The development and functional analysis of a monoclonal antibody (16C2) are reported; the antibody recognizes vasodilator-stimulated phosphoprotein (VASP; an established substrate of both cAMP- and cGMP-dependent protein kinase) only when serine 239 is phosphorylated. VASP serine 239 represents one of the best characterized cGMP-dependent protein kinase phosphorylation sites in vitro and in intact cells. Experiments with purified, recombinant human VASP and various VASP constructs with mutated phosphorylation sites (S157A, S239A, T278A) and experiments with intact cells (human/rat platelets and other cells) treated with cyclic nucleotide-elevating agents demonstrated the specificity of the monoclonal antibody 16C2. Quantitative analysis of the VASP shift from 46 to 50 kDa (indicating VASP serine 157 phosphorylation) and the appearance of VASP detected by the 16C2 monoclonal antibody (VASP serine 239 phosphorylation) in human platelets stimulated by selective protein kinase activators confirmed that serine 239 is the VASP phosphorylation site preferred by cGMP-dependent protein kinase in intact cells. Immunofluorescence experiments with human platelets treated with cGMP analogs showed that the 16C2 monoclonal antibody also detects VASP serine 239 phosphorylation in situ at established intracellular localization sites. Analysis of VASP serine 239 phosphorylation by the 16C2 antibody appears to be the best method presently available to measure cGMP-dependent protein kinase activation in intact cells. Also, the 16C2 antibody promises to be an excellent tool for the evaluation of VASP function in intact cells.

Although cGMP-dependent protein kinases (cGPKs) have been recognized as important components of major signal transduction pathways (1–3), quantitative analysis of cGPK activation in intact cells has been very difficult (1–4). This is because of the relatively low expression of cGPK in most cell types compared with the relatively high expression of its closest functional homolog, the cAMP-dependent protein kinase (cAPK), and the scarcity of specific cGPK substrates. Unfortunately, the mediating role of cGPK for a given effect/function is often implied or excluded by the use of cGPK activators and/or inhibitors alone, which is clearly insufficient to establish or rule out functional roles of cGPKs (1–4). One of the few established cGPK substrates is the 46-kDa/50-kDa vasodilator-stimulated phosphoprotein (VASP), which was initially discovered and characterized as a substrate of both cAPK and cGPK in human platelets (5–8). VASP phosphorylation in response to cyclic nucleotide-regulating vasodilators (i.e. cAMP-elevating prostaglandins and cGMP-elevating nitric oxide donors) closely correlates with platelet inhibition and in particular with the inhibition of fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ of human platelets (9–11). Molecular cloning of human, canine, and mouse VASP predicted highly homologous proteins and revealed a proline-rich protein that is organized into three structural segments of different sequence complexity (12, 13). VASP is the founding member of a new family of proline-rich proteins, which includes Enabled (Ena), a dose-dependent suppressor of Drosophila Abl- and Disabled-dependent phenotypes, its mammalian homolog Mena, and the Ena-VASP-like protein Evl (14–16). These proteins all share an overall domain organization consisting of highly homologous NH$_2$-terminal and COOH-terminal domains (Ena-VASP homology domains 1 and 2, EVH1 and EVH2), which are separated by a proline-rich central domain of low complexity (12–16). In platelets and many other cells including vascular smooth muscle cells, endothelial cells, and fibroblasts, VASP has been found to be associated with stress fibers, focal adhesions, cell-cell contacts, and highly dynamic membrane regions (16, 17). VASP colocalizes with profilins and binds directly to their poly(t-proline) binding site (18), binds to and colocalizes with zyxin and vinculin (16, 19), and also directly binds to Listeria monocytogenes surface protein ActA, which is essential for the actin polymerization-based intracellular motility of this pathogen (20). Functional evidence indicates that VASP is a crucial factor involved in the enhancement of spatially confined actin filament formation (16, 20, 21).

Three distinct phosphorylation sites were biochemically identified in VASP (serine 157, serine 239, and threonine 278) which are used in vitro and in intact human platelets by both cAPK and cGPK and by the serine/threonine protein phosphatases 2A and 2B with overlapping selectivity (8, 22). Phosphorylation of serine 157, the site preferred by the cAPK, leads to a marked shift in apparent molecular mass of VASP in SDS-PAGE from 46 kDa to 50 kDa (6, 8). In experiments with...
Generation of a Monoclonal Antibody Recognizing VASP Phosphorylated at Position Ser239—Dephospho- and phosphopeptides (RKVS(239)KQE or RKVPs(239)KQE) representing the VASP serine 239 phosphorylation site (8, 12) were synthesized on an Applied Biosystems model 431A peptide synthesizer using Fmoc chemistry. Phosphoserine was incorporated during peptide synthesis using Fmoc-Ser(PO2Bzl)OH-OH (Calbiochem). Peptides were purified to $\geq 98\%$ purity by reversed phase chromatography using a Vydac 18T5 column. The peptides were conjugated to thioltated keyhole limpet hemocyanin (nanoTools) after activation with bromoacetic acid-N-hydroxysuccinimide ester (Sigma). Female BALB/c mice (6 weeks old) were immunized subcutaneously with the keyhole limpet hemocyanin phosphopeptide immunogen (10 mg/ml; Sigma) four times at 14-day intervals with the first injection in complete Freund's adjuvant and the following injections in incomplete Freund's adjuvant. 2 weeks later, animals were given booster injections (10 mg of immunogen in PBS) on 3 consecutive days. 1 day after the last booster injection, animals were killed, and their spleens were excised. Spleen cells were fused with nonproducer myeloma cells. Hybridoma cell lines secreting antibodies specific for phosphorylated VASP were identified initially by differential screening using phosphorylated and nonphosphorylated peptides covalently bound to enzyme-linked immunosorbent assay plates (DNA-BIND, Costar). Subsequently, immobilized recombinant His$_6$-VASP or His$_6$-VASP phosphorylated in vitro by cGPK was used. Monoclonal antibodies were purified from serum-free cell culture supernatants by thiophilic adsorption chromatography (thiophilic support based on POROS 50-OH, nanoTools).

Construction of VASP Mutants—pVSV-VASP is based on the expression vector pcdNA3 (Invitrogen) and encodes human VASP (12) NH$_2$-terminally tagged with an epitope of vesicular stomatitis virus (VSV) glycoprotein G (23). The tag (amino acids YYTDEMNRLGK, preceded by methionine) was fused via a proline to the second amino acid of VASP. In this vector, site-directed mutagenesis of the phosphorylation sites (serines 157 and 239, threonine 278) to alanines was performed using splice overlap extension polymerase chain reaction. For the mutation of serine 157 to alanine (S157A), the sequence of the mutagenic primer was 5'-GGCCGGGTTGCGAGAATGCAGGAGG-3' (the mismatches with the wild-type VASP sequence are printed in lowercase). Together with the corresponding reverse primer and primers from the NH$_2$- terminal and COOH-terminal region of VASP a mutant VASP fragment was amplified and cloned back into the VASP plasmid pVSV-VASP using unique restriction sites. The sequence of the mutagenic primer for the S239A

**EXPERIMENTAL PROCEDURES**

Materials—All standard chemicals were obtained from Merck and Sigma. Restriction enzymes were from New England Biolabs and oligonucleotides from Amersham Pharmacia Biotech.
obtain the rat PRP. Ice-cold 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM imidazole buffer (pH 8.0)/1 M ammonium acetate cell mass. After overnight incubation at 4 °C without shaking, the agarose beads were collected by centrifugation at 2,000 × g for 10 min. The loaded nickel-nitrilotriacetic acid-agarose corresponding to a bed volume of 1.25 ml of a 50% slurry was then washed five times with 6 ml each of ice cold 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 50 mM imidazole buffer (pH 8.0), 0.5% (v/v) Triton X-100, including protease inhibitors. The beads were carefully drained dry and then batch eluted with 50 mM sodium phosphate buffer (pH 7.0) with 300 mM NaCl, 100 mM EDTA in three steps each of a 10-min incubation at room temperature in a total elution volume of 1.25 ml. The eluate was then dialyzed in cold against 1 liter of 25 mM HEPES buffer (pH 7.0) with 75 mM NaCl, 5% (v/v) glycerol, and 1 mM dithiothreitol for 24 h with three buffer changes. The sample was finally concentrated.

**Phosphorylation of Recombinant VASP by cAPK and cGPK**—The catalytic subunit of cAPK was purified as described (24, 25). Purified recombinant His6-VASP (25 μg/ml) was phosphorylated by a purified catalytic subunit of cAPK (11 μg/ml) or purified cGPK (24 μg/ml) at 30 °C in a 10 mM HEPES buffer (pH 7.4) containing 5 mM MgCl2, 1 mM dithiothreitol, 0.2 mM EDTA, 100 μM ATP, and (only with cGPK phosphorylation conditions) 20 μM cGMP. Aliquots were taken at various time points, mixed immediately with an SDS-containing stop solution (66.6 mM Tris-HCl buffer (pH 6.7) containing 2% SDS, 5% glycerol, 50 mM NaOH), and cells were adjusted to a final concentration of one stock solution (66.6 mM Tris-HCl buffer (pH 6.7) containing 2% SDS, 5% glycerol, 50 mM NaCl, 2 mM leupeptin, 1 mM aprotinin, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM mg/μl lysylzyme; they were incubated on ice for 30 min and lysed by ultrasonic disintegration in the presence of 5 μg/ml DNase I and 10 μg/ml RNase. The efficiency of lysis was monitored by phase-contrast microscopy. After adding 1 mM imidazole buffer (pH 8.0) to a final concentration of 50 mM and adding Triton X-100 to 0.5% the lysate was extracted by stirring on ice for 20 min. The supernatant after centrifugation at 16,500 × g for 10 min was adjusted in NaCl to a final concentration of 300 mM, recentrifuged, and added to a settled bed volume of 1.25 ml of 50% nickel-nitrilotriacetic acid-agarose (Qiagen; equilibrated previously in 50 mM sodium phosphate buffer (pH 8.0), containing 5% (v/v) glycerol, 300 mM NaCl, 50 mM imidazole buffer (pH 8.0)/1 M ammonium acetate cell mass. After overnight incubation at 4 °C with shaking, the agarose beads were collected by centrifugation at 2,000 × g for 10 min. The loaded nickel-nitrilotriacetic acid-agarose corresponding to a bed volume of 1.25 ml of a 50% slurry was then washed five times with 6 ml each of ice cold 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 50 mM imidazole buffer (pH 8.0), 0.5% (v/v) Triton X-100, including protease inhibitors. The beads were carefully drained dry and then batch eluted with 50 mM sodium phosphate buffer (pH 7.0) with 300 mM NaCl, 100 mM EDTA in three steps each of a 10-min incubation at room temperature in a total elution volume of 1.25 ml. The eluate was then dialyzed in cold against 1 liter of 25 mM HEPES buffer (pH 7.0) with 75 mM NaCl, 5% (v/v) glycerol, and 1 mM dithiothreitol for 24 h with three buffer changes. The sample was finally concentrated.

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**Transfection of PK2 Cells—**PK2 cells were grown in minimal essential medium (GIBCO, Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.) until they were about 50% confluent. Then plasmids containing the cDNA of epitope-tagged wild-type and mutated VASP under the control of a cytomegalovirus promoter (pSV-VASP) were cotransfected together with an expression vector for cGPK Iβ into PtK2 cells, which contain very low amounts of endogenous cGPK. 2 days after transfection, cells were incubated with 30 μM 8-pCPT-cGMP for 30 min and then lysed immediately with an SDS-containing stop solution and boiled. Aliquots of the lysates were analyzed by immunoblots using a polyclonal antisera against native VASP (M4) or a monoclonal antibody (16C2) prepared against the (phosphorylated) VASP phosphorylation site Ser239.

Immunoblotting—Samples were separated on 9% SDS-polyacrylamide gels. Proteins were then blotted onto nitrocellulose filters and labeled with the VASP antisera M4 (1:1,500, v/v) as described (9) followed by 125I-protein A (ICN) for radiolabeling or peroxidase-labeled anti-rabbit antibody detection by ECL (Amershams Pharmacum Biotech) or with the monoclonal antibody prepared against VASP phosphorylated at position Ser239 (16C2, 0.5 μg/ml) followed by 125I-labeled sheep anti-mouse antibody or peroxidase-labeled anti-mouse antibody. Figs. 4 and 7 are autoradiograms of 125I-labeled blots; Figs. 2 and 3 show ECL-labeled blots.

**Immunofluorescence of Human Platelets—**PRP was diluted 1:10 in PBS, and platelets were allowed to spread on glass coverslips for 45 min at room temperature as described (17). Then the cells were incubated with PBS alone or with 100 μM 8-pCPT-cGMP in PBS for 15 min at room temperature. The cells were washed once with PBS and fixed immediately with 4% (v/v) paraformaldehyde in PBS for 15 min at 4 °C, washed once with PBS, and subsequently permeabilized for 10 min with 0.2% (v/v) Triton X-100 in PBS at room temperature. The samples were then incubated with the M4 VASP antisera (1:1,000; v/v) and with the antibody 16C2 (3.3 μg/ml) for 1 h at 37 °C followed by labeling with fluorescein isothiocyanate-anti-rabbit antibody (Sigma) and Texas Red anti-mouse antibody (Molecular Probes) for 1 h at 37 °C. Photographs were taken with a Leitz Aristoplan microscope on Kodak Tri-X-400 film.

**RESULTS AND DISCUSSION**

Three phosphorylation sites have been identified previously in VASP (serine 157, serine 239, and threonine 278) which are phosphorylated by both cAPK and cGPK in vitro and in intact cells with overlapping specificity. As shown in Fig. 1, the serine 157 and serine 239 VASP phosphorylation sites are preferred by cAPK and cGPK, respectively. However, with respect to VASP phosphorylation in intact cells, only serine 157 phosphorylation could be monitored effectively by analyzing the electrophoretic shift of VASP from the 46- to the 50-kDa species in SDS-PAGE (6-9). To obtain a possible tool for the detection of VASP Ser239 phosphorylation and thereby also a possible sensitive marker for cGPK activation in intact cells, a monoclonal antibody (16C2) against a phosphorylated peptide (RKKpSL239[KQE]) representing the VASP Ser239 phosphorylation site was developed and
purified as outlined under “Experimental Procedures.”

The specificity of this 16C2 antibody was tested by analyzing the time course of the cGPK- and cAPK-catalyzed phosphorylation of purified recombinant VASP (expressed as hexahistidine-tagged protein in E. coli). Coomassie Blue staining revealed an initial shift-complete shift of VASP from 46 to 50 kDa (indicating Ser157 phosphorylation) after a 0.3-min/5-min incubation time with cAPK versus 0.6 min/15 min with cGPK, which was also observed by immunoblot analysis using the polyclonal antibody (M4) against VASP (Fig. 2). In contrast, the newly developed monoclonal antibody 16C2 did not recognize VASP at all as unphosphorylated protein (0-min aliquot in Fig. 2), but a strong signal appeared after VASP had been incubated with cAPK and cGPK using phosphorylation conditions (Fig. 2). However, a striking time course difference with respect to the appearance of a 16C2-recognized VASP signal was observed. With cGPK, a clear signal was detected at the earliest time point tested (0.3 min) which reached its maximum at about the 5-min incubation time with no apparent VASP shift (46–50 kDa form) at 0.3 min. With cAPK, a 16C2-recognized signal only appeared after 15 min and increased thereafter, whereas the VASP shift was complete within 5 min of the incubation time (Fig. 2). These data demonstrate that the 16C2 monoclonal antibody does not recognize the phosphorylation site responsible for the VASP mobility shift in SDS-PAGE (serine 157) and indicate that the 16C2-recognized phosphorylation site (serine 239) is prefered by the cGPK in vitro in agreement with our published data (6–9). Further evidence was sought to demonstrate the specificity of the 16C2 for the phosphorylated VASP sequence around serine 239. Therefore, VASP mutants lacking each of the three phosphorylation sites (mutants S157A, S239A, T278A) were constructed. Together with cGPK, these constructs and wild-type VASP were expressed in PtK2 cells, which contain very little if any endogenous cGPK (data not shown). Incubation of these transfected PtK2 cells with 30 μM 8-pCPT-cGMP resulted in the phosphorylation of VASP as indicated by the appearance of the 50-kDa VASP form and a 16C2-recognized signal (Fig. 3, wild type; first lane). Without 8-pCPT-cGMP treatment, the M4 VASP antiserum and the 16C2 antibody detected in extracts of these cells only the 46-kDa form of VASP or no signal at all, respectively (data not shown). Mutation S157A prevented the stimulus-induced shift of VASP from 46 to 50 kDa but not the appearance of a 16C2-recognized VASP signal that had the mobility of the 46-kDa species (Fig. 3). Mutation S239A abolished any reactivity of the 16C2 antibody with VASP but did not prevent the stimulus-evoked shift of VASP from the 46- to the 50-kDa species (Fig. 3). Mutation T278A had no effect on the stimulus-induced VASP shift (serine 157 phosphorylation) or the appearance of a 16C2-recognized signal (serine 239 phosphorylation). These data confirm our previous conclusions (6–9) that serine 157 (but not serine 239) is responsible for the phosphorylation-induced VASP mobility shift in SDS-PAGE (46 → 50 kDa) and demonstrate that the VASP immunoreactivity recognized by the monoclonal antibody 16C2 requires VASP serine 239 phosphorylation. These data also suggest that the three point mutations analyzed (S157A, S239A, T278A) have no gross effects on the overall VASP protein because the properties and functions of the other phosphorylation sites remained essentially unchanged.

The experiments with purified proteins and proteins/protein mutants overexpressed in PtK2 cells already indicated that the VASP shift (46 kDa → 50 kDa) and 16C2 immunoreactivity represent VASP serine 157 and 239 phosphorylation, respectively, and that the 16C2 antibody may also be used to detect VASP serine 239 phosphorylation and cGPK activity in intact cells. The latter issue was addressed more systematically in VASP phosphorylation studies with intact human platelets. Established conditions (27, 28) also confirmed by experiments with cGPK-deficient human platelets (29) for potent and specific activation of either platelet cPK (incubation with 1 μM prostacyclin or 0.5 mM 5, 6-DCl-bIMPS) or cGPK (10 μM sodium nitroprusside or 1 mM 8-pCPT-cGMP) were chosen. In extracts of washed, unstimulated human platelets the M4 VASP antiserum detected only the 46-kDa VASP species, whereas the 16C2 antibody detected no specific signal at all (Fig. 4). These data confirm that VASP serine 157 is not phosphorylated in basal, unstimulated human platelets (7–9) and demonstrate this for the first time also with respect to VASP serine 239. PGI2 (1 μM) and SNP (10 μM) induced transient and
reversible phosphorylation of VASP at serine 157 (shift analysis) and serine 239 (16C2 immunoreactivity) as shown in Figs. 4 and 5. The reversibility of VASP phosphorylation was particularly apparent with PGI2 most likely because of the short half-life of PGI2 in solution. With SNP, however, the stimulated Ser239 phosphorylation declined significantly already after 10 min and thereafter, whereas Ser 157 remained elevated, perhaps indicating differential dephosphorylation of VASP serines 157 and 239. Incubations of intact human platelets with the membrane-permeable cAPK and cGPK activators, 5,6-DCl-cBIMPS and 8-pCPT-cGMP, respectively, produced the expected and essentially irreversible extent of VASP serine 157 (shift analysis) phosphorylation and serine 239 (16C2 immunoreactivity) phosphorylation. The results shown in Figs. 4 and 5 indicate that activation of either cAPK or cGPK results in the phosphorylation of VASP serines 157 and 239. In intact human platelets, cAPK-mediated phosphorylation of VASP serines 157 and 239 is very similar with respect to extent and time course, although serine 157 phosphorylation appears to precede serine 239 phosphorylation (Figs. 4 and 5). Activation of cGPK in intact human platelets by SNP or 8-pCPT-cGMP produces the maximal extent of VASP serine 157 phosphorylation but only 50% of VASP serine 157 phosphorylation compared with the cAPK effect (Figs. 4 and 5). In all experiments performed, i.e. extracts of untreated human platelets or platelets/cells treated with cAPK or cGPK activators, the monoclonal antibody 16C2 did not specifically label or detect any protein other than VASP (see Figs. 3, 4, and 7; other data not shown).

The results obtained with the 16C2 antibody presented in Figs. 2–5 suggested the use of this antibody to detect and analyze VASP serine 239 phosphorylation in situ, i.e. at the sites of the cellular localization of VASP. Previous immunofluorescence studies with human platelets spread on glass showed VASP to be localized on the rim of the lamella and associated primarily with radial microfilament bundles representing the contact sites of the platelets with the extracellular matrix (17; see also Fig. 6, A and B). Now, the localization of VASP phosphorylated at serine 239 protein was investigated using the 16C2 monoclonal antibody. In untreated platelets, no specific signal was detected by the 16C2 antibody (Fig. 6 C), whereas a signal overlapping the one detected by the M4 antiserum clearly appeared once the glass-attached human platelets had been incubated with the cGPK activator 8-pCPT-cGMP (Fig. 6, B and D). The staining of VASP with the 16C2 antibody almost completely overlapped the staining of the polyclonal antibody M4 in double labeling experiments (not shown). The only difference observed was a somewhat weaker and less complete staining of the radial microfilaments by the phospho-VASP antibody compared with the staining with the polyclonal M4 antiserum. Also, the VASP signals detected by the 16C2 antibody by Western blot analysis (Figs. 2, 3, 4, and 7) or immunofluorescence analysis were completely prevented if the 16C2 antibody was preincubated with an excess of the phospho-epitope peptide of VASP.
monoclonal antibody was preincubated with the phosphopeptide RKVpS(239)KQE (data not shown).

The results presented indicate that monoclonal antibody 16C2 detects the phosphorylation of VASP at position serine 239 in human cells. Considering the fact that the sequence representing the serine 239 phosphorylation site is highly conserved among human, canine, and rodent VASP (12, 13) we predicted that the 16C2 antibody also detects VASP serine 239 phosphorylation in non-human cells. This was indeed demonstrated by experiments with intact rat platelets (Fig. 7). Treatment of these cells with SNP and PGE1 resulted in the phosphorylation of VASP at positions serine 157 (shift analysis) and serine 239 (appearance of a 16C2-recognized signal) as shown in Fig. 7. The extent and regulation of rat VASP phosphorylation were similar to the effects seen with human platelets, and, importantly, the 16C2 antibody did not specifically recognize any rat platelet protein other than phosphorylated VASP.

CONCLUSIONS

The results of this study demonstrate that monoclonal antibody 16C2 is able to detect and quantitate VASP serine 239 phosphorylation with purified proteins and, most importantly, in crude cell extracts (Western blots) and in fixed, permeabilized cells (immunofluorescence analysis). The results demonstrate for the first time that serine 239 of VASP is essentially dephosphorylated in basal, untreated human platelets and other cell types and rapidly and reversibly phosphorylated when the cGPK or cAPK are activated. Although VASP serines 157 and 239 are the phosphorylation sites preferred by the cAPK and cGPK, respectively, using purified proteins (8, this study) and intact cells (this study) cGPK as well as cAPK clearly use both VASP phosphorylation sites in intact human platelets. This overlapping phosphorylation may be caused by the particularly high concentration of VASP, cGPK, and cAPK known to be present in human cells (7) because a more selective cAPK-mediated VASP serine 157 phosphorylation and cGPK-mediated VASP serine 239 phosphorylation were observed in other human cell types (data not shown). Both VASP serine 157 and 239 phosphorylation can be used as parameters indicating the activation of cAPK, cGPK, or both in intact cells. In experiments with membrane-permeable cGMP analogs and selective activators of the cGPK (i.e. 8-pCPT-cGMP, used at concentrations that do not activate the cAPK) the analysis of cGMP-stimulated VASP serine 239 phosphorylation allows the sensitive detection and quantitation of cGPK activation in intact cells. In fact, we observed (data not shown) that the 8-pCPT-cGMP-induced VASP serine 239 phosphorylation detected in cultured human umbilical vein endothelial cells that do not contain endogenous cGPK (30) indicates a contamination of those cells with a small number of fibroblasts, which are known to contain both VASP and cGPK (17)). Also, the decline of 8-pCPT-cGMP-induced VASP serine 239 phosphorylation in primary cell cultures and cGPK-transfected cells indicates the loss of endogenous or transfected cGPK (30; other data not shown). Because VASP is expressed in most if not all mammalian cells, albeit at different cellular concentrations (16, 17), the analysis of VASP serine 239 phosphorylation can be performed with most mammalian cells. However, VASP and/or the VASP serine 239 phosphorylation site can also be introduced into cells containing very low amounts of endogenous VASP (see Fig. 3).

Previously, the analysis of cGPK activation in intact cells has been very difficult because of the biochemical properties and the relatively low expression of this kinase in most cell types (1–3, 31). Sensitive methods for the analysis of cGPK activity in intact cells are very much needed. Unfortunately, the mediating role of cGPK for a given physiological function/effect is often implied or excluded by the use of cGPK inhibitors claimed to be specific, which is insufficient to establish or rule out functional roles of cGPKs in intact cells. For example, the compound KT 5823 (an indole carbazole and potent inhibitor of the cGPK in vitro (32)) has never been evaluated with respect to cGPK inhibition in intact cells despite its widespread pharmacological use. In our hands, KT 5823 inhibited purified cGPK (as expected) but potentiated 8-pCPT-cGMP-induced VASP serine...
239 phosphorylation in intact human platelets, indicating that cGPK inhibition by this compound in intact cells is not established. Perhaps the analysis of cGMP-induced VASP serine 239 phosphorylation will be helpful to search for more potent and selective cGPK activators and inhibitors useful for intact cell studies.

Finally, the 16C2 monoclonal antibody allows the detection of VASP serine 239 phosphorylation at sites of the endogenous, intracellular VASP localization (i.e. focal contacts, cell-cell contacts, microfilaments) as demonstrated in Fig. 6. Of particular interest would be the analysis of VASP phosphorylation at sites of accelerated actin polymerization, i.e. at the leading edge of lamellipodia and in the tails of L. monocytogenes (16, 17, 20, 21). Although the precise functional role(s) of the VASP phosphorylation sites remain to be elucidated, increasing evidence, also obtained with the VASP-related Drosophila protein Ena, suggests that phosphorylation of Ena-VASP family members alters the interaction of these proteins with their binding partners and their in vivo function (11, 15, 16, 33). Hopefully, the 16C2 monoclonal VASP antibody will be a useful tool to analyze cGPK activation and VASP serine 239 phosphorylation in intact cells and thereby help to define the physiological function of these two proteins in intact cells.

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