Regulation of Human Endothelial Cell Focal Adhesion Sites and Migration by cGMP-dependent Protein Kinase I*

Received for publication, December 6, 1999, and in revised form, May 8, 2000
Published, JBC Papers in Press, June 12, 2000, DOI 10.1074/jbc.M909632199

Albert Smolenski, Wolfgang Poller‡, Ulrich Walter, and Suzanne M. Lohmann§

From the Institut für Klinische Biochemie und Pathobiochemie, Medizinische Universitätsklinik, Josef-Schneider Strasse 2, D-97080 Würzburg, Germany

cGMP-dependent protein kinase type I (cGK I), a major constituent of the atrial natriuretic peptide (ANP)/nitric oxide/cGMP signal transduction pathway, phosphorylates the vasodilator-stimulated phosphoprotein (VASP), a member of the Ena/VASP family of proteins involved in regulation of the actin cytoskeleton. Here we demonstrate that stimulation of human umbilical vein endothelial cells (HUVECs) by both ANP and 8-(4-chlorophenylthio)guanosine 3′,5′-monophosphate (8-pCPT-cGMP) activates transfected cGK I and causes detachment of VASP and its known binding partner (zyxin) from focal adhesions in >60% of cells after 30 min. The ANP effects, but not the 8-pCPT-cGMP effects, reversed after 3 h of treatment. In contrast, a catalytically inactive cGK Iβ mutant (cGK Iβ-K405A) was incapable of mediating these effects. VASP mutated (Ser/Thr to Ala) at all three of its established phosphorylation sites (vesicular stomatitis virus-tagged VASP-AAA mutant) was not phosphorylated by cGK I and was resistant to detaching from HUVEC focal adhesions in response to 8-pCPT-cGMP. Furthermore, activation of cGK I, but not of mutant cGK Iβ-K405A, caused a 1.5–2-fold inhibition of HUVEC migration, a dynamic process highly dependent on focal adhesion formation and disassembly. These results indicate that cGK I phosphorylation of VASP results in loss of VASP and zyxin from focal adhesions, a response that could contribute to cGK alteration of cytoskeleton-regulated processes such as cell migration.

Nitric oxide and atrial natriuretic peptide (ANP) activate soluble or particulate guanylyl cyclases, respectively. The cGMP produced by these cyclases has multiple effectors within endothelial cells (HUVECs) by both ANP and 8-(4-chlorophenylthio)guanosine 3′,5′-monophosphate (8-pCPT-cGMP) activates transfected cGK I and causes detachment of VASP and its known binding partner (zyxin) from focal adhesions in >60% of cells after 30 min. The ANP effects, but not the 8-pCPT-cGMP effects, reversed after 3 h of treatment. In contrast, a catalytically inactive cGK Iβ mutant (cGK Iβ-K405A) was incapable of mediating these effects. VASP mutated (Ser/Thr to Ala) at all three of its established phosphorylation sites (vesicular stomatitis virus-tagged VASP-AAA mutant) was not phosphorylated by cGK I and was resistant to detaching from HUVEC focal adhesions in response to 8-pCPT-cGMP. Furthermore, activation of cGK I, but not of mutant cGK Iβ-K405A, caused a 1.5–2-fold inhibition of HUVEC migration, a dynamic process highly dependent on focal adhesion formation and disassembly. These results indicate that cGK I phosphorylation of VASP results in loss of VASP and zyxin from focal adhesions, a response that could contribute to cGK alteration of cytoskeleton-regulated processes such as cell migration.

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 355/B4. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: Dept. of Cardiology and Pneumonology, University Hospital Benjamin Franklin, Free University Berlin, 12200 Berlin, Germany.
§ To whom correspondence should be addressed. Tel.: 49-30-201-3477; Fax: 49-30-201-3150; E-mail: slohmann@klin-biochem.uni-wuerzburg.de.

1 The abbreviations used are: ANP, atrial natriuretic peptide; cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; EVH, Ena/VASP homology; HUVEC, human umbilical vein endothelial cell; HEK, human embryonic kidney; VSV, vesicular stomatitis virus; Ad5, adenovirus type 5; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′,5′-monophosphate; PBS, phosphate-buffered saline; MLC, myosin light chain.

Published, JBC Papers in Press, June 12, 2000, DOI 10.1074/jbc.M909632199

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responsible for tetramerization of VASP (23), which may stabilize interactions of the VASP EVH1 domain with the focal adhesion proteins vinculin and zyxin (26).

VASP is phosphorylated by cAMP- and cGMP-dependent protein kinases on Ser157 (3), located just distal to the EVH1 domain in the central proline-rich domain of VASP, and on Ser239 and Thr278, located in the VASP C-terminal EVH2 domain (23, 25, 27). So far, only phosphorylation of Ser157 and Ser239 has been demonstrated to be of major significance in intact cells (27), and phosphorylation of these sites can be distinguished using specific antibodies (28). The phosphorylation site containing Ser157 is preferred by cAK, whereas the site containing Ser239 is preferred by cGK I in vitro and in intact cells; however, both kinases phosphorylate all three sites (27, 28). Information is, however, scarce concerning the influence of cGMP-mediated VASP phosphorylation on the described VASP interactions with other proteins or on cytoskeletal function.

Furthermore, studies on many cell types, including endothelial cells, are hampered by the rapid decline in cGK levels in cell culturing and passaging (5, 29). Therefore, in this work, we present a system for studying the effect of VASP phosphorylation, using endothelial cells lacking endogenous cGK I, in the absence and presence of expressed cGK I. cGK I phosphorylation of VASP promoted detachment of VASP (but not a triple phosphorylation site containing Ser157) and zyxin from focal adhesions, thus altering the composition and possible functional integrity of these structures. In support of this, cGK I also inhibited collagen-, fibronectin-, and fibrinogen-mediated endothelial cell migration, which is a highly dynamic process dependent on focal adhesion formation and destruction.

EXPERIMENTAL PROCEDURES

Cells—Endothelial cells were isolated from human umbilical veins and cultured as described previously (30). HUVECs from only passages 7–10) and the human embryonic kidney cell line (HEK 293) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal calf serum and 1% antibiotic solution. All cells were kept at 37 °C with 5% CO2.

Antibodies—The following antibodies against VASP were used: rabbit antiserum M3 against VASP (6), monoclonal anti-VASP antibodies IE226 and IE273 (71), monoclonal anti-phospho-Ser239 antibody 16C2 raised against a VASP peptide phosphorylated on Ser239 (28), and monoclonal anti-phospho-Ser157 antibody 5C6 (prepared by the same approach used for making the 16C2 antibody and to be described in more detail elsewhere). Other antibodies used were as follows: rabbit antiserum against cGK I Ig (31), monoclonal anti-phospho-Ser-Thr 32; and monoclonal anti-vinculin antibody hVIN-1, monoclonal anti-VSV antibody P5D4, and fluorescein isothiocyanate-labeled anti-rabbit secondary antibody (Sigma, Deisenhofen, Germany). Cy2-labeled anti-mouse and Cy3-labeled anti-rabbit antibodies were from Rockland, Inc. (Gilbertsville, PA), and Texas Red-labeled anti-mouse antibody was from Molecular Probes, Inc. (Leiden, The Netherlands).

Vectors and Constructs—The recombinant adenoviral vector Ad5-cGK I or containing human cGK I Ig cDNA (33, 34) was constructed as described previously (35). The catalytically inactive cGK I K405A mutant was newly constructed by oligonucleotide-directed mutagenesis (Transfomer site-directed mutagenesis kit, CLONTECH, Palo Alto, CA) of cGK Ig cDNA. The sequence of the mutagenic primer was 5’-GGTT TCTG TGA GAA TCCTG TCT TGA CAG TTT CG-3’. This plasmid was linearized with XhoI and ligated with the long XhoI fragment of RR5 DNA, an Ad5 mutant that carries a unique XhoI site and a deletion of the E1 region ranging from nucleotides 445 to 3333 of the wild-type adenovirus type 5 genome. Subsequently, the ligion product was transfected into E1α-transfected HEK 293 cells; and after 10–14 days, recombinant virus was recovered and plaque-purified as described previously (36).

Phosphoexpression vectors were constructed using pcDNA3 (Invitrogen, DeSchep, The Netherlands) and human VASP N-terminally tagged with an epitope of VSVG glycoprotein G. The vectors for wild-type VASP and for three mutants carrying single amino acid substitutions at each phosphorylation site (S157A, S239A, and T278A) were constructed previously (28). Now, double (AAAT) and triple (AAA) phosphorylation mutants of VASP-VSVG were generated by combining one or more singly mutated VSVGs using uninfected and altered regions sites.

Expression Experiments and Western Blot Analysis—HUVECs or human fibroblasts seeded on six-well plates were infected with Ad5 vectors at concentrations of 104 to 106 particles/ml in serum-free medium for 2 h at 37 °C. Subsequently, the virus-containing medium was replaced by fresh serum-containing medium. Alternatively, plasmid transfections of HUVECs were performed using Superfect (Qiagen Inc., Hilden, Germany) according to the instructions of the manufacturer. Later, 16–24 h after either infection or transfection, cells were washed once with serum-free medium and incubated for 5–30 min with 10 mANP (Sigma), 100 m8-pCPT-cGMP (Biolog, Bremen, Germany), or 3 m forskolin (Sigma) in serum-free medium containing 0.1% bovine serum albumin at 37 °C. Subsequently, cells were briefly washed with PBS and then lysed in SDS sample buffer for SDS-PAGE analysis or fixed for immunofluorescence as described below. For immunoblotting, samples were separated by SDS-polyacrylamide gel electrophoresis using 9% gels and subsequently analyzed on Western nitrocellulose blots. Nitrocellulose sheets were blocked overnight in blocking medium (PBS, 0.3% Triton X-100, 0.05% Tween 20, 1% hemoglobin, and 0.01% Na3VO4); incubated for 1 h at room temperature with anti-cGK I antiserum A10 (diluted 1:200), monoclonal anti-VASP antibody IE273 (diluted 1:1000), monoclonal anti-phospho-Ser239 VASP antibody 16C2 (1 μg/ml), or monoclonal anti-phospho-Ser157 VASP antibody 5C6 (2 μg/ml); and then subsequently incubated for 1 h with 3.7 kBq/ml 125I-labeled protein A or 7.4 kBq/ml 125I-labeled sheep anti-mouse antibody (both from Amersham Pharmacia Biotech, Freiburg, Germany) followed by autoradiography (see Figs. 1A and 2) or with horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies followed by ECL detection (Amersham Pharmacia Biotech) (see Figs. 1B, 4, and 10A). The amount of cGK expressed (Fig. 1A) was determined by cutting the radioactively labeled bands out of the nitrocellulose sheet and counting them in a scintillation counter. Various concentrations of purified recombinant cGK were used as a protein standard.

VASP Immunoprecipitation and In Vitro Phosphorylation—Plasmid vectors for VSVG-tagged VASP and VASP phosphorylation site mutants (Ser/Thr to Ala) were transfected into HEK 293 cells using calcium phosphate. Two days after transfection, cells were harvested and solubilized in buffer containing 20 mTris-HCl (pH 7.4), 75 mNaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mEDTA, 10 mPMSF, and 1% aprotinin. Cell lysates were immunoprecipitated using an anti-VSVG antibody and rabbit anti-mouse IgG1 coupled to magnetic beads (Dynal, Oslo, Norway). Immune complex phosphorylation was performed with the precipitates in buffer containing 10 mHEPES (pH 7.4), 5 mMgCl2, 1 mhtiothyrolthyl, 0.2 mEDTA, 4 μM ATP, 1 μC of [γ-32P]ATP, and either 50 ng of cGK catalytic subunit purified as described (37) or 50 ng of purified cGK I (38) activated with 20 μM cGMP. Samples were phosphorylated for 30 min at 30 °C and then stopped and analyzed using Western blotting as described above. Expressed VSV-VASP was detected using the monoclonal anti-VSVG antibody, a horseradish peroxidase-coupled anti-mouse secondary antibody, and ECL analysis. After washing ECL labeling from the nitrocellulose, 3P incorporation into VASP was subsequently detected by autoradiography.

Indirect Immunofluorescence—HUVECs, grown on glass coverslips previously coated with 1% gelatin for 45 min and then fixed for 15 min with 0.5% glutaraldehyde and washed five times with M199 medium, were treated with cGMP-elevating agents as described below, washed twice with PBS, fixed with 4% paraformaldehyde for 15 min on ice, washed twice with PBS, permeabilized by 0.2% Triton X-100 in PBS for 5 min, blocked with 1% BSA, and then incubated with primary antibodies (see below) for 1 h at 37 °C and with secondary antibodies for 1 h at 37 °C. The coverslips were mounted in Moviol 4-88 (Hoechst, Frankfurt, Germany)-containing mounting medium with 1% N-propyl gallate, and cells were examined with a Leitz Aristoplan microscope. Photographs were taken with Tri-X-Pan 400 or TMAX 400 film (both from Eastman Kodak Co.).
HUVECs infected with 5 × 10⁵ particles of adenoviral cGK I vectors/ml (>90% transfection efficiency) were incubated with either 100 nM ANP or 100 μM 8-pCPT-cGMP for 30 min (in some cases, also 3 h) for determining the localization of VASP, zyxin, or vinculin. cGK staining was visualized using polyclonal anti-cGK I antibody A10 diluted 1:1000 and fluorescein isothiocyanate-labeled anti-rabbit secondary antibody diluted 1:30. For cGK I-VASP colocalization studies, VASP was visualized using undiluted monoclonal anti-VASP antibody IE226 and Texas Red-labeled anti-mouse secondary antibody diluted 1:50. Vinculin was stained using monoclonal anti-vinculin antibody hVIN-1 diluted 1:50 and Cy3-labeled anti-mouse secondary antibody diluted 1:200 and Cy2-labeled anti-mouse secondary antibody diluted 1:150. For vinculin-zyxin colocalization studies, zyxin was stained using polyclonal antibody AS83-1 diluted 1:100 and Cy3-labeled anti-rabbit secondary antibody diluted 1:50. Vinculin was stained using undiluted monoclonal anti-VASP antibody IE226 and Texas Red-labeled anti-mouse secondary antibody diluted 1:50. The percentage of cGK I-expressing HUVECs (50–100 cells analyzed per experiment) staining positive for VSV-VASP (or the VSV-1:50. The percentage of cGK I-expressing HUVECs (50–100 cells analyzed per experiment) staining positive for VSV-VASP (or the VSV-tagged wild-type or mutant VASP, cGK I was stained using polyclonal anti-cGK I antibody A10 diluted 1:1000 and fluorescein isothiocyanate-labeled anti-rabbit secondary antibody diluted 1:30; expressed VSV-VASP was stained using monoclonal anti-VSV antibody F5D4 diluted 1:10,000 and Texas Red-labeled anti-mouse secondary antibody diluted 1:50. The percentage of cGK I-expressing HUVECs (50–100 cells analyzed per experiment) staining positive for VSV-VASP (or the VSV-tagged triple phospho-VASP mutant) at focal adhesions after cGK I activation was determined.

Migration Assay—Haptotactic cell migration assays were performed using modified Boyden chambers (Transwell filters, 8-μm pore size, Costar Corp., Cambridge, MA). The lower surface of the filter membranes was coated with 3 μg/ml human fibronectin, 10 μg/ml collagen I, or 10 μg/ml fibrinogen (all from Sigma). The upper surface of the membrane and the remaining free binding sites on the lower surface were blocked with 0.5% bovine serum albumin. Subconfluent HUVECs were trypsinized and resuspended in M199 medium containing 25 mM HEPES, 1 mM MgCl₂, and 0.5% bovine serum albumin, and then 30,000 cells were seeded per filter. After 5 h of incubation at 37 °C with or without 100 μM 8-pCPT-cGMP or 500 nM ANP, cells on the upper surface of the membrane were removed with a cotton swab. Migrated cells on the lower membrane surface were stained with 1% (v/v) crystal violet (Sigma) in 2% (v/v) ethanol, washed with H₂O, and dried. The stain was eluted with 10% (v/v) acetic acid, and its absorbance was measured at 600 nm.

RESULTS

Functional Expression of cGK I in HUVECs—A replication-deficient adenoviral vector containing the cDNA of human cGK I was constructed and used to evaluate the function of cGK I in HUVECs lacking endogenous cGK I. Infection of HUVECs with different amounts of vector (10⁶ to 10¹³ particles/ml) resulted in the expression of 0.2–2.0 μg of cGK I/mg of cell protein as assessed by immunoblotting (Fig. 1A) (data not shown), which is in the range of the endogenous cGK I concentration in other types of human endothelial cells (5). In subsequent experiments, adenoviral vectors were usually used at concentrations of 10¹⁰ particles/ml or less. At 10¹⁰ particles/ml (Fig. 1A), a well known cGK I degradation product (65 kDa) was observed; and at higher levels of expression, additional smaller (most likely breakdown) products were detected. Expressed cGK I was demonstrated to be functionally active by treatment of HUVECs with a cell membrane-permeant, hydrolysis-resistant, cGK-selective cGMP analog, 8-pCPT-cGMP, which activates the kinase. In response to 8-pCPT-cGMP, cGK I phosphorylated its well characterized substrate, VASP, as demonstrated by Western blot analysis (Fig. 1B). An antibody against total VASP (IE273) detected a shift in the electrophoretically determined size of VASP, from an apparent size of 46 kDa in unstimulated cells to 50 kDa in cGMP-stimulated, cGK I-expressing cells (Fig. 1B, first panel, sixth lane), a shift resulting from phosphorylation of VASP Ser¹⁵⁷. This was confirmed using a newly developed antibody (5C6) specific for Ser¹⁵⁷ in its phosphorylated state (Fig. 1B, second panel). VASP phosphorylation on Ser¹⁵⁷ was also observed with uninfected HUVECs stimulated with 3 μM forskolin, an activator of adenylate cyclase and thus cAK (Fig. 1B, first two panels, second lanes). A higher concentration of forskolin (10 μM) also caused Ser¹⁵⁷ phosphorylation (data not shown). In adenoviral vector infection studies of HUVECs, the control of catalytically inactive cGK I-K405A (third and fourth lanes) gave no VASP phosphorylation of Ser¹⁵⁷ (first two panels) or Ser²³⁹ (third panel) in response to 8-pCPT-cGMP (fourth lanes). In contrast, expressed wild-type cGK I showed some basal activity (fifth lanes) that was further stimulated by 8-pCPT-cGMP (sixth lanes) compared to both Ser¹⁵⁷ (first two panels) and Ser²³⁹ (third panel) phosphorylation. Phosphorylation of VASP on Ser²³⁹, either without (46-kDa band) or with (50-kDa band) concomitant phosphorylation of Ser¹⁵⁷, was detected by the 16C2 antibody. Shown are examples of experiments performed at least three times.
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Fig. 2. Catalytically inactive cGK Iβ-K405A mutant inhibits endogenous cGK I phosphorylation of VASP in human dermal fibroblasts. Fibroblasts were infected with increasing concentrations (1–50 × 10^5 particles/ml) of adenoviral vector for mutant cGK Iβ-K405A and 1 day later were incubated either with 100 μM 8-pCPT-cGMP (+) or with vehicle alone (−). Cell homogenates (20 μg of protein) were analyzed on Western blots labeled with specific antibodies for either cGK I (A) or VASP phosphorylated on Ser^157 (16C2 antibody) (B). Uninfected fibroblasts (first two lanes) contain endogenous cGK I (not visible on the cGK I blot shown because of short film exposure), which phosphorylates endogenous VASP after stimulation by 8-pCPT-cGMP (second lane). Infection of fibroblasts with increasing amounts of adenoviral vector for mutant cGK Iβ-K405A led to a dose-dependent increase in mutant cGK I expression (A, third through sixth lanes) and a concurrent decrease in VASP phosphorylation by endogenous wild-type cGK I (B). Examples of experiments performed three times are shown.

Fig. 3. Detachment of endogenous VASP from focal adhesions in 8-pCPT-cGMP-treated HUVECs expressing cGK Iβ. One day after Ad5-cGK Iβ infection (10^5 particles/ml), cells were stimulated with 100 μM 8-pCPT-cGMP for 30 min and fixed for immunofluorescence. In the double-labeling experiments shown, a monoclonal antibody (IE226) was used to visualize VASP (A, C, and E) in cells either expressing (bright cells) or not expressing (dark cells) cGK Iβ (B, D, and F) detected with polyclonal antiserum A10. A and B, uninfected cells, C–F, Ad5-cGK Iβ-infected cells. A–D, nonconfluent cells; E and F, confluent cells. The VASP staining of focal adhesions observed in uninfected cells (A) was absent (C) in cGK Iβ-expressing cells (bright cells in D) activated for 30 min in nonconfluent HUVEC cultures. However, at the same time in confluent HUVEC cultures, VASP remained in cell-cell contacts (E) of stimulated cGK Iβ-expressing cells (F). Representative data from at least 10 experiments are shown. Bar = 20 μm.

Other with a newly developed, catalytically inactive cGK Iβ mutant (cGK Iβ-K405A) (Fig. 1B, third and fourth lanes; and see Fig. 4, first through third lanes) or with a luciferase control vector or in uninfected HUVECs (data not shown). These results clearly demonstrate that cGMP-dependent phosphorylation of VASP does not occur in the absence of cGK and therefore is not mediated by cAK. Similar results were obtained from in vitro kinase assays performed with lysates from cells treated as shown in Fig. 1B, i.e. cGMP-activatable kinase activity was present only in cGK Iβ-infected cells (data not shown). Polymerase chain reaction analysis also detected no cGK I mRNA in pure HUVECs uncontaminated by fibroblasts, which do contain cGK I (data not shown).

Mutant cGK Iβ-K405A was not only itself catalytically inactive, but was also capable of inhibiting (Fig. 2B, third through sixth lanes) wild-type cGK I activity (second lane), as demonstrated in passage 8 human dermal fibroblasts containing endogenous cGK I (cGK I observed upon longer film exposure than that shown in Fig. 2A). VASP phosphorylation by 8-pCPT-cGMP-stimulated endogenous cGK I was completely inhibited by 5 × 10^5 particles of mutant cGK Iβ-containing adenoviral vector/ml, which produced a severalfold overexpression of the mutant kinase compared with the endogenous kinase. The mutated Lys^405 is the homolog of the essential Lys^72 in the catalytic ATP-binding site of cAK that is conserved in all protein kinases (39). The mutant’s inhibitory effect on wild-type cGK I function could be abolished by raising the concentration of 8-pCPT-cGMP from 100 to 500 μM (data not shown), suggesting that the inactive mutant competed with wild-type cGK I for the cGMP analog. In contrast, VASP phosphorylation in fibroblasts induced by 10 μM prostaglandin E_2, an agonist of endogenous cAK, was not inhibited by mutant cGK Iβ (data not shown).

Early Detachment of VASP from Focal Adhesions after cGK Iβ Activation in HUVECs—In cultured HUVECs, VASP is localized at focal adhesions, microfilaments, and cell-cell contacts (Fig. 3, A, C, and E) (5). In our present studies, VASP localization was studied by indirect immunofluorescence in HUVECs infected with the adenoviral vector for cGK I (Fig. 3, C–F). Cells were infected with 10^5 particles of viral vector for cGK Iβ/ml and 24 h later were treated with 100 μM 8-pCPT-cGMP for 30 min to activate the kinase, the procedure that produced maximal VASP phosphorylation (blot in Fig. 4). Subsequently, cells were immediately fixed for immunofluorescence. Double labeling with antibodies against cGK I and VASP demonstrated that VASP was no longer detectable at focal adhesions (Fig. 3C) in any cGK I-expressing HUVECs (bright cells in Fig. 3D) in nonconfluent monolayers. Instead, a diffuse VASP signal was observed in the cytosol, and some VASP staining was still detected along stress fibers, although it did not resemble the normal periodic pattern. Whereas complete loss of VASP from focal adhesions occurred at 30 min, a reduction in VASP at focal adhesions was observed already after 5 min of 30 μM 8-pCPT-cGMP treatment, the earliest time point and lowest cGMP analog concentration at which maximal phosphorylation of VASP was detectable by immunoblotting (data not shown). This early loss appeared as a marked general reduction in focal adhesion labeling intensity, which progressed at later times to complete loss of labeling. The specificity of the effect of cGK Iβ expression and activation on VASP localization was demonstrated by the lack of any such response to 8-pCPT-cGMP in uninfected cells (Fig. 3A), in cells at the periphery in Fig. 3C corresponding to cells not expressing detectable cGK Iβ (Fig. 3D), or in cells infected with a control (luciferase) adenoviral vector (data not shown) or with an adenoviral vector for the catalytically inactive cGK Iβ-K405A mutant (see Fig. 5A and 6A). Although cGK I activation caused a loss of VASP from focal adhesions in nonconfluent monolayers of HUVECs, it did not alter the predominant VASP localization at cell-cell contacts in confluent monolayers (Fig. 3E).

Loss of Zyxin and Vinculin from Focal Adhesions after cGK Iβ Activation in HUVECs—To investigate cGK-mediated rearrangement of focal adhesion proteins in more detail, we analyzed the localization of VASP as well as that of the established VASP-binding proteins zyxin and vinculin at different times of
cGK activation. cGK was activated either directly with 8-pCPT-cGMP or indirectly with ANP, which elevates intracellular cGMP levels via the ANP receptor guanylyl cyclase. The phosphorylation of VASP in HUVECs expressing cGK I, incubated for 5 min to 3 h with 100 nM ANP or 100 μM 8-pCPT-cGMP, is shown in Fig. 4. More important, no cGMP-dependent VASP phosphorylation was observed in HUVECs expressing the catalytically inactive cGK I-K405A mutant.

Dependence of VASP Intracellular Redistribution on VASP Phosphorylation by cGK I—The role of cGK I-dependent VASP phosphorylation in the observed redistribution of VASP was examined using VASP phosphorylation mutants in which Ser157, Ser239, and Thr278 were replaced by Ala, either singly or by double mutation of both serines (AAT) or by triple mutation of all three phosphorylation sites (AAA). To distinguish both transfected wild-type and mutant VASPs from endogenous VASP present in HUVECs, the former were expressed as VSV epitope-tagged fusion proteins and detected using an antibody directed against VSV in both Western blotting and immunofluorescence. Efficacious VASP phosphorylation site mutation was demonstrated by expressing wild-type or mutant VASP in HEK 293 cells, immunoprecipitating them, and determining their ability to be phosphorylated by purified cGK I or cAK in vitro using [γ-32P]ATP. The anti-VSV antibody detected essentially equal amounts of all VSV-VASP forms on Western blots of the phosphorylated samples (Fig. 10A); however, phosphate incorporation into the triple mutant protein (AAA) was observed.

Cotransfection of plasmids of cGK I and VSV-tagged wild-type VASP into HUVECs, followed by 30 min of 8-pCPT-cGMP
stimulation of kinase, led to detachment of VASP from focal adhesions (Fig. 11C). In contrast to the previously described experiments using adenoviral vectors, in which virtually all cGK I-expressing cells demonstrated cGMP-dependent VASP loss from focal adhesions under the same conditions used here, experiments using plasmid vectors demonstrated that only ~43% of cGK I-expressing cells lost V85V-tagged wild-type VASP from focal adhesions. However, a similar pattern of VASP relocalization was observed as in the adenoviral vector experiments, i.e., a diffuse VASP staining in the cytosol sometimes appearing as micro-aggregates, with residual stress fiber staining devoid of periodic character. Mutant VASP could localize to focal adhesions (Fig. 11E), indicating that this basic property was not disturbed by the mutations. However, after stimulation of cotransfected cGK I, the triple phosphorylation mutant of VASP was more resistant to detachment from focal adhesions (lost from only 11 ± 7% of cGK I-expressing cells) than was wild-type VASP (lost from 43 ± 9% of cells) (Fig. 11, G and C, respectively; percentages in text represent means ± S.E. of four independent experiments in which 50–100 cells were analyzed per experiment). The detachment of the double mutant of VASP from focal adhesions was not observably different from that of wild-type VASP in response to 8-pCPT-cGMP (data not shown).

Inhibition of HUVEC Migration by cGK Iβ—The functional consequence of cGK I activation on HUVEC migration was examined. In a haptotactic migration assay, HUVEC migration on fibronectin (Fig. 12A) as well as on collagen and fibronogen matrices (Fig. 12B) was inhibited by expression and activation of cGK Iβ, but not the catalytically inactive cGK Iβ-K405A mutant. This was the case when the expressed kinase was activated either directly with 100 µM 8-pCPT-cGMP or indirectly with 500 nM ANP. The inhibitory effect of ANP in the 5-h migration assay was slightly weaker than that of 8-pCPT-cGMP, perhaps reflecting the observed decline in ANP activation of cGK after 3 h (Fig. 4). Our studies demonstrate for the first time in endothelial cells that cGK, and not cAK, mediates cGMP-dependent inhibition of migration since the catalytically inactive cGK Iβ-K405A mutant did not inhibit migration. Collagen and fibronogen are known to activate the migration of HUVECs via integrins α2β1 and α5β1, respectively (40, 41). Fibronectin binds to integrins α2β1, α6β4, and α4β1 on endothelial cells (42). Thus, cGK I inhibition of haptotactic cell migration was independent of the integrins involved.

**Discussion**

Expression and activation of cGK Iβ (but not a catalytically inactive cGK Iβ-K405A mutant) in primary cultures of HUVECs resulted in depletion of several cytoskeletal proteins from focal adhesions, beginning with VASP and zyxin within 30 min and later vinculin, followed by microfilament disruption, thus causing a destabilization of motility structures that may
Regulation of Focal Adhesions and Migration by cGK I

8-pCPT-cGMP stimulation of cGK I-dependent detachment of endogenous zyxin and vinculin from focal adhesions in HUVECs. HUVECs were infected and treated with 100 μM 8-pCPT-cGMP (cGMP) as described in the legend to Fig. 6. In double-labeling experiments, zyxin (polyclonal antibody AS83-1; A, C, E, and G) was stained together with vinculin (monoclonal antibody hVIN-1; B, D, F, and H). 8-pCPT-cGMP-stimulated cells expressing the catalytically inactive Gtr-1β-K405A mutant (A and B) or unstimulated cells expressing wild-type (wt) cGK I (C and D) displayed no change in the localization of zyxin and vinculin in comparison with uninfected cells (not shown). After 30 min of 8-pCPT-cGMP stimulation of cells expressing wild-type cGK I (E and F), zyxin labeling at focal adhesions was nearly abolished, and that of vinculin was reduced. In contrast to the results obtained with ANP (see Fig. 7), a complete loss of zyxin and vinculin labeling of focal adhesions was observed after 3 h of 8-pCPT-cGMP treatment (G and H). See quantitation of data from three experiments in Fig. 9. Bar = 20 μm.

In this study, all three VASP phosphorylation sites were investigated in intact cells using a combination of site-specific antibodies and site-specific mutation. In particular, VSV-tagged VASP mutants that could be expressed and distinguished from endogenous VASP were used to demonstrate that direct VASP phosphorylation was required for VASP detachment from focal adhesions since the VSV-tagged VASP-AAA triple phosphorylation mutant was highly resistant to 8-pCPT-cGMP-dependent detachment from focal adhesions in comparison with wild-type VASP. However, in the absence of 8-pCPT-cGMP, all phosphorylation site mutants of VASP were targeted to focal adhesions and showed a periodic distribution along stress fibers, suggesting that the mutations did not induce any major changes in the tertiary structure of VASP. VASP detachment from focal adhesions was inhibited only if all three phosphorylation sites were mutated, suggesting that all three sites are important for regulation of VASP interaction with cytoskeletal components. Ser157 is located just distal to the VASP EVH1 domain, and Ser239 and Thr278 are located in the VASP EVH2 domain (12, 23). Specific functions ascribed to these domains are consistent with our results that point mutations of phosphorylation sites close to and within these domains affect VASP localization at focal adhesions. VASP truncation and other experiments have shown that both the EVH1 and EVH2 domains are involved in VASP localization at focal adhesions and that the EVH2 domain affects the function and binding properties of the EVH1 domain (discussed in Ref. 23). Addition-
Ser157 phosphorylation. Data like those shown were obtained in three experiments.

The data clearly demonstrated that both EVH1 and EVH2 domains of Ena/VASP family members are essential but alone not sufficient for the overall function of these proteins (43). Furthermore, during the review process for our manuscript, another publication appeared demonstrating that expression of the EVH2 domain in keratinocytes inhibited VASP function in keratinocytes of transgenic mice overexpressing the EVH2 domain, abnormalities in intercellular adhesion were observed (44).

Thus, it is highly conceivable that Ser278 phosphorylation, in combination with Ser157 and Ser239 phosphorylation, could affect important VASP functions, ultimately resulting in a major effect like VASP displacement from focal adhesions.

The exact role of each individual VASP phosphorylation site in either VASP loss from or recovery to focal adhesions is not clear. It was noted, however, that after 3 h of ANP treatment, VASP localization at focal adhesions began to recover concomitant with a relative loss of Ser157 phosphorylation. This was not observed after 3 h of 8-pCPT-cGMP treatment, most likely due to the greater metabolic stability of this cGMP analog in comparison with unmodified cGMP formed in response to ANP. It is possible that incomplete VASP phosphorylation is insufficient for initiating VASP removal from focal adhesions and perhaps sufficient for its recovery. All available data support the interpretation that all three VASP phosphorylation sites are critical for the interaction of VASP with focal adhesion components. This complexity is perhaps not surprising since VASP interacts via its EVH1 and EVH2 domains with at least two distinct components present in focal adhesions, FP, motif-containing proteins (e.g., zyxin and vinculin) and F-actin, as discussed below.

In comparison with VASP, VASP-related proteins show conservation of only Ser157 and Ser239 (MenA), only Ser157 (Ev1), or no sites (Ena), suggesting that VASP homologs may display different regulation in response to phosphorylation. Phosphorylation of these sites has not been extensively studied, but a cAK-induced shift in MenA migration in SDS-polyacrylamide gel electrophoresis has been shown (14), and our 16C2 antibody detects phosphorylation of MenA, probably at Ser176 homologous to VASP Ser239. Interestingly, tyrosine phosphorylation of Ena on other sites by Abl tyrosine kinase attenuates Ena association with profilin or SH3 domain-containing proteins, and mutation of these phosphorylation sites partially impairs the in vivo function of Ena, indicating that phosphorylation is required for optimal Ena function (45).

Phosphorylation state has also been shown to regulate focal adhesion localization of other proteins. Paxillin dephosphorylation on tyrosine resulted in its loss from focal adhesions in response to cAMP (46), whereas serine and threonine phosphorylation has been shown to accelerate fibronectin-induced localization of paxillin to focal adhesions (47). In contrast, serine phosphorylation of talin in response to interleukin-1β decreased talin’s focal adhesion localization (48), and phorbol ester stimulation of protein kinase C increased talin serine phosphorylation and talin removal from focal adhesions (49). Thus, cGK I regulation of focal adhesion constituents exemplifies "inside-out" regulation of adhesion sites by yet a new signal transduction pathway.
Our results in intact cells provide a physiological correlate to the previous demonstrations of in vitro interactions between VASP and either zyxin or vinculin (26, 32). These interactions were demonstrated using one solid phase (nitrocellulose, column, or microtiter plate)—bound partner to bind another from solution. More recently, VASP and vinculin have also been shown to co-immunoprecipitate after in situ cross-linking experiments (50). The direct binding partner that attaches VASP to F-actin has been shown to co-immunoprecipitate after cross-linking experiments (50). In agreement with our observations, Ikeda et al. (68) demonstrated that cGK I stimulation resulted in loss of normal functioning of the MAP kinase since this did not affect MAP kinase activity (58). Alternatively, a role for cGK I in MLC phosphatase activation has been discussed (59–61). Another potential target of cGK I is the small GTP-binding protein RhoA, which has been discussed (59–61). RhoA can be inhibited by phosphorylation through cAK, which correlates with an inhibition of lymphocyte motility by cAMP (63).

The regulation of focal adhesions is intimately involved in the process of cell migration, which is a dynamic process involving polarized formation and disassembly of focal adhesions at opposite cell ends (64, 65). Lack of focal adhesions and stress fibers in Chinese hamster ovary cells expressing a mutant integrin resulted in decreased migration in haptotactic assays like that used here, although the cells were motile in a random migration assay (65). However, others have reported that decreasing vinculin and α-actinin via antisense RNA increased focal adhesion density (7–9). The time course and concentration of cGMP that caused VASP phosphorylation were very similar to those that inhibited fibrinogen binding. However, VASP phosphorylation may be only one of the effects of cGK I on the cytoskeletal system. cGK I in platelets is known to inhibit phospholipase C and myosin light chain (MLC) phosphorylation (10, 55), which are suggested to be involved in tension- and stress fiber-mediated focal adhesion assembly (56, 57). Inhibition of MLC phosphorylation is unlikely to be mediated by cGK I phosphorylation of MLC kinase since this did not affect MLC kinase activity (58). Alternatively, a role for cGK I in MLC phosphatase activation has been discussed (59–61). Another potential target of cGK I could be the small GTP-binding protein RhoA, which has been shown to mediate the thrombin-induced increase in MLC phosphorylation in endothelial cells (62). RhoA can be inhibited by phosphorylation through cAK, which correlates with an inhibition of lymphocyte motility by cAMP (63).

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migration of rat aorta endothelial cells was inhibited by 8-Br-cGMP as well as by natriuretic peptides that elevate cGMP. However, focal adhesions and the presence of endogenous cGK I were not investigated. The migration of smooth muscle cells stably transfected with cGK I was also inhibited by cGMP analogs (69). Furthermore, overexpression of endothelial nitric-oxide synthase in balloon-injured carotid arteries resulted in impaired endothelial regeneration (70), consistent with possible nitric oxide (or cGMP)-dependent inhibition of endothelial cell proliferation and migration.

Collectively, our data demonstrate that cGMP has effects on VASP phosphorylation and localization and on cell migration that are mediated by cGK and not cAK. Use of a combination of VASP phosphorylation site-specific antibodies and mutations demonstrated the importance of cGK I-dependent VASP phosphorylation in inhibiting VASP localization at focal adhesions. Continued studies are necessary to more closely define the far-reaching consequences of cGK I-dependent inhibition of focal adhesion integrity and cellular processes that depend on them.

Acknowledgments—We thank the staff of the Theresienklinit for providing the inhibitor curcubita; Susanne Sturm, Petra Thalhammer, and Petra Honig-Liedl for expert technical assistance; Christiane Bach for single site VASP mutants; Matthias Reinhard for valuable discussions and advice; and Martin Eigenthaler for an introduction to the migration assay.

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