The identification and purification of the heterotrimeric GTP-binding protein from squid (*Loligo forbesi*) photoreceptors

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The principal GTP-binding protein (G-protein) from squid (*Loligo forbesi*) photoreceptor membranes has been identified by amino acid sequencing. The heterotrimeric protein was purified by detergent solubilization and ion-exchange chromatography. The amino acid sequence of the G-protein α -subunit (G- α) indicates that this subunit is closely related to the recently characterized Gq subgroup, whereas the G- γ subunit varies widely in sequence from other homologues.

INTRODUCTION

A role for a GTP-binding protein (G-protein) in invertebrate visual transduction has been proposed, not only because of the similarity of the photoreceptor with those of mammalian systems [1–3], but also from electrophysiological data and the use of G-nucleotide analogues [4–6]. Several different protein components of the squid retinal outer segment have been suggested to resemble a G-protein α -subunit (G- α) by their cross-reactivity in interspecies reconstitution and from peptide mapping, toxin labelling or Western-blotting [7–12]. However, conclusive identification has not been possible, and several appear to represent very minor protein components. Here we describe the purification and unambiguous identification of the three subunits of the principal heterotrimeric G-protein from invertebrate photoreceptors. In cephalopods and several other invertebrate classes, there is extensive evidence that the light-activated G-protein is linked to inositol-phospholipid metabolism [4–6,13–16]. The sequence of peptides derived from the α -subunit are most similar to regions of a recently described subclass of G-proteins [17,18]. It has been suggested that this subclass couples receptors to inositol phospholipid metabolism [19]. The G- α isolated here is by far the major G- α present in the squid photoreceptor membrane. It is present at about 10% the concentration of rhodopsin, a similar quantitative relationship to rhodopsin as in vertebrate photoreceptors [20]. Therefore, it is likely that the G-protein described here is the light-activated G-protein of cephalopod eyes, and that activation of this G-protein stimulates an inositol phospholipid-directed phospholipase C (PLC).

EXPERIMENTAL

Materials

Squid (*Loligo forbesi*) were obtained from the Marine Biological Association, Plymouth, U.K. 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.

Chemicals were of AnalaR grade or higher and were obtained from Aldrich, Sigma, BDH or from Nova Biochem.

Purification of the photoreceptor G-protein

The isolation and purification of photoreceptor outer segments (OS) were carried out as described previously [7,21] (these and all subsequent manipulations involving native rhodopsin were performed in the dark or under dim red light at 4 °C). Purified OS

were resuspended in 2% (w/v) sucrose monododecanoate (dodecyl- β -D-fructofuranosyl α -D-glucopyranoside) in 0.05 M-Tris/HCl, pH 7.0 for 1 h. Detergent-soluble (supernatant) and detergent-insoluble (pellet) proteins were separated by centrifugation (9000 g for 5 min). Detergent-soluble proteins were separated by DEAE ion-exchange chromatography over DE-52 (Whatman) equilibrated with loading buffer, 0.1% sucrose monododecanoate in 0.05 M-Tris/HCl, pH 7.0 (column size 12 $\times$ 1 cm; bed vol. 9.5 ml). The column was developed either by washing (10 ml $\cdot$ h⁻¹) with 3 bed vol. of loading buffer, followed by either a linear (0–0.5 M) NaCl gradient in loading buffer, or by discrete (2 bed vol.) steps (0.11 M; 0.25 M; 0.5 M) NaCl in the same buffer. Protein was determined by measuring the A_{280} of each fraction; peaks were then pooled and kept for further investigation.

Urea solubilization of purified OS

Purified OS were resuspended in 2 M-urea/0.05 M-Tris/HCl, pH 7.0, and thoroughly mixed on ice for 5–10 min. The urea-soluble and -insoluble fractions were then separated by centrifugation (9000 g, 10 min).

o-Iodosobenzoic acid cleavage of protein subunits

Freeze-dried protein (up to 200 pmol) was resuspended in 1 ml of 4 M-guanidinium chloride in 80% (w/v) acetic acid to which 30 μ l of *m*-cresol and 10–15 mg of *o*-iodosobenzoic acid were added. The solution was then incubated at room temperature (20 °C) for 48–72 h in the dark. Cleaved peptides were desalted either by gel filtration (Sephadex G-10), or by dialysis against water and were purified by electroelution from Tricine gels [22] before attachment to a solid-phase support for amino acid sequencing [23].

CNBr cleavage of protein subunits

Freeze-dried protein (up to 200 pmol) was resuspended in 200 μ l of 70% (w/v) formic acid to which a 100-fold molar excess of CNBr was added. The solution was then incubated at room temperature for 16 h in the dark. Peptides were recovered by freeze-drying and were purified by electroelution from Tricine gels [22] before attachment to a solid-phase support for amino acid sequencing [23].

RESULTS AND DISCUSSION

Squid (*Loligo forbesi*) photoreceptor OS membranes were purified from frozen retina by well-documented techniques

Abbreviations used: G-protein, GTP-binding protein; G- α , G- β and G- γ , α , β and γ G-protein subunits; (R) OS, (rod) outer segments; PLC, phospholipase C.

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Fig. 1. Detergent and urea solubilization of G-protein

Photoreceptor OS membranes were subjected to SDS/PAGE (5–20% acrylamide) and stained with Coomassie Blue. Samples were dissolved in reducing sample buffer and incubated at room temperature. Lane i, OS proteins soluble in urea; lane ii, OS proteins insoluble in 2 M-urea; lane iii, detergent-insoluble OS proteins; lane iv, detergent-soluble OS proteins; lane v, OS; lane vi, M_r markers. The protein loaded on to each track was derived from an equal amount of a single OS preparation, allowing comparison of relative amounts of polypeptides between lanes.

Fig. 2. DE 52 ion-exchange chromatography of detergent-soluble proteins of squid photoreceptor membranes

A profile of elution from a DE 52 column is shown. Step increases in [NaCl] were used. The 0.25 M-NaCl-eluted fraction was subjected to SDS/5–25%-PAGE and the gel stained with Coomassie Blue (inset).

[6,7,10]. The membrane-associated proteins of the OS were separated into two fractions by detergent solubilization [7] (Fig. 1). The cytoskeletal components were removed by centrifugation to leave a detergent-soluble fraction that contained rhodopsin and a few major protein species, three of which were found to be G-protein subunits. A single eye yielded membranes containing

60–100 nmol of rhodopsin, accounting for more than 60% of the detergent-soluble protein. The three G-protein subunits were recovered in this fraction (Fig. 1). Densitometry indicated a G-protein/rhodopsin ratio of approx. 1:10, similar to that observed in bovine ROS [20]. Other protein components in the membrane protein fraction were present only at concentrations an order of magnitude lower, and we conclude that the squid photoreceptors neither contain significant quantities of other G-protein-coupled receptors or of other G-proteins. By implication the G-protein described here is most likely to be the one that is coupled to photolysis of squid rhodopsin *in vivo*. This G-protein is an abundant protein; a single squid eye contains 0.5–1 mg of the heterotrimeric protein. It represents approx. 15% of the membrane protein of the isolated outer segments. As a comparison, 1 mg of rat brain membrane protein contains approx. 40 μ g of all G-proteins, and the Gq subclass amounts to less than 0.1% of membrane protein [24].

Membrane-associated protein was not released in significant quantities by washing with 2 mM-Hepes, pH 7.5, or by 1 M-NaCl/2 mM-Hepes, pH 7.5, buffers, nor by washing dark- or light-adapted membranes with these buffers containing 100 μ M-ATP, -GTP or -GDP, 20 mM- Ca^{2+} or EGTA before solubilization (results not shown). Therefore slight differences in the amounts of G-protein subunits recovered in the detergent extract (Fig. 1) are more likely to result from differences in their solubility than from losses during the preparation. Treatment of the membranes with urea released a few peripheral membrane components. The principal polypeptide that was solubilized in this way proved to be G- α (Fig. 1), and the method was used to isolate large amounts of G- α for structural analysis. The squid photoreceptor G-protein is therefore more intimately associated with the membrane than is its vertebrate counterpart. The latter is easily dissociated by salt or by light and GTP [25,26]. Thus the water-solubility of the G-protein in the present study more closely resembles that of the G-proteins involved in excitation-coupling of a variety of hormones, neurotransmitters and peptides [27]. The M_r -44 000 polypeptide released from the membrane by urea, or separated from the cytoskeleton by detergent solubilization, was not well resolved from a similar- M_r cytoskeletal component by SDS/PAGE. However, after proteolysis of the M_r -44 000 cytoskeleton component with *Staphylococcus aureus* V8 endoproteinase, we were able to identify this protein as actin by sequence analysis (results not shown).

Purification of the detergent-solubilized G-protein was achieved by ion-exchange chromatography using DEAE-cellulose. By application of a linear salt gradient, it was possible to resolve the M_r -36 000 and M_r -12 000 (G- β and G- γ) subunits from the M_r -44 000 G- α . Some losses of the M_r -44 000 component occurred during chromatography, probably due to tight binding of a fraction of this component to the resin support after dissociation of G- $\beta\gamma$. Rhodopsin, solubilized in sucrose dodecanoate, did not bind to DEAE-cellulose. After elution of rhodopsin by washing with 3 vol. of loading buffer, application of a linear 0–0.5 M-salt gradient displaced first G- $\beta\gamma$ (between 0.1 and 0.2 M salt) and then G- α (0.2–0.3 M) from resin. Use of discrete steps in the salt concentration allowed resolution of rhodopsin (in the 0.11 M fraction) from the three subunits of the G-protein, which were eluted together in the 0.25 M fraction (Fig. 2). The tight association of G- β with G- γ has been observed in all G-protein extracts that have been characterized thus far [28]. As has been shown for other G-proteins, the interaction with G- α appears weaker [25–28]. It is possible that detergent solubilization itself results in dissociation of G- α from G- $\beta\gamma$. However, it may be that electrostatic interactions between the subunits are sensitive to the increased ionic strength required for elution from the ion-exchange resin [29]. The tight interaction between G- β and G- γ

Table 1. G-protein-derived polypeptide sequences

Residues in parentheses represent the most likely of several potential residues. The unusual *o*-iodosobenzoic acid (IBA) cleavage between E and T may result from modification of the intact G- γ during electrophoresis, staining or cleavage. The three peptides derived from G- γ overlap to give a sequence LEITEQY(P)RQLAKT-DMKVSETVKL(M)EEKIKEKDPTD(M)KIAAQKXN.

Sub-unit	Cleavage method	$10^{-3} \times M_r$	Sequence
G- α	IBA	7	XQNSSVILFLNKKDLLEEXI
	IBA	14	XDPGAQEXYDRXXE (Y) QL (T) XA
	IBA	18	-
	IBA	32	-
	IBA	38	-
G- γ	IBA	6	XVKL (M) EEKIKEKDPTD (M) KIAAQKXN
	IBA	10	LEITEQY (P) RQLAKTDMKVSETVKLXEEIXI
	CNBr	4	XVSETKX (E) (E) X (I)

IBA- M_r 14000	xDPGAQExYDRxxEYQLTDxA
Mouse Gq	WN...I...C...RR...S.S.
<i>Drosophila</i> Gq	WD.A.I...C...RR...S.S.
Rat Gs	WE.E.V.AC.E.SN...T.S.
Rat Gi-1	WK.S.V.ACFN.SR...N.S.
Rat Go	WG.S.I...CFN.SR...N.S.
Bovine Gt	WK.S.I.ACF...AS...N.S.
Human Gz	WA....ACFS.SS...H.E.N.

IBA- M_r 7000	xQNSSVILFLNKKDLLEExI
Mouse Gq	WF.....K.
<i>Drosophila</i> Gq	WF.....K.
Rat Gs	WLRTI.....Q...A.KV
Rat Gi-1	WFTDT.I.....F.K.
Rat Go	FFIDT.I.....FG.K.
Bovine Gt	YFATT.IV.....VFS.K.
Human Gz	WFI.T.L.....A.K.

Fig. 3. Comparison of the partial sequences of M_r 44000 with regions of G- α

Peptide sequence data from the M_r -44000 polypeptide were aligned with those for similar regions of other G- α species. The sequences of the G- α species with which the polypeptide sequences from the squid protein were compared were obtained from [17,28]. Identical residues are indicated by a dot. Abbreviation: IBA-, *o*-iodosobenzoic acid-derived.

is, however, non-covalent, and dissociation of the subunits was achieved by denaturation with SDS. Thus subunits were separated from one another by electroelution from polyacrylamide gels.

All three of the intact G-protein subunits were refractory to Edman degradation. Therefore we subjected the individual polypeptides to proteolytic cleavage to generate internal fragments for amino acid sequence analysis. G- β was readily cleaved into a few large polypeptides by treatment with *S. aureus* V8 endoproteinase, allowing the molecular cloning of squid visual G- β cDNA [21].

Cleavage of G- α into large fragments with proteinases proved unsuccessful, due to its extreme sensitivity to enzymic digestion (results not shown). However, chemical proteolysis using *o*-iodosobenzoic acid proved more successful and allowed the isolation of five polypeptides (Table 1). The same pattern of

Fig. 4. Comparison of the partial sequence of M_r -12000 polypeptide with that of G- γ

A DIAGON analysis [33] (span = 17, score = 190) of the sequence of the overlapping peptides (Table 1) derived from G- γ by IBA or CNBr cleavage of the electroeluted M_r -12000 polypeptide against (a) bovine transducin- γ and the two cloned G- γ species [G- γ 2 (b) and G- γ 3 (c)] [35].

cleavage was obtained whether the G- α was purified from the detergent- or from the urea-solubilized fraction. The peptides were attached to DITC-glass for solid-phase sequence analysis. The sequence of polypeptide α -1 M_r 7000 (Table 1) was closely related to a conserved region of a number of G- α (around the Halliday G-box [28,30]; Fig. 3). In this region, the amino acid sequence obtained from the squid protein was identical with that of the recently characterized Gq subgroup [17] (Fig. 3). The other polypeptide from which amino acid sequence was obtained, namely that of M_r 14000 (polypeptide α -2; Table 1), was also similar to a region of the Gq subgroup (Fig. 3). The sequence of

both fragments showed significantly less similarity to other G- α s. Thus it is likely that the major G-protein of squid OS represents a new member of the Gq subgroup. The extensive evidence that squid visual transduction involves stimulation of PLC [4–6,13–16] supports the hypothesis that these proteins are involved in coupling receptors to PLC [17–19,24,31]. The other polypeptides obtained from *o*-iodosobenzoic acid cleavage were still refractory to sequencing, probably indicating that they were derived from the *N*-terminal region of G- α . As the expression of Gq in *Drosophila* is concentrated in the eye [17,18], it may be that much of the mechanism of phototransduction is shared between a wide range of invertebrates.

We found that this G- α was not ADP-ribosylated by either pertussis or cholera toxin. Sequence analysis of Gq α and its homologues indicate that they are unlikely to be substrates for pertussis toxin [17], and this insensitivity has been demonstrated for some G-qs [24] and Gq-like α -subunits [31]. Though substrates for modification by both toxins have been detected in the squid outer segment previously [8–11], the abundance of these toxin substrates relative to the G- α of the present study indicate that they represent minor components. The soluble squid visual G- α described previously [7], though not a substrate for modification by either bacterial toxin, also appears to be a distinct and unrelated protein component.

Previous reports that have demonstrated light-stimulated GTPase activity have failed to identify a major component insensitive to toxin modification [8,10,11]. This failure may result from the poor guanine-nucleotide binding of Gq [17,19]. The light-stimulated pertussis-toxin-sensitive activities that have been reported [8,10,11] may represent the activation of a G-protein that is present in the squid photoreceptor at very low levels (at least an order of magnitude less than the Gq described here). Alternatively the activity reported may result from co-purification and cross-reactivity of a G-protein from another membrane. Cross-reactivity of a variety of G-proteins and receptors has been demonstrated *in vitro* [32].

G- γ was cleaved with *o*-iodosobenzoic acid and also with CNBr. In each case two peptides were resolved on Tricine gels. The identification of G- γ stems mainly from its M_r and its tight stoichiometric association with G- β , as the sequences of peptides γ -1, γ -2 and γ -3 (M_r values 6000, 10000 and 4000 respectively) were not closely related to those of any known G- γ (Table 1). However, the sequences of the peptides overlapped and, when a low-stringency DIAGON analysis [33] was performed, a significant relationship with previously reported G- γ sequences was observed (Fig. 4).

The identification, purification and functional characterization of squid visual G-protein presents a useful system for the reconstitution of an intact signal-transduction pathway linked to PLC. Because of the heterogeneity of subunits [17,27,28,34,35] and the large number of different G-proteins present in most cell types, this level of characterization has only previously been possible for vertebrate transducin [25,26]. Sequence analysis indicates that whereas G- β is 80–90% identical in these two heterotrimeric G-proteins, neither of the other subunits show similar levels of conservation. This supports the postulate that G- β plays a crucial (though as yet unknown) role in G-protein-mediated receptor-effector coupling [21]. Finally, the squid visual G-protein is the first example of an isolated and characterized invertebrate G-protein.

We thank the Marine Biological Association, Plymouth, U.K., for

help in obtaining live squid, the Science and Engineering Research Council for financial support and Dr. Helen Saibil for helpful comments.

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