AMP-activated Protein Kinase Impairs Endothelial Actin Cytoskeleton Assembly by Phosphorylating Vasodilator-stimulated Phosphoprotein

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Vasodilator-stimulated phosphoprotein (VASP) is an actin regulatory protein that links signaling pathways to remodeling of the cytoskeleton. VASP functions are modulated by protein kinases, which phosphorylate the sites Ser-157, Ser-239, and Thr-278. The kinase responsible for Thr-278 phosphorylation, biological functions of the phosphorylation, and association with disease states have remained enigmatic. Using VASP phosphorylation status-specific antibodies, we identified AMP-activated protein kinase (AMPK), a serine-threonine kinase and fundamental sensor of energy homeostasis, in a screen for kinases that phosphorylate the Thr-278 site of VASP in endothelial cells. Pharmacological AMPK inhibitors and activators and AMPK mutants revealed that the kinase specifically targets residue Thr-278 but not Ser-157 or Ser-239. Quantitative fluorescence-activated cell sorter analysis and serum response factor transcriptional reporter assays, which quantify the cellular F-/G-actin equilibrium, indicated that AMPK-mediated VASP phosphorylation impaired actin stress fiber formation and altered cell morphology. In the Zucker Diabetic Fatty (ZDF) rat model for type II diabetes, AMPK activity and Thr-278 phosphorylation were substantially reduced in arterial vessel walls. These findings suggest that VASP is a new AMPK substrate, that AMPK-mediated VASP phosphorylation impaired actin stress fiber formation and altered cell morphology.

The regulation of actin dynamics is important for cytoskeletal remodeling, cell morphology, and associated events such as cell migration, adhesion, and motility. In this complex scenario, Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins coordinate different modes of actin organization including actin filament assembly, cross-linking, and bundling. In mammals, the Ena/VASP protein family comprises the VASP, Mena (the homolog of Drosophila Ena), and the Ena-VASP-like protein (Evl). These proteins participate in actin filament networks at sites of cell-cell and cell-matrix interactions and within lamellipodia and filopodia protrusions. All members of the Ena/VASP family share a tripartite domain organization consisting of an N-terminal Ena/VASP homology domain 1 (in VASP, residues 1–115), a central proline-rich region (PRR, 115–225), and a C-terminal EVH2 domain (225–380) (1, 2). VASP directly binds to globular actin (G-actin) and filamentous actin (F-actin) via the EVH2 domain (2, 3). Residues in the C terminus of the EVH2 domain mediate tetramerization of VASP and hetero-oligomerization with other Ena/VASP family members. These interactions are important for filament elongation and branching and, consequently, for cell migration and cell-cell or cell-matrix interactions (3, 4). Ena/VASP proteins promote actin polymerization and assembly, and several models of VASP-mediated F-actin control have been proposed, including the regulation of nucleation, bundling, branching, and capping (5, 6).

VASP effects on actin turnover are regulated by phosphorylation, and in vitro studies have identified three serine/threonine phosphorylation sites (Ser-157, Ser-239, and Thr-278) in the protein (7). Both in vitro phosphorylated VASP and VASP mutants that mimic defined phosphorylation states suggest that phosho-VASP interferes with actin filament assembly, turnover, and branching. Mechanistically, VASP phosphorylation seems to modulate F-actin binding, to reduce actin-polymerization-promoting activity (8), and to interfere with anti-capping activity (6). Originally, VASP phosphorylation was described in platelets, where both AMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) were shown to phosphorylate residues Ser-157 and Ser-239 (9). Subsequently, the importance of phospho-Ser-157 (pSer-157) and phospho-

FACS, fluorescence-activated cell sorter; TRITC, tetramethylrhodamine isothiocyanate; ACC, acetyl-CoA carboxylase; APMP, AMP-activated protein kinase; G-actin, globular actin; F-actin, filamentous actin; SRE, serum response element; SRF, serum response factor; VASP, vasodilator-stimulated phosphoprotein; 8-Br, 8-bromo-; CA, constitutively active; DN, dominant negative; wt, wild type.
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Ser-239 (pSer-239) in cyclic-nucleotide dependent-kinase signaling cascades was established in other cardiovascular cells including aortic smooth muscle cells (10) and endothelial cells (11), in vessels of animal models, and in humans (for review, see Ref. 12).

To quantify the magnitude of phosphorylation at these sites, phosphorylation-specific antibodies anti-pSer-157 (5C6) and anti-pSer-239 (16C2) were developed (11). Analyses based on these antibodies revealed that reduced Ser-157/Ser-239 phosphorylation was associated with defective nitric oxide (NO)/cGMP signaling and endothelial dysfunction (13). Although PKA and PKG may phosphorylate purified VASP at Thr-278 to a small extent in vitro (7), a role for cyclic nucleotide-dependent protein kinase phosphorylation of Thr-278 in vivo has never been convincingly demonstrated. Indeed, neither the kinase responsible for Thr-278 phosphorylation nor the functional consequences of Thr-278 phosphorylation have been resolved.

The AMP-activated protein kinase (AMPK) is a major regulator of metabolism not only at the cellular but also at the whole organism level (14, 15). The evolutionarily conserved AMPK is a ubiquitously expressed heterotrimeric protein composed of a catalytic α subunit harboring a serine/threonine kinase domain and two regulatory β and γ subunits (14, 16). AMP, generated from metabolic stress-induced ATP depletion, activates the AMPK allosterically. Two signaling pathways involving the Ca²⁺/calmodulin-dependent protein kinase and the tumor suppressor LKB1 (also referred to as STK11) activate AMPK by phosphorylating its activation loop at Thr-172 (17, 18). Once activated, AMPK phosphorylates and inactivates several key enzymes in energy-consuming biosynthetic pathways, thereby conserving ATP pools and preserving cellular energy homeostasis. Substrates of AMPK include acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl-CoA reductase, and glycogen synthase, enzymes that control the synthesis of fatty acids, cholesterol, and glycogen, respectively (for review, see Refs. 19–21). Disturbance of AMPK activity has been linked to the pathogenesis of type II diabetes mellitus and obesity (22, 23), and modulation of AMPK activity may offer novel therapeutic options for treatment of these diseases (24).

We identified AMPK in a screen for kinases that phosphorylate VASP residue Thr-278 in endothelial cells. Studies using pharmacological agents, in vitro phosphorylations, and constitutively active and dominant negative AMPK mutants confirmed that VASP is a novel AMPK substrate. AMPK specifically targets the VASP Thr-278 site. Functionally, AMPK-mediated VASP Thr-278 phosphorylation reduced F-actin assembly, resulting in defective stress fiber formation and altered cell morphology. In arterial vessel walls from Zucker Diabetic Fatty (ZDF) rats, which serve as a model for type II diabetes mellitus, AMPK activity and VASP Thr-278 phosphorylation were severely reduced. Taken together, our data show that metabolic signals via AMPK-mediated VASP Thr-278 phosphorylation may inhibit actin polymerization. In this way our study establishes a novel signaling pathway that directly links cytoskeleton dynamics to energy metabolism that is impaired in diabetic vessels.

EXPERIMENTAL PROCEDURES

Animals—4–7-month-old male ZDF (fa/fa) rats (615.0 ± 24.9 g) with overt diabetes and their non-diabetic lean controls (+/+; 392.4 ± 35.2 g) were from Harlan Winkelmann. Animal studies were approved by the Regierung of Unterfranken.

Cell Culture—Human umbilical vein endothelial cells (HUVEC, Cambrex Bioproducts) were cultivated as described (25). ECV304, HEK293, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose supplemented with 10% fetal bovine serum (26).

Generation and Characterization of Anti-pThr-278 Antibody—To generate antibodies that specifically recognize phospho-Thr-278 (pThr-278) VASP, rabbits were immunized with the following phosphopeptide coupled to keyhole limpet hemocyanin: 273CRRKApTQVGE282 (pT is phosphothreonine). The antibody was immuno-selected using columns with immobilized phosphopeptide followed by a column with unphosphorylated VASP peptide. Antibodies against the corresponding non-phosphorylated VASP-peptide (anti-Thr-278) were immunopurified from the latter column and applied to the column with immobilized phosphopeptide to remove pThr-278-cross-reacting antibodies. To test the specificity of anti-pThr-278 antibodies for 273CRRKApTQVGE282, titer plates (Maxisorb, Nunc) were coated by overnight incubation with 100 μg/ml each of the phospho- and the unphosphorylated-VASP peptide. Anti-pThr-278 antibodies were applied in a serial 1:2 dilutions (starting concentration 2 μg/ml; 13 nM) and specific bound antibody was detected with a secondary peroxidase-conjugated antibody to rabbit immunoglobulin (diluted 1:4000) and a diaminonaphthalene-azido-bis(3-ethyl-2,3-dihydrobenzthiazidine-6-sulfonate) substrate solution for 30 min. The change of absorbance was read at 405 nm. All incubations were done at 37 °C except for the coating step, which was done at 4 °C.

Cloning of VASP Mutants—VASP mutants were generated by site-directed mutagenesis using the QuikChange Multi kit (Stratagene). Human VASP cDNA in a pcDNA3 vector (Invitrogen) and the following primers were used to exchange Ser-157, Ser-239, and Thr-278 with alanine or glutamic acid, respectively: 5’-CG GAC CAC ATA GAG CGG GTG GCC AAT GCA GGA GGC CCA CC-3’ (S157A); 5’-GGA GCC AAA CTC AGG AAA GTC GCC AAG CAG GAG GAG GCC TCA GGG-3’ (S239A); 5’-G CTG GCC CCG AGA AGG AAA GCC CGG CAA GTT GGG GAG AAA ACC-3’ (T278A) and 5’-G CTG GCC CCG AGA AGG AAA GCC CGG CAA GTT GGG GAG AAA ACC-3’ (T278E). Based on the amino acids present at positions 157, 239, and 278, respectively, VASP mutants were designated AAA, AA, and AAT. All constructs were confirmed by DNA sequence analyses.

Screen for Kinases That Phosphorylate Thr-278 in Cells—To screen for kinases that may phosphorylate the VASP Thr-278 residue, confluent HUVEC, ECV304, or HEK293 cells, transiently transfected with cDNAs coding for human VASP in a pcDNA3 vector, were treated with protein kinase activators or inhibitors. After incubation, the relative amount of pThr-278 in inhibitor- or activator-treated cells was quantified by Western blot analysis with anti-pThr-278 antibody. PKA inhibitors used
were H89 (0.5–5 μM for 10–60 min, Sigma) and Rp-8Br-cAMPS (50–500 μM for 10–60 min, BioLog), and PKA activators used were Forskolin (1–10 μM for 0.5–60 min, Sigma) and 8-Br-cAMP (50–200 μM for 0.5–60 min, BioLog). PKG effects on Thr-278 phosphorylation were analyzed with the inhibitor Rp-8-Br-cGMP (50–500 μM for 10–60 min, BioLog) and with the activators sodium nitroprusside (0.20–20 μM for 1–60 min, Sigma) and 8-Br-cGMP (50–200 μM, 0.5–60 min, BioLog). PKC was analyzed using the inhibitors Bis I (0.5–50 μM for 0.5–8 h, Calbiochem) and Rottlerin (1–10 μM for 0.5–8 h, Sigma) and the activator phorbol 12-myristate 13-acetate (0.1–10 μM for 2–30 min, Sigma). To inhibit Ca2+/calmodulin-dependent protein kinase, we utilized KN93 (0.5–5 μM for 0.5–8 h, Sigma), Ca2+/calmodulin-dependent protein kinase II peptide (0.5–5 μM for 0.5–8 h, Calbiochem), or Calcimycin (1–10 μM for 10–30 min, Sigma) for stimulation. PKB (Akt) was investigated by Wortmannin (0.1–10 μM for 0.5–8 h, Calbiochem) and fetal calf serum (0.5–20% for 0.5–3 h, Invitrogen). AMPK-driven Thr-278 was assessed using the AMPK activators Metformin (1,1-dimethylbiguanide, 0.5–8 mM for 10–60 min, Sigma), Phenformin (phenethylbiguanide, 0.5–8 mM for 10–60 min, Sigma), and AICAR (5-aminomimidazole-4-carboxamide-1β-riboside, 0.5–2.5 mM for 10–30 min, Calbiochem) and AMPK inhibitors compound C (2–20 μM for 0.5–8 h, Calbiochem) and Indirubine (indirubine-3-oxime, 0.5–20 μM for 0.5–8 h, Tocris).

**Transfection Experiments**—ECV304 and HeLa cells were cultivated in 6-well plates to 70% confluence and transiently transfected with 1.0 μg of cDNA coding for an Myc-tagged constitutively active AMPK α1 subunit mutant (CAα), a dominant negative AMPK α1 form (DNα), or the wild-type α1 subunit (wta) (29) using GenePorter 2 with Booster Reagent 2 (Peqlab). After 24 h of expression washed cells were lysed, and pThr-278 levels were determined by Western blot analysis with anti-pThr-278 antibodies. Transfection efficiency was controlled by transfecting with a pcDNA3GFP vector, which encodes green fluorescent protein. Immunofluorescence microscopy revealed that ~35–40% HeLa and 20–25% ECV304 cells stained positive for GFP after 24 h of expression.

**Analysis of VASP Phosphorylation**—Cells or tissue were immediately lysed in sample buffer containing 50 mM Tris, pH 6.8, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. Lysates were separated by 8% SDS-PAGE under reducing conditions and electro-transferred to a nitrocellulose membrane (Schleicher & Schüll). Proteins on blots were probed using antibodies against AMPKα, phospho-AMPKα, phospho-ACC (Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (Chemicon), Myc (Santa Cruz Biotechnology), VASP (M4, (9)), pSer-157-VASP (5C6, (11)), pSer-239-VASP (16C2, (27)), and pThr-278-VASP as well as horseradish peroxidase-conjugated secondary antibodies. Detection was performed using a chemiluminescence technique (ECL Plus, Amersham Biosciences). The intensity of the individual bands on the x-ray films (XBA, Fotochemische Werke Berlin) was quantified by densitometric scans (Scan Pack 3.0, Biometra).

**Immunocytochemistry**—Formaline fixed and permeabilized HeLa cells in 2-well Chamberslides (Nunc) and aortic rat tissues cryosections were incubated overnight with primary antibodies against VASP (polyclonal M4 and monoclonal IE273 (28)) and phospho-VASP (see above) followed by secondary anti-mouse and anti-rabbit antibodies conjugated to Alexafluor 488 or 594 (Molecular Probes) for 1 h. All incubations were performed in 5% goat serum, phosphate-buffered saline at room temperature. Stained sections were investigated using a Nikon Eclipse E600 microscope equipped with a C1 confocal scanning head and a 100× oil immersion objective. Images were acquired using the EZ-C1 2.10 software from Nikon.

**Quantification of Cellular F-actin Content by FACS**—HeLa cells were co-transfected (GenePorter 2 and Booster Reagent 2, Peqlab) with pcDNA3 vectors coding for VASP mutants (AAA, AAE, AAT; 2.5 μg each) or Myc-tagged AMPK α1 subunits corresponding to wild-type (wta), a constitutively active mutant (CAα), or a dominant negative (DNα) mutant (5 μg each) (29). After 24 h of expression in Dulbecco’s modified Eagle’s medium with all supplements, cells were trypsinized, fixed, permeabilized (see above), and co-stained for F-actin using Alexa-fluor 647 phalloidin and for Myc-tagged AMPKα1 subunit using anti-Myc antibody followed by fluorescein isothiocyanate-conjugated anti-mouse antibody. The mean cellular F-actin content determined by phalloidin staining of overexpressing cells was quantified using FACScan (BD Biosciences) as described (3).

**Reporter Gene Assays**—We utilized a serum response factor (SRF)-based transactivation assay to quantify changes in cellular actin dynamics as described (30, 31). Briefly, HeLa cells grown on 6-well plates were transiently transfected using GenePorter2 and Booster2. Each transfection mix includes cDNAs coding for VASP mutants (0.1 μg), AMPK mutants (0.5 μg), serum response element (SRE) reporter vector (0.5 μg, pSRE, Stratagene), Renilla luciferase control vector (0.25 μg, phRL-TK, Promega), and pcDNA3 vector without insert (0.5 μg, Invitrogen). After 24 h cells were lysed, and luciferase activities were measured in the supernatants using the Dual Luciferase Reporter Assay System (Promega) with a luminometer (Lumat, Berthold). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed relative to MOCK- (set to 0%) and VASP AAA-transfected cells (set to 100%).

**In Vitro VASP Phosphorylation**—AMPK phosphorylation assays were performed at 30 °C in 40 μl reaction mixtures containing 80 ng of purified recombinant human VASP and 100 milliunits of purified AMPK (Upstate Biotechnology Inc.) in reaction buffer (5 mM Hepes, pH 7.5, 0.1 mM dithiothreitol, 0.25% Nonidet P-40, 7.5 mM MgCl2, 50 μM ATP, 300 μM AMP). Reactions were stopped at different time points by the addition of SDS sample buffer and heating at 90 °C for 10 min.

**Statistics**—Results were expressed as the means ± S.D. Comparisons between two groups were analyzed using Student’s t tests. Significant difference was defined as p values <0.05.

**RESULTS**

AMP-activated Protein Kinase Phosphorylates VASP at Position Thr-278—To analyze VASP Thr-278 phosphorylation and to identify the kinase that phosphorylates residue Thr-278, we generated a phosphorylation status-specific anti-peptide antibody (anti-pThr-278) using a phosphopeptide spanning the Thr-278 phosphorylation site (C273CRRKApTQVGE282). Immu-
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FIGURE 1. Analysis of Thr-278 phosphorylation status with phospho-specific antibodies. A, to test the phospho-specificity of anti-pThr-278, microtiter plates were coated with 100 µg/ml each of a phosphopeptide spanning the Thr-278 phosphorylation site (△) or the corresponding unphosphorylated peptide (□) followed by incubation with anti-pThr-278 antibodies in serial 1:2 dilutions starting at 2 µg/ml. Specifically bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody followed by the chromogenic substrate diaminobenzidine. Absorbance was measured at 405 nm. The figure is representative for a series of five independent experiments. B, to test the specificity of anti-pThr-278 antibody for pThr-278-VASP was analyzed using protein phosphatase 2B (PP2B) that dephosphorylates pThr-278. HEK293 cells were co-transfected with 1.0 µg of human VASP in pcDNA3 vector and 0.25 or 1 µg of CDNA coding for protein phosphatase 2B or vector without insert (MOCK). After 24 h cells were lysed and analyzed for pThr-278 (anti-pThr-278 antibody) and total VASP (anti-VASP antibody) by Western blotting using phospho-specific antibodies. The intensity of pThr-278 was measured at 405 nm. The figure is representative for a series of five independent experiments. C, ECV304 cells were treated with PKA or PKG activators Forskolin (5 and 10 µM) or 8-Br-cGMP (200 µM, 15 min), respectively. Phosphorylations at the sites Thr-278, Ser-157, and Ser-239 were analyzed and compared with untreated cells by Western blotting using phospho-specific antibodies. The phosphorylation status did not increase pThr-278 over basal levels. Summarized pThr-278 signal intensities of the doublet did not significantly differ from the pThr-278 level of VASP from unstimulated cells (100 versus 97 ± 8%, Fig. 1C, middle panel and column bar). Furthermore, relative signal intensities of the 46- and 50-kDa band from anti-pThr-278 and anti-VASP were not different (Fig. 1C, compare upper and middle panels), suggesting that phosphorylation at Thr-278 occurred independent of the Ser-157 phosphorylation status. Similarly, PKG activators such as 8-Br-cGMP failed to change Thr-278 phosphorylation in nontissue-specific bound to the phosphopeptide but not to the unphosphorylated control (Fig. 1A). Furthermore, anti-pThr-278 did not cross-react with recombinant unphosphorylated VASP in Western blotting (see Fig. 3, upper lane, t = 0 min). To confirm that anti-pThr-278 detects the phosphorylation status of cellular VASP at the third site, we overexpressed VASP and protein phosphatase 2B, which has been shown to dephosphorylate pThr-278-VASP (28). Results from Western blots incubated with anti-pThr-278 indicated that protein phosphatase 2B dose-dependently decreased pThr-278 in HEK293 cells (Fig. 1B). Together, the data confirm the specificity of anti-pThr-278 antibody for pThr-278 in vivo.

Using anti-pThr-278, we systematically screened for serine-threonine kinases that may phosphorylate VASP in intact cells. First, we selected kinases whose consensus sequences matched the Thr-278 flanking residues. Subsequently, endothelial cell lines (ECV304 and HUVEC), which express a high amount of endogenous VASP (11), as well as HEK293 cells, which transiently overexpress VASP, were incubated with specific activators or inhibitors of the selected kinases and analyzed for pThr-278 and total VASP by Western blotting (described under “Experimental Procedures” in detail). Pretreatment with the PKA activator Forskolin clearly increased pSer-157 in ECV304 cells as indicated by the phosphorylation-specific antibody (anti-pSer-157), Fig. 1C, lower panel). Phosphorylation of Ser-157 of VASP led to a shift in apparent molecular mass in SDS-PAGE from 46 to 50 kDa, and anti-VASP antibodies detected the characteristic doublet of Ser-157-non-phosphorylated and -phosphorylated protein after stimulation (anti-VASP, Fig. 1C, middle panel, the upper band was detected by anti-pSer-157). Notably, PKA activation did not increase pThr-278 over basal levels. Summarized pThr-278 signal intensities of the doublet did not significantly differ from the pThr-278 level of VASP from unstimulated cells (100 versus 97 ± 8%, Fig. 1C, middle panel and column bar). Furthermore, relative signal intensities of the 46- and 50-kDa band from anti-pThr-278 and anti-VASP were not obviously different (Fig. 1C, compare upper middle panels), suggesting that phosphorylation at Thr-278 occurred independent of the Ser-157 phosphorylation status. Similarly, PKG activators such as 8-Br-cGMP failed to change Thr-278 phosphorylation in
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73%, respectively, over basal levels. All tested AMPK inhibitors and activators similarly affected pThr-278 levels in HEK293 and ECV304 cells (data not shown), suggesting that VASP might be a novel AMPK substrate \textit{in vivo}. Notably, in non-stimulated HUVEC about 40% of total VASP was phosphorylated at Thr-278 as indicated by a comparison of signals from pThr-278 and anti-Thr-278 antibodies. Anti-Thr-278 antibody detects the corresponding peptide, which was used for generation of anti-pThr-278, in the non-phosphorylated form. Under basal conditions the ration of Thr-278-phosphorylated to total VASP was even higher in HEK293 and ECV304 (about 1:2). To confirm that the employed conditions of AMPK inhibitors and activators efficiently targeted enzyme activity in the treated cells, we analyzed phosphorylation of AMPK itself at residue Thr-172, which correlates with AMPK activity (32). AMPK Thr-172 phosphorylation paralleled pThr-278 levels (Fig. 2, A–D), supporting the hypothesis that VASP is a substrate of activated AMPK \textit{in vivo}.
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AMPK Specifically Targets the VASP Residue Thr-278 in Vitro—To examine the specificity of AMPK for any of the three VASP phosphorylation sites, recombinant purified VASP was incubated with active AMPK (Fig. 3). At 0.5, 2, 8, and 30 min, aliquots from the reaction mixture were collected and analyzed for total VASP (anti-VASP) and its phosphorylations at residues Ser-157, Ser-239, and Thr-278 using the phospho-specific anti-VASP antibodies 5C6 (detects pSer-157), 16C2 (detects pSer-239), and anti-pThr-278. Western blots revealed that VASP Thr-278 phosphorylation occurred rapidly within 0.5 min and increased for 30 min. Under these conditions, Compound C inhibited Thr-278 phosphorylation, confirming the selectivity of active AMPK-mediated VASP phosphorylation (not shown). In contrast, AMPK did not phosphorylate residues Ser-157 or Ser-239 within the incubation time. Taken together these data indicate that AMPK specifically utilized the Thr-278 phosphorylation site in vitro.

AMPK Activity Mutants Modulate VASP Thr-278 Phosphorylation—To confirm the effect of AMPK inhibitors and activators on pThr-278 levels by an independent approach, we employed AMPK mutants affecting enzyme activity. ECV304 and HeLa cells were transfected with cDNAs coding for a constitutively active mutant of the AMPK α1 subunit (CAα), a dominant negative variant (DNα), the wild-type α1 subunit (wtx), or empty vector (MOCK). CAα is a truncated form of wild type α1 subunit (amino acids 1–312) containing a mutation that alters Thr-172 to an aspartic acid (T172D), which mimics phosphorylation. Phosphorylation of Thr-172 of the α1 subunit is essential for the enzyme activity. In the DNα AMPK form, aspartic acid 157 of the α1 subunit is substituted by an alanine residue (29). After 24 h, lysed cells were analyzed for pThr-278 levels, total VASP, and expression of the constructs. Additionally, phosphorylation of the established AMPK substrate ACC (33) was analyzed as an internal measure of AMPK signaling. Compared with MOCK-transfected cells (set to 100%), CAα increased pThr-278 levels in ECV304 and HeLa cells to 123 and 129%, respectively (Fig. 4, upper lanes, A and B). Consistently, inhibition of AMPK activity using DNα reduced pThr-278 down to 60 and 88% in ECV304 or HeLa cells, respectively, whereas wtx did not significantly affect pThr-278 levels (104% in both cell lines; Fig. 4, column bars in A and B). The relative degrees of phosphorylation of Thr-278 by the various AMPK forms were similar to those observed for the AMPK substrate ACC. These results are in accordance with our findings using pharmacological AMPK inhibitors and activators (Fig. 2) and support the conclusion that AMPK phosphorylates VASP at the Thr-278 site in vivo.

AMPK-mediated VASP Thr-278 Phosphorylation Decreases Cellular F-actin Content—VASP phosphorylation modulates F-actin formation in vitro (7, 8). However, in cells the precise consequences of differential VASP phosphorylation for the F-actin ratio and cytoskele-
FIGURE 5. AMPK-mediated VASP Thr-278 phosphorylation interferes with F-actin assembly. HeLa cells were transiently co-transfected with cDNAs coding for wild-type (wt), dominant negative (DN), or constitutively active (CA) forms of the AMPKα1 subunit and VASP-mutants. A, schematic representation of the VASP mutants AAA, AAE, or AAT in the VASP domain structure. VASP consists of a central proline-rich region (PRR) that is flanked by two Ena/VASP homology domains (EVH1 and EVH2). The first phosphorylation site Ser-157 is located within the proline-rich region, whereas the second and third phosphorylation sites (Ser-239, Thr-278) are within the EVH2 domain. To block VASP phosphorylation at positions Ser-157, Ser-239, and Thr-278, we exchanged these residues to alanines. To imitate phosphorylation of Thr-278, the residue was mutated to glutamic acid. In another case it was left unchanged to allow AMPK phosphorylation specifically at this residue. In the scheme the dark, gray, or open boxes indicate an alanine, glutamic acid, or threonine residue at the indicated positions, respectively.

B, after 24 h, overexpressing cells were lysed and probed for pThr-278-VASP (anti-pThr-278), total VASP (anti-VASP), or AMPK mutants using the Myc tag (anti-Myc) by Western blotting. Representative blots from seven experiments are shown. C, after 24 h, AMPK and VASP mutants overexpressing cells were fixed, and F-actin was stained with Cy5-phalloidin and quantified using FACS-analysis. F-actin content of VASP AAA-overexpressing cells and MOCK-transfected cells was set to 100 and 0%, respectively. Column bars represent means ± S.D. (n = 7; *, p < 0.007 for the comparisons shown).

D, HeLa cells in six-well plates were transfected with the reporter pSRE, coding for SRE-driven firefly luciferase, the control plasmid phRL-TK, which codes Renilla luciferase, and expression vectors for AMPK and VASP mutants in the indicated combinations. Cells were harvested after 24 h of culture, and luciferase activities were measured in cell lysates as described under “Experimental Procedures,” with firefly luciferase activity normalized to Renilla luciferase activity. Normalized luciferase activities in VASP-AAA, and MOCK-transfected cells were assigned values of 100 and 0%, respectively. Columns present means ± S.D. (n = 4; *, p < 0.01).

E, immunocytochemistry of VASP and AMPK mutants. Transfected cells were fixed, permeabilized, and stained using antibodies against the Myc tag (anti-Myc) of the AMPK mutants and TRITC-conjugated phalloidin to stain actin fibers. The white arrowheads indicate stress fibers.
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**FIGURE 6.** AMPK-mediated VASP phosphorylation is decreased in aortic tissue in a rat model of type II diabetes mellitus. Aortas were isolated from ZDF and lean rats and rinsed, and tissue lysates were prepared immediately. Proteins were separated by SDS-PAGE and analyzed by Western blotting. A, aortic tissues were probed using antibodies against VASP phosphorylated at Thr-275 (anti-pThr-278) or polyclonal anti-VASP antibodies, which quantify total VASP. B, pThr-275 was quantified from Western blot signals using densitometric analysis and was normalized to total VASP. Columns give means ± S.D. (n = 4; *, p < 0.05, lean versus ZDF). C–F, aortas from ZDF versus lean controls were probed for AMPK (anti-AMPK) (C), active AMPK phosphorylated at Thr-172 (anti-pAMPK) (D), the AMPK substrate acetyl-CoA carboxylase phosphorylated at Ser-79 (anti-pACC) (E), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (anti-GAPDH) (F). The plots show representative results from n = 6 per group.

AMPK-mediated Thr-278 phosphorylation impairs actin polymerization by an independent approach, we utilized a SRF transcriptional reporter assay in intact adherent cells (Fig. 5D). The assay quantifies cellular G-actin pool depletion due to a rise of SRE binding SRF expression (30, 31). The conversion of G- to F-actin both by inducing F-actin assembly and inhibiting F-actin disassembly stimulates SRF activity, which increases SRE-dependent expression of the luciferase reporter gene (3). Transient VASP overexpression induces F-actin assembly (34, 35) and SRE-driven luciferase activity (3). Consistently, normalized luciferase activity of HeLa cells transfected with VASP mutants was markedly elevated as compared with MOCK-transfected control cells (set to 0%). Luciferase activity was maximal in VASP-AAA-transfected cells (set to 100%). A single negative charge at the third site in VASP-AAE reduced the signal to 30%, whereas the activity of VASP-AAT-expressing cells was in between VASP-AAA and -AAE (66%, not shown). In support of our hypothesis that phosphorylation at Thr-278 impairs F-actin assembly, co-expression of VASP-AAT with wta-AMPK and, more pronounced, with Cαα-AMPK reduced the activity (70 and 33%, respectively). In contrast, luciferase activity in Dα-AMPK- and VASP-AAT-expressing cells was high (95%) and close to the VASP-AAA level (100%). Together, these results strongly suggest that AMPK-mediated Thr-278 phosphorylation interferes with VASP-induced filament assembly in cells. Additionally, we used immunofluorescence to visualize the consequences of VASP-Thr-278 phosphorylation on the cytoskeleton in transfected HeLa cells (Fig. 5E). Consistent with the localization of endogenous AMPKα subunits (36), overexpressed Myc-tagged wta were found to be diffusely distributed in the cytoplasm. Corresponding to the FACS data and luciferase assay results, these cells presented few actin stress fibers, which were visualized by fluorescence-labeled phalloidin. Furthermore, stress fibers were amplified in VASP-AAA as compared with VASP-AAE transfected cells, which served as internal controls (supplemental Fig. 5I). Coexpression of the VASP-AAT mutant with wta-AMPK strongly increased actin filament formation as compared with MOCK-transfected cells. Importantly, actin fiber formation was further enhanced in cells expressing Dα-AMPK. In line with the hypothesis that AMPK activity negatively regulates F-actin content and stress fiber formation, actin filaments were reduced in cells that co-expressed the Cαα-AMPK and VASP-AAT. These data demonstrate that AMPK activity impairs F-actin assembly by VASP Thr-278 phosphorylation, resulting in defective stress fiber formation.
AMPK-mediated VASP Phosphorylation Is Reduced in the Aorta of ZDF Rats—It has been proposed that AMPK signaling cascades may be involved in many of the metabolic perturbations observed in type II diabetes mellitus (for review, see Ref. 37). To analyze AMPK-mediated VASP phosphorylation in diseased vessels, we utilized ZDF rats that are leptin-receptor deficient and serve as a rodent model of type II diabetes with endothelial dysfunction (38) and decreased AMPK activity (39). We investigated the degree of VASP phosphorylation in any of the three sites and localization of these differentially phosphorylated proteins in the aorta of diabetic animals. In rats, VASP residues Ser-154, Ser-236, and Thr-275 correspond to the human phosphorylation sites Ser-157, Ser-239, and Thr-278, respectively. Notably, our human VASP phospho-specific antibodies cross-react with the rat homologs (not shown). Total VASP protein in ZDF rat aortas was indistinguishable from controls (Fig. 6A, lower panel). pThr-275 signal intensities determined from Western blots were reduced to 30% as compared with control animals (lean, 100%; Fig. 6A and B). In contrast, the fraction of Ser-154-phosphorylated VASP (indicated by the shifted upper band) appeared to be independent of Thr-275 phosphorylation status (Fig. 6A). In vessels of control animals, anti-pThr-278 and anti-Thr-278 determined the ratio of Thr-278 phosphorylated to total VASP as 1:4, consistent with low pThr-278 levels in non-stimulated human platelets (7). Consistent with earlier reports (40, 41), expression of AMPK protein was not altered in diabetic animals as compared with lean controls (Fig. 6C). The amount of active, Thr-172-phosphorylated AMPK coincided with pThr-275 levels and was largely reduced in vessels of diabetic animals (Fig. 6D). Another sign of defective AMPK signaling was diminished AMPK phosphorylation of ACC (pACC levels shown) in vessels from ZDF rats (Fig. 6E), whereas glyceraldehyde-3-phosphate dehydrogenase levels were unaffected (Fig. 6F).

To further dissect the phosphorylation status of the three phosphorylation sites, we analyzed aortic ring sections for pSer-154, pSer-236, and pThr-278 using our phospho-specific anti-VASP antibodies in immunohistochemistry (Fig. 7). In accordance with the Western blot results, pThr-275 was severely diminished in vessels of ZDF rats as compared with lean controls, whereas total VASP signals were indistinguishable in both groups. The reduced pThr-275 levels were most conspicuous in the endothelial and subendothelial smooth muscle cell layers (Fig. 7, A–F). In contrast, pSer-154 did not differ in aortas of ZDF and control rats as judged from fluorescence intensities in immunohistochemical sections, confirming the Western blot analyses. Ser-154-phosphorylated VASP almost completely localized
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at the luminal plasma membrane of the endothelial layer (Fig. 7, G–M). Consistent with data from models of defective vascular NO/cGMP signaling in rabbits, rats, and humans (12, 13), VASP phosphorylation at the PKG site Ser-236 was largely reduced both in endothelial and smooth muscle cells of the aortas of ZDF rats (Fig. 7, N–S). Furthermore, in accordance with the Western blot signals, immunohistochemistry revealed reduced phosphorylation levels of ACC and AMPK, whereas AMPK antigen was unchanged (Fig. 7, T–Y). These results demonstrate that AMPK-mediated Thr-275 phosphorylation is largely decreased in the aortas of diabetic rats and suggest VASP pThr-275 as a potential vascular marker for pathological glucose metabolism.

DISCUSSION

The present study identifies VASP as a novel AMPK substrate in vitro in living cells and in a rat model for diabetes. We demonstrate that AMPK-mediated phosphorylation of the residue Thr-278 reduces F-actin accumulation activity of VASP and impairs stress fiber formation. Defective cytoskeleton organization has important implications for a variety of actin-based processes such as changes of cellular morphology and cell–cell or cell-matrix adhesion and is suspected to be important for endothelial dysfunction in diabetic vessel disorders (42, 43). We identify AMPK as the first kinase that phosphorylates the Thr-278 site of VASP in vivo. AMPK-mediated VASP phosphorylation could provide a directly link from metabolic energy regulation to cytoskeletal control.

Originally, AMPK was discovered to inhibit anabolic and initiate catabolic pathways by rapid, direct phosphorylation of their rate-limiting enzymes. Indeed, AMPK activity preserves glucose homeostasis by phosphorylating glycogen synthase and 6-phosphofructo-2-kinase (44) and switches off ATP-consuming enzymes such as acetyl-CoA carboxylase (33) or hydroxymethylglutaryl-CoA reductase in anabolic fat metabolism pathways (for a complete list of AMPK substrates see Kahn et al. (15)). Recently, the importance of AMPK-driven signaling cascades has evolved. Using transgenic mouse models, AMPK signaling was demonstrated to be involved in the regulation of food intake by integrating hormonal and nutrient signals in the hypothalamus (45). Furthermore, AMPK activity is important in the cardiovascular system since mutations in regulatory γ2 subunit are associated with hereditary heart disease in humans, i.e. the Wolff–Parkinson–White syndrome (46). Our work further expands the role of AMPK. We demonstrated that AMPK phosphorylates VASP, a protein critically involved in actin polymerization, bundling, and nucleation (1, 47). AMPK specifically targets the VASP Thr-278 site, but not Ser-157 and Ser-239 (Fig. 3), and the phosphorylation status of Thr-278 seems to be independent of PKA- and PKG-mediated VASP phosphorylation at Ser-157 and Ser-239 (Fig. 1C). Indeed, the amino acid sequence flanking Thr-278 (T) in human VASP (GLMEEMLNMLRRKATQVGEKTP) is a variant of the proposed consensus AMPK recognition motif, harboring the core motif Hyd-(basic, X)-X-X-(S/T)-X-X-Hyd (X is any amino acid; Hyd is a hydrophobic residue) (16). This AMPK site is located at the C-terminal end of the B-block (positions 259–276) within the VASP EVH2 domain. The EVH2 domain and especially residues within the B-block are known to promote F-actin binding to VASP and actin polymerization in vitro. Recently, analyses of VASP-driven F-actin accumulation confirmed the importance of residues within the B-block for actin polymerization in living cells (3, 10). Both studies utilized that the transcription factor SRF is regulated by changes of the cellular G-/F-actin equilibrium. VASP-stimulated SRE-driven transcription is dependent on the F-actin binding B-block adjacent to the Thr-278 phosphorylation site and, indeed, F-actin assembly driven by VASP mutants lacking this critical segment is defective (3). Consistent with these studies, our data indicate a crucial role of Thr-278 phosphorylation for VASP-mediated actin filament formation in cells (Fig. 5). AMPK-mediated VASP Thr-278 phosphorylation may modulate the electrostatic interactions between the nearby arginine residues and the negatively charged actin filament. Indeed, PKG- and PKA-mediated phosphorylation of Ser-239 within the A-block, another motive important for VASP-mediated F-actin formation, support the concept that phosphorylation of sites within EVH2 inhibit actin polymerization in vitro (8) and in cells (10).

Although the detailed molecular mechanisms of phosphorylation in actin assembly remain to be elucidated, VASP anti-capping and filament bundling activities in vitro are clearly inhibited upon phosphorylation by PKA (6). Interestingly, phosphorylation of Ser-239 was primarily responsible for loss of anti-capping activity, whereas phosphorylation of Ena/VASP proteins by PKA at the phosphorylation site conserved in all vertebrate Ena/VASP proteins (Ser-157 in VASP) did not interfere with anti-capping activity in vitro. Therefore, phosphorylation at the conserved site (Ser-157) might serve other functions than regulation of actin dynamics (6, 48). Previous VASP studies investigating PKA- and PKC-mediated phosphorylation at Ser-157 demonstrated an increased localization of Ser-157 phosphorylated VASP at the lamellipodium in endothelial cells (25). Although the function of VASP at this site is not completely clarified, the protein seems to be important for lamellipodia dynamics and cell motility (49) most probably by its actin bundling activity (5). At the tip of lamellipodia the VASP concentration correlates with the velocity of protrusions (50), suggesting that VASP participates in actin turnover at this site. At the leading edge, Ser-157 phosphorylation is dynamically regulated and increased during cell detachment and reattachment but reduced during cell spreading (51). As shown for the VASP Ser-157 phosphorylation site, spatially regulated phosphorylation of the Ser-239 and Thr-278 residues within the EVH2 domain by cyclic nucleotide- and AMPK-mediated signaling pathways could regulate VASP anti-capping and bundling activities in vivo. However, clarification of the precise spatial and temporal regulation of differential Ena/VASP phosphorylation patterns is a challenging topic requiring future systematic investigation.

One of the unexpected findings of this study is that the cyclic nucleotide-dependent protein kinases PKA and PKG do not phosphorylate VASP at the Thr-278 residue in living cells. Previous data, based primarily on in vitro experiments using purified PKA catalytic subunit and PKG suggested that Thr-278 might be targeted by these kinases (7). However, even after long-term incubation with PKA the magnitude of pThr-278
was minor when compared with pSer-157 and pSer-239 phosphorylation in vitro (7, 8, 10). The reasons for the disparity between results of the previously published in vitro studies and our investigations of VASP phosphorylation in cells is not entirely clear, but results obtained in intact cells provide more physiologically relevant data. Indeed, in platelets specific activation of PKA and PKG increased phosphorylations at the first and second site but did not did not alter pThr-278 (7). In contrast, AMPK specifically phosphorylates Thr-278 in vitro in primary and stable cell lines and in animal models. The basal pThr-278 levels (Fig. 2) corresponds to the basal AMPK activity observed in endothelial cells (52).

The present study focuses on AMPK-mediated VASP phosphorylation specifically in endothelial cells and vascular walls, because AMPK signaling is impaired in diabetic vessels and may contribute to diabetic retinopathy, nephropathy, or microvascular leakage. An important finding was that decrease of pThr-278 was accompanied by reduced pSer-239, but not pSer-157, in vessels of diabetic animals (Fig. 7). The concomitant decrease of pSer-239 and pThr-278 levels suggests the existence of cross-talk between NO/cGMP signaling, which regulates Ser-239 phosphorylation in the endothelium (11), and AMPK-mediated pathways in diabetic conditions (Figs. 6 and 7). Several mechanisms might explain co-regulation of NO/cGMP-dependent phosphorylation of VASP Ser-239 with AMPK-mediated phosphorylation of VASP-Thr-278. Recently, a direct link between AMPK and NO/cGMP signaling in endothelial cells was suggested by the fact that Metformin-stimulated AMPK can phosphorylate and activate endothelial nitric oxide synthase and, thus, uncouple endothelial nitric oxide synthase and, thus, decrease vascular bioavailability of NO (for a recent review, see Ref. 54) and in turn PKG activity and pSer-239. The coupling of pSer-239 and pThr-278 seems to be of functional importance for actin dynamics. In vitro studies demonstrate that phosphorylation of both Ser-239 and Thr-278 inhibit actin polymerization (8). Consistently, pSer-239 and pThr-278 synergistically interfere with F-actin formation in cells. Interestingly, actin cytoskeleton is impaired in retinal endothelial cells of diabetic rats (55), and AMPK activation using 5-aminoimidazole-4-carboxamide-1β-riboside or exercise, which activates AMPK in many tissues, prevented the development of diabetes in ZDF rats (39, 56), supporting the contribution of AMPK signaling for diabetic vessel disorders. It will be interesting to determine the phosphorylation status of the three VASP phosphorylation sites in this model using our phospho-specific antibodies. The set of phosphorylation specific antibodies offers the opportunity to quantify and to dissect PKA-, PKG-, and AMPK-mediated signaling via differential VASP phosphorylations in vitro.

In summary, this study identifies VASP as new AMPK substrate. AMPK is responsible for phosphorylation of the VASP Thr-278 site in vitro, in living cells, and in vivo. AMPK-mediated VASP Thr-278 phosphorylation interferes with actin polymerization and impairs formation of stress fibers, thus creating a novel link between glucose metabolism and regulation of the endothelial actin cytoskeleton. AMPK activity and AMPK-mediated Thr-278 phosphorylation are reduced in the vascular wall of a rat model of type II diabetes mellitus, with potential implications for diabetic actin-based vascular disorders such as microvascular leakage or diabetic retinopathy.

Acknowledgments—We are grateful to Dr. J. Bauersachs for donating rat aortas, Dr. O. Ritter for providing the protein phosphatase 2B cDNA, Dr. K. Schuh for helpful discussions, Dr. Stepam Gambaryan and Dr. S. M. Lohmann for critically reading the manuscript, and Dr. Sabine Herterich for sequencing the VASP mutants (all at the University of Würzburg).

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