Determination of interaction sites of phospholipase D_1 for RhoA

Songmin CAI and John H. EXTON[1]

Howard Hughes Medical Institute, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0295, U.S.A.

Phospholipase D (PLD) is regulated by many factors, including protein kinase C (PKC) and small G-proteins of the Rho and ADP-ribosylation factor families. Previous studies revealed that the interaction site of human PLD_1 for RhoA is located in its C-terminus, but the exact locus has not been determined. The purpose of the present study was to determine the interaction site of rat PLD_1 ($rPLD_1$) with RhoA. Selection with phage display of different peptides of $rPLD_1$ confirmed that GTP-bound RhoA interacted with a site in the amino acid sequence 873–1024 at the C-terminus of $rPLD_1$. RhoA also associated with this peptide in a GTP-dependent manner in COS-7 cell lysates and the peptide inhibited RhoA stimulation of PLD activity in membranes from COS-7 cells expressing $rPLD_1$. A series of alanine mutations of non-conserved residues were made in this sequence, and the enzymes were expressed in COS-7 cells and checked for responses to activation of PKC, which interacts with the N-terminus of PLD_1, and also to the constitutively active V14RhoA. Mutations in the C-terminus of $rPLD_1$ (K946A, V950A, R955A and K962A) caused partial loss of V14RhoA stimulation, and double mutations (K946A/K962A, K946A/V950A and K962A/V950A) caused an almost total loss. Co-immunoprecipitation studies also showed that the mutated forms of $rPLD_1$ described above failed to bind V14RhoA compared with wild-type $rPLD_1$, whereas $rPLD_1$ with mutations outside the region K946–K962 bound V14RhoA normally. It is concluded that basic amino acids in a restricted C-terminal region of $rPLD_1$ are important for binding of RhoA and its activation of PLD activity.

Key words: activation, binding, mutation, protein kinase C.

INTRODUCTION

Phospholipase D (PLD) activity is distributed widely in animals, plants, fungi and bacteria [1]. It hydrolyses membrane phosphatidylcholine to phosphatidic acid and choline. Phosphatidic acid is metabolized further to diacylglycerol or lysophosphatidic acid, but is considered an important signalling molecule itself. Many functions for phosphatidic acid have been proposed, namely promotion of mitogenesis in fibroblasts, stimulation of the respiratory burst in neutrophils, involvement in trafficking in the Golgi, regulation of the actin cytoskeleton and activation of specific protein kinases and phospholipases [2,3]. Although phosphatidic acid is its physiological product, PLD also carries out a transphosphatidylation reaction utilizing primary alcohols, which is used as a specific measure of its activity [4]. In mammalian tissues and cells, PLD is activated by a variety of agonists including growth factors, hormones, neurotransmitters and cytokines [1,2].

Knowledge of the regulation of PLD has been greatly enhanced due to the cloning of several isoforms of the enzyme [5–10]. There are two mammalian isoforms, namely PLD_1 and PLD_2, which exist as two splice variants [5]. PLD_1 is activated by Rho proteins (RhoA, Rac1 and Cdc42Hs) and ADP-ribosylation factors (ARFs), which are members of two different families of small G-protein [5–7,11,12]. This isoform is also stimulated directly by the α- and β-isoenzymes of protein kinase C (PKC) in the absence of ATP [5,11,12]. Synergistic interactions between PKC, ARF and RhoA have been observed in many studies [5,12], suggesting that the different activators interact with separate sites on PLD_1.

The molecular mechanisms by which small G-proteins activate PLD are not clear. Previous studies in our laboratory showed that conserved amino acids in the switch I region of RhoA are major PLD-interaction sites and that residues in the switch II and adjacent regions are responsible for the differential activation of PLD by RhoA and Cdc42Hs [13]. Others have found that the PLD effector region of ARF1 is in the N-terminus, including the α2 helix, part of the β2 strand and the N-terminal helix and its ensuing loop [14]. Previous studies have demonstrated that RhoA interacts with the C-terminus of human PLD_1 ($hPLD_1$) [15], and that the interaction site of PLD_2 for ARF is also in the C-terminus [16].

In the present study, we aimed to determine the interaction site of rat PLD_1 ($rPLD_1$) for RhoA utilizing both binding studies and measurements of PLD activity. We then found out which amino acids were important in this interaction. The results show that residues within the K946–K962 sequence of the C-terminal region of $rPLD_1$ are important for both RhoA binding and activation of enzyme activity.

EXPERIMENTAL PROCEDURES

Materials

PMA, fetal bovine serum, recombinant Protein G–agarose, PtdIns(4,5)P_2 and proteinase inhibitors (leupeptin and pepstatin A) were purchased from Sigma. Dipalmitoylphosphatidylcholine, phosphatidylethanolamine and phosphatidylbutanol (PtdBut) were from Avanti Polar Lipids. Guanosine 5′-[β-thio]-diphosphate (GDP[S]), guanosine 5′-[γ-thio]triphosphate (GTP[S]) and the transfection reagent FuGENE 6 were obtained from Roche. [9,10-^{3}H]Myristic acid and [2-palmitoyl-9,10-^{3}H]-phosphatidylcholine were from NEN Life Science Products. SDS/polyacrylamide gels were purchased from Novex. High-glucose Dulbecco's modified Eagle's medium (DMEM) was

Abbreviations used: PLD, phospholipase D; $hPLD_1$, human PLD_1; $rPLD_1$, rat PLD_1; GDP[S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; DMEM, Dulbecco's modified Eagle's medium; ARF, ADP-ribosylation factor; PKC, protein kinase C; PtdBut, phosphatidylbutanol; HSV, herpes simplex virus.

[1] To whom correspondence should be addressed (e-mail john.exton@mcmail.vanderbilt.edu).

from Life Technologies. T7 Select1-1b vector, pET-25b vector, DH5α BL21 competent *Escherichia coli* cells, His-Bind kit, S-protein–agarose, herpes simplex virus (HSV)-tag antibody and thrombin were from Novagen. Plasmid- and PCR-product-purification kits were from Qiagen. QuikChange mutagenesis kit and *Pfu* DNA polymerase were purchased from Stratagene. Monoclonal antibody against RhoA from mouse was purchased from Santa Cruz Biotechnology, and monoclonal antibody against the Xpress tag and pcDNA3.1 version A vector were from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies, ECL reagent and GSH-Sepharose beads were from Amersham Pharmacia Biotech. All restriction enzymes and T4 DNA ligase were from New England Biolabs.

Expression and purification of small G-proteins in *E. coli*

Glutathione S-transferase (GST)-RhoA was induced in the DH5α BL21 *E. coli* strain as described previously [13]. Briefly, temperature and time after addition of isopropyl β-D-thiogalactoside were varied to induce maximum soluble protein expression. After centrifugation, the bacterial pellet was resuspended in sonication buffer and lysed by sonication. GSH beads were added to the supernatant and incubated at 4 °C for 2 h. The beads were then transferred to a Polyprep column and washed with washing buffer. GST fusion proteins were eluted with elution buffer containing GSH, which was then removed by dialysis. The GST portion of the fusion protein was removed by thrombin digestion. The expression and purification procedure were monitored by SDS/PAGE.

Construction of the bacteriophage library and selection process

cDNA fragments encoding amino acid residues 342–617 (F1), 574–736 (F2), 723–884 (F3) and 873–1024 (F4) were generated by PCR with primers containing 5′ *Eco*R1 and 3′ *Hin*dIII sites, digested with these two enzymes and purified. The cDNA fragments for F1–F4 were ligated into T7 Select 1-1b vector and the liquid lysate amplified by infecting DH5α BL21 cultures. Screening the library was performed by biopanning in 96-well ELISA plates. RhoA protein (10 μg/ml, 100 μl/well) was coated on to the plates and incubated with 10 mM $MgCl_2$ and 50 μM GDP[S] or GTP[S]. About 3×10^{11} phages were used for each round of biopanning. After three rounds, when no further enrichment was observed (10^9 scale), PCR amplification of plaques (five plaques each time) and DNA sequencing were carried out to determine the binding region.

In vitro association of peptide F4 with RhoA

The cDNA fragment encoding F4 (corresponding to residues 873–1024) was inserted into the pET-25b (+) vector (also containing *Eco*R1/*Hin*dIII sites), transformed into DH5α BL21 competent cells, and expressed and purified with His-Bind resin according to the instructions of the manufacturer (Novagen). As F4 was expressed mainly in inclusion bodies, purification of the peptide was performed under denaturing conditions using 6 M guanidine hydrochloride and then refolding by successive decreases of guanidine hydrochloride concentration in dialysis buffer. Vector peptide or F4 peptide (2 μg) was incubated with 2 μg of RhoA pretreated with 50 μM GDP[S] or GTP[S] plus 10 mM $MgCl_2$ for 30 min at 30 °C in a total volume of 50 μl. The peptides were precipitated with a His-Bind resin and the associated RhoA and vector or F4 peptide were detected by Western blotting with antibodies to RhoA and HSV.

Table 1 ***In vivo*** **PLD activity of alanine mutants of PLD_1**

Scanning alanine mutagenesis on non-conserved amino acid residues of $rPLD_1$ was performed as described in the Experimental procedures section. cDNAs of the vector, wild-type and different $rPLD_1$ mutants were transfected into COS-7 cells. The cells were either co-transfected with V14RhoA or treated with PMA. The cells were incubated with 0.3% butan-1-ol for 30 min and, when present, PMA (100 nM) was added for 30 min before the reaction was terminated. PLD activity was assayed by measuring the percentage of total labelled lipid converted to [^{3}H]PtdBut. Because the experiments were conducted in batches, the results are normalized to the values observed with wild-type $rPLD_1$ in the presence of PMA or V14RhoA after subtraction of the values obtained with vector control. Mean values for PLD activity (percentage of total lipid corporation into PtdBut) in cells transfected with vector alone or wild-type $rPLD_1$ were as follows. Vector cells, no addition, 0.06 ± 0.01; V14RhoA, 0.06 ± 0.01; PMA, 0.72 ± 0.08. $rPLD_1$ cells, no addition, 0.18 ± 0.04; V14RhoA, 0.55 ± 0.07; PMA, 1.74 ± 0.03. The values are compiled from assays of duplicate samples from two–six experiments.

	Stimulation	
Mutant	V14RhoA	PMA
Vector	0	0
$rPLD_1$	1	1
K906A	1.19	1.02
L919A	1.0	0.92
R923A	1.16	1.02
Y928A	1.14	1.02
L929A	1.03	0.97
D931A	1.03	1.0
E934A	1.18	1.21
K943A	0.91	0.89
K946A	0.4	0.78
E947A	1.06	0.99
V950A	0.58	0.98
S951A	1.06	0.99
R955A	0.42	0.96
K962A	0.37	0.8
N969A	1.17	1.12
H973A	1.31	0.96
I976A	1.3	0.94
D980A	1.28	1.23
K984A	1.14	0.98
E990A	1.14	1.04
R994A	1.03	0.82
E997A	1.03	1.14
F1005A	1.07	1.01
Y1012A	0.94	1.04

Construction of $rPLD_1$ mutants

Full-length $rPLD_1$ was subcloned in frame with the N-terminal Xpress tag at *Kpn*I/*Xba*I sites in the polylinker region of the pcDNA3.1 version A vector as described previously [17]. This Xpress-tagged $rPLD_1$ served as the wild-type control and the template DNA for different mutants of $rPLD_1$ (described in Table 1 and Figures 3–6, see below). Mutagenesis of the plasmids was carried out using QuikChange PCR-based mutagenesis kits. Plasmids were sequenced to confirm the mutations and the integrity of the surrounding sequences for 100–150 bp.

In vivo PLD assay

COS-7 cells were cultured at 37 °C in humidified 5% CO_2 in DMEM containing 10% fetal bovine serum. The cells were transfected with plasmids using FuGENE 6 reagent according to the manufacturer's protocol. After labelling the cells with [^{3}H]-myristic acid (1 μCi/ml) and subjecting them to overnight serum starvation (0.5% fetal bovine serum in DMEM), PLD activity was assayed by incubating them with butanol for 30 min and measuring the formation of [^{3}H]PtdBut as a percentage of total

labelled lipid [18]. In studies on the effect of RhoA, the constitutively active form (V14RhoA) was co-transfected with vector, $rPLD_1$ or mutants. In studies on the effect of PMA, the labelled and serum-starved cells were incubated with 100 nM PMA for 30 min.

In vitro PLD assay

COS-7 cells were transfected with $rPLD_1$ as described for the *in vivo* PLD assay and membranes were prepared as described in [7]. The membranes were incubated with phospholipid vesicles containing phosphatidylethanolamine, PtdIns(4,5)P_2 and [palmitoyl-^{3}H]phosphatidylcholine for 30 min at 37 °C in the presence of 1% butanol as described in [7]. PLD activity was assessed by measuring the formation of [^{3}H]PtdBut as described in [11].

Immunoprecipitation of RhoA with wild-type and mutant PLD_1

Xpress-tagged wild-type $rPLD_1$ and mutated forms of $rPLD_1$ were co-expressed with V14RhoA in COS-7 cells in six-well plates (three wells for each transfection). As controls, vector, $rPLD_1$ and V14RhoA alone were also expressed. After transfection and serum-starvation as described above, the cells were washed twice with ice-cold PBS and then resuspended in lysis buffer A (25 mM Hepes, 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 0.2% Triton X-100 and 20 mM $MgCl_2$). Since preliminary experiments showed that GTP[S] enhanced the binding of wild-type $rPLD_1$ to V14RhoA, 100 μM GTP[S] was added to lysis buffer A and maintained at this concentration during the whole immunoprecipitation. The cells were scraped and passed through a 27-gauge needle five times, then centrifuged at 10000 ***g*** for 10 min at 4 °C to pellet the unbroken cells and nuclei. The supernatant was incubated with 2 μg of mouse monoclonal anti-Xpress antibody at 4 °C for 2 h and then 30 μl of Protein G beads were added for another 2 h. The beads were washed twice with lysis buffer A and the resulting pellets were resuspended in SDS sample buffer and analysed by Western blotting with RhoA antibody.

Western blotting

Protein samples of immunoprecipitates or COS-7 cell lysates were subjected to SDS/PAGE and transferred to Immobilon-P membranes (Millipore). After blocking for 1 h or overnight with 5% fat-free milk, the membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Visualization of bands was by enhanced chemiluminescence.

RESULTS

Determination of the RhoA-interacting sequence of $rPLD_1$ using a phage-display system

The interaction site in $rPLD_1$ for RhoA was first determined using the T7 phage-display system. Four $rPLD_1$ cDNA fragments encoding sequences 342–617 (F1), 574–736 (F2), 723–884 (F3) and 873–1024 (F4; Figure 1A) were inserted into the phage and enrichment for binding to GTP[S]- or GDP[S]-bound RhoA determined as described in the Experimental procedures section. A fragment corresponding to the sequence 1–318 was not examined since previous work has shown that this does not contain a binding site for RhoA [19]. After three rounds of phage display, the enrichment of phage plaques reached saturation (10^9 scale; Figure 1B). Sequencing of PCR-amplified DNA from enriched plaques showed that all of the phages (5/5) contained the cDNA sequence which encoded the F4 fragment. These results suggest that only the $rPLD_1$ sequence 873–1024 specifically binds activated RhoA.

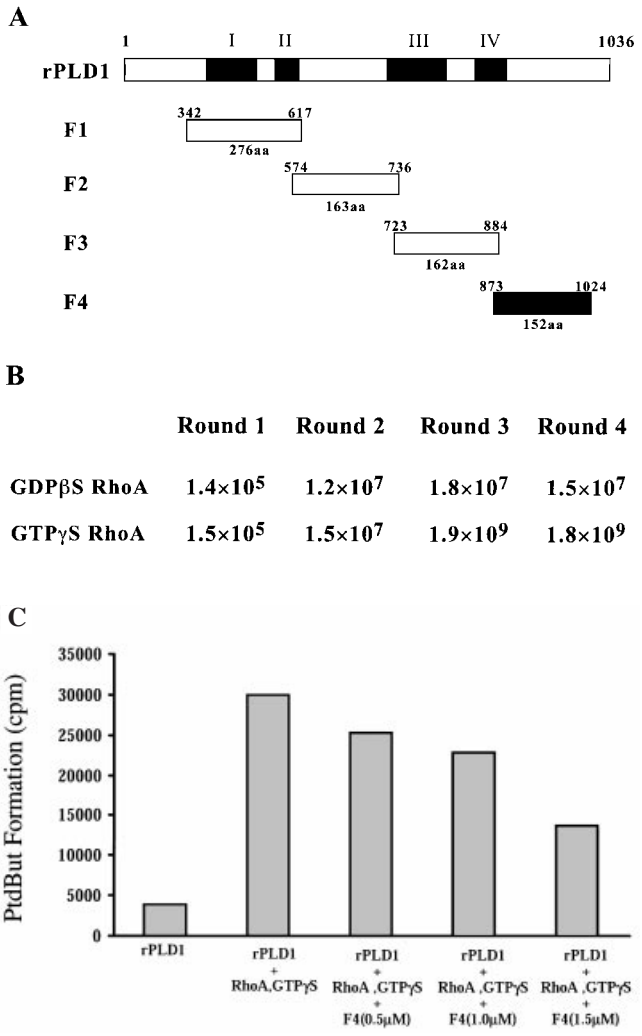

Figure 1 Determination of the amino acid sequence 873–1024 (F4 peptide) as the RhoA binding site on $rPLD_1$ using phage display and demonstration that the F4 peptide inhibits $rPLD_1$ activity

(**A**) Different $rPLD_1$ cDNA fragments encoding the sequences designated F1–F4 were inserted into T7 Select 1-1b arms of bacteriophage and packaged for selection of binding to RhoA liganded to GDP[S] (GDPβS) or GTP[S] (GTPγS). (**B**) Enrichments of phage during rounds of selection. PCR amplification of phage from the fourth round of selection and DNA sequencing showed that F4 was the fragment that preferentially bound RhoA-GTP[S]. (**C**) The PLD activity of membranes prepared from COS-7 cells expressing $rPLD_1$ was studied. The membranes (5 μg) were incubated with phospholipid vesicles containing [palmitoyl-^{3}H]phosphatidylcholine and [^{3}H]PtdBut was measured as described in the Experimental procedures section. Where present, RhoA was 0.1 μM and GTP[S] was 100 μM. F4 peptide was added at concentrations shown. The data are representative of three experiments.

The F4 fragment inhibits RhoA-stimulated $rPLD_1$ activity

To examine the effects of the F4 fragment on PLD activity, COS-7 cells were transfected with $rPLD_1$ and their membranes were incubated with RhoA and GTP[S] in the presence and absence of different concentrations of F4 peptide. Figure 1(C) shows that the peptide dose-dependently inhibited the activation of $rPLD_1$.

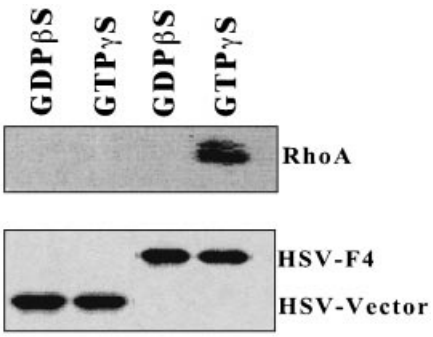

Figure 2 ***In vitro*** **association of F4 peptide with RhoA**

HSV-tagged vector and HSV-tagged F4 peptide expressed and purified from *E. coli* were incubated with RhoA in the presence of 50 μM GDP[S] (GDPβS) or GTP[S] (GTPγS) and 10 mM $MgCl_2$. The HSV-tagged proteins and RhoA were detected by Western blotting. The results are representative of three experiments.

In the absence of RhoA and GTP[S], the peptide had no effect (results not shown).

RhoA associates with the F4 fragment in a GTP-dependent manner

To confirm that the F4 fragment bound $rPLD_1$, RhoA was incubated with HSV-tagged F4 peptide or control peptide, in the presence of GTP[S] or GDP[S]. Figure 2 shows that, in the presence of GTP[S], RhoA was associated with the HSV-tagged F4 peptide, whereas little or no GDP-bound RhoA bound to the peptide. Protein produced by the vector control did not interact with either GTP- or GDP-bound RhoA. These results confirm the ability of the F4 fragment to interact with the active form of RhoA.

K946, V950, R955 and K962 in the C-terminus of PLD_1 are important residues for mediating the activation of $rPLD_1$ by RhoA

Having determined that the sequence 873–1024 contained the binding site for RhoA, we constructed a series of alanine mutations of non-conserved residues in this sequence. These mutant PLDs were checked for response to PKC (PMA stimulation) as well as to active V14RhoA. PKC activation was chosen as a control to see if any reductions in V14RhoA responses were due to a generalized loss of enzyme activity, since PKC interacts with the N-terminus, but not the C-terminus, of PLD_1 [19–21]. The results (Table 1) are normalized to the values observed with cells expressing wild-type $rPLD_1$ after subtraction of values observed with control cells, i.e. those expressing vector alone, because Table 1 is a compilation of the results from many series of experiments. Most of the mutants showed unimpaired responses to V14RhoA and PMA. However, the K946A, V950A, R995A and K962A mutants showed diminished responses to V14RhoA. Figure 3(A) shows a more extensive study of these mutants and confirms that their responses to V14RhoA were reduced. Although the responses of V950A and R955A to PMA were not significantly impaired, the responses of K946A and K962A to PMA were slightly diminished compared with the wild-type. However, the decreases in their PMA responses (22% and 20%, respectively, after correction for vector control) were not as great as those in their V14RhoA responses (60% and 63%, respectively). Figure 3(B) shows a representative experiment illustrating that the PLD mutants were all well expressed, as was V14RhoA.

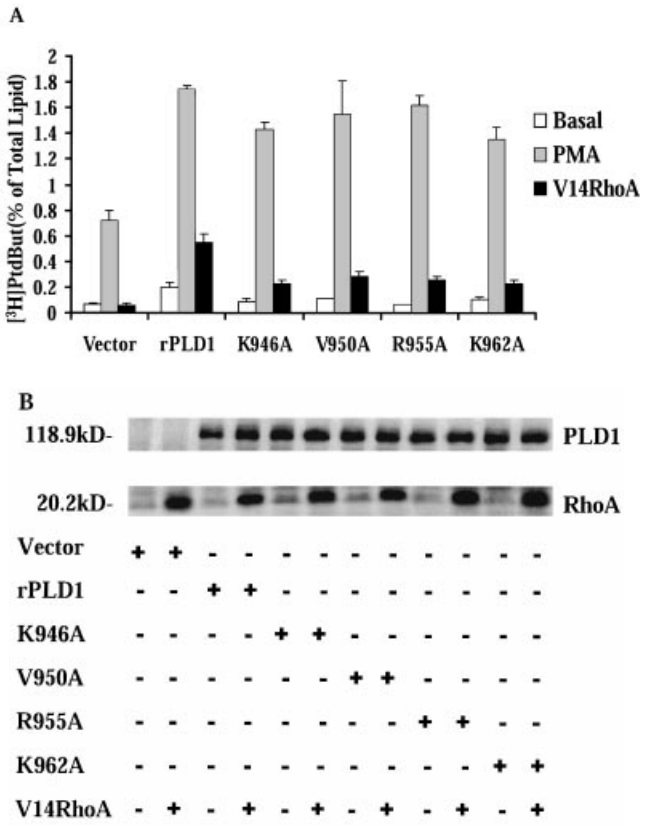

Figure 3 Effects of alanine mutagenesis on PMA and RhoA activation of PLD_1

(**A**) Wild-type or mutant forms of $rPLD_1$ cDNA were generated with N-terminal Xpress tags as described in the Experimental procedures section. These cDNAs were transfected alone or with V14RhoA cDNA into COS-7 cells. The cells transfected with wild-type or mutant $rPLD_1$ cDNA alone were also treated with 100 nM PMA for 30 min. PLD activity was assayed as described in Table 1. (**B**) Cell lysates of (**A**) were subjected to 4–20% SDS/PAGE followed by Western blotting with antibodies against the Xpress tag or RhoA. The data are means $\pm$ S.E.M. from three–six individual experiments performed in duplicate. The blots are representative of three experiments.

Effects of double mutations in PLD_1 on its activation by RhoA

Since the loss of RhoA responsiveness in the single mutations of $rPLD_1$ was only partial, the effects of double mutations were examined. As shown in Figure 4(A), the combination of K946A with K962A or V950A caused a complete or almost complete loss of the RhoA response, while having no detectable effect on the PMA response. Likewise, the combination of K962A with V950A also largely abrogated the RhoA effect with minimal impairment of the PMA effect. Thus the double mutations largely eliminated the response of the enzyme to RhoA. Figure 4(B) shows that the lack of these responses could not be attributed to a failure of V14RhoA or the PLD mutants to be expressed.

Determination of the K946–K962 PLD_1 sequence as a binding site for RhoA

The preceding mutagenesis study defined residues in PLD_1 that were required for the activation of PLD activity by RhoA. However, it is possible that these may not necessarily be involved

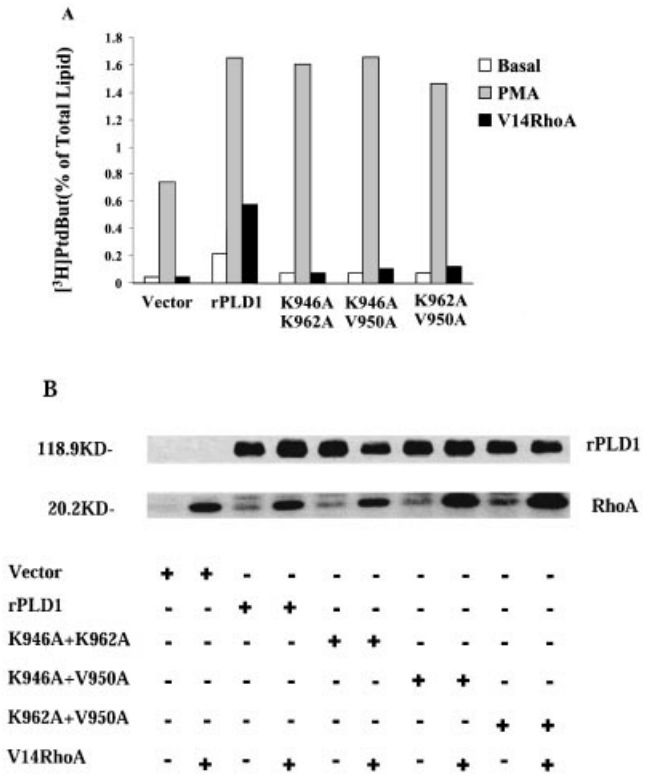

Figure 4 Effects of double mutations on RhoA activation of PLD_1

(**A**) Wild-type or mutant forms of $rPLD_1$ cDNA were generated and transfected alone or with V14RhoA cDNA into COS-7 cells as described in the Experimental procedures section. PLD activity in the absence or presence of 100 nM PMA was assayed as described in Table 1. (**B**) Cell lysates of (**A**) were Western blotted against the Xpress tag or RhoA. The data are means from two experiments performed in duplicate and the Western blot is representative of these experiments.

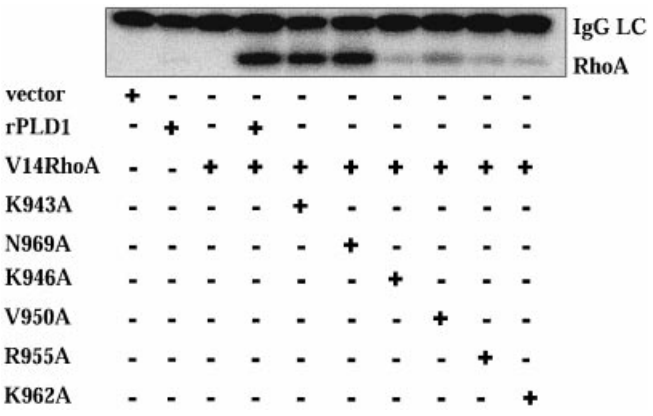

Figure 5 Immunoprecipitation of RhoA with mutant PLD_1

Xpress-tagged wild-type $rPLD_1$ and mutated forms of $rPLD_1$ were co-expressed with V14RhoA in COS-7 cells. As controls, vector, $rPLD_1$ and V14RhoA alone were also expressed. After immunoprecipitation of PLD_1 (in the presence of 100 μM GTP[S] and 20 mM $MgCl_2$ as described in the Experimental procedures section) using anti-Xpress antibody, the amount of RhoA co-immunoprecipitated was detected by Western blotting with anti-RhoA antibody. The experiment shown is representative of two. IgGLC, IgG light chain.

in the binding of RhoA. This is because some of them may be required for transducing the signal from the binding site to the catalytic centre. We therefore tested various PLD mutants in co-immunoprecipitation experiments to see if RhoA binding was altered. Figure 5 shows that mutations outside the K946–K962 sequence, namely K943A and N969A, caused no significant alteration of RhoA binding as determined by co-immunoprecipitation. On the other hand, the K946A, V950A, R955A and K962A mutants showed a substantial loss of binding. As expected, the double mutants also showed a great loss of binding (results not shown).

DISCUSSION

The designation of the 873–1024 $rPLD_1$ sequence (F4 peptide) as including the RhoA-binding site was determined by both phage display and *in vitro* RhoA-binding studies (Figures 1 and 2), and agrees with the findings of Yamazaki et al. [15] and Sung et al. [22]. Sung et al. [22] used yeast two-hybrid assay to show that the 674–1074 $hPLD_1$ sequence bound active V14RhoA but not wild-type RhoA, and Yamazaki et al. [15] used the yeast two-hybrid system and glutathione S-transferase-RhoA 'pull-down' experiments to show that RhoA bound to the 712–1074 $hPLD_1$ sequence, which corresponds to the 674–1036 $rPLD_1$ sequence. In these studies and the present study (Figure 2), the specificity of the interaction was shown by the fact that only the GTP[S]-liganded or constitutively active forms of RhoA bound to these C-terminal fragments. Yamazaki et al. [15] also showed that their C-terminal fragment inhibited the activation of $hPLD_1$ by RhoA, but not PKCα or ARF *in vitro*. We also found that the F4 peptide partly inhibited the activation of $rPLD_1$ by RhoA (Figure 1C).

Since there is evidence that PLD_2 is not activated by Rho proteins [9,16], we utilized scanning alanine mutagenesis of those residues in the 873–1024 sequence of $rPLD_1$ that were conserved between $rPLD_1$ and $hPLD_1$ [5–7], but not found in mouse, rat or human PLD_2s [8–10]. As Table 1 shows, the majority of the mutations in these residues (K906–K943 and N969–Y1012) showed no impairment of the activation of $rPLD_1$ by V14RhoA in COS-7 cells. On the other hand, four residues in the K946–K962 sequence showed significant impairment of the activation of $rPLD_1$ by V14RhoA. Not all the non-conserved residues were mutated, e.g. those adjacent to conserved sequences or to mutated residues were not altered. Thus it is conceivable that single residues that are required for RhoA activation, but are not part of a sequence, might have been missed.

As illustrated in Table 1 and Figures 3(A) and 4(A), some mutants that showed impaired responses to V14RhoA also showed some decreases in their activation by PMA. However, the magnitude of the decreases was small, indicating that the mutations did not cause non-specific (global) changes in the enzyme. It is possible that the changes responsible for the reduction in RhoA binding induced by these mutations also altered PKC binding or action. This is because other experiments have shown a synergistic interaction between RhoA and PKCα in the activation of PLD_1 [5,12], suggesting that there is some communication between the mechanisms by which these agents activate the enzymes. However, the mechanism of the synergism is unknown. It could occur by a convergence of the effects of RhoA and PKC on the catalytic site or by an interaction between their binding sites. As discussed below, the mechanism by which RhoA activates PLD_1 is unknown and how this could interact with the PKC mechanism remains speculative at this time.

The results of the RhoA-binding experiments (Figure 5) support the conclusions based on the V14RhoA activation

studies. Although it was likely that the activation studies did identify the RhoA-interaction site, this was not certain. This was because these studies could have also identified residues involved in communicating the effects of RhoA binding to the catalytic mechanism. Presumably the binding of RhoA at the C-terminus of the enzyme induces a conformational change that facilitates the catalytic reaction.

There is much evidence that the HKD motifs of PLD are required for catalytic activity and that the catalytic centre of the enzyme is formed by dimerization of the N- and C-terminal HKD domains [17,23,24]. How the binding of RhoA (or PKC or ARF) influences catalysis is unclear at this time since the three-dimensional structure of mammalian PLD isoenzymes is unknown. Deletion of the N-terminal 325 amino acids of PLD_1 does not affect its ability to be activated by RhoA [20,21], but deletions or mutations of the C-terminus and the construction of PLD_1/PLD_2 chimaeras involving this region result in inactive enzymes [16,21,25]. Thus it is difficult at present to analyse the mechanism by which RhoA binding activates the enzyme.

The interaction site for RhoA on $rPLD_1$ differs from the conserved binding motif (CRIB, which stands for Cdc42/Rac interactive binding) for the interaction of Rac and Cdc42 with the serine/threonine kinase $p65^{PAK}$ and other proteins identified in the GenBank database [26,27]. It also differs from the binding site for RhoA on another kinase $p160^{ROCK}$ [28] and has no homology with another Rho-effector motif shared by PKN, Rhophilin and Rhotekin [29]. These observations indicate that Rho-family proteins are able to interact with different motifs in their various effectors. This is consonant with the fact that different residues in these G-proteins specify which effectors they interact with [13,30–37].

In the case of RhoA, residues in the switch I activation loop (Tyr^{34}, Thr^{37}, Phe^{39}) are absolutely required for $rPLD_1$ activation [13], but specificity for RhoA versus Cdc42Hs is determined by residues in or adjacent to the switch II region (Asp^{76} and Gln^{52}). (Note: The insert region of Cdc42 has also been reported to be involved in the activation of PLD_1 by this G-protein [38]. The RhoA/Cdc42Hs chimaeras utilized in the study of Bae et al. [13] would not have revealed this additional site.) This combination of residues is different from that required for the interaction of Rho family proteins with other effectors or their elicitation of different cellular responses [30–37]. It is noteworthy that basic residues (Lys^{946}, Arg^{955}, Lys^{962}) in $rPLD_1$ are required for its interaction with RhoA and that the mutation of Ala^{954} in $rPLD_1$ to Glu causes a great loss of RhoA activation (results not shown). These results suggest that the binding of the enzyme to RhoA may involve electrostatic interactions with acidic residues in the G-protein. It is of interest that studies of the crystal structure of activated RhoA indicate that Asp^{76} protrudes from the surface of the molecule [39] and that the switch II region surrounding this residue contains several acidic residues.

Activation of PLD_1 by its regulators (PKCα, RhoA and ARF) must involve direct interaction of the regulators with the enzyme. This is because they activate the recombinant enzyme *in vitro* in a system involving lipids, but no other proteins [5,11]. The present finding that mutation of residues in the K946–K962 sequence causes a loss of both RhoA binding and impaired activation of $rPLD_1$ in COS-7 cells supports the idea that direct interaction between RhoA and PLD_1 is involved in the regulation of this enzyme *in vivo*. The use of PLD_1 mutants that are unresponsive to RhoA should prove helpful in defining the role of this small G-protein in the regulation of the phospholipase by agonist stimulation and other mechanisms *in vivo*.

Following submission of this article, another group [40] reported the requirement of specific residues in the C-terminus of $hPLD_1$ for interaction with RhoA. They utilized the yeast split-hybrid system to analyse RhoA binding and expression in COS-7 cells to examine RhoA activation of PLD activity. They identified I870 in $hPLD_1$ (corresponding to I832 in $rPLD_1$) as a major site of interaction, but since this lies outside the F4 peptide sequence (873–1024) it was not examined in the present study. Additionally, they reported that the double mutant Q975R/D999V lost interaction with RhoA [40], but that the single mutations were without effect. These residues correspond to Q937 and D961 in $rPLD_1$ and were not mutated in the present study; in particular, D961was not mutated because it is conserved between PLD_1 and PLD_2. Our finding that the K946–K962 sequence is important for RhoA binding to and activation of $rPLD_1$ does not, of course, exclude the involvement of other regions of the enzyme apart from the N-terminus.

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