A β-subclass phosphatidylinositol-specific phospholipase C from squid (*Loligo forbesi*) photoreceptors exhibiting a truncated C-terminus

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Abstract A PCR-based strategy has been used to isolate a full length cDNA encoding a phosphatidylinositol-specific phospholipase C from a sized cDNA squid (*Loligo forbesi*) retinal library. The predicted protein sequence contains 875 amino acids, with calculated M_r 98,181, and has marked similarity with PLC β-isoforms, including conservation of the 'X' and 'Y' regions. It is unique in having a major C-terminal truncation. A major protein of apparent M_r 120,000 estimated by SDS-PAGE has been isolated from squid photoreceptors and identified by partial protein sequence analysis to correspond to the protein sequence predicted from the cDNA clone. This protein has been shown to hydrolyse phosphatidylinositol 4,5-bisphosphate. It is not yet clear whether this represents the major light-activated PLC in squid vision.

Key words: Phospholipase C; Invertebrate vision

1. Introduction

In contrast to the vertebrate visual system, squid photoreceptors appear to utilise $InsP_3$ in the signal transduction pathway initiated by light activation of rhodopsin [1,2]. Identification of a phosphatidylinositol-specific phospholipase C (PLC) associated with the *norp*A visual mutation in *Drosophila* [3] supports the proposal of a role for PLC in invertebrate vision.

To date several PLC enzymes have been characterized from a variety of sources and they appear to distribute into several isoform classes (β_{1-4}, γ_{1-2}, δ_{1-2}, *norp*A) on the basis of protein sequence and different functional characteristics (for recent review see [4]). Although the protein sequences analyzed show diversity, all contain conserved 'X' and 'Y' regions which may have catalytic function [3,5–21]. The PLC β-isoforms are regulated by G-proteins and recent evidence suggests that both Gα and Gβγ components may be involved [22,23].

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The sequence depicted in Fig. 2 has been deposited in the EMBL Nucleotide Sequence Database under the accession number X76968.

Abbreviations: SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLC, phosphatidylinositol-specific phospholipase C; PVDF, polyvinylidine difluoride; PIP_2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; $InsP_3$, inositol triphosphate.

Characterisation of squid rhodopsin [24] and its homologous G-protein subunits [25–27] has revealed distinctive structural features which suggest functional differences in the phototransduction process compared with the vertebrate and insect visual systems. Molecular cloning of a cDNA encoding the PLC, together with isolation, partial purification and functional assay of a 120 kDa protein was undertaken to further characterise this attractive model system and to facilitate better understanding of PLC function.

2. Experimental

2.1. Materials

Enzymes were obtained from Promega, Boehringer Mannheim, Pharmacia or Gibco BRL Life Technologies, Inc. Radionucleotides were from New England Nuclear and all other reagents used were from Stratagene, Sigma, Aldrich, Fluka, BDH or Fisons. Oligonucleotides were synthesized on an Applied Biosystems instrument in this department. Eyes from freshly killed *Loligo forbesi* were dissected below the lens, and the lens and aqueous humour discarded. The eye-cups containing the retina were then rinsed twice in buffered saline (400 mM NaCl/5 mM HEPES (pH 7.2)/5 mM EDTA/1 mM EGTA) [26], rapidly frozen in isopentane cooled with liquid N_2 and stored in liquid N_2 until required for preparation of RNA. The eyes were processed from live animal to storage as quickly as possible.

2.2. Preparation of a squid retinal sized cDNA library

Total RNA was purified from eye-cups by the method of Chomczynski and Sacchi [29]. Poly(A^+)RNA was purified by two rounds of affinity chromatography over olido(dT)-cellulose [28] and stored at −70°C. Double stranded cDNA was synthesized (starting with 4 μg poly(A^+)RNA) using the method of Gubler and Hoffman [31], and Gibco-BRL Superscript reverse transcriptase (Part No. 8053SA). Radiolabel was incorporated into the cDNA product by inclusion of [α-^{32}P]dCTP (111TBq/mmol) in the reactions. The cDNA preparation was size-fractionated by ultracentrifuging in a potassium acetate gradient [32]. Gradient fractions were concentrated by sodium acetate/ethanol precipitation and aliquots analyzed by 1% (w/v) agarose gel electrophoresis. Fractions were pooled to an apparent cDNA lower size limit of approximately 2000 bp. The cDNA library was generated by blunt-end ligation into the *Eco*RV site of KS-pBluescript I (Stratagene), followed by transformation of Epicurian Coli XL1-Blue MRF' competent cells (Stratagene). The cDNA library was plated on Luria broth-ampicillin agar plates containing isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indoyl-β-D-galactoside for blue/white colony selection. The library contained 1×10^5 independent clones with an average insert size of 2500 bp. Cloned cDNA insert size was determined by *Eco*RI/*Hin*dIII restriction of plasmid minipreps.

2.3. Isolation and sequencing of PLC cDNA

Two degenerate oligonucleotides were designed for use as polymerase chain reaction (PCR) primers to highly conserved protein sequence within the 'X' and 'Y' domains of PLCs:
'X' domain [HYYINSSHNTY]
5'[CA(TC)TA(TC)(TC)(ATG)(TCGA)(AG)TI(AT)(AGC)I(TA)(CG)I(TA)(GC)(TCGA)CA(TC)AA(TC)AC]3' (5' primer)

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'Y' domain [CQLVALNFQT]
3'[GT(TC)TG(AG)AA(AG)TTIA(AG)IGC(TCGA)AC(TCGA)A (AGT)(TC)TG(AG)C]5' (3' primer), where I = inosine).

PCR screening of the cDNA library (48°C annealing temperature, 40 cycles) [33], using these two oligonucleotide primers, generated one unique product that was shown by sub-cloning and sequencing to be targetted to PLC. The PCR reaction generating the product was used as an assay to identify clones containing PLC cDNA. The strategy employed to screen the cDNA library was to transfer 1728 colonies from a random plating of the library to grids of clonal colonies on 10 cm × 10 cm Luria broth-ampicillin agar plates. PCR screening initially of pools containing 36 colonies from these grids subsequently led to the identification of individual PLC clones; a full-length clone was then identified by restriction analysis and DNA sequencing. To generate the complete DNA sequence, restriction fragments were sub-cloned into pBluescript, the plasmid DNA amplified and isolated, and sequenced manually using T7 DNA polymerase [34].

Two non-degenerate oligonucleotides designed to the longer PLC clone were synthesized for use as 5' PCR primers:
5'[TATTCAAGAAGGTTGTATTGCC]3' (located within the 'Y' region)
5'[GGATTATGTATCAGATGCATTCGC]3' (located to the 3' of the 'Y' region)
in conjunction with the 3' reverse (DNA sequencing) primer located on the pBluescript vector (see Fig. 3a). The PCR products generated using these primers (65°C annealing temperature, 30 cycles) were used to further characterise nucleotide sequences of the two PLC clones from the 'Y' region to the 3' end, and to check the representation of PLC clones in the whole squid retinal cDNA library.

2.4. Isolation and sequencing of PLC protein

The photoreceptor outer segment layer was lightly scraped from a thawed eye, suspended in 10 mM Tris-HCl (pH 7.0), containing 100 mM NaCl for 30 min at 4°C and then microcentrifuged for 10 min to remove suspended material. Proteins present in the supernatant were separated by preparative SDS-PAGE [35], stained lightly with Coomassie blue and destained to visualize the proteins. The major protein band of interest was excised, soaked for 5 min twice with Milli-Q water, then incubated in 3 ml SDS-PAGE stacking buffer (pH 6.8) containing 25 μg endoproteinase Glu-C (Boehringer) for 1 h at 20°C. Following digestion, the gel pieces were soaked in SDS-PAGE sample buffer (pH 6.8) for 20 min and applied to the top of a second (14%) SDS-PAGE. After electrophoresis the resolved protein digest fragments were electrotransferred [36] to polyvinylidine difluoride (PVDF) membrane (Fluorotrans, PALL Biosupport Division, Portsmouth, UK), and stained with 0.005% sulphorhodmine B (Sigma) in 30% (v/v) aqueous methanol, to visualize protein bands. Bands were excised from the PVDF blot and subjected to sequence analysis in an Applied Biosystems 477A/120A/610A protein sequencer, using standard ABI programs and reagents.

2.5. Partial purification of PLC protein and assay of PLC activity

Proteins present in the supernatant were separated by heparin agarose chromatography, eluting with an NaCl gradient from 100 mM to 600 mM. PLC activity was assayed essentially as described in [37] with final concentrations of reagents: 0.312% sodium cholate, 5 mM 2-mercaptoethanol, 38 mM PIPES (pH 6.8), 100 mM NaCl, 6 mM EGTA, 600 μM DTT, 65 μM PIP_2, 2.75 mCi/mmol [^{3}H]PIP_2 and 1 mM PE. Free calcium concentration (calculated using EQCAL) was 130 μM.

3. Results and discussion

3.1. Use of PCR to isolate cDNA clone for PLC

Two degenerate oligonucleotides for use as PCR primers were designed to highly conserved regions in PLC sequences, one as a 5' primer from the 'X' region and one as a 3' primer from the 'Y' region. PCR screening of the squid retinal sized cDNA library using these primers consistently generated a unique 380 bp product. Subsequent sub-cloning and sequencing showed that the product was similar to a region of the PLC sequence. It was found that generation of this PCR product required only the 3' primer, that mis-match primed at the 5' end. The product therefore extended from near the 5' end of the 'Y' region and into the variable region between 'X' and 'Y' (position indicated in Fig. 2).

From a random plating of the cDNA library, 1728 colonies were picked and arranged in grids on twelve 10 × 10 cm Luria broth ampicillin agar plates which were kept as master plates of clonal colonies. From these plates, 48 pools of 36 colonies were screened by the PCR reactionk, eventually generating two specific clones. An additional PCR reaction, using pBluescript forward and reverse (DNA sequencing) primers, showed that the clones contained 3.4 kb and 1.6 kb inserts. Subsequent sub-cloning of overlapping restriction fragments, followed by DNA sequencing, provided the complete DNA sequence of 3354 bp of the longer PLC clone (Fig. 1). The shorter PLC

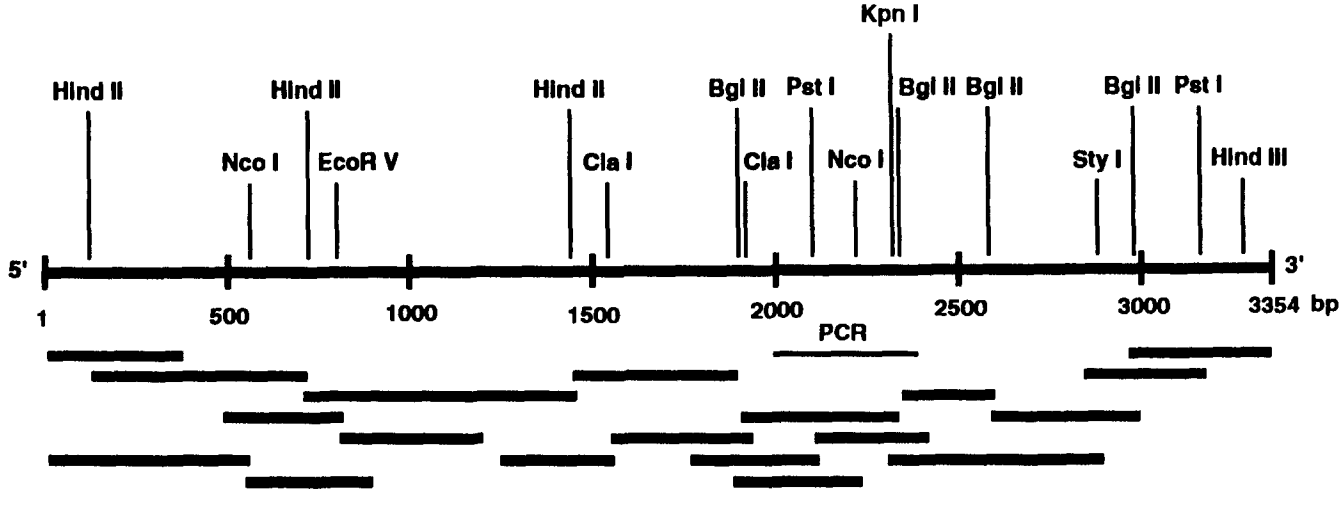


Fig. 1. Restriction map and plan of DNA sequence obtained from sub-cloned restriction fragments for the full length squid photoreceptor PLC cDNA clone. The calibrated line (5'–3') indicates the full length cDNA clone isolated; regions for which DNA sequence was obtained are indicated by the shorter bars below the line and the restriction sites used to generate DNA fragments for sequencing are indicated above the line. The position of the PCR product sequence is indicated by the bar labelled 'PCR'.

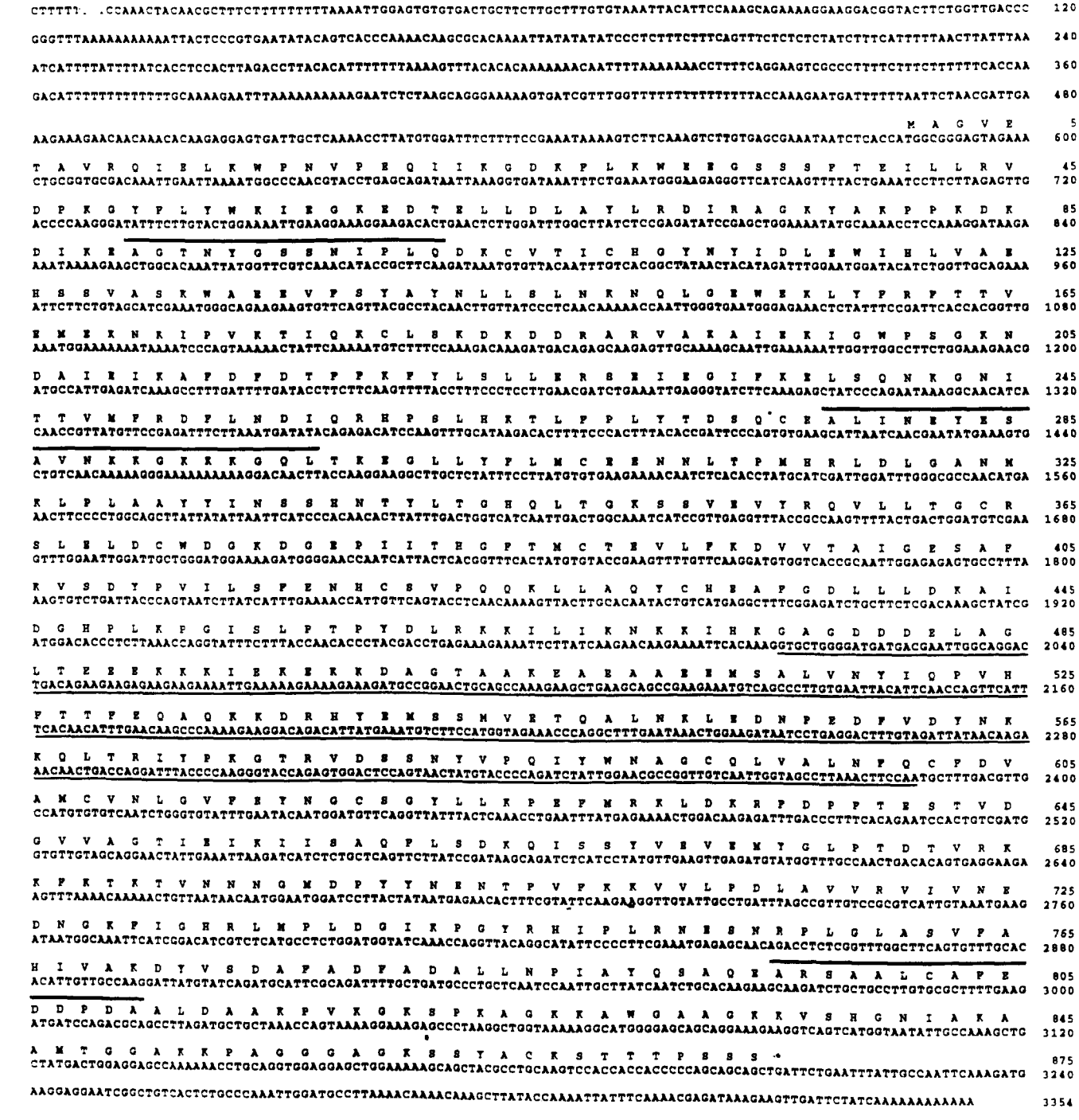
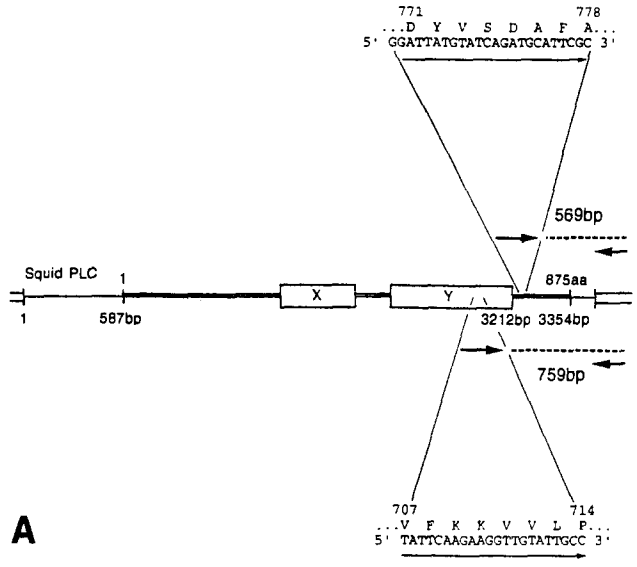


Fig. 2. Sequence of squid photoreceptor PLC cDNA (coding strand). The cDNA sequence was determined in both directions from overlapping sub-cloned restriction fragments. The protein encoded by the only long open reading frame is shown above the cDNA. The wide bars above the predicted protein sequence (residues 90–102, 278–297 and 756–770) indicate the regions where protein sequence was obtained from an endoproteinase Glu-C digest of PLC protein isolated from squid photoreceptor. The narrow bar below the DNA sequence indicates the PCR product generated.

clone, containing 1509 bp, was identical in sequence to the longer clone, starting 1899 bp from the 5′ end of the longer PLC clone and extending 53 bp past the 3′ end of the longer clone, indicating priming of the cDNA for the two clones at different poly A regions of the poly(A^+) mRNA. Computer analysis of the sequence for the longer clone for protein reading frame translation [38], generated a predicted protein sequence of 875 amino acids, with 142 bp 3′-untranslated and 586 bp 5′-untranslated regions (Fig. 2), indicating a full length clone. Although this longest open reading frame is preceded by two inframe ATG initiation codons (codons 20 and 43, positioned 5′ upstream from the assigned initiation codon), there are three in-frame termination codons (codons 13, 38 and 40, positioned 5′ upstream from the assigned initiation codon) in this region.

3.2. PCR analysis of squid PLC truncation

PCR using pBluescript reverse (DNA sequencing) primer with each of the two 5′ primers designed from the longer PLC clone (see section 2 and Fig. 3a) generated the expected products (Fig. 3b) (759 bp with the 5′ primer located in the 'Y' region and 569 bp with the 5′ primer located to the 3′ of the 'Y' region) from the longer PLC clone. The PCR products generated as above for the shorter PLC clone (Fig. 3b) were 53 bp longer at the 3′ end, consistent with the DNA sequence of the shorter PLC clone. Analysis of the whole squid retinal cDNA library with the above PCR primers generated the same major PCR products (Fig. 3b). Subcloning and DNA sequencing of all the above PCR products showed they had the same sequence consistent with that of the two PLC clones isolated. The PCR 5′ primer located in the 'Y' region was designed from the squid longer PLC clone to a region of highly conserved protein sequence to select for all PLC clone representatives in the cDNA library. The PCR 5′ primer located to the 3′ of the 'Y' region was designed from the squid longer PLC clone to less conserved protein sequence that may select for only some PLC clone representatives in the cDNA library. In both cases above the only major PCR product generated was consistent in size and DNA sequence with that of the two PLC clones originally isolated from the squid retinal cDNA library, i.e. we were not able to detect any PCR products consistent with a non-truncated PLC.



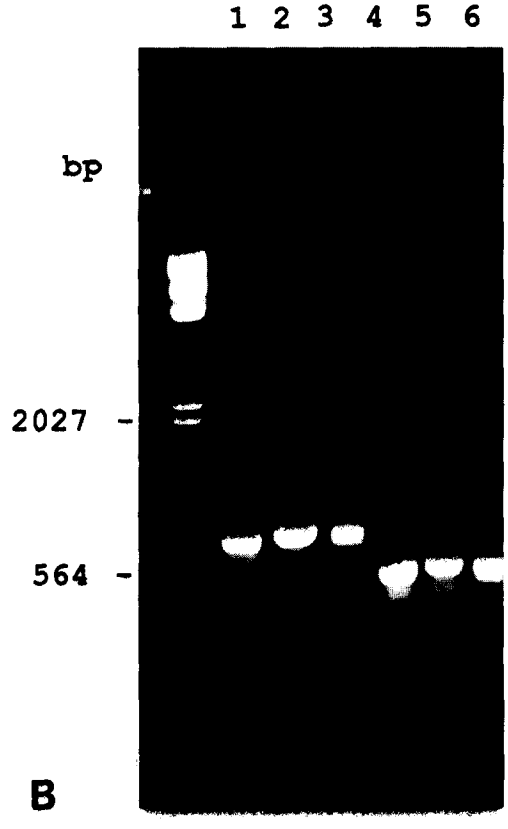


Fig. 3. PCR analysis of squid PLC truncation. (a) PCR strategy (see section 2); (b) Gel analysis. PCR reactions using a 5′ primer oligonucleotide located in the 'Y' region (lanes 1–3) and a 5′ primer oligonucleotide located in the post 'Y' region (lanes 4–6) with the 3′ reverse (DNA sequencing) primer using the full-length PLC clone (lanes 1 and 4), the shorter PLC clone (lanes 2 and 5) and the cDNA library (lanes 3 and 6). The major PCR products were subcloned and sequenced. Lambda *Hin*dIII digest fragment molecular weight markers indicate size of the products.

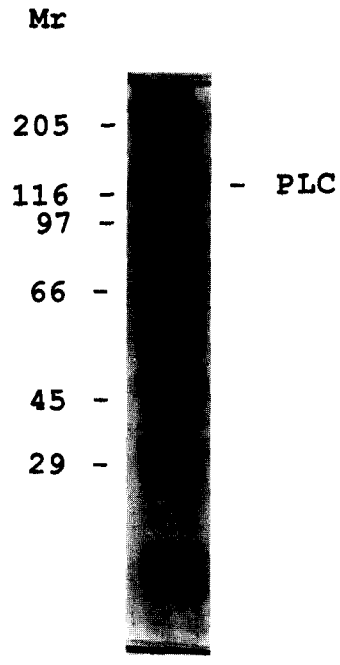


Fig. 4. Analysis of proteins extracted with salt from squid photoreceptors on SDS-PAGE. The band marked 'PLC' was excised from a preparative 10% SDS-PAGE and processed as described in section 2 to produce fragments of the PLC protein on a PVDF electroblot for sequencing. Migration positions of molecular weight markers are indicated in kDa.

3.3. Identification of PLC protein from squid microvilli

Attempts to locate a PLC in preparations of squid photoreceptor outer segments prepared by a standard procedure involving flotation on 40% (w/v) sucrose [39] were unsuccessful. Howeve,r a salt extract of photoreceptor outer segments scraped from the eye-cup contained a limited number of protein species on analysis by SDS-PAGE (Fig. 4). Subsequent amino

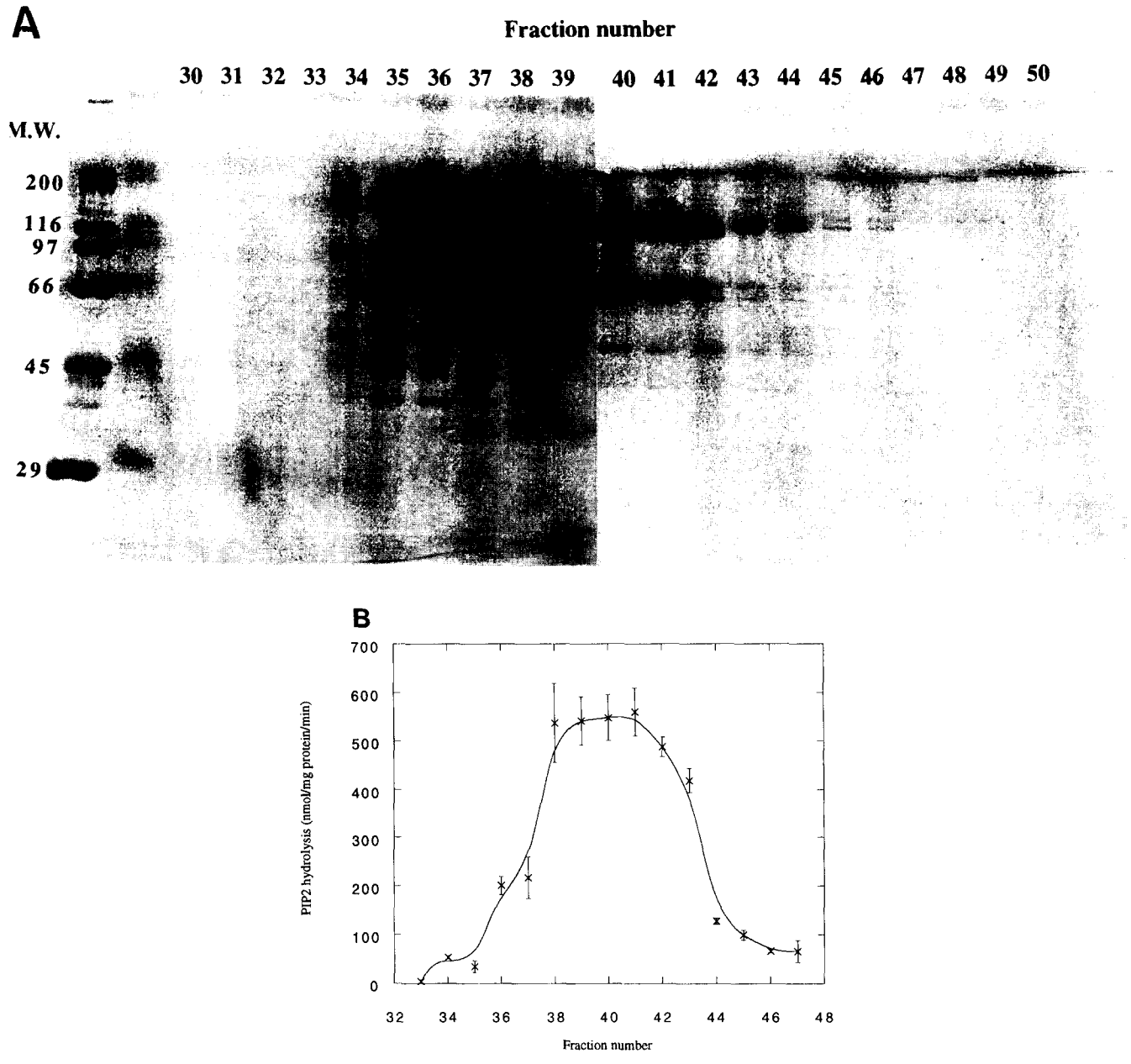


Fig. 5. Partial purification of squid PLC and in vitro assay of activity against phosphatidylinositol 4,5-bisphosphate. (a) 10% SDS-PAGE of fractions eluted from heparin agarose. The photoreceptor outer segment layer was scraped from a thawed eye and suspended in 10 mM Tris-HCl (pH 7.0), containing 100 mM NaCl for 30 min at 4°C prior to centrifugation at 13,000 rpm for 10 min. The supernatant was separated by heparin agarose chromatography, eluting with an NaCl gradient from 100 mM to 600 mM. (b) PLC activity of fractions eluted from heparin agarose. Assays were performed as described in Experimental. Free calcium concentration was 130 μM. Results are triplicate values with standard deviation shown.

acid sequence analysis of digestion fragments from a major 120 kDa protein component in this extract (see Fig. 4) generated data that were in agreement with sequence predicted from analysis of the PLC cDNA clone (see Fig. 2). The intact PLC protein was not amenable to Edman degradation and may therefore have an post-translationally modified N-terminus. No evidence was obtained for any other PLC in the library and the PLC protein identified was a major component of the soluble fraction of the photoreceptor protein complement. Partial purification of this protein (Fig. 5a) followed by PLC assay indicated greatest phosphatidylinositol 4,5-biphosphate hydrolysis in fractions enriched in the 120 kDa protein (Fig. 5b). The predicted size of the PLC from the DNA sequence is smaller than 120 kDa, however protein species running anomalously on SDS-PAGE are well reported and has been observed for the *norp*A protein in *Drosophila*.

3.4. Analysis of squid PLC protein sequence

Computer alignment (not shown) of known PLC sequences (β, γ, δ [40–42] with the squid photoreceptor PLC indicated that the squid protein most resembles the β-subclass and *Drosophila norp*A PLC. The overall sequence identity with these PLCs was 42 to 47%, 29 to 38% for the pre-'X'-region, 56 to 61% for the 'X'-region, 52 to 63% for the 'Y'-region, and 21 to

34% for the post-'Y'-region. These values are similar to the identity levels observed amongst the β-subclass PLCs alone.

However, the most striking feature of the squid PLC sequence is a major C-terminal sequence truncation. Sequence alignment indicates that there is only ~110 amino acids in the squid sequence beyond the 'Y'-region, whereas all of the β-subclass members extend ~350 to 450 amino acids beyond this point. The *norp*A PLC sequence also exhibits a smaller C-terminal region, possibly indicating that truncation may be a feature of retinal-compartmented PLCs. The sequence alignment indicates that there are no other extensive insertions or deletions in the squid PLC sequence apart form the region between 'X' and 'Y' which exhibits considerable variation in PLC members. Computer predictive methods (secondary structure, coiled-coil and diagonal alignment) provide further support for squid photoreceptor PLC being a member of the β-subclass, but appear not to indicate any differentiation as to which subtype (β_{1-4}). Recent evidence [22,23] indicates that G$\beta\gamma$ interacts with the N-terminus and Gα interacts with the C-terminus of β-PLCs. Two regions in the C-terminus of β1-PLC recently defined (designated 'P box' found to be responsible for association with the particulate fractions, and 'G box' containing sequences involved in interaction with Gαq) [43] appear not to be represented in the truncated squid PLC post 'Y' region. A cytosolic PLC was recently purified from the retina of an undefined species of squid (44). This PLC ran as a 140 kDa species on SDS-PAGE and was activated by G protein α subunit. No such PLC species is apparent in the retina of *Loligo forbesi*, where the only major PLC in the cytosol is that reported here. Thus, it is particularly important to establish whether the PLC reported here is activated by the squid visual G-protein and whether this is a new variant of the family.

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