Formin Homology Domain Protein (FHOD1) Is a Cyclic GMP-dependent Protein Kinase I-binding Protein and Substrate in Vascular Smooth Muscle Cells

Yuepeng Wang, Mohamad R. El-Zaru, Howard K. Surks, and Michael E. Mendelsohn
From the Molecular Cardiology Research Institute, Department of Medicine and Division of Cardiology, New England Medical Center Hospitals and Tufts University School of Medicine, Boston, Massachusetts 02111

Cyclic GMP-dependent protein kinase I (PKGI) mediates vascular relaxation by nitric oxide and related nitrovasodilators and inhibits vascular smooth muscle cell (VSMC) migration. To identify VSMC proteins that interact with PKGI, the N-terminal protein interaction domain of PKGIs was used to screen a yeast two-hybrid human aortic cDNA library. The formin homology (FH) domain-containing protein, FHOD1, was found to interact with PKGI in this screen. FH domain-containing proteins bind Rho-family GTPases and regulate actin cytoskeletal dynamics, cell migration, and gene expression. Antisera to FHOD1 were raised and used to characterize FHOD1 expression and distribution in vascular cells. FHOD1 is highly expressed in human coronary artery, aortic smooth muscle cells, and in human arterial and venous endothelial cells. In glutathione S-transferase pull-down experiments, the FHOD1 C terminus (amino acids 964–1165) binds full-length PKGI. Both in vitro and intact cell studies demonstrate that the interaction between FHOD1 and PKGI is decreased 3- to 5-fold in the presence of the PKG activator, 8Br-cGMP. Immunofluorescence studies of human VSMC show that FHOD1 is cytoplasmic and is concentrated in the perinuclear region. PKGI also directly phosphorylates FHOD1, and studies with wild-type and mutant FHOD1-derived peptides identify Ser-1131 in the FHOD1 C terminus as the unique PKGI phosphorylation site in FHOD1. These studies demonstrate that FHOD1 is a PKGI-interacting protein and substrate in VSMCs and show that cyclic GMP negatively regulates the FHOD1-PKGI interaction. Based on the known functions of FHOD1, the data are consistent with a role for FHOD1 in cyclic GMP-dependent inhibition of VSMC stress fiber formation and/or migration.

Nitric oxide (NO)1 is the most important endogenous vasodilator known (1–5). NO causes vasodilatation by stimulating soluble guanylate cyclase in VSMCs to increase intracellular cGMP levels. Cyclic GMP, in turn, binds and activates cyclic GMP-dependent protein kinase I (PKGI), its principal effector in vascular smooth muscle cells (6–9). Mice lacking the gene for PKGI develop systemic hypertension, and their vessels fail to relax normally (9, 10). Mouse models also demonstrate that PKGI is important in the regulation of vascular remodeling and thrombosis (9). The intracellular substrates of PKGI and the mechanisms by which PKGI regulates these substrates to alter vascular smooth muscle cell responses is, therefore, of central importance to vascular biology in health and disease.

A number of laboratories have sought proteins capable of interacting with PKGI isoforms (PKGI-interacting proteins, cGMP-dependent protein kinase-anchoring proteins, cGMP-dependent protein kinase-interacting proteins (GKIPs)). In many instances, PKG both directly binds to and phosphorylates such proteins, suggesting that one way to identify PKG substrates and to unravel PKG signaling pathways is to identify PKG-binding partners. Nearly a decade ago, using gel overlay and immunoprecipitation approaches, the intermediate filament protein vimentin was demonstrated to be a high-affinity GKIP (11), and a number of GKIPs have since been identified. In vascular smooth muscle, the NO/cGMP/PKG pathway induces relaxation by at least three mechanisms: decreased intracellular free calcium, calcium desensitization, and thin filament regulation, which may vary in importance depending upon vascular bed and the tonic or phasic contractile properties of the specific smooth muscle cells studied (12). Recently, our laboratory has identified two GKIPs for PKGIa in vascular smooth muscle that are important for the regulation of vascular smooth muscle cell tone. PKGIa binds directly to the myosin-binding subunit of myosin phosphatase (PP1M) by a leucine zipper (LZ) interaction, which is required for cGMP-mediated activation of PP1M (13). PP1M is a well characterized mediator of vascular relaxation (14–16), and the identification of PP1M as a GKIP has important implications for the molecular mechanisms of relaxation and, potentially, for drug development. The LZ of PKGIa, but not the homologous LZ in the amino termini of PKGIβ or PKGIi, binds PP1M (13), suggesting additional molecular determinants of specificity are contained in these domains. Similarly, recent studies show that the N-terminal LZ mediates the interaction of PKGIβ with the IP3R-associated cGMP kinase substrate (IRAG) protein (17), suggesting that the N-terminal LZs of the PKGI isoforms may contribute more generally to protein targeting. Consistent with this notion, we recently identified the regulator of G-protein signaling (RGS2) as a second GKIP that is important in regulation saline; FHOD1, formin-homology-2 domain-containing protein; PP1M, myosin phosphatase.

This paper is available on line at http://www.jbc.org

Received for publication, December 17, 2003, and in revised form, March 10, 2004
Published, JBC Papers in Press, March 29, 2004, DOI 10.1074/jbc.M313823200

* This work was supported in part by National Institutes of Health Grant R01 HL55309 (to M. E. M.). 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.
‡ To whom correspondence should be addressed: Tufts University School of Medicine, New England Medical Center, Molecular Cardiology Research Institute, 750 Washington St., Box 80, Boston, MA 02111. Tel.: 617-636-9370; Fax: 617-636-1444; E-mail: mmendelsohn@tufts-nec.org
1 The abbreviations used are: NO, nitric oxide; PKGI, cyclic GMP-dependent protein kinase I; GKIPs, cGMP-dependent protein kinase-interacting proteins; LZ, leucine zipper; RGS2, regulator of G-protein signaling; GST, glutathione S-transferase; FH, formin homology; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FHOD1, formin-homology-2 domain-containing protein; PP1M, myosin phosphatase.
loration of vascular contraction (18), and showed that the amino-terminal LZ domain of PKGIs mediates PKGIs-RGS2 binding (18). We have used the amino-terminal LZ-encoding domain of PKGIs in a series of vascular two-hybrid library screens to attempt to identify new PKGI-interacting proteins. We now report the identification of a new OKIP and PKGI substrate, the formin homology (FH) domain-containing protein FHOD1 (19, 20).

 MATERIALS AND METHODS

 Yeast Two-hybrid Screen—A human aortic cDNA library (Clontech) was screened using human PKGIa LZ domain, amino acids 1-58, as the bait (Fig. 1). This domain of PKGIs is known to mediate PKGIs-protein interactions (21, 22). The library was screened by sequentially transforming yeast strain Y190 (Clontech) with the bait plasmid, followed by the library DNA; it was then grown on yeast plates lacking leucine, tryptophan, uracil and hisidine and containing 50 mg/ml 3-amino-triazole. Three million independent clones were screened, and 39 positive PKGIa LZ domain-interacting clones were isolated. Positive clones were identified by their growth on selective media and confirmed by β-galactosidase activity. Positive clones were further verified by transformation into Y190 cells with the bait plasmid or empty vector.

 All cloning experiments were performed using an AHI 3100 DNA sequencer, Tufts University Core Facility. Two of these clones encoded amino acids 964–1165 in the C terminus of FHOD1 (Fig. 1).

 Cell Culture—Human coronary artery smooth muscle cells (Co396) human aortic smooth muscle cells (Ao184) were isolated by the explant method and were identified by expression of smooth muscle cell-specific α-actin. Human coronary artery endothelial cells, human aortic endothelial cells, and human umbilical vein endothelial cells were isolated by collagenase digestion and identified by expression of von Willebrand factor VIII. COS-7 and HEK293 cells were obtained from American Type Culture Collection. Cells were cultured and passaged in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

 Differential Centrifugation—Co396 cells at 80–90% confluence were serum-starved for 12 h, treated with vehicle alone or angiotensin-II (1 μM, 5 min; Sigma) and/or 8Br-cGMP (1 mM, 20 min; Sigma), and then harvested by scraping in lysis buffer (25 mM Tris-HCl, 125 mM NaCl, 10 mM EDTA, 10 mM NaF, 1% Triton X-100, pH 8.0). After centrifugation at 14,000 rpm for 20 min, the supernatant was used as a Triton X-100-soluble cell fraction, and the pellet was used as a Triton X-100-insoluble cell fraction. DNA was sheared by passing the sample through a 27-gauge needle.

 Western blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA Western Blotting—Protein concentrations were determined by BCA
The amino terminus of PKGI is important in mediating interactions between this kinase and several of its known substrates (discussed in Refs. 12, 21, 22). The initial fifty-nine amino acids of PKGⅠα contain the LZ domain of PKGⅠα, which mediates binding of PKGⅠα to two enzymes, RGS2 and PP1M (18, 21, 25). To identify other GKIPs in vascular cells, a human aortic yeast two-hybrid cDNA library was screened using a PKGⅠα construct encoding the amino-terminal 59 amino acids of PKGⅠα as bait. B, growth on selective media of yeast expressing Gal AD (empty vector) + cGK1-59-Gal BD (upper panel) or cGK1-59-Gal BD + FHOD1-Gal AD (middle panel) and corresponding β-galactosidase activity for cGK1–59 Gal BD + FHOD1-Gal AD colonies (lower panel). No β-galactosidase activity was detectable when yeast were cotransformed with either cGK1–59 or FHOD1 and empty vector (not shown). C, structure of the C-terminal amino acids 964–1165 of FHOD1 corresponding to cDNA cloned as an interaction partner with PKGⅠα. Full-length FHOD1 contains a proline-rich formin homology domain (FH1) and second and third regions of formin homology (FH2 and FH3), as well as a domain shown previously to bind Rho family small GTP-binding proteins (GBD), a coiled-coil domain (CC), and a C-terminal auto-regulatory domain (DAD).

RESULTS

The amino terminus of PKGⅠα is important in mediating interactions between this kinase and several of its known substrates (discussed in Refs. 12, 21, 22). The initial fifty-nine amino acids of PKGⅠα contain the LZ domain of PKGⅠα, which mediates binding of PKGⅠα to two enzymes, RGS2 and PP1M (18, 21, 25). To identify other GKIPs in vascular cells, a human aortic yeast two-hybrid cDNA library was screened using a construct encoding the amino-terminal 59 amino acids of PKGⅠα (Fig. 1A; Ref. 21). 56 interacting clones were identified, of which 39 proved by repeat testing to be true positives (Fig. 1B). Sequencing of these demonstrated that 2 of the 39 positive clones encoded the C-terminal domain (amino acids 964–1165) of the FH domain protein FHOD1 (Fig. 1, A and C; Ref. 19). FHOD1 is a member of the FH domain protein family that regulates actin-based cytoskeletal events, including cell migration (26). Antibodies to FHOD1 were raised and used to study FHOD1 expression in vascular cells. Substantial FHOD1 expression was observed in aortic and coronary smooth muscle extracted for 5 min at 95 °C in 2 × SDS-PAGE sample buffer, and analyzed by SDS-PAGE. After fixing the gels, autoradiography was performed at room temperature. Autoradiographs were analyzed using a PhosphorImager (Molecular Dynamics), and bands were quantified using NIH analysis software. For in vitro [32P]phosphate labeling, COS-7 cells transfected with FLAG-full-length FHOD1 wild type or its mutant S1131A, were washed and preincubated with phosphate-free medium containing 1 mM 8Br-cGMP for 30 min. Cells were scraped in TLB buffer, and the cell lysate were centrifuged to remove cellular debris. The supernatants were immunoprecipitated with M2 antibody and the immunopellets were analyzed by SDS-PAGE and autoradiography as described above.

Statistical Analysis—All data are given as the mean ± S.E. Data were analyzed by using a paired or unpaired Student’s t test. p < 0.05 was taken as a significant difference between data sets.
cells, as well as in vascular endothelial cells from both vessels (Fig. 2). FHOD1 was found principally in the Triton-soluble vascular smooth muscle cell fractions, and this distribution was not altered by exposure of cells to either angiotensin II or 8Br-cGMP (Fig. 2B). GFP-FHOD1 expressed in living coronary artery muscle cells also was distributed diffusely in the cytoplasm (Fig. 2C). FHOD1 expression in vascular cells was also quantitated by using recombinant FHOD1 protein to construct a standard protein concentration curve. Vascular smooth muscle cells contained 0.24 ± 0.06 to 2.16 ± 0.10 ng of FHOD1 per mg of cell protein; vascular endothelial cells contained 1.12 ± 0.07 to 1.44 ± 0.08 ng of FHOD1 per mg of cell protein (data not shown), supporting the idea that both vascular cell types express FHOD1 in abundance. FHOD1 has been reported to bind the small GTP-binding protein Rac (20). We used FHOD1-GST fusion protein in pull-down experiments to test whether FHOD1 binds purified recombinant monomeric GTP-binding protein. FHOD1-bound Rac1 avidly in a manner independently of GDP or GTP in this assay, but did not bind either of the related monomeric GTP-binding proteins cdc42 or RhoA (Fig. 2D).

Immunoprecipitates of PKGI from human vascular smooth muscle cell lysates also contained FHOD1 protein (Fig. 3A). The converse experiment, in which either C- or N-terminal antibodies to FHOD1 were used for immunoprecipitation, showed that a small amount of PKGI was consistently detectable in FHOD1 immunoprecipitates, but only at low levels (Fig. 3B) (see “Discussion”). To test whether a direct interaction occurs between FHOD1 and PKGI, a series of FHOD1-GST fusion proteins were constructed and used in pull-down experiments with purified recombinant FHOD1 protein (Fig. 4, A and B). C-terminal domains of FHOD1, amino acids 919–1165 and 964–1165, directly bound PKGI in these studies; the latter domain also was able to bind to endogenous PKGI from human coronary artery smooth muscle cell lysates (Fig. 4C). In addition, the FHOD1-PKGI interaction was also observed when GST-fusion proteins with portions of PKGI were used to test interactions with full-length FHOD1 (Fig. 4D). GST-fusion protein for the LZ domain of PKGI (amino acids 1–59) bound FHOD1 weakly, whereas a longer portion of the N terminus of PKGI or -Iβ (amino acids 1–236) strongly interacted with the FH domain-containing protein (Fig. 4D). Immunofluorescence confocal microscopy revealed colocalization of FHOD1 and PKGI in the cytoplasm of human coronary artery vascular smooth muscle cells, especially in the perinuclear region (Fig. 5). Together, the data of Figs. 3–5 support that PKGI and FHOD1 interact directly in the cell cytoplasm.

PKGI binds directly to a number of proteins that are substrates for phosphorylation (6, 18, 21). We examined first whether FHOD1 is phosphorylated by PKGI in standard in vitro phosphorylation assays (18, 21). Using the GST-FHOD1
fusion proteins described above, we surveyed the length of the FHOD1 molecule for potential PKG phosphorylation sites. FHOD1 contains numerous serine/threonine residues, five of which are in sequences immediately downstream from basic residues and are homologous to known PKG phosphorylation sites (6). Of the 8 FHOD1 peptides surveyed, only the two carboxyl-terminal peptides (amino acids 964–1165 and amino acids 919–1165) of FHOD1 were phosphorylated by PKGI in vitro (Fig. 6A). The specific sequences of these peptides contain a unique serine residue at amino acids 1131. An increase in phosphorylation of these fusion peptides was detected when 10 μM 8Br-cGMP was included in the reaction (Fig. 6A). Mutation of Ser-1131 → Ala abolished the ability of PKG to phosphorylate this C-terminal peptide of FHOD1 (Fig. 6B, left panel). Furthermore, epitope-tagged wild type FHOD1 was phosphorylated in intact, 32P-labeled cells, but a mutant with alanine substituted at amino acids 1131 was not (Fig. 6B, right panel). Together, these data support that Ser-1131 in the carboxyl terminus of FHOD1 is the unique site of FHOD1 phosphorylation by PKGI.

We next examined the effect of cGMP on the interaction between PKG and FHOD1 in vitro and in vivo. Activation of coronary arterial smooth muscle cell PKGI by 8Br-cGMP treatment caused a significant decrease in the amount of PKGI bound to GST-FHOD1 964–1165 from VSMC lysates (Fig. 7A). In contrast, the mutant non-phosphorylatable GST-fusion peptide, GST-FHOD1(964–1165) (Ala-1131) associated with a significantly greater amount of PKGI than was observed with the wild type (Ser-1131) peptide in this assay (Fig. 7B). Immunoprecipitation of either FHOD1 or PKGI from intact HEK293 cells treated with vehicle or 8Br-cGMP was next used to test the effect of cGMP on the FHOD1-PKGI interaction. Immunoprecipitation of either FHOD1 or PKGI from cells treated with 8Br-cGMP led to a significant reduction in the amount of FHOD1-PKGI complex recover (Fig. 8).

DISCUSSION

The data presented here demonstrate that the formin homology domain-containing protein FHOD1 interacts directly with PKGI in vascular cells. FHOD1, which is highly expressed in the cytoplasm of both human vascular smooth muscle and endothelial cells, is also a substrate for PKGI, and Ser-1131 in the carboxyl terminus of the protein is the unique site of phosphorylation of FHOD1 by PKGI. Furthermore, we find that activation of PKGI with cyclic GMP decreases the FHOD1-
PKGI interaction, and that mutating Ser-1131 to the non-phosphorylatable residue alanine reverses this effect of cGMP. The FHOD1-PKGI interaction is likely mediated by non-LZ elements in the amino termini working in conjunction with the LZs in PKG\textalpha{} and PKG\textbeta{} (compare with Fig. 4D), because shorter pieces of the PKG\textbeta{} amino terminus interacted only poorly or not at all with FHOD1. In immunoprecipitation studies, substantial FHOD1 was recovered when PKG antibody was used to immunoprecipitate protein, though immunoprecipitation of FHOD1 led to recovery of very little PKGI. This may reflect that a relatively small proportion of the total FHOD1 present in the cell is bound to PKG. Similar data have been seen in related studies of protein-protein interactions with PKGI, in which detection of substantially greater amounts of the cognate partner than PKG in the opposite immunoprecipitate protein have been observed (see, e.g. Refs. 23, 29, 33). Mutation of the PKG phosphorylation site on FHOD1 removes the ability of PKGI to phosphorylate FHOD1. However, 8Br-cGMP does not induce an increase in phosphorylation of the 961–1165 FHOD1 fragment above the resting level in our studies, because a basal level of phosphorylation of the 961–1165 FHOD1 fragment exists. The lack of any further increase in PKG phosphorylation of FHOD1 in the presence of cGMP in these studies is most likely due to substantial basal activity of the enzyme that is, at most, modestly increased by the addition of exogenous cGMP. This is not uncommon and is likely related, at least in part, to the residual cGMP bound to the purified kinase used in these assays. The effect of 8Br-cGMP in decreasing the PKG1-FHOD1 interaction was observed in two different types of experiment. Attempts to test this effect in intact, non-transfected vascular cells were unsuccessful, presumably because higher levels of protein expression were required to demonstrate the effect of 8-Br-cGMP on the interaction. However, in both GST pull-down studies with vascular cell lysates and in heterologous expression studies with HEK293 cells, cGMP stimulation led to reduced binding of PKG to FHOD.

FH domain proteins are known to interact with members of the Rho-family of small GTP-binding proteins and regulate actin cytoskeletal dynamics, cell migration, and gene expression (26, 27, 28). FH proteins typically bind Rho family GTPases at their N termini and contain a C-terminal autoregulatory domain. The formin homology domain protein FHOD1 is a relatively new mammalian member of the FH protein family that contains coiled-coil and collagen-like domains, as well as nuclear localization signals and potential phosphorylation sites for cyclic nucleotide kinases (19). FHOD1 contains all known conserved FH domains identified thus far, including a proline-rich FH1 domain and homologous FH2 and FH3 domains. FHOD1 also demonstrates autoinhibition which is thought to occur because of binding of the N-terminal 421 amino acids of the protein to its own C terminus (28). In contrast to the related FH murine diaphanous-related proteins, however, FHOD1 interacts with the small GTPase Rac1, but not with RhoA, Cdc42Hs, Rac2, or Rac3, and FHOD1 also activates transcription from the serum response element (20).

What might be the functional significance of the FHOD1-PKGI interaction? The fact that FHOD1 binds the small GTP-binding protein Rac may be relevant to this question, because the rapid turnover of actin in lamellipodia and at the edges of migrating cells is known to be regulated by Rac (29, 30). Nitric oxide and cGMP inhibit vascular smooth muscle cell migration, suggesting the hypothesis that regulation of the PKGI-
FHOD1 interaction by cGMP might mediate this inhibition (31, 32). Rac also regulates p21-activated kinase, which may mediate some of the effects of Rac on the cytoskeleton (30). p21-Activated kinase phosphorylates both myosin light chain kinase and caldesmon in vascular smooth muscle cells and may regulate calcium sensitivity and the contractile state (33–35). It is also noteworthy that the unique PKGI inhibitory domain somehow alters the regulation of FHOD1 function by this auto-inhibitory domain. Finally, it is also worth noting that PKGI opposes the contractile activating effects of another small GTPase, Rho, on myosin phosphatase. Thus, PKGI also regulates calcium sensitivity and the contractile state (33, 35).

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In summary, PKGI binds to and phosphorylates FHOD1, and the PKGI-FHOD1 complex is disrupted in the presence of cyclic GMP. The potential importance of this interaction in mediating the effects of NO and cGMP on vascular smooth muscle cell tone and migration are presently under investigation.

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