Cyclic AMP-Dependent Phosphoprotein Components I and II Interact with $\beta\gamma$ Subunits of Transducin in Frog Rod Outer Segments†

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Received August 9, 1995; Revised Manuscript Received October 6, 1995⊗

ABSTRACT: Components I and II (CI&II) in frog rod outer segments (ROS) are prominent cAMP-dependent protein kinase (PK-A) substrates. Their phosphorylation level is high in the dark, and illumination causes dephosphorylation. In order to understand their physiological role in phototransduction, biochemical characterization of CI&II phosphorylation was performed. Fractionation of phosphorylated ROS proteins showed that CI&II in the soluble fraction were highly phosphorylated by endogenous PK-A, whereas those in the membrane-associated protein fractions were not. The latter proteins could be phosphorylated by purified catalytic subunit of PK-A (PK-A_{cat}) while the former proteins were not, suggesting that membrane-bound CI&II are normally much less phosphorylated. Treatments that dissociate the α subunit (α_t) of transducin (G_t) from $\beta\gamma$ subunits ($\beta\gamma_t$) and thus produce excess free subunits of G_t in the soluble fraction caused inhibition of CI&II phosphorylation in the soluble fraction and enhancement of CI&II phosphorylation in the peripheral membrane fractions containing less G_t. Unphosphorylated CI&II tightly associated with the washed ROS membranes could be extracted after phosphorylation by PK-A_{cat}. Phosphorylation also caused elution of $\beta\gamma_t$ from the membrane under the same conditions. Cross-linking by the maleimidobenzoyl-*N*-hydroxysuccinimide ester of the peripheral membrane fraction produced a distinct phosphorylated 50 kDa product with concurrent disappearance of the β subunit of transducin (β_t) and phosphorylated CI&II. This phosphorylated cross-linked product was not recognized by a monoclonal anti-α_t antibody but was recognized by antiserum against β_t, suggesting that the 50 kDa protein is a complex of $\beta\gamma_t$ and CI&II. Amino terminal sequencing of components I and II suggests that they are identical proteins with a unique sequence unrelated to other proteins in protein data bases. Phosphopeptide mapping of phosphorylated CI&II in the soluble fraction yielded two trypsinized phosphopeptides, while that in the peripheral membrane fractions showed only one phosphopeptide. These data suggest that multiple phosphorylation of CI&II alters their cellular localization. We conclude that phosphorylation of CI&II controls their localization in frog ROS and an interaction of CI&II with subunits of G_t regulates their phosphorylation.

When photoreceptors are illuminated, activated rhodopsin initiates a cascade of signal transduction through a series of protein interactions. The photoisomerization of rhodopsin allows binding and activation of a GTP-binding protein, transducin (G_t),[1] which in turn activates cGMP phosphodiesterase (PDE). The resulting decrease in cGMP is the trigger for closure of cationic channels and membrane hyperpolarization. Cyclic GMP also may be involved in other processes. It is known that cGMP regulates GTP binding (Robinson et al., 1986) to G_t and the GTPase activating action of PDE (Arshavsky et al., 1991; Arshavsky & Bownds, 1992). It can also regulate the activity of cyclic nucleotide-dependent protein kinases present in the ROS. In both mammalian and amphibian rod outer segments, cyclic nucleotide-dependent protein kinase activity and cyclic nucleotide-stimulated phosphoproteins are prominent (Farber et al., 1979; Polans et al., 1979; Lee et al., 1987; Kamps et al., 1986; Binder et al., 1989; Hamm, 1990). Since the level of cyclic nucleotides in ROS is rapidly decreased by light-activated PDE (Woodruff et al., 1977; Cohen et al., 1978), it is expected that the activity of cyclic nucleotide-dependent protein kinases in ROS should also decrease. Illumination causes rapid dephosphorylation of two small molecular size proteins (CI&II, 13 and 12 kDa, respectively) in intact rods (Polans et al., 1979; Bownds & Brewer, 1988) and isolated ROS (Hamm, 1990) of frog photoreceptors. Components I and II are the major proteins in frog ROS whose phosphorylation is cyclic nucleotide- and light-dependent (Hamm & Bownds, 1986).

In bovine retinas, the major cyclic nucleotide-stimulated phosphoprotein of 33 kDa, phosducin, has properties similar to those of CI&II. Its phosphorylation was maximal in the dark, and light caused its dephosphorylation. This protein

† This work was supported by the American Heart Association and U.S. Public Health Service Grant EY06062.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: βME, β-mercaptoethanol; CI&II, components I and II; EDTA, ethylenediamine tetraacetic acid; G_t, transducin; α_t and $\beta\gamma_t$, the α and $\beta\gamma$ subunits of transducin; HPEC, high-performance electrophoresis chromatography; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide; PK-A, cAMP-dependent protein kinase; PK-A_{cat}, purified catalytic subunit of PK-A; PDE, cGMP phosphodiesterase; PDEγ, γ subunit of PDE; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinyl difluoride); ROS, rod outer segments.

0006-2960/96/0435-290$12.00/0

copurified as a stoichiometric complex with $\beta\gamma_t$ and was phosphorylated by PK-A (Lee et al., 1987, 1990a; Lolley & Lee, 1990). It has been shown that phosducin inhibited the light-activated GTPase activity of α_t and the activity of PDE by sequestering $\beta\gamma_t$ (Lee et al., 1992; Yoshida et al., 1994). In addition, an identical 33 kDa protein was identified in bovine brain (Bauer et al., 1992). This brain 33 kDa protein inhibited GTPase activity of the stimulatory G protein (G_s) by association with $\beta\gamma_s$. This complex formation leads to inhibition of adenylylcyclase either by preventing the function of an activated state of G_S after GTP binding or by sterically interfering with G protein−effector coupling. The inhibition was shown to be blocked by PK-A phosphorylation of the protein.

There are several lines of evidence suggesting that the CI&II phosphorylation/dephosphorylation cycle may be functionally and physically linked to the G_t activation cycle in frog ROS. The light intensity for the half-maximal dephosphorylation of CI&II in retinas (5×10^3 rhodopsin molecules per ROS per second, Hamm, 1990) is similar to those for a half-maximal decrease of cGMP content in ROS (Woodruff & Bownds, 1979). A monoclonal antibody raised against α_t (mAb 4A) blocks the light activation of G_t and also blocks phosphorylation of CI&II (Hamm & Bownds, 1984). Extra G_t introduced into ROS membranes alters the localization of phosphorylated CI&II (Hamm, 1987). These studies suggest that there is a close relationship between the G_t activation cascade and the phosphorylation of CI&II, which may play a role in phototransduction, perhaps in modulation of the G_t activation cycle or in activation of other light sensitive reactions.

The present data show a functional and physical interaction between components I and II and the $\beta\gamma$ subunit of G_t. Thus, we postulate a role for the phosphorylation/dephosphorylation cycle of CI&II in the regulation of the transducin cycle in frog ROS.

EXPERIMENTAL PROCEDURES

Materials. Carrier-free $^{32}P_i$ was from ICN. [γ-^{32}P]ATP was generated from $^{32}P_i$ by a GammaPrep-A kit from Promega. Cyclic nucleotides, GTP, and its analogues were obtained from Boehinger-Mannheim Biochemicals. Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) ester was from Sigma. Constant boiling 6 N HCl was from Pierce. The catalytic subunit of PK-A (PK-A_{cat}) purified from bovine heart was a gift from Dr. David Glass (Emory University, Atlanta, GA). All other chemicals were of the highest purity available from standard commercial sources.

Buffers and Solutions. For extraction of the ROS proteins, reaction buffer [frog Ringer's [105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.5)] containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10^2 KIU (Kallikrein inhibitor units)/mL aprotinin, 2 μM leupeptin, 1 mM β-mercaptoethanol (βME), and about 10^{-8} M Ca^{2+}] and buffer A [5 mM TRIS-HCl (pH 7.5), 0.1 mM PMSF, 1 mM βME, 100 KIU/mL aprotinin, and 2 μM leupeptin] were used. For cross-linking, buffer B [10 mM MOPS (pH 8.0), 200 mM NaCl, and 2 mM $MgCl_2$] and buffer C [10 mM MOPS (pH 8.0) and 2 mM $MgCl_2$] were used. In order to terminate the phosphorylation reaction in nondenaturing conditions, stop solution [7.5 mM phosphate (pH 7.5) and 7.5 mM EDTA] was used.

Phosphorylation of ROS Proteins. Rod outer segments from frog retinas were prepared as described elsewhere (Hamm, 1990) except using 6, 35, 45, and 70% (v/v) Percoll gradients. For endogenous phosphorylation, purified ROS were resuspended in reaction buffer. ROS were disrupted by passage through a 27-gauge needle and incubated for 10 min with 2−20 μCi [γ-^{32}P]ATP and 30 μM ATP in reaction buffer in the presence or absence of 100 μM cAMP. In the previous study, it was observed that the phosphorylation level of CI&II in retina by endogenous protein kinase reached a plateau in 8 min and started to decrease after 15 min (Polans et al., 1979; Suh, unpublished). Therefore, ROS were incubated for 10 min. 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 the dark. For exogenous phosphorylation, protein extracts of ROS were incubated for 15 min in reaction buffer containing 2.5−20 μCi [γ-^{32}P]ATP, 30 μM ATP, 3 mM $MgCl_2$, and 0.1 μg of purified PK-A_{cat}. Under these conditions, PK-A_{cat} phosphorylated CI&II to an extent similar to that observed for endogenous PK-A as described above. The reactions were terminated by either stop solution for further processing or 10% trichloroacetic acid for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE; Laemmli, 1970).

Extraction of ROS Proteins. Disrupted ROS in reaction buffer were centrifuged at 436000*g* for 10 min to obtain the soluble fraction. The resulting pellet was resuspended in buffer A and bleached on ice for 15 min under room light. The same centrifugation was performed to obtain a PDE-enriched hypotonic-wash fraction. Buffer A containing 500 μM GTP was added to the remaining membrane pellet, and the mixture was centrifuged to attain a G_t-enriched GTP-wash fraction. The final membrane pellet was resuspended in reaction buffer for a washed membrane fraction. The protein concentration was determined by the method of dye binding (Bradford, 1976) using γ-globulin as a control.

Effect of Excess G_t Subunits on CI&II Phosphorylation. Monoclonal anti-α_t antibody 4A (mAb 4A) and GTPγS are known to separate α_t from $\beta\gamma_t$ (Fung, 1983; Mazzoni & Hamm, 1989). In order to examine the effect of free G_t subunits on the phosphorylation of CI&II, 1.33 pmol of mAb 4A or 500 μM GTPγS was added separately to the disrupted ROS suspended in reaction buffer and incubated at room temperature for 30 min in the dark. The endogenous phosphorylation for 10 min was followed by addition of 20 μCi [γ-^{32}P]ATP, 30 μM ATP, 3 mM $MgCl_2$, and 100 μM cAMP to the protein mixture. The phosphorylation reaction was terminated by stop solution. Protein fractionation was performed by sequential centrifugation described as above. Each protein fraction was extracted three times. The protein extracts were pooled and precipitated by 10% trichloroacetic acid and analyzed by SDS−PAGE, autoradiography, and densitometric scanning. In the case of mAb 4A addition, the same amount of rabbit IgG was used as control.

Cross-Linking of ROS Proteins by MBS. ROS were resuspended in buffer B, disrupted first, and then endogenously phosphorylated with 20 μCi [γ-^{32}P]ATP, 30 μM ATP, and 100 μM cAMP for 10 min. The phosphorylation reaction was stopped by addition of stop solution. ROS proteins were extracted as described above using buffer C

for hypotonic extraction steps. After extraction, hypotonic-wash and GTP-wash fractions were made isotonic with 0.2 M NaCl. Each extracted protein fraction was cross-linked by 50 μM maleimidobenzoyl-*N*-hydroxysuccinimide (MBS), a heterobifunctional cross-linker specific to SH and NH_2 groups (Hingorani et al., 1988). Proteins and cross-linker were incubated in buffer B for 1 h at room temperature. The reaction was stopped by addition of 1 mM dithiothreitol. The identities of cross-linked products were analyzed by SDS−PAGE, autoradiography, and immunoblotting with antibodies.

Preparation of Antiserum and Western Blotting. The transducin $\beta\gamma$ dimer was purified from bovine ROS as previously described (Kleuss et al., 1987). Highly purified β and γ subunits were further purified on a C4 reverse-phase high-performance liquid chromatography (HPLC) column (Vydac, 4.6 × 250 mm) with a 20 to 100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Generation of antibodies against β_t and γ_t followed a previously described method (Harlow & Lane, 1988).

In order to identify the constituent proteins in cross-linked products, cross-linked proteins in each fraction were separated by SDS−PAGE and transferred onto PVDF membranes. Immunoblotting of proteins on the PVDF membrane with mAb 4A and anti-β_t antiserum was performed according to a standard peroxidase method.

Phosphopeptide Mapping of Phosphorylated CI&II. Each fraction of endogenously phosphorylated ROS proteins was separated by SDS−PAGE. Extraction of each phosphorylated component from the gel was carried out as described elsewhere (Nishikawa et al., 1987; Joseph et al., 1991). The extracted protein was mixed with 100 μg of BSA and 10% trichloroacetic acid. The precipitate was washed, suspended in reaction buffer, and lyophilized. The dried proteins were resuspended in trypsinization buffer containing 10 mM MOPS (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 25% (v/v) glycerol. Trypsinization of the proteins was performed by addition of 5 μg of TPCK−trypsin dissolved in 0.1 mM HCl and incubation on ice for 1 h. Proteolysis was stopped by 50 μg of soybean trypsin inhibitor. The proteolyzed mixture was analyzed by two-dimensional (2D) thin layer electrophoresis/chromatography. Extensive trypsinization at room temperature for 3 h showed the same results.

Partial Amino Acid Sequencing of CI&II. CI&II were purified to homogeneity by two different methods. After proteins were endogenously phosphorylated by nonradioactive ATP in reaction buffer containing 30 μM ATP and 100 μM cAMP in the dark for 10 min, the soluble fraction of phosphorylated ROS proteins was extracted and separated by SDS−PAGE. A radioactively labeled ROS protein sample was also loaded onto a separate lane as markers of phosphorylated proteins. Proteins isolated by SDS−PAGE were electroblotted onto PVDF membranes. Components I and II were localized by matching an autoradiogram on the membrane, and each band was carefully excised. In the second procedure, CI&II from the soluble fraction which was not treated with ATP were purified on an Applied Biosystem 230A high-performance electrophoresis chromatography column (HPEC, 8% polyacrylamide gel column, 3.5 × 100 mm). CI&II were separated by HPEC. The amino terminal amino acids of each component on excised PVDF membrane strips and in purified protein fractions were directly sequenced by an Applied Biosystem 477A Protein Sequencer at the Protein Sequencing/Synthesis Laboratory (University of Illinois at Chicago).

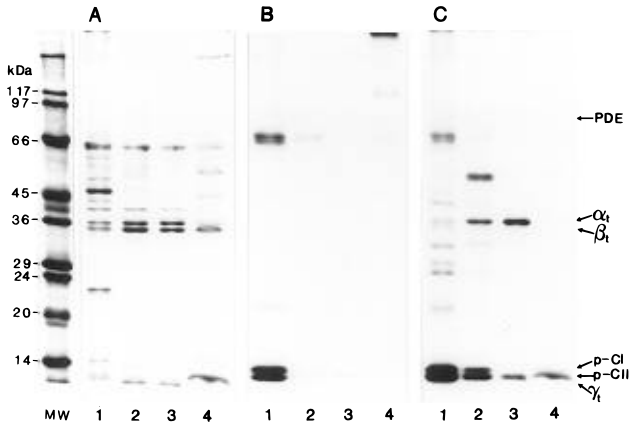

FIGURE 1: Phosphorylation of endogenously phosphorylated ROS proteins by exogenous PK-A_{cat}. ROS proteins were endogenously phosphorylated with 10 μCi [γ-^{32}P]ATP, 30 μM ATP, and 100 μM cAMP in the dark for 10 min and were fractionated into soluble (lane 1), hypotonic-wash (lane 2), GTP-wash (lane 3), and membrane (lane 4) proteins. Half of each fraction was further phosphorylated by 0.1 μg of purified PK-A_{cat}, 2.5 μCi [γ-^{32}P]ATP, 30 μM ATP, and 3 mM $MgCl_2$ for 15 min: (A) Coomassie blue staining of protein fractions, (B) autoradiogram of endogenously phosphorylated protein fractions, and (C) autoradiogram of further phosphorylated proteins by PK-A_{cat}.

RESULTS

Identification of a Pool of CI&II from ROS Membranes. It was observed previously that the endogenous cAMP-dependent protein kinase of frog ROS phosphorylated CI&II in different fractions to a different extent (Hamm & Bownds, 1986; Hamm, 1990). Therefore, it is interesting to investigate how the phosphorylation level of CI&II in each fraction is different and what the nature of this difference is. For this purpose, sequential phosphorylation of CI&II by endogenous and exogenous PK-A was performed in all fractions of ROS proteins (Figure 1). Disrupted ROS were phosphorylated in the presence of cAMP by endogenous protein kinase, and then proteins were extracted. One half of each fraction served to monitor endogenous CI&II phosphorylation. The other half was incubated with PK-A_{cat} after quenching and removal of free radioactive ATP.

Components I and II in the soluble fraction were the most highly phosphorylated by endogenous PK-A (Figure 1B, lane 1), whereas the other fractions showed very low phosphorylation of CI&II, which at this film exposure is almost undetectable (Figure 1B, lanes 2−4). However, CI&II in these three fractions could be phosphorylated significantly further by incubation with exogenous PK-A_{cat} (Figure 1C, lanes 2−4). The hypotonic-wash fraction showed a large increase in CI&II phosphorylation (compare lane 2 in Figure 1B and and lane 2 in Figure 1C), whereas the phosphorylation level of CI&II in the soluble fraction was only slightly changed by exogenous PK-A (compare lane 1 in Figure 1B and lane 1 in Figure 1C). The GTP-wash and membrane fractions also showed a large increase in CII phosphorylation by exogenous PK-A_{cat} (compare lanes 2 and 3 in Figure 1B and Figure 1C). A similar extent of increase in CI&II phosphorylation in the hypotonic-wash fraction after treatment with exogenous PK-A_{cat} was also observed after

removal of ATP by gel filtration (data not shown). The increased phosphorylation of CI&II in the membrane-associated protein fractions after postphosphorylation was observed under both native and denaturing conditions, suggesting that it occurs on physiologically relevant sites. Protein fractions which contain less G_t showed increased endogenous phosphorylation of CI&II (compare Figure 1A and Figure 1B). Since Hamm (1990) showed that these fractions do contain PKA, it may be that inhibitory factors blocking the endogenous phosphorylation of CI&II were lost during the procedure for exogenous phosphorylation, and CI&II become accessible for PK-A binding and phosphorylation. Another possibility is that endogenous p-CI&II in the membrane-associated fractions were dephosphorylated faster than those in the soluble fraction. However, dephosphorylation activity toward p-CI&II in the extracted ROS proteins has never been detected under the conditions of these in vitro experiments (Polans et al., 1979; Hamm, 1990; Suh, unpublished). This implies that there is a regulatory mechanism for CI&II phosphorylation in ROS, which may be disrupted by perturbation of the normal ROS structural integrity.

Extraction of the Membrane-Bound CI&II. Because a relatively large portion of CI&II were phosphorylated to a very small extent by endogenous PK-A and they were in membrane-associated fractions, it was investigated whether phosphorylation regulated the localization of CI&II. In addition, we attempted to search for a factor in the membranes which blocked the endogenous phosphorylation of CI&II. For this purpose, a serial extraction of ROS proteins was carried out (Figure 2). Rod outer segment proteins which were not stimulated for phosphorylation by addition of any protein kinase activator were extensively fractionated by several sequential washings into soluble (lanes 1 and 2 show the proteins in the first and last wash), hypotonic-wash (lanes 3 and 4), GTP-wash (lanes 5 and 6), and membrane fractions. Each extracted fraction was phosphorylated by the same activity of exogenous PK-A_{cat} and radioactive ATP to compare the phosphorylatability of CI&II in each extraction step. The Coomassie blue stain showed that the last wash in each extraction step removed very little protein (Figure 2A, compare lane 1 to 2, 3 to 4, and 5 to 6). A similar pattern of CI&II phosphorylation was observed, which was shown by PK-A_{cat}-catalyzed postphosphorylation (Figure 2B). The major protein in the GTP-wash fraction was G_t, and Coomassie blue staining showed that only a trace amount of G_t was extracted at the last step of GTP extraction (Figure 2A, lanes 5 and 6). The washed ROS membranes remaining after these extensive sequential extractions were next examined in two ways. First, the presence of substrates for PK-A_{cat} in the extensively washed membranes was tested by treatment with PK-A_{cat} and ATP. Second, after treatment with PK-A_{cat}, the membranes were sequentially extracted again into soluble, hypotonic-wash, and GTP-wash fractions to examine whether there was any phosphorylation-induced extraction of CI&II. There was no detectable PK-A_{cat} substrate in the washed membranes [rhodopsin, which is not a PK-A_{cat} substrate, was phosphorylated (Kühn & Dreyer, 1972)]. Interestingly, after phosphorylation of washed membranes, a significant amount of β_t and γ_t with a trace amount of α_t was found in the supernatant (Figure 2A, lane 7), although extraction of ROS proteins without phosphorylation had yielded very little

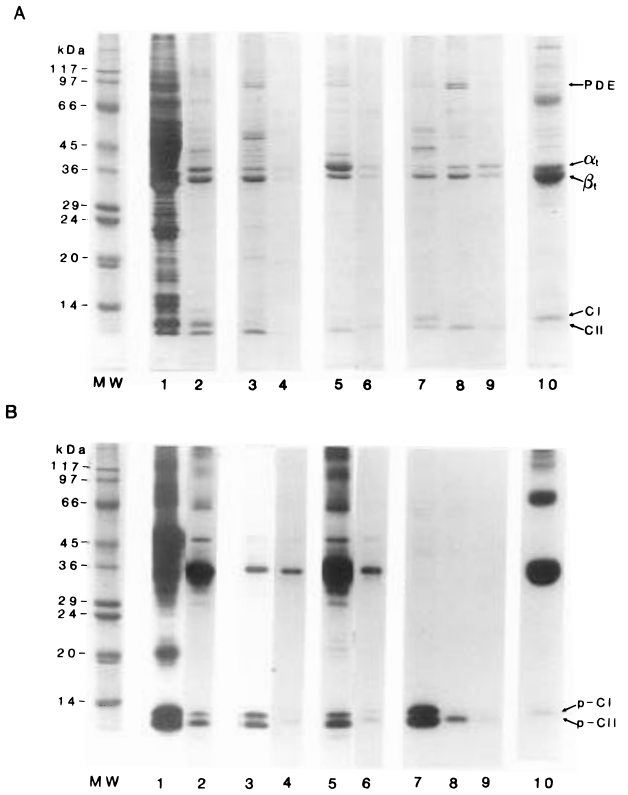

FIGURE 2: Extraction of ROS membrane proteins by treatment with PK-A and ATP. ROS proteins were fractionated into soluble proteins (lanes 1 and 2), a hypotonic-wash fraction (lanes 3 and 4), and a GTP-wash fraction (lanes 5 and 6) sequentially. The hypotonic GTP wash with buffer A was carried out six times, whereas other washing steps were performed four times each. Only the first (lanes 1, 3, and 5) and last (lanes 2, 4, and 6) extractions of each fraction are shown here. Each fraction and the resuspended washed membranes were phosphorylated by purified PK-A_{cat} with 3 μCi [γ-^{32}P]ATP for 15 min at room temperature. After the phosphorylation reaction was stopped, the phosphorylated washed membranes underwent fractionation again into a soluble fraction (lane 7), a hypotonic-wash fraction (lane 8), a GTP-wash fraction (lane 9), and an integral membrane fraction (lane 10): (A) Coomassie blue staining of extracted proteins and (B) autoradiograms of exogenously phosphorylated proteins. MW represents molecular size standard proteins.

protein (Figure 2A, lane 6). Also, significant phosphorylation of CI&II was demonstrated in that supernatant (Figure 2B, lane 7). The next hypotonic washing also extracted G_t (mostly $\beta\gamma_t$) and PDE and p-CII (lane 8 in Figure 2A,B). Washing of the exogenously phosphorylated membrane with hypotonic buffer containing GTP extracted a very small amount of G_t and p-CII (lane 9 in Figure 2A,B). In these reextraction steps after exogenous phosphorylation, only CI&II were substrates for PK-A_{cat} (Figure 2B, lanes 7−9). When the washed membrane fraction of ROS was extracted with ATP alone or without either PK-A_{cat} or ATP, no additional proteins were extracted (data not shown). These results suggested that, after serial extractions of the endogenously phosphorylated ROS membranes, there was still a pool of protein, mainly unphosphorylated CI&II and $\beta\gamma_t$, as well as some α_t and PDE, which could be eluted from the membrane by phosphorylation. In the last phosphorylated membrane fraction (lane 10), Coomassie blue staining (Figure 2A) demonstrated that there were still unextracted G_t, rhodopsin, PDE, a few other unidentified membrane proteins, and CI. However, the autoradiogram (Figure 2B) showed very little phosphorylation on CI but extensive

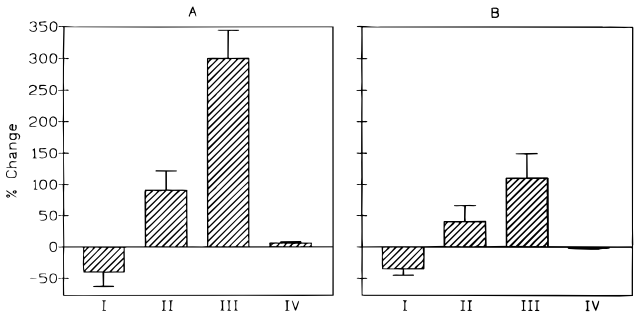

FIGURE 3: Effect of treatments that solubilize transducin on CI&II phosphorylation. Disrupted ROS were incubated with 500 μM GTPγS (A) or 1.33 pmol of mAb 4A (B) at room temperature for 30 min in reaction buffer. Phosphorylation for 10 min with 20 μCi [γ-^{32}P]ATP, 30 μM ATP, 3 mM $MgCl_2$, and 100 μM cAMP was followed. Phosphorylated ROS proteins were fractionated into four fractions: fraction I (washed with reaction buffer), fraction II (washed with hypotonic buffer A), fraction III (washed with buffer A containing 500 μM GTP), and fraction IV (washed membranes). The intensity of CI&II phosphorylation was quantitated by densitometric scanning of autoradiograms. The plotted values are the percentage of densitometric scanned values of CI&II phosphorylation after pretreatment with GTPγS or mAb 4A as compared with the values for untreated control samples.

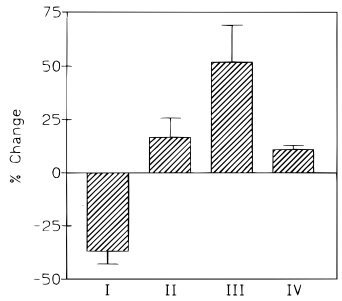

FIGURE 4: Phosphorylation of CI&II is regulated by the presence of excess G_t subunits. Disrupted ROS were either incubated with 500 μM GTPγS for 10 min and then phosphorylated with 20 μCi [γ-^{32}P]ATP, 30 μM ATP, 3 mM $MgCl_2$, and 100 μM cAMP for 10 min or phosphorylated first and then incubated with GTPγS. ROS proteins were then fractionated as in Figure 3. The intensity of CI&II phosphorylation was quantitated by densitometric scanning of autoradiograms, and the level of endogenous CI&II phosphorylation in each fraction of ROS membranes treated first with GTPγS was expressed as a percent of the control samples that were phosphorylated before treatment with GTPγS.

phosphorylation on rhodopsin and its oligomeric forms, most likely due to the bleaching of the protein during the extraction steps. This implies that CI is phosphorylated less favorably than CII. This can explain why the phosphorylation and extraction of CI&II from ROS membranes are different in many cases (Polans et al., 1979; Hamm & Bownds, 1984; Shinozawa & Yoshizawa, 1986; Hayashi et al., 1987; Hamm, 1990). We thus suggest that phosphorylation of CI&II regulates their localization in ROS; i.e., highly phosphorylated CI&II are in the soluble fraction, and less phosphorylated CI&II are membrane-bound. Coelution of G_t subunits with p-CI&II after phosphorylation from the ROS membranes supports the possibility that subunits of G_t, mostly $\beta\gamma_t$, are candidate factors which are responsible for the inhibition of endogenous phosphorylation of CI&II on the membrane and that phosphorylation of CI&II favors disruption of the interaction between G_t subunits and CI&II.

Relationship between CI&II Phosphorylation and G_t Subunits. The hypothesis that interaction of CI&II with G_t subunits regulates the phosphorylation level can be examined by altering the concentration of free G_t subunits in ROS membranes. For this purpose, ROS membranes were incubated with GTPγS or mAb 4A which are known to separate α_t from $\beta\gamma_t$ and elute them from the membranes into the soluble fraction (Fung, 1983; Mazzoni & Hamm, 1989). A nonhydrolyzable analogue of GTP, GTPγS, was incubated with ROS proteins, and the mixture underwent phosphorylation by endogenous PK-A (Figure 3A). When proteins were fractionated, a large amount of membrane-associated G_t was depleted into the soluble fraction (I) (more than 85% of G_t in fraction III was depleted). This treatment inhibited the phosphorylation of soluble CI&II by almost 40% compared to the control condition without GTPγS (Figure 3A, fraction I). The amount of G_t in the normally G_t-enriched fraction (III) was significantly decreased, and phosphorylation was increased in this fraction by 3-fold (Figure 3A, III). Similar results were observed in the hypotonic-wash fraction but to a lesser extent (Figure 3A, II). This suggests that, in the presence of excess G_t subunits in the soluble fraction, CI&II phosphorylation is inhibited, while depletion of G_t from the membrane enhanced the membrane phosphorylation of CI&II.

Similar results were obtained upon incubation of ROS membranes with mAb 4A which also causes subunit dissociation and elution from the membrane ($\sim$50% of G_t in fraction III was depleted; Mazzoni & Hamm, 1989) (Figure 3B). Monoclonal antibody 4A decreased the phosphorylation of CI&II in the soluble fraction (I) and increased it in fractions II and III. Thus, the phosphorylation of CI&II appeared to be inversely related to the amount of G_t subunits present either in solution or on the ROS membranes. These results indicate that the relative amount of free G_t subunits may control phosphorylation of CI&II.

To further confirm the above results, treatment of ROS with GTPγS was carried out either before phosphorylation, as above, or after phosphorylation (Figure 4). After fractionation, the level of endogenous CI&II phosphorylation in each fraction of ROS membranes treated first with GTPγS was expressed as a percent of the control. In the soluble fraction (I), the level of CI&II phosphorylation in ROS proteins was decreased about 35% by pretreatment with GTPγS. In fraction III, depleted of G_t by this treatment, there was a 50% increase in CI&II phosphorylation. The overall pattern of endogenous phosphorylation levels of CI&II in different fractions is similar to that in Figure 3. Therefore, phosphorylation may be regulated by the presence of free G_t subunits.

Cross-Linking of ROS Proteins. In order to examine whether CI&II interacts with other proteins, chemical cross-linking by MBS was performed on each protein fraction from ROS. Rod outer segment proteins were phosphorylated by endogenous PK-A and then fractionated. Each extracted fraction was incubated with MBS for cross-linking (Figure 5). In the soluble fraction, after MBS cross-linking, Coomassie blue staining showed no dramatic change in protein band intensities (Figure 5A, lanes 1 and 5). The densities and positions of p-CI&II as well as those of other phosphorylated proteins in the soluble fraction were little altered by cross-linking (Figure 5B, lanes 1 and 5). In the hypotonic-wash fraction, proteins were mostly cross-linked to very high molecular masses which did not enter the gel while minor bands ranging from 30 to 38 kDa appeared (Figure 5A, compare lanes 2 and 6). The radioactivity of p-CI&II as

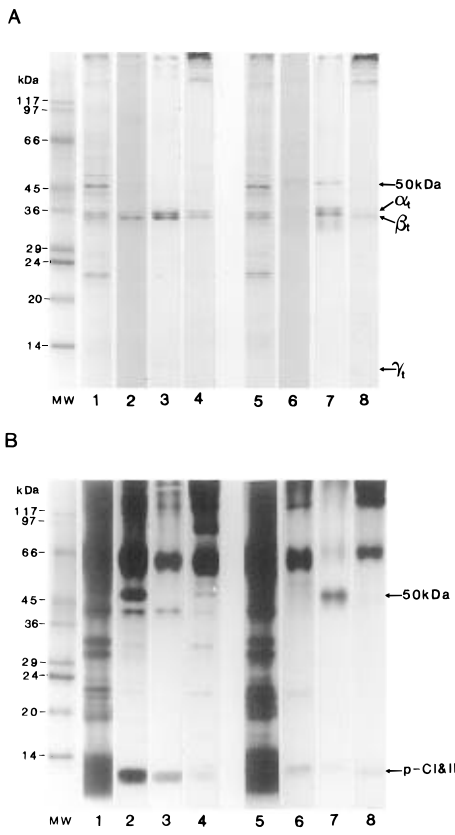

FIGURE 5: Cross-linking of ROS proteins by MBS. ROS proteins were phosphorylated by endogenous protein kinase with 20 μCi [γ-^{32}P]ATP, 30 μM ATP, and 100 μM cAMP, and then phosphorylated proteins were fractionated into a soluble fraction (lane 1), a hypotonic-wash fraction (lane 2), a GTP-wash fraction (lane 3), and a membrane fraction (lane 4). Each fraction was incubated with 50 μM MBS at room temperature for 1 h: (A) Coomassie blue staining of non-cross-linked phosphorylated ROS proteins (lanes 1−4) and cross-linked ones (lane 5−8) and (B) autoradiogram of non-cross-linked phosphorylated ROS proteins (lanes 1−4) and cross-linked ones (lanes 5−8). MW represents molecular size standard proteins.

well as phosphoproteins at 40 and 46 kDa was decreased by cross-linking in this protein fraction (Figure 5B, lanes 2 and 6). In the G_t-enriched GTP-wash fraction, the Coomassie blue-stained band densities of α and β subunits of G_t decreased with the appearance of a series of bands from 30 to 36 and 50 kDa (Figure 5A, lanes 3 and 7). This cross-linked 50 kDa band contained radioactivity (compare lane 7 in Figure 5A and lane 7 in Figure 5B), and the radioactive bands of p-CI&II were reduced in intensity (Figure 5B, lanes 3 and 7). The membrane fraction showed decreasing amounts of α_t, β_t, and 55 kDa band densities after cross-linking (Figure 5A, lanes 4 and 8). Cross-linking caused the disappearance of a few radioactive bands (23, 32, and 55 kDa), which were cross-linked into large molecules not entering the gel, with no change in p-CI&II bands (Figure 5B, lanes 4 and 8). The generation of a 50 kDa cross-linked band was observed also in the soluble and hypotonic-wash fractions. However, the intensity of Coomassie blue staining and radioactivity of 50 kDa cross-linked product was very low and further cross-linked into a larger molecule in a short time (data not shown). Thus, it appeared that CI&II were cross-linked to other proteins by MBS.

We hypothesized that the 50 kDa cross-linked product which was both Coomassie blue stained and phosphorylated in the G_t-enriched fraction might be a result of cross-linking

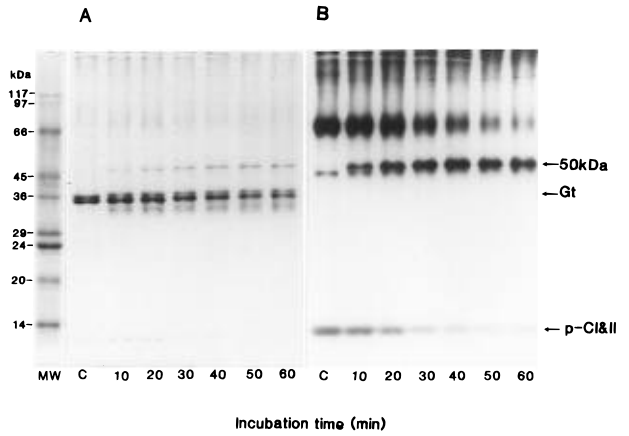

FIGURE 6: Time course of the cross-linking of ROS proteins. The GTP-wash fraction of phosphorylated ROS proteins was incubated with 50 μM MBS as described in Experimental Procedures. An aliquot was removed from the reaction mixture every 10 min, and 1 mM dithiothreitol and 10% trichloroacetic acid were added to stop the reaction. The cross-linking products were analyzed by SDS−PAGE: (A) Coomassie blue staining and (B) autoradiogram. G_t represents transducin, and p-CI&II represents phosphorylated CI&II. Cstands for the non-cross-linked control, and MW represents molecular size standard proteins.

radioactive CI&II with a target protein. To further pursue this hypothesis, we wanted to study whether this complex was the major cross-linked product without any cross-linked intermediate and how this complex was formed. To do this, the time course of cross-linking in the GTP-wash fraction was investigated (Figure 6). There was a time-dependent decrease in α_t and β_t densities with a concomitant increase in the 30−38 and 50 kDa band densities (Figure 6A). Similarly, the intensity of radioactive p-CI&II decreased, and the radioactivity of the 50 kDa band increased (Figure 6B). The intensity of the radioactive 50 kDa band was higher than that of p-CI&II. This might be due to enhancement of p-CI&II retention in the polyacrylamide gel because complex formation to a larger molecule prevented loss of small p-CI&II molecules during experimental procedures. Densitometric scans of the gel and an autoradiogram showed that there was a temporal correlation in the GTP-wash fraction between the disappearance of radioactivity in CI&II and appearance of the radioactive 50 kDa band. A radioactive band of 68 kDa also showed a time-dependent decrease in intensity as it was cross-linked into a complex too large to enter the gel.

To identify the protein components of the 50 kDa cross-linked product, immunoblotting with antisera against candidate proteins was performed. Since G_t is the major protein of this fraction, we used antibodies raised against subunits of transducin, α_t, and β_t (Figure 7). Monoclonal antibody 4A recognized α_t in all fractions (soluble, hypotonic-wash, GTP-wash, and membrane fractions) of non-cross-linked (Figure 7A) and cross-linked (Figure 7C) ROS proteins. It also recognized multiple bands ranging from 30 to 38 kDa in cross-linked proteins (Figure 7C). These bands most likely correspond to intramolecular-cross-linked products of α_t (Hingorani et al., 1988; Villancourt et al., 1990). It also recognized a band of 65 kDa which is a dimer of α_t which can persist under nonreducing conditions (lane 3 in Figure 7A,C). However, the 50 kDa cross-linked band was not recognized by mAb 4A, suggesting that it did not contain α_t.

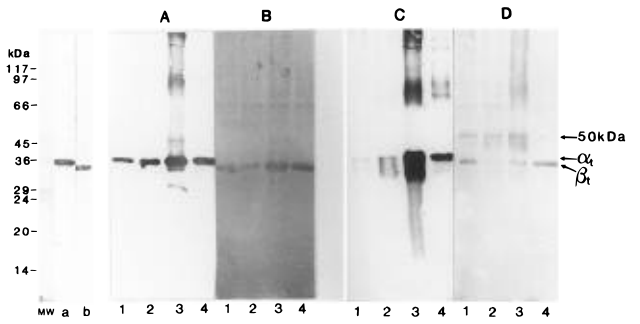

FIGURE 7: Immunoblotting of the cross-linked ROS proteins. Non-cross-linked (A and B) and cross-linked (C and D) proteins of each fraction were separated by SDS−PAGE and were transferred onto PVDF membranes and cross-reacted with mAb 4A (1:2000 dilution) (A and C) or anti-β_t antiserum (1:3000 dilution) (B and D): land 1, soluble fraction; lane 2, hypotonic-wash fraction; lane 3, GTP-wash fraction; lane 4, membrane fraction; a and b, control α and β subunits of purified G_t. MW represents molecular size standard proteins.

The anti-β_t antiserum cross-reacted with β_t in all fractions of non-cross-linked (Figure 7B) and cross-linked (Figure 7D) ROS proteins. In addition, it also cross-reacted with the 50 kDa band (Figure 7D, lanes 1−3) as well as with a smaller band at 46 kDa (Figure 7D, lanes 2 and 3) in the cross-linked proteins. The 50 kDa band comigrated with the phosphorylated 50 kDa cross-linked product (Figures 5 and 6). The minor 46 kDa band was neither stained by Coomassie blue nor radioactive and may represent a minor $\beta_t-\gamma_t$ cross-linked product (Hingorani et al., 1988). The above data suggest that the distinctive cross-linked 50 kDa band is composed of β_t (or $\beta\gamma_t$) and CI&II. A smaller amount of β_t-cross-linked band was also found in the soluble and hypotonic fractions while radioactivity was very low (Figure 7D, lanes 1−3).

Multiple Phosphorylation of CI&II. Since phosphorylation appeared to regulate the solubilization of membrane-bound CI&II, phosphorylation patterns of CI&II by endogenous PK-A might be different in the soluble fraction and in the membrane-associated protein fractions. In order to study this, phosphopeptide mapping and phosphoamino acid analysis were performed. When endogenously phosphorylated CI extracted from the soluble fraction was trypsinized, it showed two radioactive spots (Figure 8A). Endogenously phosphorylated CII from the same fraction demonstrated the same result (Figure 8B). These data suggest that CI&II are similar proteins. The endogenously phosphorylated CI&II from the combined fraction of hypotonic-wash fraction and GTP-wash fraction showed only one minor radioactive spot (Figure 8C) which showed similar mobility as spot 1 in the soluble fractions. Therefore, it was demonstrated that the endogenous phosphorylation patterns of CI&II were different between the soluble and the peripheral membrane fractions. However, phosphoamino acid analysis showed that, in both cases, phosphorylation occurs on serine residues (data not shown).

All together, CI&II phosphorylation by endogenous PK-A in the soluble fraction and the peripheral membrane fractions seems to be different in pattern and intensity. It is likely that the serine residues for phosphorylation of CI&II are located in at least two different domains. Phosphorylation on both domains of CI&II appears to cause elution of membrane-bound CI&II into cytosol.

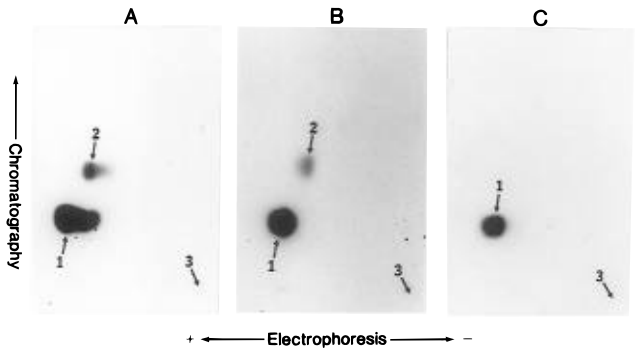

FIGURE 8: Phosphopeptide mapping of p-CI&II. ROS proteins were endogenously phosphorylated with 200 μCi [γ-^{32}P]ATP, 30 μM ATP, and 100 μM cAMP for 10 min in the dark. After the reaction was stopped, fractionation and separation of ROS proteins were followed. Proteolysis of p-CI&II extracted from polyacrylamide gel was performed on ice for 1 h by addition of 5 μg of TPCK−trypsin mixed with 100 μg of BSA. Proteolyzed peptides were analyzed on cellulose plates by electrophoresis (300 V, 45 min) and thin layer chromatography (2 h): (A) phosphopeptides of p-CI from the soluble fraction, (B) phosphopeptides of CII from the soluble fraction, and (C) phosphopeptides of CI&II from the combined fraction of PDE-enriched and G_t-enriched fractions. Lane 1, spot 1; lane 2, spot 2; lane 3, origin. Plates A and B were exposed for 2 days, and plate C was exposed for 4 days.

Table 1: Partial Amino Acid Sequences of Components I and II[a]

component	purification	sequence
I	PVDF	LRx_1YSx_2VASNEEEEYKQGNHVx_2PQ
II	HPEC	LRx_1YSx_2VASNEEEEYKQGN

[a] Each component was sequenced separately for amino terminal amino acids by an Applied Biosystem 447A Protein Sequencer. Unidentified amino acid residues are indicated as x_1 and x_2, which are chromatographically different.

Partial Amino Acid Sequences. Since the results from the phosphopeptide-mapping experiments strongly indicated that CI&II are similar proteins, we compared their amino acid sequences to examine this hypothesis. In addition, it is of interest to determine whether CI&II show sequence homology with other known proteins. Each purified component I or II contained an unmodified N-terminus so that each amino acid in the region could be identified. The amount of purified component I by electroblotting onto PVDF membrane was enough for 24 cycles of Edman degradation in the sequencing machine (Table 1), while that of purified component II by HPEC allowed 19 cycles. Residue 3 (x_1) was not identified. Other unidentified residues, 6 and 22 (x_2), showed the same chromatographic behavior during sequencing, suggesting that these two residues were the same. However, x_1 and x_2 were different in chromatographic behavior. Comparison of the amino acid sequences of CI&II from different purification procedures demonstrated the same sequence. This supports the suggestion from phosphopeptide-mapping experiments that CI&II are similar proteins. These sequences of CI&II are different from that of phosducin from bovine retinas where the N-terminal sequence is Met-Glu-Lys-Ala, and phosducin does not contain more than two consecutive glutamic acid residues (Kuo et al., 1989; Lee et al., 1990b). A search of the most current protein data bank found no other homologous proteins. Although it has been reported that CI&II contain the γ subunit of phosphodiesterase (PDEγ) (Tsuboi et al., 1994), this result clearly showed that they are not PDEγ.

DISCUSSION

On the basis of phosphorylation intensity, CI&II are the most abundant phosphoproteins in the dark-adapted ROS of frog photoreceptors (Polans et al., 1979; Bownds & Brewer, 1988; Hamm, 1990). The phosphorylation level of CI&II is high in the dark, whereas illumination caused a rapid decrease in CI&II phosphorylation within 1 s (Hamm, 1990). Their phosphorylation, which is dependent on cyclic nucleotides, is carried out by PK-A (Hamm, 1990). Although many aspects of CI&II phosphorylation behavior suggest a regulatory role for CI&II, their physiological role in the phototransduction pathway in frog ROS has not been elucidated. The stoichiometry of phosphorylation of CI&II is not known, because the proteins are very minor in ROS. Extensive efforts to purify them or to produce precipitating antibodies have not met with any success so far.

The phosphorylation of phosducin in bovine rod cells is similar to that of CI&II (Lee et al., 1987, 1990a; Lolley and Lee, 1990). Phosducin was purified from bovine retinal homogenates, and its cDNA and amino acid sequences were elucidated (Kuo et al., 1989; Lee et al., 1990b). It showed no amino acid sequence homology to any other known protein. However, no phosducin has been reported by either phosphorylation or immunological cross-reactivity in frog ROS. Conversely, no CI&II could be found in bovine ROS. Since CI&II and phosducin share many aspects of physiological regulation of phosphorylation, they may be functionally homologous proteins. Therefore, it is of interest to study the role of CI&II phosphorylation in frog ROS, where the physiological processes of vision can be studied more easily.

Partial amino acid sequences of purified CI&II and phosphopeptide mapping of p-CI&II indicated that these two components are similar proteins with slightly different molecular masses due to unknown differences, perhaps due to posttranslational modification. The limited amino acid sequences of CI&II that were obtained were different from those of bovine phosducin and did not show any homology to the sequences of any other protein reported in the data bank. Therefore, CI&II appear to be distinct proteins of amphibian photoreceptors.

A heterobifunctional cross-linker, MBS, generated a very distinct phosphorylated 50 kDa band in the G_t-enriched GTP-wash fraction. The soluble and hypotonic-wash fractions showed very low levels of the cross-linked 50 kDa band. The cross-linked 50 kDa band was recognized by an antibody against β_t and was radioactive. Therefore, it was likely that $\beta\gamma_t$ formed a complex with CI&II in this fraction. Proof that the cross-linked 50 kDa product contains CI&II requires its demonstration with an anti-CI&II antibody, but such an antibody is not available at the present time. The correlation between the disappearance of the radioactive CI&II bands and the appearance of the radioactive 50 kDa band during the time course of cross-linking suggests that the radioactive constituent of the 50 kDa band is p-CI&II. It is interesting that, among different protein fractions of frog ROS, mainly the peripheral membrane fractions containing mostly G_t showed this 50 kDa cross-linked product. Components I and II in the soluble fraction were cross-linked to a very low extent which was detected by immunoblotting, while non-cross-linked CI&II were highly phosphorylated. This result suggests that the complex mainly resides on the ROS membranes, and the formation of the complex is inhibited by CI&II phosphorylation.

The sequential phosphorylation of ROS proteins shows that there is a pool of poorly phosphorylated CI&II in membrane-associated protein fractions of frog ROS. It is possible that CI&II in these fractions are not good substrates for endogenous PK-A because they interact with other proteins, such as $\beta\gamma_t$, which may block the phosphorylation sites on CI&II. This interpretation is supported by the finding that the chemically cross-linked, less phosphorylated CI&II$-\beta\gamma_t$ complex was a poor substrate for phosphorylation by PK-A_{cat} (data not shown). Experiments which increased levels of free $\beta\gamma_t$ by dissociating G_t subunits are also consistent with this hypothesis. In the presence of increased concentrations of G_t subunits, phosphorylation of soluble CI&II was inhibited while removal of G_t subunits from membranes resulted in a higher phosphorylation level.

If the interaction between CI&II and $\beta\gamma_t$ inhibits the phosphorylation of CI&II by endogenous PK-A, what might be the role of phosphorylation? The extensively washed ROS membranes still contained a relatively large amount of CI&II and $\beta\gamma_t$ which were extractable together by phosphorylation. This indicates that the less phosphorylated or unphosphorylated CI&II and G_t reside on the ROS membranes and that increased phosphorylation decreases the affinity of these proteins for the membranes, resulting in solubilization of p-CI&II into the cytosol. On the basis of phosphopeptide-mapping studies, CI&II may have two phosphorylation domains whose extent of phosphorylation by PK-A regulates its subcellular localization. Thus, the observation that soluble CI&II are highly phosphorylated and are mostly free from other proteins and that membrane-bound CI&II are less phosphorylated or unphosphorylated and associated to other proteins may be due to the different phosphorylation state. A similar effect was also reported in the complex formation of phosducin with $\beta\gamma_t$ (Yoshida et al., 1994). Dephosphorylated phosducin binds to free $\beta\gamma_t$ with higher affinity than phosphorylated phosducin. Thus, it appears that a physical interaction between $\beta\gamma_t$ and CI&II can control the phosphorylation of CI&II, while phosphorylation in turn can control the localization of CI&II in frog ROS.

Combining all experimental data, we can propose a working model of the role of CI&II in phototransduction. The levels of cyclic nucleotides are decreased in ROS by light activation (DeVries et al., 1978; Cohen, 1982; Cohen & Brazynski, 1987). It leads to lower activity of protein kinase(s) and, under steady state conditions of phosphorylation and dephosphorylation, results in relative dephosphorylation of CI&II. Phosphorylated CI&II of frog ROS are also dephosphorylated by a phosphatase activity as a result of illumination. Dephosphorylated CI&II interact with free $\beta\gamma_t$ resulting from light activation of G_t. This component$-\beta\gamma_t$ complex then regulates the amount of $\beta\gamma_t$ available for reassociation with α_t-GDP or for activation of other effectors in frog ROS. Such a regulatory step might be postulated to play a role in an adaptational signalling process of ROS. After the restoration of cyclic nucleotides to their dark levels, endogenous protein kinases again become active which can phosphorylate CI&II and, perhaps less well, component$-\beta\gamma_t$ complexes on the membranes. At steady state, phosphorylation will cause dissociation of the complex into phosphorylated component and $\beta\gamma_t$. This freed $\beta\gamma_t$ can

reassociate with α_t−GDP to regenerate holo G_t−GDP.

ACKNOWLEDGMENT

We thank Dr. Nikolai Artemyev for valuable discussions, Dr. David Glass of Emory University for the purified catalytic subunit of protein kinase A, and Dr. Paul Morris of the Protein Sequencing/Synthesis Laboratory of the Research Resources Center at the University of Illinois at Chicago for the peptide sequencing.

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BI9518656