Mutational analysis of a putative polyphosphoinositide binding site in phospholipase C-β_2

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Abstract The phosphatidylinositol 4,5-bisphosphate (PtdIns-P_2)-regulated actin-binding protein gelsolin and most phosphoinositide-specific phospholipases C (PLCs) comprise a basic amino acid motif ((K/R)xxxKxK(K/R); x denotes any amino acid) which was previously suggested to represent a PtdInsP_2-binding site commonly present in these proteins. We have challenged this hypothesis for PLCβ_2 by replacing one or several residues of this motif (KILIKNKK; residues 457–464) and examining the functional consequences of these alterations. The results show that the integrity of the basic motif is important for PtdInsP_2 hydrolysis by PLCβ_2. Replacement of lysines 463 or 461 by arginine led to reduction or complete loss, respectively, of enzyme activity. The results provide further support to the concept that the function of the basic motif within the various PLCs is to bind the enzyme substrate PtdInsP_2.

Key words: Phospholipase C; Polyphosphoinositide; Signal transduction; Mutagenesis; Binding site; *E. coli*

1. Introduction

Phosphoinositide-specific phospholipases C (PLCs) are important elements of several major signal transduction pathways [1]. To date, at least nine distinct mammalian PLCs are known at the cDNA level. Additional forms have been identified in non-mammalian organisms. The various PLC polypeptides share two regions of high homology, which are usually designated domains X and Y and consist of about 170 and 260 amino acids, respectively. Both domains appear to be required for the hydrolysis of the PLC substrate phosphoinositol 4,5-bisphosphate (PtdInsP_2) [2,3]. The mechanisms of this hydrolysis, however, are essentially unknown.

Amino acid sequence comparison of several PtdInsP_2-binding proteins has led to the discovery of two basic amino acid motifs, one of which ((K/R)xxxKxK(K/R); x denotes any amino acid) is commonly present in the actin-binding protein gelsolin and in a large majority of the known PLCs at the carboxyl-terminus of domain X [4]. The demonstration that the latter motif of gelsolin interacts with polyphosphoinositides led to the hypothesis that a similar function might be fulfilled by the corresponding regions of the various PLCs [4]. Interestingly enough, a novel PLC has recently been identified in the brine shrimp *Artemia*, in which domain X is essentially truncated to the basic motif ([5]; cf. Fig. 9 of [6]), indicating that the basic motif might indeed represent the catalytically relevant portion of domain X.

We have previously reported that a synthetic peptide corresponding to amino acids 448–464 of PLCβ_2 and including the basic motif (LPSPEDLRGKILIKNKK) markedly stimulated the activity of this enzyme [7]. More recently, we have demonstrated that this peptide interacts directly and specifically with PtdInsP_2 and that the residues corresponding to the KILIKNKK portion of the peptide are responsible for the stimulatory effect [6]. On the basis of these results, we speculated that the role of the sequence corresponding to the stimulatory peptide within the native PLCβ_2 enzyme may be to bind PtdInsP_2 and to offer the phospholipid to the actual catalytic residues of the enzyme in a configuration more favorable to hydrolysis.

In the present study, we have challenged this hypothesis by replacing either several or single residues within the KILIKNKK motif of a variant of PLCβ_2 and investigating the functional consequences of these alterations. Our results demonstrate that the integrity of the KILIKNKK motif is important and that one of the residues of the motif, lysine-461, is indispensable for PLCβ_2-mediated hydrolysis of PtdInsP_2.

2. Materials and methods

2.1. Expression of PLC$\beta_2\Delta$ in E. coli

The cDNA of PLC$\beta_2\Delta$ [7], a deletion mutant of human PLCβ_2 [8], was cloned into the *Nco*I and *Eco*RI restriction enzyme sites of the bacterial expression plasmid pET-23d(+) [9,10]. PLC$\beta_2\Delta$ differs from the wild-type PLCβ_2 isozyme in two respects. First, it lacks a carboxyl-terminal region (F819–E1166) necessary for stimulation by α_q subunits [11]. Second, it carries a serine-to-alanine replacement in position two due to the introduction of a *Nco*I restriction enzyme site into its cDNA. PLC$\beta_2\Delta$ is indistinguishable from wild-type recombinant PLCβ_2 in terms of its interaction with PtdInsP_2, Ca^{2+}, and G-protein $\beta\gamma$-subunits [12], as well as its ability to be stimulated by a synthetic peptide corresponding the proposed PtdInsP_2 binding site [7]. Transformed BL21(DE3)pLysS bacteria were grown at 37°C in Luria-Bertani (LB) medium containing 60 μg/ml ampicillin and 34 μg/ml chloramphenicol. Expression of recombinant protein was induced in bacterial cultures at an OD_{600} of 0.6–1.0 by addition of 0.4 mM IPTG to the culture medium and continuing the incubation for 2 h at 37°C. Bacteria were harvested from the culture medium (10 ml) by centrifugation at 4,400 × *g* for 10 min at 4°C and washed once by resuspension in buffer A (10 ml) containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 100 μM PMSF, and centrifugation. The bacteria were then homogenized by resuspending the washed bacterial pellets in 1 ml of the same buffer and forcing the suspension several times through a 0.9 × 80 mm needle attached to a disposable syringe, followed by the addition of lysozyme to a final concentration 0.1 mg/ml. This suspension was incubated for 30 min at 4°C and then centrifuged at 30,000 × *g* for 60 min at 4°C. The pellets, which are referred to as particulate fraction, were resuspended to 3–5 mg protein/ml in buffer A. Soluble and particulate fractions were snap-frozen in liquid N_2 and stored at −80°C.

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; PLC, phosphoinositide-specific phospholipase C; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate.

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2.2. Random mutagenesis of the KILIKNKK motif

The cDNAs of PLC$\beta_2\Delta$ variants containing mutations in the KILIKNKK motif were generated using the polymerase chain reaction (PCR) overlap extension method [13]. Two PCR amplifications were performed using the cDNA of PLC$\beta_2\Delta$ as template and the following oligonucleotides as primers: 5′-CAAGAAGCAATTGAGGC-3′ (upstream, sense), 5′-CCTTAAGTCCTCAGGGCTGGGCAGGG-3′ (internal, antisense), 5′-CCCTGCCCAGCCCTGAGGACTTAAGG-GGC(C/A)AG(G/A)-(C/G/T)C(C/G)(C/G/T)C(G/A)(C/G/T)C(C/A)-AG(C/G/A)(C/G/A)C(C/A)AG(C/A)AGAACCAGTTTTCTGGC-3′ (internal, sense), and 5′-GCCTCCCCACCGGTATCC-3′ (downstream, antisense). The two PCR products were purified by agarose gel electrophoresis using the QIAEX extraction kit (Qiagen), reannealed, and reamplified using the upstream and downstream primers. The final PCR products and pET-PLC$\beta_2\Delta$ were digested with *Mun*I and *Age*I and the wild-type fragment was replaced by the corresponding mutant fragments, leading to the formation of a library of PLC$\beta_2\Delta$ variants containing mutations in the KILIKNKK motif (cf. Fig. 2). Expression of the mutant recombinant polypeptides was examined by SDS-PAGE and immunoblotting [14] and was similar for all mutants presented in this communication.

2.3. Site-directed mutagenesis of the KILIKNKK motif

The cDNAs of PLC$\beta_2\Delta$K461R and PLC$\beta_2\Delta$K463R were generated using the methodology described in the preceding section, except that the following internal primers were used: 5′-TCTTCCTGTTCTTGA-TTAAGATCTTGCC-3′ (antisense, K461R) or 5′-TCTTCCTGTTC-CTGATTAAGATCTTGCC-3′ (antisense, K463R) and 5′-AAGATC-TTAATCAGGAACAAGAAGAAC-3′ (sense, K461R) or 5′-AAGA-TCTTAATCAAGAACAGGAAGAAC-3′ (sense, K463R).

2.4. Phospholipase C assay

Phospholipase C activity was assayed using exogenous radiolabelled substrate essentially as previously described [15]. In brief, aliquots of the soluble fraction (20 μg protein) or the particulate fraction (100 μg protein) were incubated for 30 min or 60 min, respectively, at 25°C in a volume of 70 μl containing 28 μM [^{3}H]PtdInsP_2 *(5 Ci/mol), 280* μM phosphatidylethanolamine, 50 mM Tris-maleate, pH 7.4, 80 mM KCl, 10 mM LiCl, 10 mM 2,3-bisphosphoglycerate, 3 mM EGTA, 1 mM free Ca^{2+}, and 1.2 mM or 3.6 mM sodium deoxycholate. Preparation of the lipid substrate and extraction of inositol phosphates was done as previously described [15]. The formation of inositol phosphates was determined by liquid scintillation counting.

2.5. Miscellaneous

All PCR-amplified regions were sequenced as double-stranded DNAs using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). No mutations were found other than those desired for all mutants presented in this communication. SDS-PAGE and immunoblotting were performed as previously described [14]. Antiserum reactive against PLC$\beta_2\Delta$ was prepared as described elsewhere [16]. Protein concentrations were determined according to Bradford [17] using bovine IgG as standard. All experiments were repeated at least twice. Data from representative experiments are shown.

3. Results and discussion

In order to determine which of the structural elements of the KILIKNKK motif of PLCβ_2 are required for enzyme activity, two approaches were taken to alter its amino acid composition: random substitution of several residues and site-directed replacement of single residues within the motif. We chose to express the recombinant PLC polypeptides in *E. coli*, because these cells lack endogeneous PLC activity [3,18] and because a high number of mutant proteins can be readily produced in this system at the same time. Fig. 1 shows that transformation of BL21(DE)pLys(S) bacteria with pET-PLC$\beta_2\Delta$ caused the expression of an appropriately sized protein immunoreactive with the PLCβ_2-specific antiserum. Expression of PLC$\beta_2\Delta$ was markedly enhanced by inducing the synthesis of T7 RNA polymerase with IPTG. The recombinant protein was predominantly present in the particulate fraction of both control and IPTG-treated cells and amounted to up to 30% of total protein

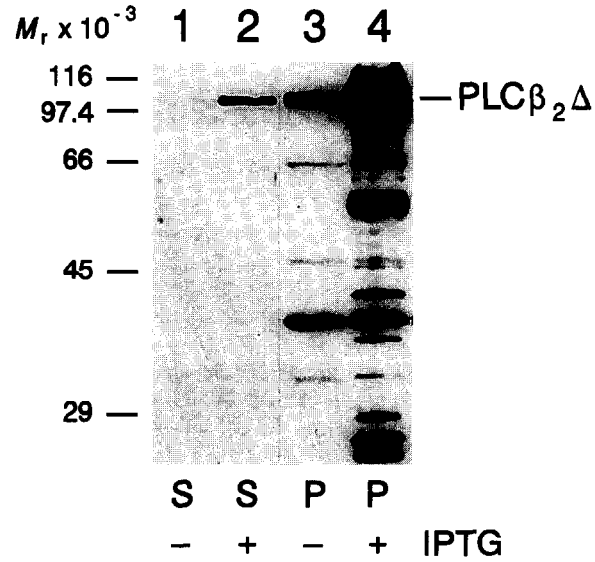

Fig. 1. Subcellular distribution of recombinant PLC$\beta_2\Delta$ in *E. coli*. BL21(DE)pLys(S) bacteria were transformed with pET-PLC$\beta_2\Delta$ and grown as indicated without (lanes 1 and 3) or with (lanes 2 and 4) induction of recombinant protein synthesis by IPTG as described in section 2. The bacteria were then lysed and fractionated into soluble (S; lanes 1 and 2) and particulate (P; lanes 3 and 4) fractions. Samples were subjected to SDS-PAGE (100 μg protein/lane) and immunoblotting was performed using antibodies reactive against PLCβ_2. Immunoreactive proteins were visualized by chemiluminescence. The positions of full-length PLC$\beta_2\Delta$ and of the molecular weight standards are indicated.

Table 1
Structure–function relationships of the putative polyphosphoinositide-binding motif of PLC$\beta_2\Delta$

Amino acid sequence	Inositol phosphate formation (pmol · min^{-1} · mg^{-1} protein^{-1})
Wild-type	
KILIKNKK	285.4 ± 24.1
Mutants	
KILVKDKK	21.7 ± 5.2
QALVKRKK	0
KAGGKAKQ	0
KVVVQSKQ	0
KVLGQRKQ	0
KVRGQTKQ	0
QIGGKGKQ	0
KSGVQHQK	0
KGGGQAQK	0
QIVIKRQQ	0
KSGVQNQQ	0
KAVAQDQQ	0
KSAAQPQQ	0

BL21(DE)pLys(S) bacteria were transformed with the expression vector pET-23d(+) containing the cDNA of either wild-type PLC$\beta_2\Delta$ or of one of the mutants listed, and grown as described in section 2. In all cases, production of recombinant protein was induced by adding 0.4 mM IPTG to the culture medium as specified in section 2. The bacteria were then lysed and fractionated into soluble and particulate fractions. Particulate fractions were assayed for phospholipase C activity in the presence of 3.6 mM as described in section 2. The values correspond to the means ± S.D. of triplicate determinations. 0 = no inotitol phosphate formation detected.

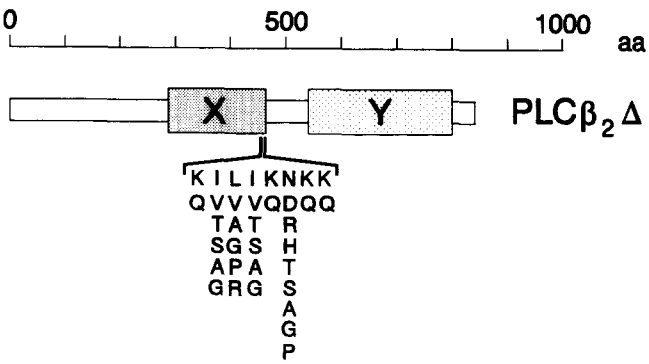

Fig. 2. Random mutagenesis of the putative polyphosphoinositide-binding motif of PLC$\beta_2\Delta$. cDNAs encoding variants of PLC$\beta_2\Delta$ carrying mutations within the the putative polyphosphoinositde-binding motif KILIKNKK were constructed as described in section 2. A linear representation of PLC$\beta_2\Delta$ specifying the positions of the two regions of high homology (X,Y) present in all phosphoinositide-specific phospholipases C and of the putative polyphosphoinositide-binding motif is presented. All amino acid permutations which could conceivably be generated by the random mutagenesis procedure are shown below the sequence of the motif. The amino acid numbering (aa) is indicated.

in the particulate fraction of the latter cells as judged by SDS-polyacrylamide gel electrophoresis (results not shown). It is likely that the additional lower molecular weight immunoreactive proteins present in the particulate fractions (Fig. 1) correspond to proteolytic degradation products of full-length recombinant PLC$\beta_2\Delta$. Soluble extracts of IPTG-induced bacteria were used as a source of PLC$\beta_2\Delta$ in most of the experiments shown here. Extracts of bacteria transformed with vector DNA without insert displayed no PLCβ_2 immunoreactivity and no PLC activity (cf. Figs. 3 and 4).

A library of mutants exhibiting amino acid variations within the KILIKNKK motif was constructed by the polymerase chain reaction using a degenerate primer which allowed for the amino acid substitutions shown in Fig. 2. Our intention was to replace the basic lysine residues by similarly sized, but non-charged glutamine residues, and to decrease the hydrophobicity of the isoleucine and leucine residues by decreasing the length of their side chains. In addition, we wished to replace the polar asparagine residue of the motif by various charged residues as well as other polar and non-polar residues of smaller size. Immunoblot analysis showed that the levels of expression were similar for wild-type PLC$\beta_2\Delta$ and the various mutant proteins discussed here (results not shown). Table 1 shows that most of the mutant proteins were inactive, which is in agreement with the notion that the KILIKNKK motif is relevant to the catalytic activity of PLCβ_2. Interestingly, some activity could be detected for the mutant KILVKDKK, which differs from the wild-type protein in two residues only (I460V, N462D). Since a valine residue is present at the corresponding position in several other PLC isoforms, e.g. rat and bovine PLCβ_1, rat and human PLCβ_3 (cf. Fig. 8 of [6]) it is more likely that the aspartic acid residue causes the observed decrease in activity by disturbing the overall positive charge of the basic motif. In the mutant QALVKRKK, the N-terminal lysine of the motif was replaced by glutamine, and the asparagine by arginine. Although these two alterations should not lead to a decrease in the overall positive charge of the motif, the corresponding mutant protein was inactive. In the mutant KVGSKTKK, which is not mentioned in Table 1 because of having an accidental serine for glycine substitution in position 456, i.e. immediately upstream of the amino-terminus of the basic motif, all four lysine residues were present and no additional negatively charged residues appeared. Nevertheless, this mutant protein was also inactive. The data obtained with the two latter mutants (QALVKRKK and KVGSKTKK) indicate that it is not merely the charge of the KILIKNKK motif, which is required for enzyme activity. Other properties of the motif, such as hydrophobicity and/or correct spacing of the basic residues appear to be important as well.

Of the four lysines present within the KILIKNKK motif, lysine-461 is unique since it is absolutely conserved in all known phospholipases C (cf. Fig. 8 of [6]). To examine the functional significance of this lysine residue, site-directed mutagenesis was used to generate the mutant PLC$\beta_2\Delta$K461R. An additional mutant, PLC$\beta_2\Delta$K463R, was contructed and analyzed for comparison. Lysine-463 is conserved in most, but not all known phospholipases C [6]. The two mutant polypeptides were produced in *E. coli* (Fig. 3) and their ability to catalyse the formation of inositol phosphates was determined (Fig. 4). It is apparent that replacement of lysine-463 by arginine led to a considerable (approximately 80%) reduction of enzyme activity. Most interestingly, a complete loss of activity was observed for the mutant PLC$\beta_2\Delta$K461R (Fig. 4). Additional experiments (results not shown) revealed that both the soluble and the particulate mutant protein were inactive even when tested at 3.6 mM deoxycholate, a condition which markedly enhances the activity of the wild-type enzyme. The fact that replacement of lysine-461 by a similarly charged arginine residue inactivates the enzyme strongly suggests that this residue plays an important functional role during catalysis. Of interest, although both lysine and arginine residues are frequently present at active sites,

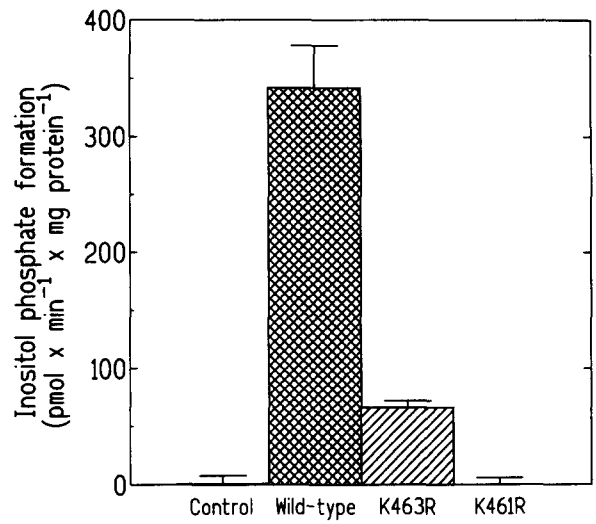

Fig. 3. Expression of soluble recombinant PLC$\beta_2\Delta$, PLC$\beta_2\Delta$K463R, or PLC$\beta_2\Delta$K461R in *E. coli*. BL21(DE)pLys(S) bacteria were transformed with the expression vector pET-23d(+) without insert (Control; lane 1), pET-PLC$\beta_2\Delta$ (Wild-type; lane 2), pET-PLC$\beta_2\Delta$K463R (K463R; lane 3), or pET-PLC$\beta_2\Delta$K461R (K461R; lane 4), and grown as described in section 2. Soluble fractions of transformed bacteria were produced as described in the legend of Table 1 and were subjected to SDS-PAGE (100 μg protein/lane) and immunoblotting. Only the $\approx$100 kDa region of the autoluminograph is shown.

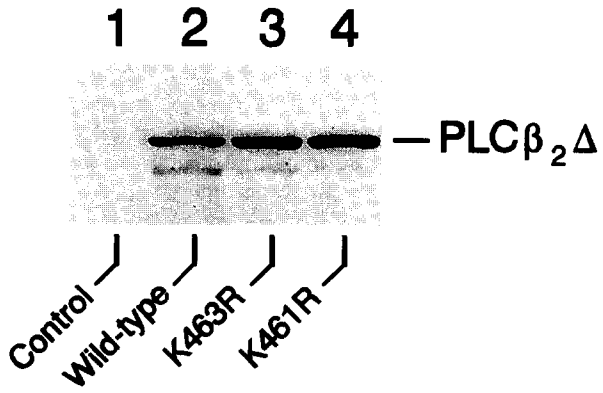

Fig. 4. Inositol phosphate formation by soluble fractions of *E. coli* expressing PLC$\beta_2\Delta$, PLC$\beta_2\Delta$K463R or PLC$\beta_2\Delta$K461R. Soluble fractions of BL21(DE)pLys(S) bacteria transformed with the expression vector pET-23d(+) without insert (Control), pET-PLC$\beta_2\Delta$ (Wild-type), pET-PLC$\beta_2\Delta$K463R (K463R), or pET-PLC$\beta_2\Delta$K461R (K461R) were produced as described in the legend of Table 1 and then assayed for phospholipase C activity in the presence of 1.2 mM sodium deoxycholate as described in section 2.

lysine residues appear to be more often involved in substrate binding, arginine residues more often in the actual catalytic process [19]. In this context, the findings reported here provide further strong support to our previous suggestion that the role of the KILKNKK motif within the native PLCβ_2 enzyme is to bind PtdInsP_2 and to offer the phospholipid to the actual catalytic residues in a configuration more favorable to hydrolysis [6]. It is tempting to speculate on the basis of the results shown here that lysine-461 interacts with one or more of the phosphate groups of PtdInsP_2, possibly in cooperation with one or more of the other positively charged residues present in the motif. The conserved aliphatic residues, on the other hand, are good candidates to mediate the hydrophobic interactions with the hydrocarbon chains of PtdInsP_2.

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