Role of Carboxyl-Terminal Charges on S-Modulin Membrane Affinity and Inhibition of Rhodopsin Phosphorylation†

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Received September 1, 1998; Revised Manuscript Received November 10, 1998

ABSTRACT: S-Modulin shows a higher affinity for urea-stripped frog rod outer segment membranes than s26 (a cone homologue of S-modulin). NaCl at a concentration of several hundred millimolar reduced the membrane affinity of S-modulin to the s26 level. Chimeric S-modulin and s26 whose respective 23 and 29 amino acids at the carboxyl terminus were swapped showed membrane affinites similar to those of s26 and S-modulin, respectively. The membrane affinity of an S-modulin mutant lacking C-terminal positive charges was reduced to the s26 level, while another S-modulin mutant lacking C-terminal negative charges has a higher membrane affinity than wild-type S-modulin. When the molar ratio of recombinant S-modulins to rhodopsin is 0.5, there was no large difference in the inhibition efficiency. However, S-modulin and mutants with high membrane affinities inhibit rhodopsin phosphorylation more efficiently than s26 and mutants with low membrane affinities at the molar ratio of 0.1. These results indicate that the C-terminal positive charges of these Ca^{2+}-binding proteins enhance the membrane affinity and the inhibitory effect on rhodopsin phosphorylation by increasing the concentration of S-modulin on the membrane.

In the dark-adapted photoreceptors of vertebrates, cGMP-gated cation channels are opened and Ca^{2+} flows into the cell (*1*, *2*). Intracellular Ca^{2+} is continuously pumped out by a Na^+-K^+/Ca^{2+} exchanger in the outer segment (*3*, *4*). Light initiates the phototransduction cascade, closes the cation channels, and blocks the Ca^{2+} influx. The result is a cytoplasmic Ca^{2+} concentration decrease in the light-adapted state. This decrease in Ca^{2+} concentration is the underlying mechanism of light adaptation of the vertebrate photoreceptor (*5*, *6*). A frog photoreceptor Ca^{2+}-binding protein, S-modulin, and its bovine homologue, recoverin (*7*), inhibit the phosphorylation of light-activated rhodopsin at high Ca^{2+} concentrations but do not interfere at low Ca^{2+} concentrations (*8*, *9*). As phosphorylation of the rhodopsin carboxyl terminus is thought to be the shutoff mechanism for the activation of transducin (*10*, *11*), S-modulin and recoverin contribute to the increased light sensitivity in the dark-adapted state (high Ca^{2+} concentrations; *12*, *13*). These Ca^{2+}-binding proteins associate with rod outer segment (ROS)[1] membrane at high but not at low Ca^{2+} concentrations, a process involving N-terminal acylation (N-myristoylation), the "Ca^{2+}-myristoyl switch" (*14*, *15*).

The basic signal transduction pathway is thought to be similar for rods and cones (*16*−*20*), but there are some differences. Rods are more sensitive than cones, and the light response of cones is faster and is terminated more rapidly than that of rods (*21*). We have found another Ca^{2+}-binding protein, s26, in frog retina which is localized to cone photoreceptor cells, suggesting that S-modulin and s26 regulate the phosphorylation of rhodopsin and cone pigment, respectively (*22*). The Ca^{2+} dissociation constants of S-modulin and s26 are only slightly different (*23*).

Large differences between S-modulin and s26 amino acid sequences are found in the C-terminal region. The S-modulin C terminus has more charged residues than s26 (*9*, *22*), and these residues may interact with membrane lipid. In this paper, we first describe the membrane affinities of recombinant S-modulin and s26, demonstrating that S-modulin has a higher affinity for ROS membrane than s26. Second, we describe the membrane affinities of mutant proteins and the efficiency of their ability to inhibit rhodopsin phosphorylation, which is shown to coincide with an increase in the number of C-terminal positive charges.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors for Chimeric Proteins and Site-Directed Mutants. cDNA fragments were exchanged between *Nco*I and *Eco*RI recognition sites of plasmid vectors pET-Smd and pET-s26 (containing S-modulin and s26 coding regions, respectively; *23*). The resulting plasmids (pET-Smd/173/s26 and pET-s26/173/Smd) were used as expression vectors for chimeric S-modulins and s26, respectively.

S-Modulin cDNA cloned into a plasmid vector (*9*) was used as the template for polymerase chain reaction to

† This work was partly supported by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture of Japan, by SUNBOR, and by JSPS Research Fellowships for Young Scientists.

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[1] Abbreviations: ROS, rod outer segment(s); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; HRP, horseradish peroxidase; EGTA, *O*,*O*′-bis(2-aminoethyl) ethylene glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid.

10.1021/bi982117u CCC: $18.00
Published on Web 12/31/1998

generate site-directed mutants. Oligonucleotide primers 5′-GCGTCGACTCGAGCTAGTGTTGTTGCTCTTGTAG-CTGGTCCTGGACTTGTTGAGGTTCGT-3′ and 5′-GCGTC-GACTCGAGCTAGTGTTTTTTCTGTTTTAGCTT-GTTCTTGACTTTTTGAGGTTCGT-3′ were made to generate S-modulin mutants Smd/del+ (K192/194/196/198/200/201Q, lysine residues at 192, 194, 196, 198, 200, and 201 of S-modulin were replaced with glutamine) and Smd/del− (D195N/E199Q, Asp195 and Glu199 were replaced with Asn and Gln, respectively). Using SMD-NTF as an external primer (*23*), mutant cDNAs were amplified and inserted between the *Nco*I and *Xho*I sites of a pET-16b (Novagen) plasmid vector (forming vectors pET-Smd/del+ and pET-Smd/del−).

Expression and Purification of Recombinant Proteins. The procedures of expression and purification of the recombinant proteins have been described in detail by Hisatomi et al. (*23*). Briefly, the expression vectors pET-Smd, pET-s26, pET-s26/173/Smd, pET-Smd/173/s26, pET-Smd/del+, and pET-Smd/del− were transfected into *Escherichia coli* BL21DE3 cells (Novagen) with (+myr) or without (−myr) pBB131, an expression vector of *N*-myristoyl transferase. The recombinant proteins were expressed by the addition of 1 mM isopropyl β-D-thiogalactopyranoside, solubilized with 8 M urea buffer, refolded by three steps of dialysis, and applied to a DEAE-Sephadex column. S-Modulin, Smd/173/s26, Smd/del+, and Smd/del− were contained in the pass-through fraction, and s26 and s26/173/Smd were eluted with 400 mM KCl. The recombinant proteins in the high-Ca^{2+} buffer were then applied to a phenyl-Sepharose column and eluted by decreasing the Ca^{2+} concentration.

HPLC. Purified recombinant proteins were loaded onto a reverse-phase C18 column. Recombinant proteins were then eluted with a linear gradient of 0 to 80% acetonitrile (1.3%/min) in 0.1% trifluoroacetic acid at a flow rate of 1.5 mL/min and detected at a monitor wavelength of 280 nm.

Preparation of Frog Rod Outer Segment Membranes. Retinas were dissected from bullfrogs, *Rana catesbeiana* (about 10 cm body length). ROS membranes were isolated by flotation on 45% sucrose in gluconate buffer [40 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 10 mM HEPES (pH 7.5)] and washed twice with gluconate buffer and then with gluconate buffer containing 4 M urea to eliminate (strip away) endogenous S-modulin, s26, and other peripheral proteins. The resulting urea-stripped ROS membranes were used for membrane binding experiments. For the phosphorylation assay, ROS membranes were isolated in phosphorylation buffer [115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 10 mM HEPES (pH 7.5)] in complete darkness with the aid of an infrared converter and washed three times with the same buffer to eliminate endogenous S-modulin, s26, and ATP.

Ca^{2+}-Dependent Membrane Association of Recombinant Proteins. Membrane binding properties of the recombinant proteins were investigated according to the method of Kawamura et al. (*24*), using plastic tubes siliconized to prevent nonspecific binding. ROS membranes containing 6 nmol of rhodopsin were mixed with 0.5 mL of gluconate buffer containing 1% bovine serum albumin, to block nonspecific binding sites. After being washed twice by centrifugation (37000*g* for 5 min at 4 °C) with 1 mL of gluconate buffer containing Ca^{2+} at various concentrations and from 0 to 500 mM NaCl (Ca^{2+}-NaCl gluconate buffer), the ROS membranes were resuspended in 20 μL of Ca^{2+}-NaCl gluconate buffer containing recombinant proteins (120 pmol). The mixtures were incubated at room temperature for 30 min and then centrifuged to obtain supernatants with the recombinant proteins that did not associate with the membranes. Extraction was repeated once to minimize the loss of unbound proteins. The two resulting soluble fractions (first extract) were mixed and analyzed by SDS−PAGE. The membrane fractions were then suspended in 20 μL of gluconate buffer containing 10 mM EGTA and from 0 to 500 mM NaCl (EGTA-NaCl gluconate buffer) and centrifuged to extract proteins liberated from the membrane by decreasing the Ca^{2+} concentration. The extraction procedure was repeated, and the resulting two combined soluble fractions (second extract) and membrane fractions were analyzed by SDS−PAGE. The integrated densities of Coomassie Brilliant Blue (CBB)-stained bands of the recombinant proteins were quantified by a two-dimensional densitometer (pdi, The Discovery Series). The amount of recombinant proteins remaining in the final membrane fractions (about 2 μL in our typical experiments) was less than 5% (data not shown), and the amount of membrane-bound protein was estimated using the ratio of (second extract)/(first extract + second extract).

Tryptophan Emission Spectra. Spectroscopic measurement was carried out as described by Hisatomi et al. (*23*). Briefly, fluorescence emission spectra were recorded from 300 to 400 nm with a fluorescence spectrophotometer (Hitachi, F-4500) with an excitation wavelength at 290 nm, in a mixture containing 2 μM recombinant protein, 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EGTA, and 100 mM HEPES (pH 7.0). The free Ca^{2+} concentration was adjusted by adding 1 M $CaCl_2$.

Rhodopsin Phosphorylation. Rhodopsin phosphorylation was assessed by the method of Kawamura (*8*) and Sanada et al. (*25*). Briefly, the reaction was carried out in 25 μL of a mixture containing rhodopsin (final concentration of 10 μM) and recombinant proteins (1 or 5 μM) in phosphorylation buffer. The free calcium concentration in the mixture was adjusted with Ca/EGTA buffer. The reaction mixtures were exposed to light for 2 min, and the phosphorylation reactions were initiated by adding ATP (0.1 mM final concentration), [γ-^{32}P]ATP (0.25 μM, 168 TBq/mmol), and GTP (0.5 mM). After incubation at room temperature for 2 min in light, the reaction was terminated by addition of 150 μL of 10% trichloroacetic acid. The reaction mixtures were then centrifuged (10000*g*) for 5 min, and the precipitates were washed with 500 μL of phosphorylation buffer and subjected to SDS−PAGE. The incorporation of ^{32}P into rhodopsin was evaluated with an image analyzer (bas 2000 Fuji Film).

RESULTS

Isolation of Recombinant S-Modulin. The recombinant proteins, either myristoylated [eS-modulin (+myr) and es26 (+myr)] or unmyristoylated [eS-modulin (−myr) and es26 (−myr)] (*23*), were expressed and purified as described in Experimental Procedures. Myristoylated chimeric and site-directed mutants were also expressed and purified. Myris-

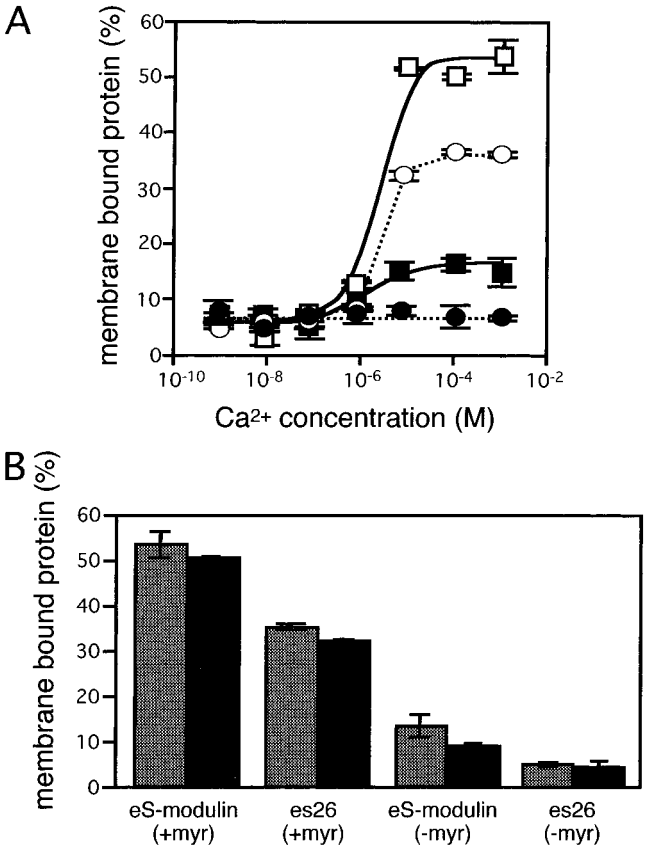

FIGURE 1: Membrane-binding properties of S-modulin and s26. (A) Binding of eS-modulin (+myr) (□), es26 (+myr) (○), eS-modulin (−myr) (■), and es26 (−myr) (●) to urea-stripped ROS membranes, quantified by densitometric analysis of CBB-stained SDS−PAGE bands. Bars represent standard deviations ($n = 2$). (B) Binding to urea-stripped (gray bars) and boiled urea-stripped (black bars) ROS membrane at 1 mM Ca^{2+}. Bars represent standard deviations ($n = 2$).

toylated recombinant proteins show a single peak with almost the same retention time from a C18 column, and the retention time is longer than that of unmyristoylated recombinants (data not shown), suggesting that myristoylated recombinants used in our experiments are indeed N-myristoylated.

Membrane Association of Recombinant Proteins. To investigate the membrane-binding properties of S-modulin and s26, myristoylated [eS-modulin (+myr) and es26 (+myr)] or unmyristoylated [eS-modulin (−myr) and es26 (−myr)] recombinant proteins were mixed with urea-stripped ROS membranes at various Ca^{2+} concentrations. First extracts (containing proteins that did not bind to ROS membranes) and second extracts (containing proteins that were liberated from the membrane by lowering the calcium concentration) were analyzed quantitatively, and the ratio of membrane-bound protein at various Ca^{2+} concentrations was determined (Figure 1A). The same extraction experiments were carried out with boiled ROS membrane at 1 mM Ca^{2+} (Figure 1B, black bars), and the results were compared with those for unboiled ROS membrane (Figure 1B, gray bars). The results indicate that (i) N-terminal myristoylation is associated with high membrane affinity of both S-modulin and s26 at a Ca^{2+} concentration of >10 μM, (ii) the membrane affinity of eS-modulin is greater than that of es26, and (iii) boiling the membrane prior to binding does not affect the binding ratio. In a Western blot analysis, a similar

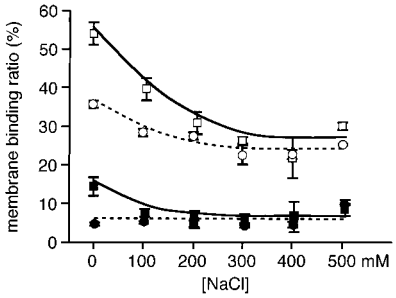

FIGURE 2: NaCl effects on membrane association. Binding ratios for binding of eS-modulin (+myr), es26 (+myr), eS-modulin (−myr), and es26 (−myr) to urea-stripped ROS membranes at 1 mM Ca^{2+} plotted against NaCl concentration. The symbols are the same as those described in the legend of Figure 2. Bars represent standard deviations ($n = 2$).

difference in membrane affinity between native S-modulin and s26 was observed in frog retina (data not shown). The difference in membrane affinity between S-modulin and s26 seems to be due to differences in the protein moiety, since a marked difference in membrane affinity is clear also between the unmyrstoylated forms eS-modulin (−myr) and es26 (−myr).

Electrostatic Interaction of Ca^{2+}-Dependent Membrane Association. To reduce the level of electrostatic interaction, the same extraction experiments were carried out in the presence of 1 mM Ca^{2+} and NaCl at various concentrations. Figure 2 shows that the membrane affinity of eS-modulin was reduced to the level of that of es26 by increasing the NaCl concentration, so it is concluded that electrostatic interaction is important for S-modulin binding to the ROS membrane as well as by hydrophobic interaction of the N-terminal myristoyl group. Figure 3 shows the effect of NaCl on the tryptophan emission spectra of S-modulin and s26 in the presence of 1 mM Ca^{2+}. The results suggest that the presence of 500 mM NaCl does not significantly change the environment of the three tryptophan residues in the Ca^{2+}-bound form of the recombinants.

Membrane Association of Chimeric and Mutant Proteins. Two myristoylated chimeric recombinants, es26/173/Smd (+myr) and eSmd/173/s26 (+myr), were made with the normal N-terminal 173 amino acids from s26 and S-modulin but with the remaining C-terminal residues swapped. The ratios of membrane-bound protein at 1 mM Ca^{2+} were 64% for es26/173/Smd (+myr) and 41% for eSmd/173/s26 (+myr) at 0 mM NaCl (Figure 4). It is therefore likely that the carboxyl terminus of S-modulin enhances ROS membrane affinity.

Two site-directed mutants of myristoylated S-modulin were also isolated, eSmd/del+ (+myr) and eSmd/del− (+myr), in which positively and negatively charged residues near the C terminus were replaced with neutral residues (K192/194/106/198/200/201Q and D195N/E199Q, respectively). The ratio of membrane-bound protein in the presence of 1 mM Ca^{2+} was 30% for eSmd/del+ (+myr) and 74% for eSmd/del− (+myr) (Figure 4), indicating that the C-terminal charges of S-modulin are important for membrane binding. The ratio of membrane-bound protein is about 5% for all the recombinants in the presence of only 1 nM Ca^{2+}, indicating that relatively high Ca^{2+} concentrations are necessary for the C-terminal charges to increase their membrane affinity.

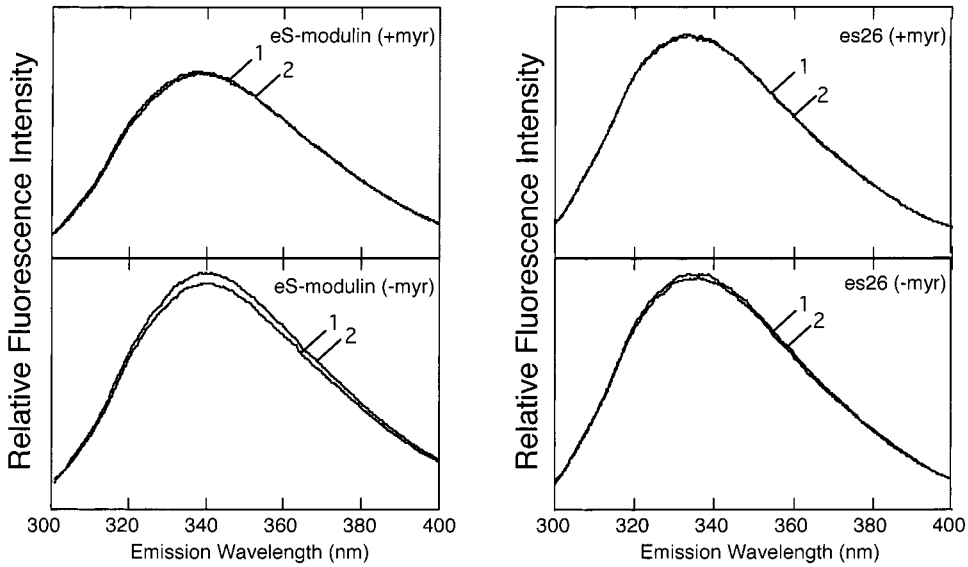

FIGURE 3: Tryptophan emission spectra of wild-type and mutant S-modulins. Fluorescence emission spectra at 0 (curve 1) and 500 mM (curve 2) NaCl.

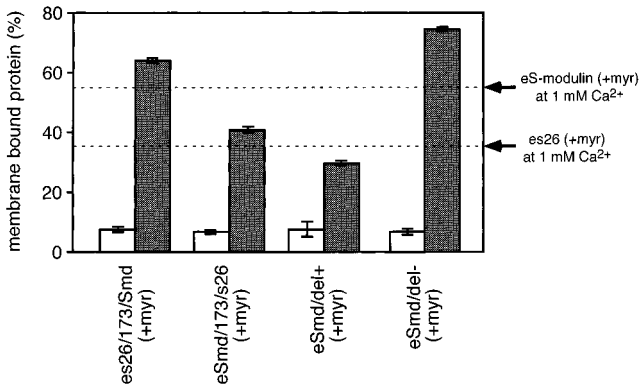

FIGURE 4: Membrane-binding properties of chimeric and mutant proteins. Binding to urea-stripped ROS membranes at Ca^{2+} concentrations of 1 nM (white bars) and 1 mM (gray bars). Error bars indicate standard deviations ($n = 2$).

Inhibition of Rhodopsin Phosphorylation by Recombinant Proteins. This study has shown that the Ca^{2+}-binding form of S-modulin has a higher membrane affinity than that of s26 and that this difference is due to charges at the carboxyl terminus. Does this difference in membrane affinity affect the efficiency of rhodopsin phosphorylation? To answer this question, phosphorylation of rhodopsin was examined in the presence of 1 or 5 μM recombinant proteins eS-modulin (+myr), s26 (+myr), s26/173/Smd (+myr), Smd/173/s26 (+myr), eSmd/del+ (+myr), and eSmd/del− (+myr) (Figure 5). All of them inhibited rhodopsin phosphorylation at high (0.1 mM) Ca^{2+} concentrations. At 5 μM (the molar ratio of S-modulin to rhodopsin is 0.5), there is only a slight diference between the recombinant proteins. However, there were certain differences in inhibitory efficiency at the S-modulin/rhodopsin raio of 0.1 (1 μM protein concentration) which is close to the molar ratio of 0.07 estimated in bullfrog retina (*22*). The recombinant proteins with high membrane affinities [eS-modulin (+myr), es26/173/Smd (+myr), and eSmd/del− (+myr)] inhibit rhodopsin phosphorylation more efficiently than proteins with low membrane affinities [es26 (+myr), eSmd/173/s26 (+myr), and eSmd/del+ (+myr)]. These results suggest that the C-terminal positive charges contribute to the inhibitory effect on rhodopsin phosphorylation perhaps

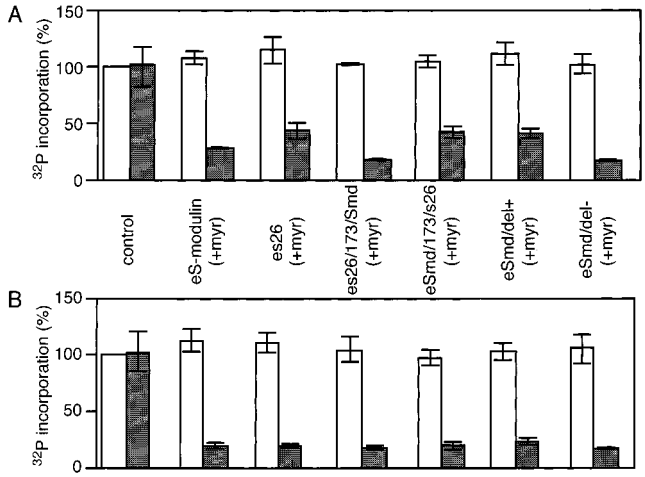

FIGURE 5: Inhibition of rhodopsin phosphorylation. ^{32}P incorporation of rhodopsin in the presence of [γ-^{32}P]ATP at Ca^{2+} concentrations of 1 nM (white bars) and 0.1 mM (gray bars). Experiments were carried out without recombinant proteins (control) or in the presence of 1 (A) or 5 μM (B) recombinant proteins. Error bars indicate standard deviations ($n = 2$).

by increasing the protein concentration on the ROS membrane.

DISCUSSION

Our results suggest that S-modulin binds to the ROS membrane not only by hydrophobic interaction with the N-terminal myristoyl group but also by electrostatic interaction with the C-terminal charges. It has been reported that S-modulin also binds to ROS membrane lipid extracted with chloroform/methanol (*26*), demonstrating that S-modulin and s26 probably bind to the membrane lipids rather than to membrane proteins. Our results in Figure 1B (showing that the ratio of membrane-bound protein is not affected by boiling ROS membranes) supports this suggestion.

Johnson et al. (*27*) have reported that the partitioning of bovine recoverin between the cytoplasmic and membrane compartments of the rod photoreceptor outer segment was unaffected by the concentration of calcium; therefore, it appears unlikely that a calcium-myristoyl switch acts alone

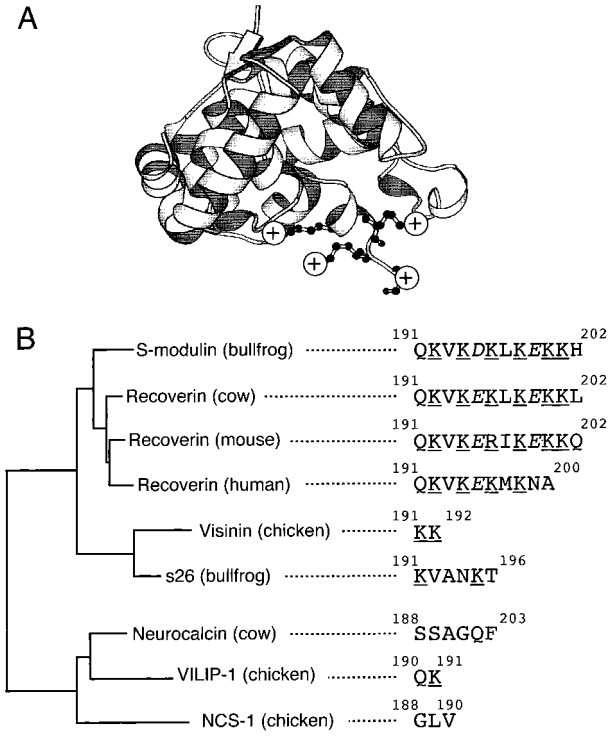

FIGURE 6: (A) Crystal structure of unmyristoylated bovine recoverin with one Ca^{2+}. Lysine residues at the carboxyl terminus (192−198) are represented by a ball-and-stick model. + indicates positive charges [constructed with MOLSCRIPT (*38*)]. (B) A phylogenetic tree calculated by the neighbor-joining method (39) and C-terminal amino acid sequences of proteins in the S-modulin family. Numbers are amino acid numbers, counting from the N terminus. Underlined and italic amino acids indicate positively and negatively charged residues, respectively.

to anchor recoverin directly to the membrane (*27*). However, frog S-modulin was isolated from frog ROS membrane washed with the 1 mM Ca^{2+} phosphorylation buffer (phosphorylation buffer containing 1 mM $CaCl_2$ instead of EGTA) by lowering the free Ca^{2+} concentration to 1 nM (*28*). There may be some difference between bovine and frog ROS membrane and/or proteins.

Although the structure of myristoylated bovine recoverin has been determined using NMR techniques (*29*), there has been no description of the structure of the carboxyl terminus. A part of the C-terminal structure (excluding residues 199−202) was determined in unmyristoylated crystalline bovine recoverin with one Ca^{2+} (Figure 6A) (*30*). In this structure, the C-terminal positive charges are at the protein surface and face in various directions. As the effect of C-terminal charge is observed only at high Ca^{2+} concentrations, C-terminal structure is thought to be changed on binding Ca^{2+}.

Recently, many calcium-binding proteins similar to S-modulin have been identified in the retina or central nervous systems (*31*−*33*). Some of these proteins, such as visinin-like protein (VILIP-1) and neuronal calcium sensor (NCS-1), show the ability to inhibit phosphorylation of rhodopsin in calcium-dependent manners (*34*). Photoreceptor-specific calcium-binding proteins consist of a subfamily of proteins, which can further be classified into two groups; group I contains mammalian recoverins and S-modulin in frog rods, and group II contains chicken visinin (*35*) and s26 in frog cones. Group I proteins have four to six positively charged residues at their C termini (*7*, *36*, *37*), whereas group II proteins have only two (Figure 6B). Chicken VILIP-1 and NCS-1 and bovine neurocalcin have one positive charge or no charge at their C termini. It is, therefore, speculated that group I proteins have evolved to bind to the photoreceptor membrane tightly and inhibit the phosphorylation of rhodopsin efficiently. It has been reported that the phosphorylation of light-activated rhodopsin is required for normal shutoff of the electrophysiological response (*11*). These positive charges seem to be likely to influence the membrane affinity and activity of these Ca^{2+}-binding proteins, which may affect the response of photoreceptor cells.

ACKNOWLEDGMENT

We thank Prof. Satoru Kawamura and Ms. Kumiko Nanda for their helpful discussions in addition to technical suggestions. We also thank Prof. Mikio Kataoka for his helpful discussion and Dr. Ian G. Gleadall for comments on the manuscript.

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BI982117U