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# Participation of GTP-binding Protein in the Photo-transduction of *Paramecium bursaria*

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*ABSTRACT.* **Analysis of the flow of light information in *Paramecium*, especially the participation of GTP-binding protein, was carried out. Injection of GDP-β-S into *Paramecium* cell abolished the light-induced inward current. The partially purified rhodopsin-like protein (RLP) that we obtained activated frog rod outer segments (ROS) GTPase. A GTP-binding protein, with Mw 57,000, was detected by immunostaining with anti-rat Gα rabbit IgG. These results suggest that the light information flows from the light-captured RLP to the GTP-binding protein.**

*Paramecium bursaria* is a photo-sensitive ciliated protozoan, that shows photo-phobic behavior upon light stimulus. An electro-physiological study has shown that the light-stimulus generates a transient depolarization of *P. bursaria* cell membrane (7, 8). We have reported the localization of a rhodopsin-like protein (RLP), with Mw 63,000, in the cilia and plasma membrane of *P. bursaria*, and the irradiation of these loci generated a transient depolarization (9). The presence of several kinds of retinal in *P. bursaria* was also reported (15). These facts suggest the participation of RLP in the photo-transduction in *Paramecium*.

The flow of light-information in vertebrate photo-receptor cells is well documented. Light-captured rhodopsin activates GTP-binding protein (Gtα in the transducin) and the activated GTP-binding protein activates cGMP phosphodiesterase. In the case of *Paramecium*, GTP affects the swimming behavior (1) and its participation in secretion has also been investigated (12). For the elucidation of the photo-transduction mechanism in *Paramecium*, the injection of GDP-β-S, the addition of partially purified RLP from *P. bursaria* to the frog ROS GTPase, and the survey of GTP-binding protein by the anti-rat Gα rabbit IgG were carried out. The results suggest the participation of GTP-binding protein in the photo-transduction of *P. bursaria*.

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Abbreviations: RLP, rhodopsin-like protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GDP-β-S, guanosine 5′-0-(2-thiodiphosphate); Con A, Concanavalin A; IgG, Immunoglobulin G; HPLC, high performance liquid chromatography; ROS, rod outer segments; CBB, Coomassie brilliant blue R-250; PVDF, Poly (vinylidene fluoride); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

## MATERIALS AND METHODS

*Reagents.* GDP-β-S was purchased from Boehringer Mannheim Co. Mw standards for the SDS-PAGE were from Pharmacia and Bio Rad. Con A-Sepharose 4B was from Pharmacia.

*Antibody.* Anti-frog ROS rhodopsin rabbit IgG was prepared as previously described (14). Anti-rat Gα (common region of the carboxyl terminal of Gα, Gtα and Gi2α) rabbit IgG was purchased from DuPont NEN Research Products (4).

*Cells.* *P. bursaria* was cultured in lettuce juice medium which was inoculated with *Klebsiella pneumoniae* one day before use. The culture was maintained under a fixed illumination cycle of 12 h light (a fluorescent light of about 1,000 lx) and 12 h dark at 25°C. Stationary phase cells were collected by low-speed centrifugation and washed in an experimental solution containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4 mM KCl and 2 mM Tris-HCl (pH 7.2). The concentrated cells were stored at −30°C. For intracellular recordings, the living cells were deciliated by incubation in the experimental solution containing 5% ethanol for 2–3 min, then returned to the experimental solution.

*Intracellular recording and light stimuli.* The mechanical set-up and general techniques were similar to those described before (9). Electrodes were filled with 1 M KCl, and their resistances were 10–40 MΩ. The cell membrane was voltage clamped with two microelectrodes. For testing GDP-β-S, this was added to two KCl electrodes at a concentration of 10

mM, and was diffused into the cell. The external solution was the experimental solution described above with its temperature controlled at 25°C. Light pulses (550 nm, 3 mW/cm$^2$) of 5 s duration were applied to the voltage clamped cell.

*Protein electrophoresis and Western blotting.* Proteins of *P. bursaria* or frog ROS GTPase was solubilized in the SDS sample solution (7% SDS, 70 mM DTT, 20 mM EDTA and 0.05% Bromophenol Blue (BPB), pH 7.5), and the mixture was boiled for 8 min. Separation of protein by SDS-PAGE was done on a 10% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. After the electrophoresis, the gel was cut and the proteins in a piece of gel were stained with CBB or silver reagent. The remaining gel was used for Western blotting as follow. Separated proteins in the gel were transferred electrophoretically to PVDF membrane (Atto) with a Horais Blot (model AE6670, Atto) in a blotting buffer (100 mM Tris-HCl, 192 mM glycine, 20% methanol and 0.1% SDS, pH 8.8). The blot was cut into a number of identical strips. The polypeptide bands on one strip were stained with CBB and the transfer of most protein bands was confirmed by this staining. The remaining strips were shaken for 1 h in a blocking solution (50 mM Tris-HCl, 150 mM NaCl and 5% Non-fat dry milk, pH 7.5) to eliminate nonspecific protein binding and were incubated with anti-frog-rhodopsin rabbit IgG (1.9 $\mu$g/ml) or with anti-rat G$\alpha$ rabbit IgG in the blocking solution for 3 h or overnight. To remove nonspecific binding of IgG, the strips were vigorously shaken on ice in the blocking solution supplemented with 0.1% Tween-20 and 0.1% SDS (pH 7.5) for 5 min and washed five times in a solution containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 and 0.1% SDS (pH 7.5). Blot-strips were incubated in horse radish peroxidase-labeled goat anti-rabbit IgG (30 $\mu$g/ml, Capel Laboratories Inc.) in the blocking solution. For the detection of the proteins which bind to anti-rat G$\alpha$ rabbit IgG, BLAST blotting amplification system (DuPont NEN Research Products) was also carried out.

*Partial purification of RLP from P. bursaria.* *P. bursaria*'s cells were sonicated in a solution of 100 mM Na-phosphate buffer (pH 6.8). After washing the sonicated sample twice with the same phosphate buffer, the precipitates of the centrifugation at 42,000 × g for 20 min were suspended into 2% digitonin solution containing 10 mM HEPES-KOH (pH 6.8), 100 mM NaCl, 1 mM DTT and 0.1 mM PMSF. The suspension was passed through a 25 gauge needle 10 times and then kept on ice for 30 min. The supernatant fraction of the centifugation at 15,000 × g for 30 min was obtained. The precipitates of this centrifugation were suspended into 2% digitonin solution again and the treatment with the needle and the centrifugation were repeated. The supernatants, obtained by the five repetitions of these treatments, were combined and supplemented with 1 mM $CaCl_2$ and 1 mM $MnCl_2$. This supernatant solution was applied to the Con A-Sepharose column. After the removal of the nonspecifically bound proteins by washing with the 0.2% digitonin solution containing $CaCl_2$ and $MnCl_2$, the adsorbed proteins were fractionated by stepwise elution using 1.5 mM, 5 mM, 10 mM, 50 mM, 100 mM, 200 mM or 500 mM of $\alpha$-methyl mannoside in 0.2% digitonin solution containing $CaCl_2$ and $MnCl_2$. The protein composition and the binding to the anti-frog rhodopsin rabbit IgG was analyzed by SDS-PAGE and Western blotting. The most purified RLP, detected by the binding to that IgG, was obtained by the elution of 10 mM $\alpha$-methyl mannoside solution. Purity of this RLP in this fraction, as deduced by the silver-reagent staining method, was approximately 50%.

*Preparation of frog ROS GTPase (Transducin).* Frog ROS were prepared as previously described (14). Elution of GTPase from ROS was carried out by washing with (GTPase) extraction buffer (100 mM GTP solution in 5 mM Tris HCl, 0.5 mM $MgCl_2$, 1 mM DTT and 0.1 mM PMSF, pH 7.2) according to the method of Kühn (5). The obtained GTPase was used after filtration through a nitrocellulose filter (pore size 0.20 $\mu$m: Corning).

*GTPase assay.* GTPase assay was carried out by the HPLC method described by Okada *et al.* (10). After the incubation of the samples (combination of the partially purified RLP, a fraction eluted by 10 mM $\alpha$-methyl mannoside from Con A-Sepharose, and frog ROS GTPase) for 10 min at 37°C, the reaction was stopped by the addition of 1/10 volume of 6 N HCl. The precipitated proteins were removed by centrifugation at 12,000 × g for 10 min followed by the harvesting of the upper-part of the supernatant. After the neutralization of supernatant, it was applied to the HPLC column (Hitachi: Inertsil ODS-2, 5 $\mu$m, 4 × 150 mm. L-6000 Pump). GTP (initially contained in the GTPase extraction buffer) and the GDP produced was detected by Hitachi L-4200 UV/VIS Detector and those amounts were calculated by Hitachi D-2500 chromato-integrator. The hydrolyzed amount of GTP corresponded to the increase of GDP. Assays were done in duplicate and agreed within 5%.

*Treatment of P. bursaria cells with Triton X-100.* *P. bursaria* cells ($10^7$) in 100 mM phosphate buffer were sonicated 10 times, for 10 sec each time. After centrifugation at 42,000 × g for 30 min, the precipitates were suspended in a solution of 1% Triton X-100 containing 10 mM Tris HCl, 100 mM NaCl, 1 mM DTT and 0.1 mM PMSF, pH 7.2. This suspension was sonicated 10 times, for 10 sec each time. The Triton X-100 insoluble (precipitate) fraction and soluble (supernatant) fraction were obtained by centrifugation at 42,000 × g for 30 min.

*Protein determination.* The amount of protein was determined by Lowry's method (6) using BSA as a standard.

## RESULTS

*Abolishment of the photo-response by the injection of GDP-β-S.* When *Paramecium* cell was voltage clamped, light stimulation induced a transient inward current (Fig. 1A). When the membrane potential was held at the hyperpolarized level (resting potential, −20 mV), amplitude of the inward current increased, corresponding to the previous report in which the amplitude

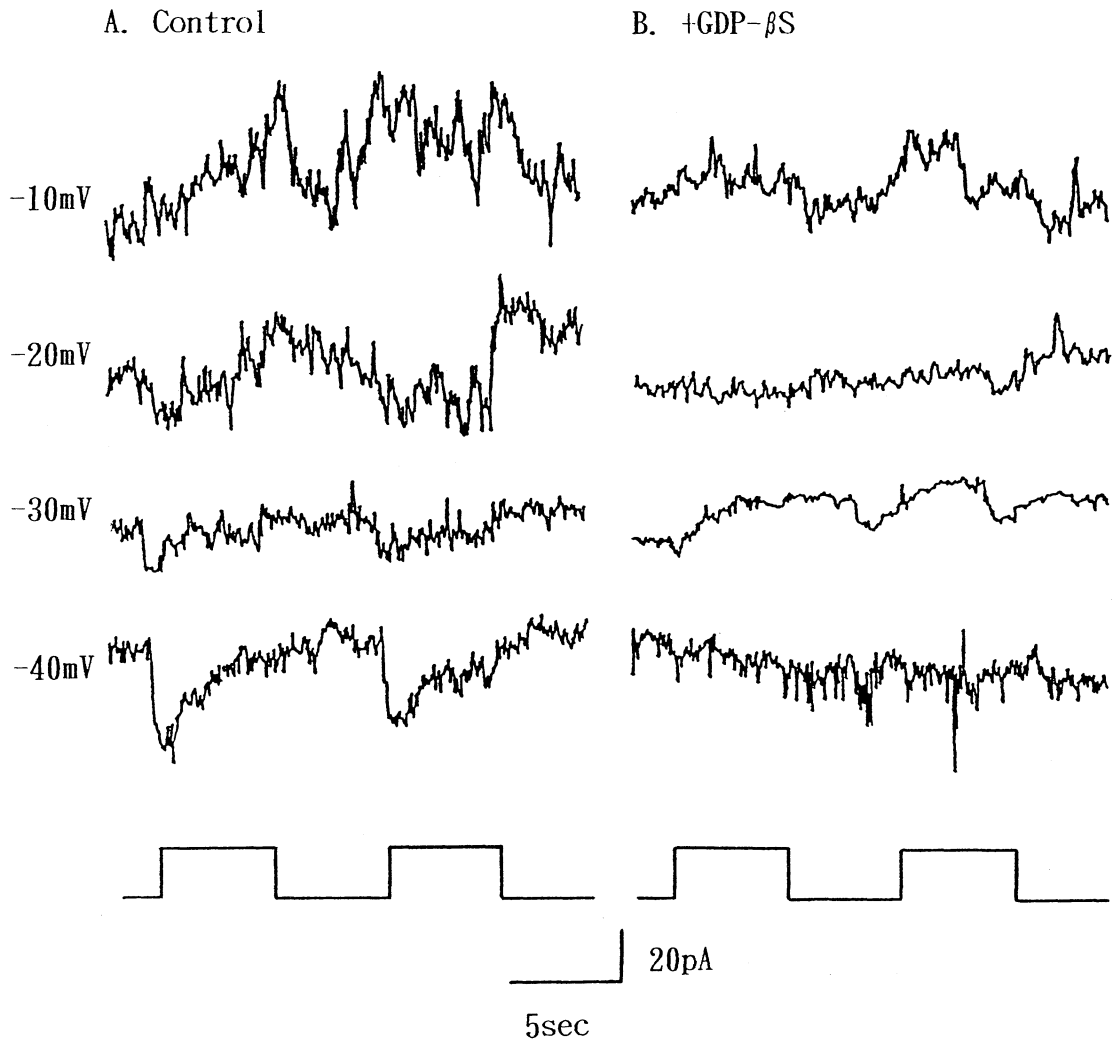


**Fig. 1.** Effect of the injection of GDP-β-S on the photo-response of *P. bursaria*. The cell was voltage clamped initially at the resting potential (about −20 mV), using KCl electrodes (A), or KCl plus GDP-β-S electrodes (B) in the experimental solution. Holding the potential at −10, −20, −30, −40 mV, light pulses were applied and current responses were recorded.

of membrane depolarization induced by light stimulation was increased by a constant current application to shift to a negative level (7). On the other hand, intracellular application of GDP-β-S inhibited the transient inward current irrespective of the voltage clamp at hyperpolarized potentials (Fig. 1B). Such inhibition suggests the possibility that GTP-binding protein is involved in the photo-transduction pathway.

*Activation of frog ROS GTPase by the partially purified RLP of P. bursaria.* Partial purification of RLP was carried out by solubilizing *P. bursaria* with digitonin and by Con A-Sepharose column chromatography. Figure 2 shows the binding of anti-frog rhodopsin rabbit IgG to *P. bursaria* proteins. RLP, detected by the IgG binding, was solubilized by digitonin and partially purified by Con A-Sepharose column chromatography. RLP was solubilized by digitonin with Mw 63,000 (Fig. 2. lane 1A, 1B) as already described (9). We could not detect this protein in a Con A-unbound fraction (Fig. 2. lane 2A, 2B). RLP was eluted with 10 mM α-methyl mannoside (Fig. 2. lane 3A, 3B). This partially purified RLP from *P. bursaria* activated frog ROS GTPase (Fig. 3). Hydrolysis of GTP by frog ROS GTPase alone (Fig. 3. Sample No.1), RLP alone (Sample No.2) or combination of RLP with heat-denatured frog ROS GTPase (Sample No.4) was very small. Contrary to that, hydrolysis of GTP by the combination of RLP with frog ROS GTPase (Sample No.3) was clearly observed. The activation of frog ROS GTPase by the fraction which did not contain RLP was at the same level as Sample No.2 (data not shown).

*Detection of GTP-binding protein of P. bursaria by*

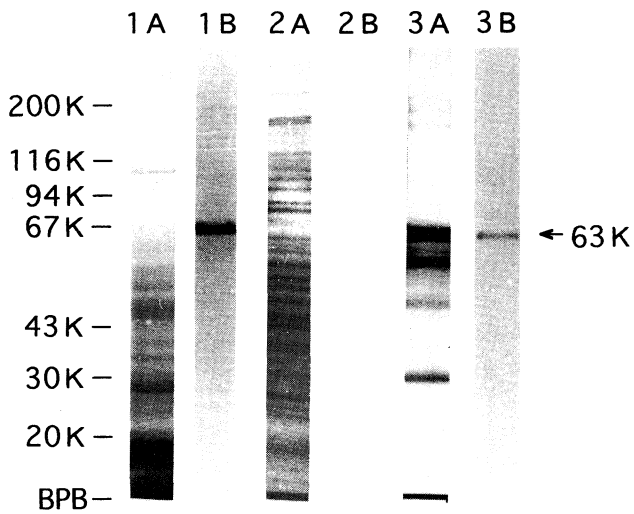


**Fig. 2.** Partial purification of RLP by Con A-Sepharose column chromatography from *P. bursaria*. Solubilized proteins by 2% digitonin solution (lane 1A, 1B, 71 μg/lane), Con A-Sepharose unadsorbed protein (lane 2A, 2B, 65 μg/lane) or Con A-Sepharose adsorbed and eluted with 10 mM α-methyl-D-mannoside (lane 3A, 3B, 12.5 μg/lane) were applied to SDS-PAGE. After the electrophoresis, the separated proteins were stained with CBB (lane 1A, 2A) or silver reagent (lane 3A) and were also electrophoretically transferred to PVDF membrane. The transferred proteins were tested for the binding to the anti-frog rhodopsin rabbit IgG using horseradish peroxidase reaction (lane 1B, 2B, 3B). Molecular weight standard proteins and BPB are shown on the left.

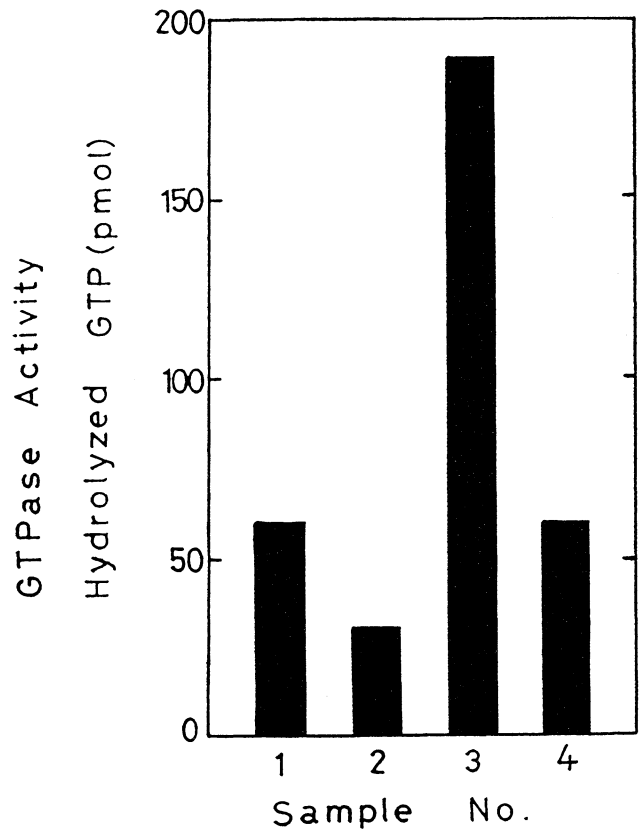


**Fig. 3.** Activation of frog ROS GTPase by the partially purified RLP from *P. bursaria*. Hydrolysis of GTP was measured by the following combinations of the sample. No.1: Elution buffer for Con A-Sepharose column chromatography + Frog ROS GTPase (4.8 μg). No.2: Partially purified RLP (1.2 μg) + Extraction buffer for the frog ROS GTPase. No.3: Partially purified RLP (1.2 μg) + Frog ROS GTPase (4.8 μg). No.4: Partially purified RLP (1.2 μg) + Heat denatured (100°C for 5 min) frog ROS GTPase (4.8 μg). Hydrolysis of GTP was measured according to the Materials and Methods.

*the anti-rat Gα rabbit IgG.* The above data suggested the presence of GTP-binding protein in *P. bursaria*. Therefore, we tried to detect that protein by the binding of anti-rat Gα rabbit IgG (Fig. 4). Antibody against rat Gα specifically bound to the α subunit of frog ROS GTPase (Gtα, Mw 36,000) (Fig. 4. lane 1B, 1C). The protein which bound to the above IgG was not detected in the Triton X-100 soluble fraction of *P. bursaria* by the horse-radish peroxidase method (lane 2B) or by the BLAST blotting amplification system (lane 2C). In the case of the Triton X-100 insoluble fraction, no binding of IgG to any protein was detected by the horse-radish peroxidase method (lane 3B). However, it was detected at Mw 57,000 by the BLAST blotting amplification system (lane 3C). In the case of the BLAST system, a diffused and irregular band appeared (lane 2C and 3C). The Mw 57,000 band was constantly (more than 10 times) and sharply detected.

The Triton X-100 insoluble, Mw 57,000 protein was solubilized with 4 M guanidine-HCl treatment (data not shown).

## DISCUSSION

Analysis of the participation of GTP in the photo-transduction of *P. bursaria* was carried out. The injection of GDP-β-S into *P. bursaria* inhibited the light-induced inward current (Fig. 1). Such an inhibition by GDP-β-S has also been reported in the case of *Limulus* photo-receptor (2). We have previously described the presence of a rhodopsin-like protein (RLP, with Mw 63,000) in *Paramecium* (9). This RLP was located in both the ciliary and the somatic membranes. Compared with the vertebrate rhodopsin (Mw 40,000), rhodopsin (− like protein) with large Mw has been described in octopus (Mw 50,300) (11) and in Planarian (Mw 65,000 and 62,000) (3). RLP in *Paramecium* was solubilized by digitonin treatment and adsorbed to Con A-Sepharose, resulting in approximately 50% purity (Fig. 2). The partially purified RLP (from *Paramecium*) activated frog ROS GTPase (Transducin) (Fig. 3). These results suggested the participation of GTP-binding protein in the photo-transduction of *P. bursaria*. The GTP-binding

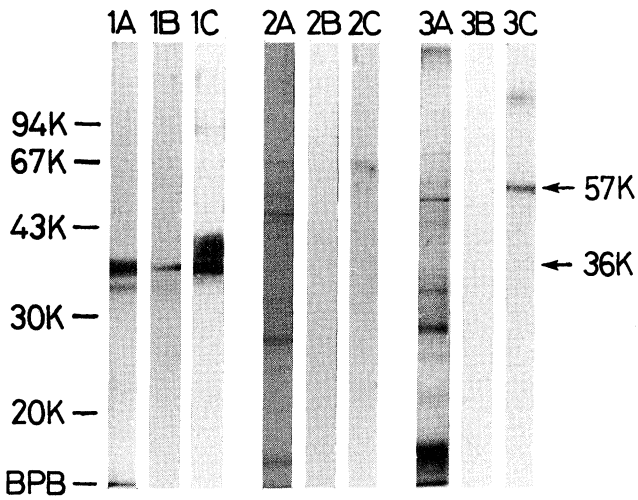


**Fig. 4.** Binding of anti-rat Gα rabbit IgG to frog ROS GTPase and to *P. bursaria* proteins. Frog ROS GTPase (lane 1A–1C, 40 μg/lane), Triton X-100 soluble *P. bursaria* proteins (lane 2A–2C, 40 μg/lane) or Triton X-100 insoluble *P. bursaria* proteins (lane 3A–3C, 40 μg /lane) were electrophoresed by SDS-PAGE. Part of the electrophoresed proteins was stained with silver reagent (lane 1A, 2A, 3A) and then the rest was electrophoretically transferred to PVDF membrane. The transferred proteins were tested for the binding to the anti-rat Gα rabbit IgG using horse-radish peroxidase reaction (lane 1B, 2B, 3B). For the detection of the proteins which bound to the above IgG, the enhancement by the BLAST blotting amplification system was also carried out (lane 1C, 2C, 3C). Molecular weight standard proteins and BPB are shown on the left.

protein with Mw 57,000 was detected in the Triton X-100 insoluble fraction of *Paramecium* by immunostaining with anti-rat Gα rabbit IgG. The Mw of this protein was larger than that of 23,000 kDa, reported by Peterson in *Paramecium tetraurelia* detected by the [$\alpha$-$^{32}$P]GTP-binding method (12). He analyzed the GTP-binding activity after SDS-PAGE. Therefore, it may be possible that the GTP-binding protein with high Mw was denatured in the SDS solution and it was not detected by that method. We used rabbit IgG against the carboxyl terminal part of rat Gα protein. Probably, the difference in the detection method might reflect the discrepancy between our data and that of Peterson. He discussed the role of this GTP-binding protein with small Mw in the response of *P. tetraurelia* to mechanical stimulation.

The Mw (57,000) of GTP-binding protein that we have detected in *Paramecium* is close to that of the protein (Mw 59,000) isolated from *Halobacterium halobium* reported by Schimz *et al.* (13). They detected it by the immunostaining with the antiserum against a synthetic peptide identical with the GTP-binding site on the α-subunit of most vertebrate G-proteins. They also suggested the participation of GTP in the light-response of that bacterium. The abolishment of the photo-response by the injection of GDP-β-S, activation of frog ROS GTPase by the partially purified RLP, and the detection of GTP-binding protein suggest the participation of GTP-binding protein in the photo-transduction in *Paramecium*. *Paramecium* has two characteristics, photo-taxis and photo-rhythm in sexual conjugation, in a single cell. Because the content of GTP binding protein in the *Paramecium* is very small, we are trying to isolate the cDNA of this protein. The clarification of this protein is the next target for the elucidatin of the above mechanism. *Paramecium* may be a unique model for the analysis of photo-receptor and its evolution.

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