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Calcium Binding, but Not a Calcium-Myristoyl Switch, Controls the Ability of Guanylyl Cyclase-activating Protein GCAP-2 to Regulate Photoreceptor Guanylyl Cyclase*

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Guanylyl cyclase-activating protein 2 (GCAP-2) is a recoverin-like calcium-binding protein that regulates photoreceptor guanylyl cyclase (RetGC) (Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* 271, 19346–19350). It was reported that myristoylation of a related protein, GCAP-1, was critical for its affinity for RetGC (Frins, S., Bonigk, W., Muller, F., Kellner, R., and Koch, K.-W. (1996) *J. Biol. Chem.* 271, 8022–8027). We demonstrate that the N terminus of GCAP-2, like those of other members of the recoverin family of Ca^{2+}-binding proteins, is fatty acylated. However, unlike other proteins of this family, more GCAP-2 is present in the membrane fraction at low Ca^{2+} than at high Ca^{2+} concentrations. We investigated the role of the N-terminal fatty acyl residue in the ability of GCAP-2 to regulate RetGCs. Myristoylated or nonacylated GCAP-2 forms were expressed in *Escherichia coli*. Wild-type GCAP-2 and the $Gly^2 \rightarrow Ala^2$ GCAP-2 mutant, which is unable to undergo N-terminal myristoylation, were also expressed in mammalian HEK293 cells. We found that compartmentalization of GCAP-2 in photoreceptor outer segment membranes is Ca^{2+}- and ionic strength-sensitive, but it does not require the presence of the fatty acyl group and does not necessarily directly reflect GCAP-2 interaction with RetGC. The lack of myristoylation does not significantly affect the ability of GCAP-2 to stimulate RetGC. Nor does it affect the ability of the Ca^{2+}-loaded form of GCAP-2 to compete with the GCAP-2 mutant that constitutively activates RetGC. We conclude that while Ca^{2+} binding plays a major regulatory role in GCAP-2 function, it does not operate through a calcium-myristoyl switch similar to the one found in recoverin.

Ca^{2+} enters outer segments (OS)[1] of vertebrate photoreceptors through cGMP-gated Na^+/Ca^{2+} channels in the plasma membranes (reviewed in Refs. 3–5). In darkness these channels allow Ca^{2+} influx, but light-induced closure of the channels lowers free intracellular Ca^{2+} because Ca^{2+} is continuously extruded from the OS by a light-independent Na^+/K^+, Ca^{2+} exchanger. The estimate of the magnitude of this effect is that light lowers free intracellular Ca^{2+} from a dark level of 500 nM to as low as 50 nM (6). The decrease in free Ca^{2+} concentration allows guanylyl cyclase activator proteins (GCAPs) (7–11) to stimulate a membrane guanylyl cyclase (RetGC) (12, 13). Two homologous GCAPs have been identified in the retina: GCAP-1 (7, 9) and GCAP-2 (8, 10). Based on immunoblot and immunocytochemical analysis, GCAP-1 (11) and GCAP-2 (10) are both specific for retina and present in photoreceptor cells. The relative abundance of GCAPs in rods and cones and their relative contributions to regulation of RetGC *in vivo* still remain to be determined. At least two different GCAP-regulated RetGCs have been identified in mammalian photoreceptors, RetGC-1 (8, 14–18) and RetGC-2 (19, 20) (or GC-E and GC-F, respectively). It has been demonstrated by independent groups that GCAP-1 and GCAP-2 can activate RetGCs in OS membranes *in vitro* within the range of free Ca^{2+} concentrations corresponding to the estimated physiological range (1, 7–12, 19). Unlike peptide ligands that regulate other known membrane guanylyl cyclases via the cyclase extracellular domains (reviewed in Refs. 21 and 22), both GCAPs interact with RetGC via the cyclase intracellular domain (23, 24). GCAP-1 is able to stimulate recombinant RetGC-1 (11, 24), while GCAP-2 stimulates both recombinant RetGC-1 and RetGC-2 (8, 19, 23). However, the question which of the two cyclases can be a target for any particular GCAP *in vivo* has not yet been properly addressed. Additional factors may also be involved in regulation of RetGCs, such as sodium concentration in the intracellular medium (25), phosphorylation (25, 26), ATP binding (23, 27), or actin binding (28). Also, an unidentified S100 protein-like factor ("CDGCAP" (29)) can stimulate RetGC *in vitro* at free Ca^{2+} concentrations that are significantly higher than estimated physiological range. Among all factors that might influence RetGC activity only GCAP-1 and GCAP-2 have been shown to stimulate RetGCs at low Ca^{2+} concentrations and to impart Ca^{2+} sensitivity to the cyclases *in vitro* within the physiological range of 50–500 nM free Ca^{2+}.

Unlike many other known members of the EF-hand superfamily (with the exception of GCAP-1), GCAP-2 acquires its activating conformation only at low Ca^{2+} concentrations. At free Ca^{2+} concentrations similar to the levels in dark-adapted vertebrate photoreceptors, GCAP-2 is not only unable to activate RetGCs, but it also inhibits RetGC. Therefore, GCAP-2 can be considered as a RetGC *regulator* rather than *activator* protein (1). Inactivation of EF hands makes GCAP-2 a constitutive activator of RetGC unable to undergo an "activator-to-inhibitor" transition (1).

GCAPs belong to the family of recoverin-like proteins that also includes a variety of neuronal Ca^{2+}-binding proteins such as neurocalcin, hippocalcin, and others (9, 10, 30). Unlike dis-

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[1] The abbreviations used are: OS, photoreceptor outer segment(s); GCAP, guanylyl cyclase-activating protein; ESI-MS, electrospray mass spectrometry; NMT, *N*-myristoyltransferase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RetGC, photoreceptor guanylyl cyclase.

tantly related calmodulin, recoverin-like proteins are C14-fatty acylated at their N termini. For mammalian retinal proteins, this acylation is heterogeneous (C14:0, C14:1, C14:2, and C12:0) (31–33). While all C14 derivatives altogether can constitute up to 75% of the total amount of fatty acyl residues, myristoyl itself represents only as much as 25% of it (32). Fatty acylation has been shown to impart to recoverin and to several recoverin-like proteins the ability to compartmentalize to membranes in the presence of calcium (34–38). The affinity of recoverin-like proteins for membranes increases as a result of fatty acyl group exposure in response to the protein transition into its calcium-bound conformation (35, 36), a mechanism referred to as a "calcium myristoyl switch" (35). The calcium-myristoyl switch in recoverin was directly demonstrated by measuring the accessibility of the fatty acylated N terminus to proteolytic cleavage (36) and by NMR (39, 40). Both GCAP-1 and GCAP-2 have a consensus sequence for the *N*-myristoylation encoded in their cDNAs (9, 10, 41). It was reported that GCAP-1 is fatty acylated (9), and the N-terminal fatty acyl group may be important for RetGC regulation; nonacylated GCAP-1 was found to have dramatically lower affinity for RetGC and lower Ca^{2+} sensitivity than the fatty acylated GCAP-1 (2).

In this study we investigated whether or not the N-terminal fatty acylation of GCAP-2 is essential for RetGC regulation. We found that GCAP-2 is fatty acylated but that its affinity for membranes is affected by Ca^{2+} in a manner opposite to that of other recoverin-like proteins. It associates with membranes at low Ca^{2+} concentrations more efficiently than at high free Ca^{2+} concentrations. We also demonstrate that neither membrane association of GCAP-2 nor various aspects of RetGC regulation by GCAP-2 require fatty acylation. We therefore conclude that Ca^{2+} induces a conformational change in GCAP-2 protein but that the calcium-myristoyl switch found in other members of the recoverin family is not essential for the ability of GCAP-2 to regulate RetGCs. We also describe an efficient bacterial expression system for producing functional myristoylated and nonacylated GCAP-2.

EXPERIMENTAL PROCEDURES

Isolation of Retinal GCAP-2—GCAP-2 was isolated from a heat-stable fraction of retinal proteins using immunoaffinity chromatography on monospecific polyclonal ΔNp24 antibodies coupled to CNBr-activated Sepharose 4B as described previously (10). Purified GCAP-2 had no detectable GCAP-1 in it as assayed by immunoblot.

Recombinant GCAP-2—GCAP-2 was expressed in HEK293 cells transiently transfected with GCAP-2 cDNA-containing vector using calcium phosphate precipitation. Protein extracts from expressing and control (vector only) transfected cells were made as described previously (1, 10). The G2A mutant was generated by introducing a GGG/GCG substitution into the second codon of GCAP-2 cDNA (10) by *Pfu* polymerase-catalyzed polymerase chain reaction using the "splice by overlap extension" approach (42). The G2A GCAP-2 mutant and a wild-type GCAP-2 were expressed in cell cultures transfected and harvested simultaneously. Protein extracts from expressing and control (vector only) transfected cells were prepared, and GCAP-2 expression was estimated by immunoblot using ΔNp24 antibody as described previously (10).

Expression of GCAP-2 in Escherichia coli and Its Purification—The GCAP-2 cDNA coding region (10) was inserted into the *Nco*I/*Bam*HI sites of the pET11d vector (Novagen) and expressed under control of the isopropyl-β-D-thiogalactopyranoside-regulated T7 promoter in the BL21(DE3)pLysS *E. coli* strain (Novagen) carrying a pBB131 plasmid encoding *N*-myristoyl transferase (NMT) (a gift from Dr. J. Gordon). To produce nonacylated GCAP-2, we used the same strain of cells but without the pBB131 plasmid. Fresh overnight 5-ml cultures of expression cells were diluted in 500 ml of standard LB media containing 50 μg/ml ampicillin or both 50 μg/ml kanamycin and 50 μg/ml ampicillin (for pBB131-containing strains). Bacteria were grown at 37 °C until the culture reached 0.2–0.3 OD units at 600 nm. Free myristic acid (100 mg/ml ethanol solution) was added into the suspension of bacterial cells to 50 μg/ml 30 min before the induction with isopropyl-β-D-thiogalactopyranoside as described earlier for recoverin (36). Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and typically in 3.5 h bacterial pellets were harvested by centrifugation at 8,000 × *g* for 20 min at 4 °C. The cells were disrupted by three cycles of ultrasonication of 30 s each. The expressed GCAP-2 and its mutants were always found in the insoluble fraction. The insoluble material was washed three times with a 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 100 μM PMSF, and 20 μg/ml leupeptin (buffer A) by centrifugation at 20,000 × *g* for 10 min. GCAP-2 was extracted from the pellet by homogenization in buffer A containing 100 mM mercaptoethanol, 1 mM EDTA, and 6 M freshly deionized urea for 30 min at 4 °C and dialyzed twice against 300–1000 volumes of buffer A overnight at 4 °C. Precipitate was removed by centrifugation at 30,000 × *g* for 10 minutes. Recombinant protein was then purified by gel filtration on a Sephacryl S100 column in 10 mM Tris-HCl buffer (pH 7.5) and 10 mM mercaptoethanol. Fractions containing GCAP-2 were combined and concentrated using Amicon YM10 membranes under nitrogen pressure to 2 mg/ml final concentration. Concentrated protein was used either immediately or after being quickly frozen in small aliquots and stored at −70 °C. Myristoylated and nonacylated EF(2/3/4)$^-$ GCAP-2 mutant, which carried substitutions E80Q/E116Q/D158N (1) was produced using the same protocol. For their functional comparisons, different GCAP-2 forms were always expressed simultaneously and purified in parallel.

Electro-spray Mass Spectrometry (ESI-MS)—ESI-MS of GCAP-2 was performed essentially as described previously for recoverin using a Sciex API III triple quadrupole instrument (31), except that purified retinal or recombinant GCAP-2 was injected into the mass spectrometer during its elution from a capillary reverse-phase high pressure liquid chromatography C18 column connected to the mass spectrometer.

Membrane Binding—For GCAP-2 extraction experiments, a fraction of OS was isolated from frozen dark-adapted bovine retinas using sucrose gradient centrifugation (43), and rhodopsin concentration was determined by measuring absorbance of an aliquot diluted in 1% Ammonix LO detergent at 500 nm before and after bleaching, using ϵ_{500} = 40,000. OS typically diluted at 1 mg/ml of rhodopsin were homogenized in 10 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μM PMSF, and 10 μg/ml of leupeptin and aprotinin (buffer B) containing 1 mM Ca-EGTA buffers using a Dounce glass-to-glass homogenizer equipped with pestle B. Only freshly isolated membranes could be used for these experiments. Homogenate was centrifuged at 80,000 rpm for 15 min at 4 °C in a Beckman T 100.1 rotor. The supernatant was aspirated, and the pellet was resuspended in the same volume of buffer. Ten microliters of soluble and membrane fractions were mixed with an equal volume of Laemmli SDS sample buffer containing 2 mM EGTA, boiled, and loaded onto 12.5% SDS-polyacrylamide gel. The addition of EGTA in sample buffer prevents the appearance of bands of Ca^{2+}-bound GCAP-2, which have higher mobility than its Ca^{2+}-free form. After electrophoresis, GCAP-2 bands were transferred to a nitrocellulose sheet, stained with a ΔNp24 antibody, and developed using goat anti-rabbit peroxidase conjugate and an Amersham ECL chemiluminescent reagent. Fluorograms of GCAP-2 bands within the quasilinear range of density were scanned using a Bio-Rad model GS-670 imaging densitometer and quantified using Bio-Rad Molecular Analyst software. For reconstitution experiments, OS membranes were first washed six times with buffer B and then incubated typically at 2.5 mg/ml rhodopsin with 2 μM recombinant GCAP-2 and 1 mM Ca-EGTA in 200 μl for 30 min at room temperature in buffer B containing 50 mM Tris-HCl. The mixture was centrifuged as indicated above, and the pellet was gently rinsed with the corresponding incubation buffer, resuspended in such buffer, and repelleted. The final pellet typically containing approximately 10% of total protein was resuspended in the original volume of water, and aliquots of membranes were analyzed in SDS-polyacrylamide gel as described above. Washed membranes incubated without added GCAP-2 were used in these experiments as controls to ensure that no endogenous protein was left in the membranes.

Liver Membrane Preparation—Fresh mouse liver was homogenized in 10 volumes of ice-cold 0.32 M sucrose containing 20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 0.2 mM PMSF and centrifuged at 5000 × *g* for 15 min. The supernatant was recentrifuged at 45,000 × *g* for 30 min. Membranes were resuspended in the same buffer without sucrose and washed three times by centrifugation. Protein concentration was determined using a Bio-Rad BCA protein assay kit.

Preparation of Liposomes—Bovine retinas were homogenized in a blender with chloroform/methanol solution for 5 min at the following volume proportions: 1.5 retinal tissue, 3.73 methanol, 1.87 chloroform. After homogenization, water (1.9 volume) and chloroform (1.9 volume)

were added to the mixture. In 20 min, the organic phase was collected, further separated from the remainders of aqueous phase by centrifugation at 10,000 × *g* for 20 min in glass centrifuge tubes, and collected using Pasteur pipettes. Lipids were dried under a constant stream of argon at room temperature, dispersed in liposome buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol), flushed with argon, and sonicated for 30 s. A fraction of liposomes sedimenting at 20,000 × *g* was collected by three cycles of centrifugation followed by resuspension of the pellet in a fresh portion of liposome buffer. The final pellet was resuspended in 200 μl of 10 mM Tris-HCl (pH 7.5) preflushed with argon, and 30 μl were taken for reconstitution with recombinant GCAP-2 and immunoblot analysis to ensure the absence of RetGC-1, RetGC-2, and rhodopsin.

The RetGC Assay—The RetGC assay was performed using [α-^{32}P]GTP as a substrate and [8-^{3}H]cGMP as an internal standard. The reaction was carried out under infrared light and analyzed by thin layer chromatography essentially as previously described in detail (10).

Ca-EGTA Buffers—Ca-EGTA buffers were calculated as in Ref. 10 and made strictly according to Ref. 44. Free Ca^{2+} concentrations were verified by a Ca^{2+}-selective electrode and Rhod-2 titration.

Immunoblot—Proteins separated by SDS-PAGE were transferred onto a Millipore nitrocellulose sheet overnight at 100 mA. Anti-rhodopsin monoclonal antibody 4D2 (a gift from Robert Molday, University of British Columbia), monospecific rabbit antibody ΔNp24 against GCAP-2 (10), antiserum against the RetGC-1 Glu^{641}–Trp^{657} peptide derived from its kinase homology domain (23),[2] and rabbit antiserum against the RetGC-2 Val^{169}–Arg^{180} peptide derived from its extracellular domain (19) were used to probe the immunoblot and developed using peroxidase conjugate and an Amersham ECL substrate.

RESULTS AND DISCUSSION

GCAP-2 Demonstrates Membrane Binding Unusual for a Recoverin-like Protein—It has been demonstrated that several members of the recoverin family (*S*-modulin, recoverin, neurocalcin, and hippocalcin) acquire stronger affinity to membranes in the presence of Ca^{2+} (34–38). In each case the membrane binding depends on the N-terminal fatty acylation (myristoylation). It has been found that in recoverin the N-terminal myristoyl group becomes exposed as a result of conformational changes caused by calcium binding (36, 39, 40), a mechanism known as "calcium-myristoyl switch" (35, 47).

GCAPs are the most closely related members of the recoverin family (9–11, 48). However, unlike other members of this family, GCAP-2 associates with membranes more efficiently when Ca^{2+} concentration is lowered below 1 μM (Fig. 1). The distribution of GCAP-2 between the soluble and membrane fractions depends on free Ca^{2+} concentrations. GCAP-2 binds more strongly to membranes when the free Ca^{2+} concentration is below 200 nM. An increase in free Ca^{2+} concentrations promotes dissociation of this protein (Fig. 1, *A* and *B*). This behavior is opposite to that of recoverin or other recoverin-like proteins (Fig. 1*B*; see also Refs. 34–38). The relative distribution of GCAP-2 between the soluble and membrane fractions also depends on ionic strength; GCAP-2 is less soluble at 100 mM NaCl than in hypotonic buffer. However, even at this ionic strength Ca^{2+} decreases the amount of GCAP-2 associated with membranes (Fig. 1*C*). This effect cannot be attributed to the nonspecific influence of divalent cations because we varied the free Ca^{2+} concentration within the micromolar range, whereas $MgCl_2$ was present at 5 mM in all of those experiments.

GCAP-2 Is a Fatty Acylated Protein—The N terminus of GCAP-2 (MGQQFS . . . ; Ref. 10), contains a consensus sequence recognized by NMT (45). Met^1 is removed during protein synthesis, and Gly^2, the actual amino-terminal residue, is acylated. To verify that such modification takes place *in vivo*, we used ESI-MS to evaluate the exact molecular mass of GCAP-2 purified from bovine retina by immunoaffinity chromatography. The results shown in Table I demonstrate that

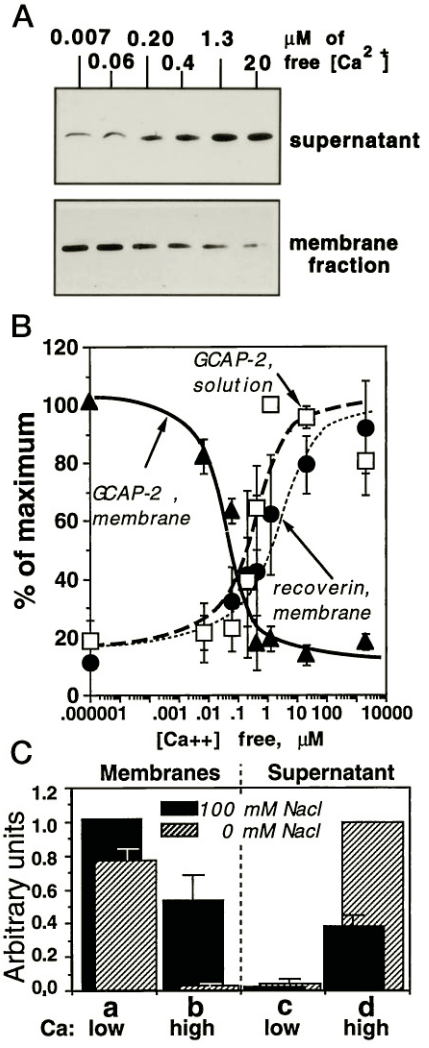

FIG. 1. **GCAP-2 is released from the OS membranes at high Ca^{2+}.** *A*, extraction of p24 and recoverin from the OS membrane fraction as a function of free Ca^{2+}. Samples containing 200 μg of protein in 250 μl were extracted and immunoblotted as described under "Experimental Procedures" and probed with anti-GCAP-2 antibody ΔNp24. *Top*, GCAP-2 in soluble fraction. *Bottom*, GCAP-2 in the membrane fraction at the indicated free Ca^{2+} concentrations. *B*, a comparison between recoverin and GCAP-2 extraction as a function of free Ca^{2+} concentration. Recoverin was detected using anti-recoverin antibody P26 (46). 100% of intensity in both cases corresponds to the maximal signal intensity of GCAP-2 in the supernatant fraction (□), GCAP-2 in the membrane fraction (▲), or recoverin in the membrane fraction (●), respectively. For other details see "Experimental Procedures." *C*, the effect of ionic strength on extraction of GCAP-2 from OS membranes. The relative amount of GCAP-2 in supernatant or in membrane is defined as a ratio between the amount of GCAP-2 in a corresponding fraction and the total amount of GCAP-2 in both membrane and supernatant. GCAP-2 extraction from OS membranes was performed as above in 1 mM EGTA (low) or 500 μM (high) free Ca^{2+} either in low salt buffer B (*left*) or in buffer B containing 100 mM NaCl (*right*). For other details see "Experimental Procedures".

GCAP-2 is C14-fatty acylated. The accuracy of this analysis performed on the whole protein does not allow us to conclude at the moment whether or not GCAP-2 has predominantly tetradecanoyl, tetradecaenoyl, or tetradecadienoyl fatty residues.

We have developed two expression systems for producing functional myristoylated and nonacylated forms of GCAP-2. One system is based on coexpression of GCAP-2 with NMT in *E. coli* that bears a plasmid encoding NMT in the presence of exogenous free myristic acid (Fig. 2, *A* and *B*). The molecular mass of GCAP-2 coexpressed with NMT equals the calculated molecular mass of the myristoylated protein and is identical to

[2] The actual size of the fragment is three amino acid residues longer than indicated in Ref. 23.

TABLE I
Molecular masses of retinal and recombinant GCAP-2

Average isotopic molecular masses of GCAP-2 forms were calculated using SHERPA software (kindly provided by A. Taylor, University of Washington). Actual average isotopic molecular masses were determined by liquid chromatography/mass spectrometry using a Sciex API III instrument. Proteins were purified as described under "Experimental Procedures" and in the legend to Fig. 2, *A–C*.

GCAP-2 form	Calculated mass
	Da
Starting with Gly^2, myristoylated	23807.0
Starting with Gly^2, nonacylated	23597.0
Starting with Met^1	23728.0
GCAP-2 form	**Mass found by ESI-MS (mean ± S.D.)**
	Da
Isolated from bovine retina	23,809.20 ± 2.80
Expressed in *E. coli*	
Coexpressed with NMT	23,807.80 ± 2.00[a]
Expressed without NMT	23,597.40 ± 2.30

[a] The major molecular species. A low level of the signal corresponding to a molecular species of 23597.00 was also detected in this preparation, which agreed with a low (less than 10%) amount of nonacylated GCAP-2 produced during coexpression with NMT as shown in Fig. 2*C*.

the molecular mass of retinal protein. GCAP-2 expressed in *E. coli* without NMT shows the molecular mass of nonacylated protein (Table I). We have also observed that the myristoylated recombinant GCAP-2 has slightly higher electrophoretic mobility than the nonacylated GCAP-2 (Fig. 2*C*). Both retinal and recombinant myristoylated GCAP-2 have the same mobility, while nonacylated recombinant GCAP-2 appears in an SDS-polyacrylamide gel as a protein approximately 1 kDa larger in size (despite the fact that its molecular mass is 210 Da lower than that of the myristoylated protein). Often a trace amount of nonmyristoylated protein can be observed in preparations of the myristoylated recombinant protein (Fig. 2*C*, *lane b*). A low level of signal from nonmyristoylated protein can be also detected by ESI-MS. Typically, at least 90% of GCAP-2 coexpressed with NMT in *E. coli* is myristoylated.

Myristoylated and nonacylated GCAP-2 expressed in human HEK 293 cell culture also have different electrophoretic mobilities (Fig. 2*D*). Because mammalian cells have an endogenous *N*-myristoyltransferase, to prevent myristoylation of GCAP-2, we expressed it as a mutant with the amino-terminal Gly^2 substituted for Ala (the N-terminal Ala is not a substrate for NMT (45)). Similar to what was found for nonacylated GCAP-2 expressed in *E. coli*, the G2A mutant expressed in HEK293 cells has a lower electrophoretic mobility as compared with the wild-type protein.

Myristoylation Is Not Essential for GCAP-2 Association with Membranes—GCAP-2 activates its target enzyme, a membrane guanylyl cyclase below 500 nM free Ca^{2+} concentration (8, 10, 19). Lowering Ca^{2+} concentration below 500 nM also promotes GCAP-2 association with the membrane (Fig. 1, *A* and *B*). It would therefore be tempting to imagine that Ca^{2+}-sensitive association of GCAP-2 with the membranes might directly reflect its interaction with the cyclase. Nevertheless, we find that recombinant GCAP-2 can associate with OS membranes in a Ca^{2+}-sensitive manner even when RetGC-1 and RetGC-2 are destroyed by trypsin. However, since the antibodies that we used were specific for regions relatively remote from the putative transmembrane region of RetGCs, there was still a possibility that some fragments of RetGCs including their transmembrane regions remain associated with the membrane after the proteolysis and that may solely account for the observed association of GCAP-2 with the membrane. Our finding that GCAP-2 is also capable of binding in a Ca^{2+}-sensitive manner

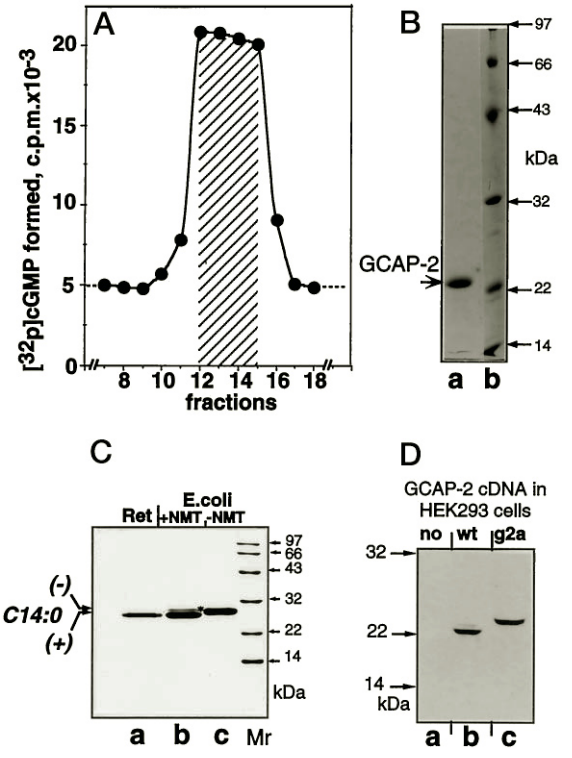

FIG. 2. **Expression of myristoylated and nonacylated recombinant GCAP-2.** *A*, purification of GCAP-2 expressed in *E. coli*. Dialyzed crude fraction of GCAP-2 expressed in pET11d(DE3)pLysS *E. coli* strain as described under "Experimental Procedures" was purified on Pharmacia HiPrep 26/60 Sephacryl S100 column, and 5 μl from each fraction was reconstituted with washed OS membranes to measure RetGC activity at 15 nM free Ca^{2+}. Fractions corresponding to the peak of activity were pooled together and concentrated. *B*, SDS-PAGE of the pooled fraction from the column (*a*) and Bio-Rad low molecular weight protein standards (*b*). *C*, 15% SDS-PAGE of the retinal immunoaffinity-purified GCAP-2 (*lane a*), recombinant myristoylated GCAP-2 expressed in *E. coli* in the presence of NMT (*lane b*), and nonacylated GCAP-2 expressed in *E. coli* without NMT (*lane c*). Details are as described under "Experimental Procedures." The *right lane* shows molecular weight markers. The *asterisk* in *lane b* marks a trace amount of nonacylated protein found in preparations of myristoylated recombinant GCAP-2. Notice that retinal GCAP-2 has an electrophoretic mobility identical to that of myristoylated recombinant GCAP-2. *D*, expression of G2A GCAP-2 mutant in HEK293 cells. HEK293 cells were transformed with an "empty" expression vector only (*lane a*) or with the same vector containing either wild-type GCAP-2 cDNA (*lane b*) or cDNA encoding G2A GCAP-2 mutant (*lane c*). Notice the up-shift of the mutant protein characteristic of nonacylated GCAP-2.

to liver membranes (Fig. 3*B*) makes such a possibility unlikely. To further exclude this possibility, we prepared liposomes from retinal lipids and found that GCAP-2 can associate with these liposomes in a Ca^{2+}-sensitive manner, although both RetGC-1 and RetGC-2 were undetectable in the artificial membranes by immunoblot (Fig. 3*C*). The liposomes used for reconstitution did not even show traces of such an abundant integral membrane protein as rhodopsin. It indicates that GCAP-2 is able to interact not only with the cyclase, but also with the lipid layer itself. We were unable to see GCAP-2 binding to liposomes prepared from commercial crude soybean phospholipids (data not shown). Therefore, it is unlikely that the aggregation of GCAP-2 would account for the observed phenomenon. This result also indicates that lipid composition of vesicles may be important for GCAP-2 association with the membranes.

We found that the overall process of GCAP-2 translocation to the membrane fraction is Ca^{2+}-sensitive; however, it does not require fatty acylation. When myristoylated and nonacylated GCAP-2 were compared for their abilities to associate with membranes, we found that myristoylation of GCAP-2 was not a

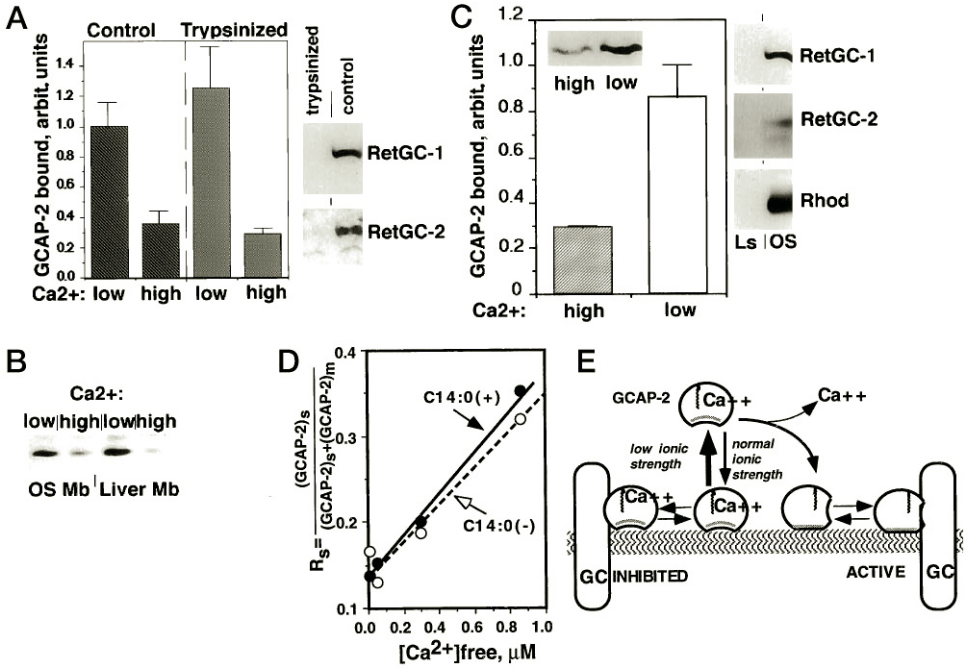

FIG. 3. **Ca^{2+}-sensitive membrane binding of GCAP-2 does not require RetGC or the *N*-myristoyl group.** *A*, *left*, relative efficiency of GCAP-2 binding to untreated and trypsin-treated membranes at low and high free Ca^{2+} concentrations. Washed OS membranes were preincubated without (*control*) or with trypsin (*trypsinized*) at a trypsin:rhodopsin ratio of 1:50 for 30 min at 30 °C followed by the addition of 1 mM PMSF. Both membranes were then washed three times with 10 mM Tris-HCl (pH 7.5) containing 100 μM PMSF and 20 mg/ml leupeptin and aprotinin. Washed membranes were resuspended in same buffer and used for reconstitution with myristoylated recombinant GCAP-2 as described under "Experimental Procedures." The amount of GCAP-2 associated with the membranes was normalized by the level of binding in control membranes at low (15 nM) free Ca^{2+} concentrations. High Ca^{2+} concentration was 10 μM. *Right*, 10-μl aliquots of untreated control and trypsinized membranes were analyzed by 7.5% SDS-PAGE, and the immunoblot was stained with antibodies against RetGC-1 or RetGC-2. The RetGC-1 and RetGC-2 present in control membranes (*control lane*) are destroyed after trypsin treatment (*trypsinized lane*). *B*, GCAP-2 binding to liver membranes. Washed OS and liver membranes prepared as described under "Experimental Procedures," containing equal amounts of total membrane protein, were reconstituted and analyzed by SDS-PAGE, and the immunoblot was stained with ΔNp24 antibody. *C*, *left*, relative efficiency of GCAP-2 binding to liposomes prepared from retinal lipids as described under "Experimental Procedures." The *inset* demonstrates an immunoblot stained with ΔNp24 antibodies. The binding assay was performed as described under "Experimental Procedures" in the presence of 500 μM EGTA (*low* Ca^{2+}) or 250 μM $CaCl_2$ (*high* Ca^{2+}). The immunoblot (*right*) demonstrates that RetGC-1, RetGC-2, and rhodopsin present in OS membranes are not detectable in liposomes (*Ls*) used for GCAP-2 binding. *D*, recombinant myristoylated (●) and nonacylated (○) GCAP-2 were reconstituted with washed OS membranes and assayed for membrane binding as described under "Experimental Procedures." After reconstitution, the relative amount of GCAP-2 released in supernatant (R_s) at different free Ca^{2+} concentrations was determined as described in the legend to Fig. 1*C*. *E*, a schematic of GCAP-2 interaction with membranes. For an explanation, see "Results and Discussion."

necessary prerequisite for its ability to interact with the membranes in a Ca^{2+}-sensitive manner (Fig. 3*D*).

The interaction with the membrane most likely plays an important role in providing compartmentalization of GCAP-2 to the membranes, where it can reach membrane RetGC via lateral diffusion (Fig. 3*E*). GCAP-2 apparently interacts with the cyclase both in low and in high intracellular Ca^{2+} concentrations based on its ability to either inhibit or activate RetGC, respectively (1). Its binding to RetGC is reversible, because GCAP-2 can be washed off the membrane (8, 13). GCAP-2 has the ability to associate with the membrane and to form a complex with the cyclase itself both at high (RetGC inhibition) and at low Ca^{2+} concentrations (RetGC activation). The affinity of GCAP-2 for membranes increases when GCAP-2 is in its "apo" form and decreases upon binding Ca^{2+}. The difference is more apparent at low ionic strength that promotes the dissociation of GCAP-2 from the membrane. However, it is most likely that *in vivo* for both forms of GCAP-2 the distribution between cytosol and membrane must be shifted toward the membrane association, because at normal ionic strength GCAP-2 tends to bind to membranes more efficiently than in hypotonic conditions (Fig. 1*C*). Calcium-myristoyl switch apparently does not play an essential role in regulating Ca^{2+} sensitivity of GCAP-2 compartmentalization to membranes. Nevertheless, because of the complex nature of GCAP-2 interaction with membrane, this does not allow us to conclude that the fatty acylation of GCAP-2 is not essential for its ability to regulate RetGC. That issue requires a direct functional comparison between the fatty acylated and nonacylated forms of GCAP-2 in a RetGC activation assay.

Nonacylated GCAP-2 Regulates RetGCs—We found that the myristoylated and the nonacylated forms of recombinant GCAP-2 are almost equivalent in their abilities to regulate RetGCs in washed OS membranes.

Nonacylated GCAP-2 expressed in *E. coli* (Fig. 4*A*) as well as nonacylated G2A GCAP-2 mutant produced in human cell culture (*insert*) stimulated RetGCs with an efficiency of at least 75% compared with the fatty acylated GCAP-2. Ca^{2+} sensitivities of RetGC regulation by myristoylated and nonacylated GCAP-2 were also very similar to each other (Fig. 4*B*).

We previously demonstrated that inactivation of EF hands made GCAP-2 a stable Ca^{2+}-insensitive activator of RetGC (1). Fig. 5 shows that the ability of Ca^{2+}-insensitive GCAP-2 mutant to become a constitutive activator of RetGC does not require fatty acylation. Myristoylated and nonacylated GCAP-2 ($EF(2/3/4)^{-}$) both stimulate RetGC even at high Ca^{2+} concentrations and with similar EC_{50}, too. The regulatory properties of GCAP-2 include not only the ability of its "apo" form to stimulate RetGCs at low intracellular Ca^{2+} concentrations but also the ability of its Ca^{2+}-loaded form to inhibit activation of RetGCs (1). We therefore tested the potential influence of the fatty acylation on this inhibitory property of GCAP-2. We found that Ca^{2+}-loaded myristoylated and nonacylated forms of GCAP-2 inhibit the stimulation of RetGCs produced by a Ca^{2+}-

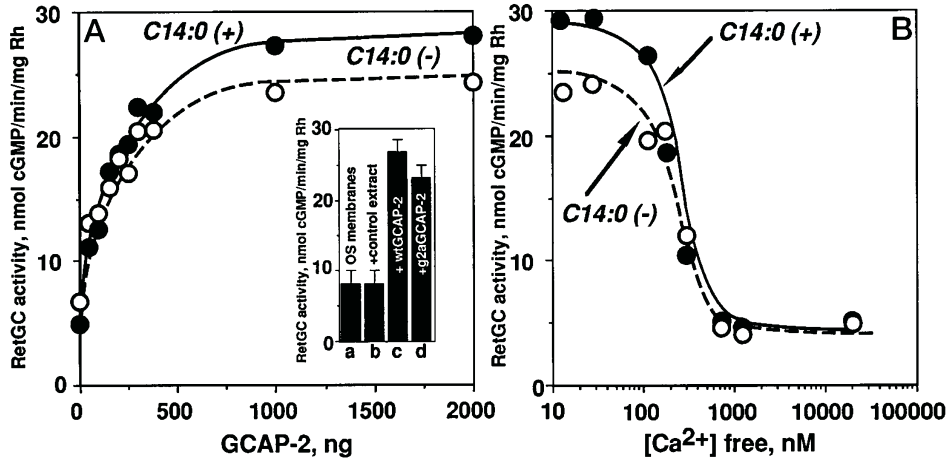

FIG. 4. **Activation of RetGCs by fatty acylated and nonacylated GCAP-2.** *A*, dose dependence of RetGC activation by recombinant GCAP-2 at 15 nM free Ca^{2+}. A RetGC assay mixture contained in 25 μl of washed OS membranes (3 μg of rhodopsin) and increased concentrations of either myristoylated (●) or nonmyristoylated (○) GCAP-2 expressed in *E. coli* as shown in Fig. 2*C*. RetGC activity was measured as described in Refs. 1 and 10. *Inset*, GC activity in washed OS membranes was measured before (*a*) and after (*b–d*) reconstitution with the extract from HEK293 cells not expressing GCAP-2 (*b*) or containing 100 ng of wild-type GCAP-2 (*c*) or Gly2Ala GCAP-2 mutant (*d*) shown in Fig. 1*D*. *B*, Ca^{2+} sensitivity of RetGC activation by myristoylated and nonacylated recombinant GCAP-2 expressed in *E. coli*. The assay contained washed OS membranes and 4 μM GCAP-2, either myristoylated (●) or nonacylated (○) and 2 mM Ca-EGTA giving final free Ca^{2+} concentrations as indicated.

insensitive myristoylated $(EF(2/3/4)^-)$ mutant equally well (Fig. 5*B*). Conversely, myristoylated and nonacylated GCAP-2 $(EF(2/3/4)^-)$ mutants demonstrate similar abilities to outcompete an inhibitory effect of Ca^{2+}-loaded wild-type GCAP-2 (Fig. 5*C*). Therefore, fatty acylation of GCAP-2 is not a critical element for RetGC regulation.

We conclude that myristoylation of GCAP-2 is not essential for its function as a regulator of RetGCs. It has been demonstrated that GCAP-2-related protein, recoverin, undergoes a conformational change called "calcium-myristoyl switch" (reviewed in Ref. 47) in which the fatty acyl group is exposed in response to calcium binding. Such a mechanism increases the affinity of recoverin and recoverin-related proteins for membranes. We find that Ca^{2+} sensitivity of GCAP-2-membrane interaction is opposite to that reported for recoverin and a number of other recoverin-like proteins (34–38). In the case of GCAP-2, we also find that all major aspects of its interaction with photoreceptor membranes and with its target RetGCs are apparently determined almost entirely by Ca^{2+}-induced changes in the protein moiety of the molecule and are not significantly influenced by the myristoyl group. It has been observed (2, 41) that a nonacylated recombinant form of GCAP-1 had much lower (near 40-fold) affinity to RetGCs and lower Ca^{2+} sensitivity compared with fatty acylated GCAP-1 (2). GCAP-1 and GCAP-2 are most closely related to each other, both genetically (48) and functionally (1, 7–11). Given our observations, it is not immediately apparent why such highly homologous proteins as GCAP-1 and GCAP-2 would demonstrate such a difference in terms of their dependence on fatty acylation. The difference in dependence may reflect a subtle difference between GCAP-1 and GCAP-2 in the structures of their activating domains. However, since we demonstrate here that the fatty acyl group itself is not an essential element of RetGC regulation by a calcium-binding protein, it also seems possible that the fatty acylation may merely favor the proper folding of recombinant GCAP-1 rather than its interaction with RetGC.

Also, despite the fact that fatty acylation of GCAP-2 is not essential for RetGC regulation *in vitro*, we cannot exclude the possibility that it might be involved in some intracellular processes such as, for example, translocation of newly synthesized GCAP-2 to the proper cell compartment. Otherwise it would be difficult to explain why the myristoylation signal in GCAP-2

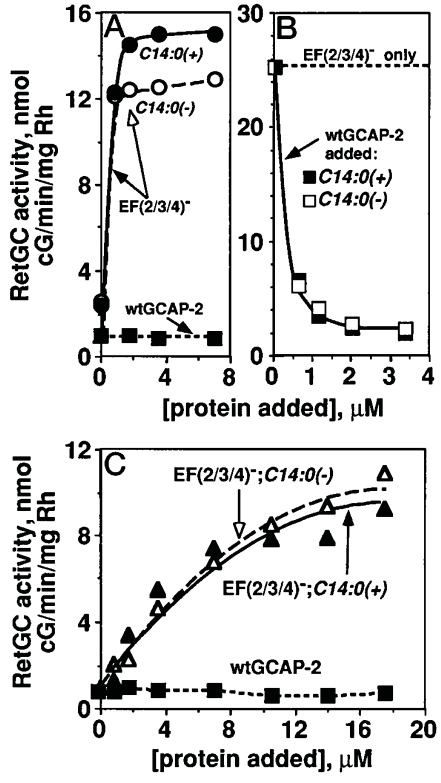

FIG. 5. **Regulation of RetGCs by myristoylated and nonacylated forms of GCAP-2.** *A*, stimulation of RetGC by myristoylated (●) and nonacylated (○) GCAP-2 with inactivated EF hands at 1 μM free Ca^{2+}. Washed OS membranes were reconstituted with increasing concentrations of wild-type (■) or constitutively active (●, ○) GCAP-2 and assayed for RetGCs activity. *B*, the inhibitory effect of Ca^{2+}-loaded myristoylated (■) or nonacylated (□) wild type GCAP-2 on RetGC activation by the Ca^{2+}-insensitive constitutive GCAP-2 mutant. Washed OS membranes were reconstituted with 0.5 μM $EF(2/3/4)^-$ GCAP-2 mutant at 1 μM free Ca^{2+}. Increasing concentrations of wild-type GCAP-2 were then added into the assay mixture, and the activity of RetGC was measured. *C*, competition of myristoylated and nonacylated constitutively active GCAP-2 mutants with a Ca^{2+}-loaded wild-type GCAP-2. Washed OS membranes were first reconstituted with 2 μM myristoylated GCAP-2 at 1 μM free Ca^{2+}. Increasing concentrations of the wild-type (■), or Ca^{2+}-insensitive activator $EF(2/3/4)^-$ GCAP-2 mutant, either myristoylated (▲) or nonacylated (△) were then added into the RetGC assay mixtures.

was not eliminated in the course of evolution of recoverin-like proteins.

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REFERENCES

1. Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* **271,** 19346–19350
2. Frins, S., Bonigk, W., Muller, F., Kellner, R., and Koch, K.-W. (1996) *J. Biol. Chem.* **271,** 8022–8027
3. Baylor, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 560–565
4. Koutalos, Y., and Yau, K.-W. (1996) *Trends. Neurosci.* **19,** 73–81
5. Yarfitz, S., and Hurley, J. B. (1994) *J. Biol. Chem.* **269,** 14329–14332
6. Gray-Keller, M. P., and Detwiler, P. B. (1994) *Neuron* **13,** 849–861
7. Gorczyca, W. A., Gray-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4014–4018
8. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* **12,** 1345–1352
9. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and Baehr, W. (1994) *Neuron* **13,** 395–404
10. Dizhoor, A. M., Olshevskaya, E. V., Henzel, W. J., Wong, S. C., Stults, J. T., Ankoudinova, I., and Hurley, J. B. (1995) *J. Biol. Chem.* **270,** 25200–25206
11. Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995) *J. Biol. Chem.* **270,** 22029–22036
12. Lolley, R. N., and Racz, E. (1982) *Vision Res.* **22,** 1481–1486
13. Koch, K.-W., and Stryer, L. (1988) *Nature* **334,** 64–66
14. Koch, K.-W. (1991) *J. Biol. Chem.* **266,** 8634–8637
15. Hayashi, F., and Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4746–4750
16. Liu, X., Seno, K., Nishizawa, Y., Hayashi, F., Yamazaki, A., Matsumoto, H., Wakabayashi, T., and Usukura, J. (1994) *Exp. Eye Res.* **59,** 761–768
17. Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., and Lowe, D. G. (1992) *Neuron* **9,** 727–737
18. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) *Biochem. J.* **302,** 455–461
19. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, O., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5535–5539
20. Yang, R. B., Foster, D. C., Garbers, D. L., and Fulle, H. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 602–606
21. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol. Chem.* **269,** 30741–30744
22. Yuen, P. S., and Garbers, D. L. (1992) *Annu. Rev. Neurosci.* **15,** 193–225
23. Laura, R. P., Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* **271,** 11646–11651
24. Duda, T., Goraczniak, R., Surgucheva, I., Rudnicka-Nawrot, M., Gorczyca, W. A., Palczewski, K., Sitaramayya, A., Baehr, W., and Sharma, R. K. (1996) *Biochemistry* **35,** 8478–8482
25. Wolbring, G., and Schnetkamp, P. P. (1996) *Biochemistry* **35,** 11013–11018
26. Aparicio, J. G., and Applebury, M. L. (1996) *J. Biol. Chem.* **271,** 27083–27089
27. Sitaramayya, A., Duda, T., and Sharma, R. K. (1995) *Mol. Cell. Biochem.* **148,** 139–145
28. Hallett, M. A., Delaat, J. L., Arikawa, K., Schlamp, C. L., Kong, F. S., and Williams, D. S. (1996) *J. Cell Sci.* **109,** 1803–18012
29. Pozdnyakov, N., Yoshida, A., Cooper, N. G., Margulis, A., Duda, T., Sharma, R. K., and Sitaramayya, A. (1996) *Biochemistry* **34,** 14279–14283
30. Subbaraya, I., Ruiz, C. C., Helekar, B. S., Zhao, X., Gorczyca, W. W., Pettenati, M. J., Rao, P. N., Palczewski, K., and Baehr, W. (1994) *J. Biol. Chem.* **269,** 31080–31089
31. Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E. Zozulya, S., Neubert, T. A., Stryer, L., Hurley, J. B., and Walsh, K. A. (1992) *J. Biol. Chem.* **267,** 16033–16036
32. Neubert, T. A., Johnson, R. S., Hurley, J. B., and Walsh, K. A. (1992) *J. Biol. Chem.* **267,** 18274–18277
33. Johnson, R. S., Ohguro, H., Palczewski, K., Hurley, J. B., Walsh, K. A., and Neubert, T. A. (1994) *J. Biol. Chem.* **269,** 21067–21071
34. Kawamura, S., and Murakami, M. (1991) *Nature* **349,** 420–423
35. Zozulya, S., and Stryer, L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11569–11573
36. Dizhoor, A. M., Chen, C.-K., Olshevskaya, E. V., Sinelnikova, V. V., Phillipov, P., and Hurley, J. B. (1993) *Science* **259,** 829–832
37. Kobayashi, M., Takamatsu, K., Saitoh, S., and Noguchi, T. (1993) *J. Biol. Chem.* **268,** 18898–18904
38. Ladant, D. (1995) *J. Biol. Chem.* **270,** 3179–3185
39. Hughes, R. E, Brzovic, P. S., Klevit, R. E., and Hurley, J. B. (1994) *Biochemistry* **34,** 11410–11416
40. Ames, J. B., Tanaka, T., Ikura, M., and Stryer, L. (1995) *J. Biol. Chem.* **270,** 30909–30913
41. Surgucheva, I., Subbaraya, I., Palczewski, K., and Baehr, W. (1996) *Invest. Ophthalmol. & Visual Sci.* **37,** S215
42. Horton, R. M, and Pease, L. R. (1991) in *Directed mutagenesis: Practical Approach* (McPherson, M. J., ed) pp. 217–250, Oxford University Press, Oxford
43. McDowell, J. H. (1993) *Methods Neurosci.* **15,** 123–130
44. Tsien, R., and Pozzan, T. (1989) *Methods Enzymol.* **172,** 230–262
45. Rocque, W. J., McWherter, C. A., Wood, D. C., and Gordon, J. I. (1993) *J. Biol. Chem.* **268,** 9964–9971
46. Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B., and Stryer, L. (1991) *Science* **251,** 915–918
47. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1996) *Cur. Opin. Struct. Biol.* **6,** 432–438
48. Surguchev, A., Bronson, J. D., Banerjee, P., Knowels, J. A., Ruiz, C., Subbaraya, I., Palczewski, K., and Baehr, W. (1997) *Genomics* **39,** 312–322