Mutations within a highly conserved sequence present in the X region of phosphoinositide-specific phospholipase C-δ_1

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Phosphoinositide-specific phospholipase C (PI-PLC) enzymes have considerable structural similarity within limited regions (X and Y) implicated in catalysis. The role of residues contained within a highly conserved sequence present in the X region was investigated by site-directed mutagenesis of PLC-δ_1 isoenzyme. Seven residues (Ser-308, Ser-309, Ser-310, His-311, Thr-313, Tyr-314, and Gln-319) were individually replaced by alanine or glutamine (His-311). Replacement of two residues, His-311 and Tyr-314, resulted in a dramatic reduction of enzyme activity. The $k_{cat.}$ of hydrolysis of phosphatidylinositol 4,5-bisphosphate by H311A and Y314A mutants was reduced 1000- and 10-fold respectively, with little effect on K_m. Further analysis of H311A and Y314A mutants, using limited proteolysis and circular dichroism, had shown that no major structural alterations had occurred. Since site-directed mutagenesis demonstrated the importance of histidine residues, their role in enzyme function was also analysed by chemical modification with diethyl pyrocarbonate. This modification of histidine residues resulted in the reduction of enzyme activity and also indicated that more than one residue could be important.

INTRODUCTION

Phosphoinositide-specific phospholipase C (PI-PLC), responsible for generation of diacylglycerol and inositol 1,4,5-trisphosphate, provides a link between a wide variety of extracellular signals and specific targets for these second messengers [1]. To fulfil this requirement and communicate with different upstream signalling components, the enzyme evolved into a superfamily of related proteins [2,3]. The three main families have been designated as PLC-β, PLC-γ and PLC-δ. Structural similarity within the superfamily is mainly restricted to the catalytic domain, while each family has characteristic features implicated in distinct types of regulation. Thus, regulation of members of the PLC-β family involves GTP-binding proteins coupled to receptors described as seven-membrane-spanning receptors; PLC-γ enzymes are generally activated by receptors that are tyrosine kinases or through interactions with non-receptor tyrosine kinases. Regulation of the PLC-δ family has yet to be clarified but seems to be different from that of PLC-β and -γ enzymes.

The amino acid sequence has been determined for more than 20 PI-PLC molecules from widely divergent species and from a variety of mammalian tissues [2–7]. Significant similarity is apparent in two separate regions designated as the X region (about 170-amino-acid residues) and Y region (about 260-amino-acid residues). Extending the previous comparison between different sequences [3] to recently described members of the superfamily [4–7], it can be calculated that about 40% and 25% of amino acid residues are invariant or conservatively substituted within the X and Y regions respectively. Analysis of the conserved regions in PLC-γ_1 [8], -γ_2[9] and -δ_1[10] by deletion mapping have shown that both X and Y regions are required for the formation of the catalytically active protein. Furthermore, experiments with PLC-δ_1 subjected to limited proteolysis by trypsin demonstrated that the two regions interact to form a stable active complex; the derived catalytic core consisted of two polypeptides, one containing the X and the other the Y region of PLC-δ_1 [10,11]. Further insight into the precise function of each region would require identification of residues involved in substrate binding and hydrolysis. The experimental approaches usually applied in these studies include screening by site-directed mutagenesis, chemical modification of specific residues and determination of the three-dimensional structures of the enzyme and the enzyme–substrate complexes. None of these studies has been carried out with members of the PI-PLC superfamily.

In this study we analysed the effect of the point-mutations and chemical modification on the enzyme activity of PLC-δ_1. This enzyme can be produced in relatively large quantities in a bacterial expression system [10]. In the absence of other information, a comparison of amino acid sequences available for different members of the PI-PLC superfamily can be used to select regions for mutagenesis experiments. Although a large number of residues appear to be conserved, they are not evenly distributed throughout the X and Y regions; several sequences contain mainly invariant residues. In addition, the only point-mutation described so far and found to reduce the activity of PLC-γ_1, was introduced in one of the conserved amino acid stretches within the X region [12]. The mutations described in this report have been introduced into a corresponding region of PLC-δ_1. Two of these mutations, as well as chemical modification of histidine residues, had a profound effect on PLC-δ_1 activity.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer (model 394). The M13mp18RF vector was

Abbreviations used: PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; GST, glutathione S-transferase; HEDTA, *N*-hydroxyethyl-ethylenediamine-triacetic acid; DEP, diethyl pyrocarbonate; CD, circular dichroism.

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from Boehringer Mannheim and pGEX-2T vector from Pharmacia. The oligonucleotide-directed *in vitro* mutagenesis system version 2.1 was from Amersham and the sequencing kit (*Taq* Dye Deoxy Terminator Cycle) was from Applied Biosystems. DNA purification systems, Magic mini preps and Magic M13DNA purification system were obtained from Promega.

Phosphatidyl[^{3}H]inositol ([^{3}H]PI) and phosphatidyl[^{3}H]-inositol 4,5-bisphosphate ([^{3}H]PIP_2) were obtained from New England Nuclear. Sodium salts of soybean PI and bovine brain PIP_2 were purchased from Sigma Chemical Co. Glutathione–Sepharose 4B and Mono Q column (PC 1.6/5) were from Pharmacia. Trypsin (sequencing grade) was from Promega and human thrombin from Sigma Chemical Co. Diethyl pyrocarbonate (DEP) was obtained from Aldrich Chemical Co. Imidazole was from Sigma Chemical Co.

Plasmid construction and site-directed mutagenesis

The 1.5 kb *Bam*H1/*Kpn*1 fragment from rat brain PLC-δ_1 cDNA [10] was subcloned into an M13mp18RF vector. Subsequently, mutations were introduced using an oligonucleotide-directed *in vitro* mutagenesis system. The following oligonucleotides were used for mutagenesis: T/G 923 (S308A), 5′-TACTTAGTG*G*CTTCTTCC-3′; T/G 926 (S309A), 5′- TTAGTGTCT*G*CTTCCCAC-3′; T/G 929 (S310A), 5′- GTGTCTTCT*G*CCCACAACA-3′; C/G 934 (H311Q), 5′- CTTCTTCCCA*G*AACACCTA-3′; C/G 932 and A/C 933 (H311A), 5′-GTGTCTTCTTC*CG*CCAACACCTACCTGC-3′; A/G 398 (T313A), 5′-TCCCACAAC*G*CCTACCTGC-3′; T/G 941 and A/C 942 (Y314A), 5′-TCCCACAACACC*GC*CCTGCTGGAAGAC-3′; C/G 956 and A/C 957 (Q319A), 5′-CTGCTGGAAGAC*GC*GCTCACAGGGCCC-3′. The altered nucleotides are italicized. Mutants were verified by sequencing single-stranded DNA prepared from isolated phage, on an Applied Biosystems 373A automated sequencer using *Taq* Dye Deoxy Terminator Cycle sequencing chemistry. Inserts recloned into plasmids encoding mutants with severely reduced activity were completely sequenced to ensure only desired base changes were present. The 1.5 kb insert was sequenced using four custom made primers and -21M13 Forward primer [ABI]: Base 898–916, 5′-CAGCCACTGAGTCACTAC-3′; Base 1261–1279, 5′-GTTACCACGAGCCTGCCT-3′; Base 1621–1639, 5′-CGTCATAACGTGAGCTGT-3′; Base 2014–2032, 5′-CAGACAGCAGTTATTACC-3′; -21M13Forward, 5′-TGTAAAACGACGGCCAGT -3′.

The *Acc*1/*Kpn*1 digestion of the M13mp18RF constructs, containing different PLC-$\delta_1$mutations, yielded 1.3 kb fragments; these were subcloned into plasmid, also digested with *Acc*1/*Kpn*1, encoding the control enzyme as a glutathione S-transferase (GST)-fusion protein. Construction of the control plasmid (construct 3) has been described previously [10].

Expression and purification of recombinant proteins

Expression and purification of recombinant PLC-δ_1 protein lacking the first 58 amino acid residues has been described previously [10]. The same procedure was used to obtain mutant enzymes. Briefly, GST-fusion protein was isolated from the *Escherichia coli* extract by affinity chromatography on glutathione–Sepharose. The PLC-δ_1 protein was separated from GST and removed from the affinity matrix by thrombin cleavage. Soluble PLC-δ_1 was further purified from minor contaminants by chromatography on a Mono Q column (PC 1.6/5) using a SMART system (Pharmacia). The concentration of the protein in peak fractions was 2–5 mg/ml; aliquots of these fractions were stored at −80 °C for several months without loss of enzyme activity.

Assay of PLC activity

Assay of hydrolysis of PIP_2 was essentially the same as described previously [13,14]. The reaction mixture (50 μl) contained 20 mM Tris/maleate, pH 7.0, 0.4 mg/ml BSA, 5 mM 2-mercaptoethanol, 100 mM NaCl, 0.5% sodium cholate, Ca/HEDTA buffer for the final concentration of free Ca^{2+} of 10 μM and 220 μM PIP_2 (0.025 μCi). The same conditions were used for hydrolysis of PI, except that the calcium buffer was replaced by 1 mM $CaCl_2$. Incubation was at 37 °C for 10 min.

For kinetic analysis, concentrations of PIP_2 were as described in Figure 5 and incubations were carried out for 0, 2.5, 5, 10 and 20 min. The calcium-dependence of PIP_2 and PI hydrolysis was analysed as described before [13].

Circular dichroism (CD) spectral measurements

CD spectra were recorded with a Jasco J-720 spectropolarimeter fitted with a thermostatically controlled cell holder and interfaced with a Neslab RTE-110 water-bath. Measurements were performed at 25 °C using a scan speed of 20 nm/min with a 1 nm slit and 8 s response time, averaging six scans. The sample concentration was 0.4 mg/ml and the cuvette path length was 0.5 mm.

Chemical modification with DEP

The concentration of commercial DEP was determined from the increase in absorbance at 240 nm when an aliquot of the DEP solution was added to a solution of 10 mM imidazole in 100 mM potassium phosphate buffer, pH 6.5, using an absorbance coefficient of 3200 $M^{-1} \cdot cm^{-1}$ [15]. Before each experiment, the reagent was freshly diluted in ice-cold ethanol. Modification was carried out by incubating PLC-δ_1 with DEP in 100 mM potassium phosphate buffer, pH 6.5, at 25 °C. The final concentration of ethanol in the reaction mixture (75 μl) never exceeded 4% (v/v). The difference absorbance spectra of DEP-modified versus native PLC-δ_1 was obtained with a Beckman DU-650 spectrophotometer using a micro-cuvette, path length 10 mm, volume 75 μl.

Other methods

Electrophoresis in polyacrylamide gels in the presence of SDS was performed according to Laemmli [16]. Determination of protein was according to Bradford [17] with BSA as a standard.

RESULTS

Expression and enzyme activity of PLC-δ_1 mutants

Within the sequence of 17 amino acid residues, 304–320 in PLC-δ_1, nine residues are invariant and one is conservatively substituted within the PI-PLC superfamily (Figure 1). To test the role of individual residues within this sequence in the formation of an active enzyme, mutations were introduced to replace selected residues with an alanine residue. In addition, His-311 was also mutated into an glutamine residue since the corresponding mutation in PLC-γ_1 has been reported to influence enzyme activity [12]. Among the selected residues, Ser-309, Ser-310, His-311, Thr-313, Tyr-314 and Gln-319 are conserved while the position corresponding to Ser-308, although not conserved, is usually occupied by serine or asparagine in the PI-PLC superfamily. All mutations were introduced into a PLC-δ_1 with a deletion of 58 amino acid residues from the N-terminus rather than into a full-length protein. Since this deletion mutant retains

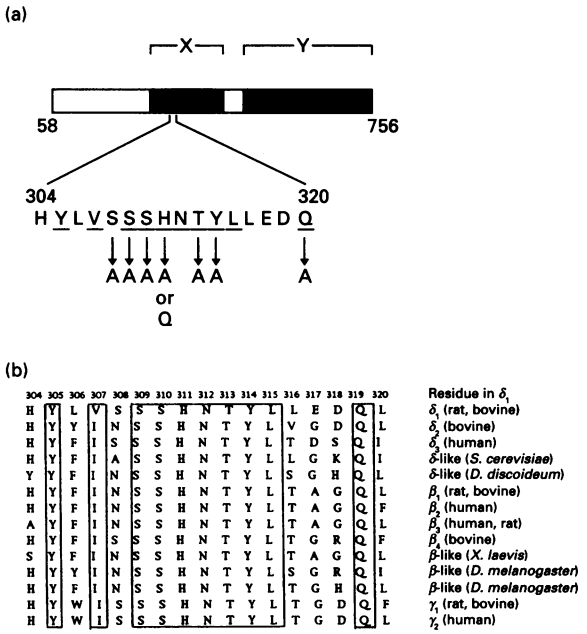

Figure 1 (a) Schematic representation of the structure of PLC-δ_1 and mutations that were analysed and (b) alignment of the selected sequence in PLC-δ_1 with corresponding regions of other PI-PLC enzymes

(**a**) The locations of two conserved regions (X and Y) in PLC-δ_1 protein (residues 58–756) are denoted by black boxes and the sequence selected for mutational analysis (residues 304–320) shown. The arrows indicate individual replacements of the specified residues by alanine or glutamine. Residues conserved between different sequences listed below (in **b**) are underlined. (**b**) This alignment includes sequences compared previously [2,3] and the sequences obtained more recently for *S. cerevisiae* [4], *X. laevis* [7], *D. discoideum* [5] and mammalian PLC-β_4 [6]. Applying the criteria for conservation described earlier [3], the comparison shows that residues Tyr-305, Ser-309, Ser-310, His-311, Asn-312, Thr-313, Tyr-314, Leu-315 and Gln-319 are invariant and Val-307 conservatively substituted in PLC-δ_1 (boxed). Some other positions are selectively occupied. Position corresponding to residue 304 in PLC-δ_1 is usually histidine, 306 is an aromatic residue, 308 serine or asparagine and 320 leucine, isoleucine, or phenylalanine. A notable exception from the sequences considered above is a putative PI-PLC from *Artemia* lacking most of the X region, including the selected sequence [27].

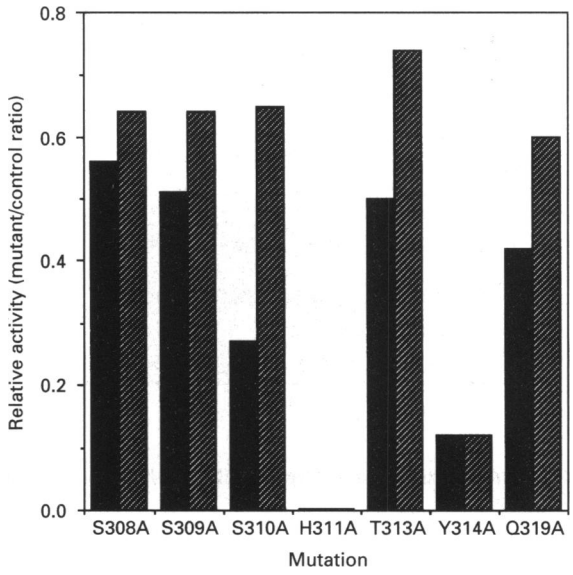

Figure 2 Hydrolysis of PI and PIP_2 by mutant proteins of PLC-δ_1

Preparations of purified proteins of different mutants were analysed under conditions optimized for the hydrolysis of PI (black bars) and PIP_2 (hatched bars) by the control (or wild type) PLC-δ_1 (see the Materials and methods section). Two (S308A, S309A, T313A and Q319A) or three (S310A, H311A, Y314A) preparations were tested for each mutant. The activities are expressed as mean values; the values are within $\pm 25\%$.

full enzyme activity in the detergent–lipid mixed micelle assay used in this study, it is referred to as the control PLC-δ_1.

Control and mutated PLC-δ_1 were expressed as GST-fusion proteins, separated from GST by thrombin cleavage and purified to apparent homogeneity on a Mono Q column (see the Materials and methods section and Figure 3). The yields of pure proteins obtained for all mutants were similar to that of the control PLC-δ_1 (0.5–1 mg from 1 litre of bacterial culture).

The enzyme activity of the purified proteins was analysed in the presence of 0.5% sodium cholate, using PI and PIP_2 as substrates. In order to achieve maximal hydrolysis, the concentrations of free calcium were 1 mM for PI and 10 μM for PIP_2 hydrolysis. Under these conditions, the specific activity of the control protein was about 1000 μmol/min per mg with PIP_2 and 150 μmol/min per mg for PI as a substrate. The ratio of hydrolysis of the two substrates (7:1) and the values calculated for specific activities are within the range previously determined for mammalian PLC-δ enzymes [18–20] or recombinant PLC-δ_1 protein expressed in bacteria [21]. A comparison of the enzyme activities of different mutants with that of the control protein is presented in Figure 2. Mutations of conserved residues at positions 309, 310, 313, 319, as well as non-conserved 308, had little effect on enzyme activity; a reduction less than 3-fold was observed. Mutations of residues at positions 314 and especially 311, had a greater effect on enzyme activity resulting in 10-fold and 500–1000-fold reduction of the activity respectively. Replacement of the histidine at position 311 by glutamine had a similar effect to the replacement by alanine (results not shown).

The reduction of enzyme activity determined with two different substrates was generally similar (Figure 2). Some selectivity was observed for the S310A mutant where reduction of activity towards PI was about 3-fold and towards PIP_2 less than 2-fold. In the case of H311A, the reduction was more pronounced with PIP_2 as a substrate (about 1000-fold) than with PI (up to 500-fold).

One general feature for all members of the PI-PLC superfamily, including an evolutionary distant enzyme from *Saccharomyces cerevisiae* [4], is calcium dependence for substrate hydrolysis *in vitro*. To test the effect of mutations on a requirement for calcium, the purified proteins were tested for PI and PIP_2 hydrolysis over a range of free calcium concentrations (10^{-8} to 10^{-3} M). This analysis did not reveal any significant differences between control and mutant PLC-$\delta_1$proteins.

The effect of pH on PIP_2 hydrolysis by several phospholipases of the PI-PLC superfamily has been described [18–21]. It has been found that the pH profile varies for the different enzymes when compared directly [18]. Due to the complexity of the factors that might be involved no 'educated guess' was made concerning the identity of residues involved in catalysis. In this study pH profiles of the control protein and H311A mutant have been compared; significant differences have not been detected (results not shown).

Analysis of H311A and Y314A mutants of PLC-δ_1 for changes in protein structure

The reduction of enzyme activity of H311A and Y314A mutants could be due to a requirement of these residues for correct protein folding and stability, or they could be more specifically

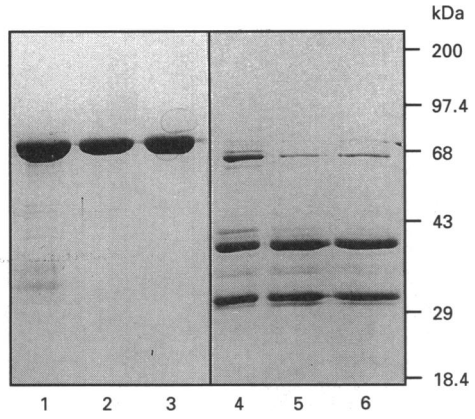

Figure 3 Limited proteolysis by trypsin of the control PLC-δ_1 and H311A and Y314A mutants

Samples of the purified proteins before (lanes 1–3) and after (lanes 4–6) limited proteolysis by trypsin were analysed by SDS/PAGE. Incubation with trypsin was carried out for 30 min at 37 °C; 1–2 μg of the protein was incubated with 0.016 μg of trypsin. Lanes 1 and 4 contain control protein, lanes 2 and 5 H311A mutant, and lanes 3 and 6 Y314A mutant. The positions of molecular-mass markers (kDa) are indicated on the right-hand side.

involved in catalysis. To analyse the first possibility, we used two different methods that could detect conformational changes of proteins: limited proteolysis by trypsin and CD spectral measurements.

When control PLC-δ_1 was subjected to limited proteolysis by trypsin two fragments of 40 and 30 kDa were generated (Figure 3, lanes 1 and 4). In our previous study [10] we have shown that the 40 kDa fragment contained the X region (amino acid residues 139–475) while the 30 kDa fragment contained the Y region (from amino acid residue 475 to the C-terminus) of PLC-δ_1. These two fragments form a stable complex and only after prolonged incubation with trypsin do they become further fragmented into short polypeptides. Limited proteolysis of H311A and Y314A mutants produced the same distinct pattern (40 and 30 kDa polypeptides) as observed for the control (Figure 3, lanes 2, 3, 5 and 6). These results demonstrated that the overall structure of these mutants was not drastically altered.

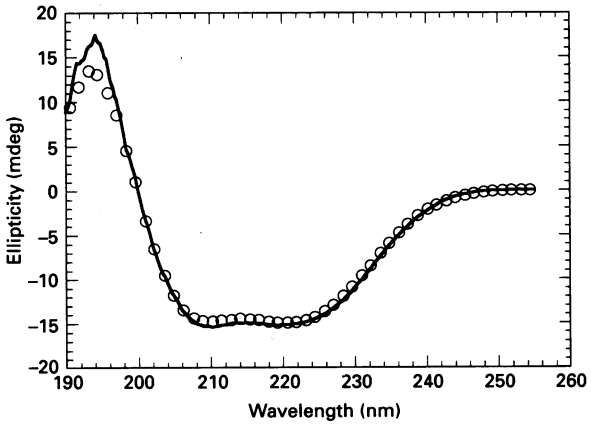

Figure 4 Circular dichroism spectra

Samples of the control (—) and H311A (○) protein were subjected to CD measurements as described in the Materials and methods section.

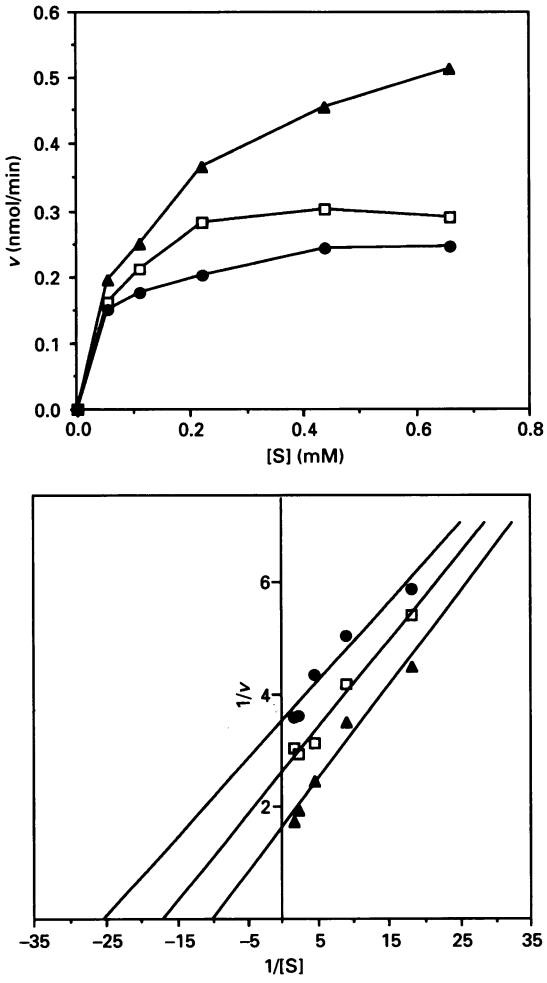

Figure 5 Kinetic analysis of H311A and Y314A mutants

The concentration of the control protein and H311A and Y314A mutants was adjusted so that under standard conditions for PIP_2 hydrolysis (see the Materials and methods section) analysed samples had comparable activities. Initial velocities were measured over the range of PIP_2 concentrations (upper panel) and presented as the double-reciprocal Lineweaver–Burk plot (lower panel). ▲, Control; ●, H311A; □, Y314A.

In order to further confirm structural integrity of the mutant with the greatest reduction of activity (H311A), it was analysed using CD (Figure 4). PLC-δ_1, or any other member of the superfamily, has not previously been analysed by this method. The CD spectrum is typical for a folded protein with dominant CD of the α-helix. Spectra of control PLC-δ_1 and H311A mutant, measured over the range 190–260 nm, were effectively superimposable.

Kinetic analysis of H311A and Y314A mutants of PLC-δ_1

In order to determine the apparent K_m for the control and mutant proteins, initial velocity was measured over the range (55–660 μM) of PIP_2 concentrations (Figure 5). Previous measurements of apparent K_m for PLC-δ purified from mam-

Table 1 Kinetic parameters for the control PLC-δ_1 and H311A and Y314A mutants

Hydrolysis of PIP_2 by the purified proteins was analysed as described in the legend of Figure 5. Results are shown as means of two sets of measurements. Values are within ±10% for K_m and ±20% for $V_{max.}$.

Enzyme	K_m (μM)	$V_{max.}$ (μmol·min^{-1}·mg^{-1})	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Control	102	1350	1800	1.7×10^7
H311A	39	1.2	1.6	4.1×10^4
Y314A	59	134	179	3.0×10^6

malian sources gave values within the range of 30–300 μM [18–20]; the K_m for bacterially expressed full-length protein [21] was within this range (170 μM). Some of these variations are likely to reflect differences in assay conditions, particularly type and concentrations of detergent which affected the true micellar concentration of the substrate. Under the conditions used in this study (0.5% sodium cholate) the double-reciprocal plot was linear over the selected range of PIP_2 concentrations. The apparent K_m for the control protein was estimated to be 102 μM. The mutant proteins approached saturation at lower concentrations of PIP_2 (Figure 5) and K_m values of 39 and 59 μM were calculated for H311A and Y314A mutants respectively (Table 1). Thus, both mutations have a similar effect on the apparent K_m, resulting in slightly decreased values.

Kinetic parameters summarizing the effects of the mutations on hydrolysis of PIP_2 are presented in Table 1. While differences in K_m values were no greater than 2.6-fold, the turnover number ($k_{cat.}$) of the H311A mutant was reduced from 1800 to 1.6 s^{-1}; a smaller decrease of 10-fold was calculated for the Y314A mutant. Consequently, differences in $k_{cat.}/K_m$ values mirrored those of $k_{cat.}$.

The assay system used in this study simplifies, to at least some degree, interactions that are taking place in the more physiological situation (further described in the Discussion). Due to the absence of detailed kinetic studies of PI-PLC enzymes, there is no information concerning the existence of intermediate complexes that could be formed under these conditions and what could be the relative magnitudes of the various rate and equilibrium constants. Thus, the physical significance of K_m and $k_{cat.}$ cannot be stated with certainty. However, if it is assumed that a simple kinetic mechanism is obeyed, the data presented here would suggest that dissociation of the substrate (or other enzyme-bound species) has not been affected by H311A and Y314A mutations. Similarly, if the $k_{cat.}$ could be assigned to the process of product formation this would be the function that is most affected by the mutations.

Chemical modification of PLC-δ_1 with DEP

Site-directed mutagenesis in the selected region of PLC-δ_1 demonstrated the importance of the His-311 residue for the enzyme activity. As a complementary approach to mutagenesis, chemical modification of specific residues can be used to assess their role in enzyme function. DEP is commonly used for modifying histidine in proteins. Although DEP can react with functional groups of several amino acids, the usual group modified in neutral or slightly acidic media is histidine. Furthermore, the modification of histidine with DEP can be confirmed by difference spectroscopy between 230 and 250 nm, which directly monitors the carbethoxylation of histidine residues [22]. The spectral

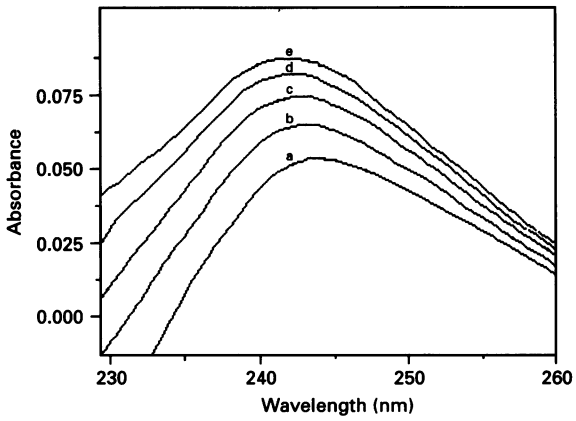

Figure 6 The UV difference absorption spectrum of native versus DEP-modified PLC-δ_1

PLC-$\delta_1$control protein (0.2 mg/ml in 100 mM potassium phosphate, pH 6.5) was placed in two cuvettes; ethanol was added into the reference cuvette instead of DEP/ethanol solution. The incubation was with 0.2 mM DEP and the difference spectrum monitored after 5 min (a), 15 min (b), 25 min (c), 35 min (d) and 45 min (e).

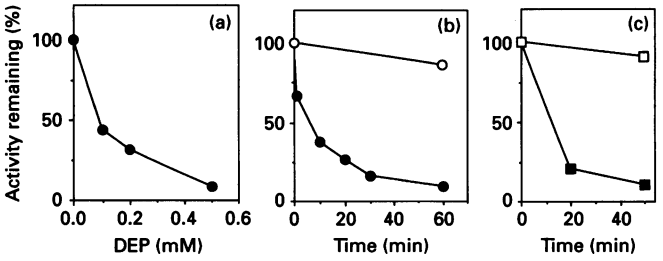

Figure 7 Hydrolysis of PIP_2 by DEP-modified PLC-δ_1 proteins

Samples of PLC-δ_1 control protein (○,●) and H311A mutant (□,■) were analysed. Incubation of the control protein with different concentrations of DEP (**a**) was for 30 min. For the time course experiment (**b**) the concentration of DEP was 0.2 mM (●); the protein solution was also incubated without DEP for 60 min (○). H311A mutant protein (**c**) was incubated with 0.2 mM DEP (■) for 20 and 50 min or without DEP (□) for 50 min. After incubation, an aliquot of each sample was diluted and assayed to determine remaining PLC activity using PIP_2 as a substrate.

changes of PLC-δ_1 after incubation with 0.2 mM DEP at pH 6.5 for different periods of time are presented in Figure 6; the absorbance between 230 and 250 nm markedly increased in a time-dependent manner. Similar spectral changes were found afer incubation of PLC-$\delta_1$with different concentrations of DEP (results not shown). Modification of some other residues in addition to histidine cannot be completely ruled out; however, the spectral data could be used to demonstrate modification of tyrosine known to decrease the absorbance at 280 nm. No such changes were observed in these studies of PLC-δ_1 modification by DEP.

The effect of DEP on the enzyme activity of PLC-δ_1, under conditions resulting in the spectral changes, was also analysed. The incubation in 100 mM potassium phosphate buffer at pH 6.5 resulted in a concentration- and time-dependent reduction of enzyme activity. The reduction to about 10% of the control level was observed after 30 min with 0.5 mM DEP or 60 min with 0.2 mM DEP (Figures 7a and 7b). In order to achieve complete inactivation of some enzymes, a concentration of DEP higher

than those used here (up to 0.5 mM) was needed; concentrations up to 60 mM have been reported [23].

Chemical modification by DEP was also performed using H311A protein; the conditions were similar to those used in studies of the control protein. Since the protein has 21 histidine residues, it was not surprising that the removal of His-311 did not significantly alter spectral changes at 230–250 nm (not shown). However, activity of this mutant, already severely impaired, could be reduced further after incubation with DEP (Figure 7c). It is therefore likely that some other residues (likely to be histidine) accessible to react with DEP have an important role in the enzyme function.

DISCUSSION

We describe here analysis of eight mutations introduced within a highly conserved sequence present in the X region of PLC-δ_1 that identified two residues important for the enzyme function. The selected sequence (residues 304–320 in PLC-δ_1) contains 10 residues that are identical (nine residues) or conservatively substituted (one residue) in all mammalian β, γ, and δ isoenzymes; the same residues are also present in corresponding positions of PLCs from *Xenopus laevis*, *Drosophila melanogaster*, *Dictyostelium discoideum* and *S. cerevisiae*, thus showing evolutionary conservation (Figure 1). Several other positions within this region, although not conserved, are restricted to a subset of specific residues. Some similarity between the X region of the PI-PLC superfamily and *Bacillus cereus* PI-PLC has also been reported [24]. One of several residues identified as invariant by the alignment corresponds to Gln-319 in PLC-δ_1, a residue included in this study. Despite such striking conservation throughout the studied sequence, the effect of different mutations was selective; when Ser-308, Ser-309, Ser-310, His-311, Thr-113, Tyr-314 and Gln-319 in PLC-δ_1 were individually replaced by alanine, only mutations H311A and Y314A had a pronounced effect on the enzyme activity (Figure 2). The effect of replacing His-311 was much greater than Tyr-314. This histidine corresponds to His-335 in PLC-γ_1 previously subjected to mutational analysis [12]. It was estimated that the activity of H335Q PLC-γ_1 expressed in COS cells, was reduced at least 10-fold. Thus, it seems likely that replacement of this conserved histidine in other members of the PI-PLC superfamily could have a similar effect.

Further analysis of the H311A and Y314A mutants demonstrated that these residues are not critical for overall protein folding and stability. Thus, the level of expression of mutant and control proteins was similar. Furthermore, their susceptibility to limited hydrolysis by trypsin was not changed (Figure 3). Consistent with these observations was analysis of H311A mutant by CD, demonstrating that no major structural alteration had occurred in the mutant protein (Figure 4).

Enzymic properties of H311A and Y314A mutants have been examined in more detail. The mechanism of catalysis of PIP_2 by PI-PLC enzymes has not been studied in great detail. Limited studies of PLC-δ_1 [11] have suggested that this enzyme may hydrolyse the substrate processively or in the 'scooting' mode. Two substrate-binding sites have been proposed. One site requires residues at the N-terminus (1–60) and anchors the protein to the membrane surface; once bound, the enzyme can catalyse numerous cycles of PIP_2 hydrolysis through a separate catalytic site formed by conserved regions X and Y. For our mutational analysis of PLC-δ_1, the residues required for non-catalytic binding of PIP_2 were removed. Furthermore, in the assay used here the substrate has been presented as cationic detergent (sodium cholate)/PIP_2 mixed micelles rather than micelles with a non-ionic detergent or, even more physiological, large unilamellar vesicles used in studies described above. Under these conditions, the presence of the sequences from the N-terminus of PLC-δ_1 have no significant influence on substrate hydrolysis and kinetic analysis seems to be simplified to functions of the catalytic site only. Since the mutations were introduced into the X region of the protein, this assay system was suitable for their kinetic analysis (Figure 5, Table 1). The effect of H311A and Y314A mutations was generally similar and major changes were observed in values for $k_{cat.}$. H311A mutation had a much greater effect than Y314A and the values calculated for $k_{cat.}$ were reduced about 1000- and 10-fold respectively. The effect on apparent K_m values was marginal.

There are numerous examples illustrating the essential role of histidine residues in the function of different enzymes [25]. A histidine residue has been found in the catalytic centre of a number of enzymes using the catalytic triad (histidine/asparagine/serine) where it participates in deprotonation of serine. Histidine can also participate as a direct nucleophile in reactions such as those catalysed by phosphotransferases. In different zinc-metalloenzymes histidine residues are essential for zinc binding; phosphatidylcholine-specific PLC from *B. cereus* requires zinc ions for substrate binding and catalysis [26]. Although His-311 is clearly a critical residue for the enzyme function, the experimental evidence presented here does not provide conclusive evidence that this histidine residue contributes to the catalytic centre of the PLC. Although overall structural changes have not been detected, replacement of this histidine could cause limited changes that indirectly result in reduction of catalysis. However, it seems unlikely that the effect of the H311A mutation is caused by a 'hole' in protein structure where a residue with a larger side chain has been replaced by a much smaller one, since replacement of His-311 in PLC-δ_1 or the corresponding histidine in PLC-γ_1 by glutamine had a similar effect on the enzyme activity.

Regardless of the mechanism involved, mutations resulting in a substantial decrease of an enzyme activity without causing overall structural changes could be useful tools in studies of protein function *in vivo*. Thus, H335Q PLC-γ_1 mutant has been used to demonstrate that increased inositol phosphate production by platelet-derived growth factor requires an intact catalytic domain and is not some indirect effect caused by overexpression of the regulatory region [12]. In PLC-δ_1, the regulatory region has not been identified but it seems likely that such a region lies outside the catalytic core and, like the regulatory region of PLC-γ_1, may retain its function(s) in an H311A mutant.

The important role of the histidine residue in PLC-δ_1 and PLC-γ_1 has been demonstrated using site-directed mutagenesis. In this study we further examined the role of histidines for PLC function using a complementary approach to site-directed mutagenesis, chemical modification of histidine by DEP. Under conditions that minimize modification of other residues, the incubation of PLC-δ_1 control protein with DEP resulted in a reduction of enzyme activity (Figures 7a and 7b). When His-311 was replaced by alanine, the mutant protein was still modified on histidine residues and its ability to hydrolyse PIP_2 reduced a further 10-fold (Figure 7c). These data suggest that other histidines, in addition to His-311, play an important role in enzyme function. These other modified residues responsible for the reduction of activity still remain to be identified. The cDNA for rat PLC-δ_1 predicts the presence of 22 histidines. However, only three are conserved among the different isoenzymes and widely divergent species. If histidine(s) is involved in catalysis, these would be the most likely candidates. In our further studies we are mutating these residues and assessing their effect on reduction of enzyme activity by DEP. We are also trying to

identify relevant histidines by direct labelling and peptide sequencing.

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