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Ral GTPase interacts with the N-terminal in addition to the C-terminal region of PLC-δ1

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ABSTRACT

Previously, we have shown that RalA, a calmodulin (CaM)-binding protein, binds to the C2 region in the C-terminal of PLC-δ1, and increases its enzymatic activity. Since PLC-δ1 contains a CaM-like region in its N-terminus, we have investigated if RalA can also bind to the N-terminus of PLC-δ1. Therefore, we created a GST-PLC-δ1 construct consisting of the first 294 amino acids of PLC-δ1 (GST-PLC-$\delta1_{1-294}$). *In vitro* binding experiments confirmed that PLC-$\delta1_{1-294}$ was capable of binding directly to RalA. W-7 coupled to polyacrylamide beads bound pure PLC-δ1, demonstrating that PLC-δ1 contains a CaM-like region. Competition assays with W-7, peptides representing RalA and the newly identified RalB CaM-binding regions, or the IQ peptide from PLC-δ1 were able to inhibit RalA binding to PLC-$\delta1_{1-294}$. This study demonstrates that there are two binding sites for RalA in PLC-δ1 and provides further insight into the role of Ral GTPase in the regulation of PLC-δ1 function.

Introduction

Intracellular calcium, as a second messenger, plays an important role in normal cell signaling and alterations in calcium regulation are associated with the development of various diseased conditions including, cardiac hypertrophy [1]. Phosphoinositide-specific phospholipase C (PLC) is a cellular protein that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid found in the cell membrane, into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [2]. DAG activates protein kinase C, while IP_3 causes release of calcium from the endoplasmic reticulum. There are five different isoforms of PLC (PLC-β, PLC-γ, PLC-ε, PLC-δ, and PLC-ζ). PLC-δ is one of the most sensitive isoforms to calcium and is activated by increase in intracellular calcium due to the activation of PLC-β, PLC-γ, and PLC-ε, and is responsible for the amplification of initial calcium spike [3]. The mechanism responsible for regulating PLC-δ1, however, is not well understood.

PLC-δ1 is composed of several distinct domains [3]. The pleckstrin homology (PH) domain (29–130 amino acid residues) is required for membrane localization [4]. The EF-hand domain, composed of residues 133–279, is required for catalytic activity [5–7]. The X (296–430) and Y (492–609) catalytic domains are separated by a linker region [7]. The C2 domain, which binds calcium, is found at the C-terminus encompassing amino acid residues 647–756 [7]. The EF-hand motif of PLC-δ1 is composed of a helix-loop-helix arrangement [7] and, on its own, has a weak affinity for calcium [8]. However, in the presence of arachidonic acid and the PH domain, the calcium affinity increases [8]. PLC-ζ, the most recent member of the PLC family, is also regulated through its calcium sensitive EF-hand domain [9–11]. Structurally, the EF-hand domain of PLC-δ1 is similar to the EF-hand domain found in calmodulin (CaM) [12]. CaM, an important cell signaling protein, has four calcium binding EF-hand motifs, allowing it to exist in the calcium free state (apo-CaM), and the calcium bound state (Ca^{2+}-CaM) [12]. CaM-binding sequences, typically ranging from 16 to 30 amino acids in length, form amphipathic alpha-helices that have a basic and a hydrophobic face [13], and thus can bind both Ca^{2+}-CaM and apo-CaM [14]. W-7 is a potent small molecule inhibitor of CaM, and has been shown to inactivate PLC-δ1 *in vitro* [15,16]. IQ motifs, which bind CaM in a calcium independent fashion, have amphipathic character [14]. We have identified an IQ motif in PLC-δ1 and demonstrated that CaM binds to this region and inactivates the enzyme [17]. Previously it has been shown that CaM-binding peptides and CaM inhibit PLC activity [18,19].

Small GTP-binding proteins belonging to the Ras superfamily (Ral, Ras, and Rho), which are important in cell signaling pathways controlling cell proliferation, differentiation, trafficking, and cytoskeleton organization [20] have been shown to bind and regulate various isoforms of PLC. Thus, PLC-β isoforms through a long C-terminal extension, interact with heterotrimeric G-proteins [5,21]. The N-terminus of PLC-β also binds CaM [22]. PLC-ε has

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separate Ras and Rho binding domains [23,24]. Ral, a member of the Ras subfamily, consists of two closely related members, RalA and RalB. The two proteins are 85% identical, and most of this difference is in the C-terminal region [25]. We have shown, *in vitro* and *in vivo*, that both RalA and RalB have a calcium dependent CaM-binding site in their C-terminal region, and a calcium independent binding site in their N-terminal region [26]. A peptide comprised of 18 C-terminal amino acids of RalA has been shown to bind CaM [27]. Recently, we have shown that RalA binds PLC-δ1 at the C2 domain, and causes PLC-δ1 activation [17].

In the present report we have investigated the existence of a second RalA binding site in PLC-δ1. The results demonstrate that RalA binds to the N-terminal region of PLC-δ1. In addition, W-7 and a peptide representing the IQ region in PLC-δ1 also bind PLC-δ1 and inhibit RalA binding to the N-terminus of PLC-δ1. Along with our previous findings, the current study offers more insight into the complex regulation of PLC-δ1.

Materials and methods

Reagents and plasmids. Rat PLC-δ1 in pGEX-2T (pGEX-2T–PLC-δ1) was a generous gift of Dr. M. Katan (The Institute of Cancer Research, London, UK). Restriction enzymes, DNA Polymerase I Large Fragment (Klenow), T4 Quick Ligation Kit were obtained from New England Biolabs, while Wizard® DNA Clean-Up System was from Promega. RalA (NH_2-SKEKNGKKKRKSLAKRIR-COOH) and RalB (NH_2-KSSKNKKSFKERC-COOH) C-terminal peptides, and IQ (NH_2-VRSQVQHKPKEDKLKLVPELS-COOH) peptide were commercially synthesized. Bovine brain calmodulin, W-7 covalently coupled to polyacrylamide beads, W-7·HCl and W-5·HCl was purchased from Calbiochem. DH5α *Escherichia coli* chemically competent cells, isopropyl 1-thio-β-D-galactopyranoside (IPTG), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin were supplied by Invitrogen. Glutathione (GSH)-agarose beads, lysozyme, and thrombin were from Sigma. Culture plates were purchased from Corning. CNBr-activated Sepharose™ 4 Fast Flow beads, PVDF membrane, and ECL plus Western Blotting Detection System were from Amersham Biosciences. Mouse anti-human-RalA (1:5000), mouse anti-human-PLC-δ1 (1:250) were purchased from BD Biosciences, while mouse anti-human-calmodulin (0.2 μg/mL) was purchased from Upstate Biotechnology. Goat anti-mouse IgG horseradish peroxidase conjugate was obtained from Bio-Rad. All other chemicals were reagent grade.

Plasmid constructs. pGEX-2T–PLC-δ1 was restricted with AccI and SmaI. The resulting DNA fragments were separated on a 1% agarose gel. Two bands were visualized (~5781 bp and ~1386 bp). The larger band encoding for pGEX-2T and PLC-δ1 consisting of amino acids 1–294 was cut out of the gel. This DNA fragment was isolated using steel wool, and cleaned using Wizard® DNA Clean-Up System. The purified DNA fragment was Klenow-blunted, ligated, and transformed into DH5α *E. coli* chemically competent cells. The transformed cells were plated on LB plates supplemented with 100 μg/mL ampicillin, and incubated overnight at 37 °C. The resulting truncated GST-PLC-δ1 construct was termed, GST-PLC-$\delta1_{1-294}$.

Glutathione-S-transferase (GST) recombinant protein expression and purification. The GST-fusion proteins were expressed in DH5α *E. coli* cells in the presence of 0.5 mM IPTG as previously described [17,26]. A small aliquot was used to test for protein expression and purity using 12% SDS–PAGE and Coomassie staining, while the rest was used in binding experiments.

Cleavage of GST-fusion proteins by thrombin. Purified GST-fusion proteins were resuspended in phosphate buffered saline (PBS) (20 mM NaH_2PO_4, pH 7.4, 300 mM NaCl) in the presence of 0.5 U/ml of thrombin and incubated at room temperature overnight. The protein mixture was centrifuged at 325*g* for 10 min. The supernatant containing the cleaved protein was aliquoted and stored at −20 °C.

Cell culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (v/v), 1.5 g/L $NaHCO_3$ and 100 U of penicillin/streptomycin at 37 °C and 5% CO_2 on 6 well or 100 mm plates.

Peptide coupling to CNBr-activated Sepharose beads. RalB peptide (2.5 mg/ml) was coupled to CNBr-activated Sepharose beads as per manufacturer's protocol.

Binding assays—endogenous protein. Cells were lysed in ice cold lysis buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol) containing protease inhibitors. Cell lysate was centrifuged at 13,000*g* at 4 °C for 10 min. The supernatant containing soluble proteins was kept for further experiments. Approximately 500 μg of protein was incubated with 50 μg of an appropriate GST-fusion protein or the CNBr-RalB-peptide coupled beads in the presence or absence of EGTA and varying concentrations of $CaCl_2$ for 2 h at 4 °C with gentle rocking. The binding reaction mixture was centrifuged at 1300*g* for 1 min, washed three times in the lysis buffer containing appropriate concentration of EGTA and $CaCl_2$, separated by 12% SDS–PAGE [28] and analyzed by Western blotting using appropriate antibodies.

Binding assays—pure protein. An aliquot of pure cleaved GST-fusion protein or pure calmodulin was incubated with the appropriate GST-fusion protein or the CNBr-RalB-peptide coupled beads or W-7 covalently coupled to polyacrylamide beads or appropriate control beads in the presence or absence of EGTA and $CaCl_2$ for 2 h at 4 °C with gentle rocking. The binding reaction mixture was centrifuged at 1300*g* for 1 min, washed three times in the lysis buffer containing appropriate concentration of EGTA and $CaCl_2$, separated by 12% SDS–PAGE and analyzed by Western blotting using appropriate antibodies.

Competition assays. RalA or RalB or IQ peptides, W-7·HCl or W-5·HCl were incubated with 50 μg of GST-PLC-$\delta1_{1-294}$ in the presence of 5 mM EGTA and 1 mM $CaCl_2$ for 15 min at 4 °C with gentle rocking, after which thrombin cleaved GST-RalA was added and the mixture incubated for additional 2 h at 4 °C with gentle rocking. The binding reaction mixture was centrifuged at 1300*g* for 1 min, washed three times in the lysis buffer containing appropriate concentration of EGTA and $CaCl_2$, separated by 12% SDS–PAGE and analyzed by Western blotting using appropriate antibodies.

Results

RalA but not CaM bind to PLC-δ1$_{1-294}$

We hypothesized that since RalA is capable of binding CaM, then RalA would be capable of binding to the CaM-like domain in the N-terminal of PLC-δ1. In order to test this hypothesis we created a GST-PLC-δ1 construct (GST-PLC-$\delta1_{1-294}$) consisting of the first 294 amino acids, thus encompassing the PH domain and the entire EF-hand domain. We used this construct to pull-down RalA from HeLa cell lysate (Fig. 1A). The binding of RalA to PLC-$\delta1_{1-294}$ increased with increasing calcium concentration (Fig. 1A). Since Ral, CaM, and PLC-δ1 can exist in a complex [16], we investigated if CaM was also present in the pull-down sample. However, we did not detect any CaM (Fig. 1A). This suggests that a CaM-binding region is not present in PLC-$\delta1_{1-294}$. To assess if RalA can interact directly with PLC-$\delta1_{1-294}$, pure RalA was incubated with GST-PLC-$\delta1_{1-294}$. The results demonstrated that RalA interacts directly with GST-PLC-$\delta1_{1-294}$ in a calcium dependent manner (Fig. 1B). GST-PLC-$\delta1_{1-294}$ did not cross-react with the antibodies used to detect RalA or CaM (Fig. 1A and B). GST did not bind RalA (Fig. 1B).