The Pleckstrin Homology Domain of Phospholipase C-β₂ as an Effector Site for Rac

Received for publication, February 10, 2003, and in revised form, March 19, 2003
Published, JBC Papers in Press, March 25, 2003, DOI 10.1074/jbc.M301418200

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Increasing evidence links the activation of Rho family GTPases to the stimulation of lipid hydrolysis catalyzed by phospholipase C (PLC)-β isozymes. To better define this relationship, members of a library of recombinant Rho GTPases were screened for their capacity to directly engage various purified PLC-β isozymes. Of the 17 tested members of the Rac family, only the active isoforms of Rac (Rac1, Rac2, and Rac3) both stimulate PLC-β activity in vivo and bind PLC-β₂ and PLC-β₃ but not PLC-β₁, in vitro. Furthermore, the recognition site for Rac GTPases was localized to the pleckstrin homology (PH) domain of PLC-β₂ and this PH domain is fully sufficient to selectively interact with the active versions of the Rac GTPases, but not with other similar Rho GTPases. Together, these findings present a quantitative evaluation of the direct interactions between Rac GTPases and PLC-β isozymes and define a novel role for the PH domain of PLC-β₂ as a putative effector site for Rac GTPases.

Pleckstrin homology domain of PLC-β isozymes convert a variety of external stimuli into intracellular signaling events by hydrolyzing the minor membrane lipid component phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) into the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (see Refs. 1 and 2). This important step in receptor-mediated signal transduction initiates an increase in cytoplasmic calcium ion levels and activates protein kinase C (3, 4). Whereas there are currently four distinct classes of phospholipase C (β, γ, δ, and ε), only the PLC-β (2) and PLC-ε (5, 6) members are known to catalyze lipase activity in response to extracellular ligand binding to heptahelical, transmembrane G protein-coupled receptors (7).

The four known mammalian PLC-β isozymes (PLC-β₁-₄) are tightly regulated by G protein-coupled receptors via direct interactions with activated Gq (8–10) and Gq/11 subunits (11–13) of the heterotrimeric G protein family. Structural data from several PLC homologs suggest that PLC-β isozymes possess a modular domain architecture containing an N-terminal pleckstrin homology (PH) domain adjacent to calcium-binding EF hand domains, followed by the catalytic αβ barrel domain, connected to a C2 domain, terminating with a C-terminal coiled-coil helical domain (14–17). Whereas the C-terminal region unique to PLC-β isozymes is required for engaging Goq (18), the binding surface of Gβγ is less clear. Conflicting reports document the PH domain, a portion of the putative EF hands, and a region within the catalytic domain of PLC-β as important for recognizing Gβγ (19–23).

Both myristylated Goq and isoprenylated Gβγ independently regulate PLC-β at cellular membranes (24), where PLC-β engages its phosphoinositide substrate, PtdIns(4,5)P₂. Interestingly, PH domains from many proteins bind phosphoinositide membrane lipids (25), and for the case of PLC-β, the PH domain recruits the enzyme to the lipid bilayer via specific interactions with PtdIns(4,5)P₂ (26). Thus, whereas PLC-β isozymes are soluble in aqueous solution, their regulation and catalytic function are intimately linked with membrane-associated heterotrimeric G protein subunits and particular membrane compositions, including specific lipid rafts (27).

The tacit assumption that inositol lipid signaling is solely regulated by heterotrimeric G proteins has grossly underestimated the complexity of PLC-β-related signaling. Illenberger et al. (28, 29) have identified several members of the Rho family of small GTPases (Cdc42, Rac1, and Rac2) that stimulate PLC-β catalyzed inositol phosphate production. Rho GTPases comprise a major branch of the Ras superfamily of GTPases, and these oncogenic molecular switches are best known for their capacity to direct the organization of the actin cytoskeleton (30, 31). Regulation of Rho GTPases by Rac GTPases appears to utilize portions of PLC-β distinct from regions involved in promoting Goq and Gβγ signaling (29). Furthermore, Rac2 also regulates the membrane association of PLC-β₂ (32), and a lipase-competent chimera of the putative PH domain of PLC-β₂, with the remaining portion of PLC-β₂, failed to respond to Rac stimulation and membrane recruitment (29).

The spectra of Rho GTPases that stimulate PLC-β have not yet been defined. Moreover, whether Rho proteins and PLC-β isozymes interact via direct protein-protein interactions, and which portions of PLC-β are required for this activation are not yet known. To investigate the mechanism of activation of PLC-β by Rho GTPases, a biosensor screen was developed to audition the Rho family GTPases for their capacity to directly recognize various full-length and truncated fragments of PLC-β isozymes. This analysis reveals that PLC-β₂ and PLC-β₃ possess remarkable specificity for only the Rac members of the Rho family of GTPases, and these detected interactions are strictly dependent upon the activated, GTP-bound state of Rac. Importantly, the PH domain of PLC-β₂ is sufficient to engage...
Rac GTPases in an identical, nucleotide-specific manner with similar affinity. Thus, our findings quantify the direct interactions between PLC-β isoforms and Rac GTPases and redefine the role of the PH domain of PLC-β2 as fully competent to bind activated Rac GTPases.

EXPERIMENTAL PROCEDURES

Protein Production—The coding sequences for all known human Rho family GTPases (Fig. 1A) were amplified by PCR and ligated into pProExHTa. With the exception of RhoBTB1 and RhoBTB2, all Rho GTPase constructs were terminated at the site of C-terminal isoprenylation and substituted with serine for the cysteine normally isoprenylated. For the multidomain RhoBTB1 and RhoBTB2 proteins, only the putative Rho GTPase portions (residues 1–247) were produced. Escherichia coli strain BL21(DE3) cells were grown in LB broth until mid-log phase, and Rho proteins were induced from the pProExHTa vector with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The bacterial expression plasmid (pET28a) encoding the cDNA encoding the PH domains of human PLC-β1 (residues 1–144) and rat PLC-β3 (residues 111–140) were subcloned into pProExHTa, expressed in E. coli strain BL21(DE3) cells until mid-log phase, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside at either 30 °C for 6 h or 22 °C for 12 h. The soluble portion of the cellular lysate from each culture was applied to nickel-charged chelating Sepharose resin (Amersham Biosciences), and recombinant proteins were purified by immobilized metal ion affinity chromatography. Fractions rich in soluble, purified GTPase were dialyzed overnight against 20 mM Hepes (pH 7.5), 150 mM NaCl, 5% glycerol, and 5 mM MgCl2, and stored at −80 °C until needed. Guanosine 5′-O-(3-thiotriphosphate) (GTPγS) (GTP analog) into GTPases and Rac GTPases were subsequently loaded onto the sensor chip surface with an injection of 1:1 mix of NHS/EDC. Each protein was captured on individual flow cells using injections of 0.2 mg/ml protein solutions followed by an injection of 1 μl ethanolamine to block remaining active surface groups as described previously (34). For the screening of Rho GTPases, 8000–10,000 response units of proteins were attached to each sensor surface, whereas lower surface densities (1000–1500 response units) were created for the titration studies. Rho GTPases were injected in a random order over the sensor surfaces using kinjets of 20–35 μl, with buffer flowed over the sensor chip to promote dissociation of each GTPase with dissociation times extending to 1000 s. Rho GTPase proteins routinely completely dissociated from each sensor surface as judged by the sensor signals returning about the baseline and remained in this state throughout the experiments. Thus, to preserve the integrity of the covalently immobilized proteins, no regeneration steps were performed. Unless noted, all sensorgrams were always normalized to the signal achieved due to binding a control “blank” flow cell surface. For kinetic evaluations, sensorgrams from each set of titrations were aligned, and the normalized data were globally fit to a 1:1 binding model (R1 = 0) with BLAevaluation 3.2 software (Biacore). Resulting thermodynamic constants are the result of three independent sets of titrations.

Phospholipid binding was measured using a Biacore 2000 in a manner similar to the description above, with the following noted exceptions. Phospholipids were purchased from Biomol, dissolved in a 1:1 chloroform/methanol solution dried under a stream of nitrogen, resuspended in Hepes-buffered saline (Biacore) by sonication. His6-tagged PLC-β1 PH domain was attached to a CM5 chip (Biacore) coated with covalently attached anti-penta-histidine antibody (Qiagen) and washed with buffer to remove nonspecific binding before phospholipid injections were performed. Between lipid injections, the PLC-β1 PH domain surface was regenerated with a pulse of 1 mM imidazole before immobilizing fresh protein.

COS-7 Cell Transfections and Phospholipase C-β Activity Measurements—pcDNA3.1 vectors (Invitrogen) encoding Rho GTPases activated with a G12V mutation and fused to hemagglutinin tags were obtained from the Guthrie cDNA Resource Center. Gβ1, Gβ2, and Goα pcDNA vectors have been described previously (35). COS-7 cells were seeded in 12-well culture dishes at a density of ~60,000 cells/well and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in an atmosphere of 95% air/5% CO2. The indicated DNA vectors were transfected using FuGENE 6 (Roche Applied Science) transfection reagent (3 μg DNA per well). Approximately 24 h after transfection, media were removed and replaced with myro-[3H]inositol (1 μCi/ml) in control-media containing 1× dialysis buffer of Dulbecco’s modified Eagle’s medium supplemented with 150 mM NaCl. After 1 h incubation, media were removed, and cell membranes were prepared for isotope neutralization with 150 mM NH4OH. [3H]Inositol phosphates were measured using a Biacore 2000 apparatus (Biacore) according to the manufacturer’s protocol. Six hundred ng total DNA/well was transfected, with empty vector added as necessary to maintain an equal amount of DNA for each well. Approximately 24 h after transfection, media were removed and replaced with myro-[3H]inositol (1 μCi/ml) in isoinositol-free Dulbecco’s modified Eagle’s medium. After 12 h, [3H]inositol phosphate accumulation was initiated by addition of LiCl to a final concentration of 10 mM. The reaction was stopped after 1 h incubation of the medium and addition of 50 μM formic acid followed by neutralization with 150 mM NH4OH. [3H]Inositol phosphates were quantified by Dowex chromatography as described previously (36).

RESULTS

Bioassay GTPase Screen—To define the spectrum of Rho family GTPases that directly bind PLC-β isoforms, 17 recombinant Rho GTPases were produced and purified (Fig. 1, A and B). Each purified GTPase is indeed functional because these Rho proteins all exhibit the capacity to load guanine nucleotides (see “Experimental Procedures”) (37). Specifically, the integrity of the Cdc42 protein was further validated by demonstrating that intersectin, a known Cdc42-specific guanine nucleotide exchange factor, but not Tiam1, a Rac1-specific ex-
change factor, will stimulate removal of bound nucleotide within Cdc42 as described previously (33).

Full-length PLC-β1, PLC-β2, and PLC-β3 were immobilized to individual flow cells on a Biacore CM5 sensor chip as a means to identify GTPases that directly interact with PLC-β isozymes using surface plasmon resonance (Fig. 1C). To verify the integrity of each immobilized PLC-β surface, we demonstrated that, as reported previously (24), an active form of Goαq (GDP + AlF4), but not inactive Goαq (GDP), specifically binds to each PLC-β isozyme (Fig. 1D). For each Rho GTPase displayed in Fig. 1B, both the active (GTPγS-loaded) and inactive (GDP-loaded) states were screened for binding to each PLC-β surface (Fig. 1E). Surprisingly, only GTPγS-loaded Rac GTPases (Rac1, Rac2, and Rac3), but no other tested Rho protein, displayed differential binding to the PLC-β surfaces compared with a blank control surface. Moreover, the activated Rac GTPases, but not the GDP-loaded forms, exhibited an observed specificity for PLC-β2 and PLC-β3, with no detectable binding to PLC-β1 (Fig. 1F). These data suggest that Rac1, Rac2, and Rac3, but not other members of the highly conserved Rho family of GTPases, can directly interact with PLC-β isozymes in a nucleotide-specific manner.

In Vivo PLC-β2 Stimulation by Rac GTPases—The capacity of Rac GTPases to specifically stimulate PLC-β2-catalyzed inositol phosphate formation in COS-7 cells was next evaluated (Fig. 2). As expected, increasing amounts of cDNA for Goαq (Fig. 2A) and Gβγ (Fig. 2B) both stimulated inositol phosphate accumulation, but only after co-transfection with PLC-β2. Significantly, and in a fashion consistent with our surface plasmon resonance biosensor screen, constitutively active GTPase-deficient Rac1 (Fig. 2C), Rac2 (Fig. 2D), and Rac3 (Fig. 2E) G12V mutants (38) considerably enhanced the lipase activity of PLC-β2. In contrast, constitutively active Cdc42 (G12V) failed to stimulate detectable inositol phosphate accumulation (Fig. 2F) despite being expressed to significant levels. These data indicate that the capacity of Rac GTPases to stimulate PLC-β2 activity in vivo is not shared by Cdc42 and are consistent with the lack of observable binding between Cdc42 and the PLC-β isozymes tested without using surface plasmon resonance.

Quantification of Affinities between Rac GTPases and PLC-β Isozymes—To quantify interactions between recombinant Rac GTPases and PLC-β2 and PLC-β3, a concentration series of each Rac GTPase (loaded with GTPγS) was injected across the immobilized PLC-β isozyme surfaces using Biacore technology (Fig. 3, A and B). The resulting sensorgrams display concent-
titration-dependent binding events between the Rac GTPases and PLC-β₂ and PLC-β₃. To estimate the dissociation constants for these interactions, the steady state equilibrium responses ($K_{eq}$) were plotted against Rac concentration and fit to simple binding isotherms (39) (Fig. 3C). As displayed in Table I, Rac₁, Rac₂, and Rac₃ each bind PLC-β₂ ($K_D = -5 -10 \mu M$) with greater affinity than PLC-β₃ ($K_D > 25 \mu M$) and display no detectable binding to PLC-β₁ at concentrations of up to 50 μM GTPase.

**Phosphoinositide Binding by the PLC-β₂ PH Domain**—Previous studies implicate the PH domain of PLC-β₂ as important for Rac-mediated stimulation of PLC-β₂ (29, 32). Thus, we produced and assessed the functional properties of the isolated PH domain of PLC-β₂. Because the most attributed biochemical function of PH domains is their capacity to bind phosphoinositides (25), the lipid binding properties of the PLC-β₂ PH domain were examined using micelles composed of individual phospholipids. Significantly, the PLC-β₂ PH domain preferably bound PtdIns(4,5)P₂ and phosphatidylinositol 4-phosphate over other negatively charged membrane lipids, phosphatidylinositol and dipalmitoyl phosphatidylethanol (Fig. 4A). Therefore, as observed for the majority of the previously characterized PH domains (40), the PLC-β₂ PH domain promiscuously binds various phosphoinositides with no significant binding to other phospholipids. The estimated dissociation constant is $-15 \mu M$ for the interaction of PtdIns(4,5)P₂ and the PH domain of PLC-β₂ (Fig. 4B). These data indicate that the refolded PH domain from PLC-β₂ is biochemically functional and suitable as a target for assessing the binding of Rho GTPases.

**Rac GTPase Binding by the PLC-β₂ PH Domain**—To define the region of PLC-β that is sufficient to recognize activated Rac GTPases, several soluble fragments of PLC-β₂ were generated (Fig. 5A). These include the C-terminal domain, a fragment spanning the PH and C2 domains, and the above-mentioned isolated PH domain. The capacity of the PLC-β₂ fragments to recognize Rac GTPases was analyzed via surface plasmon resonance. Whereas the C-terminal fragment of PLC-β₂ did not bind any of the Rho GTPases from our recombinant library, the isolated PH domain and the PH-C2 protein both mimicked the binding characteristics of full-length PLC-β₂ by interacting with GTP₆S-loaded Rac GTPases (Fig. 5B), but not with GTP₆S-loaded RhoA (Fig. 5C) or any of the other screened Rho proteins (Fig. 1B). Titration experiments revealed that the PH domain and the PH-C2 proteins bound Rac-GTP₆S isomers ($K_D = -10 -20 \mu M$) with affinities comparable to the full-length PLC-β₂ ($K_D = -5 -10 \mu M$) (Table I). In contrast, the C-terminal domain, which has been shown to be properly folded by circular dichroism (17), did not bind Rac or any other Rho family GTPase.

To further verify the specific interaction of activated Rac GTPases with the isolated PH domain of PLC-β₂, Rac binding to a set of similar PH domains was compared. Equivalent surface levels of purified PH domains from the β-adrenergic receptor kinase 1, PLC-δ₂, and PLC-β₂ were created. Injections of Gβ₂γ₂ resulted in a robust binding signal for the PH domain of β-adrenergic receptor kinase 1 relative to the PH domains of PLC-δ₂ and PLC-β₂ (Fig. 5D). This observation confirms that the β-adrenergic receptor kinase 1 PH domain is functional and competent to engage Gβ₂γ₂ as described previously (41). The weak signal observed for Gβ₁γ₂ binding to the PLC-δ₂ and PLC-β₂ PH domains did not increase with increasing Gβ₁γ₂ concentrations and is thus likely to be nonspecific (data not shown). Using this same system, Rac-GTP₆S isoforms displayed remarkable specificity for the PH domain of PLC-β₂ over the highly homologous PLC-δ₂ and β-adrenergic receptor kinase 1 PH domains (Fig. 5E). The PH domain of PLC-β₂ was highly selective for Rac-GTP₆S over Rac-GDP and all other tested Rho proteins in either nucleotide state (Fig. 5F). All three GTP₆S-loaded Rac isoforms bound the PLC-β₂ PH domain.

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**Fig. 3.** Representative data sets for the analysis of Rac3-GTP₆S interacting with PLC-β isoforms. A and B, sensorgrams from titrations of Rac3-GTP₆S (0.05, 0.1, 0.25, 1, 5, 10, 25, and 50 μM) injected over PLC-β₂ (A) and PLC-β₃ (B) surfaces. Dashed lines indicate the global fits obtained from a 1:1 interaction model, $A + B = AB$, and arrows indicate interval of injections. C, plots of the steady state equilibrium binding signal against the concentration of Rac3-GTP₆S fit to a binding isotherm for each indicated PLC-β interaction. Estimated dissociation constants from these titrations and also for the Rac1 and Rac2 titrations are presented in Table I.

**Table I**

| PLC-β₂ | PLC-β₃ | PLC-β₁ | PLC-β₂ PH-C2 | PLC-β₂ PH | PLC-β₂ CT |
|--------|--------|--------|--------------|-----------|-----------|
| Rac1   | NB     | 5.3 ± 5.6 | 30 ± 11      | 16 ± 5.3  | 13 ± 6.7  | NB        |
| Rac2   | NB     | 7.0 ± 1.9 | 58 ± 2.1     | 17 ± 8.4  | 11 ± 5.4  | NB        |
| Rac3   | NB     | 9.3 ± 5.6 | 88 ± 1.4     | 14 ± 10   | 18 ± 11   | NB        |
main in a similar concentration-dependent manner with an estimated dissociation constant of ~13 μM for the Rac1 interaction (Fig. 5G). Therefore, although the PH domain of PLC-β2 does not display significant binding to Gβγ2, it is sufficient to selectively engage Rac1, Rac2, and Rac3 in a nucleotide-dependent manner.

**DISCUSSION**

This study defines Rac1, Rac2, and Rac3 as the sole members of the highly conserved family of Rho GTPases capable of directly engaging PLC-β2 and PLC-β isoforms in a GTPγS-dependent manner. Our *in vitro* direct binding and *in vivo* transfection approaches both concur in specifically distinguishing only the Rac isoforms as modulators of PLC-β. Quantitative affinity measurements clearly delineate all three Rac GTPases as similar in their differential ability to recognize PLC-β2 > PLC-β3 > PLC-β1. This trend mimics the pattern of PLC-β stimulation observed with Rac1 and Rac2 by others (29). Moreover, our findings demonstrate that both PLC-β2 and PLC-β3 bind the very similar Rac isoforms with comparable affinities, with no observed binding to GDP-loaded GTPases. Previous reports identified Cdc42, Rac1, and Rac2 as stimulators of PLC-β activity (28, 42). In contrast, Cdc42 does not interact with PLC-β2 as measured by surface plasmon resonance (Fig. 1E), and we have been unable to observe enhanced lipase activity upon co-transfection of activated Cdc42 with PLC-β2 (Fig. 2F). Whereas interactions between Cdc42 and PLC-β isoforms were not detected in this study, Illenberger et al. (29) reported that Cdc42 stimulates PLC-β2 10-fold less efficiently than Rac2. Thus, it is plausible that our biosensor assay is insufficiently sensitive to detect weak binding of Cdc42. Nonetheless, the *in vivo* stimulation of PLC-β2 by Cdc42 appears insignificant relative to activation by Rac GTPases.

An important outcome of this analysis is the delineation of the PH domain of PLC-β2 as sufficient for binding Rac GTPases. The isolated PH domain differentially recognizes GTPγS-loaded Rac1, Rac2, and Rac3 over the GDP-loaded Rac isoforms and all other screened Rho proteins in either nucleotide state. Thus, the isolated PH domain of PLC-β2 mimics the full-length enzyme in selectively binding the activated forms of Rac with dissociation constants of ~5–10 μM. This finding coincides with the capacity of a previously designed chimera of PLC-β2 containing a substituted PH domain from PLC-β1 to retain lipase activity yet fail to respond to GTPγS-loaded Rac2 (29).

Whereas we readily detect binding of Gβγ to the PH domain of β-adrenergic receptor kinase 1 using surface plasmon resonance, Gβγ does not display significant affinity for the PH domains of PLC-β2 or PLC-δ under similar conditions. This observation directly conflicts with previous studies showing lateral association of fluorescence-tagged PH domains of PLC-β and PLC-δ with Gβγ in lipid vesicles (21). However, it must be noted that these previous studies used larger fragments (e.g. 1–171 of PLC-β2 and 1–155 of PLC-δ) that also encompass portions of the first EF hand. Therefore, the discrepancies in binding may result from different fragment sizes. Alternatively, potential interactions of Gβγ with PH domains of PLC-β and PLC-δ isoforms are sufficiently weak, such that we are unable to detect these interactions using surface plasmon resonance. Therefore, the increased effective concentrations afforded by sequestering the binding partners to a two-dimensional lipid membrane may be necessary to observe these interactions (21). A strong mechanistic consequence of the interaction between the PH domain of β-adrenergic receptor kinase 1 and Gβγ has been defined (41, 44). In contrast, Gβγ has been demonstrated to bind a plethora of PH domains from diverse signaling molecules, including the PH domain of PLC-β (21, 45–48); however, the functional significance of these interactions is still unclear.

These studies also demonstrate a clear selectivity of the PH domain of PLC-β2 for phosphoinositides over other negatively charged lipid components. This observation classifies the PLC-β2 PH domain as similar to the majority of characterized
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PH domains that also have weak, promiscuous interactions with various phosphoinositides (40). However, both isolated PH domains from PLC-β isozymes and the full-length enzymes target to lipid vesicles independent of phosphoinositide composition (21, 49). Thus, whereas PH domains from PLC-β isozymes and other membrane-associated proteins (Dbll family guanine nucleotide exchange factors) recognize phosphoinositides (50), these interactions are insufficient to target the host proteins to the cellular membrane (49, 51).

Despite the uncertain functional roles of PLC-β PH domains in recognizing Gαy and phosphoinositides (52), this study clearly defines the PH domain of PLC-β2 as a binding site and perhaps an effector site for Rac GTPases. Whereas PLC-β has no recognized CRIB motif common to well-understood Rho GTPase effectors (p21 activated kinase, ACK, and Wiskott Aldrich syndrome protein) (53–55), several lines of evidence provide a precedent for GTPases directly engaging PH domains.

Acknowledgments—We thank M. Pham and S. Gershburg for help with protein production.

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