The γ-subunit of the principal G-protein from squid (*Loligo forbesi*) photoreceptors contains a novel N-terminal sequence

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The squid (*Loligo forbesi*) visual system presents as accessible a system for study of G-protein mediated signal transduction as the vertebrate rod outer segment with the added advantage that the major G-protein is a member of the Gq-class. Here the cDNA clone encoding the γ-subunit of this G-protein is reported, thereby completing the molecular cloning of the heterotrimeric G-protein. The deduced protein structure of G-γ has relatively little sequence identity with known mammalian counterparts particularly in comparison with the relatively high degree found for both the α- and β-subunits of this protein. In particular, the N-terminus of the squid visual G-γ contains a repetitive, highly charged region, rich in lysine and glutamate, that has no parallel in other G-proteins. The amino acid sequence of a number of peptides derived by chemical cleavage of G-γ accounted for much of the protein sequence predicted from the cDNA, including the unusual N-terminal region.

Invertebrate vision; Molecular cloning; G-protein subunit; *Loligo forbesi*

1. INTRODUCTION

Heterotrimeric G-proteins play a central role in many signal transduction pathways [1–3 for reviews]. All three G-protein subunits show heterogeneity and it has been postulated that different combinations of G-α, G-β and G-γ may result in the generation of the diverse functional properties of this family of proteins [2]. Different classes of G-α have been shown to be involved in coupling specific receptors to effector enzymes [1–3]. However, only in very few cases has it been possible to determine whether the composition of the βγ-subunits of a particular signalling pathway are also homogeneous. One such example is the light-sensitive region of squid photoreceptor where the three subunits of the major GTP-binding protein have been identified and purified [4]. The high concentration of this G-protein in the light-sensitive membranes (approx. 10% that of rhodopsin) strongly suggests a function for it in squid phototransduction [4]. The molecular cloning of both the α- and β-subunits of this G-protein has been reported previously [5,6] as has the molecular cloning of the squid rhodopsin [7]. The G-protein α-subunit is closely related to proteins of the Gq subclass [8–10], that have been proposed to function in activation of PLC-β [11–14]. This too suggests that the major G-protein of the squid photoreceptor is involved in visual transduction as there is considerable evidence to support a critical role for PLC-β in invertebrate vision [14–17]. Here we report the sequence of the cDNA for the γ-subunit of this G-protein.

2. MATERIALS AND METHODS

Squid (*Loligo forbesi*) were obtained from the Marine Biological Association, Plymouth, UK. Eyes from freshly killed animals were dissected, washed with saline buffer (600 mM NaCl, 5 mM HEPES, pH 7.4, 5 mM EDTA, 1 mM EGTA) and were rapidly frozen in isopentane cooled with liquid nitrogen. Enzymes were obtained from Boehringer Mannheim, Gibco BRL, Promega or Pharmacia; plasmid pBluescript was from Stratagene. Radionucleotides were from New England Nuclear or ICN Flow. Sucrose monolaurate was obtained from Nova Biochemicals. All other reagents were obtained from Sigma, Fisons or BDH and were all AnalaR grade or higher.

2.1. Protein purification and sequencing

Photoreceptor membranes were purified as described previously [7,20] and were resuspended in 2% (w/v) dodecyl-β-Δ-fructofuranosyl-α-Δ-glucopyranoside in 50 mM Tris-HCl, pH 7 (1 h, 4°C). The detergent-insoluble cytoskeletal fraction was removed by centrifugation (9,000×g, 5min). The detergent-soluble fraction contained rhodopsin

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Abbreviations: G-protein, GTP-binding protein; PLC, phospholipase C; SML, sucrose monolaurate; PCR, polymerase chain reaction; SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7; NET, 15 mM Tris-Cl, 1 mM EDTA, pH 8.3; SSPE, 150 mM NaCl, 10 mM NaH$_2$PO$_4$, 1 mM EDTA, pH 7.4.

The cDNA sequence referred to in this paper was submitted to the EMBL Database and has been given the accession number Z15112.

and its associated G-protein, along with other components. The γ-subunit was resolved from other protein components by SDS-PAGE and was electroblotted onto a PVDF-membrane filter. The protein was cleaved by incubation of the filter with a 100-fold molar excess of CNBr in 70% formic acid. CNBr and formic acid were removed by evaporation and the cleaved protein was subjected to automated amino-acid sequencing.

2.2. Isolation and sequencing of G-γ cDNA

Standard molecular biology techniques were carried out as described in [21] and [22]. An oligonucleotide probe (oligo-1) was designed on the basis of peptide sequences [4] and was used to screen a portion (2×10⁴ plaques lifted onto nitrocellulose) of a cDNA library in λgt10 made previously from squid eye poly(A)⁺ mRNA [6,23].

    Oligo 1:   5'-ACC/T-TTC/T-TCC/T-TTI-ATC/T-TTC/T-TCC/T-TC-3'

Hybridization with 5' ³²P-labeled oligo-1 was in 6×NET, 0.5% SDS, 5×Denhardt's reagent and 100 μg/ml sheared salmon-sperm DNA (48°C for 12–16 h). Washes were carried out in 6×SSC, 0.1% SDS (2×5 min, 45°C). Insert DNA was excised from recombinant λ by incubation with EcoRI; inserts which hybridized to oligo-1 on Southern analysis were subcloned into pBluescript and were sequenced using T7 DNA polymerase. Insert cDNA from recombinant phage was also prepared using the polymerase chain reaction (PCR). Oligos-2 and -3 were designed as primers to hybridize either side of the EcoRI cloning site of λgt10. Recombinant plaques were cored and vortexed in 500 μl of SM buffer, 2 μl of which was used as template for PCR (30 cycles, 55°C annealing temperature). Products were incubated with EcoRI and were subcloned into pBluescript for sequencing.

    Oligo 2: 5'-GAG-TAT-TTC-TTC-CAG-GG-3'
    Oligo 3: 5'-CAG-CAA-GTT-CAG-CCT-GG-3'

2.3. Northern analysis

Total RNA was isolated from a frozen eyecup using acid phenol/chloroform extraction [22]. RNA was denatured and separated on a 1.2% formaldehyde agarose gel and was transferred to nitrocellulose for Northern analysis. Hybridization was carried out in 50% formamide, 5×SSPE, 2×Denhardt's reagent, 0.1% SDS, 100 μg/ml sheared salmon-sperm DNA (42°C for 12–16 h) and stringent washes (0.1×SSC, 0.1% SDS, 42°C, 12–16 h) were subsequently performed.

3. RESULTS AND DISCUSSION

The identification of the γ-subunit of the major G-protein of squid photoreceptor membranes was based on its tight and stoichiometric association with the β-subunit, similarity of its size to mammalian G-γs and limited identity in sequence of proteolytic fragments to regions of sequence of these subunits [4]. We synthesized the degenerate oligonucleotide (oligo-1) on the basis of peptide sequence obtained previously [4] and used this as a probe to screen a squid-eye cDNA library. Using this approach an insert of approximately 850 bp that hybridized with the probe on Southern analysis was isolated. The sequence of this insert contained an open reading frame of 87 amino acids, representing the full amino acid sequence of G-γ (Fig. 1). Subsequent screening of the library using this cDNA as a probe showed that G-γ clones are represented at a frequency of about 0.3%. A similar abundance of both the G-α and G-β cDNAs has been determined previously [5,6], and is consistent with the high concentration of the three subunits of this G-protein found in the squid photoreceptor membranes.

The sequences of the peptide fragments of G-γ reported previously [4] can be identified in the sequence predicted from the cDNA. In order to confirm that the cDNA sequence that was isolated encoded the only G-γ that is present at high abundance in the squid eye, inserts from a number of clones were isolated using PCR. Several of these inserts were subcloned into pBluescript and were sequenced. All sequences determined confirmed that of the original clone. The full sequence of the longest insert found is also shown (Fig. 1). The G-γ
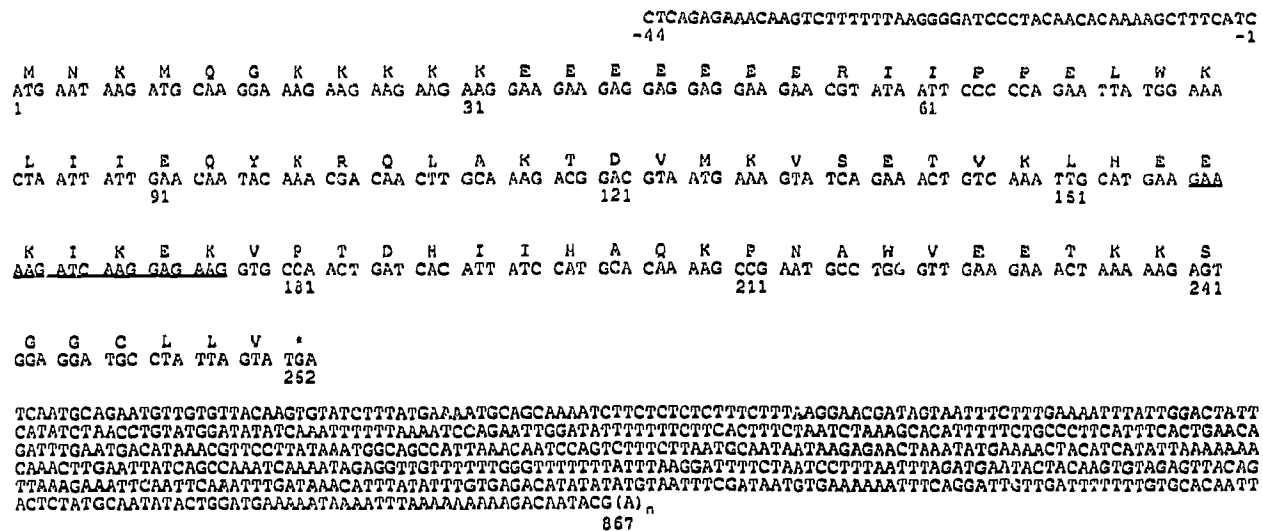

Fig. 1. Sequence of G-γ cDNA. Sequence of coding strand of longest insert shown, determined from two independant clones. The protein sequence encoded by the only long open reading frame is shown above the cDNA. The amino acid sequence used for design of oligonucleotide probe 1 is underlined.

cDNA was also used as a probe for northern analysis of total RNA from squid eyecup. A single RNA species of approximately 1.4 kbp was detected by hybridization at high stringency (Fig. 2).

A stop codon was found in the cDNA sequence 5' to the starting methionine. However, the N-terminus of the predicted protein contains two closely spaced methionine residues followed by the sequence (lysine)$_5$(glutamate)$_7$ and bears no similarity to the sequences of the N-termini that have been reported for other G-γ subunits. Therefore we were concerned that the first methionine might not represent the true start site of G-γ translation. The sequencing studies had indicated that the N-terminus of G-γ was refractory to Edman degradation [4] and was therefore probably modified in some way. Thus, we made use of CNBr treatment to remove the starting methionine to obtain amino acid sequence beginning at residue 2. The primary structure of G-γ as encoded by its cDNA contained only one other internal methionine residue and as the predicted sequence at each of the three cleavage sites was available, it was not necessary to purify the peptides generated by cleavage with CNBr. Therefore G-γ was gel-purified, blotted onto a solid support (PVDF), cleaved by treatment with CNBr and the peptide mix was reattached to this support for sequence determination. The amino acid sequences determined in

Fig. 2. Northern analysis of squid G-γ. Total RNA from squid eyecup was probed with the G-γ cDNA and washed at high stringency. RNA ladder from Gibco-BRL was used to determine size of hybridizing band.

this fashion contained the lysine and glutamate rich stretch predicted from the cDNA for the N-terminus of G-γ (Fig. 1). It is impossible to speculate as to the precise function of this domain, but the lack of a similar region in a recently cloned *Drosophila* G-γ subunit (found at high concentrations in the head and eye and therefore potentially the insect counterpart of the squid visual G-γ) [23] may indicate that it is a feature unique to the cephalopod visual G-γ.

The squid visual G-γ sequence ends cysteine, leucine, leucine, valine, which fits the generalized -cysteine-(aliphatic)$_2$-any ('-CAAX') motif shown to be a signal for cleavage of the polypeptide C-terminal to the cysteine, carboxylmethylation of the new C-terminus and isoprenylation of the cysteine residue [24]. All previously reported cDNA sequences for G-γ subunits have this sequence motif, and where it has been investigated the motif has been demonstrated to result in G-γ isoprenylation [25–27]. In the case of the yeast mating factor pathway G-γ homologue STE18, farnesylation of the C-terminal cysteine has been shown to be necessary for membrane attachment of the G-$\beta\gamma$ complex and for G-protein function [27]. The isoprene derivative attached to proteins with different '-CAAX' motifs has been identified as either a farnesyl or the longer, more hydrophobic geranyl-geranyl group. It appears that it is the sequence of the '-CAAX' motif that determines which isoprenoid group is attached [24]. Although the '-CAAX' sequence of squid G-γ does not exactly match any where the nature of the modifying group has been determined, it is far more similar to those that result in geranyl-geranylation than the ones that lead to farnesylation. Taken together the evidence implies that squid G-γ is likely to be modified by C-terminal geranyl-geranylation and that this hydrophobic group may contribute to the binding which necessitates the use of detergent to dissociate the squid visual G-$\beta\gamma$ complex from the membrane [4].

Comparison of the amino acid sequence of the squid G-γ with those of previously cloned or sequenced mammalian and insect γ-subunits [23, 28–31] (Fig. 3) reveals some similarity in primary structure between the proteins. However, the degree of identity is not great, in marked contrast to the very high sequence identity between the squid visual β-subunit and its mammalian counterparts [6].

Except in *Saccharomyces cerevisiae* where the G-$\beta\gamma$ activates the mating response pathway [32], the role of G-$\beta\gamma$ subunits in signal transduction is not well understood. On the other hand, the existence and high level of interspecies sequence identity of different sub-classes of both these G-protein subunits [31, 34] suggest more than a passive role for G-$\beta\gamma$ in signal transduction. Recently several diverse functions have been suggested for these subunits in mammalian cells. For example the activity of different adenylate cyclases appear to be variably sensitive to addition of mixed populations of G-$\beta\gamma$
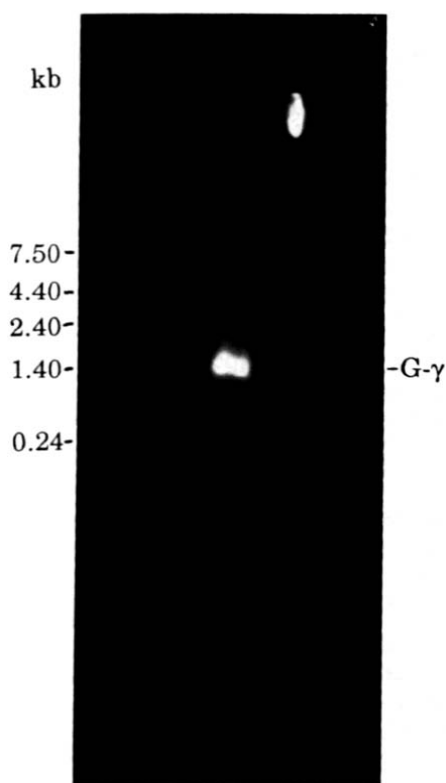
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squid: MNKMQGKKKKKEEEEEEEERIIPPELWKLTIEQYKRQLAKTDVMKVSETVKLHEEKIKEKVPTDHIIHAQKPNAWVEETKKSGGCLLV
dros:  ---------------mDvmssshqqqRVVVEQ1RRe-AaTDrqtdSESCakmmkyItEHeqeDyLLtgftsQkvnpfreKSs-CtVL
trans: -----------mpviniEdLtekDk1KMeVDQ1KKe-VtlErM1VSkcceefrDyVeERsgeDpLVKg-iPedknpfkelkGGCVIs
gam2:  --------------masnntAsiaqaRkLVEQ1Kme-AniDrIKVSkaAadlmayceaHAkeDpLLtp-vPasenpfreKkffCAIL
gam3:  ----------mkgEtpvnstMsigqaRkMVEQ1Kie-AslcrIKVSkaAadlmtycdaHAceDpLItp-vPTsenpfreKkffCALL
gam5:  ---------------msgsssvaAmKkVVqQ1Rie-Ag1nrVKVSqaAadlkqfclqnAqhDpLLtg-vsSstnpfrpqkv-CsfL
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Fig. 3. Alignment of the sequence of squid G-γ with other G-protein γ subunits. Where amino acid residues are identical to the squid sequence, they are shown in bold capital letters. Conservative replacements are shown in upper case, the rest of the sequence in lower case. Dros, *Drosophila* G-γ [23]; trans, bovine transducin γ [28]; gam2, gam3, bovine brain G-γs [29,30]; gam5, bovine liver γ [31].

subunits [35]. There have also been several reports that G-βγ may stimulate K$^+$-channels either directly [36] or by activation of phospholipase-A$_2$ [37]. However, the reproducibility of experiments between laboratories has been low, and in part this may be because the undefined composition of the G-βγ that has been used [2]. Thus the characterization of all the subunits of the squid visual Gq, that probably mediates the coupling of light absorption by rhodopsin to PLC activation, is likely to help define the roles of G-protein subunits in signalling. Increasing attention has also been focused on the interactions between the G-β and G-γ subunits. These interactions seem critical for the structural stability of the of both these subunits [38]. Therefore the molecular cloning of the squid visual G-γ, with its unique N-terminus, low overall identity to other G-γs and known interacting G-β [6] may also prove very useful in defining interactions that are important between G-protein subunits.

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