Functional Analysis of Type 1α cGMP-dependent Protein Kinase Using Green Fluorescent Fusion Proteins*

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The cGMP-dependent protein kinases (PKGs) are ubiquitous effector enzymes that regulate a variety of physiological processes in response to nitric oxide and natriuretic agonists. We have constructed green fluorescent fusion proteins (GFP) using full-length (PKG-GFP) and truncations encoding either the regulatory domain of PKG1α (G1αR-GFP) or the catalytic domains of PKG1α (GFP-G1C) to examine the enzymatic properties and intracellular location. When transiently transfected into mammalian cells, these constructs were detected on Western blots at the expected sizes using anti-GFP antibodies. The GFP-G1C and the full-length PKG1α-GFP fusion proteins were found to have constitutive activity both in vitro and in vivo. The G1αR-GFP protein was found to dimerize with endogenous type 1 PKG and behaved in a dominant negative manner both in vitro and in vivo. When expressed transiently in either HEK-293 or A549 epithelial cells, the fusion proteins encoding the amino-terminal regulatory domains (PKG-GFP, G1αR-GFP) were present in the cytosol and were rarely observed in the nucleus. In contrast, the GFP-G1C (lacking regulatory domains) concentrated in the nucleus. Of the fusion proteins containing the regulatory region, the constitutive PKG-GFP protein was present in a more centralized location, whereas the G1αR-GFP protein co-localized with F-actin on stress fibers and in dynamic regions of the plasma membrane. Microscopic and immunoprecipitation studies indicated that both the G1αR-GFP and the PKG-GFP fusion proteins colocalized with vasodilator-stimulated phosphoprotein (VASP). These constructs thus represent novel tools with which to visualize inactive, and activated, PKG1α in vivo, and we have used them to demonstrate two functionally independent domains. In addition, we show for the first time in living cells that PKG is found in dynamic membrane regions in association with VASP.

The cGMP-dependent protein kinases, also known as protein kinase G (PKG),1 are the main effector enzymes activated by elevated cGMP levels due to stimulation by nitric oxide and natriuretic peptides (reviewed in Refs. 1–3). These enzymes have many essential regulatory roles in diverse physiological processes, including platelet activation, kidney function, smooth muscle relaxation, gene expression, and chemotaxis (for recent reviews, see Refs. 4–6). In mammals there are three isoforms of PKG that are transcribed from two distinct genes. The α and β isoforms of type 1 PKG are widely distributed and originate from alternative splicing of the same gene (7). The expression of type 2 PKG is more restricted to the kidney, the intestinal brush border, and certain tissues of the brain (8–11).

The primary structure of PKG is highly homologous to cAMP-dependent protein kinase (PKA), particularly in areas that encode nucleotide binding domains in the amino-terminal half of the molecule, and in regions involved in ATP binding and phosphotransferase activity in the COOH-terminal half of the molecule (see Refs. 4–6). A major difference between the two cyclic nucleotide-dependent protein kinases is that the PKA holoenzyme is composed of regulatory and catalytic subunits encoded by distinct genes, whereas the corresponding functional units of PKG are encoded as a contiguous polypeptide. Activation of PKA results in dissociation of the catalytic subunit, which then accumulates in the nucleus where some important substrates are located (1, 12). Several of the functional domains of PKG deduced by comparison with PKA (such as nucleotide binding, active site) have been described in great detail by extensive biochemical analyses of purified protein (1, 13, 14). The area of least homology between PKA and PKG lies in the first 100 amino acid residues and constitutes the principal difference between the two splice variants of type 1 PKG (15). The first 54 amino acids encode a leucine zipper structure that is thought to mediate homodimer formation. Proteolytic removal of this region and part of the adjacent pseudosubstrate motif from purified enzyme results in monomers with constitutive activity. More recently it was shown that the length of PKG greatly increases upon activation by cGMP, suggesting that a PKG monomer might exist in a closed conformation that is hidden within the catalytic region. It has been reported that activation of PKG by cGMP leads to unmasking of this pseudosubstrate motif from the catalytic subunit which then accumulates in the nucleus where some important substrates are located (1, 12).

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1The abbreviations used are: PK, protein kinase; 8-Br-cGMP, 8-bromo-cyclic AMP; PBS, phosphate-buffered saline; VASP, vasodilator-stimulated phosphoprotein; GFP, green fluorescent protein; GKAP, G-kinase-anchoring protein; PAGE, polyacrylamide gel electrophoresis; NLS, nuclear localization sequence.

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which are often also a substrate (22–24). The concept of anchoring proteins is a common theme for the serine/threonine protein kinase superfamily where they serve the important role of localizing the kinase in the vicinity of its specific substrates (25, 26). Supportive evidence for the importance of the leucine zipper motif in targeting PKG has recently emerged using the yeast two-hybrid assay, which has identified several muscle-associated proteins that function as GKAPs by binding to this region (22–24). Although in the past, type 1 PKG was widely believed to be a soluble enzyme, immunofluorescence studies, largely with leukocytes, have indicated that PKG also localizes to discrete cellular compartments (27, 28). Similar evidence has demonstrated that stimulation of cells can lead to alterations in PKG localization that may be important to enzyme function. Other laboratories have not reproduced similar studies, and whether such compartmentalization occurs in nonleukocyte cells remains to be determined.

This study used a molecular approach to investigate the functions of specific domains of PKG1α in the form of GFP fusion proteins. Results shown here demonstrate that the COOH-terminal catalytic region is capable of nuclear translocation but is restricted from nuclear entry by interaction with the amino-terminal regulatory region. Visualization of GFP fluorescence in different cell types indicates that both active and inactive forms of PKG colocalize with the known substrate for PKG: vasodilator-stimulated phosphoprotein (VASP). Both dominant negative and constitutive fusion proteins described here should provide useful tools for future studies of physiological roles for PKG.

**EXPERIMENTAL PROCEDURES**

**Cell Culture/Transfection and Reagents—**Cyclic nucleotides, BD-PETide, and H89 were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). The phosphatase inhibitor mixture and protease inhibitor mixture were from Calbiochem. Nonidet P-40 and Tween 20 were from Fisher. The p81 phosphocellulose was obtained from Upstate Biotechnology Inc. (Lake Placid, NY), and [32P]ATP was from Amer sham Pharmacia Biotech. Unless specified, all other chemicals were from Sigma.

Human lung epithelial cells (A549) and human embryonic kidney (HEK-293) cells were passaged by trypsin treatment prior to confluence and maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Omega Scientific) and supplemented with streptomycin, penicillin, and l-glutamine. The day before transfection, cells were plated at 50% confluence on either 100-mm dishes or six-well plates. The cells were transfected to express exogenous DNAs using LipofectAMINE Plus™ (Life Technologies, Inc.) according to the manufacturer’s instructions. A routine experiment involved addition of DNA liposome mixture to cells in RPMI (1.5 ml/5 ml/100-mm dish, 0.3 ml/1 ml/well) for 4 h followed by addition of an equal volume of normal medium for a further 36 h.

**Construction and Enzyme Assay of Green Fluorescent Fusion Proteins—**The cloning of the cDNA encoding human PKG1α from lung RNA using reverse transcriptase polymerase chain reaction has been described previously (29). A region encompassing amino acids 1–347 that contains the entire regulatory region homologous to the PKA-R1α subunit was generated using polymerase chain reaction. This 1-kilobase fragment was subcloned in frame with GFP into the EcoRI/BamHI sites of the pEGFP-N1 vector (CLONTECH, Palo Alto, CA). The full-length PKG1α coding region and a catalytically inactive mutant (T256A, see Ref. 29) were similarly subcloned into the pEGFP-N1 vector using the same forward primer and a reverse primer that removed the stop codon and produced a fusion protein with EGFP at the COOH terminus. The catalytic regions of PKG1α encompassing amino acids 348–671 were amplified by polymerase chain reaction and subcloned into the EYFP-C1 vector (CLONTECH, Palo Alto, CA) such that the PKG sequence was in frame at the COOH terminus of GFP. Similar fusion proteins of PKGα were generated by adding a primer encoded FLAG epitope (DYKDDDDK) in place of GFP. The FLAG-tagged PKG fusion proteins were all subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). The murine FLAG epitope-tagged VASP protein was a gift from Dr. Michael Uhler (University of Michigan) and has been described elsewhere (21, 29). The kinase activity of the expressed enzymes was measured in cell homogenates using BDFEteiose as substrate as detailed previously (21, 29).

**Immunoprecipitation and Western Blotting—**To prepare crude extracts from transfected cells, monolayers were washed briefly with PBS followed by lysis in extraction buffer (50 mM HEPES, pH 7.0, 1% Nonidet P-40, 1× protease inhibitor mixture) by rocking for 20 min at 4 °C. Homogenates were clarified by centrifugation at 10,000 × g for 10 min and then frozen at −80 °C until needed. For direct electrophoretic analysis 30 μl of homogenates were mixed with 10 μl of 5× PAGE sample buffer, boiled for 5 min, then 10 μl was loaded per lane. For immunoprecipitation studies, antibodies against GFP (Molecular Probes, Eugene, OR) were added to 1 ml of lysis for 1 h at 4 °C, followed by addition of 30 μl of protein A-Sepharose (50% v/v in PBS) for 30 min. Alternatively, 30 μl of anti-FLAG (M2)-conjugated Sepharose beads (Sigma) was added directly to 1 ml of lysis for 1 h at 4 °C. Immunoprecipitates were subsequently washed twice in extraction buffer and one time in PBS. The pellets were resuspended in 30 μl of PBS sample buffer, and 25 μl was loaded per lane. In some cases the proteins were eluted from the beads with 100 μM FLAG peptide prior to electrophoretic analysis.

Electrophoresis of proteins was performed on 10% mini-gels (Bio-Rad) followed by electrophoretic transfer to nitrocellulose. The blots were blocked with 5% bovine serum albumin in PBS buffer (PBS containing 0.05% Tween 20) for 30 min at room temperature, then antibodies were added (1 μg/ml for monoclonals, 1/1000 for serum) for 2 h at 4 °C. Following addition of 1/3000 peroxidase-conjugated secondary antibody (Calbiochem) for 30 min, the bands on the blots were visualized using chemiluminescence according to manufacturer’s instructions.

**Fig. 1.** Properties of GFP fusion proteins encoding full-length and truncated PKG1α. The amino acid sequence homology is compared for PKA type 1α regulatory and catalytic genes and type 1α PKG. Identical residues are indicated as dark shading. The fusion proteins were made with GFP (light shaded area) at the COOH terminus of full-length PKG, with the regulatory regions corresponding to R1α or with the catalytic regions corresponding to C1α. Domains with reported functions such as nucleotide binding, PKG dimerization (variable), and catalysis are indicated above.
At least three washes (5 min each) using excess PTS buffer were performed between each incubation step.

**VASP Dephosphorylation**—To demonstrate the effect of the phosphorylation state of VASP on electrophoretic mobility, HEK-293 cells were transfected to express FLAG-VASP, which was immunoprecipitated using anti-FLAG-Sepharose as detailed above. Following precipitation and washing with lysis buffer, the beads were washed two times with phosphatase buffer (50 mM Tris-HCl, pH 7.2, 5 mM MgCl₂, 1 mM dithiothreitol). The immunoprecipitates were subsequently treated with 1.5 μg/ml protein phosphatase 2A (Calbiochem), in the same buffer for 20

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**Fig. 2. Expression of the PKG fusion proteins in HEK-293 cells.** PKG fusion constructs were transiently transfected into HEK293 cells. A, protein expression was determined by Western blotting with anti-PKG (left panel) and anti-GFP (right panel) antibodies. B, the catalytic properties of the PKG fusion proteins in homogenates from transfected HEK-293 cells were examined using *in vitro* kinase assays as detailed under “Experimental Procedures.” The PKG-specific phosphotransferase activity in the absence (open bars) or the presence of 10 μM 8-Br-cGMP (closed bars) was determined. The error bars represent S.E. from three independent experiments, each with duplicate samples.
min at 30 °C. Control tubes containing phosphorylated VASP were incubated in phosphatase buffer without protein phosphatase 2A. The VASP was subsequently eluted from the beads with 100 μM FLAG peptide before analysis by Western blotting with anti-FLAG antibodies.

**Immunofluorescence Microscopy**—All epifluorescence microscopy and digitization of images was done using a Nikon Eclipse inverted microscope (model TE300) equipped with Hoffman optics and appropriate filter sets for epifluorescence microscopy. The image capture system was purchased from C-Imaging Systems (Cranberry Township, PA) and included a Hamamatsu CCD digital camera (model C4742-95) and SimplePCI software. For colocalization experiments, images captured using the 100× oil immersion lens were overlaid using Photoshop 4 software (Adobe, San Jose, CA).

For live imaging of fluorescence, cells were grown in six-well plates and transiently transfected to express GFP fusion proteins. After 24 h the plate was sealed with parafilm and placed directly on the microscope stage. Visualization was not continued beyond 30 min. High magnification and immunohistochemistry was performed cells grown on coverslips in six-well plates. Following transfection the coverslips were briefly washed in PBS followed by fixation in 3.7% paraformaldehyde (Electron Microscopy Sciences, Washington, PA) for 30 min at room temperature. Coverslips were subsequently washed in PBS, extracted with 0.1% Triton X-100 for 5 min, and then blocked with 10% bovine serum albumin in PBS for 1 h at room temperature. Monoclonal antibodies against FLAG epitope were used at 10 μg/ml in PBS containing 1% fetal bovine serum for 20 h at 4 °C. After four washes with PBS containing 1% fetal bovine serum, rhodamine-conjugated antimouse IgG antibodies were added for 1 h. After additional washing, the coverslips were mounted on glass slides using Vectashield™ fluorescence mounting medium (Vector Laboratories, Burlingame, CA). Visualization of F-actin was accomplished by growing and fixing cells on coverslips as detailed above, followed by staining with rhodamine-phalloidin according to the manufacturer’s instructions (Molecular Probes, Eugene, OR).

**Antibody Production**—Antiserum specifically recognizing the COOH terminus of type 1 PKG or phosphorylated serine 239 of VASP was generated by immunizing rabbits with synthetic decapetides coupled to KLH using standard methods. The peptide used as antigen had the sequence CAKLRKVS*KQEE, where a phosphorylated serine was included as indicated. The peptide used as antigen for PKG had the sequence CADDNSGWIDF. In both cases the amino-terminal Cys-Ala served to couple with carrier. Sera were tested by enzyme-linked immunsorbent assay and Western blotting after sequential booster inoculations until the effective titer reached 1/1000.

**RESULTS**

Generation and Expression of Green Fluorescent PKG Fusion Proteins—To gain some insight into the functions of different structural regions of type 1 PKG in vivo, we sought to create GFP fusion proteins that would permit visualization without fixation/extraction artifacts. The largest construct incorporated the full-length coding sequence for PKG1α with GFP fused at the COOH terminus (PKG-GFP). The design of other PKG constructs was based upon known biochemical properties of purified PKG and its homology with the closely related PKA enzymes (Fig. 1). Truncations of PKG1α were generated such that either the amino-terminal regulatory regions (GFP-G1C) or the COOH-terminal catalytic regions (G1αR-GFP) were essentially replaced with GFP. With PKG-GFP as the exception, because of similarity in the size of GFP compared with the regulatory and catalytic regions of PKG (35–40 kDa), the corresponding fusion proteins were expected to be similar in size to the endogenous PKG monomer (75, 85, and 110 kDa for GFP-G1C, G1αR-GFP, and PKG-GFP, respectively). When transiently transfected into HEK-293 fibroblasts all of the constructs were translated into proteins of the expected sizes as...
detected by Western blots probed with either anti-GFP or anti-COOH-terminal-specific anti-PKG antibodies (Fig. 2). As expected for the G1aR-GFP construct in which the COOH-terminal catalytic region was replaced with GFP, this protein was not detected by the PKG-specific antibodies that recognize the COOH terminus, but was recognized by anti-GFP antibodies. The catalytic properties of these proteins were examined in homogenates from transfected HEK-293 cells using BPDEtide as a substrate for in vitro kinase assays. These cells have previously been shown to express modest amounts of type 1 PKG (29), and in mock-transfected cells the presence of endogenous enzyme was reflected in a 2.5-fold increase in kinase activity in response to cGMP. Homogenates transfected to express PKG-GFP exhibited constitutive activity that could be only slightly increased by incorporation of cGMP in the assay. As would be expected by the lack of a catalytic region, overexpression of G1aR-GFP did not produce any basal kinase activity, but notably this construct was able to block the cGMP-stimulated activity of the endogenous kinase. In the absence of regulatory regions, the GFP-G1C protein exhibited a constitutive activity that was unaffected by the presence of cGMP.

The amino-terminal 100 residues of type 1 PKG encode a leucine zipper motif that has been demonstrated as essential for homodimerization as well as interaction with specific GKAPs (22–24). Within this region there is also a pseudosubstrate sequence that is thought to interact with the active site of the kinase. Experiments were performed to determine whether the truncated regulatory domain could dimerize with endogenous PKG and whether the catalytic domains could interact with the regulatory region. To facilitate immunoprecipitations, the GFP component of the PKG fusion proteins was replaced with FLAG epitope (Fig. 3). In these experiments the FLAG-G1aR protein, presumable through leucine zipper interactions, was able to interact with cotransfected PKG-GFP, indicating proper folding of these proteins. The regulatory domains were also found to bind to the catalytic regions as measured by cotransfection of either FLAG-G1C with G1aR-GFP or by FLAG-G1C with G1aR-GFP (Fig. 3A). These experiments suggested that the PKG fusion proteins containing the amino-terminal regulatory regions should be able to form dimers with endogenous PKG. To examine this possibility, increasing amounts of FLAG-G1aR were transiently transfected into HEK-293 cells followed by precipitation of the expressed regulatory region with FLAG antibody (Fig. 3B). The endogenous PKG in the precipitates was subsequently determined by quantitative Western blotting with anti-PKG antibody. Since this antibody was raised against a COOH-terminal peptide, it does not recognize the expressed truncation in which the COOH-terminal region was deleted (see Fig. 2A). These experiments revealed that the regulatory region expressed alone could indeed bind to endogenous PKG as an 80-kDa band. When plotted as a function of the amount of FLAG epitope, it was found that the relative amount of endogenous PKG found in the precipitates decreased with increased expression of the regulatory region (Fig. 3C). Based upon this finding it was hypothesized that lower concentrations of G1aR protein formed heterodimers with endogenous PKG, but higher expression levels resulted in a greater proportion of homodimers composed of truncated regulatory region only.

Visualization of PKG Fusion Proteins in Vivo—Although widely appreciated as cytosolic, the intracellular localization of type 1 PKG has gained little attention. The experiments described here demonstrate that the domains of the G1aR-GFP and PKG-GFP fusion proteins involved in dimerization were functional, indicating that these proteins would also be likely to interact with endogenous GKAPs. Furthermore, as suggested by the in vitro kinase assays, these fusion proteins should behave similarly to inactive and active PKG (respectively). We set out to examine the intracellular localization of the GFP fusion proteins by transiently transfecting several cell types (Fig. 4). When transfected to express either PKG-GFP or G1aR-GFP, HEK-293 cells showed fluorescence throughout the cytosol, and in many cells there was significantly more fluorescence of both constructs in the perinuclear region. Importantly, even at high levels of expression, neither construct was detected in the nucleus (<1%). A striking feature of the HEK-293 cells expressing the G1aR-GFP protein was the presence of pseudopodial extensions with a concentration of fluorescence at the distal end. In several instances filaments were observed, which when examined using time-lapse video microscopy were very dynamic. In contrast, the HEK-293 cells expressing the constitutive PKG-GFP protein had fewer pseudopodia, and these structures were quiescent and were devoid of visible filaments.

Two epithelial cell lines, A549 (type II human lung) and HeLa (human cervical), were also transiently transfected to express the GFP fusion proteins. Similar to the fibroblasts, in both epithelial cell lines the G1aR-GFP and PKG-GFP proteins were observed throughout the cytoplasm and were rarely present in the nucleus (Fig. 4). Interestingly, there was a pronounced localization of G1aR-GFP at the leading edge of many cells, and in some cells the G1aR-GFP protein was observed on filaments resembling stress fibers. Similar to the pseudopodia of the HEK-293 cells, time-lapse video microscopy revealed a dynamic movement of these regions relative to the cell body with a rolling, retrograde movement of the GFP. The PKG-GFP protein did not localize to the membrane in any cell line tested.
FIG. 5. Localization of GFP-G1C to the nucleus. A549 cells (A, B) or HEK-293 cells (C, D) were transiently transfected followed by visualization of expressed GFP in live cells using epifluorescence microscopy. Cells were transfected with EYFP-C1 vector (A), with a cDNA encoding the COOH-terminal catalytic regions of PKG1α fused to GFP (GFP-G1C; B, C), or were cotransfected with the GFP-G1C construct and the NH\(_2\)-terminal regulatory regions of PKG1α (FLAG-G1αR; D). The fields shown are representative of observations from at least two experiments. The bar represents 25 μm.

but instead was more concentrated in a perinuclear region away from the plasma membrane and cortical zones. Notably, many cells transfected to express PKG-GFP appeared to be more rounded with less prominent extensions.

Expression of the EGFP protein alone in either HEK-293 or A549 cells was identical with the fluorescence uniformly distributed throughout the cell without any distinguishing structures. Like PKG-GFP, the GFP-G1C protein has constitutive kinase activity, but in contrast is devoid of regulatory regions involved in dimerization and anchoring to GKAPs. When either the HEK-293 or the A549 cells were transiently transfected to express GFP-G1C, the fluorescence was present throughout the cytosol but was much more concentrated in the nucleus (Fig. 5). Cells expressing this construct were also more rounded and had less pseudopodial extensions compared with EGFP vector transfected cells. The lack of dynamic regions in the GFP-G1C transfected cells was similar to those expressing PKG-GFP except that the former were typically smaller and contained less cytosol. The GFP-G1C construct was shown to interact with a FLAG epitope-tagged construct encoding the regulatory regions of PKG1α (Fig. 3A). This interaction was confirmed in living cells, as coexpression of FLAG-G1αR was able to prevent the concentration of the cotransfected GFP-G1C protein from entering the nucleus.

Specific Interaction of Inactive PKG1α with F-actin—The appearance of spontaneous, prominent lamellipodia in A549 cells prompted us to use this cell line for further study. The location of G1αR-GFP in the live cells suggested that this catalytically inactive truncation of PKG might associate with the microfilament component of the cytoskeleton. To test this, cells that were transiently transfected to express G1αR-GFP were subsequently fixed, and the F-actin was stained using rhodamine-phallicidin (Fig. 6). These experiments revealed colocalization of G1αR-GFP with F-actin in membrane ruffles and in the active membrane regions found at the leading edge. In some of the cells G1αR-GFP clearly colocalized with stress fibers proximal to these active membrane regions, and occasionally it was observed in the focal adhesion complexes. When transfected to express the constitutive PKG-GFP or the free catalytic half of PKG (not shown), A549 cells were more rounded in morphology, and there was a marked overall loss of F-actin and stress fibers. Furthermore, in these cells, focal contacts and active membrane regions observed in the mock-transfected or the G1αR-GFP transfected cells were rarely observed. The F-actin in the cells expressing constitutive PKG-GFP localized predominantly to the cell cortex and randomly throughout the cytoplasm. In support of the visualization of PKG-GFP in live cells, in these fixed cells the fusion protein was more centrally located, discretely separate from the plasma membrane, and did not associate with the existing actin filaments.

Effect of PKG Fusion Proteins on VASP Phosphorylation—The VASP family members are structurally similar proteins that are considered to be important regulatory components of the actin cytoskeleton in most cell types (30). Reflecting the ability of VASP members to bind focal adhesion and actin cytoskeleton-associated proteins, including vinculin, zyxin, profilin, and actin itself, these proteins localize to focal adhesions, stress fibers, and dynamic membrane regions of the leading edge (30–36). The prototypic member of this family is the VASP, and its phosphorylation by PKA and PKG has been well documented (37, 38). Having examined the catalytic properties of the PKG fusion proteins \textit{in vitro} using a peptide substrate, it was of interest to determine the effects of the proteins described here on the phosphorylation of VASP \textit{in vivo}. Furthermore, since the localization G1αR-GFP in epithelial cells paralleled that reported for VASP, we also sought to determine whether this well characterized substrate for PKG might be involved in localizing the G1αR-GFP protein \textit{in vivo}.

Initial studies involved transfection of both HEK-293 and A549 cells with a cDNA encoding murine VASP, tagged at the amino terminus with the FLAG epitope (21). Phosphorylation...
of the transfected VASP was measured as a shift in electrophoretic mobility on Western blots probed with anti-FLAG antibodies (Fig. 7). These experiments confirmed the presence of endogenous PKG in the HEK-293 cells but revealed its absence in the A549 cells, as there was no cGMP-induced shift in VASP mobility in the latter. Cotransfection of a cDNA encoding wild-type human PKG1α produced a basal level of VASP phosphorylation in both cell types. In addition, exogenous expression of wild-type PKG enabled the A549 cells to respond to cGMP treatment with a shift in VASP mobility and greatly augmented the magnitude of VASP phosphorylation in the HEK-293 cells. Cotransfection of a previously described catalytic mutant of PKG1α (T516A) did not produce any background VASP phosphorylation or confer cGMP responsiveness to the A549 cells. Surprisingly, transfection of HEK-293 cells with the T516A mutant produced a high background level of VASP phosphorylation, similar in magnitude to that of cGMP-stimulated cells transfected with VASP alone. A similar result was obtained by transfection of a truncated mutant of PKG that expressed only the COOH-terminal catalytic regions (data not shown). The electrophoretic shift of VASP produced by either the T516A mutant or by stimulation with 8-Br-cGMP was similarly due to phosphorylation, since treatment of the precipitated VASP with protein phosphatase 2A (39) elicited a pattern of VASP migration corresponding to the basal state (Fig. 7C).

Because of the existence of endogenous PKG, HEK-293 cells were subsequently used to assess the effects of G1αR-GFP and PKG-GFP on VASP phosphorylation. In these experiments, treatment of the cells with 50 μM 8-Br-cAMP was found to cause a much more dramatic shift in the mobility of VASP on PAGE gels than similar concentrations of 8-Br-cGMP. Despite the relatively small shift in VASP induced by treatment of the cells with 8-Br-cGMP, there was a pronounced labeling of VASP with an anti-phospho-Ser239 antibody that was undetectable in the cAMP-treated cells (Fig. 8). These findings support several previous studies indicating that Ser239 is a preferred phosphorylation site for PKG but produces a lesser mobility shift, whereas modification of Ser157, which is preferred by PKA, results in a larger electrophoretic shift (37, 38). When cells were cotransfected with increasing amounts of FLAG-G1αR, the ability of cGMP to phosphorylate VASP at Ser239 was greatly diminished. In contrast, the shift in VASP mobility induced by treatment with cAMP was not affected by expression of equal amounts of FLAG-G1αR. Identical results were obtained using the G1αR-GFP construct (data not shown). This effect paralleled the ability of this construct to inhibit the cGMP-stimulated phosphorylation of BDPEptide by endogenous PKG in vitro. In support of in vitro kinase assays, transfection of HEK-293 cells to express PKG-GFP resulted in a marked (~60%) shift of the VASP on PAGE gels in the absence of exogenously added cGMP. Furthermore, this constitutive level of activity was enhanced by treatment of the cells with cGMP (Fig. 9). In cotransfection experiments this fusion protein was also able to diminish the phosphorylation of VASP by the cGMP-independent activity of the PKG-GFP protein.

Interaction of PKG with VASP in Vivo—The ability of the G1αR-GFP fusion protein to inhibit the cGMP-independent phosphorylation of VASP by PKG-GFP suggested a competitive mechanism. The possibility that the PKG might localize to...
VASP in vivo was tested by cotransfection of the PKG fusion proteins with FLAG-VASP into A549 cells followed by fixation and staining with anti-FLAG antibodies (Fig. 10). In these studies the exogenously expressed VASP protein was found to localize to focal adhesions, membrane ruffles, and stress fibers as has been reported previously (30–32). Although there was significant cytosolic staining, the G1αR-GFP protein was found to colocalize with the VASP in active membrane regions, but interestingly, it was only found in a subset of the focal adhesions. As detailed earlier, the PKG-GFP protein is constitutively active and its expression resulted in a general decrease in the amount of cellular F-actin. When cotransfected with VASP, the VASP also showed almost exclusively cytosolic staining, as did the PKG-GFP. Similar results were obtained by cotransfection of VASP with FLAG-G1C (data not shown). These data suggested that the GFP fusion proteins are always

**Fig. 7.** Phosphorylation of vasodilator-stimulated phosphoprotein in transfected cells. A549 epithelial cells (A) and HEK-293 fibroblasts (B, C) were transiently transfected with a cDNA encoding FLAG epitope-tagged murine VASP together with either wild-type or mutant PKG1α as indicated above. After 24 h, the transfected cells were either left untreated or stimulated for 20 min with 100 μM 8-Br-cGMP as indicated. The phosphorylation state of the expressed VASP was measured by Western blotting with anti-FLAG antibodies as detailed under “Experimental Procedures.” The lower panels are parallel Western blots probed with anti-PKG antibodies (A and B). The effect of phosphorylation on the electrophoretic mobility of VASP was determined by treating expressed VASP with protein phosphatase 2A (PP2A) (C) as detailed under “Experimental Procedures.” Results shown are representative of at least two experiments.

**Fig. 8.** Specific inhibition of cGMP-stimulated VASP phosphorylation by G1αR-GFP in vivo. HEK293 cells were transiently transfected to express FLAG-VASP and increasing quantities of FLAG-G1αR as indicated below. The cells were stimulated with 8-Br-cAMP or 8-Br-cGMP (as indicated above) for 20 min. The cell extracts were then analyzed by Western blotting with anti-FLAG antibodies (upper panel) and anti-VASP(Ser239(P)) (lower panel) as detailed under “Experimental Procedures.” The identity of the detected bands is indicated on the right. Blots shown are representative of at least three independent experiments.

**Fig. 9.** Constitutive phosphorylation of VASP on Ser239 by PKG-GFP. HEK-293 cells were transiently cotransfected to express FLAG-VASP with either vector alone (basal) or with PKG-GFP (as indicated above). The effect of cGMP treatment (100 μM, 20 min) or expression of G1αR-GFP (as indicated below) on VASP phosphorylation was determined by Western blotting with anti-FLAG antibodies (upper panel) or with anti-VASP(Ser239(P)) antibodies (middle panel). To confirm expression of the GFP fusion proteins the homogenates were also analyzed by Western blotting with anti-GFP antibodies (lower panel). Results shown are representative of two independent experiments.

**Fig. 10.** Colocalization of GFP fusion proteins with VASP in vivo. A549 cells were transiently cotransfected to express FLAG-VASP and either G1αR-GFP (B, D, F) or PKG-GFP (A, C, E). After 36 h, cells were fixed and stained for VASP expression with anti-FLAG antibodies followed by Texas Red-conjugated anti-mouse IgG secondary antibodies as detailed under “Experimental Procedures.” The localization of VASP is shown in the upper panels (A, B) as red fluorescence, the middle panels show GFP fluorescence, and the lower panels are overlays with colocalization visualized as yellow. Representative cells from duplicate transfections are shown. The bar represents 25 μm.
Interactions between the regulatory and catalytic parts of nuclear localization sequence (NLS) that has previously been diffusion. These data thus support the existence of a functional 70 kDa, entry into the nucleus is not likely to occur by random dynamic state. When expressed alone, the GFP-G1C protein indicated that this enzyme is in a pseudosubstrate domains of the holoenzyme, as has been suggested to occur with isolated peptides corresponding to amino acids 546–576 of the enzyme (40). This idea is supported by the observed interaction between the catalytic half of PKG (either FLAG-G1C or GFP-G1C) with the regulatory half of PKG in immunoprecipitation studies. The inhibitory properties reported for the T516A mutant of PKG1α are likely explained as above, by its ability to behave as a cGMP sink when overexpressed. These studies indicate that the T516A mutant of PKG1α should not be used as a dominant negative enzyme to investigate PKG function in future studies.

Type-1 PKG is widely recognized as predominantly cytosolic, but the recent identification of several GKAPs suggests that this enzyme might exist in discrete cellular compartments (25, 41). Low expression levels of PKG relative to other kinases make specific visualization in situ difficult, and few reports have described the intracellular localization (27, 28). In studies shown here, dimerization of fusion proteins containing the amino-terminal regulatory regions (FLAG-G1αR, G1αR-GFP), and heterodimer formation between FLAG-G1αR and endogenous PKG, demonstrated that the leucine zipper domain is functional in these fusion proteins. Because the amino-terminal leucine zipper domain has also been shown to mediate the interaction of PKG with several GKAPs, the G1αR-GFP protein would be expected to localize to intracellular regions in a manner expected for inactive PKG (since this construct does not express the catalytic regions of the enzyme). Similarly, because the PKG-GFP protein also contains the leucine zipper domain but is constitutively active, it is reasonable to assume that this protein would mimic activated PKG in its intracellular distribution. The G1αR-GFP protein was found to be associated with F-actin in dynamic membrane regions and stress fibers in several cell types. This contrasted with the localization of the PKG-GFP protein, which did not associate with the membrane, and appeared to reduce the amount of F-actin and dynamic membrane regions in transfected cells. This difference in local-
IZATION is a direct result of enzyme activity, since the fluorescence in cells transfected with PKG(T516A)-GFP more closely resembled that of the G1aR-GFP (data not shown). These observations support recent findings where investigators studying VASP found that long term activation of PKG lead to loss of stress fibers and focal adhesions with a concomitant localization of VASP to more centralized regions (42). In the same study the authors demonstrated that phosphorylation of VASP was central to this process. It was shown here for the first time that both the active and inactive forms of PKG fusion protein colocalize with VASP, suggesting binding to alternative sites.

VASP family members have recently been shown to have a binding to phosphorylated VASP, suggesting binding to alternative sites. More work is required to determine the precise binding site for PKG on VASP. Immunoprecipitation studies shown here did not detect a significant decrease in PKG fusion protein binding to phosphorylated VASP when compared with unphosphorylated VASP, suggesting binding to alternative sites. VASP family members have recently been shown to have a negative regulatory role in chemotaxis, possibly by inducing the formation of stable contacts with the substratum (30, 42, 43). In addition to the regulation of chemotaxis, the relocalization of PKG-VASP complexes upon activation of PKG might therefore affect other cellular processes, as substrates at these new locations would be available for modification.

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