Simple Purification and Functional Reconstitution of Octopus Photoreceptor G_q, Which Couples Rhodopsin to Phospholipase C†

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Received June 10, 1996; Revised Manuscript Received August 22, 1996⊗

ABSTRACT: In invertebrate photoreceptors, illuminated rhodopsin activates multiple G proteins, which are assumed to initiate multiple phototransduction cascades. In this paper, we focused on one of the phototransduction cascades, which utilizes rhodopsin, a G_q-like G protein, and phospholipase C (PLC). A G_q-like G protein from octopus photoreceptors was successfully purified to apparent homogeneity as an active form by simple two-step chromatography. The purified G protein had an $\alpha\beta\gamma$-trimeric structure consisting of 44-kDa α, 37-kDa β, and 9-kDa γ subunits. The 44-kDa α subunit was assigned to the G_q class by western blot with antiserum against mammalian $G_q\alpha$ and by partial amino acid sequencing of its proteolytic fragments. Light-dependent binding of GTPγS was observed when the purified octopus G_q was reconstituted with octopus rhodopsin that had been integrated into phospholipid vesicles. Octopus G_q activated PLCβ_1 purified from bovine brain dose-dependently in the presence of AlF_4^-. Finally, light- and GTP-dependent activation of PLCβ_1 was observed in a reconstitution system consisting of octopus rhodopsin, G_q, and bovine PLCβ_1.

Photon absorption by rhodopsin triggers a visual transduction cascade, leading to alteration of ionic permeability of photoreceptor cell membranes. The molecular mechanism of phototransduction of vertebrate photoreceptors is now established that a heterotrimeric GTP-binding protein (transducin) couples the photoexcited rhodopsin to cGMP hydrolysis, which results in cell hyperpolarization due to the closure of cGMP-gated cation channel in the rod plasma membranes (Stryer, 1986). On the other hand, cGMP hydrolysis is not induced by light in invertebrate photoreceptors (Tsuda, 1987a), so the underlying phototransduction machinery appears to be quite distinct from that operating in vertebrate photoreceptors (Tsuda, 1987b). Electrophysiological studies in *Limulus* photoreceptors show that an injection of inositol trisphosphate (IP_3)[1] (Brown & Rubin, 1984; Brown *et al*., 1984; Fein *et al*., 1984; Payne & Fein, 1987) or cGMP (Bacigalupo *et al*., 1991; Johnson *et al*., 1986) mimics the effect of light, leading photoreceptors to depolarization, and suggest that they both could be the final messengers. Moreover, there are three components of the light-activated current in ventral photoreceptor of *Limulus*, which have been reported to have different physical and pharmacological properties (Nagy, 1993). These findings imply the existence of multiple transduction pathways. Further evidence for multiple phototransduction pathways in invertebrate photoreceptors has been shown in *Drosophila* eyes. Phospholipase C (PLC) activity is abundant in normal *Drosophila* eyes while drastically reduced in photoresponse-defective *norpA* mutants (Inoue *et al*., 1985), suggesting that the PLC and phosphoinositide cascade could be involved in phototransduction. On the other hand, recent cloning of *Drosophila* cDNA encoding a cGMP-gated ion channel expressed in eyes and antennae supports the notion that cGMP acts as an intracellular messenger (Baumann *et al*., 1994).

Additional evidence for multiple signaling pathways in invertebrate phototransduction is the finding that multiple G proteins are coupled to illuminated rhodopsin. Light-dependent GTP hydrolysis in invertebrate photoreceptors was first reported for octopus photoreceptor membranes by Calhoon *et al*. (1980). We have found two light-activated octopus photoreceptor G proteins, termed G_{ip} and G_{34}, which are ADP-ribosylated by pertussis toxin (Tsuda & Tsuda, 1990; Tsuda *et al*., 1986). In several invertebrate photoreceptors, pertussis toxin insensitive G proteins have been identified and their cDNAs cloned (Lee *et al*., 1990; Pottinger *et al*., 1991; Ryba *et al*., 1993). Their deduced amino acid sequences indicate marked similarity to mammalian G_q, suggesting that these G proteins carry a photosignal from activated rhodopsin to PLC. Purification of a G_q-like G protein and cloning of its cDNA have been reported for squid photoreceptors by Findlay *et al*. (Pottinger *et al*., 1991; Ryba *et al*., 1993). However, they have not tested its biological ability to couple with rhodopsin and/or PLC. It is indispensable to prove direct interaction among these signal coupling proteins in a defined system.

In this work, we purified a G_q-type G protein in a biochemically active state from an extract of octopus photoreceptor membranes. The purified octopus G_q bound GTP analogue in a light-dependent manner when reconstituted with phospholipid vesicles containing purified octopus

† This work was supported in part by research grants to M.T. (0740819, 08257219) and S.K. (7780579, 8780631) from the Ministry of Education, Science, Sports and Culture of Japan.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: G protein, guanine nucleotide-binding protein; PLC, phospholipase C; APMSF, 4-(amidinophenyl)methanesulfonyl fluoride; App(NH)p, adenosine 5′-($\beta\gamma$-imido)triphosphate; DTT, dithiothreitol; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

S0006-2960(96)01360-8 CCC: $12.00

rhodopsin. In addition, we successfully demonstrated that PLCβ_1 from bovine brain was activated by octopus G_q which was activated by either AlF_4^- or light-activated rhodopsin and GTPγS. The presence of two populations of G_q, tightly membrane-bound and loosely bound, and their biological relevancy are also discussed.

EXPERIMENTAL PROCEDURES

Purification of Octopus G_q from Detergent Extract of Octopus Photoreceptor Microvillar Membranes. Octopus photoreceptor microvillar membranes were prepared from about 200 eyes of *Paraoctopus defrini* as described previously (Tsuda *et al*., 1992). Isolated microvillar membranes were washed three times with 10 mM Tris-HCl (pH 7.4), 0.4 M KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine hydrochloride, 20 μM APMSF (isotonic buffer), and then six times with 10 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM benzamidine hydrochloride, 20 μM APMSF (hypotonic buffer). The microvillar membranes washed with the hypotonic buffer, which contains about 0.8 g of total proteins, were solubilized in 100 mL of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM benzamidine hydrochlroide, 20 μM APMSF, and 1% (w/v) sucrose monolaurate and then centrifuged at 240000*g* for 1 h. The clear supernatant was applied with a plastic syringe to a column (2.5 × 2 cm) of DEAE-cellulose (bps, U.K.), and the column was washed with 50 mL of the solubilization buffer. Rhodopsin, which was the most abundant protein in the extract, passed through the column while G proteins bound under low ionic conditions. G proteins were then eluted with 0.5 M NaCl in 35 mL of the same buffer but containing 1% (w/v) sodium cholate as a detergent. The eluate was concentrated to about 3 mL by an ultrafiltration system of NM-3 (Asahikasei, Tokyo) and applied to a Sephacryl S-300 HR gel filtration column (2.2 × 90 cm, Pharmacia Biotech Inc.) which had been equilibrated with 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 mM benzamidine hydrochloride, 20 μM APMSF, 1 μM GDP, and 1% sodium cholate at 0.7 mL/min. The eluate was collected in each fraction of 7 mL, and the fractions were assayed by western blot with anti-$G_q\alpha$ and Gβ antibodies. For separation of α and $\beta\gamma$ subunits, trimeric G_q fractions from Sephacryl S-300 were diluted 3-fold with 20 mM Tris-HCl (pH 7.4), 1 mM DTT, and 0.1% sucrose monolaurate. The diluted sample was applied to high-resolution Mono Q PC 1.6/5 anion exchange column for the SMART System (Pharmacia Biotech Inc.), which had been equilibrated with 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 40 mM NaCl, and 0.1% sucrose monolaurate at 0.1 mL/min. The subunits were eluted with a 4-mL linear gradient of 40−400 mM NaCl in the same buffer, and the eluate was collected in fractions of 0.2 mL.

The loosely bound form of octopus G_q used in GTPγS-binding experiments was partially purified from the soluble fraction of isotonic wash of the microvillar membranes by essentially the same chromatography procedures. G_q and its subunits thus purified to apparent homogeneity were stored at −80 °C until use.

Amino Acid Sequencing. The subunits of the purified G_q were separated on SDS−PAGE (11%), and the bands of $G_q\alpha$ in the gel were cut out. The protein (about 1 nmol) was electrically eluted from gels, dialyzed against 8 M urea, and digested overnight by *Achromobacter* protease I (EC 3.4.21.50) in 50 mM Tris-HCl (pH 9.5) and 4 M urea. The digested peptides were separated by reversed phase HPLC, and amino acid sequences of the peptides in the peak fractions were analyzed by model 473A Protein Sequencer (Applied Biosystems).

Photoaffinity Labeling of G Proteins in Octopus Microvillar Membranes. Photoaffinity probe, [γ-^{32}P]-4-azidoanilido-GTP was prepared as described by Thomas and Pfeuffer (1991). Microvillar membranes (about 12.5 μg of total protein), which had been either kept in dark or illuminated by a panchromatic light, were incubated with 1 μM [γ-^{32}P]-4-azidoanilido-GTP at 15 °C for 30 min in the presence or absence of 100 μM cold GTP in 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM GMP, and 0.1 mM App(NH)p. The reaction was terminated by dilution with 10 volumes of an ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM $MgCl_2$, and 100 mM NaCl (referred to henceforth as TMN). The membranes in the mixture were collected by centrifugation and then resuspended in 20 μL TMN, and the bound [γ-^{32}P]-4-azidoanilido-GTP was covalently attached by UV-irradiation at 254 nm on ice for 5 min. The samples were solubilized with 20 μL of SDS−PAGE sample buffer, and the radioactivity incorporated into proteins was visualized by autoradiography after SDS−PAGE.

Reconstitution of Octopus Rhodopsin in Phospholipid Vesicles. Octopus rhodopsin was affinity purified from the flow-through fraction of DEAE-cellulose column on ConA Sepharose (Pharmacia Biotech). The rhodopsin-containing fraction was applied to a ConA column which was equilibrated with 10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.1% sucrose monolaurate, and the column was thoroughly washed with the equilibrate buffer. Rhodopsin was then eluted from the column with 250 mM α-methyl-D-mannopyranoside in equilibrate buffer.

Reconstitution of the rhodopsin in phospholipid vesicles was carried out as described by Cerione *et al*. (1984). Briefly, the purified rhodopsin (approximately 8 μM) in a buffer containing 2.5 mg/mL azolectin (Sigma), 1% (w/v) octyl glucoside, and 1 mg/mL BSA was passed through a 1-mL column of Extractigel-D (Pierce) which had been equilibrated in TMN. Turbid fractions after void volume were collected. Rhodopsin in the reconstituted vesicles retained an ability to convert to metarhodopsin upon illumination.

GTPγS-Binding Assay. The reaction mixture containing octopus G_q (about 45 nM), 0.5 μM [^{35}S]GTPγS (4×10^3 cpm/pmol), and the rhodopsin-reconstituted vesicles (400 nM rhodopsin) was incubated at 20 °C in the dark or light in TMN. At the indicated time, a 20-μL aliquot was withdrawn and diluted with 250 μL of ice-cold TMN further supplemented with 1 μM cold GTPγS. The diluted mixture (the final concentration of sucrose monolaurate was below 0.002%) was filtered through nitrocellulose filters, and the filters, after being washed three times with ice-cold TMN, were counted for ^{35}S by liquid scintillation. At least 99% of $G_q\alpha$ applied was held on the filters under these conditions.

Measurement of PIP_2 Hydrolysis. Assay for hydrolysis of PIP_2 was conducted as described by Smrcka *et al*. (1991). Briefly, reaction was initiated by addition of PLCβ_1 purified from bovine brain membranes to the mixture containing 50 μM [^{3}H]PIP_2, 500 μM phosphatidylethanolamine, 0.16%

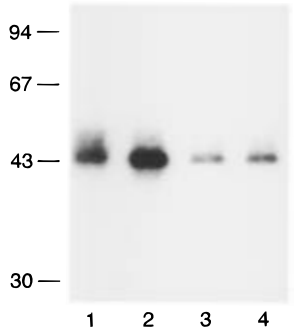

FIGURE 1: Photoaffinity labeling of a light-activated G protein in octopus photoreceptor membranes. The microvillar membranes were incubated with 1 μM [γ-^{32}P]-4-azidoanilido-GTP in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 100 μM GTP in the dark (lanes 1 and 3) or light (lanes 2 and 4). [γ-^{32}P]-4-Azidoanilido-GTP bound to proteins was covalently attached by UV irradiation, and the samples were analyzed as described under Experimental Procedures.

sodium cholate, 0.83 mM $MgCl_2$, 20 μM $AlCl_3$, 6 mM NaF, and various concentrations of the purified G_q. After incubation at 30 °C for 10 min, the reaction was terminated by addition of a chloroform/methanol/0.1 N HCl (40:60:1, by volume) mixture and vortex mixing. Chloroform and 0.1 N HCl were further added, and then a portion of the aqueous phase, which contains hydrolyzed IP_3, was measured by liquid scintillation counter for ^{3}H. To measure rhodopsin-dependent activation of PLCβ_1, octopus G_q was preincubated with rhodopsin vesicles and nucleotides in the dark or light, and assays were carried out without $AlCl_3$ and NaF.

Electrophoresis and Immunoblot Analysis. SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Protein blotting to PVDF membrane was performed as Towbin's method (1979) using a tranfer buffer containing 0.1% (w/v) SDS and 15% (v/v) methanol. For immunological detection, horseradish peroxidase-conjugated anti-IgG antibodies and the ECL chemiluminescence detection system (Amersham Corp.) were used according to manufacturer's directions.

Miscellaneous. Purified bovine brain PLCβ_1 and anti-$G_q\alpha$ polyclonal and anti-Gβ (porcine brain) monoclonal antibodies were generous gifts from Dr. T. Haga (Institute for Brain Research, University of Tokyo), and anti-Gβ (*Drosophila*) monoclonal antibody was kindly provided by Dr. J. B. Hurley (University of Washington); sources of antigen and their immunological specificity were described elsewhere (Nakamura *et al*., 1991; Yarfitz *et al*., 1991). Sephacryl S-300 HR, Mono Q, and ConA Sepharose were purchased from Pharmacia Biotech Inc., DEAE cellulose was from Funakoshi (Tokyo), and Extractigel-D was from Pierce. [γ-^{32}P]GTP, [^{35}S]GTPγS, and [^{3}H]PIP_2 were obtained from DuPont-New England Nuclear. *Achromobacter* protease I was purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents used were the highest grade commercially available.

RESULTS

G_q-Like G Protein in Octopus Photoreceptors. To identify light-activated G proteins in octopus photoreceptor microvillar membranes, we carried out photoaffinity labeling of the membrane proteins with [γ-^{32}P]-4-azidoanilido-GTP. As shown in Figure 1, a 44-kDa protein was radiolabeled upon illumination of the membranes, and labeling was markedly decreased by addition of an excess amount of cold GTP in the mixture. These results suggest that the 44-kDa protein specifically binds GTP and that this protein was activated by illuminated rhodopsin. Photoaffinity labeling by [γ-^{32}P]-4-azidoanilido-GTP of the 41-kDa α subunit of G_{ip}, which is ADP-ribosylated by pertussis toxin in a light-dependent manner (Tsuda *et al*., 1986), could not be detected in these experiments probably due to its lower abundance in microvillar membranes.

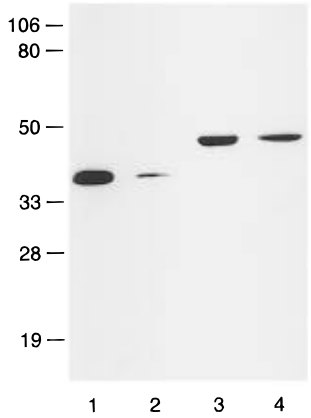

FIGURE 2: Western blot of octopus photoreceptor membrane proteins with anti-$G_q\alpha$ and anti-Gβ antibodies. The microvillar membranes were washed with 10 mM Tris-HCl buffer (pH 7.4) containing 0.4 M KCl, 10 mM $MgCl_2$, 1 mM DTT, and 20 μM APMSF. Particulate (lanes 1 and 3) and solubilized (lanes 2 and 4) fractions were subjected to western blot with an antiserum against the synthetic peptide corresponding to the C-terminal region of mouse $G_q\alpha$ (lanes 3 and 4) or a monoclonal antibody against Gβ purified from porcine brain (lanes 1 and 2).

Figure 2 shows an immunoblot of microvillar membranes with antibodies recognizing the subunits of G_q. The antiserum raised against a synthetic peptide corresponding to the carboxyl terminus of mammalian $G_q\alpha$ strongly crossreacted with the 44-kDa protein; thus the 44-kDa protein shares antigenic determinants common to mammalian $G_q\alpha$. In addition to the presence of the 44-kDa α subunit, a 37-kDa protein was detected in microvillar membranes by monoclonal antibodies against Gβ. Interestingly, as shown in Figure 2, $G_q\alpha$-like immunoreactivity was detected not only in the membrane-bound fraction but also in the soluble supernatant of the retinal homogenate. From these results, a fraction of the octopus photoreceptor G_q pool, unlike mammalian $G_q\alpha$, exists as a loosely bound membrane protein, which is readily extracted in the solution without detergent. On the other hand, the pertussis-toxin substrate was solely detected in the membrane fraction (Tsuda & Tsuda, 1990; Tsuda *et al*., 1986).

Purification of Octopus G_q from Detergent Extract of Photoreceptor Microvillar Membranes. To further characterize octopus photoreceptor G_q, we intended to purify it from microvillar membranes. We started with a 1% sucrose monolaurate extract of washed microvillar membranes, which we had also been utilizing as a starting material for purification of rhodopsin. The extract was loaded onto a DEAE-cellulose column, through which rhodopsin passed. G_q bound to the DEAE-cellulose and eluted from the column with an elution buffer containing 0.5 M NaCl and 1% sodium cholate. The detergent was exchanged to sodium cholate at this step because cholate makes relatively small micelles and is suitable for the next purification step, gel filtration chromatography.

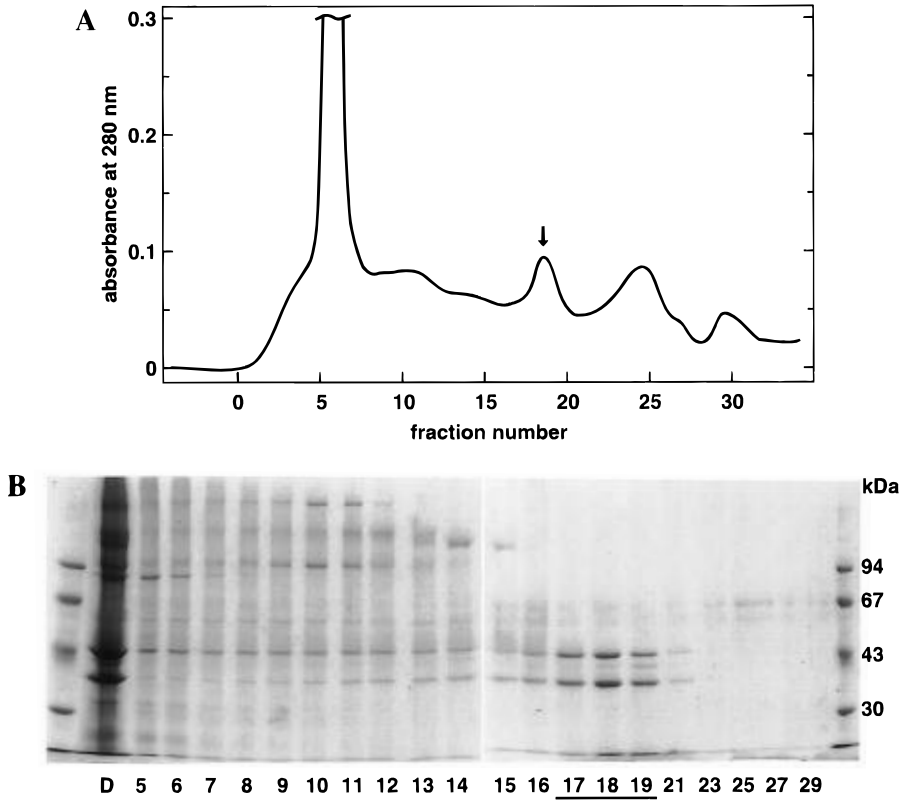

FIGURE 3: Elution profile of octopus G_q from a Sephacryl S-300 HR column. The concentrated extract from octopus photoreceptor microvillar membranes was applied to a Sephacryl S-300 HR column (2.2 × 90 cm). (A) Absorbance at 280 nm of eluted proteins was monitored. The peak containing G_q detected by the antibodies is indicated by an arrow. (B) SDS−PAGE patterns of the fractions on 11% polyacrylamide gels. Lane D, DEAE cellulose eluate; lanes 5−29, fractions from Sephacryl S-300. Pooled fractions for the purified protein are underlined.

The eluate was concentrated and next applied to a Sephacryl S-300 HR gel filtration column. The elution profile of proteins from the column is shown in Figure 3A. Fractions were assayed by western blotting with anti-$G_q\alpha$ and Gβ antibodies (data not shown). The proteins that crossreacted with anti-$G_q\alpha$ and Gβ antibodies were eluted from the Sephacryl S-300 HR column in the same fractions (Figure 3B, fractions 17−19). G_{ip} was not detected at all in these fractions according to pertussis toxin-catalyzed ADP-ribosylation assay, which is the most sensitive method to detect G_{ip}. Thus, purification to apparent homogeneity was achieved in this step, although a minor 42-kDa degradative product of $G_q\alpha$ was occasionally observed. The pooled fractions were used as a purified G_q preparation in later experiments.

Separation of α and $\beta\gamma$ subunits was performed by high-resolution anion exchange Mono Q PC 1.6/5 column chromatography. The G_q trimer fraction eluted from the Sephacryl S-300 HR column was diluted 3-fold and loaded onto a Mono Q column equilibrated with 20 mM Tris-HCl (pH 7.4), 40 mM NaCl, 1 mM DTT, and 0.1% sucrose monolaurate. Elution was performed with a 4-mL linear concentration gradient of 40−400 mM NaCl. The elution profile of the subunits is shown in Figure 4A. The α and $\beta\gamma$ subunits were eluted at about 280 and 160 mM NaCl from the Mono Q column, respectively.

When the purified G protein was analyzed by SDS−PAGE and stained with Coomassie blue, and α and β subunits of ocotpus G_q migrated as 44- and 37-kDa polypeptides, respectively (Figure 5A). The purified G_q preparation also contained a low molecular mass 9-kDa polypeptide, which is assumed to be the γ subunit from its size (Figure 5B).

Amino Acid Sequence of Octopus $G_q\alpha$. As we failed to identify the amino-terminal sequence of intact $G_q\alpha$ probably due to some modification, we digested the purified full-length protein with *Achromobacter* protease I, a lysine-specific endoprotease. Sequencing the fragmented peptides separated by reversed phase HPLC, we obtained the amino acid sequences over 250 residues. These data were aligned with several deduced amino acid sequences of cloned $G_q\alpha$ as shown in Figure 6. The sequence of the fragments from octopus $G_q\alpha$, which covered some 70% of the predicted size, exhibited high homology to $G_q\alpha$ of several species.

Activation of GTPγS-Binding to the Purified Octopus G_q by Illuminated Rhodopsin. To test whether the purified G_q binds to GTP analogue in a rhodopsin-dependent manner, we conducted GTPγS-binding experiments in the presence of the reconstituted rhodopsin vesicles. Figure 7 shows the time course of GTPγS-binding to the purified octopus G_q. Basal GTPγS-binding in the absence of rhodopsin was not observed. In contrast, GTPγS-binding to the purified G_q occurred in the presence of the phospholipid vesicles containing rhodopsin. These results clearly show that the purified octopus G_q coupled with and was activated by octopus rhodopsin. Next, we compared the potency to couple with rhodopsin between two populations of G_q; tightly bound G_q that had been purified from sucrose monolaurate extract of the washed microvillar membranes, and loosely bound G_q that was readily extracted from the membranes by washing with the isotonic buffer. As shown in Figure 7, GTPγS bound to the tightly bound G_q much faster than the loosely bound G_q, suggesting that the tightly bound G_q couples more potently with illuminated rhodopsin.

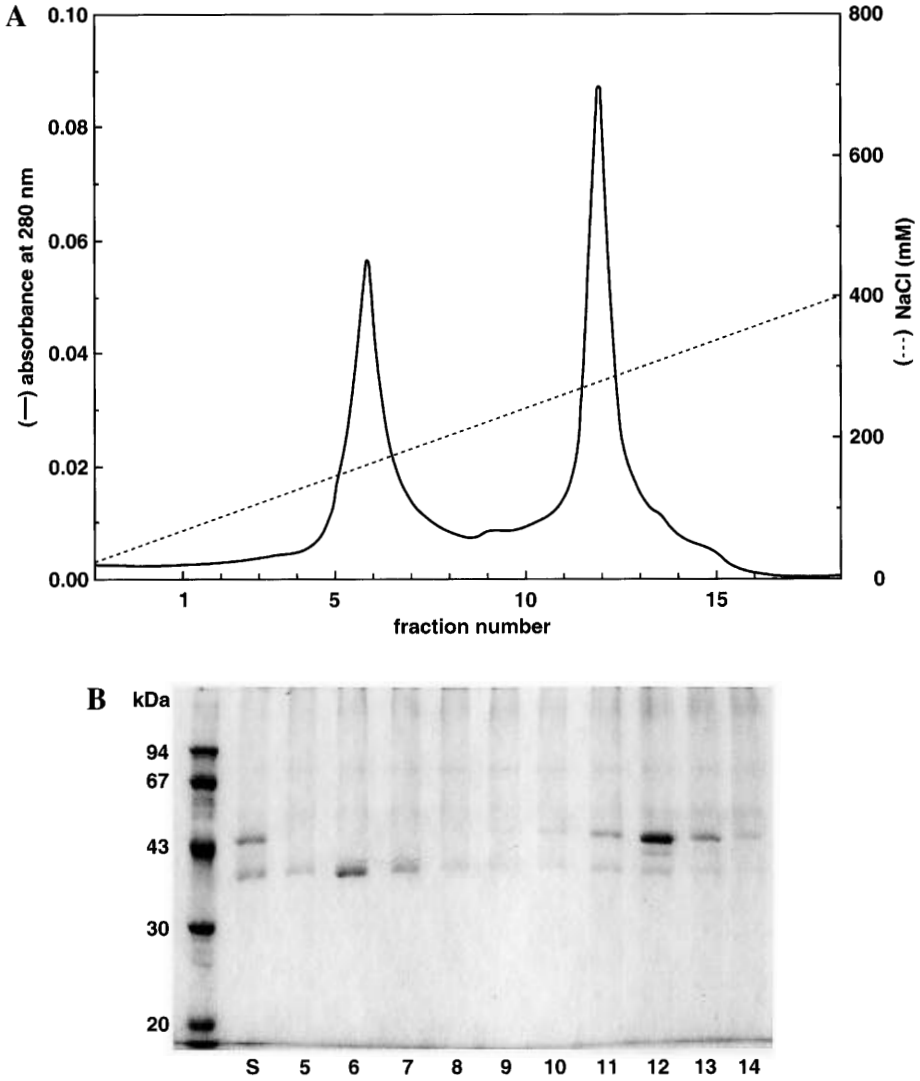

FIGURE 4: Separation of α and $\beta\gamma$ subunits of octopus G_q with a Mono Q PC 1.6/5 column. The purified trimeric G_q was applied to and eluted from a high-resolution anion exchange Mono Q PC 1.6/5 column (1.6 × 50 mm). (A) Absorbance at 280 nm of eluted proteins was monitored. (B) SDS−PAGE patterns of the fractions. Lane S, purified G_q; lanes 5−14, fractions from the column.

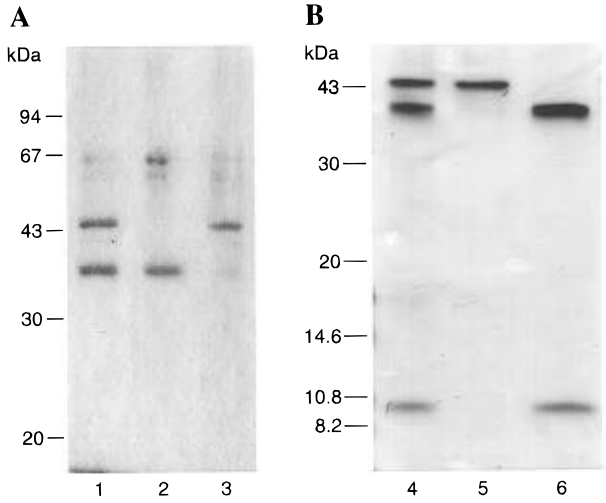

FIGURE 5: SDS−PAGE of purified octopus G_q and the subunits. The purified proteins (2 μg) were analyzed by SDS−PAGE on either 11% (A) or 16% (B) gel and stained with Coomassie Blue. Lanes 1 and 4, G_q; lanes 3 and 5, α subunit; lanes 2 and 6, $\beta\gamma$ subunits.

Activation of Bovine Phospholipase C by Activated Octopus G_q. Our next attempt was to examine whether the purified octopus G_q directly activates PLCβ. We investigated PIP_2 hydrolysis by PLCβ_1, which had been purified from bovine brain membrane extract, in the presence of activated octopus G_q. As shown in Figure 8, octopus G_q, in the presence of AlF_4^-, activated bovine PLCβ_1 in a dose-dependent manner, which is comparable to that of mammalian G_q (Smrcka *et al.*, 1991). G_q alone, even at a maximum dose, exerted only slight activation without AlF_4^-. To address whether octopus G_q can couple photoactivated rhodopsin with PLC, we next tested light-activation of PLCβ_1 in the presence of the reconstituted octopus rhodopsin and G_q. G_q was incubated with GTPγS or GDP in the presence of rhodopsin vesicles prior to the assay. The entire assay including preincubation was carried out under either dim red light (dark) or room light (light). The results were shown in Figure 9. In the reconstituted systems containing purified G_q and rhodopsin, PLCβ_1 activity was markedly enhanced upon illumination of rhodopsin in the presence of GTPγS but not GDP. When an assay was conducted in the dark, only slight activation of PLCβ_1 was observed even in the presence of GTPγS, indicating that activation of G_q with GTPγS depends on illuminated rhodopsin. The extent of light-dependent activation of PLCβ_1 in the presence of GTPγS was comparable to that of receptor-independent activation in the presence of AlF_4^-.

FIGURE 6: Amino acid sequences of the digested peptide fragments from octopus $G_q\alpha$ aligned with the sequences deduced from squid and mouse $G_q\alpha$ cDNA. Purified octopus $G_q\alpha$ was digested with *Achromobacter* protease I, and the digested fragments were sequenced as described under Experimental Procedures. Sequence data were searched for similarity against the SWISS-PROTEIN data bank. Sequence alignment was shown with squid and mouse G_q. The asterisk (*) and the dot (·) added above indicate identical position and conservative substitution, respectively. Conservative substitutions are grouped as follows: cysteine; serine, threonine, proline, alanine, and glycine; asparagine, aspartic acid, glutamic acid, and glutamine; histidine, arginine, and lysine; methionine, isoleucine, leucine, and valine; phenylalanine, tyrosine, and tryptophan.

DISCUSSION

In this work, we have first purified a G_q-class G protein in an active state from invertebrate photoreceptors, which couples with illuminated rhodopsin. Purification to apparent homogeneity was achieved from a sucrose monolaurate extract of retinal microvillar membranes by simple two-step chromatography. Successful purification is attributed to use of thoroughly washed membranes as a source. In addition, use of sodium cholate, which makes relatively small micelles suitable for gel filtration, facilitated better separation in Sephacryl S-300 chromatography. The purified protein consisted of α, β, and γ subunits whose molecular masses on SDS−PAGE were 44, 37, and 9 kDa, respectively (Figure 5). The 44-kDa α subunit strongly crossreacted with the antibody raised against the synthetic peptide corresponding to C terminus of mouse $G_q\alpha$. This suggests that the purified octopus G protein is a member of the G_q family. Occasionally, a 42-kDa protein, which crossreacts with anti-$G_q\alpha$ antibody, was observed in the G_q preparation (see Figure 3B). From amino-terminal sequence analysis, we identified this 42-kDa protein as a proteolytic product of 44-kDa $G_q\alpha$ subunit, which is likely to occur artificially during the preparation. The similarity to G_q was further confirmed by amino acid sequencing of the α subunit of the purified protein. A striking identity to deduced amino acid sequences from cloned cDNA of other species was observed over entire regions of some 250 residues sequenced from the purified octopus protein (Figure 6). From these results we concluded that the octopus photoreceptor G protein that we purified belongs to the G_q family. In addition, antibodies that recognize mammalian and *Drosophila* Gβ equally crossreacted with the 37-kDa β subunit of octopus G_q, which shows structural similarity among those polypeptides, although the β subunit of octopus G_q has a slightly larger size than those of mammalian G proteins. The size of the γ subunit of ocotopus G_q is also in good agreement with those of other G proteins.

Identification of G proteins in invertebrate photoreceptors has been reported for several species, and a G_q-class G protein has been isolated from squid photoreceptor (Pottinger *et al.*, 1991). However, functional coupling of those G proteins with rhodopsin has not yet been reported in a defined system. In this study we demonstrated light-dependent activation of GTPγS-binding to purified octopus G_q in a well-defined reconstitution system. As shown in Figure 7, GTPγS-binding to purified G_q was observed upon illumina-

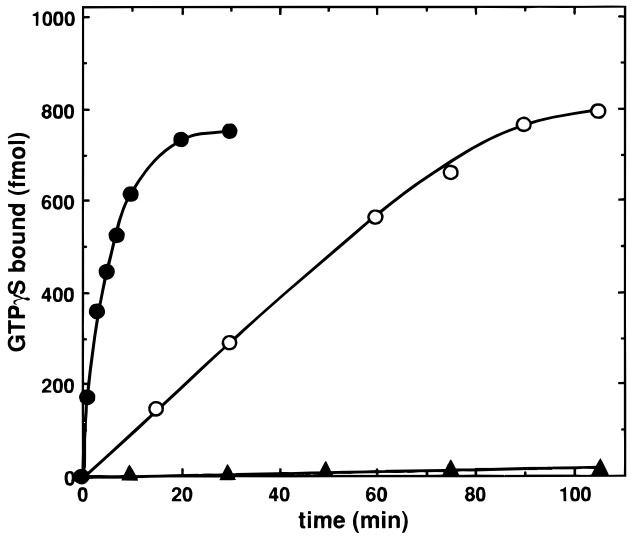

FIGURE 7: Time course of GTPγS binding to the tightly or loosely bound G_q in the presence of the rhodopsin-reconstituted vesicles. Octopus G_q (about 45 nM), either purified from the sucrose monolaurate extract (●) or partially purified from the isotonic buffer extract (○), was incubated with 0.5 μM [^{35}S]GTPγS (4×10^3 cpm/pmol) and the rhodopsin-reconstituted vesicles (400 nM rhodopsin) at 20 °C in light. At the indicated time, an aliquot was withdrawn and GTPγS bound was assayed as described under Experimental Procedures. Specific binding was not detected in the absence of rhodopsin in either case (▲).

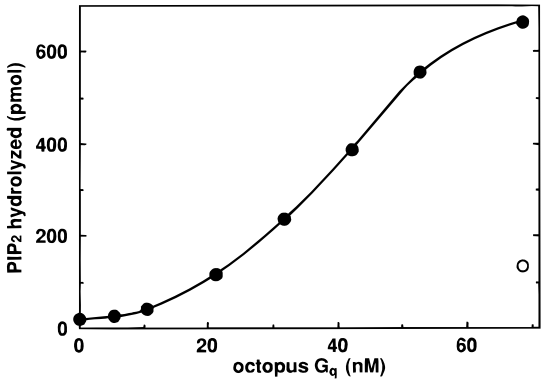

FIGURE 8: Activation of bovine brain PLCβ_1 by AlF_4^--activated octopus G_q. PIP_2 hydrolysis catalyzed by purified bovine brain PLCβ_1 was assayed in the presence of indicated concentrations of purified octopus G_q with (●) or without (○) AlF_4^- as described under Experimental Procedures.

tion of the purified octopus rhodopsin, which has been reconstituted into phospholipid vesicles. No significant binding was observed in the control experiments in which rhodopsin was not included, These results clearly show that purified octopus photoreceptor G_q directly couples with and is activated by illuminated rhodopsin in a reconstitution system.

We found that a part of octopus photoreceptor G_q is readily stripped off the microvillar membranes by washing membranes with an isotonic buffer (Figure 2). To address the biological relevancy of this, we compared the ability of the tightly bound G_q (i.e., purified from the detergent extract) and the loosely bound G_q to couple with rhodopsin. The results demonstrated in Figure 7 show that the tightly bound G_q is more potent in coupling with rhodopsin than the loosely bound G_q. It has been reported in expressed cell systems that mammalian G_q undertakes palmitoylation at cysteine residues near the amino terminus and that dynamic palmi-

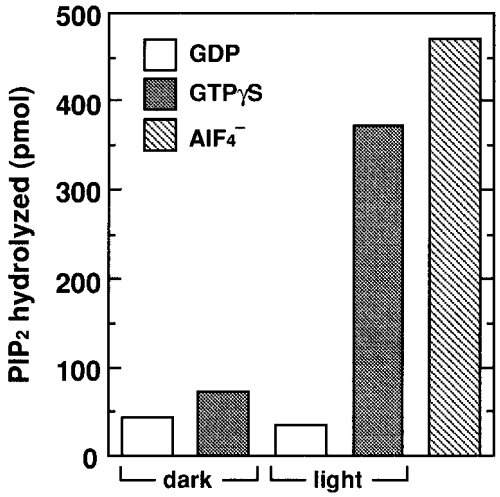

FIGURE 9: Light activation of bovine brain PLCβ_1 in the presence of purified octopus rhodopsin and G_q. Purified octopus G_q was preincubated at 20 °C with 50 μM GDP (open bar) or GTPγS (filled bar) in the presence of rhodopsin vesicles for 20 min in the dark or light. To the mixture was added an assay solution containing PIP_2, and then hydrolysis was initiated by adding PLCβ_1. IP_3 released was assayed as described under Experimental Procedures. As a positive control, PLC activity was measured in the presence of G_q activated by AlF_4^- without rhodopsin vesicles as shown in Figure 8 (hatched bar).

toylation may regulate membrane localization and physiological activity of G_q (McCallum *et al*., 1995; Parenti *et al*., 1993; Wedegaertner *et al*., 1993). Since we have not yet been able to isolated the amino terminus peptide from *Achromobacter* protease I fragments of $G_q\alpha$, we have not determined if octopus G_q is acylated. However, a highly conserved amino terminal region among cloned G_q suggests that octopus G_q is also palmitoylated. Thus, the dynamic state of acylation, especially palmitoylation, may alter the affinity of G_q for microvillar membranes. Interestingly, Terakita *et al*. have reported light-dependent translocation of G_q from a membrane region to the cytosol in crayfish photoreceptors (Terakita *et al*., 1996). Together with those findings, our results suggest that octopus G_q may be present in two forms, acylated or deacylated, in photoreceptors, and that light stimulation may cause deacylation of G_q, resulting in a decrease in its potency to couple with rhodopsin. It has been also reported that deacylated G_q loses its ability to activate PLC (Wedegaertner *et al*., 1993), and we are now investigating this point for octopus proteins.

In many tissues and cultured cell systems, it has been reported that stimulation of the receptor which couples with G_q-type G proteins leads to activation of the β isoform of PLC. Furthermore, PLC is thought to be a candidate for the effector enzyme that is activated upon photoexcitation in invertebrate photoreceptors. To clarify whether the purified octopus G_q has an ability to activate PLC, we conducted reconstitution experiments in which PLCβ_1 purified from bovine brain was used as the coupling enzyme. As clearly demonstrated in Figure 8, purified octopus G_q, in the presence of AlF_4^-, activated bovine PLCβ_1 in a dose-dependent manner. The potency of octopus G_q to activate PLCβ_1 was comparable to those of mammalian G_q reported (Smrcka *et al*., 1991). Thus, octopus G_q is likely to share common structural and functional properties with mammalian G_q.

Next, we tried light-dependent activation of PLCβ_1 in the presence of octopus G_q and rhodopsin. Figure 9 shows activation of PLCβ_1 by light in the reconstitution system. PIP_2 hydrolysis was enhanced upon illumination of rhodopsin in the presence of GTPγS. This is apparently due to formation of active GTPγS-bound $G_q\alpha$ stimulated by illuminated rhodopsin, since no significant activation was observed either in the presence of GDP instead of GTPγS nor in experiments conducted in the dark. These results indicate that octopus G_q activates the β isoform of PLC in a receptor-dependent manner and strongly suggests that octopus G_q carries signals from activated rhodopsin to PLC in photoreceptor cells.

Several problems still remain to be solved. In this work, we used a bovine PLCβ_1 isoform, which is activated by the α subunit but not by $\beta\gamma$ subunits of G_q, while *Drosophila* NorpA has been reported to share high sequence homology with the mammalian PLCβ_4 isoform. Therefore, molecular characteristics of the octopus photoreceptor PLC are of our interest. We have succeeded in purifying endogenous octopus photoreceptor PLC (unpublished experiments) and are now attempting to isolate the cDNA encoding it. In addition, we have identified a pertussis toxin-sensitive G protein, G_{ip}, in octopus photoreceptors. Since ADP-ribosylation of G_{ip} by pertussis toxin is light-dependent, G_{ip} also appears to couple with rhodopsin (Tsuda & Tsuda, 1990; Tsuda *et al*., 1986). Thus, the possible relevancy of signaling pathways mediated by G_q and G_{ip} in phototransduction is also of our interest.

In conclusion, we hereby report for the first time the coupling of the invertebrate photoreceptor rhodopsin to PLC via a G protein in a defined reconstitution system, and this will pave the way for precise understanding of the molecular mechanism of invertebrate visual signal transduction.

ACKNOWLEDGMENT

We thank Dr. T. Haga for his generous gifts of purified PLCβ_1 enzyme and specific antibodies and Dr. J. B. Hurley for providing an anti-Gβ antibody and critical reading of the manuscript.

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BI961360V