Sequence of the β -subunit of the phosphatidylinositol-specific phospholipase C-directed GTP-binding protein from squid (*Loligo forbesi*) photoreceptors

Nicholas J. P. RYBA, John D. D. POTTINGER, Jeffrey N. KEEN and John B. C. FINDLAY

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

The β -subunit (G- β) of the squid (*Loligo forbesi*) visual GTP-binding protein (G-protein), thought to be associated with a phosphatidylinositol-specific phospholipase C, has been identified and the sequence of the protein determined from its cDNA. The predicted polypeptide has a very marked sequence similarity with its mammalian counterparts (80–90 % identity). Squid G- β also has somewhat lower similarity to the yeast protein STE4 (approx. 40 % identity). The role of G- β in signal transduction is discussed in the light of its pronounced structural conservation.

INTRODUCTION

A functional role for a GTP-binding protein (G-protein) in invertebrate vision has been proposed, not merely because the photoreceptor, rhodopsin, is closely related to its mammalian counterparts [1–3], but also because of the influence that GTP and its analogues have on the electrophysiological response of the photoreceptor cells [4,5]. Evidence from a variety of invertebrates implies that the activation of the visual G-protein is coupled to generation of the second messenger InsP_3 via the activation of a PtdIns-specific phospholipase C [6,7]. However, conclusive identification of the subunits of this G-protein has not yet been confirmed. Biophysical data, antibody binding and toxin labelling have indicated that a variety of polypeptides may function as the α -subunit of the squid photoreceptor G-protein [8–10]. The β -subunit (G- β) has proved even more elusive, and, unlike in mammals where three separate sub-classes of G- β have been identified [11–13], only a single gene for an invertebrate G- β has been identified in *Drosophila* [14]. Thus identification and sequencing of G- β in the squid (*Loligo forbesi*) photoreceptor has a wide and general significance. It presents a unique opportunity to study a PtdIns-specific, phospholipase C-directed G-protein in a relatively simple and accessible model system. It also allows examination of the relationship between invertebrate and mammalian G-proteins and of the exact roles of G- β and its subtypes in signal-response coupling.

EXPERIMENTAL

Materials

Enzymes were obtained from Boehringer Mannheim, Cetus or Pharmacia. Radionucleotides were from New England Nuclear, and all other reagents used were from Stratagene, Sigma, Aldrich, Fluka, Nova or BDH and were Analar grade or higher. Oligonucleotides were synthesized in the Biotechnology Unit, University of Leeds. Eyes from freshly killed *Loligo forbesi* were dissected below the lens, and the lens and aqueous humor were discarded. The eye-cups containing the retina were then rinsed twice in buffered saline [600 mM-NaCl/5 mM-Hepes (pH 7.2)/5 mM-EDTA/1 mM-EGTA] and were either rapidly frozen in isopentane cooled with liquid N_2 or were used immediately for preparation of RNA.

Polypeptide purification and sequencing

Photoreceptor outer segments were isolated from the frozen retina by flotation on 40 % (w/v) sucrose [8], and were washed twice in 100 mM-NaCl/5 mM-MgCl₂/10 mM-Hepes (pH 7.2)/1 mM-EGTA. Outer segment membranes were solubilized with 2 % (w/v) sucrose monolaurate in 100 mM-NaCl/5 mM-MgCl₂/10 mM-Hepes (pH 7.2)/1 mM-EGTA (2 h, 40 °C). The insoluble cytoskeleton was removed by centrifugation (9000 g, 5 min). The β -subunit of the visual G-protein was purified using SDS/PAGE [5–20 % (w/v) acrylamide] followed by electroelution [15]. As the intact G- β was N-terminally blocked to Edman degradation, the polypeptide was digested with V8 protease from *Staphylococcus aureus* (Boehringer). G- β was incubated in 67 mM-phosphate buffer (pH 7.4)/0.2 % SDS with *S. aureus* V8 protease [2 % (w/w), 45 min, 37 °C] [16]. The fragments generated by proteolysis were purified by SDS/PAGE and electroelution and were covalently coupled to a solid-phase support for sequencing [15].

Generation of a G- β -specific polynucleotide probe

Total RNA was purified from eye-cups by the method of Chirgwin *et al.* [17]. Poly(A)⁺ RNA was purified by two rounds of affinity chromatography over oligo(dT)-cellulose [18]. This poly(A)⁺ RNA was analysed by electrophoresis through 1 % agarose containing 6 % formaldehyde, and was transferred to Gene-Screen-Plus for Northern hybridization [19]. Double-stranded cDNA was synthesized using the method of Gubler & Hoffman [20]. Gel filtration over Sepharose CL4B was used to separate longer cDNA from unincorporated oligo(dT) primer and cDNA shorter than ~ 500 bp. G- β -specific oligonucleotides were constructed on the basis of the peptide sequence. Two degenerate oligonucleotides: d[C-A-Y-G-A-Y-A-A-Y-A-T-H-A-T-H-T-G-Y-G-G] (20-mer, 5'-primer), and d[R-T-T-R-C-A-R-T-T-R-A-A-R-T-C-R-T-C-R-T-A] (21-mer, 3'-primer), were synthesized. The oligonucleotides were used as primers for amplification of cDNA by the polymerase chain reaction (PCR) (45 °C annealing temperature, 25 cycles) [19]. The PCR product was asymmetrically amplified for a further 15 cycles and was then analysed by direct sequencing using 5'-[³²P]oligonucleotide primer and T7 DNA polymerase. The PCR product was also used to generate a high specific radioactivity probe: [α -³²P]dCTP

Abbreviations used: G-protein, GTP-binding protein; G- α , G- β , G- γ , α -, β - and γ -subunits of G-proteins; SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0; PCR, polymerase chain reaction.

The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X56757.

(3000 Ci/mmol) was incorporated by primer extension, using 20 ng of PCR-amplified cDNA and T7 DNA polymerase (10 min, 37 °C); partial products were extended to the full length of the PCR-generated template by a chase with 100 μ M-dCTP (10 min, 37 °C).

Isolation and sequencing of G- β cDNA

A portion of a λ gt10 cDNA library (10^4 plaques) made from the same squid eye mRNA was transferred to Gene-Screen-Plus and was screened using the PCR-generated probe. Pre-hybridization was carried out in 50% formamide, 6 $\times$ SSC (1 $\times$ SSC = 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0), 0.5% SDS, 1 $\times$ Denhardt's reagent, 6.75 mM-sodium pyrophosphate, pH 6.5, and 100 mg of denatured sheared salmon-sperm DNA/ml (reagents prepared as described in [19]; 42 °C for 2 h). For hybridization, 10 ng of labelled PCR product ($\sim 1 \mu$ Ci/ng) was denatured in boiling water, and was added to the pre-hybridization mix. Hybridization was carried out for 8 h at 42 °C.

Stringency washes were carried out in 0.1 $\times$ SSC/0.5% SDS (3 $\times$ 10 min, 45 °C). Recombinant λ DNA was prepared from small liquid lysates [19] and was digested with *Eco*RI to liberate cDNA inserts. DNA fragments were separated on 1% (w/v) agarose gels and were transferred to Gene-Screen-Plus for Southern analysis [19]. Insert DNA was subcloned into pBluescript for restriction analysis and sequencing. Nested deletions were generated using *Escherichia coli* exonuclease III and S1 nuclease [19]. Double-stranded DNA was prepared for sequencing using T7 DNA polymerase [21].

RESULTS AND DISCUSSION

The squid visual G-protein is present at about 10% of the concentration of rhodopsin in squid retinal outer segments (Fig. 1a). The intact polypeptide (M_r approx. 36000) was recalcitrant to sequencing and therefore it was subjected to limited proteolysis to produce fragments with free N-termini. Sequence data from three peptides generated by digestion with *S. aureus* V8 protease were obtained. It is interesting to note that the cleavage site for Peptide II is not C-terminal to a glutamate or aspartate residue, but to a threonine residue. We have recently observed this cleavage for several electroeluted polypeptides, and believe that it reflects an extended specificity of *S. aureus* V8 protease in the presence of SDS. On the basis of the amino acid sequence (Peptide III), degenerate oligonucleotide primers were constructed for amplification of G- β -specific cDNA. The residues which were not identified in the peptide sequence were assumed to be cysteines by comparison with the mammalian protein (DNA sequencing later showed this to be correct). Amplification of squid eye cDNA by the PCR yielded a single product of about 90 bp. Direct sequencing of the amplified product confirmed that it was an oligonucleotide encoding the sequence obtained for the region of Peptide III between the primer sites. Northern analysis of poly(A)⁺ RNA of the eye-cup, using the PCR-generated probe to G- β (Fig. 1), revealed a single 1.8 kbp species which hybridized stringently. Thus it appears that, unlike the mammalian eye in which at least three distinct G- β subtypes are found [13], only a single transcript size predominates in the cephalopod eye. In the *Drosophila* genome it appears that there is only a single-copy gene which hybridizes with probes based on mammalian G-proteins [14]. Therefore invertebrates may only have a single gene for G- β , which consequently must have a role in a variety of critical transduction pathways.

Approx. 1% of the λ gt10 library contained cDNA which hybridized stringently with the G- β probe. Southern analysis

Fig. 1. Analysis of G- β of squid photoreceptors

(a) SDS/PAGE (stained with Coomassie Blue) of detergent-solubilized squid outer segments showing the relative amounts of G- β and of rhodopsin (rho). (b) Northern analysis of squid eye RNA hybridized to the PCR-generated probe DNA; λ -DNA digested with *Hind*III and the full-length clone (cG- β) were used as markers.

demonstrated that the majority of these cDNA inserts were between 1.3 and 1.8 kbp long. The sequence of one of these contains the single long open-reading frame of 341 amino acids representing the full amino acid sequence of G- β (Fig. 2). Compared with mammalian counterparts, the predicted squid G- β has no inserted or deleted amino acids, apart from a single extra threonine residue immediately after the starting methionine (Fig. 3). The protein has slightly greater similarity to the human and bovine type I protein (87% identity) [11] than to human or bovine type II (84%) [12], *Drosophila* G- β (84%) [14] or human type III (79%) species [13]. The majority of substitutions in the invertebrate G- β s are conservative, with the changes mainly clustered in regions where the mammalian proteins differ from one another. In contrast, the sequence of the yeast protein STE4 [22] is somewhat longer, and is without the extensive regions of very tight sequence conservation characteristic of the other G- β s.

The differences between invertebrate and mammalian proteins mean that approx. 40×10^6 years are required for a 1% change in the amino acid sequence of G- β , more than twice the time required for a similar change of cytochrome *c* [23]. The latter is generally considered to have a highly preserved sequence, reflecting the multiple interactions that it must make. Histones, with an even slower evolutionary rate, make interactions with the regular repeated units of DNA that are thought to be a major factor of their tight conservation. Therefore it might be concluded that the interactions of G- β with G- α and G- γ lead to pronounced conservation of the primary structure of G- β . However, the other subunits of G-proteins, and indeed the receptors to which they are coupled, appear to be much more variable than G- β [24–27]. Moreover, G- β is considerably larger than either cytochrome *c* or the histones and thus, if globular, has a smaller surface/volume ratio, decreasing the degree to which interaction with other macromolecules would be expected to influence sequence conservation. Both of these aspects suggest that G- β may be responsible for further important features of the system not apparent so far.

The conventional model of G-protein-mediated signal-response coupling is that the heterotrimeric G-protein interacts with an activated receptor, resulting in guanine nucleotide exchange at a site on G- α , with dissociation of the whole complex to release active receptor, G- α -GTP and G- $\beta\gamma$. Deactivation of

Fig. 2. Sequence of G- β cDNA (coding strand) determined in both directions from overlapping nested deletions and sub-cloned restriction fragments

The protein encoded by the only long open reading frame is shown above the cDNA. The sequences of proteolytic fragments (Peptides I, II and III) of G- β are indicated below the cDNA sequence. The amino acid sequence used for design of PCR primers is underlined. The sequence 45–1345 obtained from clone 53 was identical with the complete sequence shown, obtained from clone 43.

G- α requires the hydrolysis of GTP and recombination with G- $\beta\gamma$ to form the heterotrimeric protein. In this model, G- $\beta\gamma$ is required for the receptor-mediated activation of G- α . In addition, it has been shown that G- $\beta\gamma$ can inhibit coupling of G- α to its effector enzyme [28,29]. A more active role for G- $\beta\gamma$ has been proposed in the control of phospholipase A₂ [30]. This controversial suggestion has gained support from the activity ascribed to STE4 and STE18, the G- β and G- γ analogues in the yeast mating factor system [22,31]. In yeast, null mutants of the G- α equivalent lead to a state of constitutive mating response, whereas null mutants of STE4 or STE18 result in insensitivity to mating pheromones [22]. However, the sequence of squid G- β is much more similar to the conventional G- β s than to yeast STE4, and there is no evidence that it has such a function. Recently

there have been indications that, although it is soluble, transducin- α does not leave the membrane during phototransduction [32,33]. This may be consistent with another report that the three subunits of G_s do not dissociate from one another during adenylate cyclase activation [34]. If this is true, current views on G-protein coupling and the role of G- β may need revision.

Although three sub-classes of mammalian G- β have been characterized, it is not known whether they are always associated with specific G- γ and G- α , or if they are involved in coupling specific receptor and effector systems [13]. The differences between invertebrate G- β and their mammalian counterparts are not much greater than those between the three sub-classes and are clustered in the same regions of the sequence (Fig. 3). Thus

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MTSELEALRQETELKQKIREAKAAADTTLAMATANVEPV--GRIQMRTRRLRGLHA
...dQ...a...d...C...a...sQI.N.id...
...Q...a...r...d...Cg.s...tQI.Gld...
...g.m.Q...a...K..Ad...C..V...ELVSGI.V...v...
...W..ds...a...s...d...C..s.LQ.atSI..i...
**IQNKI..A...sK..hA..NK.khKIQ.as.FQMANK.Ts1TKhk.nkpNIV.k..NN
KIYAMHWASDRNLVSASQDGLVMDGYTTNKVHAIPLRSSWMTCAYPAGSG-NYVACGG
...gt...L...s...i...s...s...f...
...gt...L...s...i...s...s...f...
...gt...L...s...i...s...s...f...
...gN...L...s...i...s...s...f...
...SDFr.SR..kRil..Fm...sAagl.QN...D.Q..ls..Is..sSTL..Sa
LDNICSYISL-KTREGNVRVSRLEPGHTGYLSCCRFIDNQIVTSSGDMTCALMNIETGNO
...N...t...RvS.ENRVAQN.aSIFK...C.i.DIE.T.nAH.l.a...d.PKAKR
ITSFICHTGDVMSLS**LAFDMRTFVSGDASAKFLIDRGICKQTFTGHESDINAITYFP
T.t.t...s...**..T.L...d...w.v.e.m.r...Cf..
TVg.a...s...nG...i...w.v.sm.r...I...vAf..
K.V.V...C...a**vs..FNL.i...w.v.e.T.r...Cf..
v...L...a...gCK...i...e.v...P...v.f..
vREyad.L...la.a**..ENSSN..A.Cgs.gTtYiw.S.SpSAV.s.YVnd...IRf.K
NGFAFATGDDATCRILFIRADQIEGHS**HDNIICGITSVAFSKSGRLLGGYDDFNCNV
...y..t...l...lMT...s...a...
...N...l...lL...f...a...i...
...E.IC...s...l...lICf...eS...L...Fa...
...Q...l...l...a...a...
d.MsIVa...ngaiNmy.l.s.CS.Atf.**SYLDNQ.vv.ID.A...mYsC.T.IG.V.
MDVLKQERAGVLGHGDNVSCGLVTEGDGMAVATGSMDFLKIIN
...A..Ad...d...
...Am.Gd...d...
...Sm.S..V.i.s...A...
...Tm.A..s.i...n...rv...
...G.IV.K.E..GG..tGVRSsP..l..C...Tm...SPGYQ

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Fig. 3. Comparison of the sequence of squid G- β with human/bovine type I, human type II, human type III, *Drosophila* G- β and yeast STE4

Identity with the residue in the squid protein is denoted by a dot (·). Hyphens correspond to regions where there are insertions of only one or two amino acids in the sequence of STE4, whereas longer insertions in STE4, bearing no similarity to the sequences of the other proteins, are indicated by *. Conservative replacements [35] are denoted by the lower case letter representing the particular amino acid, whereas more radical changes are shown in upper case.

it is tempting to speculate that the type of G- β as well as the type of G- α may be related to G-protein function.

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