generating mutants of VASP mimicking phosphorylation (phospho-mutants), in which residues Ser153 and Ser235 were replaced by aspartic acid residues (S153D and S235D) and Thr274 was replaced by a glutamic acid residue (T274E). Phospho-mutants comprising any combination of mutated phosphorylation sites were cloned, giving rise to seven mutants in total (a schematic overview of all VASP constructs is given in Fig. 7A). First only the single phospho-mutants (S153D, S235D, and T274E) in which only one of the three residues had been replaced, were tested for actin nucleation by fluorimetric analysis in comparison with VASPwt at a molar ratio VASP:actin of 1:4 (Fig. 4A). All three phospho-mutants reduced actin polymerization, but to a far lesser extent than in vitro phosphorylated VASP (compare Fig. 3A). However, the phospho-mutant S235D, in which the serine residue located within the actin-binding domain (EVH2) had been replaced, always had the largest effect as was determined in four independent experiments using different protein preparations.

To test for a possible cumulative effect of the three phosphorylation sites, we repeated the experiment using the triple phospho-mutant, in which all three residues had been exchanged for acidic residues (S153D/S235D/T274E). There was a marked reduction in signal intensity, but actin polymerization was still observed (Fig. 4B). Because even this mutant did not abolish actin nucleation, as had been observed with VASPwt phosphorylated by PKA, the double mutants bearing two acidic residues were not tested in this assay.

Similar results were obtained, when the same four phospho-mutants were investigated for their binding to actin filaments as compared with VASPwt (Fig. 5). Co-sedimentation assays

Fig. 3. The influence of VASP phosphorylation on actin polymerization and binding of VASP to pre-polymerized F-actin. A, fluorescence spectroscopy monitoring the polymerization of pyrenelabeled actin in the presence of VASP phosphorylated for different periods of time as indicated by numbers: 1, 0 min; 2, 0.5 min; 3, 1.5 min; 4, 3 min; 5, 10 min; 6, 60 min; 7, actin control. B, sedimentation analysis of 2 μM prepolymerized actin with equimolar amounts of unphosphorylated VASP (left panel) phospho-VASP (P-VASP) that had been phosphorylated by PKA for similar amounts of time as in A as well as controls (right panels). The positions of PKA, actin (A), VASP (V), and phospho-VASP (P-V) are indicated. C, densitometric analysis of VASP binding. The ratio of VASP remaining in the supernatant (S) versus the total amount of VASP (S+P) with respect to phosphorylation time is given in percentages.
Fig. 4. The influence of VASP constructs bearing mutations that mimic phosphorylation on actin polymerization. A, the effect of VASP wt (1) and the single phospho-mutants T274E (2), S153D (3), and S235D (4) on actin polymerization was monitored by fluorescence spectroscopy. 1 μm pyrenyl-labeled actin was polymerized in the absence (S) or presence of equimolar amounts of VASP mutants, where a single serine/threonine residue had been replaced by aspartic acid or glutamic acid to mimic phosphorylation. B, a similar experiment was performed with the triple phospho-mutant (S153D/S235E/T274E) in which all three known phosphorylation sites had been replaced by acidic residues. 1, VASP wt; 2, triple mutant; 3, actin control.

Fig. 5. Sedimentation analysis of VASP wt, the single phospho-mutants S153D, S235D, and T274E and the triple phospho-mutant S153D/S235E/T274E. Each VASP construct (1.5 μM) was added to 2 μM prepolymerized actin at 15 mM (A, upper panel) and 50 mM KCl (B). Controls are shown in the lower panel of A, C and D, densitometric analyses of three independent sedimentation experiments for 15 mM (C) and 50 mM KCl (D). The percentage of VASP remaining in the supernatant is given as the ratio of VASP protein remaining in the supernatant (S) and total VASP (S+P). Note the diminished F-actin binding of the triple mutant with respect to the single phospho-mutants. E, comparison of the electrophoretic mobilities of VASP wt, the triple phospho-mutant, and VASP phosphorylated by PKA. The difference in electrophoretic mobility is better resolved in glycine gels than in tricine gels (compare E with A and B).

with 2 μM actin and 1.5 μM VASP protein revealed that under low salt conditions (15 mM KCl) the single phospho-mutants were indistinguishable from the wild type protein. In contrast, a significant fraction of the triple phospho-mutant remained in the supernatant (Fig. 5A). Increasing the salt concentration to 50 mM KCl (Fig. 5B) enhanced this effect. Binding of the triple mutant was significantly reduced under these conditions, whereas the single phospho-mutants were comparable with VASP wt. A densitometric analysis of the Coomassie-stained gels is shown in Fig. 5 (C and D, respectively).

Taken together, the data obtained with the phospho-mutants support our earlier findings that the VASP-actin complex is mainly based on electrostatic interactions (8). Introducing an acidic amino acid does probably not yield a negative charge comparable with that obtained after phosphorylation as is reflected by a reduced shift of the triple phospho-mutant in comparison with phospho-VASP (compare electrophoretic mobilities in Fig. 5E). It is also important to note that the shift was far better resolved on glycine gels than on tricine gels (Figs. 5, compare A, B, and E). However, these experiments still demonstrate that increasing the net negative charge causes a reduction in the VASP-actin complex formation. Furthermore, there seems to be indeed a cumulative effect when increasing the number of phosphorylation sites, with Ser153 Having a greater effect on actin nucleation than Ser153 or Thr274. These data are consistent with our results obtained from VASP phosphorylated in vitro.

VASP Phosphorylation Alters Actin Filament Organization—VASP not only binds to actin filaments, but it also organizes them into bundles. In a previous study with rhodamine-phalloidin-labeled actin filaments (8), we demonstrated that both VASP and the EVH2 domain alone display a potent actin bundling activity. However, the morphology of the bundles induced by either protein differed markedly; when actin was allowed to polymerize in the presence of VASP at a molar ratio of 1:4 (VASP/EVH2:actin), VASP induced numerous short bundles emanating from a distinct center, thus giving the aggregates a star-like appearance, whereas the EVH2 domain led to the formation of long, flexible bundles. We performed similar assays in this study to investigate whether VASP phosphorylation would alter filament organization (Fig. 6). First we noticed that when VASP was added to prepolymerized actin filaments, only bundles, but no star-shaped aggregates were observed (data not shown). Thus these stellar structures pres-
ent after co-polymerization reflect actin nucleation in addition to mere bundling. Next we assayed actin filament formation in the presence of either VASPwt, the single phospho-mutants (S153D, S235D, and T274E), the triple phospho-mutant (S153D/S235D/T274E), or phospho-VASP that had been phosphorylated by PKA for 60 min, respectively, at a molar ratio of VASP:actin of 1:4. Actin filaments were stained with rhodamine-phalloidin and analyzed by fluorescent microscopy. In contrast to the actin control (Fig. 6A), VASPwt (Fig. 6B) induced the star-like aggregates as described previously (8). In agreement with our results obtained from the actin polymerization assay (compare Fig. 4A), the single phospho-mutants were indistinguishable from VASPwt (data not shown), and even the triple mutant was still capable to nucleate actin. However, there were fewer star-like aggregates with the centers being less distinct and the actin filaments radiating from the latter being longer (Fig. 6C). No star-like aggregates were observed for VASP phosphorylated by PKA for 60 min, respectively, at a molar ratio of VASP:actin of 1:4. Actin filaments were stained with rhodamine-phalloidin and analyzed by fluorescent microscopy. In contrast to the actin control (Fig. 6A), VASPwt (Fig. 6B) induced the star-like aggregates as described previously (8). In agreement with our results obtained from the actin polymerization assay (compare Fig. 4A), the single phospho-mutants were indistinguishable from VASPwt (data not shown), and even the triple mutant was still capable to nucleate actin. However, there were fewer star-like aggregates with the centers being less distinct and the actin filaments radiating from the latter being longer (Fig. 6C). No star-like aggregates were observed for VASP phosphorylated by PKA for 60 min, respectively, at a molar ratio of VASP:actin of 1:4. Actin filaments were stained with rhodamine-phalloidin and analyzed by fluorescent microscopy.
Subcellular distribution of VASPwt and the triple phospho-mutant. C2C12 myoblasts were transiently transfected with EGFP-fusion proteins of VASPwt (A–C) and the triple phospho-mutant (D–E). Both proteins targeted to focal contacts and showed a punctate pattern along stress fibers as was determined by counterstaining for vinculin (B and E) and actin (C and F). The insets in A and D represent higher magnifications (2.5-fold) of the areas indicated by arrowheads. Bar, 10 μm.

Analysis of VASP-profilin-actin interactions. A, fluorescence spectroscopy following actin polymerization in the presence of VASPwt and profilin II (1), VASPwt and profilin I (2), VASPwt (3), and either profilin isoform alone (5 and 6); actin control (4). B, similar experimental setup as in A. Actin polymerization was monitored in the absence (1) or presence of phospho-VASP that had been phosphorylated by PKA in vitro for 60 min (2), phospho-VASP and profilin I (3), and phospho-VASP and profilin II (4). Although either profilin isoform promotes actin polymerization in the presence of VASPwt, no polymerization is observed after addition of phospho-VASP. C and D, surface plasmon resonance analysis of VASPwt with profilin I (C) and profilin II (D) as analytes. The concentrations of profilin isoforms are in descending order 30 μM (1), 10 μM (2), 3 μM (3), and 1 μM (4). Note the differences in binding affinities as well as in reaction rates between the two profilin isoforms for their interaction with immobilized VASPwt.

Phosphorylated VASP Still Binds to the Profilin-Actin Complex but Cannot Promote Actin Polymerization—Several authors have proposed a model in which VASP serves as an actin nucleator and organizer at cell adhesion sites (1, 7, 29, 30). It is targeted to the latter by binding to vinculin or zyxin via the EVH1 domain and may then recruit the profilin-actin complex, thus favoring actin polymerization. To test this hypothesis biochemically, we first confirmed VASP-profilin-actin complex formation for both profilin isoforms in co-sedimentation assays (data not shown). Actin polymerization was investigated in the presence of both profilin isoforms with or without VASPwt present in the sample (Fig. 10A). 1 μM G-actin in polymerization buffer showed no significant polymerization. Adding equimolar amounts of either profilin isoform further diminished the fluorescence intensity, probably because of the sequestering of G-actin. With VASPwt present in the sample at a molar ratio of 1:4 (VASP-profilin-actin), both profilin isoforms enhanced actin polymerization compared with VASPwt alone. Hence, the sequestering effect of profilin is overcome by VASP recruiting profilin-actin complexes. When the same experiment was performed using phospho-VASP that had been phospho-

Dissociation phase of the curves, $k_{off}$ was determined as $0.45/s$ for profilin I and $0.03/s$ for profilin II, and $k_{off}$ values as well as the maximum increase of response units that can be obtained by complete binding of the analyte to the immobilized ligand ($RU_{\text{max, exp}}$) were calculated with the Biacore evaluation software III. Best fits were obtained assuming a 1:2 VASP-profilin complex, which is in good agreement with the crystal structure of a poly-proline (Pro10) peptide and profilin (31) as well as gel filtration experiments with a VASP (GP15) peptide (15). A global fitting procedure was applied for profilin I data. For profilin II a local fitting procedure was applied for the curve at 1 μM, because profilin II oligomerized at higher concentrations (data not shown). The results are summarized in Table I. Profilin II binds VASP with much higher affinity than profilin I ($K_{\text{d, I}} = 0.136/0.825 \mu M$ for profilin II compared with $K_{\text{d, I}} = 63/65 \mu M$ for profilin I). The different slopes after addition of either profilin isoform further argue for a difference in binding kinetics. The steep rise for profilin I indicates that although it binds to VASP with lower affinity, the complex formation is much faster. The higher affinity of profilin II for VASP was also confirmed in an ELISA assay, where wells had been coated.
with 50 pmol of either profilin isoform and increasing amounts (0.1–100 pmol/well) of VASPwt were added. Repeating this experiment with VASP phosphorylated by PKA (60 min) did not show any significant differences (data not shown).

In conclusion, VASP may recruit profilin-actin complexes irrespective of its state of phosphorylation. In contrast, actin nucleation and polymerization as well as actin filament organization are negatively affected by VASP phosphorylation.

**DISCUSSION**

The present study investigates how VASP and its phosphorylation may contribute to the regulation of actin dynamics. VASP phosphorylation by cyclic nucleotide-dependent kinases and dephosphorylation by protein phosphatases I and II in vitro and in intact human platelets are well established (11, 32, 33). Both PKA and PKG phosphorylate VASP at three residues: Ser153, Ser235, and Thr274 (numbering according to murine VASP) with overlapping selectivity as is depicted in Fig. 1. Our results obtained for VASP phosphorylation by PKA match previous results (11); Ser153 is the site preferred by PKA and is readily phosphorylated. This leads to a shift in the apparent molecular mass in SDS-PAGE. In contrast, phosphorylation of Ser235 and Thr274 cannot be monitored accordingly, because it does not cause any changes in electrophoretic mobility.

VASP is a multi-ligand protein that targets to the cytoplasmic face of cell-cell and cell-matrix contact sites in a variety of cells (4, 29). It is thought to associate at these sites with other focal adhesion proteins like zyxin and vinculin and to promote actin polymerization through its recruitment of profilin-actin complexes. In the present study, we therefore investigated the influence of phosphorylation on the binding of VASP to these ligands. Complex formation with vinculin and zyxin was observed irrespective of the state of phosphorylation in vitro. VASP mutants mimicking phosphorylation showed unaltered targeting to focal contacts in C6C12 cells, supporting the observation that no changes in the subcellular distribution of VASP take place after treatment with cyclic nucleotide-elevating agents (34).

Regarding VASP-profilin interactions, again no significant differences were observed with respect to phosphorylation, indicating that even the close proximity of Ser235 to the (GP5)3 module (residues 165–182) in the central VASP domain that binds to profilin (15) has no effect in vitro and in vivo, which is in good agreement with previous data (7). We did, however, note a difference in binding of profilins with respect to the isoform. Although the affinity of profilin II for VASPwt lies in the upper nanomolar range and is significantly higher than that of profilin I, complex turnover is much faster for the latter. Similar binding affinities and binding kinetics have been shown recently using a (GP5)3 peptide (35).

Binding of VASP to actin is mediated via the EVH2 domain primarily through electrostatic interactions (8). The EVH2 domain harbors two of the three phosphorylation sites, Ser235 and Thr274. Because Thr274 is only phosphorylated to a minor extent by PKA and PKG in vitro and in intact human platelets (11) (and this report) its influence on VASP-actin interaction remains elusive. In contrast, actin nucleation is clearly affected by VASP phosphorylation at the two serine residues. Although phosphorylation of Ser153 alone has little effect on actin polymerization, additional phosphorylation at Ser235 prevents filament formation. However, to answer the question of whether this is merely due to phosphorylation at Ser235 or whether both serine residues need to be phosphorylated requires further investigation. Evidence drawn from the analysis of phospho-mutants of VASP indicates that phosphorylation of Ser235 alone has a greater effect on actin polymerization, but a cumulative effect is observed for the triple mutant. The larger effect of Ser235 may be due to its location within a highly conserved basic region neighboring a KLRK motif (residues 230–233), similar to the one critical for G-actin binding in β4-thymosin (36). Unfortunately, we were unable to demonstrate VASP-G-actin complexes in vitro, but their direct interaction is suggested by the actin nucleating activity of VASP.

Binding to prepolymerized F-actin is also negatively affected by phosphorylation. Although VASPwt almost quantitatively co-sediments with actin filaments, VASP phosphorylated by PKA for 60 min remains mainly in the supernatant after high speed centrifugation. At present we cannot separate the effect of single phosphorylation sites from simple accumulation of negative charges by multiple phosphorylation. It was previously suggested that basic stretches within the EVH2 domain mediate VASP binding to F-actin (9) and that this complex relies on electrostatic forces (8). This may explain why the introduction of negative charges by phosphorylation weakens VASP-F-actin interactions.

In a recent report, it was shown that recombinant VASP from baculovirus-infected Sf9 cells, phosphorylated to approximately 30%, co-sedimented to a higher extent with actin filaments when compared with the unphosphorylated, faster migrating VASP protein present in the same sample (37). However, as phosphorylation was only judged by the electrophoretic shift and the putative heterogeneity because of differential phosphorylation at Ser235 and Thr274, respectively, was not assessed, these data cannot be compared with the ones presented here.

VASP not only binds to actin filaments, but it also organizes them into distinct bundles (8, 9). When actin is polymerized in the presence of VASPwt, nucleation, polymerization, and filament bundling take place simultaneously and result in the
formation of star-shaped aggregates (8) (and this report). Although replacing Ser158, Ser235, and Thr274 with acidic residues only leads to a decrease in nucleation, but filament bundling is still observable, phosphorylation by PKA abolishes both effects. Our data show that this is not due to reduced oligomerization of VASP, which has been found to be confined to amino acids 277–380 (9).

We finally investigated the effect of VASP phosphorylation on the recruitment of profilin-actin complexes. Although both profilin isoforms increased actin polymerization by VASPwt, no actin polymerization was observed in the presence of phospho-VASP. Because binding of a VASP-derived (Gp)3 peptide to the poly-proline binding site of profilin does not significantly change the affinity of profilin for actin (35), the effects observed in the present study are probably due to direct VASP-actin interactions.

Given the prominent salt sensitivity of the interaction of VASP with actin in vitro (8), the observed association of both proteins under physiological conditions in cells remains to be explained. Full-length VASP is mainly found at focal contacts, but it also decorates stress fibers in a punctate pattern reminiscent of the zyxin distribution (38), indicating that VASP may be targeted to microfilaments even outside the focal contact area through its binding to zyxin. However, the isolated EVI2 domain that cannot interact with zyxin exclusively binds to stress fibers in fibroblasts after overexpression, arguing for a direct VASP-actin interaction (8), even at the ionic strength of the cytoplasm. Similar observations have been reported for calponin (39). Although this protein only bundles F-actin under low ionic strength in vitro, it associates with the actin cytoskeleton in different cell types (40, 41). It has been proposed that the local concentrations of actin and calponin may be sufficient high to allow complex formation even under physiological ionic strength. An analogous explanation may apply to VASP.

High local concentrations of VASP and a high ratio of VASP to actin may be assumed either during specific phases of cell differentiation or in special cell types. An example for the first may be the observed essential role of VASP in the microfilament organization and the formation of adhesion zippers during cell-cell contact formation in keratinocytes (29).

An example for the latter may be the situation found in blood platelets that are rich in cyclic nucleotides and cyclic nucleotide-dependent kinases, especially PKG (10), as well as in actin and VASP. However, they are virtually devoid of the VASP relatives Ena/Mena and Evl (14), which may interfere with a VASP-actin-based regulation of microfilament assembly in adhesion complexes in other cells (42). The “all-or-nothing” response during platelet activation requires the immediate, synchronous rearrangement of the microfilament system. Platelets of VASP-deficient mice show enhanced agonist-induced aggregation (13, 43), indicating that in these cells, VASP is indeed important as a negative regulator of actin polymerization. Our data suggest that this may directly be mediated by VASP phosphorylation.

In conclusion we propose a model of how VASP phosphorylation might regulate actin dynamics at cell adhesion sites (Fig. 11). VASP can form complexes with its known ligands vinculin, zyxin, and profilin irrespective of its state of phosphorylation. It may thus target to cell adhesion sites and recruit profilin-actin complexes. However, phosphorylation by PKA and PKG inhibits actin polymerization from profilin-actin complexes; thus polymerization is only initiated after VASP dephosphorylation, which is probably achieved by serine/threonine phosphatases 1 and 2 (33). The formation of VASP hetero-oligomers with respect to its state of phosphorylation allows further regulation of actin polymerization at cell adhesion sites. The advantage of this model is that the cell may assemble all components necessary for adhesion prior to actin polymerization, with VASP phosphorylation serving as an important regulatory switch.

Acknowledgments—We thank Dr. J. Wehland (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) for VASP antibodies and T. Messerschmidt for expert technical assistance.

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Phosphorylation of the Vasodilator-stimulated Phosphoprotein Regulates Its Interaction with Actin
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doi: 10.1074/jbc.M005066200 originally published online July 5, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005066200

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