Kinship of cephalopod photoreceptor G-protein with vertebrate transducin

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G-protein, a signal coupling protein in invertebrate photoreceptors, was characterized by toxin-labeling and antibody-binding experiments. A 41 kDa protein of octopus photoreceptors is specifically ADP-ribosylated by pertussis toxin. Labeling is maximal in the dark in the presence of GDPβS, as observed with vertebrate transducin. Furthermore, an antiserum prepared against the β- (35 kDa) and γ- (8 kDa) subunits of bovine transducin cross-reacts with a 36 kDa protein in octopus photoreceptors. These results indicate that the α- and β-subunits of octopus photoreceptor G-protein are akin to those of vertebrate transducin and other G-proteins.

G-protein Transducin (Cephalopod) Photoreceptor

1. INTRODUCTION

In vertebrate rods the participation of cGMP in photoreception is mediated by a light-activated enzymatic cascade [1,2]. Transducin, a member of the G-protein family, is the amplified signal-coupling protein in this cascade [3–5]. Although less is known about the molecular mechanism of phototransduction in invertebrates, some common features are becoming evident. The finding of a light-activated GTPase activity in octopus, squid, and fly photoreceptors suggests that a transducin-like protein also participates in invertebrate vision [6–8]. Moreover, octopus and squid photoexcited rhodopsin can trigger the activation of mammalian transducin [7,9,10]. The octopus is evolutionarily far from the vertebrates. Thus, it is of great interest to ascertain how much of the transduction machinery is preserved in these two visual systems. Here we have explored homologies between invertebrate and vertebrate photoreception by studying the labeling of octopus photoreceptors by pertussis toxin [11] and antibodies.

2. MATERIALS AND METHODS

2.1. *Preparation of octopus photoreceptor membranes*

Freshly dissected octopus eyes were hemisected, the surface of retinas washed with buffer A (400 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-Cl, pH 7.4, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride), the retinas removed and homogenized with a small volume of buffer A. Microvillar membranes were purified by mixing the homogenized retinas in 40% sucrose in buffer A (40 ml per 20 retinas) and placing this mixture in a 50 ml centrifuge tube with 1 ml buffer A layered on top. After centrifugation (18000 rpm, 1 h) the pellet was discarded and the entire supernatant mixed so that it had a volume of approx. 38 ml with a sucrose concentration of approx. 38%. This

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was transferred to a new centrifuge tube, another 1 ml buffer A layered on top and the sample recentrifuged. This procedure, with each flotation step leading to slight dilution of the sucrose concentration, was repeated 4–6 times until a black spot was absent from the bottom of the tube. The supernatant (now in about 34% sucrose) was then diluted with an equal volume of buffer A and centrifuged. The pellet contained crude microvillar membranes (MV). The microvillar membranes were then washed with 10 vols buffer A. The supernatant (SA) from the first wash was used for recombination experiments. The washing with buffer A (40 ml) was continued 4 times to give a preparation of microvillar membranes devoid of many soluble proteins (MVA). This preparation was then washed 4 times with 40 ml buffer B (5 mM Tris-Cl, pH 7.4; 1 mM DTT, 0.2 mM PMSF). The pellet from these low ionic strength washings formed two distinct layers: a heavier layer (MVBH) and a lighter layer (MVBL). The lighter fraction was washed once with 10 ml of 200 μM GTP in buffer B, then 3 times with buffer B to remove residual GTP, to give a GTP-washed membrane fraction (MVBG).

2.2. *ADP-ribosylation by pertussis toxin*

Membrane fractions were incubated with [^{32}P]NAD (5 μM, 1 Ci/mmol) and 100 μg/ml pertussis toxin in 100 μl ADP-ribosylation buffer (50 mM Tris-HCl, pH 7.5; 10 mM thymidine, 1 mM EDTA, 2 mM dithiothreitol) at 23°C for 20 min. The reaction was quenched by adding 1 vol. gel sample buffer. After an overnight incubation the membrane fractions were run on 12% SDS-polyacrylamide gels.

2.3. *Preparation of antiserum Tα and Tβγ and Western blot*

Transducin was purified from bovine retinas and its α- and $\beta\gamma$-subunits separated by ω-aminooctylagarose chromatography [13]. Polyclonal antibody was raised in BALB/C mice by immunization with the purified Tα or T$\beta\gamma$ emulsified in Freund's complete adjuvant. Mice were boosted with the proteins 30 days later, and after 3 days, antisera were obtained. Western blots were performed by transferring proteins from a slab gel to a nitrocellulose sheet by electroelution at 7 V/cm in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol. The sheet was cut out and subjected to an enzyme immunoassay. Each strip was blocked by TBS (50 mM Tris-HCl, 200 mM NaCl, 2 mM $MgCl_2$, pH 7.4) containing 3% (w/v) bovine serum albumin, and reacted with mouse antiserum. Peroxidase-labeled goat anti-mouse IgG was then added as a second antibody. After each sheet was washed with TBS containing 0.05% (v/v) Tween 20, substrate solution (0.05% diaminobenzidine, 0.03% hydrogen peroxide in TBS) was added to reveal the bands on the gels that reacted with the antibody.

3. RESULTS AND DISCUSSION

Fig.1 shows the radiolabeling in the dark by pertussis toxin and [^{32}P]NAD$^+$ of proteins from several different preparations of photoreceptors, freshly isolated from dark-adapted eyes of octopus (*Paroctopus defleini*). Whole retinal tissue (lane 1) was not radiolabeled. However, microvilli isolated by sucrose flotation (MV) contained a weakly labeled 41 kDa polypeptide (lane 2). This band increased in intensity when whole microvilli were

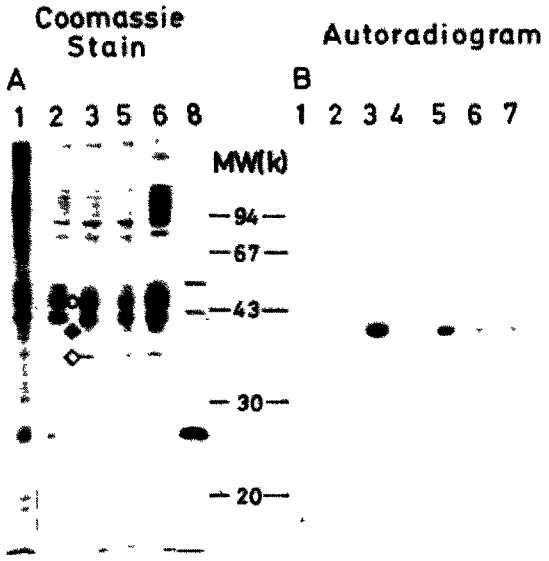

Fig.1. Labeling of octopus photoreceptor proteins by pertussis toxin. (A) Coomassie blue-stained gel of photoreceptor membrane fractions. (B) Autoradiogram of pertussis toxin-labeled fractions. Lanes: 1, retina; 2, MV; 3, MVA; 4, MVA + SA; 5, MVBH; 6, MVBL; 7, MVBG; 8, SA; (○) octopus rhodopsin, (♦) Gα, (◇) Gβ.

washed several times in modified physiological cephalopod saline (MVA, lane 3), suggesting that an inhibitory component was removed by washing. Indeed, when the supernatant from the initial wash (SA) was recombined with MVA, the labeling was very much reduced (lane 4). The inhibitory factor could be an enzyme that hydrolyzes NAD^+ or removes ADP-ribose (or ADP or AMP) from the labeled protein. Alternatively, it could be an inhibitor of the labeling reaction itself. When the MVA membranes were washed in a low ionic strength buffer and fractionated into light (MVBL) and heavy (MVBH) membrane layers, it was found that MVBH (lane 5) was more heavily labeled than MVBL (lane 6). The protein substrate was not removed by washing MVBL membranes with 200 μM GTP prior to treatment with pertussis toxin (lane 7). GTP elutes transducin from vertebrate ROS but not the G-proteins from octopus membranes. Thus, the pertussis toxin target in octopus photoreceptors is membrane-bound, like the G-proteins of hormone systems.

The effect of light on radiolabeling of the 41 kDa polypeptide by pertussis toxin is shown in fig.2. Whole microvilli (MV) were weakly labeled, regardless of the illumination conditions (lanes 1,2). A large difference, however, was seen when high salt washed membranes (MVA) were treated similarly. The labeling by pertussis toxin was strong in the dark, but greatly reduced by light (lanes 3,4). The light-dark difference seen in fig.2 is reminiscent of the labeling of vertebrate transducin by pertussis toxin [12]. In vertebrate rod outer segments, the substrate for labeling is the inactive GDP-bound form of transducin (T-GDP). The effect of light there is to activate rhodopsin, forming a species R* which binds tightly to T-GDP,

$$R^* + \text{T-GDP} \longrightarrow R^*\text{-T} + \text{GDP}$$

Light inhibits labeling by pertussis toxin because R*-T is not a substrate for ADP-ribosylation.

We next looked at the nucleotide dependence of pertussis toxin labeling in octopus photoreceptors as another criterion of its homology with vertebrate transducin. Octopus microvilli were mixed with 100 μM of one of several guanyl nucleotides, then treated with pertussis toxin, in either the absence or presence of light. As with vertebrate transducin, labeling was observed with

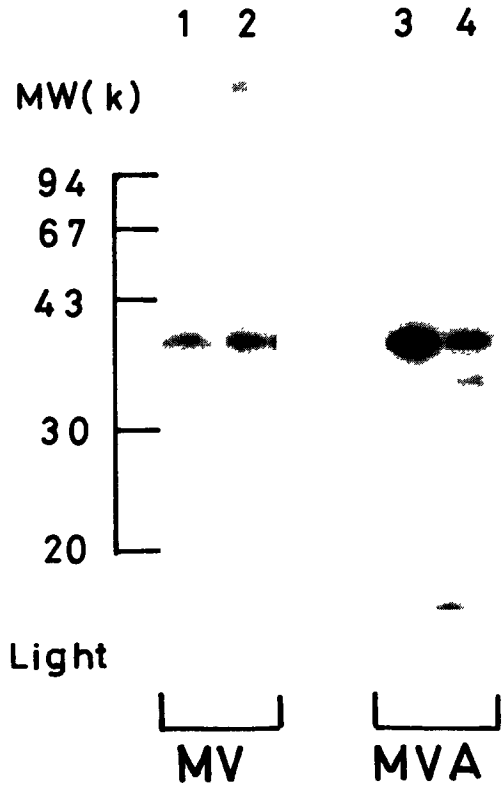

Fig.2. Effect of light on the labeling of octopus photoreceptor proteins by pertussis toxin. During the labeling period, samples were kept in the dark or were illuminated with a 200 W slide projector positioned at a distance of 50 cm.

both GDP and GTP (not shown), where GTP was probably hydrolyzed to GDP during the reaction. The non-hydrolyzable GDP analog GDPβS stimulated pertussis toxin labeling the most (fig.3, lane 2), as observed with vertebrate transducin [12]. In contrast, both GTPγS and GppNHp were inhibitory (lanes 6,7). In the vertebrate visual system, these non-hydrolyzable GTP analogs also inhibit pertussis toxin labeling. They do so by exchanging with bound GDP, resulting in a conformational change in transducin and its dissociation into free α- and $\beta\gamma$-subunits [13]. These results suggest that the pertussis toxin substrate in octopus photoreceptors, as in vertebrate systems, is a guanyl nucleotide-binding protein, and that the preferred substrate is the GDP-bound form. Furthermore, this target for pertussis toxin labeling is most likely responsible for the light-activated

GTPase activity previously seen in octopus photoreceptor preparations [6].

Because of these similarities between the octopus pertussis toxin substrate and vertebrate transducin, we next looked for immunologic cross-reactivity between these proteins. Purified α- and $\beta\gamma$-subunits of bovine transducin were used to prepare antisera for Western blot analyses. Crude transducin and octopus microvilli (MV and MVBG) were electrophoresed in polyacrylamide gels, then blotted onto nitrocellulose membranes. Individual lanes were cut out and treated with mouse antisera specific for the α(ASα-1) or $\beta\gamma$(AS$\beta\gamma$-1, AS$\beta\gamma$-2, and AS$\beta\gamma$-3) subunits of transducin. ASα-1 reacted with the α-, but not the β- or γ-subunits of transducin, whereas AS$\beta\gamma$-1 strongly reacted with the β-subunit and weakly with the γ-subunit of transducin (fig.4, lanes d,g).

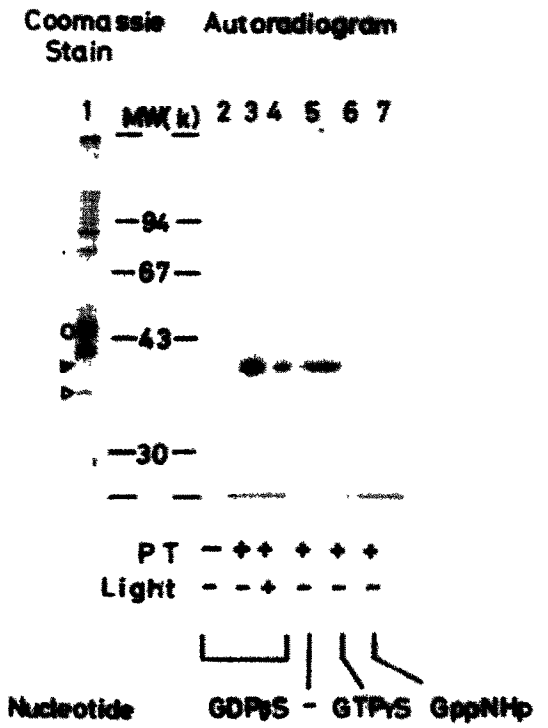

Fig.3. Effect of nucleotides on the labeling of octopus photoreceptor proteins by pertussis toxin. Lane 1, Coomassie-stained gel. Autoradiogram – lanes: 2–4, 100 μM GDPβS; 5, no added nucleotide; 6, 100 μM GTPγS; 7, 100 μM Gpp(NH)p; (○) octopus rhodopsin, (►) Gα, (▷) Gβ.

ASα-1 did not react with any of the polypeptides present in octopus photoreceptors (lanes e,f). In contrast, AS$\beta\gamma$-1 reacted with a polypeptide of 36 kDa in the octopus photoreceptor membranes (lanes h,i). The 36 kDa polypeptide is probably the cephalopod homolog of the β-subunit of vertebrate transducin. We also tested two other antisera raised against T$\beta\gamma$ (AS$\beta\gamma$-2 and AS$\beta\gamma$-3) with octopus photoreceptor membranes. These antisera did not react with the 36 kDa band (lanes k,l; not shown with AS$\beta\gamma$-3), indicating structural differences between this polypeptide and Tβ. The observed cross-reactivity using AS$\beta\gamma$-1 indicated that these proteins share a common antigenic determinant.

The pertussis toxin-labeling and immunological experiments show that octopus photoreceptors contain a protein-like vertebrate transducin. The 41 kDa octopus protein labeled by pertussis probably corresponds to vertebrate Tα. The 36 kDa octopus protein that cross-reacts with anti-bovine T$\beta\gamma$ antibody is probably like vertebrate Tβ. It is known that antibody to bovine transducin also cross-reacts with the β-subunits of the stimulatory (G_s) and inhibitory (G_i) G-proteins [14]. Furthermore, there is structural and functional evidence that the β-subunit of G_s is identical or nearly so to the β-subunits of G_i and vertebrate transducin [15,16]. Hence the β-subunit has broad distribution in nature and is highly conserved. In contrast, the α-subunits of the G-protein family are more diverse. The common labeling by pertussis toxin of the subunits of G_i and both vertebrate transducin and octopus photoreceptor G-protein probably defines a subfamily of G-proteins that share a distinct receptor-binding region. Both bovine transducin and G_i are labeled by pertussis toxin and interact strongly with bovine rhodopsin, whereas G_s does not [16,17]. G_s interacts strongly with the β-adrenergic receptor, whereas G_i and bovine transducin do not [17]. The fact that bovine transducin also interacts with octopus rhodopsin [6] suggests that this latter receptor is similar to vertebrate rhodopsin. Thus, octopus photoreceptor G-protein probably belongs to the G_i subfamily.

Finally, it is interesting to consider what possible role octopus photoreceptor G-protein might play in invertebrate photoreceptors. Unlike the vertebrate case, so far there is no evidence for a

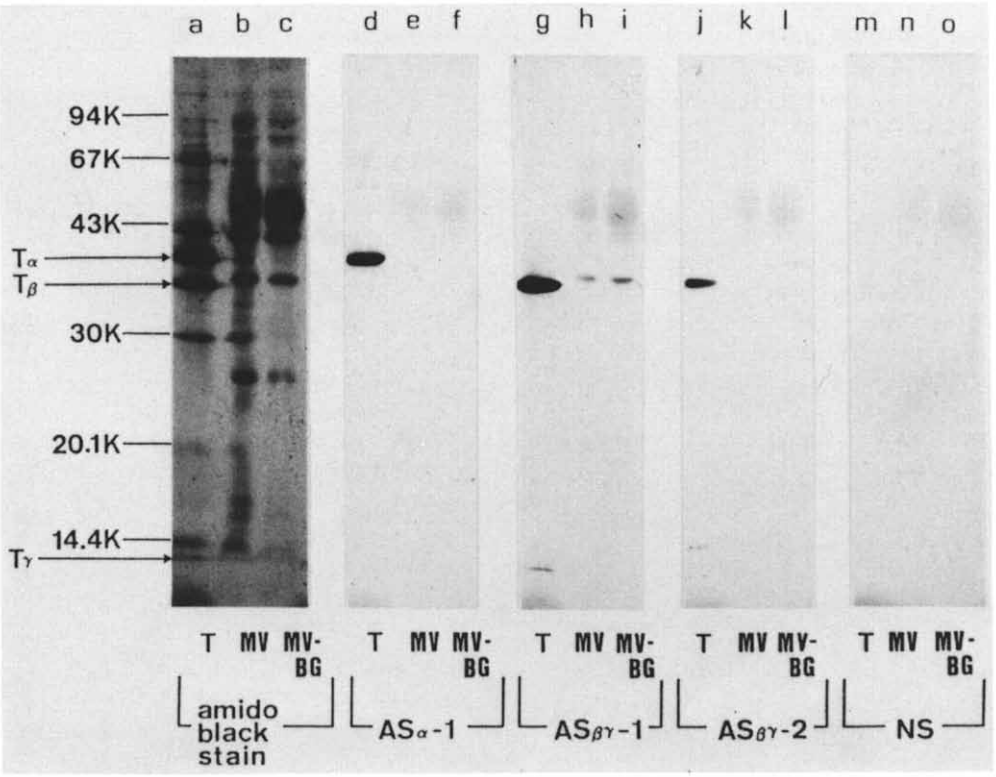

Fig.4. Cross-reaction of octopus photoreceptor proteins with antisera specific for the α- and $\beta\gamma$-subunits of bovine transducin. Lanes: a–c, stained with amido black; d–f, with ASα-1 (diluted 1000-fold); g–i, with AS$\beta\gamma$-1 (diluted 2000-fold), j–l, with AS$\beta\gamma$-2 (diluted 2000-fold); m–o, with normal serum (NS diluted 1000-fold).

striking light-regulated phosphodiesterase (or guanylate cyclase) activity in these cells [6,7]. Yoshioka et al. [18] and Vandenberg and Montal [19] have reported a light-activated inositol triphosphatase activity (which is also a polyphosphoinositol polyphosphodiesterase). More recently, a similar enzymatic activity under light control has been reported in *Limulus* photoreceptors [20]. Little is known about how light regulates the enzymes (or substrates) involved in inositol metabolism, but it seems possible that octopus photoreceptor G-protein, once activated by photolyzed rhodopsin, might control this set of enzymes.

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REFERENCES

[1] Miller, W.H. (1981) Curr. Top. Membranes Transp. 15.
[2] Stryer, L. (1985) Annu. Rev. Neurosci. 9, in press.
[3] Fung, B.K.-K., Hurley, J.B. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.
[4] Kuhn, H. (1980) Nature 283, 587–589.
[5] Chabre, M. (1985) Annu. Rev. Biophys. 14, 331–360.
[6] Calhoon, R., Tsuda, M. and Ebrey, T.G. (1980) Biochem. Biophys. Res. Commun. 94, 1452–1457.

[7] Vandenberg, C.A. and Montal, M. (1984) Biochemistry 23, 2339–2347.
[8] Blumenfeld, A., Erusalimsky, J., Heichal, O., Selinger, Z. and Minke, B. (1985) Proc. Natl. Acad. Sci. USA, in press.
[9] Ebrey, T.G., Tsuda, M., Sassenrath, G., West, J.L. and Waddell, W.H. (1980) FEBS Lett. 116, 217–219.
[10] Saibil, H.R. and Michel-Villaz, M. (1984) Proc. Natl. Acad. Sci. USA 81, 5111–5115.
[11] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[12] Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R.D., Manclark, C.R., Stryer, L. and Bourne, H.R. (1984) J. Biol. Chem. 259, 23–26.
[13] Fung, B.K.-K. (1983) J. Biol. Chem. 258, 10495–10502.
[14] Gierschik, P., Codina, J., Simons, C., Birnbaumer, L. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. USA 82, 727–731.
[15] Manning, D.R. and Gilman, A.G. (1983) J. Biol. Chem. 258, 7059–7063.
[16] Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E.L., Moss, J. and Vaughan, M. (1984) J. Biol. Chem. 259, 7378–7381.
[17] Cerione, R.A., Staniszewski, C., Benovic, J.L., Lefkowitz, R.J., Caron, M.G., Gierschik, P., Somers, R., Spiegel, A.M., Codina, J. and Birnbaumer, L. (1985) J. Biol. Chem. 260, 1493–1500.
[18] Yoshioka, T., Inoue, H., Takagi, M., Hayashi, F. and Amakawa, P. (1983) Biochim. Biophys. Acta 755, 50–55.
[19] Vandenberg, C.A. and Montal, M. (1984) Biochemistry 23, 2347–2352.
[20] Brown, J., Rubin, L.J., Ghalayini, A.J., Tarver, A.P., Irvine, R.F., Berridge, J.J. and Anderson, R.E. (1984) Nature 311, 160–163.