Functional Significance of Triton X-100-insoluble Constituents in Signal Transduction System of Vertebrate Photoreceptors

妹尾, 圭司

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Functional Significance of Triton X-100-insoluble Constituents in Signal Transduction System of Vertebrate Photoreceptors

脊椎動物視細胞の信号伝達系における Triton X-100 不溶性成分の重要性

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神戸大学大学院自然科学研究科

妹尾　圭司

CONTENTS

要約

脊椎動物視細胞の光情報伝達系は、G タンパク質仲介型細胞内情報伝達系として知られているが、光信号の忠実な細胞内 cGMP 信号への変換や、その伝達効率の調節のためにさまざまな調節機構が用意されている。こうした種々の調節機構を含めて光情報伝達系を構成するタンパク質の多くは膜結合タンパク質である。一般に、膜タンパク質の研究のためにはその可溶化が不可欠であり、通常 Triton X-100 をはじめとする非イオン性の界面活性剤が用いられてきた。しかし、細胞内にはこれらの界面活性剤に不溶性のタンパク質が多く存在しており、取扱いの困難さから界面活性剤不溶性の成分に関する研究は遅れてきた。しかし、最近になって、これらの界面活性剤不溶性の成分が様々な細胞膜の機能に重要な役割を果たしていることが明らかになり、特にカベオレやラフトといった界面活性剤不溶性膜分画がにわかに注目されるに至っている。私は、視細胞の光情報伝達系でも、これまで研究されてこなかった界面活性剤不溶性の成分が何らかの重要な役割を果たしているのではないかと考え、本研究を実施した。

その結果、1）視細胞光受容膜の Triton X-100 不溶性分画に存在する cGMP 合成酵素が同じ分画に存在する RGS9 という活性型 G タンパク質の寿命を調節するタンパク質と特異的に相互作用することを発見し、信号変換に必要な cGMP 合成と分解の両過程の間に直接的な相互作用が存在する可能性を始めて示唆することができた。また、2）視細胞光受容膜からはラフト様の Triton X-100 不溶性膜分画（detergent-resistant membrane: DRM）が調製できることを始めて明らかにし、さらに G タンパク質やその標的酵素である cGMP 分解酵素（PDE）が光情報伝達の特定の段階でこの DRM 上に凝集したり離散することを発見した。界面活性剤不溶性膜分画やその上に局在するタンパク質が信号伝達反応の場として重要な意味を持っている可能性を始めて示唆することができた。

まず第 1 章では、ROS の界面活性剤不溶性分画中に cGMP 合成酵素（guanlate cyclase ：GC）結合タンパク質を検索し、新規な GC 結合タンパク質を発見した。GC が界面活性剤不溶性であることは良く知られているが、アミノ酸配列から予測すると一回膜貫通型タンパク質で、界面活性剤不溶性は予想されない。そこで視細胞外節の Triton X-100 不溶性分画中でこのタンパク質と相互作用するタンパク質を検索した。方法としては、膜オーバーレイ法を採用した。Triton X-100 不溶性分画に含まれるタンパク質を 2 次元電気泳動法

によって分離し、PVDF 膜に転写した後、比較的弱い界面活性剤共存下に精製 GC をオーバーレイした。結合した GC は GC ペプチド特異抗体を用いて検出した。その結果、55 k Da の未知のタンパク質が GC と選択的に結合し、その活性を阻害することが明らかになった。このタンパク質はほどなく G タンパク質の活性型寿命を調節するタンパク質である RGS9（regulator of G-protein signaling 9）であることが判明した。この結果は、光依存的に cGMP 濃度を変化させる cGMP 分解系と cGMP 合成系のタンパク質間に直接的な相互調節機構が存在することを強く示唆した。

第２章では、視細胞の光受容膜で発見したラフト様膜分画について検討した。タンパク質が界面活性剤に不溶となる仕組みとしては、タンパク質間相互作用によって細胞骨格タンパクなどと結合することが考えられてきたが、近年、膜中に“DRM”と呼ばれる Triton X-100 不溶性の領域が存在することが明らかになり、シグナルを効率よく伝達する場を提供する仕組みとして注目されるようになった。そこで、視細胞にも DRM が存在し、光情報伝達に重要な働きをしているのではないかと考え、視細胞外節での DRM の調製を試みた。その結果、他の細胞で見られる DRM と同様に、コレステロールやガングリオシド（GD3）を比較的多く含む膜分画を得ることができた。この DRM の光情報伝達系における役割を明らかにするために、光信号変換の諸段階でのシグナルタンパク質の DRM への分布を調べた。視細胞の G タンパク質であるトランスデューシンは暗状態では Triton X-100 可溶性だが、そのかなりの部分が光刺激で DRM へ移行した。さらに光とともに GTP の非水解性アナログである GTPγS を与え、トランスデューシンαサブユニット上でのヌクレオチド交換を起こし、トランスデューシンを活性化させると、トランスデューシンは再び可溶性分画に移行することが明らかになった。これと対照的に、トランスデューシンの標的酵素である PDE は暗状態では Triton X-100 可溶性だが、活性化されるとほとんどが DRM へ移行するという結果が得られた。これら以外のタンパク質の分布に関しては光刺激および GTPγS による顕著な差は見られなかった。以上述べた研究の結果、視細胞においても信号伝達にラフト様膜領域が重要な働きをしていることが強く示唆される結果となった。

ラフト様膜領域とそれ以外の膜領域での諸タンパク質の相互作用を明らかにすることによって光情報変換分子機構のより深い理解が得られるものと期待される。

Abstract

Vertebrate phototransduction is a typical G-protein-mediated system. It has been known that various proteins are functioning for the regulation of this system, and that many of them are intrinsic or peripheral membrane proteins. To study such proteins, membrane solubilization by neutral detergents is essential. In numerous studies, the neutral detergent Triton X-100 has been used. However, there are many proteins, that are insoluble in detergents such as Triton X-100. Studies of these insoluble proteins have been limited because it has been difficult to analyze them biochemically. Recently, importance of these insoluble proteins in membrane functions has been recognized. Especially, detergent-resistant membrane domains in the plasma membrane, such as caveolae and rafts, have attracted the attention of many researchers in various fields.

In this paper, I report two new findings with Triton X-100 insoluble components in bovine photoreceptor rod outer segments (ROS). 1) I found that the cGMP-synthesizing enzyme, guanylate cyclase (GC), can specifically interact with a G-protein regulating protein (RGS9) that is localized in the detergent-insoluble fraction of ROS. It has been suggested that the cGMP synthesizing and degrading systems are closely coordinated for phototransduction. 2) I found that a raft-like detergent-insoluble membrane fraction can be prepared from ROS. Further, I found that G protein and its target enzyme, cGMP-phosphodiesterase (PDE), are recruited to, or removed from, DRM during phototransducition. These findings indicated that the DRM plays an important role as a platform for signal transduction.

In Chapter I, I describe the identification of a guanylate cyclase (GC)-binding protein in a detergent-insoluble fraction of bovine-ROS. GC has been known to be detergent-insoluble, although it was estimated from its amino acid sequence to be a typical single-pass membrane protein and seemed to be detergent soluble. First, I investigated GC-binding proteins in a Triton X-100-insoluble fraction of bovine ROS by employing a membrane-overlay technique. Proteins in the Triton X-100-insoluble fraction were separated by two-dimensional gel electrophoresis and blotted to PVDF membrane. The membrane was overlaid with purified bovine GC in the presence of a milder detergent (Tween 20), and bound GC was detected by the use of an anti-GC peptide antibody. A novel 55-kDa protein was found that specifically binds GC, and it could inhibit GC in vitro. The 55-kDa protein was soon identified as the ninth member of regulator of G-protein signaling (RGS9), which regulates the lifetime of activated G protein. These

results indicated that there may be a mutual regulatory mechanism that directly links the cGMP synthesizing and the cGMP-hydrolyzing system in ROS.

In Chapter II, I describe the studies on a raft-like membrane fraction obtained from photoreceptor ROS. Although it has been thought that the detergent-insolubility of a certain protein is due to the interaction of the protein with cytoskeletal proteins, recent studies on the Triton X-100-insoluble membrane domain, named detergent-resistant membranes (DRM), have suggested that such membrane is a place where at least a certain group of detergent-insoluble proteins are localized. The DRM is also implicated in the signal transduction of the plasma membrane. I though there may be DRMs in photoreceptor, and they function in phototransduction. Therefore, I tried to prepare DRM from photoreceptor ROS by the conventional DRM preparation method. As a result, a buoyant Triton X-100 insoluble fraction was obtained. This fraction was relatively enriched in cholesterol and glycosphingolipids (sphingomyelin and ganglioside GD3). In order to clarify the role of DRM in phototransduction, I examined the distribution of signaling proteins in DRM at each step of phototransduction. In the dark, transducin was soluble in Triton X-100. However, light stimulus caused translocation of transducin to DRM. In contrast, under the transducin activating condition of the presence of light and an unhydrolyzable analog of GTP, transducin became soluble in Triton X-100 again. On the other hand, PDE in the dark was TritonX-100 soluble, and activated PDE was recruited to DRM. No translocation was observed in other proteins. These data suggest that the translocation of G protein and PDE between the tightly packed raft-like domains and fluid membrane domains are closely coincided with the functional steps in the process of phototransduction.

Further studies on the detergent-insoluble membrane domains in ROS will give us important information that will shed light on the molecular mechanism of phototransduction in vertebrate photoreceptor cells.

Abbreviations

BAPTA : O,O'-bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetra-acetic acid

BHT : butylated hydroxytoluene

CBB : coomassie brilliant blue

cdk5 : cycline dependent kinase 5

cGMP : cyclic guanosine monophosphate

DMS : dimethyl suberimidate

DOC : deoxycholic acid

DRM : detergent resistant membrane

DTT : dithiothreitol

DW : distilled water

ECL : enhanced chemiluminescence

EDC : 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride

EDTA : ethylenediamine-tetra-acetic acid

EGTA : ethyleneglycol-bis-N,N,N',N',-tetra-acetic acid

GAP : GTPase activating protein

GC : guanylate cyclase

GCAP : guanylate cyclase activating protein

GCBP : guanylate cyclase binding protein

GDP : guanosine diphosphate

GTP : guanosine triphosphate

GTPγS : guanosine 5'-O-(3-thiotriphosphate)

HPTLC : high-performance thin layer chromatography

HRP : horseradish peroxidase

IgG : immunoglobulin G

KLH : keyhole limpet hemocyanin

MCD : methyl-β-cyclodextrin

MOPS : 3-(N-morpholino)propanesulfonic acid

Pα : α-subunit of PDE

Pβ : β-subunit of PDE

Pγ : γ-subunit of PDE

PAGE : polyacrylamide gel electrophoresis

PDE : cGMP-phosphodiesterase

PMSF : phenylmethanesulfonyl fluoride

PVDF : polyvinylidene fluoride

RGS : regulator of G-protein signaling

ROS : rod outer segment

Rho : rhodopsin

Rho* : activated rhodopsin

SDS : sodium dodecylsulfate

T_α : α-subunit of transducin

T_β : β-subunit of transducin

T_γ : γ-subunit of transducin

General Introduction

Rod type and cone type photoreceptor cells exist in the deepest layer of vertebrate retina. A photon passes through transparent neuronal layers, and reaches the photopigment (rhodopsin) in the outer segment of a photoreceptor cell (Fig. 1). Phototransduction occurs in the membrane systems in the outer segments of rod and cone cells. Rod cells are more abundant in retina and operate optimally at low light intensity. Cone cells take charge of color vision by three kinds of photopigment with different spectra. Outer segments of rod and cone cells are developed specifically for phototransduction. Rod outer segments (ROS) contain highly stacked disk-shaped membranes, which are densely packed with rhodopsin (Fig. 2). ROS can be purified easily by sucrose density gradient centrifugation methods. Purified ROS have a complete phototransduction system, and almost all proteins in ROS are phototransduction-related. Because of these characters, the phototransduction system is a very good resource for biochemical investigations of signal transduction, and phototransduction is one of the best understood systems of intracellular signal transduction. The process from light absorption to cell excitation is well known as phototransduction cascade (Fig. 3). The phototransduction cascade is a typical G-protein-mediated signal transduction system (for reviews see Hargrave and McDowell 1992; Ebrey and Koutalos 2001). Rhodopsin consists of a protein component termed opsin and a chromophore termed retinal. In ROS, the photopigment, rhodopsin, absorbs photon, and its chromophore, 11-cis retinal transforms to all-trans form. This transformation leads to a conformational change and activation of rhodopsin. Activated rhodopsin stimulates the G-protein, transducin. Transducin is a heterotrimeric G-protein, and consists of α, β and γ subunits. The

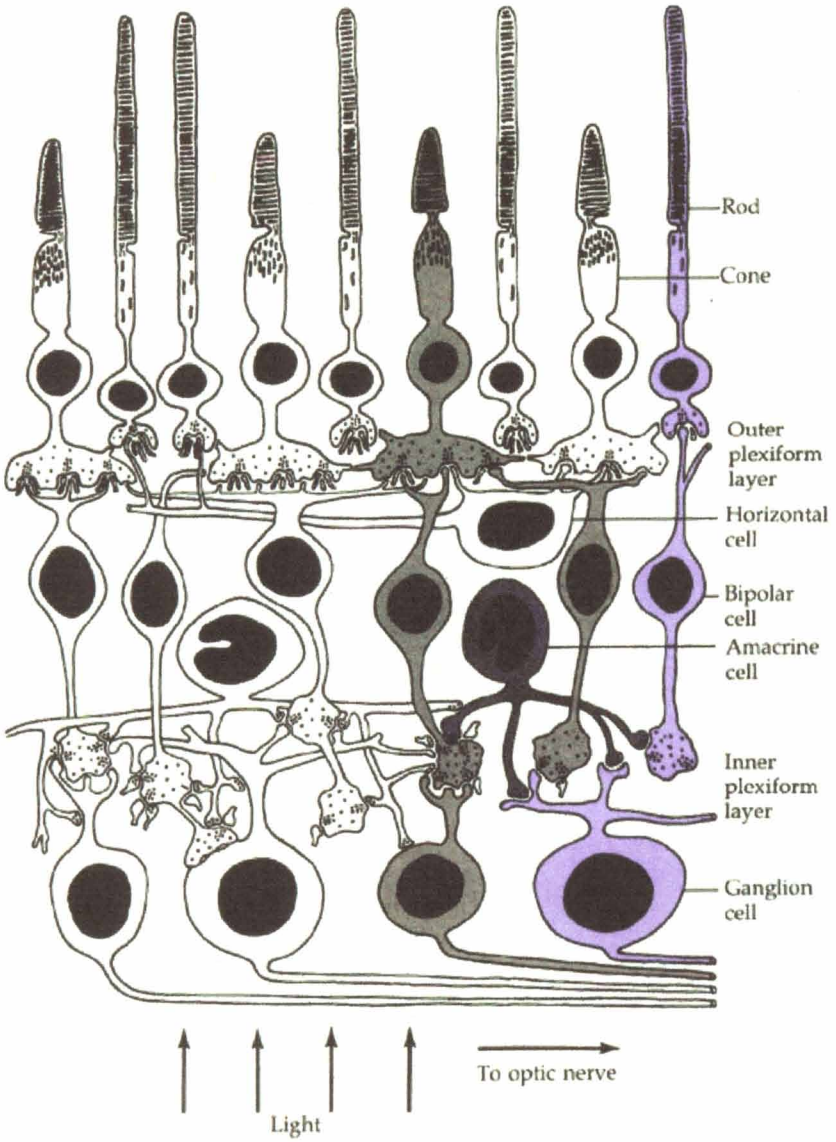


Fig. 1 Schematic view of vertebrate retina

Schematic view of vertebrate retina (Nicholls et al. (1992)). Light enters the retina and passes through transparent nerve cells, and reaches rod and cone photoreceptor cells.

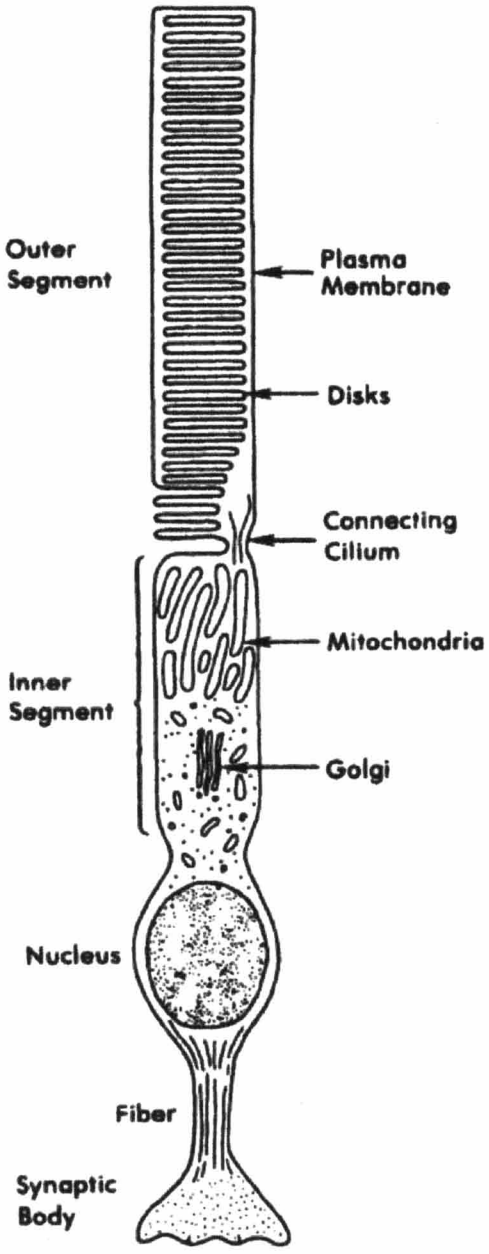
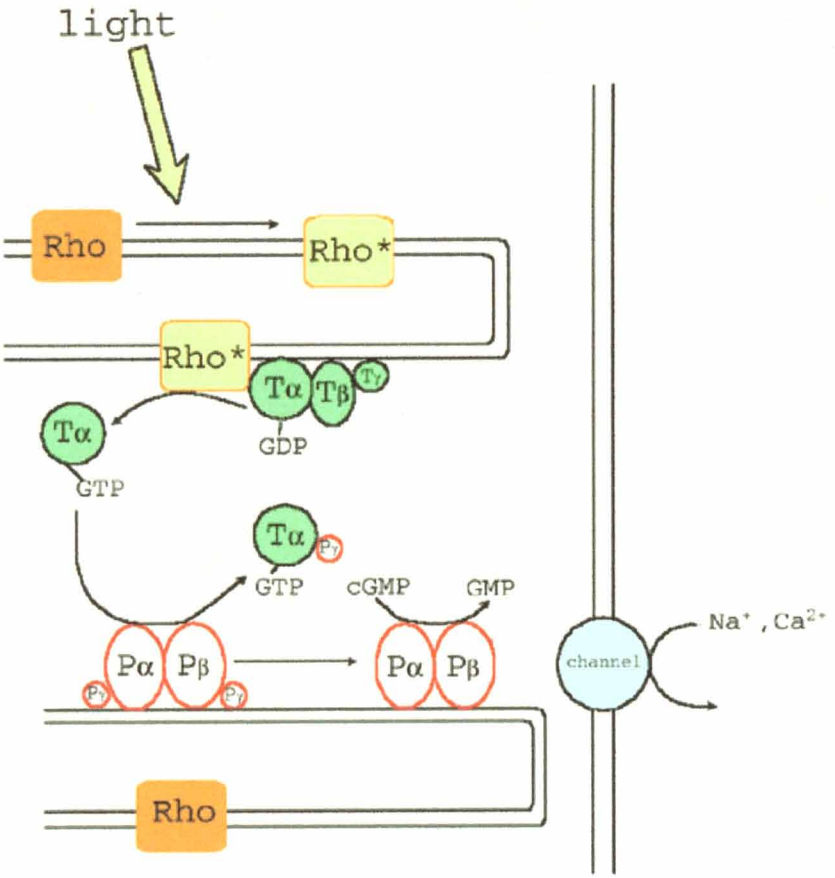


Fig. 2 Schematic view of rod photoreceptorcell (Hargrave and McDowell 1992)

in the light condition



in the dark condition

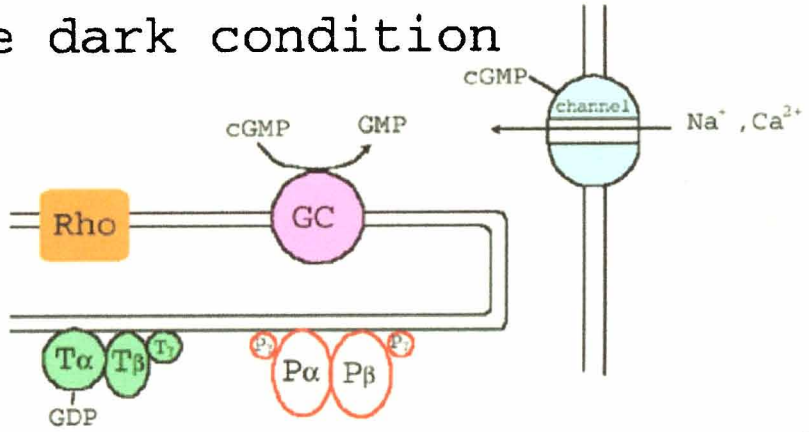


Fig.3

Fig.3 cGMP cascade model of vertebrate photoreceptor

Summary of phototransduction mechanism is schematically illustrated. Inactive rhodopsin (Rho) is activated (Rho*) by the absorption of light. Transducin binds activated rhodopsin as a trimeric complex (Tαβγ). Activated rhodopsin stimulates GDP-GTP exchange on the α subunit of transducin. GTP- Tα binds and releases PDE γ subunit from the catalytic αβ subunit of PDE. Since Pγ is an inhibitory subunit, active Tα activates PDE by binding and releasing the constraint of Pγ on Pαβ. Activated PDE hydrolyzes cGMP and reduces concentration of cGMP in ROS. The reduction of the cGMP concentration leads to closure of cGMP dependent cation channels in the plasma membrane. The closure of the channels lead to hyperpolarization of ROS and excitement of photoreceptor cell. In dark condition, cGMP is synthesized by guanylyl cyclase. The cGMP binds and opens the cation channel.
Tα, transducin α subunit; Tβ, transducin β subunit; Tγ, transducin γ subunit; Pα, phosphodiesterase α subunit; Pβ, phosphodiesterase β subunit; Pγ, phosphodiesterase γ subunit; GC, guanylyl cyclase; channel, cGMP dependent cation channel.

transducin α subunit (Tα) binds guanosine diphosphate (GDP) in its inactive form, while the transducin β (Tβ) and γ (Tγ) subunits bind tightly onto the disk membrane surface. GDP-bound Tα binds with Tβγ and this binding results in the localization of Tα in the disk membrane surface, at which rhodopsin is present. Light-activated rhodopsin binds to GDP-Tαβγ and stimulates the exchange of GDP to guanosine triphosphate (GTP) on Tα. The resultant activated Tα (GTP-Tα) is released from Tβγ and binds with the γ-subunit of cyclic GMP-phosphodiesterase (PDE). PDE consists of one α (Pα), one β (Pβ) and two γ (Pγ) subunits. Pα and Pβ have putative catalytic regions and their activities seems to be inhibited by binding with Pγs. Active Tα activates PDE by binding and releasing the constraint of Pγ from Pαβ. Activated PDE hydrolyzes cGMP and reduces the concentration of cGMP in ROS. The reduction of cGMP concentration leads to the closure of cGMP dependent cation channels in the plasma membrane. The closure of the channels leads to hyperpolarization of ROS, which yields an electrical light response. This hyperpolarization results in termination of the transmitter release of rod cell. Reduced cGMP concentration is restored by the synthesis of guanylate cyclase from GTP.

Owing largely to the highly integrated adaptation system summarized above, vertebrate photoreceptor cells can deal with a very wide range of light intensity from that in a daytime field to at a crescent night. By constant adaptation, rod cell can cover a 10^3-fold range of light intensity. This dynamic range is same as simple Michaelis-Menten type enzyme reaction. But light condition shifts the dynamic range 10^3. As a result, rod cell can recognize 10^6 range of light intensity. Adaptation also changes the time course of photoresponse. Light adapted ROS recovers faster against flashlight. Various regulatory mechanisms of the phototransduction cascade are known to ensure the adaptation. Some adaptation mechanisms are based on changes

in the cytoplasmic Ca^{2+} concentration, because the cytoplasmic [Ca^{2+}] depends light condition (Matthews et al., 1988; Nakatani and Yau, 1988). In dark-adapted condition, Ca^{2+} ions flow in through the cGMP dependent cation channel, and a high concentration of Ca^{2+} is maintained. In light adapted conditionon the other hand, the cation channel is closed and Ca^{2+} inflow is stopped, while the outflow of Ca^{2+} continues by Na+, K+/Ca^{2+} exchanger. The regulation of rhodopsin function is known as one of the Ca^{2+} concentration-dependent systems. Under the light condition, activated rhodopsin is phosphorylated by rhodopsin kinase (Bownds et al., 1972; Frank et al., 1973) and the phosphorylated rhodopsin is bound with the regulator protein arrestin. The arrestin-bound rhodopsin is not able to activate transducin. The rhodopsin kinase activity is regulated by the Ca^{2+}-binding protein recoverin, which binds and inhibits rhodopsin kinase when the cytoplasmic [Ca^{2+}] is high (Kawamura, 1993). Therefore, these mechanisms lower the sensitivity of ROS in light adapted condition. Gating of the cGMP dependent cation channel is regulated by the calcium binding protein calmodulin (Hsu and Molday, 1993). When the cytoplasmic calcium concentration is high or in the dark condition, a calcium-calmodulin complex binds channel associated protein, and increases the Michaelis constant of the channel for cGMP. This results in a decrease of cation influx into ROS and increases the sensitivity. Regulation of guanylate cyclase is also known to be Ca^{2+} dependent. To regulate the activity of guanylate cyclase, GCAP (guanylate cyclase activating protein) binds to it and accelerates the synthesis of cGMP when cytoplasmic Ca^{2+} concentration is low (Gorczyca et al. 1994). Consequently, hyperpolarization in light condition of ROS recovers rapidly to the static states and lower the sensitivity.

The activity of transducin is regulated by RGS9 (regulator of G-protein signaling 9) through the acceleration of intrinsic GTPase activity, which causes hydrolysis of transducin bound GTP

to GDP (He et al. 1998). This hydrolysis leads to a conformation change of transducin to an inactive form. Binding of the PDE γ subunit to GTP-Tα enhances the effect of RGS9. In order to facilitate the inactivation of PDE, phosphorylation of Pγ by cdk5 (cycline dependent kinase 5) when Pγ is bound by Tα is proposed (Matsuura et al. 2000; Hayashi et al. 2000). Phosphorylated Pγ easily reassociates with Pαβ and inhibits the PDE activity. Although many regulatory mechanisms are studied, the whole picture of the phototransduction regulating system is still vague.

Most of biochemical processes such as column chromatography, assay reactions, etc. are carried out in aqueous solutions. For this reason, solubilization of proteins is very important for biochemical analyses. Nonionic detergent such as Triton X-100 has been used to solubilize various membrane proteins, though it has been known that some components are hardly solubilized by such nonionic detergents and are, therefore, very difficult to analyze biochemically. However, there are many reports on the components of Trion X-100 insloluble fraction that function in the signal transduction. Tubulin, which is a major component of Triton X-100 insoluble fraction, takes part in the signal transduction through G-protein (Ravindra 1997). One of the Triton X-100 insoluble membrane constituents, known as lipid raft, function as a platform to concentrate signaling molecules (Harder and Simons 1997). In ROS, for example, guanylate cyclase is very difficult to solubilize in nonionic detergent (Hayashi and Yamazaki 1991). Nevertheless guanylate cyclase is a very important enzyme, which synthesizes the second messenger cGMP, and the regulation of guanylate cyclase is important for adaptation of photoreceptor cells. I observed the presence of many Triton X-100 insoluble proteins in ROS, almost all of which were unidentified. Therefore, I thought that the study of proteins in the fraction that is difficult to solubilize by nonionic detergent is important. In this thesis work, I investigated the significance of detergent-insoluble membrane proteins and raft-like lipid phase in phototransduction of vertebrate photoreceptor cells.

Chapter I

Introduction

In vertebrate photoreceptor cells, the cyclic nucleotide, cGMP, plays the role of a second messenger. In the phototransduction system, photosignals are transdued to changes in the activity of cGMP-phosphodiesterase (PDE), which hydrolyzes cGMP with an extraordinarily high reaction rate. The reduced cGMP concentration leads to closure of the cGMP gated cation channel on the plasma membrane. Channel closure causes hyperpolarization of photoreceptor cells and stops the release of chemical transmitters from their synaptic termini. On the other hand, recovery of cGMP concentration to the dark state level is achieved by cGMP synthesis by an intrinsic membrane protein, guanylate cyclase (GC). It has been known that the channel closure causes reduction of the cytoplasmic Ca^{2+} concentration, and that a Ca^{2+}-sensitive GC regulator, GCAP, activates GC on the disk membrane. In addition to this Ca^{2+}-mediated regulation of GC, there seems to exist more complex mechanism(s) for the regulation of GC. For example, the direct inhibition of GC by $T\beta\gamma$ has recently reported (Wolbring et al. 1999). Although it has been accepted that more intense studies should be done on the regulatory mechanism of GC in the photoreceptor cells, biochemical analysis of GC has been hampered by the difficulty arising from its insolubility in neutral detergents. For biochemical study of membrane-binding proteins, solubilization of the membrane is a critical process. Nonionic detergents like Triton X-100 have been used to solubilize membrane proteins, since they can solubilize a large majority of membrane proteins and do not affect most of the enzyme activities. Photoreceptor GC is a single membrane spanning protein being suggested to be soluble in neutral detergents if they stand alone in the membrane. However, Triton X-100 could hardly solubilize GC under usual ionic strength. Solubilization of GC

required a high ionic strength environment as 1.5 M KCl (Hayashi and Yamazaki 1991). It could be expected that GC is bound by detergent insoluble elements, possibly cytoskeletal proteins through ionic interactions. There had been two proteins known as GC binding proteins in ROS; one was GCAP (guanylate cyclase activating protein) (Gorczyca et al. 1994), and the other was GC itself (Yu et al 1999). However, neither of them could explain the insolubility of GC. The GCAP is water soluble in a high Ca^{2+} concentration range corresponding to the cytoplasmic Ca^{2+} level in the dark condition, and in tern, it becomes hydrophobic in a low Ca^{2+} concentration range corresponding to that in light adapted ROS. Hydrophobic surfaces of GCAP associate with GC inducing dimerization and activation of GC (Yu et al. 1999). In contrast to such Ca^{2+}-dependent association of GCAP to GC, the detergent-insolubility of GC itself is not Ca^{2+}-dependent. So far, no factor making GC detergent-insoluble has been identified. There seems to be another GC binding components on disk membranes. These were the reasons why I started my project to find GC binding component, which anchors GC to Triton X-100-insoluble fraction of ROS.

I used protein overlay method, also known as "far western" or "west western" method, to seek the GC-binding protein(s). To study protein-protein interaction, the "two-hybrid system" is known to be useful method. However, I did not employ this method. In the two-hybrid assay system, a high accessibility of bait and target proteins to the binding site on transcription factor is critical. However, putative GC- binding protein(s) are water-insoluble. Therefore, the accessibility seemed to be low, and the reaction seemed to be unlikely to happen.

Materials and Methods

Materials.

Dark-adapted frozen bovine retinas were purchased from Lawson Co. Ltd., Nebraska. Chemicals used were obtained from the following sources: [α-^{32}P] GTP from NEN Life Science Products Inc.; alumina N-Super I from ICN; GTP-agarose resin from Sigma.

Preparation of antibodies.

Antibodies were prepared by immunizing rabbits with peptides crosslinked to keyhole limpet hemocyanin (KLH). For anti-GC antibody preparation, a peptide corresponding to the amino acid sequence of human retinal GC from ^{636}His to ^{658}Thr (Shyjan et al., 1992) was synthesized and used for immunization. An antibody against 55kDa-GC-binding-protein (GCBP-55, later we found it was identical with RGS9) was raised against its N-terminal 15 amino acid sequence. To the both peptides were added a cysteine residue at their C-termini. A chemical crosslinker, 3-maleimidobenzonic acid N-hydroxysuccinimide ester was used to crosslink C-terminal SH-group of the peptides with KLH. For the first injections, KLH conjugated peptides were mixed with complete Freund's adjuvant containing 5mg of desiccated *Mycobacterium butyricum* to make emulsion, and immunized to rabbits by intradermal injections. Inactive *Bordetella pertussis* suspension ($2x10^{10}$ cells) was also injected intradermally. Four weeks after the first injection, we injected KLH conjugated peptides mixed with incomplete Freund's adjuvant every 2 weeks until titer of serum rose enough.

Preparation of ROS.

Bovine ROS were prepared from dark-adapted frozen retinas as described (Yamazaki et al. 1988). All preparation steps were done under dim red light until ROS were purified by ultracentrifugation. One hundred retinas were thawed in a water bath at room temperature. After the thawing step, all procedures were carried out at 4°C. Retinas were gently

homogenized with 60 ml of buffer A (10mM MOPS, 60mM KCl, 30mM NaCl, 5mM $MgCl_2$, 1mM DTT, 200μM PMSF, 0.0025% BHT) containing 30% sucrose in a loose-fit plastic homogenizer (100ml plastic graded cylinder (diameter 22.3 mm), and Teflon pestle (diameter 28.4 mm)). Homogenate was centrifuged at 2,000xg, 5 min at 4°C, and the supernatants were pooled. This step was repeated 3 times, and the supernatants were diluted to 3 fold by buffer A, and centrifuged at 3500 x g, at 4°C 10 min. The precipitate was collected and suspended by buffer A containing sucrose (density 1.10). The suspension was placed on a step density gradient (1.10, 1.13 and 1.15) of sucrose in buffer A. The gradient was centrifuged 10,000xg, 4°C, 40 min by a swing rotor (Beckman SW 28). ROS was recovered from the interface between density 1.10 and 1.13, and diluted with buffer A. Diluted ROS was precipitated by centrifuge (12,000xg, 4°C, 10min). The precipitation was suspended with buffer A, and stored in liquid nitrogen until use.

SDS-PAGE.

SDS-PAGE was performed as described (Laemmli 1970). As an exception, when rhodopsin was contained in samples, DTT concentration was raised to 70mM and heating step was omitted to minimize rhodopsin aggregation.

Western Blotting.

Proteins are separated by SDS-PAGE and blotted on nitrocellulose membrane (BioRad, Trans-Blot Transfer Medium pure nitrocellulose membrane (0.45 μm)) by using a blotting buffer (20% methanol, 40mM Tris, 40mM glycine, 0.1% SDS) and a semi-dry blotter (BIO-RAD, TRANS-BLOT SD) at 10V for 90 min. Blotted membrane was blocked by appropriate blocking buffer, and immersed in specific 1st antibody solution more than 1 hour. The membrane was washed with T-TBS (20 mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% Tween 20) and immersed in 2nd antibody solution more than 1 hour. The membrane was

washed by T-TBS and developed. When color development method was used to visualize the antigens, Goat anti rabbit IgG conjugated alkaline phosphatase (Bio-Rad) was used as the 2nd antibody, and an alkaline phosphatase (AP)-development solution (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl (pH 9.5), 165μg/ml Nitro Blue Tetrazolium, 330μg/ml bromochloroindolyl phosphate) was used as colorimetric substrate. When the enhanced chemiluminescence (ECL) method was used, donkey anti rabbit IgG, HRP-Linked whole antibody (Amersham Pharmacia Biotech) was used as the 2nd antibody, and for development, membrane was immersed in ECL western blotting detection reagents solution (Amersham Pharmacia Biotech) for 1 min, and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech). Then the film was developed

Purification of guanylyl cyclase.

Purification of GC was performed as described (Hayashi and Yamazaki 1991). All steps were done on ice. Frozen stock of ROS purified from 50 retinas was thawed and homogenized with 7 ml of buffer B (100 mM Tris/HCl (pH 7.5), 5 mM DTT, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.0025% butylated hydroxytoluene (BHT)) by passing 21 G needle. Following centrifugation (100,000xg 20min 4°C), precipitation was collected. This washing process was repeated 7 times. Then, the membrane fraction was washed 7 times with buffer C (100 mM Tris/HCl (pH 7.5), 5 mM DTT, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.0025% BHT, 400μM GTP). The precipitation was washed 7times in buffer D (5 mM Tris/HCl (pH 7.5), 5 mM DTT, 1 mM EGTA, 0.1 mM PMSF, 0.0025% BHT). The membrane fraction prepared from 50 retinas was solubilized with 15 ml of buffer E (1.5 M KCl, 5 % Triton X-100, 20 mM Tris/HCl (pH 7.5), 1 mM DTT, 0.2 mM PMSF, 0.005 % BHT, 5 μM aprotinin, 5 μM leupeptin, 5 μM pepstatin). Homogenization was done by passing the solution through #21 gauge needle 7 times. The homogenate was left on ice 3 hours. Solubilized sample was centrifuged at 100,000 x g for 20min at 4°C and the supernatant was

collected. To the solution, were added glycerol and $MnCl_2$ to make 25% (w/v) and 2 mM in final concentration, respectively. Then, the solution was dialyzed against buffer F (0.2 % Triton X-100, 20 mM Tris/HCl, 2 mM $MnCl_2$, 1 mM DTT, 0.2 mM PMSF, 0.0025 % BHT, 25% glycerol). Dialyzing buffer was exchanged 3 times with intervals of more than 3h. Dialyzed sample was applied to GTP agarose column (2 ml in bed volume, Sigma), which has been washed with buffer G (0.2 % Triton X-100, 20 mM Tris/HCl, 2 mM $MnCl_2$, 1 mM DTT, 0.2 mM PMSF, 0.0025 % BHT, 25% glycerol, 10 mM GTP), and buffer H (0.2 % Triton X-100, 20 mM Tris/HCl, 2 mM $MnCl_2$, 1 mM DTT, 0.2 mM PMSF, 0.0025 % BHT, 25% glycerol, 2 M NaCl), and equilibrated by buffer F in advance. The column was washed with 10 ml of buffer F, and eluted with a GTP concentration gradient. Five hundred micro liters of eluate containing 5, 20, 50, 100, 200, 300, 400, 500, and 600 μM GTP. The fractions #4~7 were peak fractions. These peak fractions were applied on G50 gel filtration columns (4 ml bed volume) separately. Columns had been equilibrated with buffer I (100 mM KCl, 0.1 % Tween 20, 20 mM Tris/HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM PMSF, 0.0025 % BHT) in advance. Purified GC was eluted from the column with buffer I (500 μl x 10 fractions). Fraction #5, 6, and 7 were peak fractions. Purity and concentration of the samples were checked by densitometric scanning of SDS-PAGE gel and GC activity assay. We used GC of more than 95% purity on the basis of CBB staining. These samples are stored at –80°C until use.

Preparation of Triton X-100 insoluble fraction

Frozen stock of purified ROS was thawed and washed with buffer B, C and D, in the same as GC purification. Washed membrane was solubilized with buffer J (5 % Triton X-100, 20 mM Tris/HCl (pH 7.5), 1 mM DTT, 0.2 mM PMSF, 0.005 % BHT, 5 μM aprotinin, 5 μM leupeptin, 5 μM pepstatin) contains 1.5M KCl or nothing, and incubated on ice 3 hours. Samples were centrifuged at 100,000 x g for 20min. Precipitate was suspended with buffer K

(20 mM Tris/HCl (pH7.5), 500 mM NaCl), and stored at –20°C until use.

Detection of GC-binding protein by immuno detection of GC overlaid

Proteins in Triton X-100 insolubule fraction (50 μg) were separated by SDS-PAGE using 8~18% gradient gel and blotted on PVDF membrane (Immobilon-P, millipore). Membrane was incubated in buffer M (50 mM Tris/HCl, 5 mM mercaptoethanol, 6 M guanidine HCl) more than 30 min. Then, the membrane was cut into each lane and washed 5 times with buffer N (50 mM Tris/HCl (pH 8.0), 5 mM mercaptoethanol, 0.05% Tween 40) at room temperature. Membranes were incubated in buffer L (20 mM Tris/HCl, 100 mM KCl, 1 mM DTT, 5 mM $MgSO_4$, 0.1% Tween 20, 0.2 mM PMSF, 0.0025% BHT) for 30 min at 4°C. The membranes were, then, incubated overnight at 4°C in buffer L containing 0.3 μg/ml of purified GC. Incubated membrane was washed 3 times with buffer L at 4°C for 5 min. Then, the membrane was washed with T-TBS 10min, and blocked in 3% gelatin in T-TBS of 1 hour. As the 1st antibody 1/3000-fold diluted anti-GC serum with 1% gelatin in T-TBS was applied the membrane 1 hour at room temperature. The membrane was washed with T-TBS 5 min, and then anti-Rabbit IgG (HRP-linked whole antibody 1/3000 diluted with 1% gelatin in T-TBS) was applied 2 hours as a 2nd antibody. The membrane was immersed in ECL western blotting detection reagents solution and GC on the membrane was detected by exposing the membrane to X-ray film (Hyperfilm-ECL , Amersham Pharmacia Biotech).

Detection of GC-binding protein after its separation on 2D gel electrophoresis

The first dimensional gel electrophoresis was carried out by using cylindrical gels (3 mm diameter, 11 cm length) equipped on PROTEAN II system (BIO-RAD). Triton X-100-insoluble fraction (100 μg of protein in 100 μl) was mixed with 25 μl of isoelectric focusing sample buffer (1 % NP-40, 1 % appropriate pH ampholine, 1% SDS, 100 mM DTT) and 100 mg urea. The sample was applied on the first dimensional gel that contains 8M urea

and 2% (w/v) of appropriate pH ampholine. Then electrophoresis was done at 400 V for 14 hours, and then at 800 V 2 hours, sequentially. The gels were extruded from glass tubes and immersed in SDS-PAGE sample buffer 20 min with gentle agitation. For 2nd dimensional electrophoresis, gels were applied on SDS-PAGE 8-18 % gradient gels (14cm (w) x 10 cm(h)) and electrophoresis were done. Two dimensionally separated proteins were blotted on a PVDF membrane and stained by CBB by using CBB-staining solution for PVDF membranes (0.1 % CBB R250 in methanol). After protein profile was recorded, the membrane was completely destained with methanol, and then washed twice with distilled water for 5 min. Washed membrane was immersed in buffer M over 1 hour at room temperature, and washed with buffer N (5min x 5 times, on ice). The membrane was incubated in buffer L for 30 min twice on ice. Purified GC solution was overlaid in the same procedure described in the section of "Detection of GC-binding protein by immuno detection of GC overlaid", and the GC bound by GC-binding protein on the membrane was detected by ECL detection kit.

Detection of GC binding protein by GC activity of GC-overlaid

Proteins in Triton X-100 insolubule fraction (50 μg) were separated by SDS-PAGE using 8~18% gradient gel and blotted on PVDF membrane. Membrane was incubated in buffer M more than 30 min. Then, the membrane was cut into each lane and washed 5 times with buffer N at room temperature. Membranes were incubated in buffer L for 30 min at 4°C. The membranes were, then, incubated overnight at 4°C in buffer L containing 0.3 μg/ml of purified GC. Then membranes were washed 3 times with buffer L at 4°C for 5 min. The membranes were cut into 3 mm width in order to separate bound proteins into different molecular weight strips. Bound GC on each strip was measured by GC activity assay.

GC activity assay

GC activity was measured as described (Hayashi and Yamazaki 1991) with some

modification. Reaction was carried out in 100 μl solution containing 20 mM Tris/HCl (pH7.5), 1 mM DTT, 2 mM $MnCl_2$, and 2.5% dodecyl maltoside. Samples were mixed with buffer and incubated on ice 1hour. Reaction was started by adding substrate solution ([α-32P] GTP 1μCi, 10 mM GTP, 20 mM cGMP) 10 μl and incubated at 35°C for 3 hours. Reactions were stopped by adding 50 μl of 100 mM EDTA and the heated at 80°C for 2 min. Each sample was applied on 1 ml of alumina columns (ICN alumina N-super I), respectively. Unbound cGMP was eluted with 18 ml of 50 mM Tris/HCl (pH 7.5), and bound by an anion-exchange column (AG1x8 (BIO-RAD), 2 ml). AG1x8 columns were washed with 30 mM HCl, and cGMP was eluted with 500 mM HCl. Radio activity of each sample were measured by liquid scintillation counter, following mixing with scintillation cocktail (Scintisol EX-H, dojindo laboratories).

Amino acid sequencing

N-terminal amino acid sequencing of GC-binding protein separated on two-dimensional gel electrophoresis was done as follows. Proteins in Triton X-100 insoluble fraction of bovine ROS were separated by SDS-PAGE. In order to suppress the artificial blockage of N-termini of peptides, electrophoresis was carried out by using electrode buffer containing 0.002 % mercapto acetic acid. Proteins were blotted on PVDF membrane (ProBlot, Applied Biosystems) and stained with CBB. The target protein on the membrane was excised, and applied to a protein sequencer (Applied Biosystems 473A).

To determine the internal amino acid sequence, proteins were separated by SDS-PAGE, and stained with a no-fix stain solution (0.2 % CBB R 250, 20 % methanol, 0.5 % acetic acid), and then destained with 30 % methanol. The protein band was excised, and the protein was extracted by homogenizing in buffer O (Tris/HCl (pH8.0), 1% SDS). After 5 hours mixing, gel and solution was separated by centrifugation. Remaining proteins in the gels were extracted by overnight agitation in buffer O. Extracted solution was collected, and small gel

particles were removed by centrifugal filtration (ultrafree C3GV (0.22 μm) Millipore). To remove SDS, acetone precipitation was carried out, and precipitation was dried by air. To get the internal peptide, I used cyanogen bromide or V8 protease (Boehringer mannheim). For cyanogen bromide cleavage, protein was dissolved in 70 % formic acid, and cyanogen bromide was added to make 1 % solution. Then incubated at 4°C for 15~24 hours in the dark. After the cleavage, sample was freeze-dried and the peptides were separated by SDS-PAGE with 15 % gel. The cleaved peptides were blotted on PVDF membrane and stained by CBB, then some of them were analyzed by a protein sequencer. For the experiment using V8 protease, extracted proteins were dissolved with buffer P (100 mM NH_4HCO_3 (pH 7.8), 0.1 % SDS), and the pH was adjusted to 7.5~8.5 by adding HCl or NaOH. To improve protein cleavage efficiency, DTT was added to 4 mM and incubated at 50°C for 15min. Samples were cooled to room temperature, and solid iodoacetoamide (8mM in final concentration) was added to the sample. Following 15 min incubation at room temperature, V8 protease was added and incubated at 25°C over night. After the cleavage, samples were freeze-dried and applied to SDS-PAGE with 15 % gels. Peptides were blotted on PVDF membranes and stained by CBB, then some of them were analyzed by protein sequencer.

Antibody affinity purification of GCBP-55

GCBP-55 was found by GC overlay. In order to study the effect of GCBP-55 on GC activity, GCBP-55 was purified from ROS using anti-GCBP-55 antibody affinity column. GCBP-55 specific IgG was purified from serum of a rabbit immunized by peptide corresponding to N-terminal 16 amino acids of GCBP-55. Anti serum was applied on a protein A sepharose column (Sigma), and IgG was eluted by 100 mM glycine/HCl (pH 3.0). Eluate was quickly mixed with 1/10 volume 1 M Tris/HCl (pH8.0) and applied to an antigen peptide-conjugated column, which had been made by conjugation of antigen peptide on EAH-sepharose (Amersham Pharmacia Biotech) through a chemical cross-linker EDC

(1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride, Pierce). Purified antibody was eluted from the column with 200 mM glycine/HCl (pH 2.8). Eluate was mixed with 1 M Tris (300 μl for 1 ml fraction). Purified antibody was mixed with protein A sepharose and cross-linked through DMS (Pierce). This antibody-conjugated sepharose was packed in a column and used as anti-GCBP-55 affinity column.

ROS was solubilized with buffer Q (150 mM NaCl, 1 % NP-40, 0.5 % DOC, 0.1 % SDS, 50 mM Tris/HCl (pH 8.0)) and incubated for 1h on ice. Then centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant was applied on a protein A sepharose column, and the flow through was applied on an anti-GCBP-55 antibody column. Column was washed with buffer Q and equilibrated with an equilibration buffer (0.2 % Triton X-100, 20 mM Tris/HCl (pH 7.5), 2 mM $MnCl_2$, 1 mM DTT, 25 % glycerol, 0.2 mM PMSF, 0.0025 % BHT). RGS9 was eluted by an elution buffer (0.2 % Triton X-100, 200 mM glycine, 2 mM $MnCl_2$, 1 mM DTT, 25 % glycerol, 0.2 mM PMSF, 0.0025 % BHT adjusted pH2.8). Eluent was mixed with 1/5 volume of 1 M Tris/HCl (pH 8.0) immediately.

Results

Detection of GC binding proteins by GC-overlay method

a. Detection by immunological technique.

a-1. GC-binding proteins separated by 1D- PAGE

Retinal GC was insoluble to 5% Triton X-100 without the addition of a high concentration of KCl, while a major fraction of GC could be solubilized in the presence of 1 M or higher concentration of KCl. These results suggested that GC binds to Triton X-100-insoluble

component(s) of disk membranes or cytoskeletal proteins on the surface of disk membrane through both hydrophilic and hydrophobic interactions. Thus, I examined the difference in the composition of Triton X-100-insoluble fraction prepared in the presence or absence of KCl. I employed GC-overlay technique to identify GC-binding proteins in these fractions. GC on the GC-binding protein was detected by several methods including immunological or activity assay. First, I detected GC binding proteins by an immunological technique. Proteins were separated by SDS-PAGE, and blotted onto PVDF membranes. Then purified GC was overlaid, and GC-binding proteins were identified using a GC-specific antibody (Fig. 4). Several bands were detected on the membranes. In all lanes, 110 kDa protein was observed (Fig. 4, lane3-6). Since this protein was detected without overlaying GC, it was highly likely that the 110 kDa protein was endogenous GC. In contrast, two proteins, i.e., 55 kDa and 32 kDa proteins, were detected only when the Triton X-100-insoluble fraction was prepared in the absence of KCl, and only when PVDF membranes were overlaid by GC (Fig. 4, lane 5).

The other two proteins (53 and 35 kDa) were slightly detected by GC antibody in any experimental condition (Fig. 4, lane 3 and 5). Thus, they seemed to be proteolytic products of GC or just non-specific cross-reactive components.

a-2. GC-binding proteins separated by 2D- PAGE

For more precise separation of Triton X-100-insoluble proteins, I used two-dimensional gel electrophoresis. I tried to detect GC binding proteins blotted on PVDF membranes by using anti-GC-antibody following GC-overlay (Fig. 5). In the alkaline region (pI > 8.0), the GC-binding protein of molecular mass 55kDa (GCBP-55) was detected (Fig. 5, panel C). This protein was also recognized by CBB staining (Fig. 5, panel A). In comparison to this data, I could not detect this 55kDa protein in the detergent-insoluble fraction prepared in the presence of 1.5 M KCl.

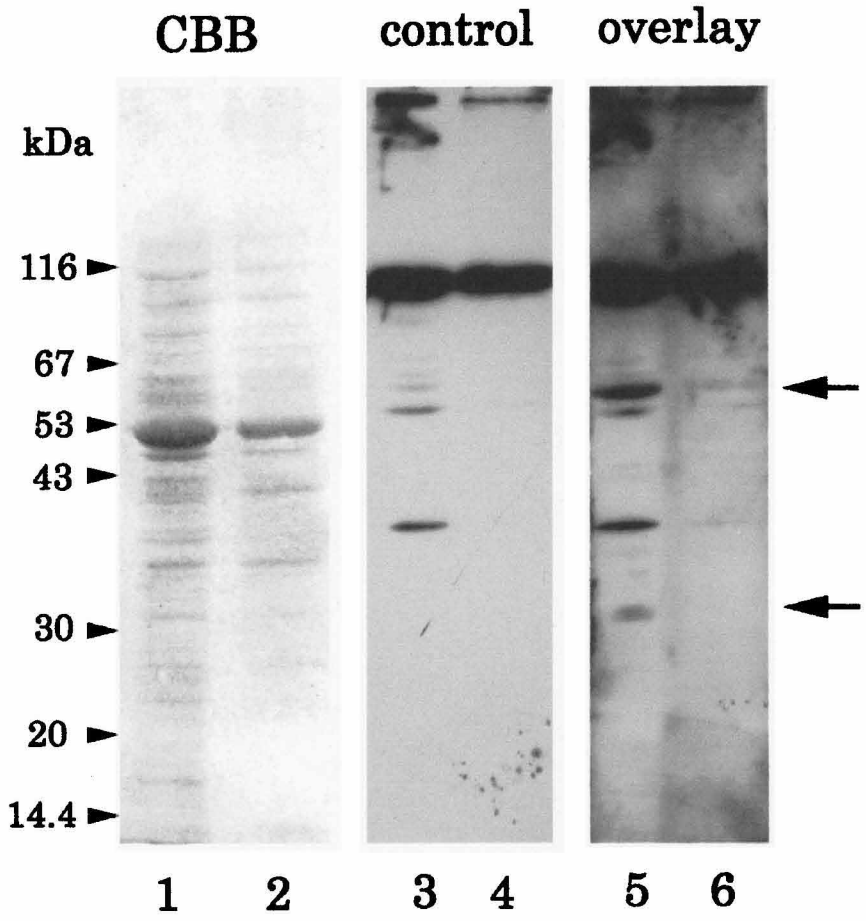


Fig.4 GC overlay with immunological technique

Proteins in Triton X-100 insoluble fraction (lane 1,3,5) or 1.5M KCl-Triton X-100 insoluble fraction (lane 2,4,6) were separated by SDS-PAGE with 8-18% gradient gel, and blotted on PVDF membrane. Membranes were incubated with or without purified GC (0.3 μg/ml) overnight at 4°C, and then GC bound to GC binding proteins were detected using anti-GC antibody. Lane 1 and 2, proteins stained with CBB; lane 3 and 4, membranes without incubation with GC; lane 5 and 6, GC detection after overlay of purified GC to the membrane. Arrowheads indicate the position of molecular mass standards. Arrows indicate position of detected GC-binding proteins.

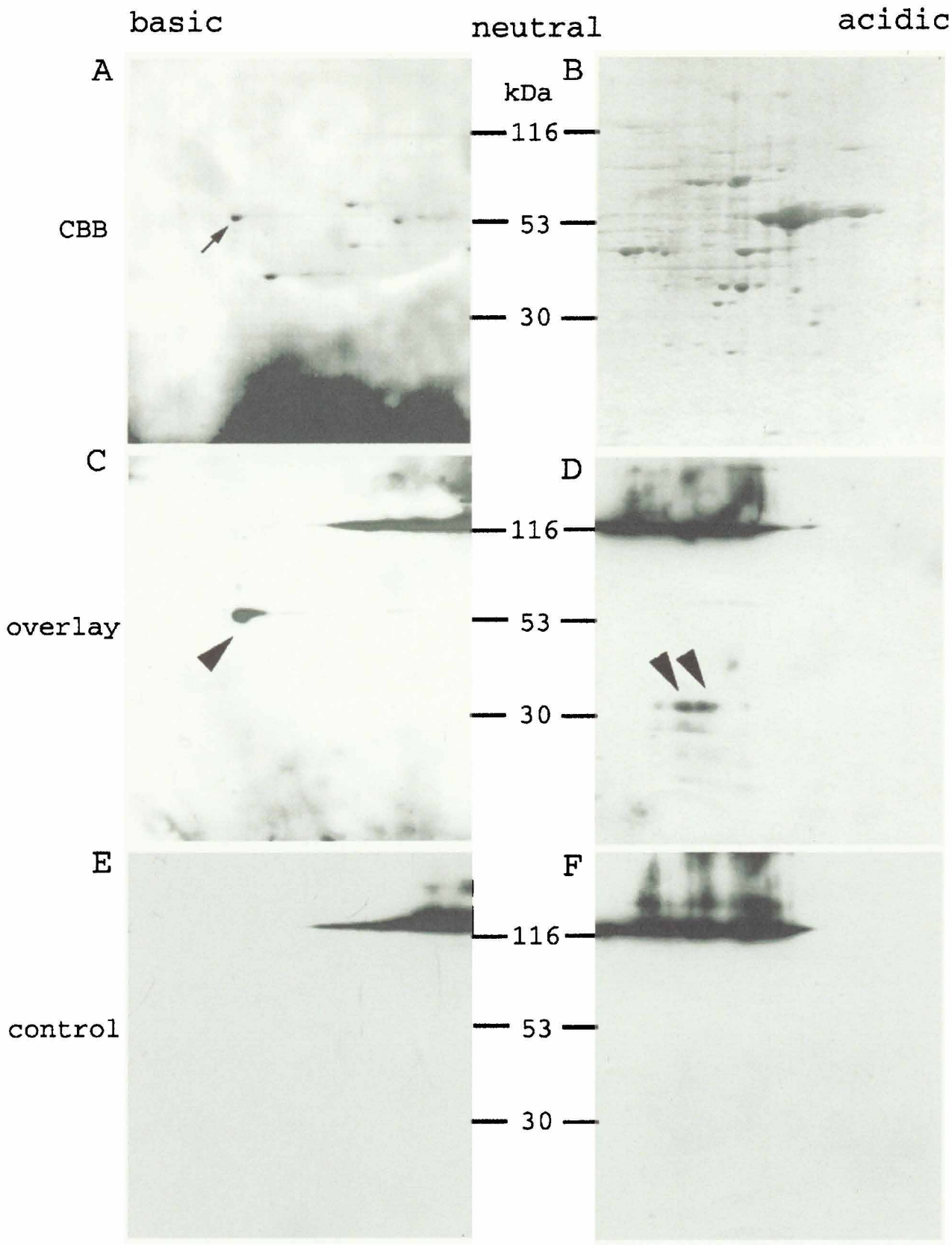

basic
neutral
acidic
A
B
kDa
116
53
30
CBB
C
D
116
53
30
overlay
E
F
116
53
30
control

Fig. 5

Fig. 5 GC overlay with 2D-PAGE

Proteins in Triton X-100 insoluble fraction (100μg) were separated by two dimensional gel electrophoresis using appropriate ampholine (pH 6-9 for A, C and E; pH 4-7 for B, D and F) and 8-18% gradient gel. Separated proteins were blotted on PVDF membranes and these membranes were incubated with or without purified GC (0.3 μg/ml) overnight at 4°C. Then GC bound to GC binding proteins were detected using anti-GC antibody. A and B, proteins stained with CBB; C and D, GC detected after overlay of purified GC to the membrane. E and F, GC detected without overlay of purified GC. Arrowheads indicate detected GC binding proteins. An arrow indicates 55kDa protein, which detected at the same position of 55 kDa GC binding protein.

In the acidic region, two major signals of molecular mass around 32 kDa, and several minor signals of 25, 20 and 12 kDa proteins were found (Fig. 5, panel D). However, these acidic GC-binding proteins appear to be less abundant than the GCBP-55 because these proteins were not detected by CBB staining. To identify the 32 kDa proteins, the acidic region of the two dimensional electrophoresis gel was stained with silver staining method (Fig. 6). Since many minor proteins were observed at the position where the 32 kDa GC-binding proteins resided, I could not identify the GC-binding proteins.

b. Detection by GC activity assay of GC-binding proteins separated by one-dimensional SDS-PAGE

I also tried to detect these GC-binding proteins by measuring the activity of GC bound to these proteins. Proteins in Triton X-100-insoluble fraction of ROS were prepared, separated and blotted as described above. Then purified GC was overlaid to these proteins. After the overlay, membranes were cut into peaces to separate the blotted proteins by their molecular weights. And the activity of bound GC was assayed. As shown in Fig.7, there was one major peak at fraction 22. Molecular mass of this fraction corresponds with 32kD-GC-binding protein. However, GCBP-55 was not detected by this method. These data suggested that GC bound to GCBP-55 is inhibited. To examine the effect of GCBP-55 on the activity of purified GC, I tried immuno affinity purification of the GCBP-55 as described in following section "Purification of GCBP-55 by antibody affinity column".

N-terminal amino acid sequence of GCBP-55

N-terminal amino acid sequence of GCBP-55 was directly analyzed by a protein sequencer. I have succeeded to know the N-terminal 20 amino acid sequence of GCBP-55. The sequence was "TIRHQGQQYRPRMAFLRKIE". By the similarity search on NCBI protein data base, I found that the sequence was exactly identical with ^{2}T-^{21}E of

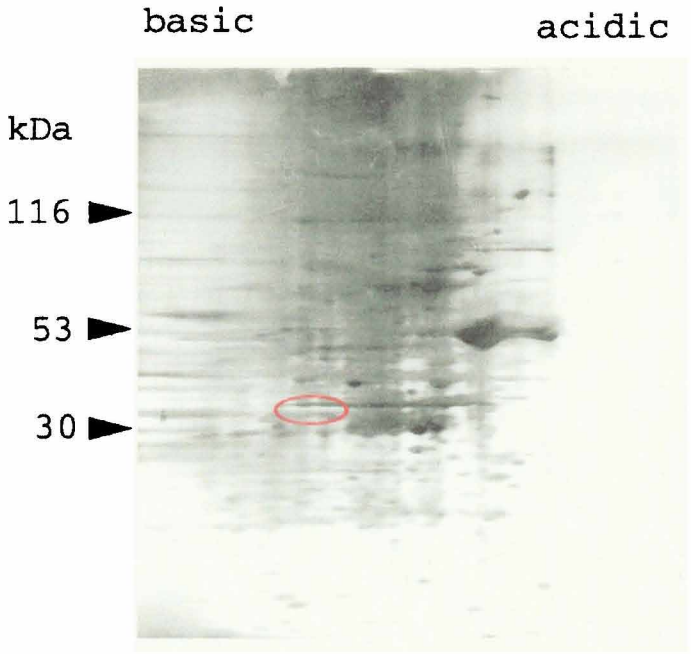


Fig. 6 Silver staining of proteins in acidic area of Triton X-100 insoluble fraction with two-dimensional gel electrophoresis.

Proteins in Triton X-100 insoluble fraction (100μg) were separated by two dimensional gel electrophoresis with acidic pH ampholine (4-7) and 8-18% gradient gel. Separated proteins were stained with silver staining method. Red circle indicates the position where 32 kDa GC binding protein appeared by GC overlay.

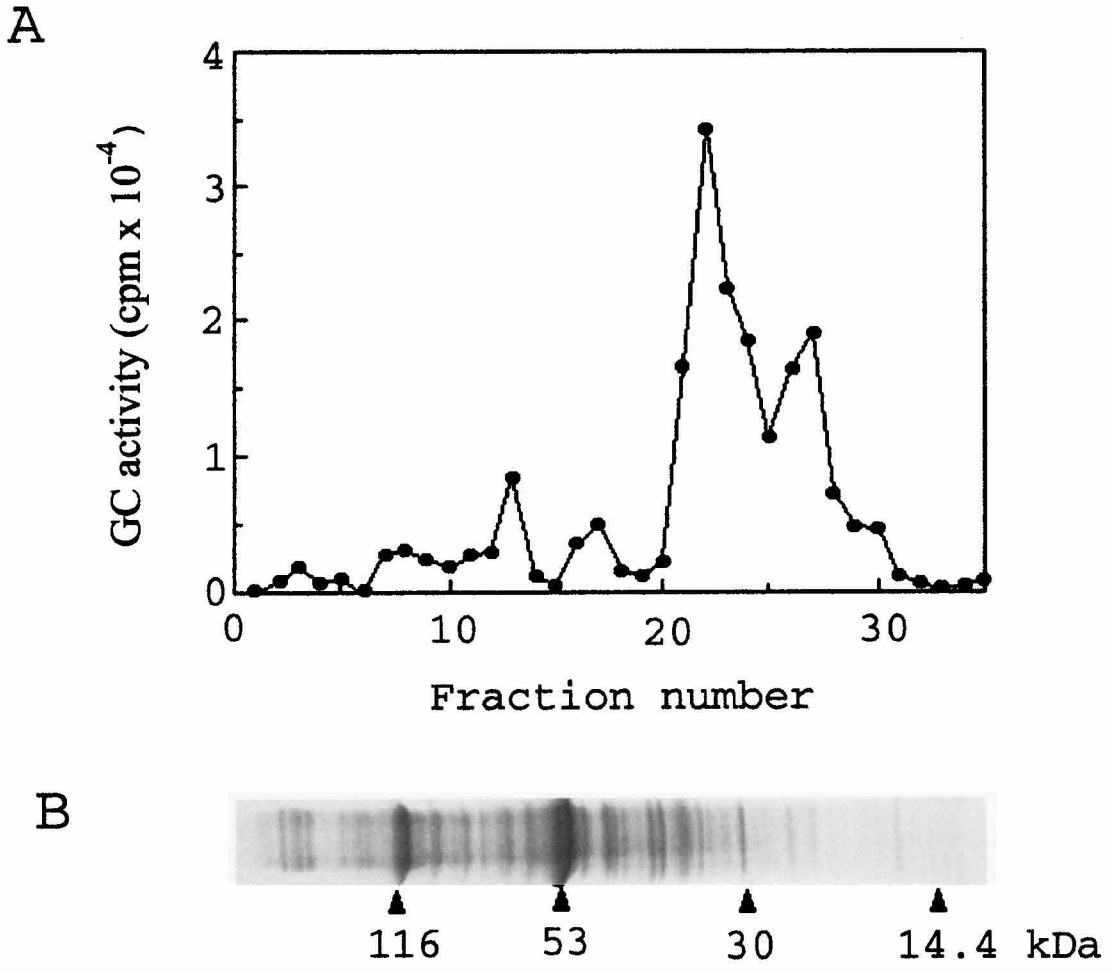


Fig.7 Detection of GC binding protein by GC activity

A, Proteins (50 μg) of a Triton X-100 insoluble fraction were separated by SDS-PAGE (8-18%). After blotting to a PVDF membrane, the membrane was incubated with purified GC (0.3 μg/ml) for overnight at 4°C. Membrane was cut into 3-mm strips and washed. The activities of bound GC on GC-binding proteins were measured using [α-^{32}P] GTP. Fraction numbers were defined from higher molecular weight to lower molecular weight on blotted PVDF membrane.

B, Protein profile of Triton X-100 insoluble fraction. Proteins on the PVDF membrane were stained with CBB. Arrowheads indicate the position of molecular mass standards.

photoreceptor-specific ninth member of the regulator of G protein signaling (RGS9), which had been cloned by He et al. (1998) at almost the same time with my finding of GCBP-55.

Internal amino acid sequence of GC binding protein

I purified the GCBP-55 using an antibody affinity-column as will be described in later section. The purified protein was degraded chemically or enzymatically. Then, I got several peptide sequences as shown in Fig.8. A computer search indicates that all amino acid residues that I identified were identical to the corresponding amino acid residues of RGS9. These sequences contain 134 amino acid residues of RGS9, i.e. more than 25% of the amino acid sequence of RGS9. These data suggest that the GCBP-55 is identical with RGS9.

Preparation of antibody against GC binding protein

I made an anti-peptide antibody against the GCBP-55. N-terminal 16 residues peptide (Fig. 8 gray box) was synthesized and immobilized on KLH, and injected to rabbit. I successfully obtained an antibody against N-terminal peptide of GCBP-55 (RGS9). Western blotting of Triton X-100 insoluble proteins separated on SDS-PAGE by using this antiserum showed specific immunoreactivity to the 55 kDa protein (Fig. 9 lane 2).

Western blotting of Triton X-100 insoluble protein separated on two-dimensional PAGE by using this antiserum showed the 55 kDa protein having pI=8 (data not shown).

Purification of GCBP-55 by antibody affinity column

The specific antibodies against GCBP-55 were cross-linked on protein A Sepharose beads, and the immobilized antibody column was utilized for the purification of GCBP-55. By using this column, I could obtain GCBP-55 of the purity higher than 95% directly from solubilized bovine ROS (Fig. 10).

Fig. 8 Amino acid sequence of GCBP-55 (RGS9)

Full-length amino acid sequence of RGS9 (He et al. 1998). Gray box is identical sequence of the GCBP-55's N-terminal sequence. Black framed sequence peptide was synthesized and used as antigen. Red letters' sequences were identical with the sequences, which I read from GCBP-55.

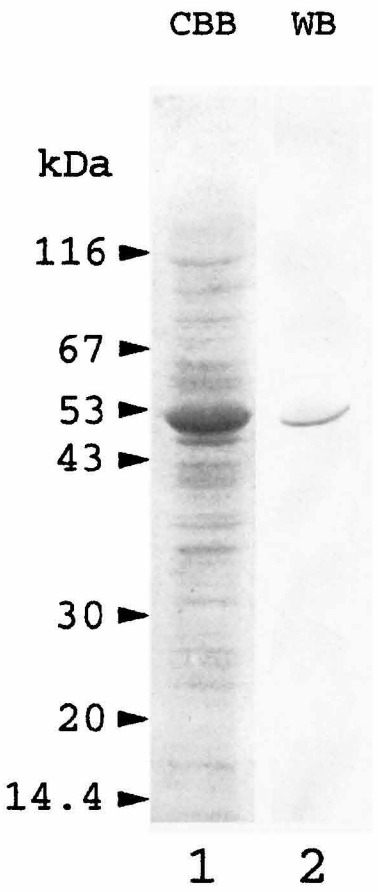


Fig. 9 Western blotting using anti-GCBP55 antibody.

Proteins in Triton X-100 insoluble fraction (30μg) were separated by SDS-PAGE (8-18 % gel) and blotted on nitrocellulose membrane. Then GCBP-55 was detected with specific antibody using color development method. Lane 1, Protein profile by CBB staining; Lane 2, Immuno-detection by anti-GCBP55 antibody. Arrowheads indicate the position of molecular mass standards.

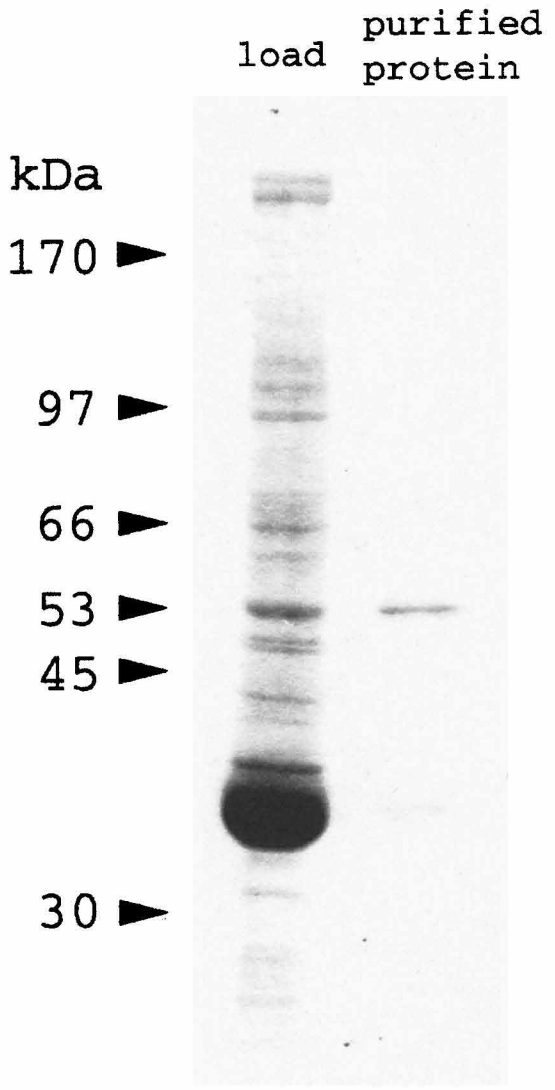


Fig. 10 Affinity purification of GCBP-55

Purified ROS was solubilized with buffer Q, and applied to anti-GCBP-55 anfibody conjugated column. The column was washed with buffer Q and equilibration buffer, and bound proteins were eluted by lowering pH. Eluted sample was neutralized with Tris/HCl (pH 8.0) and stored. Proteins are analyzed by SDS-PAGE (8-18 % gel) with CBB staining. Left lane, ROS proteins; Right lane, eluted GC-binding protein. Arrowheads indicate the position of molecular mass standards.

Effect of GCBP-55 on GC activity

I examined the effect of the GCBP-55 of purified GC. As shown in Fig.11, GCBP-55 inhibited GC activity in a dose dependent manner. Boiled GCBP-55 was inactive. At 50 nM GCBP-55 inhibited about half of the GC activity suggesting that the affinity of this binding protein to GC is fairly high.

Discussion

Identification of GCBP-55

In order to elucidate the functional significance of these GC-binding proteins, I have tried to identify these proteins by sequencing their N-termini and internal peptides. A computer search indicated that all amino acid residues of GCBP-55 peptides were identical with the corresponding amino acid residues of RGS9 (Fig. 8). These sequences contained 134 out of 484 amino acids of RGS9 indicating that the GCBP-55 is RGS9. Thus, it was strongly suggested that the GCBP-55 I found was the photoreceptor-specific regulator of G protein signaling, RGS9. The following observations also support the idea that GCBP-55 is RGS9; 1) The molecular mass of GCBP-55 is similar to that of RGS9. The nucleotide sequence of the open reading frame of bovine RGS9 indicates that RGS9 is a 484-amino acid protein with a molecular mass of 56.7 kDa, 2) The pI value of RGS9 calculated from its amino acid sequence is 9.54. The pI value of GCBP-55 was estimated to be higher than 8.0 from the data on two-dimensional gel electrophoresis (Fig 5, panel A and C). However, I should note that it is difficult to get accurate pI value in the range higher than 8.0 under my experimental conditions, 3) By using anti-GCBP-55 antibody I prepared, it was clarified that GCBP-55 was localized in the outer segments of rod and cone photoreceptors in mammalian retinas in terms

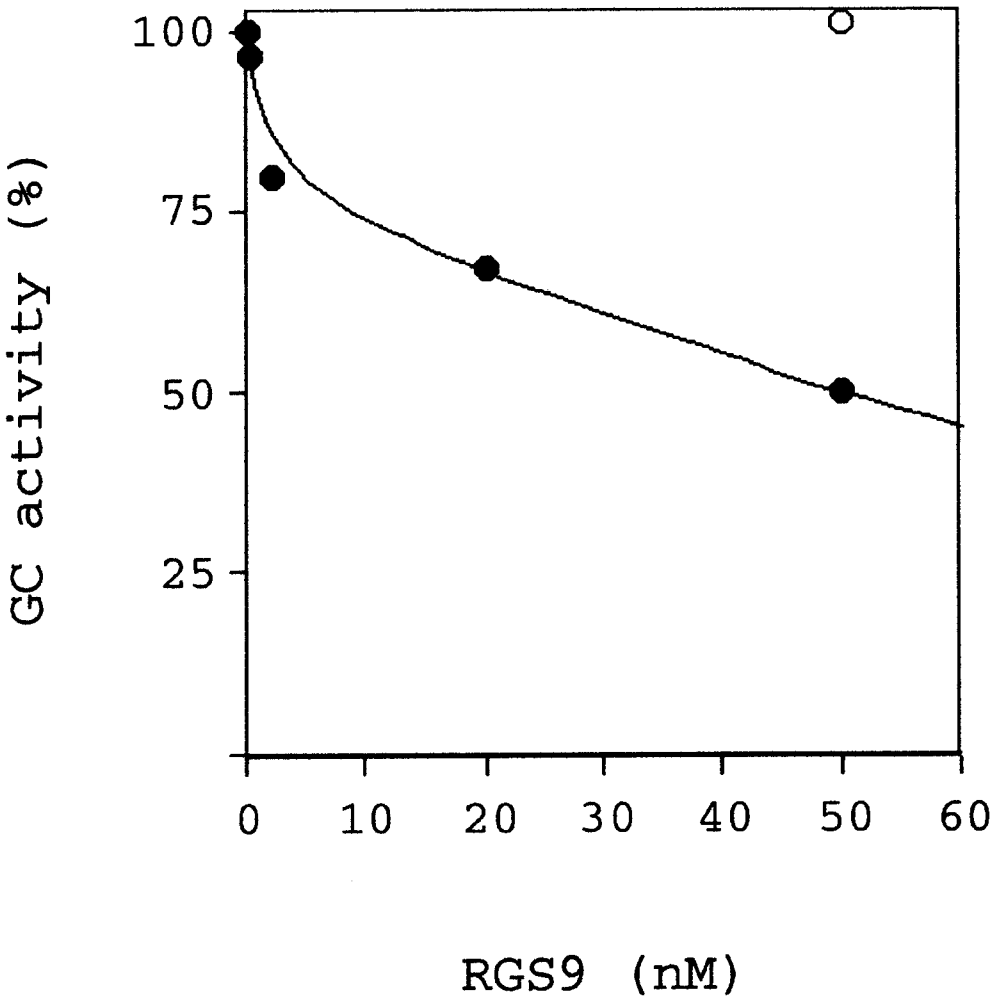


Fig. 11 Dose dependent inhibition of guanylyl cyclase activity by addition of purified GCBP-55

Various amounts of antibody affinity purified GCBP-55 was added to purified GC (15ng) and the GC activity was assayed. One hundred % of GC activity indicates 25 pmol of cGMP synthesized/min/tube. GC activity was inhibited by GCBP-55 in dose dependent manner. Black circle, GC activity with GCBP-55; white circle, GC activity in the presence of 50 nM of heat denatured GCBP-55.

of both light and electron microscopy by using our anti-GCBP-55 antibody (Nishizawa et al. 1998). RGS9 is also localized in ROS, although higher expression was observed in cone photoreceptor (Zhang et al. 1999), 4) Both GCBP-55 and RGS9 are membrane-bound proteins. Although peripheral membrane proteins such as transducin and PDE can be extracted from ROS membranes by washing with a hypotonic solution, either GCBP-55 or RGS9 are not extracted by the treatment (He et al. 1998). GCBP-55 was hardly extracted by any detergent-free buffers containing either a low (5 mM), moderate (100~200 mM), or high (1~1.5 M) concentration of 1-1 electrolyte irrespectively of the presence or absence of GTP (data not shown).

New adaptation model based on GC-RGS9 interaction.

There has been no concrete evidence that supports the presence of direct relationship between the regulatory system for the light-dependent reduction of cGMP level, and cGMP synthesizing system on the disk membranes. However, my finding that purified GCBP-55, i.e., RGS9, inhibited the GC activity in a dose dependent manner (FIG. 11) suggest that the association of RGS9 and GC may participate in the mutual regulation of the functions of these two proteins. Although there has been no evidence for such relationship between the two proteins in vivo, mutual regulation between two opposite reactions may be appropriate for the adaptation mechanism of phototransduction.

Thus, I dare propose a new model of the light-adaptation mechanism based on the mutual regulation between RGS9 and GC.

At first, I have to assume that GC is bound by RGS9 and is inhibited only in the dark conditions. By my experimental results, it has been shown that RGS9 can bind and inhibit GC. Although I have never been able to examine whether in the light or dark conditions the association of the two proteins happens, it is likely that the GC-RGS9 binding occurs in the

dark condition because RGS9 is thought to be free from its regulatory proteins only in the dark conditions. RGS9 is supposed to react with its target, the α subunit of transducin (Tα), in the light conditions. The GTPase activating protein (GAP)-activity of RGS9 is revealed only when it binds with GTP-Tα/Pγ complex, which is brought about by the activation of PDEαβγγ by GTP-Tα. Of course, the GTP-Tα is released from the holoenzyme of transducin (Tαβγ) when the transducin is activated by light-bleached rhodospin. If we assume that RGS9 inhibits GC in the dark condition, light-induced decrease in cyctoplasmic [cGMP] in a low background light would be larger than that expected in a higher background light condition, in which GC is free from the constraint of RGS9, and can replenish cGMP in a high reaction rate.

On the other hand, in the light conditions, RGS9 exerts its activity as a GAP for transducin (He et al. 1998). It is obvious that the activation of GAP activity of RGS9 results in the shortening the lifetime of active transducin (GTP-Tα), and brings about the suppression of the amplitude of photoresponse in the cytoplasmic cGMP level. Such shuttling of RGS9 between GC and transducin can be one of the key processes of the adaptation of photoreceptor cells (Fig.12). When the photoreceptor cell is adapted to a blight light, RGS9 would be in a state in which RGS9 is relatively easy to access to GTP-Tα-Pγ complex. In this context, our finding, which will be described in the latter part of this thesis, indicating the presence of RGS9 in a DRM in the disk membrane, and the light-dependent translocation of transducin to the DRM, will give us a new horizon to explore the molecular mechanisms of light- and/or dark-adaptation in vertebrate photoreceptors.

GCBP-32.

I also found 32 kDa GC binding protein (GCBP-32) (Fig. 4, lane 5). GCBP-32 binds GC but not inhibit the activity (Fig. 7). The function of GCBP-32 is not clear. Judging from the

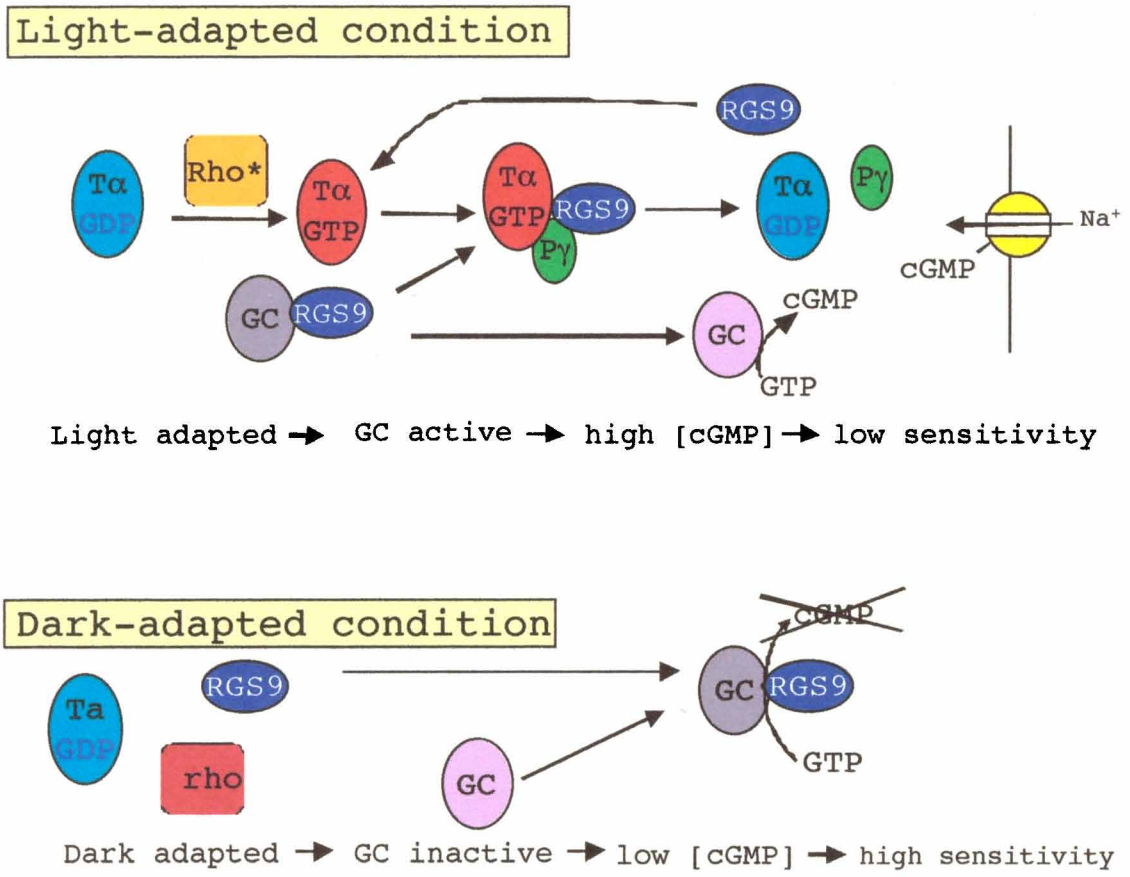


Fig. 12 Schematic view of new adaptation mechanism by GC-RGS9 interaction

Under light adapted condition, RGS9 is released from guanylyl cyclase, and binds with transducin α subunit. As a result, guanylyl cyclase activity increases and cGMP-dependent channels open. In a whole, rod outer segment has low sensitivity. Under dark-adapted condition, RGS9 binds guanylyl cyclase, and inhibits guanylyl cyclase activity. As a result, cGMP dependent channel closes easily. In a whole, rod outer segment has high sensitivity. Tα, transducin α subunit; Pγ, PDE γ subunit; Rho, rhodopsin; GC, guanylyl cyclase; ch, cGMP dependent cation channel.

silver staining data (Fig. 6), quantity of GCBP-32 is very small. Purification and identification of GCBP-32 will be difficult. May be different method needed to the study.

In summary, I report two new guanylyl cyclase-binding proteins in bovine ROS. One of the proteins is RGS9. I found the inhibition of guanylyl cyclase activity by RGS9. This inhibition can participate in the adaptation mechanism of phototransduction in ROS. Many studies are required to clarify the role of this interaction.

Chapter II

Introduction

The principal constituents of biomembranes are phospholipids, cholesterol, membrane proteins, and glycolipids. The most popular model for the structure of biological membrane is the fluid mosaic model proposed by Singer and Nicholson, in which membrane proteins are floating in the sea of lipid bilayer. In this model, lipids have been thought to move freely on the membrane, and spread uniformly. However, it has recently been proposed that there is a lateral organization on biological membranes. Lipids having largely saturated fatty acids, such as sphingolipids, tend to assemble with cholesterols to make distinct micro membrane domains called "rafts" since they are floating in the sea of highly unsaturated glycerophospholipids (Fig. 13). These distinct membrane domains are characterized by their insolubility to neutral detergent. Therefore, they are also called as detergent resistant membranes (DRM), detergent–insoluble glycolipid-enriched domain (DIG), triton-insoluble floating fraction (TIFF) or glycolipid-enriched membrane (GEM). In general, the DRMs are lateral assemblies of glycosphingolipids and cholesterol on membranes, which associate with specific proteins (for review Brown and London 2000; Kurzchalia and Parton 1999). In the DRMs, lipid-lipid interactions restrict lateral diffusion of proteins and lipids in the membrane. Increasing evidence has indicated that the DRM plays important roles in various cellular functions. One of them would be a role as a platform on which signaling molecules are concentrated (Harder and Simons 1997). Many of the signaling proteins are identified in DRMs. They include both small and heterotrimeric GTP- binding proteins (Sargiacomo et al 1993), Src family kinases (Kasahara et al. 1997), EGF receptors (Smart et al. 1995), PDGF receptors (Liu et al. 1996), Grb2 (Mineo et al. 1996), Shc (Liu et al. 1996), MAPkinase (Lisanti et al. 1994) and so on. DRM also seems to work for trafficking system from Golgi apparatus to apical membrane (Simons and Ikonen 1997).

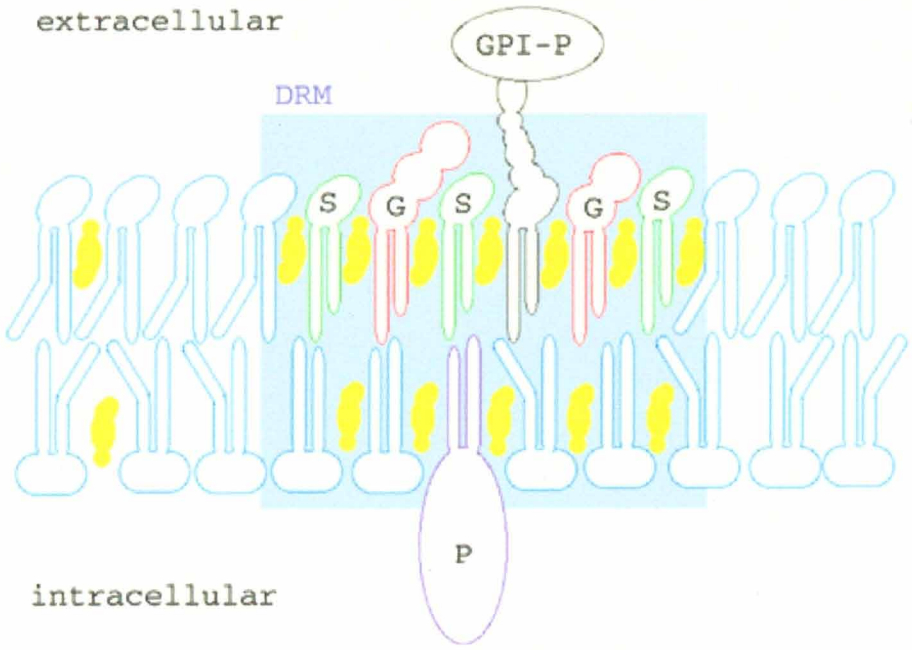


Fig. 13 Schematic view of DRM.

Blue background area indicates DRM area. Yellow parts represent cholesterol. Blue parts represent phospholipids. Green parts represent sphingomyelin. Red parts represent ganglioside. Black part represents PGI-anchored protein. Purple part represents DRM located protein.

Accumulation of glycosylphosphatidylinositol (GPI)-anchored protein, like prion protein (Naslavsky et al. 1997), to the DRM is also known. T-cell activation requires clustering of signaling molecules in DRM (Rodgers and Rose 1996). Budding of many kind of virus, including HIV-1 (human immunodeficiency virus type I), occurs selectively from DRMs (Nguyen and Hildreth 2000).

One of the most intriguing issues in membrane biology is whether reft-like distinct domain actually exist in the plane of the plasma membrane. It has not been thoroughly answered the question whether the phase-separation of such lipid domains occurs in living cells as well as in artificial membranes. But recently, some investigations on living cells using fluorescence resonance energy transfer (FRET) microscopy (Varma and Mayor 1998) or chemical crosslinker (Friedrichson et al. 1998) have showed that GPI-anchored proteins are organized in small membrane domains suggesting the existence of raft on the plasma membrane of living cell.

Although the emerging importance of the DRMs in signal transduction systems has been envisaged, there has been no report about the DRM in rod photoreceptor outer segments (ROS). ROS is comprised of numerous number of bilayer membranes; they comprise densely packed disk membrane and surrounding plasma membrane. Most of the principal proteins in the phototransduction system of ROS are intrinsic or peripheral membrane proteins. Furthermore, some of them are Triton X-100-insoluble, e.g., guanylate cyclase. Thus, I thought that the investigation of DRM of ROS would give us a deeper insight in the molecular mechanism of phototransduction in vertebrate photoreceptor cell.

In this chapter, I report on the detergent-resistant membranes (DRM) prepared from bovine ROS membranes. I found that principal components of phototransduction, i.e., transducin and its effector, cGMP-phosphodiesterase exert drastic translocation between DRM and detergent soluble domains of ROS membranes. These data suggest that the raft-like membrane domains in ROS plays very important role in phototransduction.

Materials and methods

Antibodies

Anti-peptide antibodies against bovine PDEα (^{24}N-^{40}A), bovine PDEβ (^{24}G-^{40}C) and bovine PDEγ (^{16}V-^{28}P) were raised against KLH-coupled peptide in rabbits. Procedures were written in chapter I. Antibody against Tα (^{2}G-^{15}L) was obtained from Calbiochem. Antibodies against Tβ ($G\beta_1$; C-16) and Tγ ($G\gamma_1$; P-19) were obtained from Santa Cruz Biotechnology, Inc.

Preparation of ROS

Preparation of ROS was done as described in chapter I with some modifications. All steps were done under complete darkness using infrared lamp and an image converter (NEC, Noctovision).

SDS-PAGE

Most of SDS-PAGE was done as the same with chapter I. As an exception, 8-18 %T, 0.08 %C gels were used to separate PDEα and β subunit.

Western blotting

Western blotting was done as described in chapter I.

Preparation of DRM

DRM was purified from bovine ROS as described previously for rat olfactory epithelium (Schreiber et al. 2000) with some modifications (Fig 14). Purified dark-adapted ROS membranes (12.5 mg/ml) were stored in the dark at –95°C. An aliquot of 400 μl was thawed at room temperature. After this step, all procedures were done on ice. One hundred μl of buffer A (10mM MOPS (pH 7.2), 60mM KCl, 30mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1mM BAPTA, 1mM PMSF, 5μM aprotinin, 1μM leupeptin, 1μM pepstatin, 1μM E64), and 2 μl of 100 μM

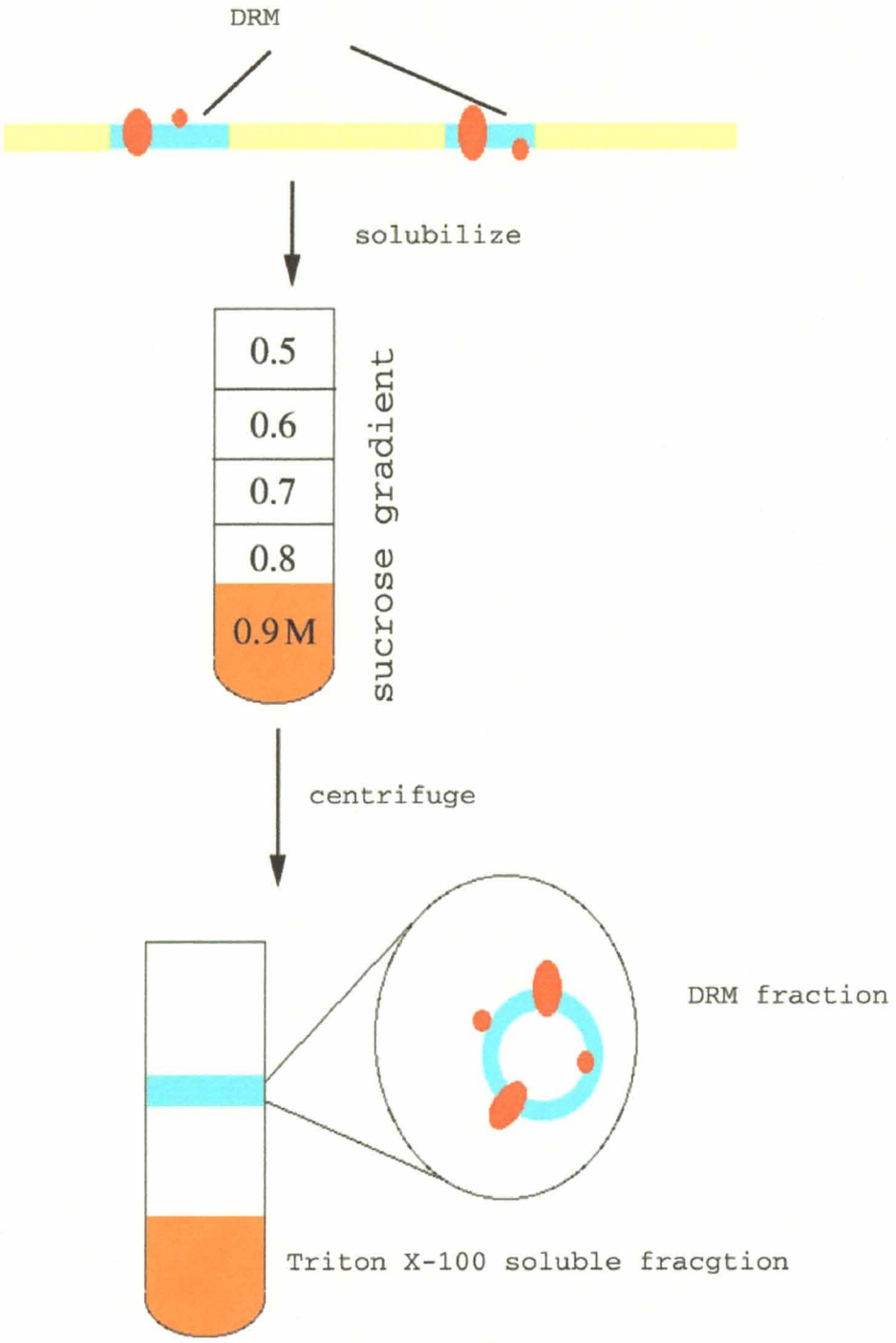


Fig. 14 schematic view of DRM purification procedure.

Lipid bilayer is composed DRM (blue area) and other area (yellow area). Membrane was solubilized with 1% Triton X-100 containing buffer. Sucrose was added to the solubilized sample to make sucrose concentration 0.9 M. Samples were overlaid with 0.8, 0.7, 0.6 and 0.5 M sucrose solution, and subjected to ultracentrifugation (300,000 x g for 20 hours at 4°C). After the centrifugation, sample was fractionated from the top of the centrifuge tube downwards.

GTPγS or DW, were added and the mixture was incubated at 0°C for 30 min in the dark or under room light. The following procedures were all carried out at 4°C, and for dark-adapted ROS all procedures were done in complete darkness. After incubation, each suspension was mixed with 62.5 μl of 10 % Triton X-100, and 160 μl of buffer A containing 1 % Triton X-100, and then 433.5 μl of buffer A containing 2.4M sucrose. Final concentrations of Triton X-100 and sucrose were 1 % and 0.9M, respectively. Samples were overlaid with 0.8, 0.7, 0.6 and 0.5 M sucrose solution in Buffer A (900 μl each) and subjected to ultracentrifugation (SW55-Ti, 46,000 rpm for 20 h at 4°C) (Fig. 14). 500-microliter fractions were collected from the top of the centrufuge tube downwards and stored at 0°C.

Preparation of fresh retina

Fresh bovine eyeballs were purchased from local slaughterhouse. The eyeballs were kept in the dark for more than 2 h on ice. Following all steps were done under complete darkness using infrared lamp and an image converter (Noctovision, NEC). Frontal hemisphere of the eyeballs were eliminated with a razor blade, and the glass bodies were removed by turning screlas and retinae inside out. Retinas were detached from pigment epitheria on reversed scleras by gentle scraping with a back of a blade of scissors. Fresh ROS was purified from these retinae by the method described in the previous chapter, and used directly for the purification of DRM.

Lipid composition analysis on two-dimensional high performance thin layer chromatography

Whole lipid was extracted from DRM fraction of ROS or ROS itself. Sucrose in DRM fraction was removed by dialysis against cold water, and samples were lyophilized. For the extraction of lipids, samples were suspended with 2 ml of chloroform/methanol (2:1) solution and sonicated for 10 min. Then, two-phase system was made by adding 0.5 ml of 1.5 M KCl. Mixed sample was centrifuged (2000 x g for 5 min at 4°C), and the aqueous phase was removed by aspiration. The lower phase was washed with 1 ml of a theoretical upper phase (chloroform/methanol/1.5 N KCl, 1:50:49). The aqueous phase and fluffy layer were removed, and residual solution was dried under N_2 gas stream. Extracts were solubilized with 50 μl of chloroform/methanol/water (2:1:0.015). Lipids were analyzed by two-dimensional high performance thin layer chromatography (HPTLC) on HPTLC plates (Silica gel 60 HPTLC,

Merck), which had been pretreated with chloroform/methanol/water (65:25:4), and dried completely by heating at 110°C for 20 min. Samples were applied on a corner of the plates with 1 cm margins, and separated with chloroform/methanol/water (65:25:4) for the first run. Then the plate was dried up, and the lipids were separated with 1-butanol/acetic acid/water (60:20:20) for the second run. In order to visualize lipids, plates were dried up, then wetted by spraying with sulfuric acid / acetone (1:1), and heated at 110°C for 30 min.

Extraction of transducin α subunit (Tα) from DRM by GTPγS

DRM fraction (100 μl) prepared under the light without GTPγS was diluted with the same volume of buffer A and spun down by centrifugation (100,000 x g for 5 min at 4°C). The precipitation was suspended with 100 μl of buffer A, and centrifuged the same condition. The precipitation was suspended with 0 or 400 μM GTPγS contained buffer A and incubated on ice for 30 min. Then the samples were centrifuged 100,000 x g for 5 min at 4°C. The precipitations were suspended with 100 μl of buffer A. Proteins in 5 μl aliquots of both supernatant and precipitation were analyzed by SDS-PAGE.

Cholesterol depletion from DRM

Cholesterol in DRM was removed by methyl-β-cyclodextrin (MCD) treatment as described previously (Maekawa et al 1999) with some modification. DRM fraction was washed with buffer A without DTT, and suspended with buffer A containing 0-15 mM MCD. After the incubation on ice for 1 hour, the suspension was centrifuged (100,000 x g for 30 min at 4°C). Proteins in extracts and residues were analyzed by SDS-PAGE. Cholesterol in these samples were assayed by using cholesterol test kit T-Cho I CII (Wako Pure Chemical Industries, LTD). By the requirement from the assay method, DTT was omitted from buffer A.

Results

Preparation of DRM from ROS

I examined whether the detergent-resistant membrane could be isolated from bovine ROS employing a typical Triton X-100/sucrose gradient method. Suspensions of ROS were incubated in the dark or under room light, with or without GTPγS, and then homogenized in a buffer containing 1% Triton X-100. After the centrifugation, one could see additional yellow-colored light-scattering layers emerged at low-density region (1.1 ± 0.05 g/ml at 4°C) of sucrose gradient (Fig. 15).

Lipid composition of DRM

Since DRMs are known as a cholesterol- and glycosphingolipid-rich domains, lipid composition of the low-density light-scattering layer fraction was examined by two-dimensional thin layer chromatography, and compared with that of whole ROS (Fig. 16). In ROS, glycerophospholipids, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), were major components (Fig. 16, panel A). On the contrary, the low-density light-scattering layer fraction showed different lipid composition. It was characterized by a relatively high percentage of cholesterol, sphingomyelin (SM) and a ganglioside, GD3 (Fig. 16, panel B). Judging from these lipid composition and density, the low-density fraction is highly likely the photoreceptor DRM.

Effect of light and GTPγS to protein compositions in DRM

After the sucrose density gradient centrifugation, samples were fractionated and analyzed by SDS-PAGE with CBB staining (Fig. 17). A characteristic protein profile was observed in the DRM fractions. In the fractions most abundant protein was rhodopsin, although most of the rhodopsin was distributed in the soluble fractions in all conditions. A similar protein composition was observed in the DRM of all conditions examined, while three proteins with molecular mass of 90, 88 and 40kDa seemed to vary their distribution drastically in a stimulus-dependent manner (Fig. 18). In the absence of GTPγS, light exposure of ROS elicited massive translocation of the 40 kDa protein from the detergent soluble to the DRM fraction (Fig. 17 panel D and Fig. 18, lane 3). This light-dependent accumulation of the 40 kDa protein in the DRM was prevented by the

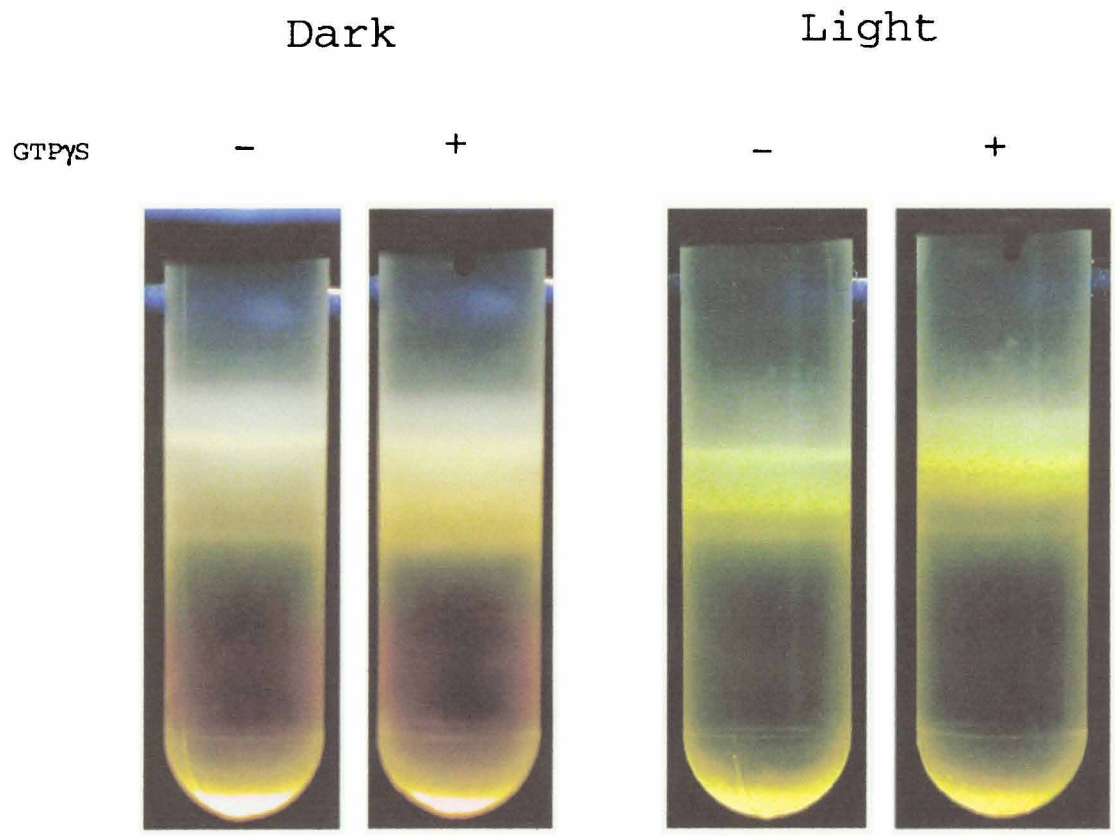


Fig. 15 DRM separation by sucrose density gradient centrifugation.

Dark-adapted or light-bleached ROS was incubated for 30 min at 4°C in the presence or absence of 400 μM GTPγS. After the incubation, ROS were solubilized with 1% Triton X-100 containing buffer, and subjected to sucrose density gradient centrifugation. Low density buoyant fraction revealed by light illumination after centrifugation.

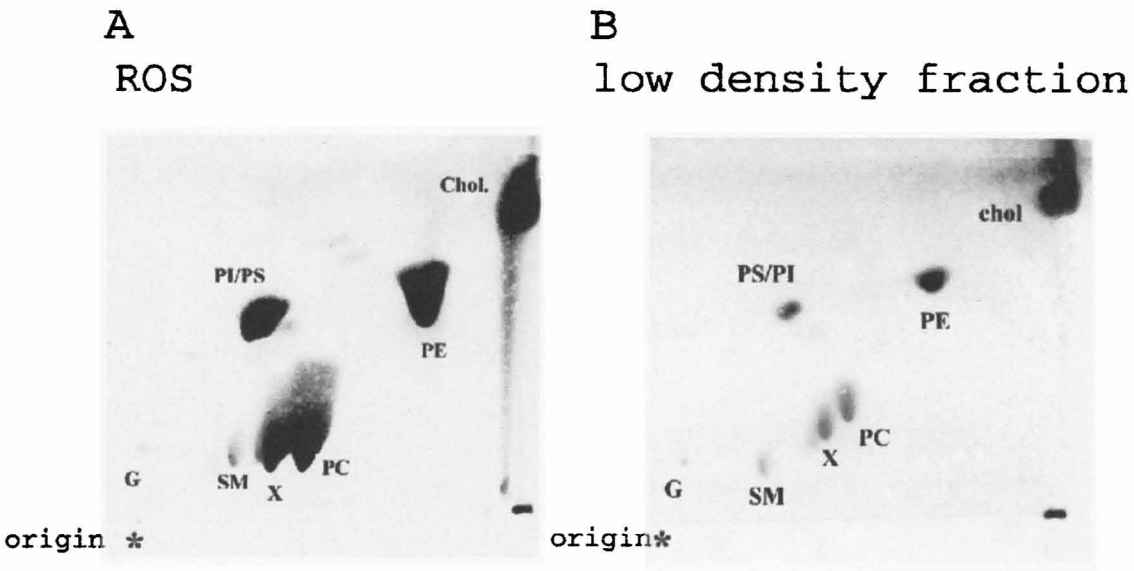


Fig. 16 Two dimensional thin layer chromatographic lipid analysis of ROS and low density fraction.

Whole lipid was extracted from DRM of ROS or ROS itself. Sucrose in DRM was removed by dialysis. Lipids were visualized by carbonization with surfonic acid at 120°C. Lipids were identified by comparison with standard lipid. PC, phosphatidyl choline; chol, cholesterol; PE, phosphatidyl ethanolamine; PI/PS, mixture of phosphatidyl inositol and phosphatidyl serine; SM, sphingomyelin; G, ganglioside; X, unidentified, probably PC derivative with different fatty acid.

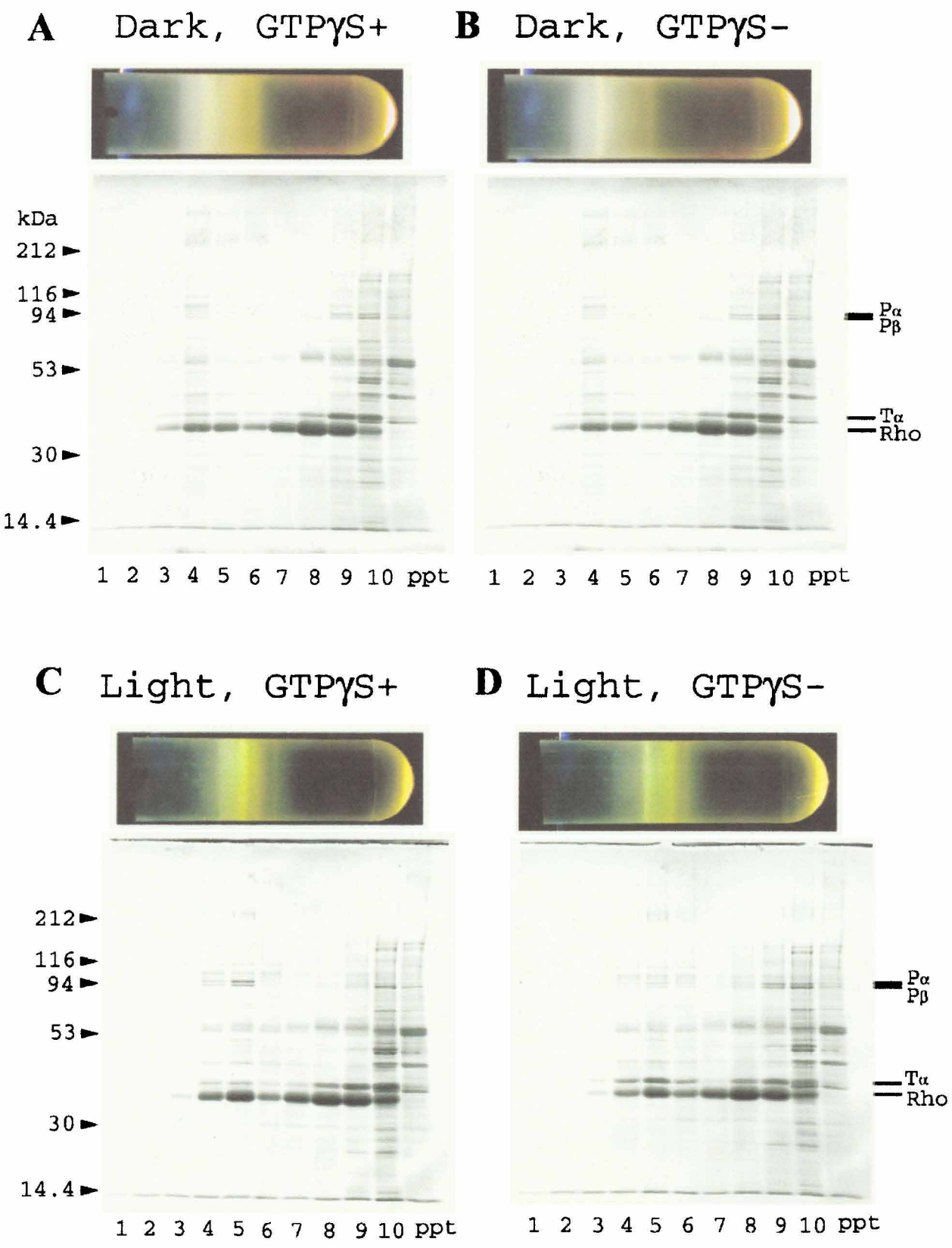

A Dark, GTPγS+
B Dark, GTPγS-
kDa
212
116
94
53
30
14.4
Pα
Pβ
Tα
Rho
1 2 3 4 5 6 7 8 9 10 ppt
C Light, GTPγS+
D Light, GTPγS-

Fig. 17

Fig. 17 protein composition analysis after sucrose density gradient centrifugation.

Dark-adapted or light-bleached ROS was incubated for 30 min at 4°C in the presence or absence of 400 μM GTPγS. After the incubation, ROS was solubilized by buffer A containing 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Then samples are fractionated from top to bottom with 500 μl each, and precipitation was suspended with 500 μl of the same buffer. Upper panels, the appearances after centrifuge (top to bottom of fraction is shown from left to right); lower panels, protein profile of each fraction. Five μl of each fraction was applied SDS-PAGE (8-18 %T, 0.08 %C gels), and proteins were stained with CBB. Arrowheads indicate the position of molecular mass standards. Molecular mass of Pα, Pβ, Tα and rhodopsin were indicated with bars at right side of the panel.

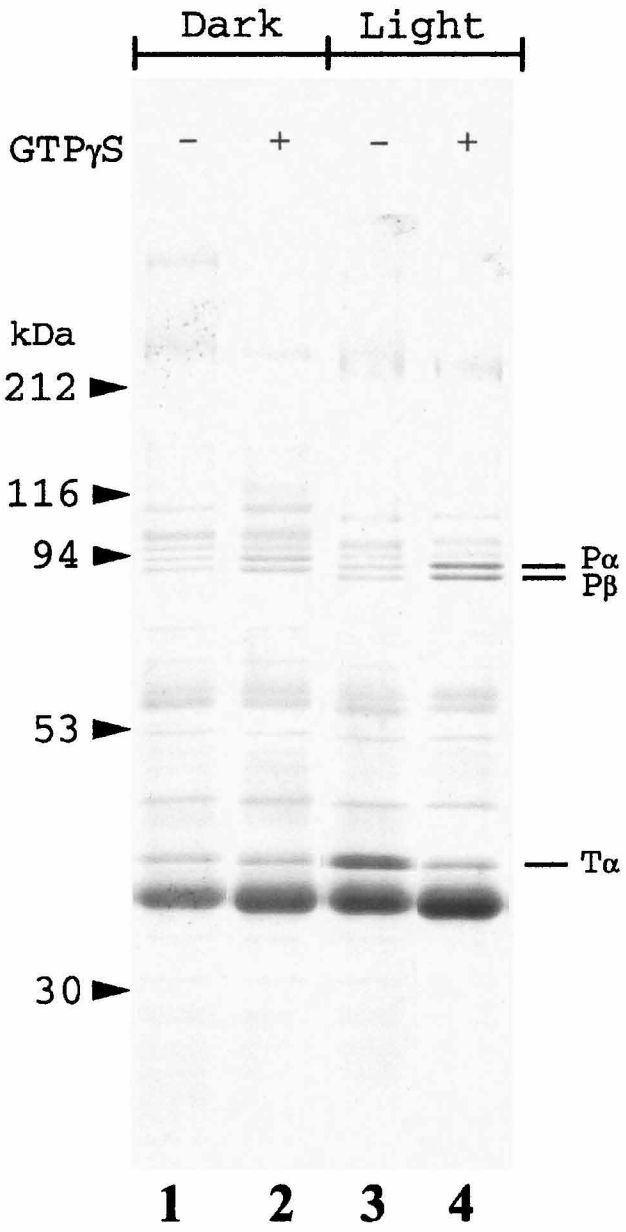


Fig. 18 Effect of light and GTPγS on the protein composition of DRM fraction

DRM fractions, that were purified under four different conditions (lane 1 and 2, under complete darkness; lane 3 and 4, under room lightning; lane 1 and 3 absence of GTPγS; lane 2 and 4, presence of 400 μM GTPγS). DRM proteins were separated with SDS-PAGE using 8-18 %T, 0.08 %C gel, and the proteins were stained with CBB. Three proteins (90, 88 and 40 kDa) had different appearance. Molecular weight of Pα, Pβ and Tα were indicated at right side bars. Arrowheads indicate the position of molecular mass standards.

addition of GTPγS to light-bleached ROS (Fig. 17 panel C and Fig. 18, lane 4). In contrast, no effect of GTPγS on the distribution of the 40 kDa protein was observed in the dark-adapted ROS (Fig. 17 panel A and B, and Fig. 18, lane 1 and 2). On the basis of its molecular weight and its quantity relative to rhodopsin, we thought that the 40 kDa protein was likely to be the transducin α subunit. On the other hand, the 90 and 88 kDa proteins accumulated in DRM following simultaneous stimulation of ROS by light and GTPγS (Fig. 17 panel C and Fig. 18, lane 4), while protein bands of these molecular weights diminished in the detergent-soluble protein fractions. Even in dark-adapted ROS, GTPγS induced indistinct but detectable translocation of 90 kDa and 88 kDa proteins to the DRM fractions (Fig. 17 panel A and B, and Fig. 18, lane 1 and 2). I thought that these bands were likely to be the α and β subunits of PDE.

Identification of Translocating Components by Western blotting

I have tried to identify the proteins that translocated in response to the stimulus by light and/or GTPγS. In order to examine the distribution of proteins in concern, I have used the specific antibodies against three subunits of transducin (Tα, Tβ and Tγ), and also against three subunits of PDE (Pα, Pβ and Pγ). The immunoblotting data indicated that Tα showed exactly the same behavior as 40 kDa protein shown in Fig.17. In dark-adapted ROS, all subunits of transducin were detergent soluble. In light bleached ROS, 30~50% of all transducin subunits were located in the DRM fractions. Upon addition of GTPγS to light-bleached ROS, these subunits become detergent-soluble again (Fig. 19). Pα and Pβ were detected at the same positions as the CBB-stained 90 kDa and 88 kDa bands. In light-bleached ROS, addition of GTPγS elicited a massive translocation of all PDE subunits from detergent soluble fractions to the DRM fractions (Fig. 20). More than 80% of all PDE subunits were in the DRM fractions. Even in dark-adapted ROS, GTPγS induced small but detectable translocation of all PDE subunits to the DRM fractions.

DRM is not the artifact arisen from freezing and thawing of ROS membranes; DRM preparation from fresh bovine retina.

As I described above, raft-like membrane fraction, i.e., DRM, could be obtained from frozen

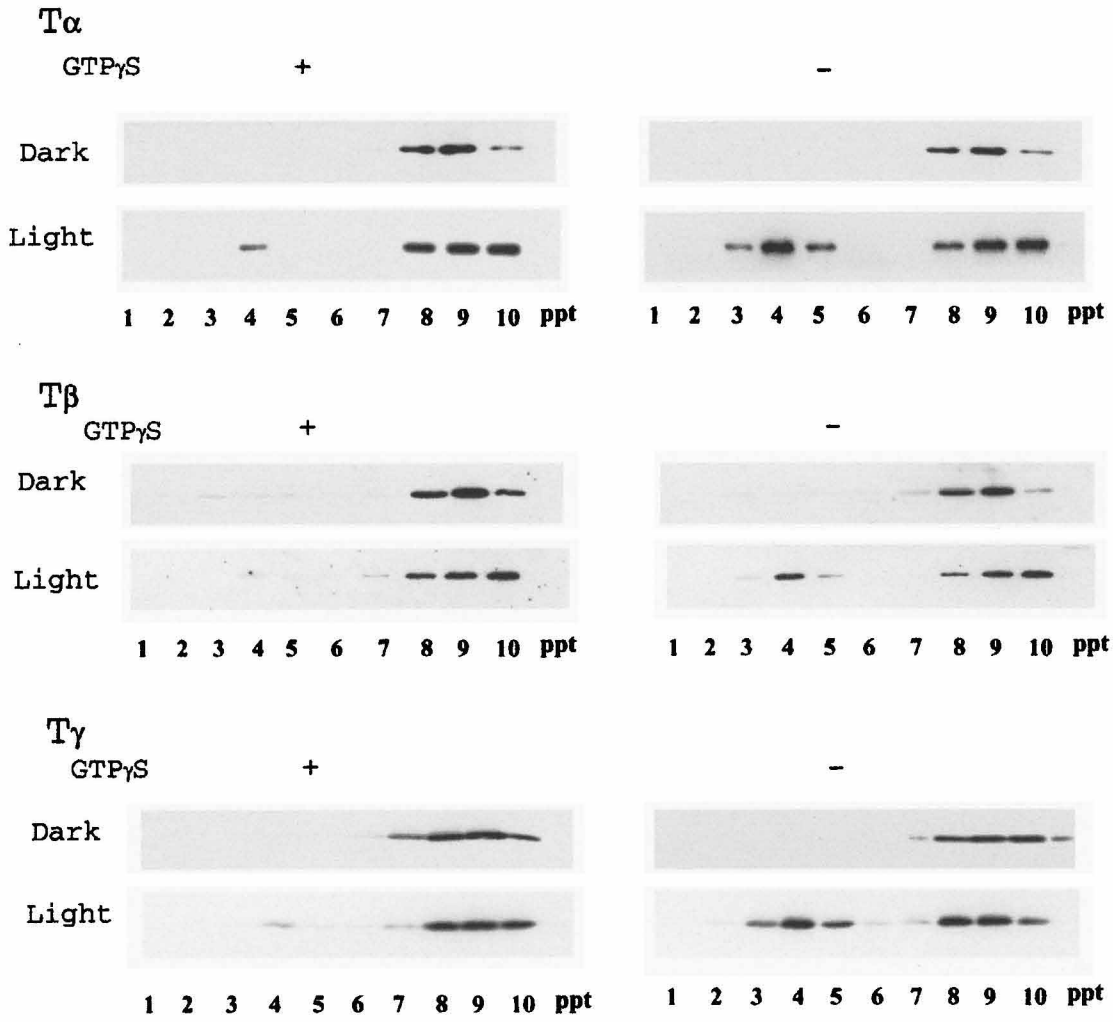


Fig. 19 Effect of light and GTPγS on the distributions of three subunit of transducin to the DRM.

Dark-adapted or light-bleached ROS was incubated for 30 min at 4°C with or without 400 μM GTPγS. After the incubation, ROS was solubilized by buffer A containing 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Then samples are fractionated from top to bottom with 500 μl each, and precipitation was suspended with 500 μl of buffer. Proteins in 0.2 μl of each fraction were separated on SDS-PAGE (8-16 % gels) and blotted onto nitrocellulose membranes. Distributions of three subunits of transducin were visualized by western blotting by using specific antibodies against each subunit of transducin.

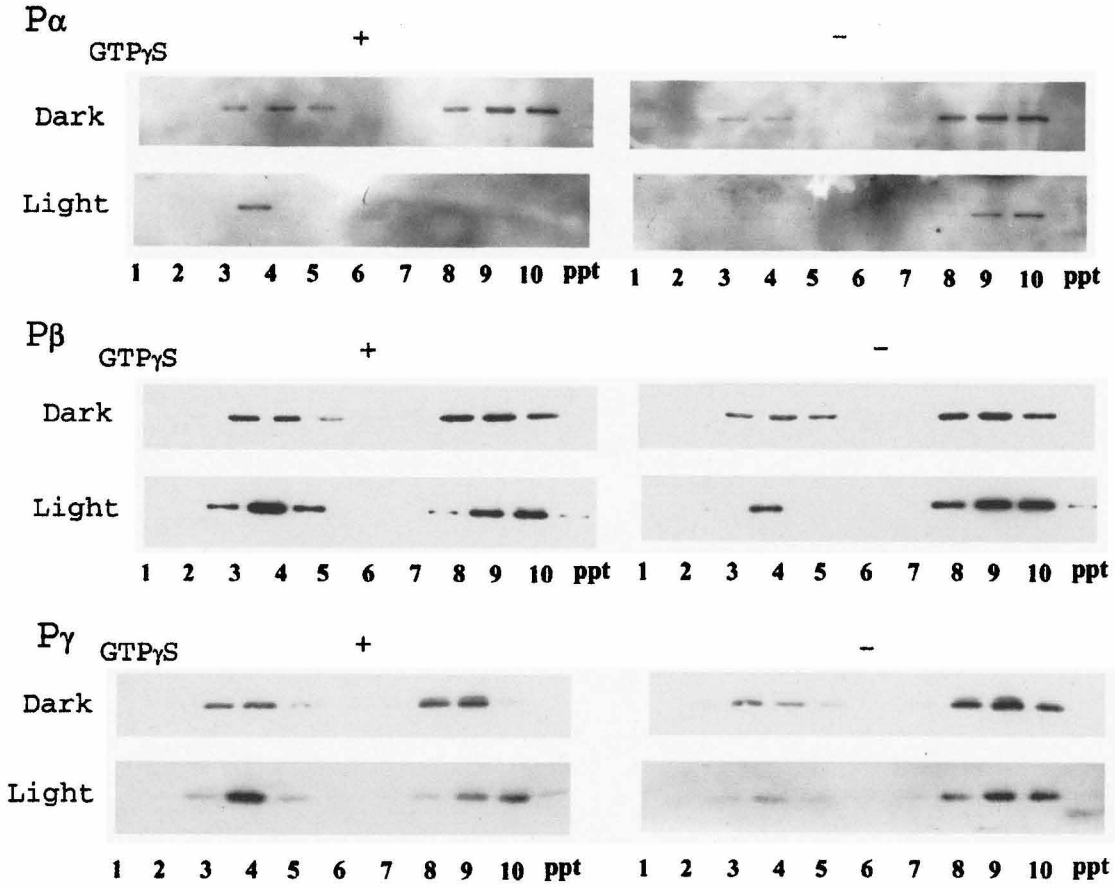


Fig. 20 Effect of light and GTPγS on the distributions of three subunit of PDE to the DRM.

Dark-adapted or light-bleached ROS was incubated for 30 min at 4°C with or without 400 μM GTPγS. After the incubation, ROS was solubilized by buffer A containing 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Then samples are fractionated from top to bottom with 500 μl each, and precipitation was suspended with 500 μl of buffer A. Proteins in 0.2 μl of each fraction were separated on SDS-PAGE (8-16 % gels) and blotted onto nitrocellulose membranes. Distributions of three subunits of PDE were visualized by western blotting by using specific antibodies against each subunit of PDE.

ROS membranes purified from frozen retinas. However, it has been known that the presence of DRM in the membrane is highly temperature-sensitive, and in addition to this, their presence in the membrane might be strongly affected by freezing and thawing that may disrupt lateral organization of biological membranes. Because of these reasons, there was a possibility that the existence of DRM could be due to an artifact arisen from the use of frozen retinas and frozen ROS membranes. To eliminate this possibility, I tried to prepare DRM from fresh bovine retinas. Since I could purchase only 10 fresh eyeballs, I purified only limited quantity of ROS. From such quantitative limitation of ROS membranes, I was forced to use a relatively higher Triton X-100/ROS lipids ratio for the solubilization of ROS membranes in comparison to usual procedure. Irrespective of such bad situation, I was able to get DRM from fresh bovine ROS membranes that had never been frozen in any process of preparation. The appearance of light-scattering buoyant fraction obtained in the sucrose gradient centrifugation was slightly different from that obtained from frozen ROS membranes. The appearance of DRM obtained from light-exposed ROS in the presence of GTPγS was not cloudy, but it looked like a paste (Fig. 21, panel A). No such difference between native and frozen ROS membranes was observed in the other conditions. Irrespective of the difference in the appearance of DRM fractions, the protein composition and their distribution pattern along the sucrose concentration gradients were almost the same being independent of the history of ROS membranes (Fig. 21, panel B).

Transducin in DRM was released by the addition of GTPγS.

Since transducin distribution under light condition was affected by the addition of GTPγS, it was suggested that the nucleotide exchange on the transducin presumably determines the fate of the protein in distributing in DRM or other detergent-soluble membrane domains. In order to study whether the nucleotide exchange occurs on the transducin recruited to the DRM of light-bleached ROS membranes, I examined the effect of GTPγS on the DRM that was purified from bleached ROS without the addition of GTPγS. As a result, transducin α subunit was extracted specifically from the DRM fraction in a GTPγS dependent manner (Fig. 22, lane 1). It is well known that, in ROS, transducin (GDP-Tαβγ) binds with light-activated rhodopsin, and then nucleotide-exchange occurs on the Tα in the hetero-tetrameric complex. In tern, activated Tα (GTP-Tα) is released from the complex. The result of this experiment indicated that the same

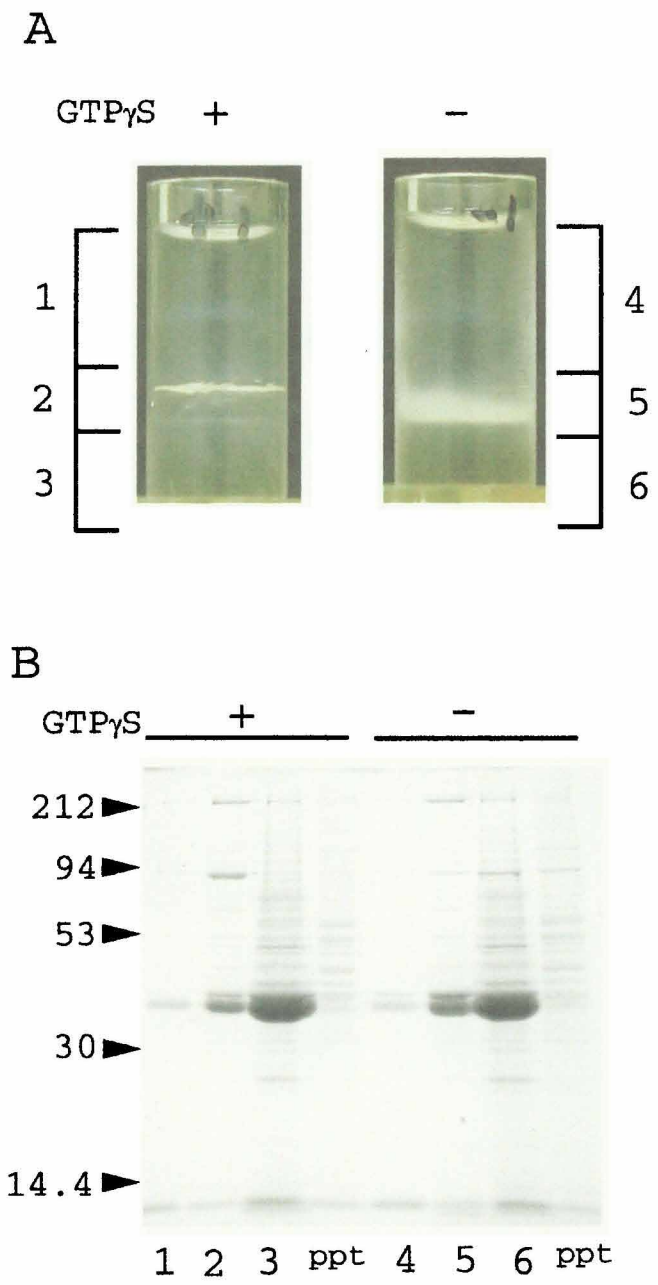


Fig. 21 DRM purification from fresh retinas.

A, Fresh ROS was purified from fresh retinas purified from fresh eyeballs. Fresh ROS (7.5 mg/ml, 60μl) was incubated for 30 min at 4°C in the presence or absence of 400 μM GTPγS. After the incubation, ROS was solubilized by 1% Triton X-100 containing buffer to make 80 μl, and subjected to sucrose density gradient ultracentrifugation (overlaid 0.8M and 0.5M sucrose buffer, 50 krpm for 16h at 4°C with TLS 55 rotor). DRM was purified under room lightning without freeze-thaw step from these fresh ROS. After centrifugation, low-density DRM fractions were appeared. **B**, Samples were fractionated as indicated in panel A, and 2 % of each fractions were subjected to SDS-PAGE (8-16 % gel), and stained with CBB. Arrowheads indicate molecular mass standard.

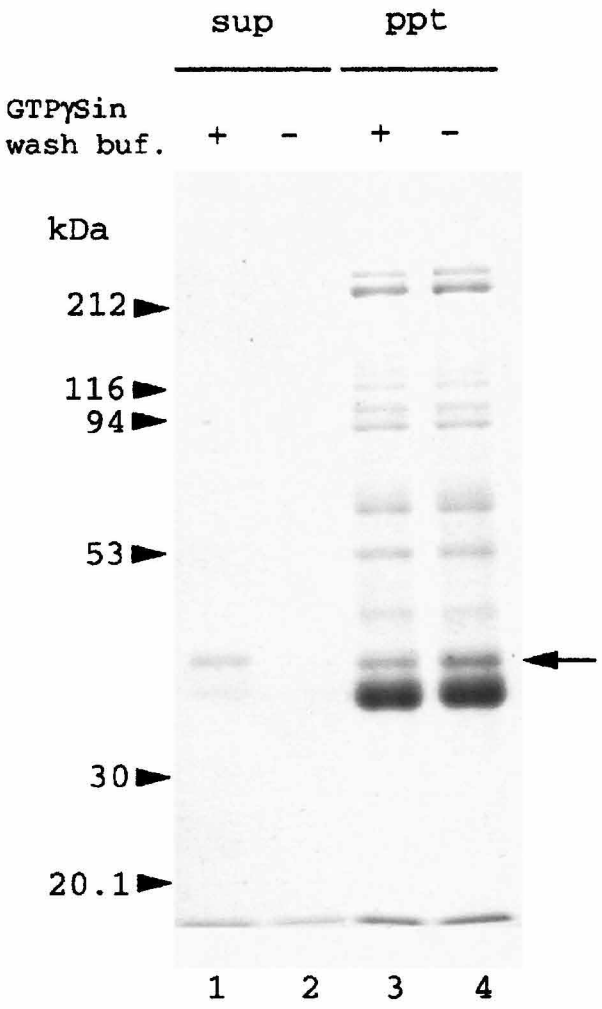


Fig. 22 GTPγS wash of DRM

One hundred micro liter of DRM fraction purified under light condition without GTPγS was diluted and precipitated by centrifugation, and washed in the presence (lane 2, 4) or absence (lane 1, 3) of 400 μM GTPγS containing buffer (100μl). After the centrifugation, 5 μl of supernatants (lane 1, 2) or precipitations (lane 3, 4) were subjected to SDS-PAGE (8-16 % gel), and stained with CBB. Arrowheads indicate molecular mass standard. An arrow indicates molecular weight of transducin α subunit.

process happened in the DRM.

Cholesterol-depletion from DRM by cholesterol removing reagent methyl-β-cyclodextrin (MCD)

Since cholesterol was a major component of DRM, it was expected that cholesterol plays very important role in DRM. Thus, I examined the effect of the depletion of cholesterol by cholesterol-binding molecule, methyl-β-cyclodextrin (MCD), on the protein composition of DRM. I tried to deplete the cholesterol of DRM by adding MCD to DRM prepared from light-bleached ROS membranes, and examined protein composition of the DRM by SDS-PAGE (Fig 23). Almost all cholesterol in DRM was extracted by MCD in a dose dependent manner, and MCD extracted PDE partially but selectively.

Discussion

There had been no evidence to support the presence of raft-like distinct domains in ROS membranes. However, I succeeded to observe and to prepare the detergent-resistant membranes (DRM) from bovine ROS membranes (Fig. 15). I used a typical preparation method of DRM, which had been employed for the preparation of DRM from various cellular membranes. Preparation of DRM was performed under four different conditions, with or without light exposure, and with or without non-hydrolysable GTP analogue, GTPγS. The fact that the translocation of signal proteins between DRM and Triton X-100 soluble fraction is dependent on photo-stimuli and unhydrolyzable analog of GTP suggests DRM plays very important role in phototransduction (Fig 17 and 18).

DRM-recruitment mechanism for rhodopsin and transducin.

Effect of light and GTPγS to the redistrubution of transducin and PDE gave very important information for elucidating the molecular mechanism of the recruitment of these proteins to

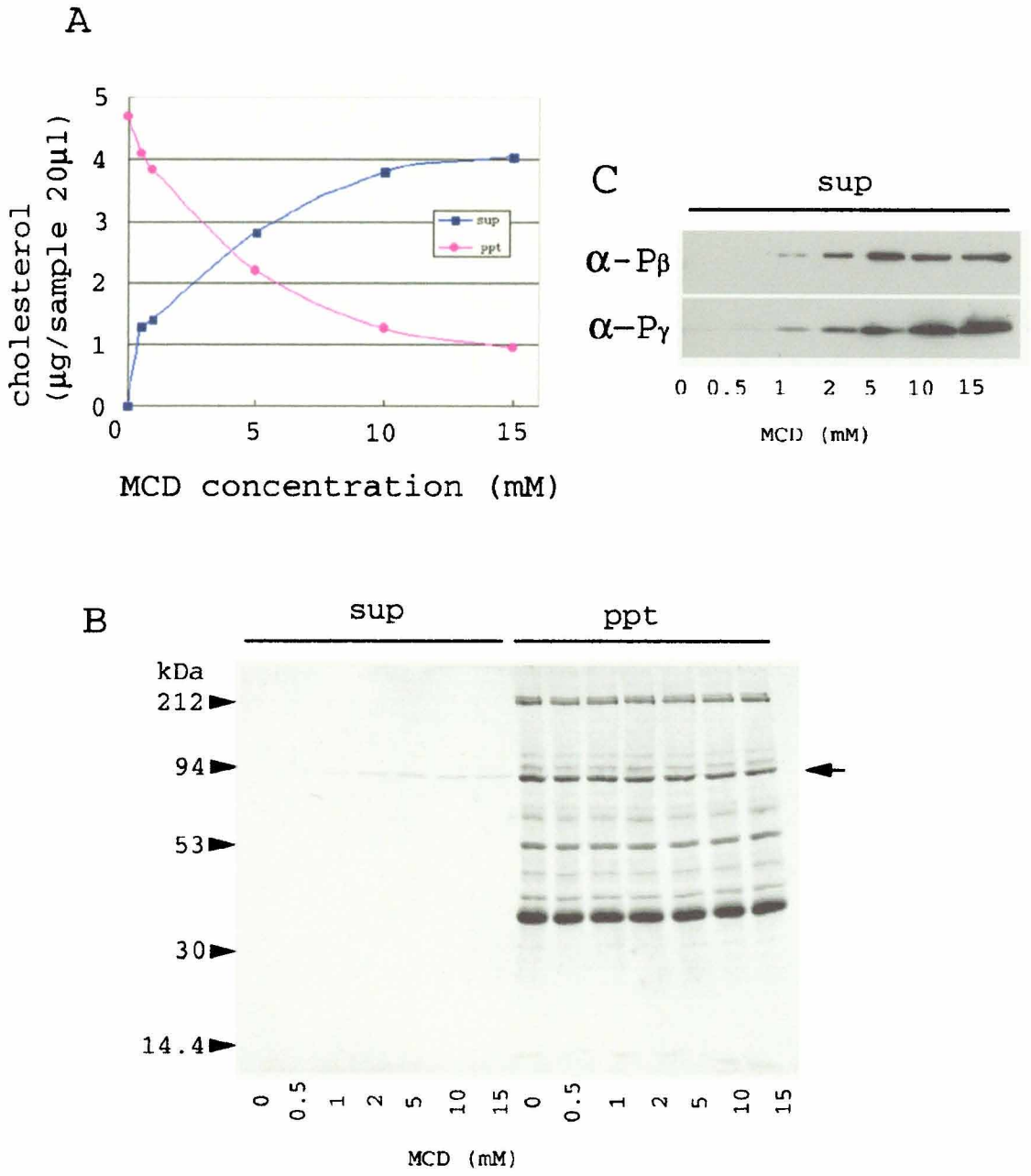

A
cholesterol (μg/sample 20μl)
5
4
3
2
1
0
0
5
10
15
MCD concentration (mM)
sup
ppt
C
sup
α-Pβ
α-Pγ
0 0.5 1 2 5 10 15
MCD (mM)
B
sup
ppt
kDa
212
94
53
30
14.4
0 0.5 1 2 5 10 15 0 0.5 1 2 5 10 15
MCD (mM)

Fig. 23

Fig. 23 Cholesterol-depletion from DRM by MCD

Light and GTPγS-induced DRM (32-μg protein) was washed with buffer A, and incubated with indicated concentration of MCD in 100-μl of buffer A for 60 min on ice. After the incubation, the samples were centrifuged at 100,000 x g for 30 min at 4°C. Supernatants (sup) were recovered. Precipitates (ppt) were resuspended in the original volume. Cholesterol contents in these samples were assayed by using cholesterol test kit T-Cho I CII (Wako Pure Chemical Industries, LTD). Cholesterol contents of supernatants and precipitates were plotted as a percentage of the total cholesterol content (0.67-nmole) in DRM (panel A). The same sample volume (10μl) of supernatants and precipitate suspensions were analyzed on a CBB-stained SDS-PAGE gel (8-16 %T, 0.4%C) (panel B). Proteins in supernatants were separated by SDS-PAGE and detected by western blotting using Pβ- and Pγ-specific antibodies (panel C). By the requirement from the cholesterol assay method, DTT was omitted from buffer A.

DRM. Transducin was located to DRM in breached ROS without GTPγS (Fig 17 and 18), and was extracted by GTPγS wash (Fig 22). These data suggest that transducin is recruited to DRM by binding with the rhodopsin in DRM. Then, I have to analyze the mechanism of rhodopsin recruitment to DRM. Rhodopsin is modified by tandem palmitoyl residues at the carboxyl terminal end of the fourth cytoplasmic loop. It has been known that the lipid-modification of membrane protein is a crucial factor for determining its distribution in closely packed raft-like liquid-ordered (l_d) phase of membrane lipids or fluid liquid-disordered phase of membranes (Melkonian et al. 1999). Tandem palmitoylation has been shown as a quite strong protein-targeting signal to DRM (Arni et al. 1998). The localization of rhodopsin in DRM as the most abundant protein component might be ascribable with these palmitoyl residues extruded from rhodopsin. However, by now, I have no answer to the question why a major part of rhodopsin is still remained in the detergent-soluble fractions. By elucidating the difference between these two components of rhodopsin, one may be able to obtain a deeper insight into the dynamic performance of phototransduction system in vertebrate photoreceptors.

Tα and Tγ are modified with a fatty acyl chain and farnesyl group, respectively, but they hardly seem to contribute to the recruitment of transducin to the DRM. Since the acyl chain on Tα is relatively short and varies in the degree of unsaturation, the affinity of Tα to DRM seems to be low. Although the farnesyl group on Tγ is as hydrophobic as acyl chains, it has a bulky branched structure that would not fit well into the liquid ordered (l_d) phase in DRM. These points would explain why Tα and Tβγ were preferentially distributed in detergent-soluble fractions in dark-adapted ROS, and in light-bleached ROS after Rho*/GDP-Tαβγ complex was dissociated by the addition of GTPγS.

Since the modifications on Tα and Tγ are not the targeting signals to DRM, the interaction between Tα and activated rhodopsin seems to make transducin locate to DRM (Fig 17 and 18). However, the ratio of Tα and rhodopsin between DRM and detergent soluble fraction showed a discrepancy. Rhodopsins were localized in both DRM and the soluble fractions; the ratio was about 1:10. However, about half of transducin was recruited to DRM when ROS was illuminated by light (Fig. 24). Two hypotheses can explain the discrepancy in the distribution pattern of two proteins; 1) Some rhodopsins are originally localized on DRM, and when bleached, they

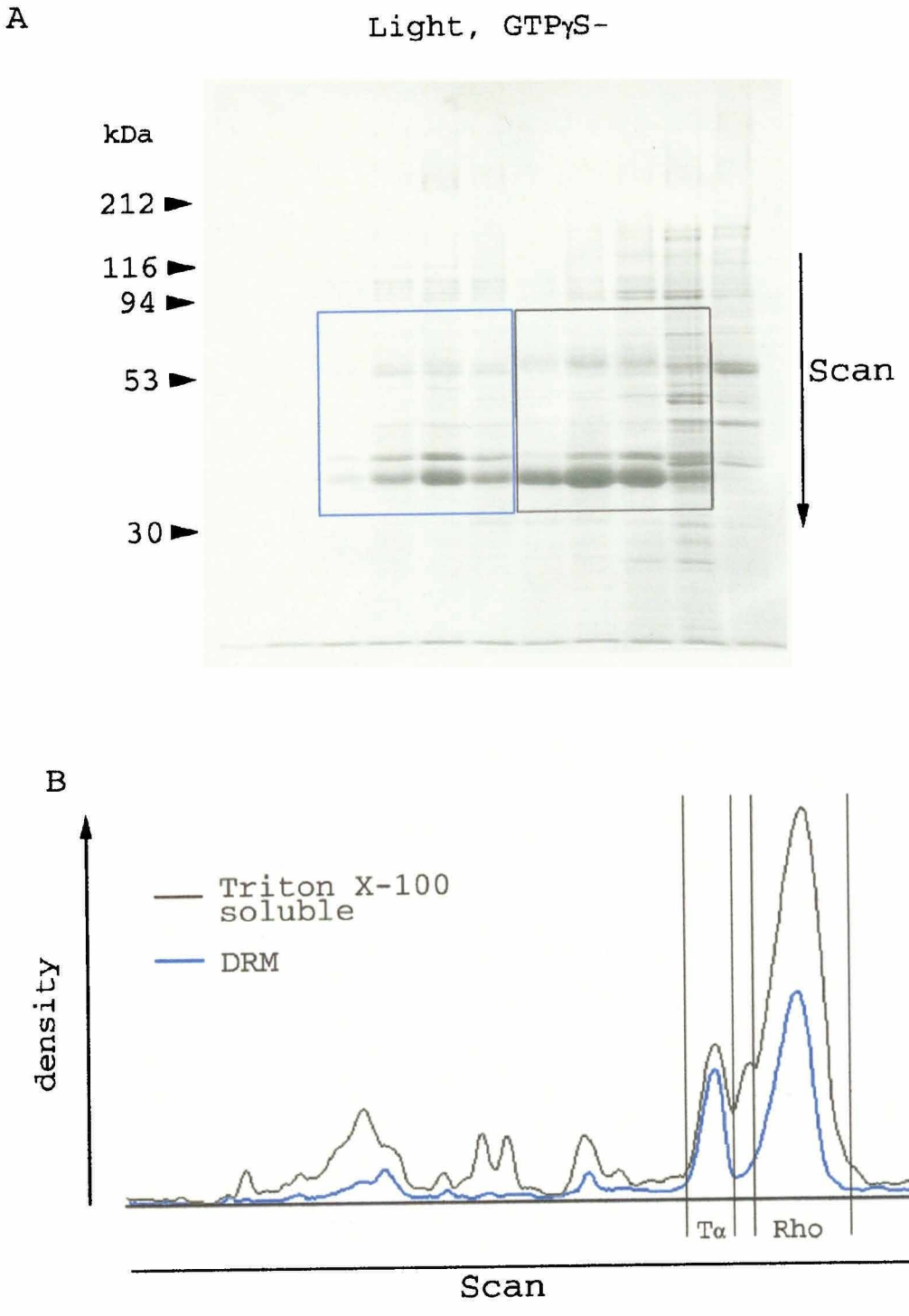

A
Light, GTPγS-
kDa
212
116
94
53
30
Scan
B
density
Triton X-100 soluble
DRM
Tα
Rho
Scan

Fig. 24

Fig. 24 Densitometric analysis of distribution of Ta and rhodopsin

A, DRM was purified from light-bleached ROS in the absence of GTPγS, and subjected to SDS-PAGE (8-18 %T, 0.08 %C gels) like Fig. 18. Then, proteins were stained with CBB. Protein distributions were analyzed between DRM fractions (black frame) and detergent soluble fractions (blue frame) with densitometric scanning. Scanning direction was indicated by arrow. Arrowheads indicate the position of molecular mass standards. **B**, Density of stained proteins were plotted. Black line indicates DRM proteins' density. Blue line indicates Triton X-100 soluble proteins' density. Molecular mass of Tα and Rhodopsin (Rho) were separated with vertical lines.

preferentially recruit GDP-Tαβγ from detergent-soluble domains of disk membranes. 2) Activated rhodopsin binds with GDP-Tαβγ in the detergent-soluble regions of ROS membranes, and then the complex was recruited to DRM. In the latter case, light-dependent increase in rhodopsin should be observed with the assembly of transducin to the DRM, though no increase in the amount of rhodopsin in DRM was detected (Fig. 17). In addition, DRM purification with additional transducin didn't affect the rhodopsin recruitment to DRM (data not shown). These data correspond with the first hypothesis. This hypothesis suggests that there are two rhodopsin pools in ROS, one in DRM and one in the detergent soluble membranes. Activated rhodopsin in DRM seems to have a priority to make contact with GDP-Tαβγ, in comparison to activated rhodopsin in the detergent-soluble membranes. Although heterogeneity in rhodopsin's affinity for transducin has not been reported, it is possible that unknown factor(s) in the DRM will enhance the association of these two proteins. The factors involved in the apparent heterogeneity of transducin activation by rhodopsin remain to be elucidated in future studies.

DRM recruitment mechanism of PDE.

PDE showed light- and GTPγS-dependent translocation from the detergent-soluble to the DRM fractions (Fig 17 and 18). The direction of the translocation of PDE was reversal to the direction of the translocation of transducin. My coworker Kishimoto reported that, the activity of PDE localized in DRM is considerably high (Kishimoto 2001), though it seemed to have two inhibitory subunits (Pγ) with its catalytic subunits (Pαβ) (Fig. 20). This result indicated that the PDE was activated by some factors in the DRM, which may presumably be the active Tα (GTPγS-Tα). Although the most of the transducin in the DRM was released upon addition of GTPγS, a little portion of transducin remained in DRM (Fig. 19). The molar ratio of transducin and PDE in native ROS has been known to be 10:1. Thus, only the little portion of transducin remaining in DRM might be enough to activate PDE in DRM.

I would like to discuss again on the possible contribution of GTPγS-Tα on DRM for the recruitment of active PDE to DRM. Notably, almost the same proportion of Pγ and Pαβ in ROS translocated to the DRM, despite the fact that ROS were stimulated by GTPγS. In classical model, active Tα binds Pαβγγ. and then Tα/Pγ complex is freed from Pαβ. However, it has also been hypothesized that active Tα and active PDE form a multi-protein complex, i.e.,

GTP-Tα/Pαβγγ, on disc membranes (Cote et al. 1994). Our data supported this hypothesis because a part of Tα, which seemed to be enough to make 1:1 complex with PDE, was localized in DRM after the addition of GTPγS (Fig. 19). Thus, it is highly likely that GTP-Tα located in DRM recruit PDE to DRM.

In addition to transducin and PDE, I observed a major fraction of retina specific regulator of G-protein signaling (RGS9; 55kDa) and a novel Gβ subunit (Gβ5L; 44kDa) in DRM (data not shown). RGS9 and Gβ5L have been reported to form a 1:1 protein complex, which activates the GTPase of Tα. It has been proposed that Pγ is an essential factor for the GAP activity of the RGS9/Gβ5L complex. Since they have a high-affinity to AlF_4^--GDP-Tα/Pγ complex, it is highly likely that they form multi-protein complex with RGS9/Gβ5L/GTPγS-Tα/Pαβγγ on the DRM on the disk membrane. This suggests the RGS9/Gβ5L complex on the DRM as a candidate for the site of transducin and PDE translocate.

Cholesterol depletion of the DRM by MCD extracted PDE specifically (Fig 23). This suggests that the interaction of PDE and cholesterol in DRM may contribute to the recruitment of PDE to DRM.

Functional significance of isoprenyls of PDE

In the context of raft-targeting signal of membrane protein, the lipid modification of PDE should be considered. Pα and Pβ subunits are isoprenylated at each C-terminal region (Anant et.al. 1992). Isoprenyl moieties on peripheral membrane proteins are considered to be a negative targeting signal to DRMs (Melkonian et al. 1999). The two isoprenyl residues on Pαβ may ascribe its exclusion from the DRM domains on the disk membrane in its resting state, i.e. in the absence of GTPγS. In its inactive state, PDE presumably extrudes two isoprenyl moieties to the outside, and thus distributes in the liquid-disordered (l_d) phase of the disk membrane. Proteins in l_d phase membranes are soluble in Triton X-100, which is in agreement with the high detergent-solubility of unexcited PDE. Further, I speculate that the activation of PDE may bring about a conformational change of PDE, which might reduce its targeting signal to the l_d phase presumably by rearranging the two isoprenyl moieties to an unexposed location.

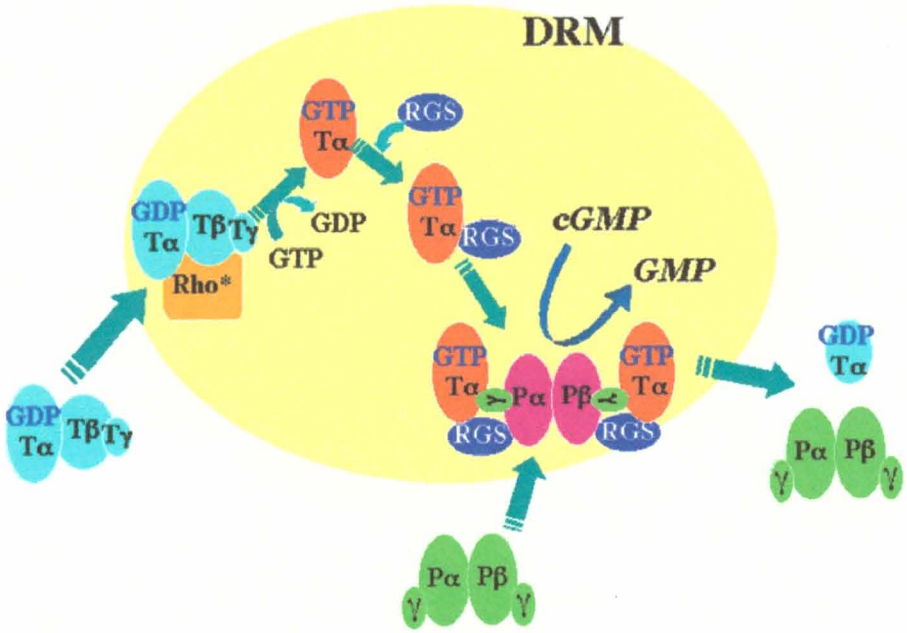
New hypothetical phototransduction mechanism with DRM.

Our new findings on the raft-like membranes in photoreceptor ROS will give us a new insight in the phototransduction mechanism in vertebrate photoreceptors. I would like to discuss the physiological significance of such raft-like phase in the phototransduction system on disk membranes. (Fig. 25)

First, it can be expected that the transient location of transducin and PDE, and the stable location of RGS9 in DRM ensure the high performance in time-resolution of phototransduction system in ROS. Recruitment of transducin (GDP-Tαβγ) to DRM by binding with Rh* in DRM will result in the increase in the concentration of active Tα around the DRM. Since a major fraction of GTPase activating protein, RGS9, with the long splice variant of type 5 G protein β subunit, Gβ5L, are located in DRM, the active Tα recruited to DRM will be easily captured by the terminator. However, since the photoreceptor RGS9 needs binding with Pγ for exerting its GAP (GTPase activation protein) activity, it is expected that GTP on Tα will not be hydrolysed until the GTP-Tα binds PDE. Because inactive PDEs are localized outside of the DRM, GTP-Tα-RGS9 complex may be waiting for the inactive PDE at the border of DRM and detergent soluble membrane area. The association of GTP-Tα/RGS9 with inactive PDE will lead activation of PDE and stimulation of GAP activity on RGS9 at the same time. This sequential but almost simultaneous activation of PDE and GTPase activating protein may allow the photoreceptor cells to undergo an effective signal transduction with a high performance in pulse-response characteristics. If there was no DRM and no assembly of these proteins, activation of PDE by GTP-Tα will continue for considerably a long period. Thus, the presence of raft-like phase is highly likely indispensable for the sequential formation of signaling protein complex, by which phototransduction system exerts signaling of high temporal resolution.

So far, I have no data about the relationship between light adaptation and DRM formation. However, if light-exposure of ROS, by any means, alters the shape, size, localization or protein composition of the raft-like phase in disk membranes, both the effectiveness in the activation and inactivation of transducin and PDE may be changed. These changes may shade a light to the molecular mechanism of the adaptation of phototransduction system. Thus, the characterization of raft-like phase in disk membrane must be an argent issue for understanding the molecular

phototransduction with DRM



phototransduction without DRM

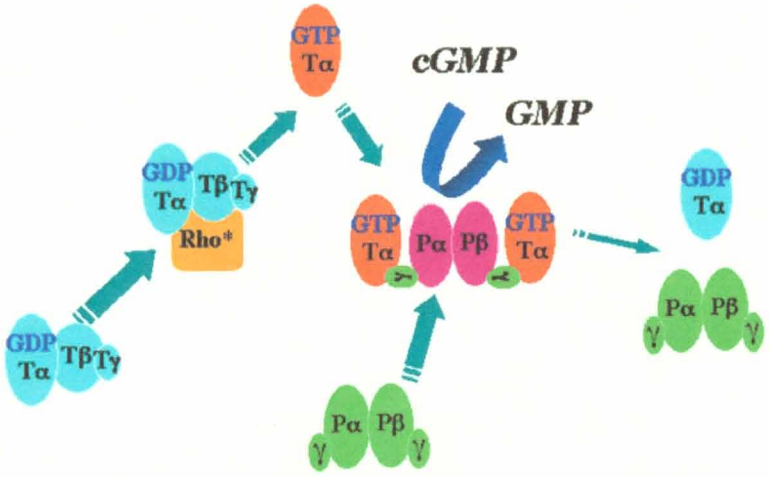


Fig. 25

Fig. 25 schematic view of new phototransduction mechanism with DRM

Upper panel shows the hypothetical phototransduction mechanism in the presence of DRM. Recruitment of transducin to DRM by binding with Rh* in DRM. Since a major fraction of RGS9 is located in DRM, the active Tα recruited to DRM will be easily captured by the terminator. However, since the photoreceptor RGS9 needs binding with Pγ for exerting its GAP activity, it is expected that GTP on Tα will not be hydrolysed until the GTP-Tα binds PDE. The association of GTP-Tα/RGS9 with inactive PDE will lead activation of PDE and stimulation of GAP activity on RGS9 at the same time. This sequential but almost simultaneous activation of PDE and GTPase activating protein may allow the photoreceptor cells to undergo an effective signal transduction with a high performance in pulse-response characteristics.

Lower panel shows the phototransduction mechanism in the absence of DRM. If there was no DRM and no assembly of these proteins, activation of PDE by GTP-Tα will continue for considerably a long period.

Tα, transducin α subunit; Tβ, transducin β subunit; Tγ, transducin γ subunit; Pα, PDE α subunit; Pβ, PDE β subunit; γ, PDE γ subunit; Rho, rhodopsin; RGS, RGS9

mechanism of light-adaptation of photoreceptor cells.

Conclusion

In this chapter, I reported on the existence of raft-like detergent-resistant membranes (DRM) in photoreceptor rod outer segment, and that there are various important proteins involved in phototransduction in the raft-like membranes. Light-dependent assembly of transducin to the DRM seemed to be appropriate for rapid quenching of active transducin by RGS9, which is localized in DRM. Further, the assembly of PDE to the DRM seemed to also be appropriate for transient activation of PDE by forming PDE/GTP-Tα/RGS9/Gβ5L. In this multi-protein complex, the constraint of the inhibitory subunit (Pγ) of PDE is released by GTP-Tα, and in tern, Pγ bound by GTP-Tα turns on the switch of GAP activity of RGS9. Thus, the raft-like domains containing RGS9 and Gβ5L seemed to be indispensable for quick quench of active Tα, and also for quick inactivation of PDE catalytic subunits. However, there are still many issues to dissolve. Not all of the DRM located proteins are identified. Mechanism of DRM proteins' distribution and translocation are not clear. Effects on various enzyme activities by DRM are not clear. Many experiments are needed to solve these subjects. The results will give us various important information not only for full understanding of phototransduction system, but also for understanding the physiological significance of raft-like phase in signaling systems in various cellular membranes.

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