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Functional Expression and Characterization of Frog Photoreceptor-Specific Calcium-Binding Proteins

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S-modulin (sensitivity-modulating protein) is a photoreceptor-specific calcium-binding protein which plays an important role in the light adaptation process by controlling rhodopsin phosphorylation in rods. S-modulin and its cone homologue, s26, were expressed at high level (more than 30% of total protein) in *Escherichia coli* and then purified. They both inhibited rhodopsin phosphorylation in a calcium dependent manner. Myristoylated recombinants of S-modulin and s26 showed calcium-dependent changes in tryptophan emission spectra with half-maxima at about 0.7 μM free calcium concentration. However, the spectral changes are distinctive from each other, suggesting that there is some difference in the structural change between S-modulin and s26. © 1997 Academic Press

In rod cells, light activates rhodopsin, which triggers the phototransduction cascade, resulting in the closure of cation channels. Ca^{2+} influx through this cation channel is then stopped, and consequently the intracellular Ca^{2+} concentration decreases. The light-activated rhodopsin is inactivated probably with phosphorylation by rhodopsin kinase, cGMP-gated cation channels are then opened, and the intracellular Ca^{2+} concentration is then restored to a high level. This change in the Ca^{2+} concentration has been suggested to be the underlying mechanism of the light-dark adaptation of vertebrate photoreceptor cells (1, 2). Kawamura and Murakami (3) found a photoreceptor-specific Ca^{2+}-binding protein, S-modulin (sensitivity-modulating protein), which controls the phosphorylation of light-activated rhodopsin in frog rods. S-modulin inhibits rhodopsin phosphorylation at high Ca^{2+} concentration (that is, in the dark-adapted state), but not at low Ca^{2+} concentration (light-adapted state) (4).

The visual transduction cascade in cones is less well understood than that in rods. However, cones are reported to have similar isozymes of phototransduction proteins, including the cGMP-sensitive channel (5, 6), opsins (7), transducin (8), and phosphodiesterase (PDE) (9). It was suggested that the basic signal transduction pathway of cones and rods is similar, even though cones are less sensitive than rods. In primates, cones require 100 times as many as photons to elicit a half maximal signal in comparison with rods (10). The time course of the light response is faster in cones than rods, and light adaptation is much more pronounced in cones than in rods (11). However, the biochemical bases underlying these physiological differences are still unclear.

We have reported the isolation of s26 cDNA, a homologue of S-modulin, which exists in frog cones (12). The deduced amino acid sequence shows 68% and 77% identity with, respectively, S-modulin and chicken visinin, a cone-specific Ca^{2+}-binding protein. Functional expression of these recombinant proteins will provide useful tools for studying the molecular mechanism of adaptation in rods and cones. In this paper, we report the functional expression of S-modulin and s26 in *Escherichia coli* (*E. coli*), and the characterization of these photoreceptor-specific Ca^{2+}-binding proteins.

EXPERIMENTAL PROCEDURES

Construction of expression vectors for S-modulin and s26. S-modulin cDNA was cloned into a plasmid vector as described by Kawa-

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Abbreviations used: S-modulin, sensitivity modulating protein; IPTG, isopropyl-b-*D*-thiogalactopyranoside; HPLC, high pressure liquid chromatography; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulphonylfluoride; SDS, sodiumdodecylsulfate; MOPS, 4-morpholinepropanesulfonic acid; NMT, *N*-myristoyl transferase; LB, L-broth; PDE, phosphodiesterase; CD, circular dichroism; CBB, Coomassie brilliant blue.

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mura *et al.* (13), and used as the template for polymerase chain reactions. The coding region of S-modulin was amplified using as primers SMD-NTF (5′-CGCCCGGGCTCGAGCCACCATGGGTAA-CACCAA-3′) and SMD-CTR (5′-GCGTCGACTCGAGCTAGTGTT-TTT-3′) (14), inserted between the NcoI and BamHI sites of a pET-16b (Novagen) plasmid vector (forming vector pET-Smd).

s26 cDNA was isolated and cloned into a pUC18 plasmid vector as described in Kawamura *et al.* (12). To make additional restriction sites in the 3′ end of the s26 coding region (for insertion into a pET-16b vector), 40 cycles (94-50-72°C) of amplification were carried out, using a frog retinal cDNA as the template and s26-F3 (5′-TGATAA-AATTGCTGAAGG-3′) and s26-CTR (5′-CCTAAGCTTGGATCC-TCAAGTCTTGTTAGC-3′) as primers. The amplified 3′ fragment was cloned and ligated with a 5′ (PstI-EcoRI) fragment of s26. The coding region of s26 was then inserted between the NcoI and BamHI sites of a pET-16b plasmid vector (forming vector pET-s26).

Expression of eS-modulin and es26 in E. coil. In this paper, S-modulin and s26 expressed in *E. coli* are designated eS-modulin and es26, respectively, to discriminate them from the native S-modulin and s26 isolated from frog retinas. BL21(DE3) cells were transformed with pET-Smd or pET-s26. Cells were pre-cultured in 10-50 ml of Luria-broth (LB) medium containing 50 μg/ml of ampicillin at 37°C for 4-5 h, and were then added to 1 l of LB medium. After optical density at 600 nm reached 0.6-1.0 (about 3-4 h later), 1 mM (final concentration) of isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to the medium, and the cells were further cultured for 8-12 h.

For the expression of myristoylated S-modulin and s26, BL21(DE3) cells previously transformed with pBB131 (kindly provided by Prof. Jeffrey I. Gordon; an expression vector of *N*-myristoyl transferase), were co-transformed with pET-Smd or pET-s26 (15). Cells were cultured in LB medium containing ampicillin and kanamycin (final concentration 50 μg/ml each), and 50 μM (final) of myristic acid was added to the culture medium 1 h before the addition of IPTG.

E. coli cells were harvested by centrifugation (2,700 ×g for 15 min), washed with 0.9% NaCl aqueous solution and recentrifuged. The pellet was suspended in 100 ml of lysis buffer (50 mM Tris-HCl, 200 mM KCl, 1 mM DTT, 1 mM EDTA, 0.1 mg/ml phenylmethylsulfonyl-fluoride, pH 8.0), and incubated at 0°C for 30 min. The lysates were then sonicated 5 times for 5 min, with intervals of 5 min on ice, and centrifuged at 6,500 ×g for 30 min.

Purification of eS-modulin and es26. eS-modulin expressed in BL21(DE3) cells was mainly found in the insoluble fraction. Cell debris from centrifugation was suspended in 200 ml of buffer A (50 mM Tris-HCl, 50 mM KCl, 5 mM EGTA, 5 mM DTT, pH 8.5) containing 8 M urea, and the soluble fraction was then isolated by re-centrifugation. In order to remove urea, the supernatant was dialyzed stepwise (3h each) against buffer A containing 4 M urea, buffer A containing 2 M urea, and then buffer B (50 mM Tris-HCl, 1 mM DTT, 1 mM EGTA, pH 8.0, 50 mM KCl). A small amount of precipitated protein was removed by recentrifugation.

The centrifugal supernatant from the dialysate was applied to a DEAE-Sephadex (Pharmacia) column equilibrated with buffer B, and the recombinant proteins were eluted with a linear gradient of 0.05-1M KCl in buffer B. The fractions containing eS-modulin were dialyzed against buffer C (50 mM HEPES, 1 mM DTT, pH 7.5) containing 500 mM KCl and 5 mM $CaCl_2$ at 4°C for 3 h, and applied to a phenyl-Sepharose (Pharmacia) column equilibrated with buffer C. eS-modulin was eluted with buffer C containing 50 mM KCl and 5 mM EGTA, and stored at 4°C until use. The refolding and purification protocols of es26 were performed likewise.

Phosphorylation assay. Rhodopsin in ROS (rod outer segment) membranes of frog retinas washed with the low (1nM) Ca^{2+} buffer, was bleached in the presence of (γ-^{32}P)ATP and the exogenous recombinant proteins (4). After separation by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), the radioactivity of ^{32}P incorporated into the bleached rhodopsin was measured by Cerenkov counting and densitometric analysis of the autoradiographs.

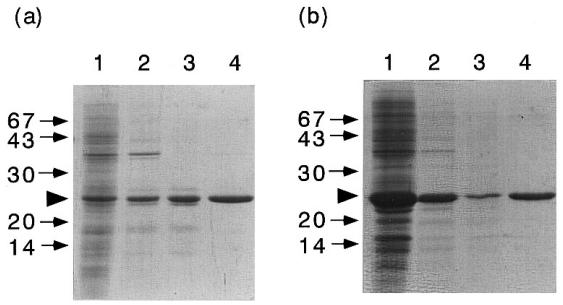

FIG. 1. Expression and purification of eS-modulin (a) and es26 (b), expressed in BL21(DE3) cells: total protein (lane 1); insoluble fraction (lane 2); and soluble fraction after purification by DEAE-Sephadex (lane 3) and phenyl-Sepharose (lane 4) column chromatographic steps. Arrows indicate mobility of molecular weight markers (kD), and arrowheads the bands of recombinant proteins.

Spectroscopic measurements. The amounts of purified eS-modulin and es26 were quantified by spectrophotometrical measurement at 280 nm using the specific absorbance ($E_{1mg/ml}$ = 1.9). Fluorescence emission spectra were recorded from 300 to 400 nm by a fluorescence spectrophotometer (Hitachi, F-4500) with an excitation wavelength at 290 nm, in mixtures containing 2 μM recombinant protein, 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EGTA, 100 mM HEPES (pH 7.0) and $CaCl_2$ of various concentrations, adjusted with the Ca^{2+}/EGTA buffers described by Sitaramayya and Margulis (16). Free Ca^{2+} concentration in the buffers was determined by using the fluorescent indicator Fura-2 (17). Circular dichroism (CD) spectra were measured by a CD spectrophotometer (Jasco, CD-J600), using mixtures containing 10 μM recombinant protein, 20 mM KCl, 10 mM HEPES and 4 mM DTT (pH 7.0), in the presence of 1 mM EGTA or 0.5 mM $CaCl_2$.

RESULTS

Expression and purification of eS-modulin and es26. For the high-level expression of eS-modulin, we constructed an expression vector, pET-Smd, and transformed BL21(DE3) cells with the vector. Judging from the SDS-PAGE pattern stained with Coomassie brilliant blue (CBB), the amount of eS-modulin was estimated to comprise more than 30% of the total protein (Fig.1, lane 1). Although the native S-modulin being a soluble protein, eS-modulin was found in the insoluble fractions (Fig.1, lane 2) and was considered unlikely to have the native conformation. Therefore, we solubilized it with buffer A containing 8 M urea, and refolded the protein by three steps of sequential dialyses (see Experimental Procedures). This stepwise dialysis was required to avoid aggregation induced by the dialysis of the 8M urea solution directly against buffer B. The centrifugal supernatant from the dialysate was applied to a DEAE-Sephadex column. Similar to native S-modulin isolated from frog retinas, eS-modulin was found in the pass-through fraction (Fig.1a, lane 3). The eS-modulin fraction was further purified with a phenyl-Sepharose column, and the resulting eS-modulin showed a single band on SDS-PAGE analysis (Fig.1a, lane 4). We ordinarily obtained 20-50 mg of purified

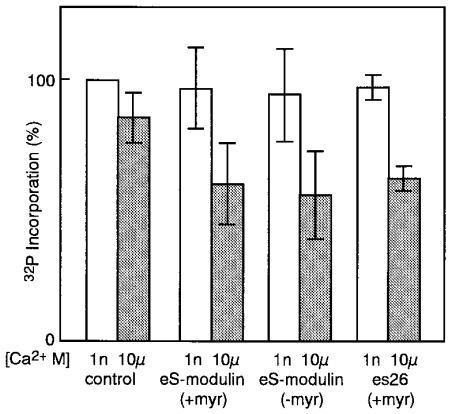

FIG. 2. ^{32}P-incorporation of rhodopsin in the presence of γ-^{32}P-ATP at low (1 nM; open bars) and high (10 μM; shaded bars) Ca^{2+} concentrations. Experiments were carried out without recombinant proteins (control), in the presence of 8 μM of myristoylated (+myr) or unmyristoylated (−myr) eS-modulin, or myristoylated (+myr) es26. Bars represent standard deviations from the means of experiments.

eS-modulin from 1 l of culture. es26 was purified in the same way, except for the DEAE-Sephadex column step, where es26 was eluted with buffer B containing 400 mM KCl (Fig1b, lane 3).

The amino acid compositions of eS-modulin and es26 showed values reasonably close to those calculated from their amino acid sequences. The N-terminal amino acid sequences of eS-modulin and es26 expressed without pBB131 were directly confirmed by peptide sequencing. N-terminal myristoylation was suggested by the fact that the N-termini of eS-modulin and es26 expressed with pBB131 were blocked against the Edoman degradation (myristoylated and unmyristoylated recombinant proteins are discriminated by the notations (+myr) and (−myr), respectively). The existence of myristoyl groups in the N-termini of eS-modulin (+myr) and es26 (+myr) was further confirmed by the fact that the retention time from a C-18 column for the (+myr) species was longer than that for the (−myr) species. Judging from HPLC patterns, the presence of (−myr) species in purified eS-modulin (+myr) or es26 (+myr) samples, if present, was less than 1 % (data not shown).

Physicochemical properties of eS-modulin and es26. Figure 2 shows the inhibition of rhodopsin phosphorylation in the presence of eS-modulin and es26. Both (+myr) and (−myr) species of eS-modulin and es26 (+myr) inhibited the phosphorylation at a high (10 μM) Ca^{2+} concentration, as do native S-modulin and s26 isolated from frog retinas (4, 12).

The CD spectra of these proteins indicated helical structures because they exhibited the typical pattern of a α-helix with paired troughs at 208 and 222 nm. The effects of the myristoylation were observed on the CD spectra of eS-modulin (Fig.3). In the region from 200 to 250 nm, the CD spectra of (+myr) and (−myr) species of eS-modulin had different profiles in the absence of Ca^{2+} (Fig.3a, solid line), but those of es26 showed less difference (Fig.3b). The CD spectra of both (+myr) and (−myr) of both proteins species were almost the same in the presence of 0.5 mM Ca^{2+}. In all cases, negative peaks around 222 nm were enlarged in the presence of Ca^{2+}, suggesting that the α-helical content of these proteins was increased by binding Ca^{2+}. Similar CD spectral changes were observed in our analysis of bovine recoverin (18). It seems that these Ca^{2+}-binding proteins undergo similar structural changes when they bind Ca^{2+}.

N-terminal myristoylation also affected the tryptophan emission spectra of eS-modulin (Fig.4a) and es26 (Fig.4b). eS-modulin (+myr) showed emission maxima at 322 in the absence of Ca^{2+}, and shifted their maxima to a longer wavelength 337 by increasing Ca^{2+} concentration to 1 mM. eS-modulin (−myr) had emission maxima at 332 and 340 nm in the absence and presence of Ca^{2+}, respectively. In the case of es26, emission maxima of (+myr) and (−myr) species appeared to be at 324 and 331 nm, respectively, and also underwent red-shifts (at 334 and 336 nm, respectively) by increasing free Ca^{2+}. Similar emission spectra were reported for (+myr) and (−myr) species of recombinant recoverin, which had maxima at 333 and 339 nm, respectively, in the absence of Ca^{2+}. Emission maximaof both (+myr) and (−myr) species of recoverin underwent red-shift to at 339 nm in the presence of Ca^{2+} (19). The half maximal Ca^{2+} concentrations of the emission changes at 370 nm were estimated at 0.73 μM for eS-modulin (+myr)

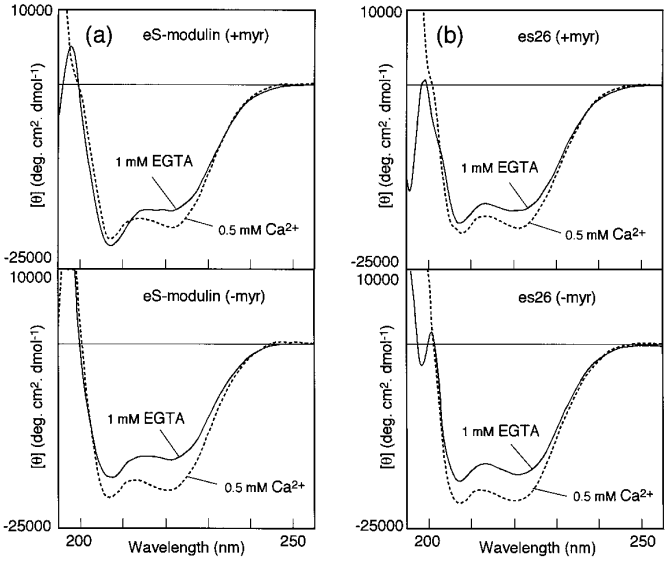

FIG. 3. CD spectral changes of myristoylated (+myr; upper panels) and unmyristoylated (−myr; lower panels) eS-modulin (a) and es26 (b), in the presence of 1 mM EGTA (solid lines) or 0.5 mM $CaCl_2$ (dashed lines).

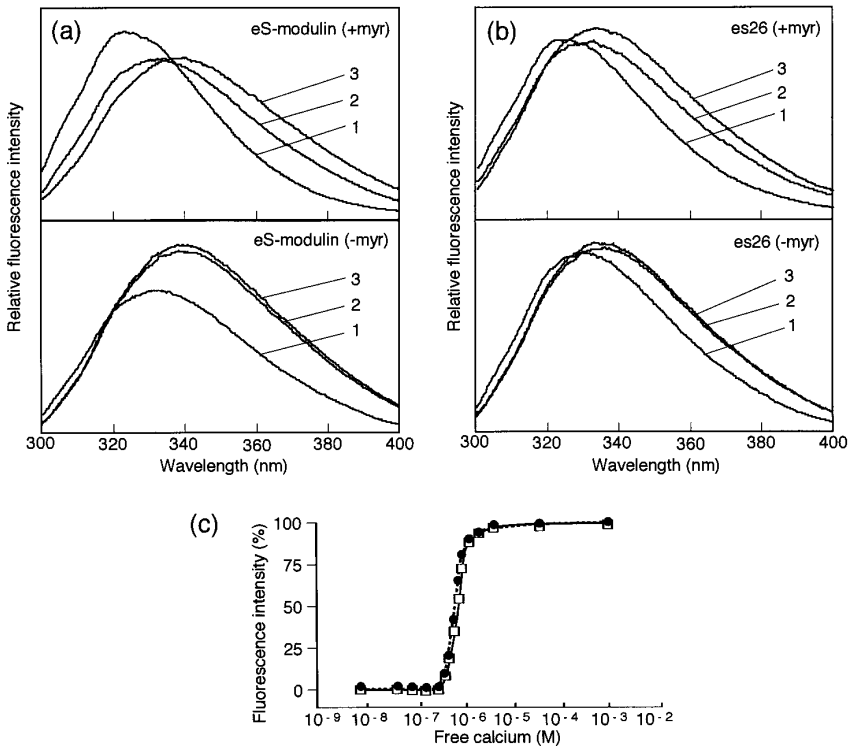

FIG. 4. Fluorescence emission spectra of myristoylated (+myr; upper panels) and unmyristoylated (−myr; lower panels) eS-modulin (a) and es26 (b), in the presence of 1 nM (curve 1), 0.77 μM (curve 2), or 1 mM (curve 3) free Ca^{2+}. (c) Emission intensity at 370 nm and titration curves for eS-modulin (+myr; open squares and solid line) and for es26 (+myr; closed circles and dashed line).

and 0.67 μM for es26 (+myr) (Fig. 4c). Similar values were calculated on monitoring either wavelength or integration of emission intensity from 350 to 400 nm.

DISCUSSION

es26 (+myr) inhibited rhodopsin phosphorylation to almost the same extent as eS-modulin (+myr) (Fig.2). This indicates that s26 is involved in the adaptation mechanism in cones, that is, by binding to the cone outer segment membrane, inactivates cone kinase (if it exists) and inhibits phosphorylation of cone pigments.

Recoverin is thought to bind two Ca^{2+} ions per molecule (20). In our preliminary experiments by the rapid flow dialysis method (21), both eS-modulin and es26 were shown to bind two Ca^{2+}. The peptide segments of S-modulin and s26 corresponding to the EF-2 and EF-3 regions of recoverin may bind Ca^{2+}. The half-maximal Ca^{2+} concentrations of bovine recoverin, which affect binding to phenyl agarose resin and the inhibition of rhodopsin phosphorylation, are reported to be 1.0 μM and 0.56 μM, respectively (22, 23). These are close to the values obtained for eS-modulin and es26 in the present experiments of the half-maximal emission changes (about 0.7 μM). S-modulin and s26 seem to have similar Ca^{2+}dependent physical properties irrespective of their localization in the retina. However, some difference between es26 and eS-modulin was found in response to the change of the local environment; that is, the Ca^{2+}-bound form of eS-modulin showed a slightly larger shift of the emission maximum than that of es26. Furthermore the CD spectral changes induced by Ca^{2+} concentrations also showed some difference between S-modulin and s26 (Fig.3). It is not yet clear if these differences have some relationship with the physiological differences between rods and cones.

A variety of proteins with diverse biological activities have their N-termini linked to fatty acids such as myristic acid (24). The (−myr) species of eS-modulin inhibited rhodopsin phosphorylation to almost the same extent as the (+myr) species, but the fatty acid modification of S-modulin is more likely to influence the membrane binding properties (22, 25) rather than the direct interaction with target proteins (26, 27). However, it has been suggested recently that *N*-myristoylation of recoverin enhances its efficiency as an inhibitor of rhodopsin kinase (28, 29).

It has been suggested that the myristoyl group is in contact with hydrophobic residues, including tryptophan, of recoverin in the Ca^{2+}-free form and is exposed to solvent in the Ca^{2+}-bound form (30). This conformational transition of recoverin is not grossly affected by the presence or absence of myristoylation (30). Bovine

recoverin, frog S-modulin and s26 have three conserved tryptophan residues at corresponding positions. The change of fluorescence emission spectra leads us to speculate on the Ca^{2+} dependent structural changes of S-modulin and s26 as follows. One or more tryptophan residues of both eS-modulin and es26, irrespective of the myristoylation, are in the more polar environments in the Ca^{2+}-bound form than in the Ca^{2+}-free form. The difference of the local environments between (+myr) and (−myr) species is larger in the Ca^{2+}-free form than in the Ca^{2+}-bound form. In these Ca^{2+}-binding proteins, the exposure of hydrophobic surfaces to solvent may play a more essential role in the inhibition of rhodopsin kinases than fatty acid modification of the proteins.

Our overexpression system of S-modulin or s26 with and without myrstoylation should enable more detailed structural analyses, by providing large enough samples for multidimensional nuclear magnetic resonance spectroscopy and X-ray crystallography. It may provide informations to understand the physiological differences between rods and cones.

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