Analytical Biochemistry 431 (2012) 106–114

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Analysis of the phospholipase C-δ1 pleckstrin homology domain using native polyacrylamide gel electrophoresis

Michikazu Tanio [a], Katsuyuki Nishimura [a,b,*]

[a] Institute for Molecular Science, Myodaiji, Okazaki 444-8585, Japan
[b] Graduate University for Advanced Studies (SOKENDAI), Myodaiji, Okazaki 444-8585, Japan

ARTICLE INFO

Article history:
Received 16 August 2012
Received in revised form 4 September 2012
Accepted 10 September 2012
Available online 18 September 2012

Keywords:
Phospholipase C-δ1
Pleckstrin homology domain
Ligand affinity
Thermal stability
Native PAGE

ABSTRACT

The phospholipase C (PLC)-δ1 pleckstrin homology (PH) domain has a characteristic short α-helix (α2) from residues 82 to 87. The contributions of the α2-helix toward the inositol 1,4,5-trisphosphate (IP_3) binding activity and thermal stability of the PLC-δ1 PH domain were investigated using native polyacrylamide gel electrophoresis (PAGE). Native PAGE analyses of gel migration shift induced by IP_3 binding and of protein aggregation induced by heating indicated that disruption of the α-helical conformation by replacement of Lys86 with proline resulted in reduced affinity for IP_3 and in thermal destabilization of the IP_3-binding state. Although the mutant protein with replacement of Lys86 with alanine showed a slight reduction in thermal stability, the IP_3-binding affinity was similar to that of the wild-type protein. Replacement of Phe87 with alanine, but not with tyrosine, also resulted in reduced affinity for IP_3 and in thermal instability. These results indicated that the helical conformation of the α2-helix and the phenyl ring of Phe87 play important roles in the IP_3-binding activity and thermal stability of the PLC-δ1 PH domain. Based on these results, the biological role of the α2-helix of the PLC-δ1 PH domain is discussed in terms of membrane binding.

© 2012 Elsevier Inc. All rights reserved.

Phospholipase C (PLC)[1] binds to phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cell membrane through the pleckstrin homology (PH) domain and hydrolyzes PIP_2 to produce two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), by the catalytic domain [1–4]. Diacylglycerol activates protein kinase C, and IP_3 releases calcium from internal stores, leading to further signal transduction pathways [5,6]. PH domains consist of approximately 120 residues and are found in many proteins involved in cellular signaling [7]. Although most PH domains bind weakly and nonspecifically to phosphoinositides [7], the PLC-δ1 PH domain stereospecifically recognizes a phosphoinositide ligand with high affinity [8,9]. PLC-δ1 is ubiquitously expressed in most tissues [10,11] and is shuttled between the cytoplasm and the nucleus [12,13]. Although the crystal structure of the full-length PLC-δ1 remains unresolved, the individual structures of its PH domain alone and the near full-length form without the PH domain have been reported [14,15]. The core structure of the PLC-δ1 PH domain has a 7-stranded β-sandwich structure with a C-terminal α-helix, and the β1–β2 and β3–β4 loops mainly form the ligand-binding site (Fig. 1) [14]. This core structure is essentially conserved in all PH domains, although some PH domains have a different ligand-binding site [3,16]. In the PLC-δ1 PH domain complexed with IP_3 [14], the positive side chains of Lys30, Lys32, and Lys57 bind directly to the 4- and 5-phosphate groups of IP_3 (Fig. 1B). In particular, Lys30 and Lys57 bind simultaneously to both phosphate groups. In addition, the 1-phosphate group of IP_3, which connects to a diacylglycerol moiety in PIP_2, directly hydrogen bonds to the side chains of Trp36 and Arg38. Mutations at these residues in the PLC-δ1 PH domain completely abolished or markedly reduced its ligand-binding ability [17,18].

Unlike other PH domains, the PLC-δ1 PH domain has two additional short α-helices at the N terminus and in the β5–β6 loop, and neither of these helices makes direct contact with the ligand (Fig. 1) [14]. Although the roles of these additional α-helices in the protein activity are unclear, solid-state ^{13}C nuclear magnetic resonance (NMR) studies of the PLC-δ1 PH domain indicated that conformational changes involving Ala88 in the β5–β6 loop are induced in the protein bound to PIP_2 embedded in the membrane, suggesting that the short amphipathic α-helix at residues 82 to 87 (α2-helix) in the β5–β6 loop nonspecifically interacts with the hydrophobic layer of the membrane due to the membrane localization of the protein [19]. This may be related to membrane insertion

* Corresponding author. Fax: +81 564 55 7415.
E-mail address: nishimur@ims.ac.jp (K. Nishimura).

[1] *Abbreviations used:* PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology; IP_3, inositol 1,4,5-trisphosphate; NMR, nuclear magnetic resonance; native PAGE, nondenaturing polyacrylamide gel electrophoresis; cDNA, complementary DNA; GST, glutathione *S*-transferase; DTT, dithiothreitol; EC_{50}, 50% effective concentration; T_{agg}, 50% effective aggregation temperature; BSA, bovine serum albumin; ΔT_{agg}, increment of the T_{agg} value.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.09.012

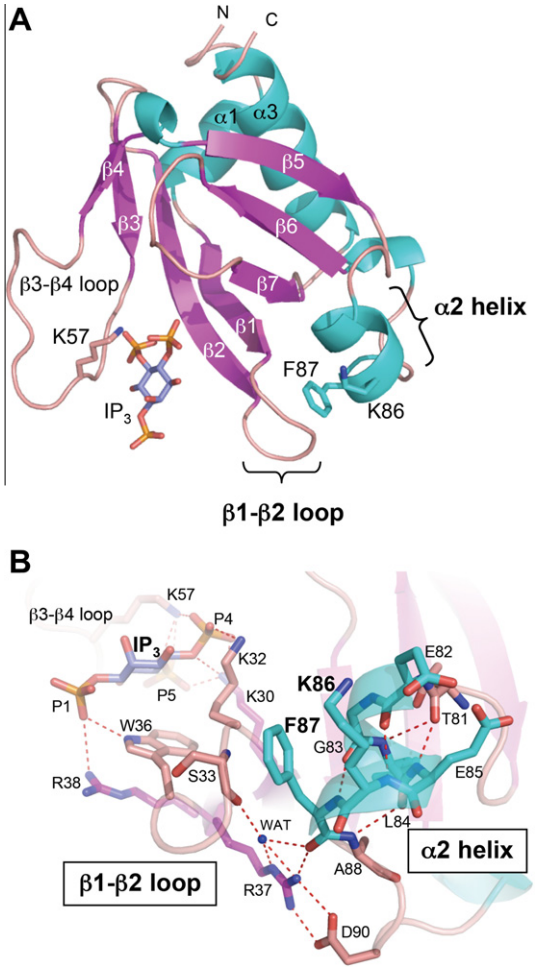

Fig.1. Crystal structure of the rat PLC-δ1 PH domain complexed with IP_3 (PDB ID: 1MAI [14]). (A) IP_3 and the residues mutated in the current study are shown as sticks. (B) Focus around the α2-helix and IP_3-binding site. Important residues in the current study are shown as sticks. Figures were prepared using PyMOL (http://www.pymol.org). Although the primary sequence of the PLC-δ1 PH domain is essentially conserved between rat and human, Pro17, Ser61, Ile91, Gln123, and Arg126 in the rat protein are replaced with Glu, Thr, Val, Leu, and His in the human protein, respectively.

of the protein [20,21]. It was also found that the conformation of the α2-helix in the membrane-binding protein is influenced by the membrane environment [22,23]. Molecular dynamic simulations also suggested conformational changes involving the α2-helix induced by IP_3 or PIP_2 membrane binding [24]. Interestingly, the PLC-δ1 PH domain shows approximately 10-fold higher affinity for isolated IP_3 than for PIP_2 embedded in the membrane [25,26]. Different conformations of the α2-helix may account for the different binding affinities of IP_3 and PIP_2.

In the current study, we investigated the contributions of the α2-helix toward the IP_3-binding activity and thermal stability of the PLC-δ1 PH domain. Because the strong binding of highly negatively charged IP_3 to the protein was expected to form a more negatively charged complex than the ligand-free protein, we used a gel shift assay method on nondenaturing polyacrylamide gel electrophoresis (native PAGE) [27–29] to evaluate the IP_3-binding abilities and thermal stabilities of the wild-type and mutant proteins of the PLC-δ1 PH domain. From the native PAGE analyses, we found that conformational disruption of the α2-helix resulted in reduced affinity for IP_3 and in thermal destabilization of the IP_3-binding state and that the phenyl ring of Phe87 in the α2-helix effectively stabilizes IP_3 binding to the protein. These findings provide the first evidence that the conformation of the α2-helix is strongly coupled with the ligand-binding activity and thermal stability of the PLC-δ1 PH domain.

Materials and methods

Preparation of the PLC-δ1 PH domain and its mutants

The complementary DNA (cDNA) encoding the PLC-δ1 PH domain, corresponding to residues 1 to 142 of human PLC-δ1 (NCBI database accession no. AAA73567), was amplified using a human cDNA library (Clontech) as the template, with a forward primer (5′-CGGGATCCATGGACTCGGGCCGGGACTTC-3′), containing a *Bam*HI site, and a reverse primer (5′-CCGCTCGAGTCACTGTAGCTTCTGACGCTGGTCCATGGAGC-3′), containing an *Xho*I site. The *Bam*HI to *Xho*I fragment containing the PLC-δ1 PH domain gene was cloned into *Bam*HI and *Xho*I sites of the pGEX-6P2 vector (GE Healthcare). The protein expressed by this construct consisted of glutathione *S*-transferase (GST) at the N terminus, an LEVLFQGPLGS sequence containing the PreScission Protease (GE Healthcare) cleavage site, and residues 1 to 142 of human PLC-δ1 at the C terminus. Site-directed mutagenesis was performed using a KOD-Plus Mutagenesis Kit (Toyobo) according to the manufacturer's instructions. The resulting plasmid was transformed into *Escherichia coli* BL21(DE3). The transformed *E. coli* was precultivated in 5 ml of LB medium with 50 μg/ml ampicillin at 37 °C overnight. The preculture was diluted 100-fold with 400 ml of LB medium containing 50 μg/ml ampicillin, and the medium was cultured until the optical density at 600 nm was 0.7 to 1.0. To induce gene expression, 0.4 ml of 1 M isopropyl β-D-thiogalactopyranoside was added to the medium, followed by further incubation for 5 h. The harvested *E. coli* cells were suspended in 50 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl and 1 mM dithiothreitol (DTT) and were disrupted by sonication. The lysate was centrifuged, and the supernatant was loaded onto a column containing 5 ml of COSMOGEL GST-Accept resin (Nacalai Tesque). After washing, the GST tag was cleaved on the column by PreScission Protease overnight at 4 °C. The eluted protein was further purified by size exclusion chromatography using a HiPrep 16/20 Sephacryl S-200 column (GE Healthcare) in Mes buffer containing 10 mM Mes–NaOH (pH 6.5), 20 mM Na_2SO_4, 1 mM DTT, and 0.025% NaN_3. Monomer fractions as estimated from the elution profile of standard proteins (Bio-Rad) were pooled and concentrated to 2.5 to 5 mg/ml using an Amicon Ultra-15 filter (Millipore, 3000 molecular weight cutoff [MWCO]). The purity of the PLC-δ1 PH domain (17.1 kDa), containing an additional GPLGS sequence at the N terminus, was confirmed by SDS–PAGE (see Supplemental Fig. S1 in supplementary material). Protein concentrations were determined by the Bradford method using a Bio-Rad Protein Assay Kit (Bio-Rad). The typical yield of the purified wild-type protein in the current method was approximately 3 to 5 mg/L culture medium.

Native PAGE

The purified PLC-δ1 PH domain was diluted to 1 mg/ml (58 μM) in Mops buffer (50 mM Mops–NaOH [pH 7.4], 300 mM NaCl, and 2 mM DTT). To assess the IP_3-binding activity of the PLC-δ1 PH domain and its mutants, 3 μl of the protein solution was mixed with 3 μl of 0 to 600 μM IP_3 (Sigma) solution. After incubation for more than 30 min on ice, 1.5 μl of sample buffer (5×BPB: 0.4% bromophenol blue, 50% glycerol, and 5 mM Tris–HCl, pH 7.5) was added to the reaction mixture. To analyze the thermal stability of the proteins, the mixture of 2 μl of the protein solution and 2 μl of 0 or 600 μM IP_3 solution was incubated in a 0.2-ml polymerase chain reaction (PCR) tube at 40 to 70 °C for 10 min, and 1 μl of 5×BPB

Download English Version:

https://daneshyari.com/en/article/10533254

Download Persian Version:

https://daneshyari.com/article/10533254

Daneshyari.com