NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM $MgCl_2$, 1 0 mM dithiothreitol, 1 0 mM sodium adenosine triphosphate, 1 0 μM guanosine triphosphate, 0 05% sodium cholate, and citralva at appropriate concentrations (pH 7 4) The free Ca^{2+} in this solution was 45 nM as measured by a Ca^{2+} electrode (Orion, Boston, MA) The third syringe contained 13 5% trichloroacetic acid A time course with intervals ranging from 20 ms to 500 ms was used, and the quenched samples were collected on ice and spun 10 min in a microfuge at 4°C The supernatant was collected, and the cAMP was assayed with the Amersham ^{125}I-labeled cAMP assay system The protein concentration in the first syringe averaged 150 μg per milliliter of cilia buffer, as determined by the method of Lowry [O H Lowry, N J Rosebrough, A L Farr, R J Randall, *J Biol. Chem* 2, 265 (1951)]

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Role of the Acylated Amino Terminus of Recoverin in Ca^{2+}-Dependent Membrane Interaction

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Recoverin, a calcium ion (Ca^{2+})–binding protein of vertebrate photoreceptors, binds to photoreceptor membranes when the Ca^{2+} concentration is greater than 1 micromolar. This interaction requires a fatty acyl residue covalently linked to the recoverin amino (NH_2)-terminus. Removal of the acyl residue, either by proteolytic cleavage of the NH_2-terminus or by production of nonacylated recoverin, prevented recoverin from binding to membranes. The acylated recoverin NH_2-terminus could be cleaved by trypsin only when Ca^{2+} was bound to recoverin. These results suggest that the hydrophobic NH_2-terminus is constrained in Ca^{2+}-free recoverin and liberated by Ca^{2+} binding. The hydrophobic acyl moiety of recoverin may interact with the membrane only when recoverin binds Ca^{2+}.

A variety of proteins with diverse biological functions have their NH_2-termini linked to short-chain fatty acids such as myristic acid (*1–11*). Membrane association of $p60^{v\text{-}src}$, which is necessary for $p60^{v\text{-}src}$ oncogenic activity (*2*) and for its interaction with a plasma membrane receptor (*3*), requires that its NH_2-terminus be myristoylated. Intracellular membrane transport may be mediated through a guanosine triphosphate (GTP)– and myristic acid–dependent interaction of adenosine diphosphate (ADP) ribosylation factor (ARF) with membranes (*4*). The NH_2-termini of two vertebrate photoreceptor proteins, transducin and recoverin, are heterogeneously acylated at their NH_2-termini with one of four types of fatty acyl residues, including myristoyl (C14:0, a fatty acyl residue with 14 carbons and no double bonds), C14:1, C14:2, and C12:0 moieties (*9–11*).

Recoverin is a 23-kD Ca^{2+}-binding protein that is soluble and may influence the recovery phase that follows photoexcitation of vertebrate photoreceptors (*12, 13*). During photoexcitation, guanosine 3′,5′-monophosphate (cGMP) is hydrolyzed by means of a guanine nucleotide–binding protein (G protein)–mediated cascade of reactions (*14, 15*). Because Ca^{2+} enters photoreceptors primarily through cGMP-gated channels, cGMP hydrolysis disrupts Ca^{2+} influx. The consequent net loss of intracellular Ca^{2+} by means of a plasma membrane Na^+-Ca^{2+} exchanger stimulates resynthesis of cGMP by activating a Ca^{2+}-sensitive guanylate cyclase. Preparations of recoverin have been reported to activate this guanylate cyclase in vitro only when the free concentration of Ca^{2+} is below ~400 nM (*12, 13*). The mechanism by which recoverin participated in guanylate cyclase regulation in those experiments remains unknown. Another possible role of recoverin may be to regulate the rate of inactivation of photoreceptor cGMP phosphodiesterase (*16*).

We investigated the possible role of NH_2-terminal acylation in recoverin function. NH_2-terminal fatty acyl residues may enhance protein-protein (*11*) or protein-membrane interactions (*17*). Alternatively, they may function as covalently bound allosteric regulators of enzyme activity. The studies described here show that the NH_2-terminal fatty acyl residue of recoverin participates in Ca^{2+}-dependent membrane interaction.

Ca^{2+}-dependent interaction of recoverin with rod outer segment (ROS) membranes was initially observed in an experiment designed to identify Ca^{2+}-dependent membrane-binding proteins from bovine photoreceptors. We washed photoreceptor membranes several times with buffer containing Ca^{2+} (0.5 mM) to remove proteins that were loosely bound in the presence of Ca^{2+}. Subsequent washes with buffer containing EGTA (1 mM) continued to elute loosely bound proteins. However, they also specifically eluted six additional proteins that had resisted elution in the presence of Ca^{2+} (Fig. 1). One was a 26-kD protein that reacts with affinity-purified antibody to bovine recoverin (*12*). The remaining five proteins of 20, 21, 31, 70, and 80 kD have not yet been identified.

Ca^{2+}-dependent binding of recoverin to membranes was enhanced by high concentrations of protein and membrane. Most of the recoverin in ROS membranes could be eluted even without EGTA if highly diluted membranes were washed many times (*18*). We used this method to strip ROS membranes of endogenous recoverin for the studies described here. Others have also observed Ca^{2+}-dependent membrane binding of recoverin (*19*) and of S-modulin (*16, 20*), a frog photoreceptor protein similar to bovine recoverin.

We characterized the interaction of recoverin with the membrane by reconstituting purified bovine retinal recoverin with photoreceptor membranes stripped of endogenous recoverin. The stripped membranes bound recoverin in the presence of Ca^{2+} but not in its absence (Fig. 2A). Recoverin binding to the membranes was saturated in these experiments at ~40% of the total recoverin as the concentration of Ca^{2+} was raised to 10 μM (Fig. 2B). The partial binding of retinal recoverin to membranes appears to reflect a weak affinity for membranes, rather than an effect of acyl group heterogeneity. Myristoylated recoverin binds to membranes with approximately the same efficiency as heterogeneously acylated retinal recoverin (Fig. 3A).

Ca^{2+}-dependent binding of recoverin to membranes does not appear to be a general divalent cation effect because the Mg^{2+} (5 mM) present in all of these assays did not promote binding. Experiments suggest that recoverin may bind to the phospholipid component of the membranes because Ca^{2+} also promotes recoverin binding to liposomes made from a 1:1 mixture of phosphatidylserine and phosphatidylcholine (*18, 19, 21*). However, our experiments do not rule out the possibility that recoverin also interacts with a protein in ROS membranes.

To determine if the NH_2-terminal acyl residue participates in the interaction of recoverin with ROS membranes, we measured membrane binding of acylated and nonacylated recoverin produced in *Esche-*

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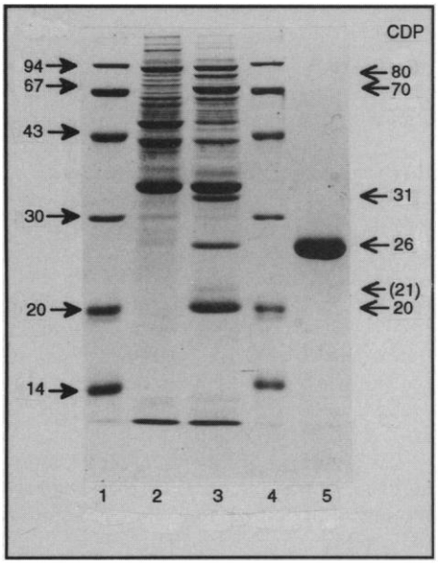
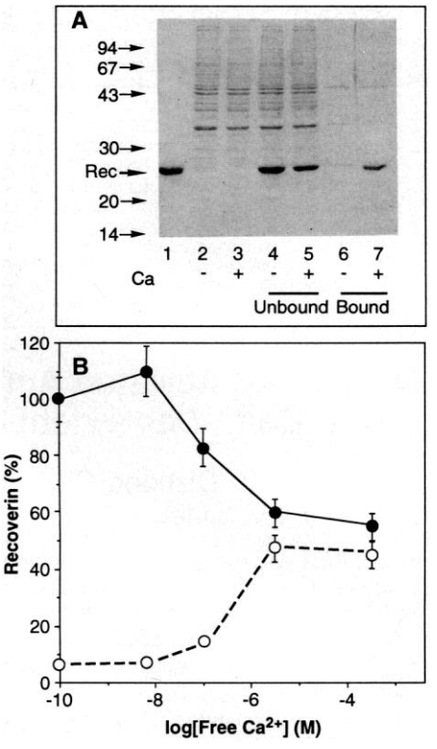


Fig. 1. Ca^{2+}-dependent binding of proteins to ROS membranes. We extracted Ca^{2+}-dependent membrane-binding proteins (CDPs) from ROS by first washing the membranes seven times with buffers (*30*) containing 0.5 mM Ca^{2+} and then washing with buffer containing 2 mM EGTA. The eluted proteins were concentrated and then analyzed by SDS-PAGE and Coomassie blue staining. Lane 1, molecular size markers are indicated at left (in kilodaltons); lane 2, 20 μg of protein from the final Ca^{2+} wash; lane 3, protein (20 μg) from the EGTA wash; lane 4, as in lane 1; and lane 5, purified recoverin (10 μg) (*31*). The apparent molecular sizes of the CDPs are shown on the right. The 21-kD protein appears to be less abundant than the others.

Fig. 2. Ca^{2+}-dependent binding of purified recoverin (Rec) to ROS membranes. (**A**) SDS-PAGE analysis of binding. Purified retinal recoverin (lane 1) and recoverin-depleted ROS membranes (*32*) (lanes 2 and 3) were mixed and incubated (*33*) (lanes 4 through 7). Proteins were incubated in the presence of 2 mM EGTA (lanes 2, 4, and 6) or 1 mM $CaCl_2$ (lanes 3, 5, and 7). Membranes were separated by centrifugation and portions of the supernatants were analyzed (lanes 2 through 5). Lanes 2 and 3 show low amounts of proteins that elute from the membranes without added purified recoverin. Membrane-bound proteins were eluted with EGTA and analyzed (lanes 6 and 7). Molecular size standards are shown at left (in kilodaltons). (**B**) Ca^{2+}-dependence of purified recoverin binding to stripped ROS membranes. The distribution of recoverin between supernatant (closed circles) and membranes (open circles) was determined as described (*33*). The values shown are the means ± SD of two measurements of the percentage of total recoverin.



richia coli (*22*). Both recombinant myristoylated and nonacylated recoverin were purified for these experiments by phenyl-Sepharose chromatography (*23*). In the presence of 10 μM Ca^{2+}, myristoylated recombinant recoverin bound to ROS membranes as efficiently as retinal recoverin did, but nonacylated recombinant recoverin bound only weakly to the membranes (Fig. 3A).

NH_2-terminal acylation influences recoverin structure. Proteolysis studies in the presence of EGTA (Fig. 3B) revealed that nonacylated, Ca^{2+}-free recoverin (lanes 7 and 8) is rapidly degraded by trypsin. NH_2-terminal acylation of recoverin (lanes 5 and 6) makes Ca^{2+}-free recoverin more resistant to trypsin, presumably by stabilizing recoverin structure. In the presence of Ca^{2+}, acylated and nonacylated forms of recoverin are equally resistant to trypsin (lanes 1 through 4).

The observation that the hydrophobic acyl residue promotes the membrane binding of Ca^{2+}-acylated recoverin but not the binding of Ca^{2+}-free acylated recoverin (Figs. 2 and 3) suggested that the acylated recoverin NH_2-terminus might be accessible for membrane interaction only when Ca^{2+} is bound. In Ca^{2+}-free acylated recoverin, the NH_2-terminus might be constrained within the protein and inaccessible for membrane interactions. To estimate the accessibility of the acylated NH_2-terminus, we tested the susceptibility of the NH_2-terminus of myristoylated recoverin to digestion with trypsin in the presence and absence of Ca^{2+}. This experiment was performed with recombinant recoverin labeled at its NH_2-terminus with [^{3}H]myristic acid. Incorporation of [^{3}H]myristic acid into recoverin was dependent on coexpression with *N*-myristoyl transferase (NMT) (Fig. 4B), suggesting that the label is present only at the recoverin NH_2 terminus. Polyacrylamide gel electrophoresis (PAGE) followed by fluorography revealed that the recoverin NH_2-terminus is susceptible to tryptic cleavage only in the presence of >1 μM Ca^{2+} [Fig. 4A, concentration effect (*18*)]. The observation that all radioactivity was cleaved from Ca^{2+}-recoverin, together with mass spectrometry analysis, confirmed that the radioactive label in the protein was only in the NH_2-terminal [^{3}H]myristoyl residue.

Trypsin removes both the NH_2- and COOH-termini from a large core fragment of Ca^{2+}-recoverin by cleaving after lysines 5 and 194 (*9*). We measured the abilities of the [^{3}H]myristoylated NH_2-terminal peptide and of the recoverin core fragment to bind to membranes. Intact [^{3}H]myristoyl recoverin bound to membranes in the presence of Ca^{2+} (Fig. 4C, lanes 1 and 2), whereas the core polypeptide did not bind to the membranes (Fig. 4C, lanes 3 and 4).

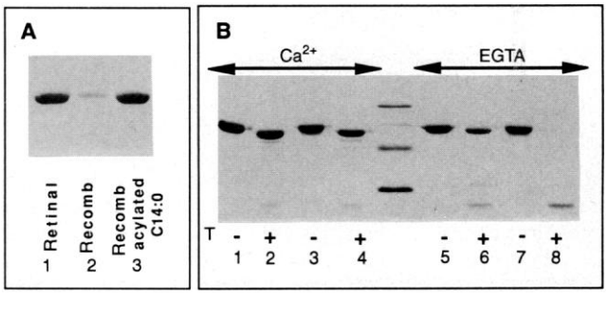


Fig. 3. Effect of NH_2-terminal fatty acylation on Ca^{2+}-dependent binding of recoverin to membranes and on the stability of Ca^{2+}-free recoverin. (**A**) Coomassie-stained gel showing the amounts of retinal recoverin (lane 1), recombinant (Recomb) nonmyristoylated recoverin (lane 2), and recombinant myristoylated recoverin (lane 3) that bound to recoverin-depleted ROS membranes in the presence of 100 μM $CaCl_2$. EGTA eluates of the membranes to which recoverin had been bound in the presence of Ca^{2+} are shown (*33*). (**B**) Myristoylated (lanes 1, 2, 5, and 6) or nonmyristoylated (lanes 3, 4, 7, and 8) recoverin was incubated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of trypsin (T) (*29*) in either 5 mM $CaCl_2$ (lanes 1 through 4) or 5 mM EGTA (lanes 5 through 8). Products of these digests separated by SDS-PAGE are shown. Molecular size standards in the middle (from top to bottom) are 31, 21, and 14 kD.

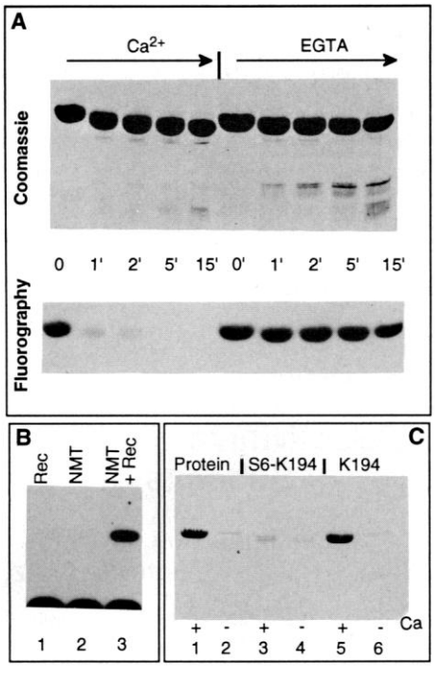


Fig. 4. Effect of Ca^{2+} on trypsin digestion of the recoverin NH_2-terminus. (**A**) Products of limited tryptic digestion of recoverin acylated at its NH_2-terminus with a [^{3}H]myristoyl residue (*34*). Recoverin was incubated with trypsin (*29*) either in the presence of $CaCl_2$ (100 μM) or EGTA (5 mM). Samples were taken at the indicated times. Proteins were separated by SDS-PAGE and analyzed by Coomassie staining and fluorography. Electrospray mass spectrometry (*9*) was used to verify that recoverin cleaved in the presence of Ca^{2+} lacked both the NH_2-terminal myristoyl-Gly-Asn-Ser-Lys acyl peptide and the COOH-terminal Lys^{194}-Leu^{202} peptide. Recoverin cleaved in the presence of EGTA lacked only the COOH-terminal peptide. (**B**) [^{3}H]Myristic acid was incorporated into recoverin during expression in *E. coli* (*22*) and portions of the crude *E. coli* lysates (20 μg of protein) were analyzed by SDS-PAGE followed by fluorography. No ^{3}H was incorporated into recoverin when only recoverin (lane 1) or yeast NMT (lane 2) were expressed. However, when they were coexpressed, [^{3}H]myristic acid was incorporated into recoverin. The dark band at the bottom of each lane is formed by unincorporated [^{3}H]myristic acid label. (**C**) Ca^{2+}-dependent binding of tryptic fragments of recoverin to stripped ROS membranes. Intact recombinant myristoylated recoverin (lanes 1 and 2) and fragments truncated either at both the NH_2- and COOH-termini (lanes 3 and 4; Ser^6-Lys^{194}) or only at the COOH-terminus (lanes 5 and 6; cut after Lys^{194}) were incubated with stripped ROS membranes in the presence of 0.5 mM $CaCl_2$ (lanes 1, 3, and 5) or 5 mM EGTA (lanes 2, 4, and 6). The membrane-bound proteins were eluted with EGTA, separated by SDS-PAGE, and detected by Coomassie staining.

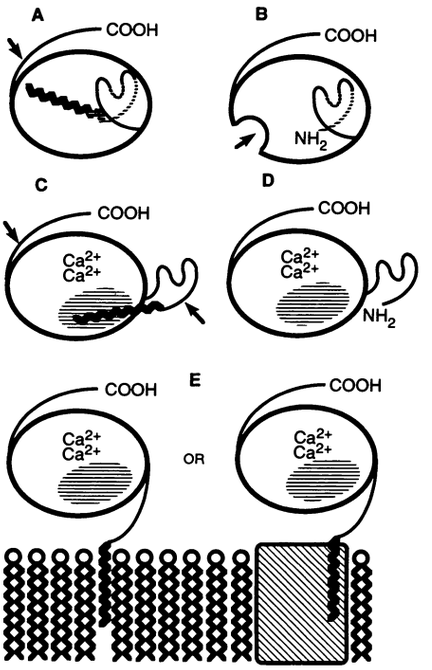


Fig. 5. Model for the fatty acid and Ca^{2+}-dependent interaction of recoverin with membranes. The striped oval area indicates the proposed hydrophobic patch. Arrows indicate areas susceptible to trypsin digestion. The thick zig-zag line represents a myristoyl residue. (**A**) Myristoylated recoverin without Ca^{2+}. (**B**) Nonacylated recoverin without Ca^{2+}. (**C**) Myristoylated recoverin with Ca^{2+}. (**D**) Nonacylated recoverin with Ca^{2+}. (**E**) Myristoylated recoverin with Ca^{2+} interacting with the lipid bilayer (at left) or a protein (at right).

We also observed that nearly all of the [^{3}H]myristoyl NH_2-terminal peptide released from recoverin by proteolysis partitioned into the membranes (*24*).

These results demonstrate that a fatty acyl residue covalently linked to the recoverin NH_2-terminus is essential for Ca^{2+}-dependent membrane binding and that the acyl peptide interacts with membranes. To ensure that proteolytic removal of the COOH-terminus did not influence these experiments, we removed only the COOH-terminus by cleaving recoverin with trypsin in the presence of EGTA. Electrospray mass spectrometry analysis (9) confirmed that the recoverin NH_2-terminus is intact in these preparations, whereas the COOH-terminus is truncated at Lys^{194}. The truncated recoverin with its intact myristoylated NH_2-terminus bound efficiently to membranes (Fig. 4C).

The fatty acyl residue at the recoverin NH_2-terminus appears to function in a Ca^{2+}-dependent association of recoverin with ROS membranes (Fig. 5). In Ca^{2+}-free recoverin, the acylated NH_2-terminus is constrained. The attached fatty acyl residue may be accommodated within a site in the protein that may help protect Ca^{2+}-free acylated recoverin from trypsin (Fig. 5A). When acylated or nonacylated recoverin binds Ca^{2+}, a change in conformation may expose a hydrophobic patch (Fig. 5, hatched regions). Such a hydrophobic surface exists in a related Ca^{2+}-binding protein, calmodulin (*25*), and would account for the observed (*22*) interaction of both acylated and nonacylated recoverin with phenyl-Sepharose. In the absence of membranes, the hydrophobic NH_2-terminal fatty acyl residue may bind to this surface (Fig. 5C). However, in the presence of ROS membranes, the fatty acyl residue could also partition into the more hydrophobic interior of the phospholipid bilayer or interact with a protein to hold recoverin to the membrane (Fig. 5E). Recoverin lacking the NH_2-terminal fatty acyl residue does not bind to membranes (Fig. 5D).

These findings identify two factors, bound Ca^{2+} and an NH_2-terminal fatty acyl residue, that promote recoverin binding to membranes. NH_2-terminal myristic acid–dependent protein-membrane interactions are also regulated by phosphorylation of MARCKS protein (*26*) and by GTP-binding to ARF (*4*). The Ca^{2+}-dependence of recoverin-membrane interaction occurs over what may be a physiological range of Ca^{2+} concentrations (*27*) and may therefore influence the biological activity of recoverin. Effects of the heterogeneity of NH_2-terminal acylation of recoverin on Ca^{2+}-dependent membrane binding, Ca^{2+} binding to recoverin, or the cellular photoresponse have not yet been investigated.

Purified preparations of recoverin have been reported to stimulate a membrane-associated guanylate cyclase when added to photoreceptor membranes in low Ca^{2+} buffers (*12*, *13*). We find that under these conditions, even in the presence of guanylate cyclase assay buffers and reagents, recoverin dissociates from the membrane. Together, these findings suggest that recoverin does not stimulate guanylate cyclase by direct association.

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21. Liposomes were prepared from a mixture of phosphatidylserine and phosphatidylcholine (dipalmitoyl, Sigma) (1:1) by extrusion under argon pressure through a membrane with a 100-nm pore size. The membrane-binding assays contained 200 to 500 μg of phospholipid, 100 mM KCl, 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.5), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 2 mM Ca^{2+}-EGTA buffer (*28*), and 10 μg of retinal recoverin.
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24. After cleavage from recoverin, the NH_2-terminal myristoyl peptide partitions into membranes. We treated [^{3}H]myristoyl recoverin (54 μg per assay) with trypsin for 15 min (*29*) in the presence of 1 mM $CaCl_2$ to cleave off the NH_2-terminal [^{3}H]myristoyl peptide. The resulting mixture was incubated with stripped ROS membranes (100 μg of rhodopsin) and centrifuged Radioactivity in the supernatant and membranes was analyzed by scintillation counting. More than 90% of the radioactivity was found to be associated with the membranes independently of the Ca^{2+} concentration.
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29 Purified recoverin (4 μg) was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (0.04 μg) (Sigma) for 60 min at 37°C. Reactions were stopped by the addition of 0.1 mM phenylmethysulfonyl fluoride (PMSF) and analyzed by SDS-PAGE.
30. ROS membranes from fresh or frozen (Hormel) bovine retinas were isolated on a sucrose step gradient as described (*12*). To extract Ca^{2+}-dependent membrane-binding proteins, we first homogenized bleached ROS membranes containing rhodopsin (2 mg/ml) three times on ice in 30 mM tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM PMSF, and 0.5 mM $CaCl_2$. The membranes were then extracted three more times with 5 mM tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 μM GTP, and 0.5 mM $CaCl_2$. The membranes were washed once more with the same buffer without GTP and then with the same buffer containing 1 mM EGTA instead of $CaCl_2$. The extracts were concentrated by pressure filtration and analyzed by SDS-PAGE.
31. Recoverin was isolated from bovine retinas either by affinity purification with a rabbit antibody to bovine recoverin (*9, 12*) or by phenyl-Sepharose chromatography (*23*) Recoverin was produced in *E. coli*, either with or without coexpression of yeast NMT, with plasmids pET11a-mr21 and pBB131 in BL21(DE3) strains, as described (*22*)
32 ROS membranes were stripped of most of their endogenous recoverin by three extractions with 5 mM tris (pH 8.0) and rhodopsin (0 1 mg/ml)
33 Purified recoverin and membranes were mixed in a 50-μl total volume containing 30 mM tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 2 mM Ca^{2+}-EGTA buffer (*28*), rhodopsin (200 to 300 μg), and recoverin (20 μg) This mixture was incubated for 5 min at 37°C and centrifuged at 200,000*g* for 5 min (TLA-100 centrifuge, Beckman) The supernatants were gently aspirated, and the sedimented material was rinsed and resuspended in 45 μl of the same buffer containing 2 mM EGTA This sample was centrifuged, and the supernatants were saved for analysis Amounts of recoverin bound to the membranes were estimated by densitometric analyses of Coomassie blue–stained gels (Fig. 2)
34 To produce [^{3}H]myristoyl recoverin, we cultured *E coli* strain BL21(DE3) containing pBB131 and pET11a-mr21 in the presence of 200 μCi (50 Ci/mmol) of [^{3}H]myristic acid (Amersham) The cells were harvested and lysed, and recoverin was purified as described (*22*).
35 We thank M. Chabre for his support in the initial phase of this project, L Ericsson and R. Johnson for electrospray mass spectrometry analyses, and S. Zozulya and L. Stryer for discussions and for sharing their findings and ideas with us before publication Supported by NIH grant EY06641 and by a grant from the Human Frontiers in Science organization

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Selectivity in Signal Transduction Determined by γ Subunits of Heterotrimeric G Proteins

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Various heterotrimeric guanine nucleotide–binding proteins have been identified on the basis of the individual subtypes of their α subunits. The βγ complexes, composed of β and γ subunits, remain tightly associated under physiological conditions and have been assumed to constitute a common pool shared among various guanosine triphosphate (GTP)–binding (G) protein heterotrimers. Particular α and β subunit subtypes participate in the signal transduction processes between somatostatin or muscarinic receptors and the voltage-sensitive L-type calcium channel in rat pituitary GH_3 cells. Among γ subunits the γ_3 subtype was found to be required for coupling of the somatostatin receptor to voltage-sensitive calcium channels, whereas the γ_4 subtype was found to be required for coupling of the muscarinic receptor to those channels.

G proteins undergo a cycle in which they switch between active and inactive states by guanine nucleotide exchange and GTP hydrolysis (*1*). The inactive G protein $\alpha_{GDP}\beta\gamma$ is stimulated by a ligand-activated receptor to exchange guanosine diphosphate (GDP) for GTP. In the active form α_{GTP} dissociates from the βγ complex. The α_{GTP} and the βγ complex are then able to interact specifically with cellular effector molecules to evoke the cellular response. Until recently it had been thought that the function of a particular G protein was solely determined by the α subunit, and no specific functions had been assigned to the β and γ subunits. The contribution of β or γ subunits to receptor-effector coupling is difficult to investigate because these subunits are functionally inactive when separated from each other. Nevertheless, the βγ complex may directly interact with effector molecules (*2–4*). Inhibition of adenyl cyclase by a stimulatory (G_s)-type G protein depends on whether the G protein is reconstituted with βγ complexes from retina or brain tissue (*5*).

Four different β polypeptide sequences are known (*6*) and all have similar sequences. Three of five identified γ cDNAs have been cloned (*7–9*). The sequence of a fourth γ cDNA, γ_4 (*10*), as well as that of a fifth γ cDNA (*11*) has been established, and additional γ subtypes may exist (*12*). Because of the apparent sequence heterogeneity in the γ subunits, functional differences of the βγ complexes have been attributed to the γ subunits (*12, 13*).

We have examined the role of individual subtypes of γ subunits in selective receptor-effector coupling. We studied the effects of γ subtype on the inhibition of voltage-sensitive Ca^{2+} channels through activation of muscarinic (M_4) or somatostatin receptors. These modulatory effects of receptor agonists are mediated by pertussis toxin–sensitive G_o proteins (*14*), and the subtypes of α_o and β subunits have specific effects in this system (*15, 16*). We detected mRNAs for γ_2, γ_3, and γ_4 subtypes in the rat pituitary tumor cell line GH_3 (Fig. 1). The γ_1 subtype has only been found in retina.

We have previously established (*15*)

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