J. Biochem. Mol. Biol. Vol. 29, No. 4 pp. 372~379 July 31, 1996

# Biochemical Characterization of the Interaction between Small Phosphoproteins and Transducin in Frog Photoreceptors

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(Received April 10, 1996)

**Abstract:** Components I and II (CI&II) are major phosphoproteins in the frog rod outer segments (ROS) of retina, whose phosphorylation is light- and cyclic nucleotide-dependent. Although it was reported that CI & II could be chemically cross-linked to $\beta\gamma$-subunit of transducin ($\beta\gamma_t$), it was not clear whether CI&II physically interact with $\beta\gamma_t$ under native conditions. CI&II extracted by hypotonic washing fo ROS membranes showed an overlapped migration with $\beta\gamma_t$ in sucrose density gradient centrifugation. The elution profile of CI&II in the peripheral membrane fractions from gel filtration chromatography also overlapped that of $\beta\gamma_t$. These hydrodynamic parameters indicate that the native molecular state of CI&II in the peripheral membrane fraction appears to be within a complex, most likely with $\beta\gamma_t$. CI&II coeluted with $\beta\gamma_t$ showed no phosphorylation by endogenous kinase which phosphorylates a serine of CI&II in other fractions. The purified CI&II were not able to inhibit trypsin-activated cGMP-phosphodiesterase, and CI&II were not recognized by a monoclonal antibody against the $\gamma$-subunit of transducin, indicating that CI&II are not $\gamma$-subunit of PDE or transducin. Thus, it is likely that native CI&II, which undergo a light-dependent phosphorylation/dephosphorylation cycle, can associate with $\beta\gamma_t$ in frog photoreceptor membranes, and the complex formation has an inhibitory effect on the endogenous phosphorylation of CI&II.

**Key words:** components I and II, phosphorylation, photoreceptor, rod outer segments, transducin.

Signal transduction in photoreceptor cells is carried out by a cascade of protein interactions which results in a large amplification of the light signal. Several important aspects of this transduction mechanism have been elucidated (for a review, see Gilman, 1987; Lolley and Lee, 1990; Stryer and Bourne, 1986). The photoactivation of rhodopsin leads to binding of transducin ($G_t$) to it. After exchange of GTP for GDP on the $\alpha$-subunit of $G_t$ ($\alpha_t$), the complex dissociates into rhodopsin and two functional subunits of $G_t$, $\alpha_t$ and $\beta\gamma_t$. Active $\alpha_t$-GTP interacts with the $\gamma$ subunit(s) of cGMP-phosphodiesterase (cGMP-PDE) to dislocate the inhibitory activity from the catalytic $\alpha\beta$ subunits of PDE. The disinhibited PDE rapidly hydrolyzes cGMP which holds cation channels open along the plasma membrane of ROS. The hydrolysis of cGMP results in the closure of cation channels leading to membrane hyperpolarization.

In addition to the role of cGMP in regulating channels, cyclic nucleotides are also involved in phosphorylation reactions during phototransduction. It was reported that there are cyclic AMP-dependent protein kinase (PK-A) and a few cyclic-nucleotide dependent phosphoproteins in amphibian rod outer segments (ROS) (Polans *et al.*, 1979; Hamm and Bownds, 1986; Hamm, 1990). Since the level of cyclic nucleotides in ROS in rapidly decreased by illumination (Woodruff *et al.*, 1977; Cohen *et al.*, 1978), it is expected that the activity of cyclic nucleotide-dependent protein kinase in ROS should also decrease resulting in a change of phosphorylation level of ROS proteins. In particular, two small molecular-size proteins, components I and II (CI&II, 13 and 12 kDa, respectively), showed rapid dephosphorylation upon illumination in osmotically intact ROS (Polans *et al.*, 1979; Bownds and Brewer, 1988) and isolated ROS (Hamm, 1990). Although their molecular sizes and protein levels are small and very low, respectively, CI&II are the major phosphoproteins in frog ROS whose phosphorylation is cyclic nucleotide- and light-dependent (Hamm and Bownds, 1986).

There are several reports indicating a functional relationship between CI&II phosphorylation and transducin ($G_t$) subunits, which may explain the physiological role of CI&II in the photosignal transduction system in frog ROS. The light intensity for the half maximal dephosphorylation of CI&II was within the range of those for

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$G_t$ activation and cGMP hydrolysis (Woodruff and Bownds, 1979). A monoclonal antibody against $\alpha_t$ blocked the light activation of $G_t$ and also blocked phosphorylation of CI&II (Hamm and Bownds, 1984). The localization of phosphorylated CI&II in ROS membrane was altered by the introduction of extra $G_t$ (Hamm, 1987; Suh and Hamm, 1988). Recently, it was shown that the level of endogenous phosphorylation of CI&II varied in different subcellular fractions of ROS. The peripheral membrane fraction containing $G_t$ as a most abundant protein species showed no phosphorylation of CI&II, and only these CI&II were chemically cross-linked to $\beta\gamma_t$ (Suh and Hamm, 1996). These results suggest that there is a close functional relationship between $G_t$ and phosphorylation of CI&II, which may play a role in phototransduction. However, it is still not clear whether CI&II in native state physically interact with subunits of $G_t$, and whether this interaction has any effect on the endogenous phosphorylation of CI&II. This report presents data from hydrodynamic studies to answer these questions.

## Experimental Procedures

### Materials

Carrier-free $^{32}P_i$ was from Amersham (Arlington Height, USA). [$\gamma$-$^{32}$P]-ATP was generated from $^{32}P_i$ by GammaPrep-A kit from Promega (Madison, USA). Purified catalytic subunit of PK-A (PK-$A_{cat}$) was also from Promega (Madison, USA). Cyclic nucleotides, GTP and its analogues, leupeptin and aprotinin were obtained from Boehinger-Mannheim Biochemicals (Mannheim, Germany). Sephadex G-100 was from Pharmacia P/L Biochemicals (Uppsala, Sweden). Polyvinylidene difluoride (PVDF) membrane was from Applied Biosystems (Foster city, USA). Constant boiling 6 N HCl and CF50A membrane were from Pierce and Amicon (Bevery, USA), respectively. Cellulose thin-layer plate (20×10 cm) without fluorescent indicator was a Sigma (St. Louis, USA) product. Frozen dark-adapted frog ROS, and purified cGMP-phosphodiesterase (PDE) from bovine ROS and its $\gamma$-subunit ($PDE_\gamma$), and monoclonal antibody against $\gamma_t$ were kind gifts from Dr. H. Hamm (University of Illinois at Chicago). All other chemicals were of the highest purity available from standard commercial sources.

### Buffers and solutions

For extraction of the ROS proteins: frog Ringer's (105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.5), reaction buffer [Frog Ringer's containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), $10^2$ KIU (Kallikrein Inhibitor Units)/ml aprotinin, 2 μM leupeptin, 1 mM β-merceptoethanol (βME) and about $10^{-8}$ M $Ca^{2+}$] was used. For sucrose density gradient centrifugation: solution A (0.1 mM EDTA, 1 mM βME, 0.1 mM PMSF, pH 7.5), buffer B (5 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 1 mM βME, 500 μM GTPγS and 50 mM $MgCl_2$), and buffer C (same as Buffer B without GTPγS and $MgCl_2$) were used. For gel filtration: buffer D (10 mM Tris-HCl, pH 7.5, 1 mM βME and 1 mM $MgCl_2$) was used. For thin layer electrophoresis: solution B (7.8% acetic acid, 2.2% formic acid, pH 1.9) and solution C (5% acetic acid, 0.5% pyridine, pH 3.5) were used. In order to terminate phosphorylation reaction in non-denaturing conditions, a stop solution (7.5 mM phosphate, pH 7.5 and 7.5 mM EDTA) was used.

### Phosphorylation of ROS proteins

For endogenous phosphorylation, frozen ROS were thawed on ice and resuspended in reaction buffer. ROS were disrupted by passage through a 27 gauge needle and incubated for 10 min with 20~30 μCi [$\gamma$-$^{32}$P] ATP and 30 μM ATP in reaction buffer in the presence or absence of 100 μM cAMP. The reaction was stopped by addition of stop solution for further extraction of proteins. Unless otherwise indicated, all endogenous phosphorylations and extractions of soluble proteins were performed in dim red light. For exogenous phosphorylation, protein extracts of ROS were incubated for 15 min in reaction buffer containing 2~3 μCi [$\gamma$-$^{32}$P]ATP, 30 μM ATP, 3 mM $MgCl_2$ and 0.1 μg of purified PK-$A_{cat}$. The reactions were stopped by either stop solution for further processing or 10% trichloroacetic acid for SDS-PAGE. The separation of proteins by SDS-PAGE and the phosphorylation intensity of proteins were analyzed by densitometric scanning of dried gels and autoradiographies, respectively.

Phosphoamino acid of endogenously phosphorylated CI&II was analyzed according to the method of Kamp and Sefton (1989).

### Sucrose density gradient centrifugation

ROS proteins were fractionated into soluble and peripheral membrane fractions by sequential washings of ROS membranes in reaction buffer for the soluble fraction and solution A for the peripheral membrane fraction. A high yield of peripheral membrane-bound proteins (enriched in $G_t$ and PDE) was obtained by washing ROS membranes with solution A. Each fraction was concentrated by Amicon CF50A membrane. The concentrated soluble (300 μg) or peripheral membrane proteins (100 μg) or ROS were loaded onto a 5~20% (w/w) linear gradient of sucrose and centrifuged at 105,000 ×g for 16 h. Gradients of sucrose density were made

in different buffers: isotonic buffer (reaction buffer), hypotonic buffers were used to maintain the protein environment close to that of extraction procedures for peripheral membrane proteins. In order to facilitate the separation of $\alpha_t$ from $\beta\gamma_t$, 50 mM $MgCl_2$ and 500 μM GTPγS were added (Mattera *et al.*, 1987). After centrifugation, proteins were fractionated and each fraction was phosphorylated by 0.1 μg PK-$A_{cat}$ with 3 μCi [γ-$^{32}$P]ATP, 30 μM ATP and 3 mM $MgCl_2$ for 15 min.

### Gel filtration chromatography

ROS proteins were phosphorylated by an endogenous protein kinase(s) with 30 μCi [γ-$^{32}$P]ATP, 30 μM ATP and 100 μM of cAMP and cGMP. The phosphorylated ROS proteins were then fractionated into the soluble and peripheral membrane fractions by sequential washing of ROS membranes with reaction buffer and hypotonic buffer D containing 500 μM GTP. Each fraction was concentrated by an Amicon CF50A membrane to 1 mg/ml of soluble proteins (2.3 mg total protein) and 0.75 mg/ml (1.4 mg total protein) of peripheral membrane proteins. Each concentrated protein was loaded onto a Sephadex G-100 column (1.5×150 cm) and eluted by the same buffer found in each protein extract at a flow rate of 0.5 ml/min. Every fraction was assayed for protein content and extent of phosphorylation by SDS-PAGE, Coomassie blue staining and autoradiography.

### cGMP-phosphodiesterase assay

In order to study whether CI&II can inhibit the activity of PDE, purified PDE was treated with trpsin as previously indicated (Liebman and Evanczuk, 1982), which performed a limited proteolysis of γ-subunit of PDE to activate the enzyme. Trypsin-activated PDE (tPDE), and CI&II from the soluble and hypotonic-wash fractions were homogenously purified respectively by C4 reversed-phase chromatography (Vydac, 4.6×250 mm). The cGMP-hydrolysis activity of tPDE was measured using the proton-evolution assay (Liebman and Evanczuk, 1982). The assay was carried out at room temperature in a final volume of 200 μl in 10 mM HEPES buffer (pH 8.0) containing 150 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol. The tPDE concentration in the assay was 2.5 nM. The reaction was initiated by the addition of cGMP (4 mM). Each purified CI&II (150 nM) was added to the assay mixture after 1 min to study whether CI&II inhibited active tPDE. As a control for complete inhibition of the enzyme activity, 5 nM purified γ-subunit of PDE was added.

## Results

### Native molecular state of CI&II

In order to reveal the native molecular state of CI&II, the hydrodynamic properties of CI&II were studied. For this purpose, experiments of membrane filtration, sucrose density gradient centrifugation and gel filtration chromatography were performed. Extracts of soluble and peripheral membrane-bound proteins from frog ROS were concentrated in a 50 kDa cut-off membrane, and most CI&II were retained, suggesting that the native molecular masses of CI&II were larger than 50 kDa (data not shown). The denatured molecular masses of CI&II estimated by SDS-PAGE were 13 and 12 kDa, respectively. Therefore, it appeared that in the native state CI&II existed either as an oligomer of themselves or as a complex with other protein(s).

In order to study the identity of this complex further, protein fractions of ROS membranes were separated by sucrose density gradients and the co-migration with other known ROS proteins was studied. When soluble proteins were separated in isotonically buffered sucrose density gradients, CI&II migrated to a density of approximately 4.2 S (Fig. 1A). The molecular size of the complex estimated from the calibration curve of standard proteins was approximately 64 kDa. On the other hand, other soluble proteins seemed to migrate to the density corresponding to their molecular mass (e.g., 48 kDa protein to 3.3 S and 22 kDa protein to 2.1 S). The migration pattern of CI&II in the peripheral membrane fraction in hypotonically buffered sucrose density gradients containing GTPγS and $MgCl_2$ was similar to that of soluble proteins (data not shown). When the peripheral membrane proteins were centrifuged in hypotonically buffered sucrose density gradient without $MgCl_2$ and GTPγS, $\alpha_t$ migrated to a different density than $\beta\gamma_t$ (Fig. 1B). This separation was specific to $G_t$ because proteins of molecular mass 45 kDa (3.1 S), PDE (9.2 S) and other proteins did not change their mobilities in this condition. The α-subunit of $G_t$ moved to a very high density of sucrose (~11.6 S). Under this condition, the migration profile of CI&II overlapped that of $\beta\gamma_t$, which also moved to a higher density than in other conditions. The calculated sedimentation coefficients of proteins in the different conditions are summarized in Table 1.

Gel filtration chromatography was performed on extracted ROS proteins to characterize further the native molecular state of CI&II and their corresponding phosphorylation states (Fig. 2). Proteins in ROS were phosphorylated by endogenous cyclic nucleotide-dependent protein kinase(s) and fractionated into the soluble and peripheral membrane proteins. Each concentrated fraction was separated by Sephadex G-100 column. Chro-

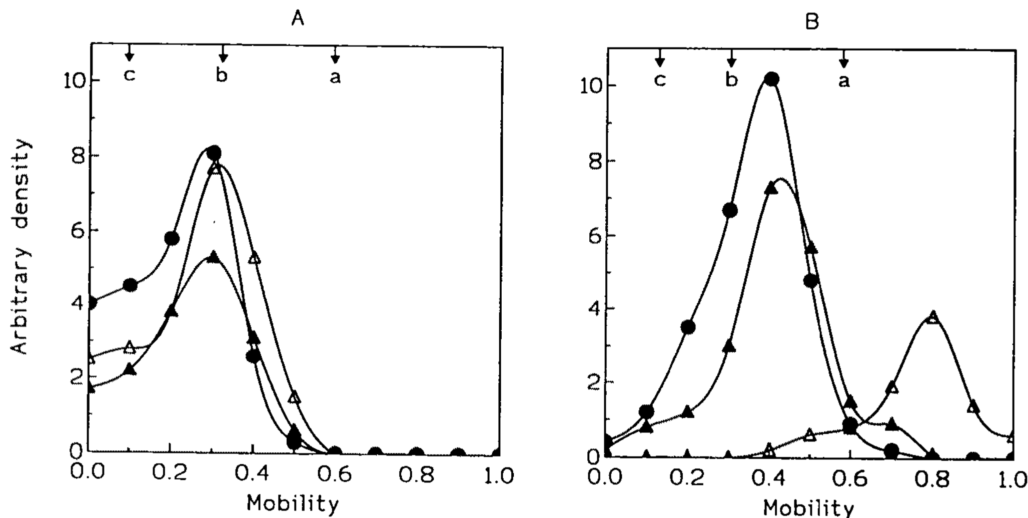


**Fig. 1.** Migration of ROS proteins in sucrose density gradient centrifugation. ROS proteins were extracted into soluble and peripheral membrane fractions by washing ROS membranes in isotonic reaction buffer and solution A, sequentially. Each concentrated fraction (300 μg soluble proteins, 100 μg peripheral proteins) was loaded onto corresponding 5~20% (w/w) linear gradient of sucrose which was made with reaction buffer or hypotonic buffer B or C. Centrifugation was carried out at 105,000×$g$ for 16 h. Proteins were fractionated and phosphorylated by PK-$A_{cat}$ as described in "Experimental Procedures". The migration profiles of $G_t$ subunits were determined by densitometric scanning from Coomassie blue stained gels and those of CI&II were from autoradiograms. (A) Migration pattern of the soluble proteins in isotonically buffered sucrose gradient. (B) Migration pattern of the peripheral membrane bound proteins in hypotonically buffered gradient containing no GTPγS and $MgCl_2$. Phosphorylated CI&II (filled circle), $\alpha_t$ (open triangle) and $\beta_t$ (filled triangle). The standard proteins used were: a, alcohol dehydrogenase (159 kDa, 7.2 S); b, bovine serum albumin (66 kDa, 4.3 S); c, carbonic anhydrase (29 kDa, 2.7 S).

**Table 1.** Calculated sedimentation coefficients for frog ROS proteins in different conditions[a]

| Conditions | Frog ROS proteins observed | Sedimentation coefficient (S) |
|---|---|---|
| Isotonic | $\alpha_t$, $\beta\gamma_t$ | 4.15± 0.05 |
| Hypotonic, Buffer B[b] | $\alpha_t$, $\beta\gamma_t$ | 4.20± 0.2 |
| Hypotonic, Buffer C[c] | $\alpha_t$ | 11.57± 0.24 |
| Hypotonic, Buffer C | $\beta\gamma_t$ | 5.75± 0.05 |
| Isotonic | p-CI&II | 4.2[d] |
| Hypotonic, Buffer B | p-CI&II | 4.2± 0.2 |
| Hypotonic, Buffer C | p-CI&II | 5.75± 0.05 |

[a] The mobility of each protein in sucrose density gradient was determined by comparing its separation profile or phosphorylation pattern to those of standard proteins. The S values are averages from three separate experiments.
[b] Buffer B: 5 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 1 mM βME, 500 μM GTPγS, and 50 mM $MgCl_2$.
[c] Buffer C: same as Buffer B except no additions of GTPγS and $MgCl_2$.
[d] Not enough number of experiments.

matography of the soluble fraction showed a phosphorylated CI&II (p-CI&II) peak at an elution volume of 111 ml with molecular mass of ~75 kDa. Under these conditions, $G_t$ was eluted at 120 ml (~65 kDa) (Fig. 2A). In the peripheral membrane fraction endogenously phosphorylated CI&II were not detected. The β-subunit of $G_t$ was the major protein stained by Coomassie blue, and the staining density of $\alpha_t$ was too low to be measured by densitometry. To determine whether CI&II were present but unphosphorylated or were absent from the elution, an additional exogenous phosphorylation with the catalytic subunit of PK-A (PK-$A_{cat}$) was performed on each fraction. Phosphorylated CI&II were then found in several fractions some of which overlapped the peak fractions of $\beta\gamma_t$ (63 kDa, Fig. 2B).

### Phosphorylation state of CI&II

The lack of phosphorylation of CI&II from peripheral membrane proteins was studied more in detail. When the peak $G_t$-containing fractions from the soluble fraction were analyzed by SDS-PAGE, proteins of 36, 35 and 48 kDa corresponding to $\alpha_t$, $\beta_t$, arrestin as well as other minor proteins in the soluble fraction were visualized by Coomassie blue staining (Fig. 3, lane 1). Autoradiography of $^{32}P$-labelled phosphoproteins showed that CI and a smaller amount of CII in the soluble protein fraction were phosphorylated by the endogenous protein kinase (lane 2). In the peak fraction of peripheral membrane proteins, $\beta_t$ was the major protein stained by Coomassie blue whereas $\alpha_t$ was minor (lane 4).

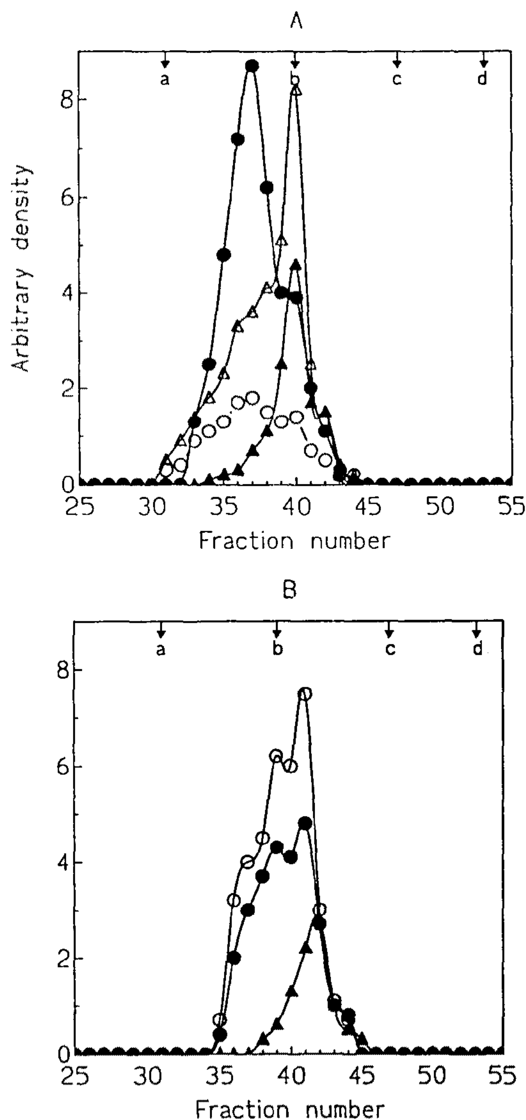


**Fig. 2.** Elution profile of ROS proteins from Sephadex G-100 gel filtration column. ROS proteins were phosphorylated by endogenous protein kinase with 30 μCi [$\gamma$-$^{32}$P]ATP, 30 μM ATP, 100 μM cAMP and cGMP for 10 min. The phosphorylated proteins were fractionated into the soluble and peripheral membrane proteins by washing ROS membranes in reaction buffer and hypotonic buffer D, respectively. Each concentrated protein fraction was loaded onto a column (2.3 mg soluble proteins, 1.4 mg peripheral proteins). Proteins were eluted by the same buffer at a flow rate of 0.5 ml/min. Each eluted fraction was analyzed by densitometric scanning of Coomassie blue stained gels ($\alpha_t$ and $\beta_t$ and autoradiograms (p-CI&II). (A) Elution profile of endogenously phosphorylated soluble proteins. (B) Elution profile of endogenously phosphorylated peripheral membrane proteins which were detected by the second phosphorylation with purified PK-A catalytic subunit. The CI&II profiles can not be quantitatively compared to $G_t$ due to densitometric scannings of different objects: dye-stained gels for $G_t$ and autoradiograms for CI&II. Phosphorylated CI (filled circle), p-CII (open circle), $\alpha_t$ (open tri-angle), and $\beta_t$ (filled tri-angle). Molecular size standard proteins were: a, alcohol dehydrogenase (159 kDa); b, bovine serum albumin (66 kDa); c, carbonic anhydrase (29 kDa); d, cytochrome C (14 kDa).

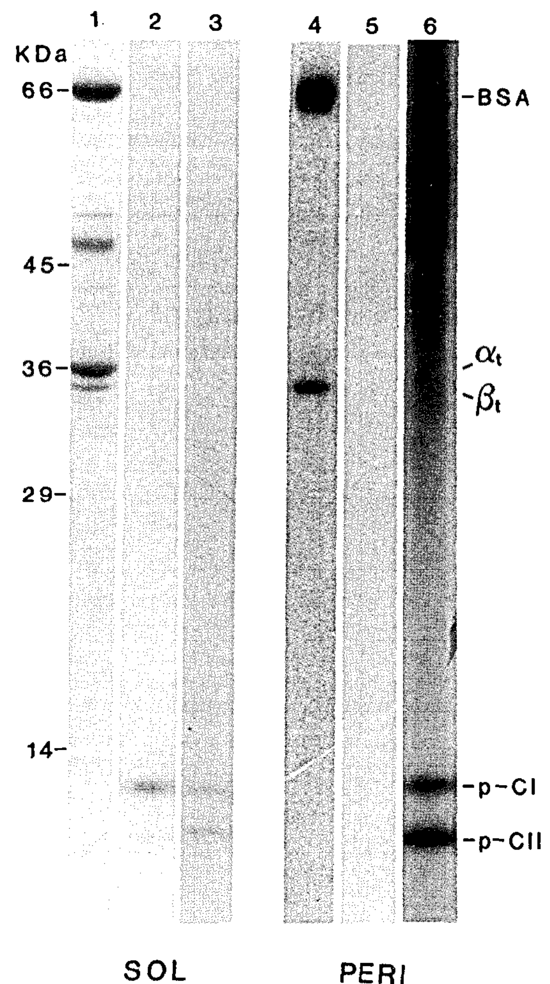


**Fig. 3.** Double phosphorylation of CI&II by endogenous and exogenous PK-A. Proteins were phosphorylated as in Fig. 2 legend and loaded on gel filtration column. Fractions from Sephadex G-100 column were phosphorylated again by PK-$A_{cat}$ (0.1 μg), 2 μCi [$\gamma$-$^{32}$P]ATP, 30 μM ATP and 3 mM $MgCl_2$. Only the peak CI&II fraction of each separation is shown here (fraction number 37 of the soluble proteins and number 41 of the peripheral membrane proteins). Proteins were separated on 10~20% gradient gels. Lanes 1 and 4 show Coomassie blue staining of the peak fractions from soluble proteins (SOL) and peripheral membrane proteins (PERI). Lanes 2 and 5 show the phosphorylations of CI&II by endogenous protein kinase. Lanes 3 and 6 show additional exogenous phosphorylation of endogenously phosphorylated CI&II. Bovine serum albumin (BSA, 20 μg) was used as a carrier for TCA precipitation. $\alpha_t$ and $\beta_t$, $\alpha$ and $\beta$ subunits of transducin; p-CI and p-CII phosphorylated CI&II.

Interestingly, the fraction from peripheral membrane proteins did not contain any radiolabelled CI&II (lane 5). In order to examine whether this lack of phosphorylated CI&II was due to absence of CI&II or simply absence of phosphorylation by endogenous protein kinase, each fraction was phosphorylated again by purified PK-$A_{cat}$. After this treatment, the fractions of peripheral membrane proteins showed phosphorylated CI&

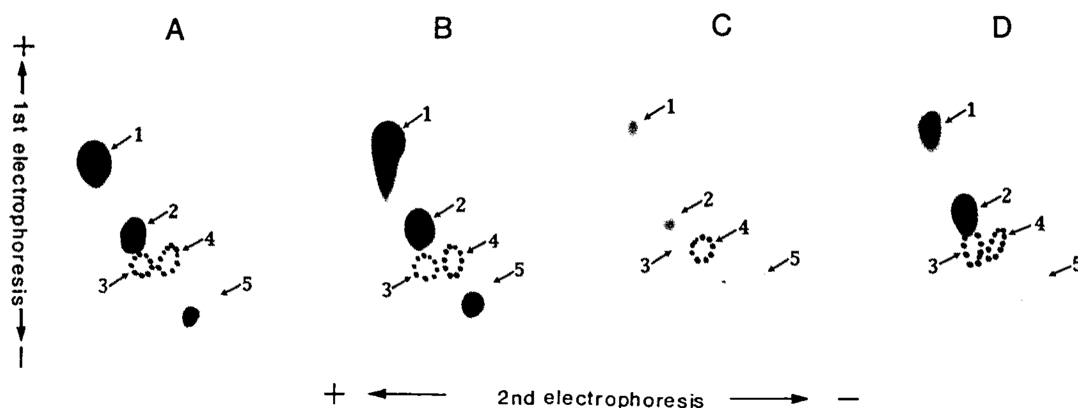


**Fig. 4.** Phosphoamino acid analysis of endogenously phosphorylated CI&II. ROS proteins were phosphorylated by endogenous protein kinase with 30 μCi [γ-$^{32}$P]-ATP, 30 μM ATP, 100 μM cAMP for 10 min. The phosphorylated proteins were fractionated into the soluble, PDE-enriched, and $G_t$-enriched fractions and separated by SDS-PAGE. After electro-transfer of proteins from the gel to PVDF membrane, the membrane pieces containing p-CI or/and p-CII were excised. Each membrane piece containing p-CI or/an p-CII was put into 200 μl 5.7 N HCl for acid hydrolysis (115℃, 2 h). Acid lysate was freeze-dried and resuspended in 20 μl solution B. Five μl of sample was mixed with each 0.5 μg of standard phosphoamino acids (phosphoserine, phosphothreonine and phosphotyrosine) and was spotted on cellulose plate. The first dimensional electrophoresis was conducted for 1.5 h (300 V) and the second dimensional was carried out in the solution C for 45 min (300 V). Spots on the plate were identified by comparing Ninhydrin staining and autoradiography. Each panel shows phosphoamino acid of p-CI (A) and p-CII (B) from the soluble fraction, and combined p-CI&II from PDE-enriched (C) and $G_t$-enriched fraction (D). Panel A and B were exposed for 1 day and C and D were for 3 days. Spot 1, inorganic phosphate; spot 2, phosphoserine; spot 3, phosphothreoine; spot 4; phosphotyrosine; spot 5, origin.

II (lane 6) whereas those of soluble proteins showed an increase of phosphorylation on CII with little change in CI (lane 3). Therefore, the soluble CI was already phosphorylated by endogenous protein kinase, whereas CI&II in the peripheral membrane fraction were not endogenously phosphorylated but could be phosphorylated by exogenous PK-$A_{cat}$. These results suggest that the peripheral membrane-bound CI&II were less susceptible to phosphorylation by endogenous PK-A. It is also possible that CI&II in the peripheral membrane proteins loast their incorporated phosphate during gel filtration. However, in extensive experiments designed to measure dephosphorylation of p-CI&II, none has been seen, in agreeement with similar findings from Hamm (1990) and Polans *et al.* (1979).

When phosphoamino acid of endogenously phosphorylated CI&II was analyzed, it was shown that a serine residue was a major target for the phosphorylation (Fig. 4). Phosphorylated CI and CII from the soluble fraction respectively showed phosphoserine as phosphoamino acid species (Fig. 4A nad B). Combined CI&II from the PDE-enriched fraction (Fig. 4C) and $G_t$-enriched fraction (Fig. 4D) also showed similar results with much less intensity of phosphorylation, and especially the CI&II from PDE-enriched fraction demonstrated a trace level of phosphorylation on a threonine residue.

## Molecular identity of CI&II

Structural and functional assays were performed (Fig. 5) to see whether CI&II are γ-subunits of transducin ($\gamma_t$, 5~8 kDa) or PDE (PDE$_\gamma$, 14~13 kDa), because the molecular sizes of these proteins are very similar and they migrated close to each other in SDS-PAGE. In addition, it has been reported that CI&II might contain PDE$_\gamma$ (Tsuboi *et al.*, 1994). When the proteins from several fractions of ROS, the soluble, hypotonic buffer-washed (PDE-enriched), and hypotonic GTP-washed fractions ($G_t$-enriched), were electro-transferred onto PVDF membrane, a monoclonal antibody against $\gamma_t$ did not recognize any protein band near 12 and 13 kDa molecular size where CI&II migrated (Fig. 5A, lanes 1, 2, and 3). Instead, the antibody recognized $\gamma_t$ very well at 6 kDa position in every fraction of ROS proteins.

The γ-subunit of PDE is a potent inhibitor of catalytic PDE$_{\alpha\beta}$ with picomolar affinity (Hurley and Stryer, 1982). Thus, if CI&II were identical to PDE$_\gamma$, thdy should inhibit active PDE at a very low concentration. In order to examine whether CI&II are identical to PDE$_\gamma$, CI&II from the soluble and hypotonic-wash fractions including PDE- and $G_t$-renriched fractions were purified by C4 reversede-phase chromatography. Purified CI&II were phosphorylated by PK-$A_{cat}$ and separated by SDS-PAGE to assess their purities. There was a single Coomassie blue stained band (Fig. 5B, lane 1) and radioactive band (lane 2) at 12~13 kDa position for the soluble CI&II. Purified CI&II from the hypotonic-wash fraction were not visible on Coomassie stained gel (Fig. 5B, lane 3) whereas exogenous phosphorylation showed clearly two radioactive bands (lane 4) at 12~13

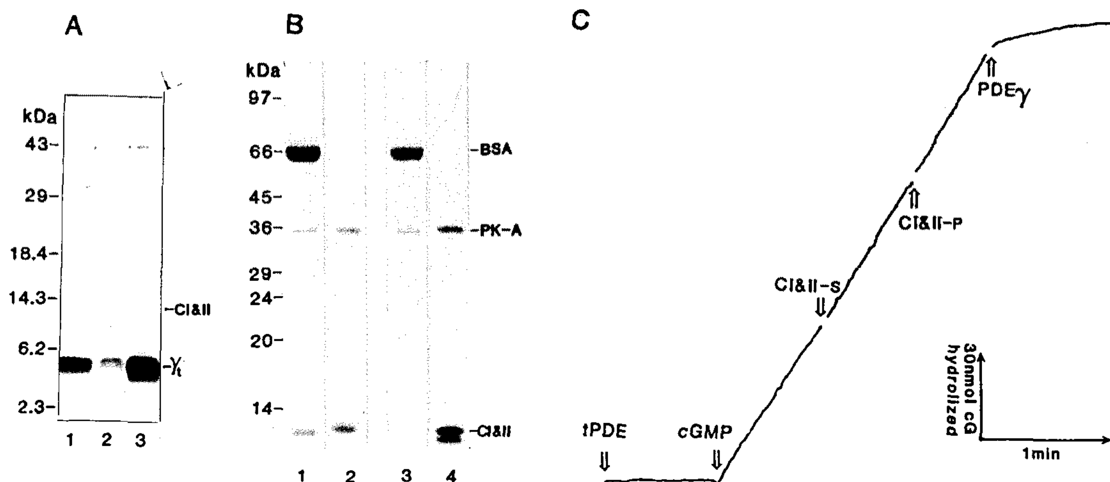


**Fig. 5.** Are CI&II γ-subunits of transducin or PDE ? (A) Immunoblotting of ROS protein fraction with a hybridoma supernatant containing mAb 2H3 against $\gamma_t$ (lane 1, soluble fraction; lane 2, hypotonic buffer-wash fraction; lane 3, hypotonic GTP-wash fraction). Proteins were separated by 10~20% gradient polyacrylamide gel of SDS-PAGE. (B) Purification of CI&II from the soluble fraction (lane 1) and the peripheral membrane fraction (lane 3) by C4 reverse-phase HPLC column. Purified CI&II from the soluble (lane 2) and peripheral membrane (lane 4) fractions were identified by exogenous phosphorylation with PK-$A_{cat}$ (C) The ability of the purified CI&II (150 nM) from the soluble (CI&II-s) and peripheral (CI&II-p) fractions to inhibit tPDE. The enzyme activity was measured as change of pH as described in "Experimental Procedures". Five nanomolar purified $PDE_\gamma$ completely inhibited tPDE activity. The concentration of purified CI&II was estimated by comparasion Coomassie blue-stained band density of CI&II to that of cytochrome C. BSA, bovine serum albumin; p-CI&II, phosphorylated CI&II.

kDa positions. Highly purified CI&II from the soluble (CI&II-s) and hypotonic-wash (CI&II-p) fractions did not inhibit tPDE (Fig. 5C). There was not any inhibition of tPDE when even micromolar CI&II were added. However, authentic purified $PDE_\gamma$ (5 nM) completely inhibited the enzyme activity. These results indicate that CI&II are not functionally related to the γ-subunits of PDE. In addition, neither the γ-subunit of $G_t$ or PDE is phosphorylated by PK-A (Hamm and Bownds, 1986; Hamm, 1990; Udovichenko *et al.*, 1993). However, it was reported that $PDE_\gamma$ was phosphorylated by a unknown kinase which is not stimulated by cAMP (Tsuboi *et al.*, 1994).

## Discussion

Components I and II are the major cyclic nucleotide-dependent phosphoproteins and their phosphorylation is regulated by light, thus they may play an importory role in phototransduction. Although the physiological functions of CI&II phosphorylation/dephosphorylation in frog ROS have not been identified, previous studies indicated a possible involvement of CI&II phosphorylation/dephosphorylation in phototransduction (Brodie and Bownds, 1976; Woodruff *et al.*, 1977; Polans *et al.*, 1979; Hamm and Bownds, 1984; Hamm, 1987; Binder *et al.*, 1988).

In this report it was clearly shown that CI&II were not γ-subunits of $G_t$ and PDE, since a monoclonal anti $\gamma_t$ antibody did not recognize p-CI&II and highly purified CI&II did ot inhibit active PDE. Gamma subunit of $G_t$ and $PDE_\gamma$ were not phosphorylated by endogenous protein kinase nor by exogenous PK-$A_{cat}$. In addition, Polans *et al.* (1979) extensively investigated CI&II to showed that they were not phospholipids nor proteolyzed fragments of larger proteins in frog ROS.

The native molecular sizes of CI&II estimated from migration in sucrose density gradient and gel filtration were larger (~60 kDa) and they interact with other ROS protein (most likely $\beta\gamma_t$) mainly in the peripheral membrane fractions of ROS proteins. In addition, the CI&II showing a physical interaction with other ROS proteins was not phosphorylated by endogenous PK-A. It is likely that the phosphorylation site(s) of CI&II in native state was blocked when the proteins were in a complex.

Interestingly, phosducin (33 kDa) in bovine retinal protein has similar properties to CI&II (Lee *et al.*, 1987; Lee *et al.*, 1990; Lolley and Lee, 1990; Lee *et al.*, 1992). This protein was phosphorylated by PK-A and light caused its dephosphorylation. Purification of this protein from retinal homogenates yielded a stoichiometric complex with the $\beta\gamma_t$. I have investigated whether a phosphorylated phosducin exists in frog ROS, and whether CI&II are present in mammalian ROS, and have no evidence for either. An antibody against phos-

ducin did not recognize CI&II and when phosducin from bovine retina was treated with cross-linkers, there was no cross-linked protein observed (Ting *et al.*, 1991). Instead, binding of phosducin to $\beta\gamma_t$ blocked the cross-linking among $\alpha_t$, $\beta_t$, and $\gamma_t$ which was previously described by Hingorani *et al.* (1988). This indicates that CI&II and phosducin are structurally different. On the other hand, CI&II in frogs and phosducin in cows may have similar functional properties, and thus could be functional homologs of each other.

In conclusion, the hydrodynamic properties of the peripheral membrane-bound CI&II show that they form a complex with larger proteins, most likely $\beta\gamma_t$, in native state, and this complex is not a good substrate for endogenous PK-A. Therefore, CI&II phosphorylation/dephosphorylation has a role in the photosignal transduction process in frog photoreceptors by the complex formation with $\beta_t$ on RoS membranes.

**Acknowledgement**

This project was financially supported by Central Research Fund from Pai Chai University for the year of 1995. The author thanks Dr. H. Hamm for the supply of important materials and valuable discussions.

## References

Binder, B. M., Brewer, E. and Bownds, M. D. (1988) *J. Biol. Chem.* **264**, 8857.

Bownds, M. D. and Brewer, E. (1988) The Molecular Mechanism of Photoreception, Springer-Verlag, New York.

Brodie, A. E. and Bownds, M. D. (1976) *J. Gen. Physiol.* **68**, 1.

Cohen, A. I., Hall, I. A. and Ferrendelli, J. A. (1978) *J. Gen. Physiol.* **71**, 595.

Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615.

Hamm, H. E. (1987) *Biophysical J.* **51**, 270a.

Hamm, H. E. (1990) *J. Gen. Physiol.* **95**, 545.

Hamm, H. E. and Bownds, M. D. (1984) *J. Gen. Physiol.* **84**, 265.

Hamm, H. E. and Bownds, M. D. (1986) *Biochemistry* **25**, 4512.

Hingorani, V. N., Tobias, D. T., Henderson, J. T. and Ho, Y.-K. (1988) *J. Biol. Chem.* **263**, 6916.

Hurley, J. B. and Stryer, L. (1982) *J. Biol. Chem.* **257**, 11094.

Kamp, M. P. and Sefton, B. M. (1989) *Anal. Biochem.* **176**, 22.

Lee, R. H., Brown, B. M. and Lolley, R. N. (1990) *J. Biol. Chem.* **265**, 15860.

Lee, R. H., Liebman, B. S. and Lolley, R. N. (1987) *Biochemistry* **26**, 3983.

Lee, R. H., Ting, T. D., Lieberman, B. S., Tobias, D. E., Lolley, R. N. and Ho. Y.-K. (1992) *J. Biol. Chem.* **267**, 25104.

Liebman, P. A. and Evanczuk, T. (1982) *Methods Enzymol.* **81**, 532.

Lolley, R. N. and Lee, R. H. (1990) *FASEB J.* **4**, 3001.

Mattera, R., Codina, J., Sekura, R. D. and Bimbaum, L. (1987) *J. Biol. Chem.* **262**, 11247.

Polans, A. S., Hermolin, J. and Bownds, M. D. (1979) *J. Gen. Physiol.* **74**, 595.

Stryer, L. and Boum, H. R. (1986) *Annu. Rev. Cell Biol.* **2**, 19.

Suh, K. H. and Hamm, H. E. (1988) *Inv. Ophthal. Vis. Sci.* **29**, 956.

Suh, K. H. and Hamm, H. E. (1996) *Biochemistry* **35**, 290.

Ting, T. D. and Ho, Y.-K. (1991) *Biochemistry* **30**, 8996.

Tsuboi, S., Matsumoto, H., Jackson, K. W., Tsujimoto, K., Williams, T. and Yamazaki, K. (1994) *J Biol. Chem.* **269**, 15016.

Udovichenko, I. P., Cunnick, J., Gonzales, K. and Takemoto, D. J. (1993) *Biochem. J.* **295**, 49.

Woodruff, M. and Bownds, M. D. (1979) *J. Gen. Physiol.* **73**, 629.

Woodruff, M. L., Bownds, M. D., Green, S. H., Morrisey, J. L. and Shedlovsky, A. (1977) *J. Gen. Physiol.* **69**, 667.