Cloning, Sequencing, and Expression of a 24-kDa Ca\textsuperscript{2+}-binding Protein Activating Photoreceptor Guanylyl Cyclase*  

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Two vertebrate photoreceptor-specific membrane guanylyl cyclases, RetGC-1 and RetGC-2, are activated by a soluble 24-kDa retinal protein, p24, in a Ca\textsuperscript{2+}-sensitive manner (Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) Neuron 12, 1345–1352; 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). The primary structure of bovine p24 has been derived from peptide sequencing and from its cDNA. p24 is a new EF-hand-type Ca\textsuperscript{2+}-binding protein, related but not identical to another guanylyl cyclase-activating protein, GCAP (Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekra, B. S., Ruiz, C. C., Ohguro, H. Huang, J., Zhao, Crabb, J. W., J ohnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and Baehr, W. (1994) Neuron 13, 395–404) and other members of the recoverin family of Ca\textsuperscript{2+}-binding proteins. Antibodies against a truncated fusion protein and against a p24-specific synthetic peptide specifically recognize retinal p24 on immunoblots. Both antibodies inhibit activation of photoreceptor membrane guanylyl cyclase by purified p24. p24 is found only in retina, and it copurifies with outer segment membranes. Immunocytochemical analysis shows that it is present in rod photoreceptor cells. An immobilized antibody column was used to purify p24 from a heat-treated retinal extract. Purified p24 appears on SDS-polyacrylamide gel electrophoresis as a homogenous protein not contaminated with GCAP, and it activates photoreceptor guanylyl cyclase in vitro at submicromolar concentrations. Ca\textsuperscript{2+} inhibits this activation with an EC\textsubscript{50} near 200 nM and a Hill coefficient of ~1.7. Recombinant p24 expressed in 293 cells effectively stimulates photoreceptor guanylyl cyclase. These findings demonstrate that p24, like GCAP, imparts Ca\textsuperscript{2+} sensitivity to photoreceptor membrane guanylyl cyclase. We propose that p24 be referred to as GCAP-2 and that GCAP be referred to as GCAP-1.
McDowell and Kuhn (1977) with modifications, p24 was isolated from a crude retinal extract using heat denaturation, phenyl-Sepharose chromatography, preparative polyacrylamide gel electrophoresis (PAGE), and reverse phase HPLC as described (Dizhoor et al., 1994). The only modification was that 5 mM CaCl₂ was added, and NaCl was omitted during homogenization of retinas. Sequencing of p24—Purified p24 had a blocked N terminus, which made it unavailable for direct Edman degradation, so it was cleaved by trypsin and cyanogen bromide (CNBr). Purified p24 was transferred onto Millipore Immobilon-PSQ membranes (Matsudaïra, 1987), reduced and alkylated with isopropylacetamide (Krutzsch and Inman, 1993), followed by digestion in 25 µl of 1 M ammonium bicarbonate, 10% acetonitrile with 0.2 µg of modified trypsin for 12 h (Henzel et al., 1994). The solution was concentrated in a Speed-Vac and injected onto a C18 0.22 × 100 mm capillary column (LC Packings, Inc.). Peptides were eluted using a linear gradient of 0–80% acetonitrile (solution A contained 0.1 aqueous trifluoroacetic acid, and solution B was acetonitrile containing 0.07% trifluoroacetic acid) at a flow rate of 3.5 µl/min and detected by absorbance at 195 nm. Purified p24 was cleaved in 50 µl of 0.1 M HCl at 45°C for 3 h using a single crystal of CNBr. The peptides were electroblotted onto polyvinylidene difluoride membrane after separation in a Tris-tricine SDS gel. Automated protein sequencing was performed on a model 470A Applied Biosystems sequencer equipped with an on-line PTH analyzer using modified cycles (Henzel et al., 1994). Sequence interpretation was performed on DEC Alpha DEC workstation (Digital, 1987). Additional sequencing was performed on a H-Boehringer sequencing machine using mass spectrometry of Lys-C fragments of p24. HPLC-purified peptides were mixed with α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile and 2% trifluoroacetic acid. Mass spectra were obtained with a Vestec (Houston, TX) LaserTec Research laser desorption linear time-of-flight mass spectrometer equipped with a 337 nM VSL-337 ND nitrogen laser (Laser Science, Inc.). The DNA product was inserted into the expression vector pET15b (Novagene) and expressed in an E. coli strain BL21(DE3) to produce a fusion protein containing the N terminus to a 20-arginine His-Tag-containing peptide. Protein induced by isopropyl-1-thio-β-D-galactopyranoside reacted with the antibody P24SVE and demonstrated a Ca²⁺-dependent shift of electrophoretic mobility in SDS-PAGE. This shift was insignificant and could be solubilized in 6 M urea and purified on a Ni²⁺-bound His-bond column (Novagen). All buffers for the purification contained 6 M urea. Purified protein was dialyzed against 20 mM phosphate buffer, pH 7.5, containing 100 mM NaCl. The main part of the protein precipitates during this procedure. Protein was solubilized again at pH 9.5, and more than 50% of it remained soluble after subsequent dialysis at pH 8. Antibody was produced in rabbits and purified on the recombinant Pro²⁰²-Phe²⁰⁴ fragment cross-linked to CNBr-activated Sepharose 4B at pH 8.3. Unreacted protein was removed by extensive washing with 100 mM Tris buffer, pH 10.5. The affinity column was neutralized to pH 8 and used for purification of the antibody. The column was stable at 4°C and efficient for purification of up to 10 mg of antibodies from 30 ml of immune serum. The antibody strongly reacted with p24 on immunoblot and was suitable for immunocytochemical analysis. Only a trace of cross-reactivity of Np24 antibody with recombinant GCAP was found using immunoblot. To compete away this residual cross-reactivity, a soluble recombinant N-truncated fragment Asp¹¹⁰,Gly²⁰⁵ of GCAP was expressed in E. coli as a His-Tag fusion protein using pET15b vector and used as an immunizing antigen as above. Recombinant GCAP and anti-GCAP antibody UW14 were provided by K. Palczewski (University of Washington).

Immunochemistry—Cryosections of paraformaldehyde-fixed bovine retina were a gift from Dr. Ann Milam (University of Washington). Sections were air dried for 40 min, blocked in a solution of 1% horse serum in phosphate-buffered saline containing 0.1% Triton X-100 for 1 h, incubated overnight at 4°C with primary Np24 antibody (0.25 µg/ml), washed with phosphate-buffered saline 2 × 15’, incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Cappel) for 1 h at room temperature, and washed with phosphate-buffered saline and photographed using a Nikon fluorescence microscope (objective ×20 or ×40). The antibody was removed by a 30 min at room temperature with 2 µM recombinant fragment Pro²⁰²-Phe²⁰⁴ of p24 or recombinant recoverin or 4 µM N-truncated recombinant GCAP. Both preincubated and non-preincubated antibody solutions were centrifuged for 10 min at 10,000 × g, 4°C, prior to using them on retinal sections.

Immunoblot—After electrophoresis in 15% SDS-PAGE, proteins were transferred onto nitrocellulose membrane and probed with anti-p24 antibodies. Bovine neurocalcin, recoverin, and hippocalin were expressed in E. coli as described in (Teng et al., 1994). The blot was developed using alkaline phosphatase-conjugated secondary antibody with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-indolyl-phosphate as a substrate.

Guanylyl cyclase was assayed under native conditions using washed OS membranes and analyzed by TLC (Dizhoor et al., 1994). Bovine rod outer segments were washed in the dark three times in 5 mM Tris-Cl buffer (pH 7.5) containing 1 mM MgCl₂, 0.05 mM CaCl₂, 10 mM 2-mercaptoethanol, 0.05 mM phenylmethylsulfonyl fluoride, and 5% (v/v) glycerol. Membranes were pelleted at 30,000 × g for 30 min, aliquoted, and frozen at -70°C. Before the analysis, 40 µl aliquots of washed membranes were thawed on ice and resuspended in 600 µl of 2 × buffer (100 mM MOPS-KOH, pH 7.5, 16 mM NaCl, 200 mM KCl, 20 mM MgCl₂, 14 mM 2-mercaptoethanol, and 10 mM dipyridamol). Reaction mixture contained 12.5 µl of resuspended membranes (∼7 µg of rhodopsin) in the final volume of 25 µl. To start the reaction, 5 µl of substrate solution containing 5 mM GTP, −1 µl Ci of [α-³²P]GTP (Amersham) was added. This solution also contained 20 mM cGMP, ∼100,000 dpm of [³²P]-cGMP (Amersham), and 0.5 mM ATP. Each reaction mixture was incubated in a closed Eppendorf tube for 10 min at 30°C and then heated for 2 min at 100°C to stop the reaction. The reaction tubes were centrifuged at 10,000 × g for 10 min (Sorvall SS-34 rotor, 2°C). The pellets were re-suspended in 20 µl of either 10 mM NaCl, 50 µM D-galactopyranoside, and 0.5 mM dipyridamol, or 30°C. 0.5 µl of each sample was loaded onto a 10% SDS-PAGE gel, and the gel was fixed and transferred onto nitrocellulose membrane and probed with anti-p24 antibodies. The antibody strongly reacted with p24 on immunoblot and was suitable for immunocytochemical analysis. Only a trace of cross-reactivity of Np24 antibody with recombinant GCAP was found using immunoblot. To compete away this residual cross-reactivity, a soluble recombinant N-truncated fragment Asp¹¹⁰,Gly²⁰⁵ of GCAP was expressed in E. coli as a His-Tag fusion protein using pET15b vector and used as an immunizing antigen as above.

Recombinant GCAP and anti-GCAP antibody UW14 were provided by K. Palczewski (University of Washington).
Fig. 1. A, primary structure of a novel 24-kDa Ca2+ -binding protein. Peptides of p24 purified from retina were generated by cyanogen bromide cleavage (CNBr14kd, CNBr20kd, and CNBrCaBP) and tryptic digest (T7, T12, T21, T23) and sequenced by Edman degradation. The sequence between Glu26 and Phe204 was found in cDNA clone 9–3 from a bovine retinal cDNA library. Region M1-F41 was encoded by a cDNA product of 5'-rapid amplification of cDNA ends (see "Material and Methods" for the details). B, p24 belongs to the family of recoverin-like proteins. The sequence of p24 was aligned to sequences of GCAP (Palczewski et al., 1994), bovine neurocalcin (Okazaki et al., 1992), rat hippocalcin (Kobayashi et al., 1992), and recoverin (Dizhoor et al., 1991; Hurley et al., 1993). Amino acids identical to p24 are shadowed.
and purified using QIAGEN plasmid DNA purification columns. HEK293 cells were transfected using calcium-phosphate-precipitated DNA. 10 μg of DNA was used to transfet cell cultures at 50% confluence. After the transfection, cells were grown for 72 h until almost confluent. Control cells were treated simultaneously without adding the expression DNA construct. Before harvesting, the cells were briefly washed with an extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 10 mM β-mercaptoethanol), mechanically removed from the plate, and homogenized in 2 ml of the extraction buffer using a Dounce homogenizer. The homogenate was centrifuged at 80,000 rpm in a Beckman TLA100.3 rotor for 10 min. Equal volume aliquots of soluble and mem- brane fractions were loaded onto 15% SDS-PAGE, electroblotted onto nitrocellulose membrane, and probed with antibodies to proteins (Johnson et al., 1992) and other photoreceptor

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**FIG. 2. Antibodies against p24.** A, reactivity with related Ca2+-binding proteins. Ca2+-binding proteins were transferred from SDS-PAGE onto nitrocellulose membrane and probed with Np24 antibodies (0.25 μg/ml). Upper panel: a, recombinant rat hippocalcin (0.1 μg); b, recombinant bovine neurocalcin (0.1 μg); c, recombinant bovine recoverin (1 μg); d, purified retinal p24 (0.1 μg). B and C, specificity of anti-p24 antibodies for p24 compared to GCAP. B, antibody P24SVE (0.5 μg/ml) was used to stain an immunoblot containing 0.5 μg of recombinant GCAP (left) or p24 (right). C, Np24 antibody (0.25 μg/ml) was used to stain an immunoblot containing 0.5 μg of recombinant GCAP (a, c, e) or affinity-purified p24 (b, d, f). The Np24 antibody was also preincubated for 10 min with 2 μM of N-truncated recombinant p24 (c, d) or 4 μM of N-truncated recombinant GCAP (e, f).

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**Fig. 3. Tissue specificity of p24.** Homogenates of different bovine tissues were subjected to electrophoreses in SDS-PAGE, transferred onto nitrocellulose membrane, and probed with Np24 antibodies. Approximately 20 μg of total protein in extracts from heart (a), adrenal (b), kidney (c), lung (d), liver (e), brain (f), retina (g), and outer segments fraction (h). No signal was found in the spleen (not shown). Essentially the same result was obtained with P24SVE anti-peptide antibody (not shown).

Primary Structure of p24 (GCAP-2)—We determined the primary structure of p24 by Edman degradation of peptides cleaved from purified p24 (Dizhoor et al., 1994) and by sequence analysis of p24 cDNA (Fig. 1A). p24 is a novel Ca2+-binding protein most closely related to a family of recoverin-like pro- teins within the EF-hand protein superfamily. Fig. 1B demonstrates the close similarity between several recoverin-like proteins. p24 has four EF-hand-like motifs, but the first two align poorly with the consensus motif for EF-hands. Only EF-hands 3 and 4 are likely to bind Ca2++. A consensus glycine is substituted with asparagine in the second potential EF-hand, and two oxygen-containing amino acid residues are missing from the first EF-hand-related motif.

p24 is 41, 38, and 29% identical to bovine GCAP, neurocalcin, and recoverin, respectively. Like other members of this family, p24 has a consensus sequence for N-terminal myristoylation. It has yet to be determined if it is heterogeneously fatty acylated like recoverin (Dizhoor et al., 1992) and other photoreceptor proteins (Johnson et al., 1994).

**Tissue Specificity of p24—**We produced two types of antibodies that specifically recognize p24. The first, Np24, was raised against a large 152-amino acid fragment Pro26-Phe204 expressed in E. coli as a His-Tag fusion protein. The second antibody, P24SVE, was generated against a synthetic peptide, Cys13-Leu155, highly specific for p24 and significantly different from the corresponding region in GCAP (Fig. 1B). Both antibodies were raised in rabbits and affinity purified. They reacted with PPLC-purified p24 and did not react with recom- binant bovine recoverin, neurocalcin, or hippocalcin (Fig. 2A). Special attention was paid to the possibility of cross-reactivity with GCAP. P24SVE does not react with recombinant GCAP on immunoblots (Fig. 2B). Only very weak cross-reactivity with GCAP was detected with the Np24 antibody on immunoblots. This was completely suppressed by pre-adsorption of Np24 antibody with a recombinant fragment (Asp110-Gly205) of GCAP (Fig. 2C).

Immunoblot analysis using both antibodies showed p24 immunoactivity only in an extract from retina (Fig. 3) and not in extracts from kidney, liver, adrenal, lung, spleen, heart, or brain. This is consistent with our previous finding that GC-stimulating activity was not detected in those tissues (Dizhoor et al., 1994).

Immunolocalization of p24—We originally isolated p24 from
a crude retinal extract (Dizhoor et al., 1994). However, immuno- blot analysis demonstrates that p24 is also present in a preparation highly enriched in OS (Fig. 3). This suggests that a substantial fraction of this protein copurifies with OS. Direct immunocytochemical localization of p24 was done using affinity-purified antibody ΔNp24, which specifically recognized outer and inner segments of photoreceptors on cryosections of fixed bovine retina. A weak staining of synaptic termini of photoreceptors was also detected (Fig. 4A). Similar results were obtained with fixed paraffin sections of retina (not shown). Preincubation of the antibody with recombinant fragment of p24 eliminated the signal from the photoreceptor cells (Fig. 4A). Similar results were obtained with fixed paraffin sections of retina (not shown). Preincubation of the antibody with recombinant fragment of p24 eliminated the signal from the photoreceptor cells (Fig. 4A). Preincubating the antibody with a recombinant N-truncated fragment of GCAP did not block the signal in the photoreceptors (Fig. 4D). Neither full-length recombinant GCAP nor recombinant competed with the specific signal from photoreceptors (data not shown). Cones did not appear to stain with the antibody (Fig. 4A, B, C, and D). The most intense signal was detected in the outer segments and in the upper part of the inner segments of rods. Although this suggests that p24 is a rod-specific protein, the possibility that the epitope for antibody recognition is masked in cone cells cannot be excluded. Further studies using additional antibodies will be required to determine the exact localization of p24 in photoreceptor cells.

Antibodies Against p24 Inhibit Activation of GC—A crude heat-treated retinal extract containing p24 can be completely depleted of GC-stimulating activity by passing it through a column of immobilized affinity-purified antibody ΔNp24 (Fig. 5A). The activator retained on the column can be eluted at low pH as an apparently homogeneous 24-kDa protein (Fig. 5B). In our SDS gel system, p24 and GCAP have almost identical electrophoretic mobility. Therefore, we used antibodies against GCAP to examine our preparations for the presence of GCAP (Fig. 5C). A highly specific antibody against GCAP, UW14, Strongly recognizes recombinant GCAP on an immunoblot (right lane). This preparation of p24 activates GC in washed OS membranes (Fig. 4D) with the same Ca2+-sensitivity (EC50 = 200 nM) and cooperativity (Hill coefficient, 1.7) as HPLC-purified p24 (Dizhoor et al., 1994). Both anti-ΔNp24 and anti-p24VE antibody efficiently inhibit activation of GC by purified p24 in vitro (Fig. 6). Since p24VE antibody does not cross-react with GCAP (Fig. 2C), we can say with certainty that GC-stimulating activity in purified p24 preparations derives from p24 itself. This conclusion is consistent with the fact that GC-stimulating activity copurifies with p24 through several purification steps (Dizhoor et al., 1994; Lowe et al., 1995). Based on these results, p24 is a Ca2+-sensitive regulator of photoreceptor GC distinct from GCAP.

Recombinant p24 Stimulates Activation of GC in Vitro—Several attempts to express full-length p24 in E. coli gave us only insoluble protein (data not shown). However, we successfully expressed recombinant p24 in HEK293 cells. Fig. 7A demonstrates that p24 is expressed in p24 cDNA-transfected cells as a soluble protein. Only a small amount of it was found in the particulate fraction of homogenized cells. We compared extracts from p24 cDNA-transfected and control, mock-transfected cells for their ability to stimulate GC. As shown in Fig. 7B, only the extract containing recombinant p24 effectively activates GC in washed OS membranes.

**DISCUSSION**

The data presented in this paper demonstrate that p24 is a Ca2+-binding protein that regulates photoreceptor membrane...
GC. It is present in the outer and inner segments of photoreceptor cells and, when expressed as recombinant protein in 293 cells, effectively stimulates photoreceptor GC in vitro.

Based on its primary structure, p24 is closely related to another recently identified GC activator referred to as GCAP (Gorczyca et al., 1994; Palczewski et al., 1994). p24 and GCAP are both members of the recoverin family of EF-hand proteins, but they are structurally and functionally more similar to each other than to other members of the recoverin family. Therefore, we propose that GCAP be referred to as GCAP-1 and p24 as GCAP-2. It has been proposed that the very N-terminal domain of GCAP-1 participates in activation of GC (Palczewski et al., 1994). However, GCAP-2 is clearly distinct from GCAP-1 within the first 20 amino acid residues (Fig. 1B). This implies that a domain other than the very N terminus is involved in GC activation. GCAP-1 and GCAP-2 are most similar within the Ca$^{2+}$-binding domains, es-
Established. Two Ca\textsuperscript{2+} ions in the EF-3 and EF-4 sites. Amino acid residues in EF-1 and EF-2 of GCAP-2 do not match well to the EF-hand consensus sequence. In EF-1, two oxygen-containing residues (positions 35 and 41) of the consensus are substituted with Cys and Phe, respectively. EF-2 has oxygen-containing residues (positions 35 and 41) of the consensus. Amino acid residues in EF-1 and EF-2 of GCAP-2 do not bind Ca\textsuperscript{2+} with the same affinity as in EF-3 and EF-4.

Heterogeneous fatty acylation at their N-termini (Dizhoor et al., 1992) plays an important role in the structure of recoverin referred to as the "calcium-myristoyl switch" (Zozulya and Stryer, 1992; Dizhoor et al., 1993; Ames et al., 1995). Other proteins from retina that have this motif are also heterogeneously acylated with C14 and C12 saturated and non-saturated fatty acids (Johnson et al., 1994). A mass spectrometry analysis is being done to verify if GCAP-2 is also heterogeneously acylated.

At the N terminus of GCAP-2, there is a motif known to be recognized by N-myristoyl transferase. Two photoreceptor Ca\textsuperscript{2+}-binding proteins, recoverin and GCAP-1, are known to be heterogeneously fatty acylated at their N termini (Dizhoor et al., 1992; Palczewski et al., 1994). This fatty acylation plays an important role in the structure of recoverin referred to as the "calcium-myristoyl switch" (Zozulya and Stryer, 1992; Dizhoor et al., 1993; Ames et al., 1995). Other proteins from retina that have this motif are also heterogeneously acylated with C14 and C12 saturated and non-saturated fatty acids (Johnson et al., 1994). A mass spectrometry analysis is being done to verify if GCAP-2 is also heterogeneously acylated.

Both GCAP-1 and GCAP-2 are present in photoreceptor cells, but their precise intracellular localization has not yet been established. Two Ca\textsuperscript{2+}-sensitive membrane cyclases, RetGC-1 and RetGC-2, are also present in photoreceptor cells (Dizhoor et al., 1994; Lowe et al., 1995). RetGC-1 is present in outer segments and inner segment and appears more abundant in cones than in rods (Dizhoor et al., 1994; Liu et al., 1994). RetGC-2 protein in photoreceptor cells has not been localized. GCAP-2 appears to be more abundant in rods, whereas GCAP-1 immunoreactivity is both in rods and cones. Further studies are required to establish the relationship between RetGC-1 and -2 and GCAP-1 and -2.

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2 K. Palczewski, personal communication.

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