Calcium–myristoyl protein switch
(recoverin/EF-hand superfamily/retinal rod cell/membrane binding/hydrophobic interactions)

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ABSTRACT Recoverin, a recently discovered member of the EF-hand superfamily of Ca\(^{2+}\)-binding proteins, serves as a Ca\(^{2+}\) sensor in vision. The amino terminus of the protein from retinal rod cells contains a covalently attached myristoyl or related N-acyl group. We report here studies of unmyristoylated and myristoylated recombinant recoverin designed to delineate the biological role of this hydrophobic unit. Ca\(^{2+}\) induces the binding of both the unmyristoylated and myristoylated proteins to phenyl-agarose, a hydrophobic support. Binding was half-maximal at 1.1 and 1.0 \(\mu\)M Ca\(^{2+}\), respectively. The Hill coefficients of 1.8 and 1.7, respectively, indicate that binding was cooperative. In contrast, Ca\(^{2+}\) induced the binding of myristoylated but not of unmyristoylated recoverin to rod outer segment membranes. Binding to these membranes was half-maximal at 2.1 \(\mu\)M Ca\(^{2+}\), and the Hill coefficient was 2.4. Likewise, myristoylated but not unmyristoylated recoverin exhibited Ca\(^{2+}\)-induced binding to phosphatidylcholine vesicles. These findings suggest that the binding of Ca\(^{2+}\) to recoverin has two effects: (i) hydrophobic surfaces are exposed, allowing the protein to interact with complementary nonpolar sites, such as the aromatic rings of phenyl-agarose; and (ii) the myristoyl group is extruded, enabling recoverin to insert into a lipid bilayer membrane. The myristoyl group is likely to be an active participant in Ca\(^{2+}\) signaling by recoverin and related EF-hand proteins such as visinin and neurocalcin.

Recoverin, a recently discovered 23-kDa Ca\(^{2+}\)-binding protein, activates guanylate cyclase in bovine retinal rod outer segments when the Ca\(^{2+}\) level is lowered in the submicromolar range (1, 2). This Ca\(^{2+}\) sensor is a new member of the EF-hand superfamily, which includes calmodulin, troponin C, and parvalbumin (3, 4). The cDNA for recoverin has been cloned and expressed in high yield in Escherichia coli (5). Bovine recoverin is 59% identical in amino acid sequence to visinin, a Ca\(^{2+}\)-binding protein found in chicken cone cells (6). Homologs of recoverin and visinin have recently been found in mammalian brain (7-10). Interest in this Ca\(^{2+}\) sensor has been further heightened by the finding that the amino-terminal glycine of retinal recoverin is linked to a myristoyl (C14:0) or related fatty acyl group (C14:1, C14:2, or C12:0) (11). Numerous eukaryotic and viral proteins contain a covalently attached myristoyl group (12, 13). The importance of this covalent modification is evident in the viral src protein pp60\(^{v-src}\), which is oncogenic only when myristoylated (14).

Human retinal recoverin (isolated as the p26 antigen in cancer-associated retinopathy) (15) and S-modulin, a recoverin-like protein from frog retinas (16, 17) are known to bind more tightly to membranes or hydrophobic supports in the presence of Ca\(^{2+}\) than in its absence. A similar effect has been observed with calmodulin (18) and the annexins (19), which lack a myristoyl group. Is myristoylation essential for the Ca\(^{2+}\)-dependent binding of recoverin and homologs to membranes? We answered this question by preparing both myristoylated and unmyristoylated recombinant recoverin in E. coli and by measuring their binding to phenyl-agarose, retinal rod outer segment (ROS) membranes, and phosphatidylcholine vesicles. The Ca\(^{2+}\)-induced binding of myristoylated but not of unmyristoylated recoverin to lipid bilayers shows that hydrophobicity conferred by this covalently attached fatty acyl group can be dynamically controlled by Ca\(^{2+}\). Recoverin contains a Ca\(^{2+}\)-myristoyl switch akin to the GTP-myristoyl switch recently found in ADP-ribosylation factor, a myristoylated guanylate cyclase binding protein that regulates protein transport (20, 21).

MATERIALS AND METHODS

Recoverin. Recombinant myristoylated and unmyristoylated recoverins were expressed in the overproducing E. coli strains pTrec2/pBB131/DH5\(\alpha\)F\(\alpha\) and pTrec2/DH5\(\alpha\), respectively, and were purified by a modification of a previously described procedure (5, 15). Cells from 20 liters of a bacterial culture were resuspended in 200 ml of 100 mM KCl/1 mM dithiothreitol/1 mM MgCl\(_2\)/50 mM potassium Hepes, pH 7.5 (buffer A) supplied with 1 mM EGTA and disrupted by sonication. Streptomycin sulfate was added with stirring over a 10-min period to a final concentration of 0.1% from a 5% stock prepared in buffer A, and the cell debris was removed by centrifugation at 4°C for 30 min at 18,000 \(\times\) g. The cleared lysate was filtered through filter paper (Whatman 1), brought to 2 mM CaCl\(_2\), and applied to an 80-ml phenyl-Sepharose CL-4B (Pharmacia) column previously equilibrated with buffer A containing 2 mM CaCl\(_2\). The column was washed with 400 ml of the same buffer, and the fraction containing recoverin was eluted with 100–150 ml of buffer A containing 2 mM EGTA. The eluate was diluted with 3 volumes of cold water, applied to a HiLoad 26/10 Q-Sepharose HP column (Pharmacia), and chromatographed with a gradient of 0–200 mM KCl in 500 ml of 20 mM Tris-HCl, pH 8.0/1 mM dithiothreitol/1 mM MgCl\(_2\)/0.1 mM EGTA. Purified recoverin was concentrated to 10–20 mg/ml using Centriprep 10 centrifugal concentrators (Amicon), flash-frozen in liquid nitrogen, and stored at \(-70^\circ\)C.

To produce tritium-labeled myristoylated recoverin, the overproducing strain pTrec2/pBB131/DH5\(\alpha\)F\(\alpha\) containing the yeast N-myristoyltransferase expression vector (22) was grown in minimal medium with Casamino acids (M9CA) and supplied with \(^{3}H\)-myristic acid [New England Nuclear; 39.3 Ci/mmol, 10 \(\mu\)Ci/ml of culture (1 \(\mu\)Ci = 37 GBq)] at the moment of induction with isoprropyl \(\beta\)-d-thiogalactoside. The specific activity of the resulting tritiated myristoylated recoverin was 0.31 Ci/mmol.

Membrane Preparations. Liposomes were formed from soybean phosphatidylcholine (type IV-S; Sigma) or from a mixture of phosphatidylcholine and bovine brain phosphatidylserine (Avanti Polar Lipids) by sonicating a lipid suspension in buffer A. Stock suspensions of liposomes (10–20 mg of lipid per ml) were stored at 4°C and used within several

Abbreviation: ROS, rod outer segment.

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days. ROS were isolated in the dark from frozen bovine retinas as described (23). The concentration of rhodopsin in ROS membrane suspensions was calculated from the change in absorbance at 500 nm after bleaching in 1.5% Ammonyx LO (Onyx Chemical, Jersey City, NJ); an absorbance coefficient of 40,600 M⁻¹·cm⁻¹ was used.

Free Ca²⁺ Concentrations. Ca²⁺/EGTA buffers were prepared as described (24). The free Ca²⁺ concentration in these buffers was determined by using the fluorescent indicator rhod-2 (Molecular Probes). Fluorescence measurements were made with an SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, IL). The free Ca²⁺ concentration was calculated from the fluorescence emission intensity at 576 nm on excitation at 553 nm by using a K_d of 1.0 μM for the Ca²⁺-rhod-2 complex (24, 25).

Binding of Recoverin to Membranes and Phenyl-Agarose. ROS membranes (100 μM rhodopsin) were bleached and washed twice in the light with 50 vol of 10 mM potassium Hepes (pH 7.5). Myristoylated or unmyristoylated recombinant recoverin (10 μM) in buffer A containing 2 mM EGTA ("low Ca²⁺") or 50 μM CaCl₂ ("high Ca²⁺") was incubated with ROS membranes (10 μM rhodopsin) or liposomes (1 mg lipid per ml) for 20 min at 24°C. Membranes were pelleted by centrifugation for 5 min at 10,000 × g for ROS) or 20 min at 100,000 × g (for liposomes), and the supernatant was removed. The pellet was gently rinsed with buffer and then analyzed by SDS/polyacrylamide gel electrophoresis.

The Ca²⁺ dependence of the binding of unmyristoylated or myristoylated recoverin to phenyl-agarose was measured by incubating the protein (2 μM) with 50 μl of phenyl-Sepharose 4B (Pharmacia) in 250 μl of each of a series of Ca²⁺/EGTA buffers (2 mM EGTA) containing 40 mM NaCl, 1 mM MgCl₂, and 80 mM potassium Hepes (pH 7.4) (buffer B). These mixtures were vigorously shaken for 30 min at 25°C and then centrifuged for 5 min at 10,000 × g. The supernatants were removed and diluted with 10 volumes of buffer B containing 2 mM EGTA and no added Ca²⁺. The concentration of recoverin in each supernatant was determined from its tryptophan fluorescence intensity.

The Ca²⁺ dependence of the binding of myristoylated recoverin to ROS membranes was measured by incubating membranes (25 μM rhodopsin) with tritiated myristoylated recoverin (0.5 μM, 690 cpnm/pmol) in 250 μl of Ca²⁺-EGTA buffer, as described above, for 30 min at 25°C. Membranes were pelleted by centrifugation for 5 min at 10,000 × g and dissolved in 0.4 ml of 10% Ammonyx-LO. Tritium radioactivity was measured by scintillation counting in 10 ml of Ecolume (ICN Biomedicals). In control experiments, 32P_i (6 × 10⁶ cpnm per tube) was added to incubation mixtures to determine how much of the supernatant volume adhered to the pellets. All binding experiments were performed in triplicate.

RESULTS

Binding of Recoverin to Phenyl-Agarose. Retinal recoverin is known to bind in a Ca²⁺-dependent manner to phenyl-agarose, a hydrophobic support (15, 17). Indeed, this property is exploited in the purification of recoverin. The question arises as to whether the Ca²⁺-sensitive binding of retinal recoverin is conferred by its myristoyl group or instead is an expression of a Ca²⁺-induced exposure of hydrophobic residues, as occurs in calmodulin or troponin C (4, 18). The availability of recombinant unmyristoylated and myristoylated recoverin enabled us to distinguish between these possibilities. Both forms of recombinant recoverin bound to phenyl-agarose at high but not at low Ca²⁺ (Fig. 1). Binding was half-maximal at 1.1 μM Ca²⁺ for unmyristoylated recoverin and at 1 μM Ca²⁺ for myristoylated recoverin. The Hill coefficients are 1.8 and 1.7, respectively, indicating that multiple Ca²⁺ must be complexed to a recoverin molecule to induce binding to the hydrophobic matrix. The myristoyl group is clearly not required for the binding of recoverin to the phenyl groups of the support. Rather, binding is a consequence of the Ca²⁺-induced exposure of hydrophobic side chains of the protein.

Binding of Recoverin to ROS Membranes and Liposomes. A contrasting result was obtained for the binding of the two forms of recoverin to ROS membranes. The gel electropho-

![Fig. 1. Ca²⁺ dependence of the binding of recoverin to phenyl-agarose. (A) Unmyristoylated recombinant recoverin. (B) Myristoylated recombinant recoverin. ( Insets) Hill plots of the data (f denotes the fraction of bound recoverin).](image-url)
Fig. 2. Ca\textsuperscript{2+}-and myristoylation-dependent binding of recoverin to ROS membranes and liposomes. Gel electrophoresis patterns (12.5% SDS/polyacrylamide gel) of sedimented ROS membranes (A) or phosphatidylcholine vesicles (B) incubated with unmyristoylated (lanes 1 and 2) or myristoylated (lanes 3 and 4) recoverin in the presence of 2 mM EGTA (lanes 1 and 3) or 50 \mu M CaCl\textsubscript{2} (lanes 2 and 4). The positions of recoverin (Rec) and rhodopsin (Rho) are indicated.

The positions of phosphatidylinositol protein kinase C (PI-3 kinase) and poly(ADP-ribose) polymerase (PARP) in the pattern depicted in Fig. 2A shows that myristoylated but not unmyristoylated recoverin exhibits Ca\textsuperscript{2+}-induced binding to ROS membranes. Does recoverin bind to a specific ROS protein or does it interact with the lipid bilayer? Myristoylated but not unmyristoylated recoverin likewise binds to phosphatidylcholine vesicles or phosphatidylcholine/phosphatidylserine (1:1) vesicles in a Ca\textsuperscript{2+}-dependent manner (Fig. 2B). Thus, a protein receptor is not required. The simplest interpretation is that the myristoyl group of acylated recoverin inserts into the lipid bilayer when Ca\textsuperscript{2+} is bound to the protein. Hurley and coworkers have recently obtained similar results in experiments that complement ours (A. M. Dizhoor, C.-K. Chen, E. Olshevskaya, V. V. Sinelnikova, P. Phillipov, and J. B. Hurley, personal communication).

The dependence of the binding of recoverin on the concentration of ROS membranes at low and high Ca\textsuperscript{2+} is shown in Fig. 3. At low Ca\textsuperscript{2+}, the proportion of recoverin retained in the pellet after removal of the supernatant was about 5%, nearly independent of the membrane concentration. Most of this background came from a layer of supernatant that adhered to the pellet, as shown by control experiments in which \textsuperscript{32}P\textsubscript{i} was used as an unbound label. At high Ca\textsuperscript{2+}, the binding of myristoylated recoverin to ROS membranes was nearly linear with membrane concentration from 5 to 50 \mu M rhodopsin. The extrapolated dissociation constant at high Ca\textsuperscript{2+} is about 220 \mu M rhodopsin. It was also evident that the affinity of myristoylated recoverin for ROS membranes is at least 10-fold higher at high Ca\textsuperscript{2+} than at low Ca\textsuperscript{2+}.

The Ca\textsuperscript{2+} dependence of the binding of myristoylated recoverin to ROS membranes is shown in Fig. 4. Binding of recoverin was half-maximal at a Ca\textsuperscript{2+} concentration of 2.1 \mu M. The Hill coefficient of 2.38 (Fig. 4 Inset) indicates that binding is cooperative with respect to Ca\textsuperscript{2+}. For this preparation of ROS membranes, the calculated dissociation constant at high Ca\textsuperscript{2+} is 127 \mu M rhodopsin.

**DISCUSSION**

The binding of Ca\textsuperscript{2+} to recoverin has two consequences. First, hydrophobic groups other than the myristoyl unit become exposed, enabling recoverin to bind tightly to phenyl-agarose. Second, the myristoyl group of the acylated protein probably becomes accessible, as evidenced by the binding of myristoylated recoverin to ROS membranes and phosphatidylcholine vesicles. The Ca\textsuperscript{2+}-bound form of unmyristoylated recoverin does not bind to lipid bilayer membranes, most likely because its exposed hydrophobic groups do not have the finger-like shape of the myristoyl group. The fatty acyl chain introduced by myristoylation, in contrast, is

Fig. 3. Dependence of the binding of tritiated myristoylated recoverin to ROS membranes on the membrane concentration (expressed in terms of the rhodopsin concentration).

Fig. 4. Ca\textsuperscript{2+} dependence of the binding of myristoylated recoverin to ROS membranes (25 \mu M rhodopsin). (Inset) Hill plot of the data.

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Fig. 5. Schematic diagram of a Ca\textsuperscript{2+}-myristoyl switch. The binding of two or more Ca\textsuperscript{2+} is postulated to promote the extrusion of the myristoyl group (or a related N-acyl group) of recoverin and the exposure of other hydrophobic residues (marked by the stippled area).
well-suited for inserting a protein in a lipid bilayer. Our binding data strongly suggest that recoverin contains a Ca\(^{2+}\)-myristoyl switch (Fig. 5). The binding of two or more Ca\(^{2+}\) to recoverin could have several functional consequences: (i) the protein could be translocated from the cytosol to the disk membrane or plasma membrane; (ii) the extruded myristoyl group could be recognized by another protein, leading to the formation of a complex; and (iii) hydrophobic groups clustered around the myristoyl unit in the Ca\(^{2+}\)-free form of recoverin could become available for interaction with another protein. It will be interesting to learn which of these Ca\(^{2+}\)-induced changes are utilized in interactions of recoverin with its targets. The challenge now is to delineate the mechanism by which recoverin activates guanylate cyclase at low Ca\(^{2+}\).

Ca\(^{2+}\) promotes the binding of recoverin to membrane; hence, membranes must promote the binding of Ca\(^{2+}\) to recoverin. The linkage between Ca\(^{2+}\) binding and membrane binding can be represented by a simple scheme:

\[
\begin{align*}
V & \xrightarrow{K_1} V\text{-Ca}_2 \\
M\text{-V} & \xrightarrow{K_4} M\text{-V}\text{-Ca}_2 \\
K_2 & \\
K_3 & \\
M\text{-V} & \xrightarrow{K_2} M\text{-V}\text{-Ca}_2
\end{align*}
\]

in which V denotes recoverin; M, a membrane site; Ca\(_2^+\), two bound Ca\(^{2+}\); and \(K_i\), a dissociation constant. The binding of two Ca\(^{2+}\) is presumed to induce a strong interaction with the membrane. Suppose that \(K_1 = (1 \mu M)^2\), \(K_2 = 100 \mu M\), and that \(K_3 = K_4/1000\) (i.e., the binding of the Ca\(^{2+}\)-bound form of recoverin to membranes is 1000 times as strong as that of the Ca\(^{2+}\)-free form). For these values of the dissociation constants, the calculated binding curve (Fig. 6) for a ROS suspension containing 25 \(\mu M\) rhodopsin resembles the experimentally observed one (Fig. 4). Now let us increase the membrane concentration to 3 mM rhodopsin, the actual value in vivo. Binding of recoverin would then be centered at 180 nM Ca\(^{2+}\), which would place it in the physiologic range.

The Ca\(^{2+}\)-myristoyl switch found in recoverin probably also occurs in other members of the EF-hand superfamily. Calcineurin B, the regulatory subunit of protein phosphatase 2B, is known to be myristoylated and to undergo Ca\(^{2+}\)-dependent binding to membranes (26–28). Visinin and other homologs of recoverin are probably myristoylated (Fig. 7). It seems likely that calcineurin B and the recoverin branch of the EF-hand superfamily have a common Ca\(^{2+}\)-myristoyl switch.

Myristoyl switches are likely to be used in transducing diverse signals. ADP-ribosylation factors (ARFs) are small GTP-binding proteins that play key roles in vesicular trafficking (20, 21). Members of this family are myristoylated and bind tightly to membranes in the presence of hydrolysis-resistant analogs of GTP. Kahn et al. (21) have found that deletion of the amino-terminal region of ARF eliminates its GTP-dependent binding to membranes and vesicular transport functions. Reciprocally, the binding of guanine nucleotides to this mutant is independent of added membrane. This important study revealed that ARFs contain GTP-myristoyl switches. A related switch is probably present in the myristoylated alanine-rich C kinase substrate (MARCKS) protein), which participates in macrophage activation and growth control (29). The dephosphorylated form of MARCKS binds tightly to membranes, whereas the phosphorylated form is released into the cytosol. This reversible binding could be mediated by a phosphoryl–myristoyl switch that couples phosphorylation to a change in accessibility of the myristoyl group. It should be interesting to learn whether covalently attached prenyl and palmitoyl groups also are dynamically switched in signal transduction processes.

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Fig. 7. The amino-terminal sequence of recoverin and several other members of the EF-hand superfamily meet the criteria for myristoylation: a glycine residue next to the amino-terminal methionine of the nascent protein, and a serine (or threonine) four residues away. The sequences shown are those of bovine recoverin (1), chicken visinin (6), bovine neurocalcin (7), rat 21-kDa CABP (calcium-binding protein) (8), Drosophila melanogaster frequenin (J. Lindemeier, I. Krah-Jentgens, and O. Pongs, personal communication), and bovine calcineurin B (25).

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