Photoreceptor Protein s26, a Cone Homologue of S-modulin in Frog Retina*

(Received for publication, May 6, 1996, and in revised form, June 7, 1996)

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A frog retinal protein named s26 is a 26-kDa protein found during purification of S-modulin in frog retina (Kawamura, S. (1992) Photochem. Photobiol. 56, 1173-1180). To identify its role in frog retina, first s26 was purified to nearly homogeneity with three chromatographical steps. Based on the partial amino acid sequences of the proteolysed fragments of s26, we isolated cDNAs that encode s26. The analysis of its amino acid sequence revealed that s26 is an S-modulin-like protein, while its N-terminal region is much more homologous to visinin than to S-modulin. S-modulin is a Ca\(^{2+}\)-binding protein reported to be present in chicken cones, but its localization in the retina had been a subject in dispute. The present study showed that s26 is present in cone photoreceptors. The study also showed that s26 inhibits phosphorylation of rhodopsin after a light flash at high Ca\(^{2+}\) concentrations as S-modulin does. From these results, we concluded that s26 is a cone homologue of S-modulin. The result is consistent with the idea that each type of photoreceptors expresses each cell-type specific version of phototransduction proteins.

** This work was supported in part by Grants 07458173 and 07309013 from the Ministry of Education, Science and Culture of Japan and by grants from the Tokyo Biochemical Research Foundation and the Human Frontier Science Program Organization (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article therefore must be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** The abbreviations used are: R*, light-activated rhodopsin; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.

On light absorption, vertebrate rods hyperpolarize through a well-characterized phototransduction cascade (1-3). Photoreceptors not only respond to on and off of light, but also adapt to environmental light. The underlying mechanism of the light adaptation has been shown to be the decrease in the intracellular Ca\(^{2+}\) concentration in the rod outer segment during light. Previous studies showed that newly found frog retinal s26 (Lisson et al., 1994) is a cell type specific version of phototransduction proteins.

A protein named s26 (small 26-kDa protein) was found during purification of S-modulin (12). The molecular characteristics of s26 are similar to those of S-modulin in several aspects. For example, their apparent molecular weights are slightly different but close to 26 kDa on our SDS-PAGE gel and they both bind to a hydrophobic column, phenyl-Sepharose, in a Ca\(^{2+}\)-dependent manner (12). The major difference is that s26 is eluted from a DEAE column at higher salt concentrations than S-modulin. These results raised the possibility that s26 is either a chemically modified form of S-modulin or a molecule different from S-modulin having a similar or different function in photoreceptors or other cells in frog retina. A chicken photoreceptor protein, visinin, has been reported to be present in cones (13) and have similar activity as S-modulin (14). Therefore, one possibility is that visinin is a cone homologue of S-modulin and that s26 is frog visinin. However, the possibility that visinin is the cone homologue has been questioned because only the recoverin immunoreactivity has been found in both rods and cones in mammals (5, 15-17). Another possibility is that s26 localizes in some bipolar cells, because a recoverin-like protein is suggested to be present in these cells in human and monkey retinas (17). In the present study, to clarify these points, we first obtained purified s26 and then cloned its cDNAs. Furthermore, we examined a possible function and localization of s26 in frog retina.

** Experimental Procedures

Preparation of Frog Photoreceptor Proteins—Retinas were removed from 100 frogs (Rana catesbeiana) under room light. Photoreceptor outer segments were brushed off the retinas in 50 ml of a potassium gluconate buffer (115 mM potassium gluconate, 2.5 mM KCl, 2 mM MgCl\(_2\), 0.2 mM EGTA, 0.1 mM CaCl\(_2\), 10 mM HEPES, 1 mM dithiothreitol, pH 7.5; K-gluc buffer). The outer segments in the suspension were fragmented by passing through a 26-gauge needle several times, and then centrifuged (50,000 × g, 15 min). The supernatant was used for the source of photoreceptor soluble proteins.

Column Chromatography—A Phenyl Sepharose CL-4B (Pharmacia) column (inner diameter = 1 cm, length = 15 cm) was preequilibrated with a K-gluc buffer supplemented with 1 mM CaCl\(_2\) (1 mM Ca-K-gluc buffer). The Ca\(^{2+}\) concentration was also made 1 mM in the photoreceptor soluble protein fraction by adding 1 mM CaCl\(_2\) just before application to the column. After applying the sample to the column, the column was washed with 300 ml of the 1 mM Ca-K-gluc buffer. Then, to elute the bound s26 and S-modulin, a 40 mM K-gluc buffer supplemented with 3 mM EGTA (3 mM EGTA, 40 mM K-gluc buffer) was applied. (In the 40 mM K-gluc buffer, only the concentration of potassium gluconate was reduced to 40 mM in the K-gluc buffer.) A DEAE Sepharose (Pharmacia) column (inner diameter = 1 cm,
length (15 cm) was preequilibrated with the 3 mM EGTA, 40 mM K-gluc buffer. The S26 and S-modulin fraction obtained in the phenyl-Sepharose column chromatography was applied to the DEAE column. S-modulin was obtained in the pass-through fraction, and S26 was eluted by increasing NaCl concentration (see "Results").

A Mono Q column (Pharmacia; HR 5/5) placed in our HPLC system was preequilibrated with the 3 mM EGTA, 40 mM K-gluc buffer. After the salt in the S26 fraction obtained above was removed on an ultrafilter (Molcut L. LGC, Millipore), S26 was purified with the Mono Q column by increasing NaCl concentration from 0 to 200 mM in the 3 mM EGTA, 40 mM K-gluc buffer.

Recoverin was prepared as described previously (7). Determination of Partial Amino Acid Sequence of s26—Purified s26 was digested with lysyl endopeptidase (Wako) according to the manufacturer’s protocol. The peptide fragments were isolated by reversed phase HPLC (C18 ODS column, Nakalai) by applying 0–100% gradient of CH3CN in the presence of 0.1% trifluoroacetic acid. Major peak fractions were collected, and the amino acid sequences of these peptides were analyzed by a protein sequencer (Applied Biosystems model 473A).

Isolation of cDNA Clones of s26—We synthesized degenerate nucleotides corresponding to the region between the site specific to s26 and the region conserved among S-modulin, visinin, and recoverin (see "Results"). The synthesized degenerated oligonucleotides were used as the primers for PCR. The PCR products were then used for the search of cDNA encoding s26. Since the full-length cDNA of s26 was not found in our cDNA library, RACE-PCR (18, 19) was applied to determine the complete nucleotide sequence at both ends. In this PCR, frog retinal cDNA was prepared according to an ordinary method (20), and RVF1 (CGAAGCTT CATC-AGAGTATGA) and RVF2 (CGAAGCTTGGGAGGCTTCTAAG) primers were used for amplifications of the 3' and the 5' regions, respectively (21).

Rhodopsin Phosphorylation—Rhodopsin phosphorylation was conducted as described previously (6, 7). Calcium concentrations were adjusted by EGTA/CaCl2 buffer (6).

Antibody Production—A Japanese White rabbit was immunized by a synthetic peptide of s26. An s26 peptide (see "Results") was conjugated with maleimide-activated KLH (Pierce) through cysteine attached to the amino terminus of the peptide. Anti-s26 peptide antibody was affinity purified by its elution from a Western blot of purified s26. Anti-S-modulin antibody was raised against S-modulin in a mouse. Due to cross-reactivity of this antibody with s26, S-modulin antibody was preadsorbed by s26 before use.

Immunohistochemistry—Frogs were dark-adapted for at least 2 h, and the retina was removed with the aid of the IR converter. A piece of the retina was fixed in the dark night at 4 °C either with Omni Fix (Xenetics Biomedical Inc.) or Bodian II solution (70% ethanol, 5% acetic acid) and then cryosectioned. Immunoreactivity of anti-s26 antibody was detected by fluorescein isothiocyanate-labeled anti-rabbit IgG antibody (Zymed Laboratories Inc.) and anti-S-modulin antibody by Texas Red-labeled anti-mouse IgG antibody (Zymed Laboratories Inc.). Fluorescence was detected using a confocal microscope (Bio-Rad MRC 600 and MRC 600).

Evolutionary Distance Analysis among S-modulin in Family Proteins—Evolutionary distances of the sequences (k) were calculated for 192 amino acids in the regions from Met-1 to Val-192 of s26. In this calculation, we used the proportion value (p) determined for the two amino acids and took multiple substitution into consideration with an equation of 

\[
\ln (1 - p) \approx -p^2 + p/2
\]

(22). A phylogenetic tree was constructed by the neighbor-joining method (23) using chicken VILIP (24), bovine neurocalcin (25), and human hippocalcin (26) as outgroups.

results

Purification of s26—From a phenyl-Sepharose column, a fraction containing both S-modulin and s26 was eluted by the 3 mM EGTA K-gluc buffer (Fig. 1A). Then, s26 was separated from S-modulin by a DEAE column (Fig. 1B). S-modulin was eluted in the pass-through fraction at 40 mM potassium gluconate (indicated with both in the elution profile and SDS-PAGE in Fig. 1B), and s26 was eluted at higher salt concentrations (b). The protein s26 was further purified by a Mono Q column (Fig. 1C). We found two peaks, a and b, that showed the same apparent molecular mass on our SDS-PAGE (Fig. 1C, inset). Purified proteins in both peaks showed an apparent molecular mass slightly smaller than that of S-modulin (Fig. 1D). For this reason, we had named this protein small 26-kDa protein, s26 (12). The amount of s26 in the first peak of Mono Q column chromatography (Fig. 1C) was generally in excess to that of the second peak, and therefore, we used the first peak in the following study.

The molar abundance of s26 to that of rhodopsin or S-modulin was determined after the purification with DEAE Sepha-rose. The protein s26 was present at a population of 4.8 ± 1.0% of rhodopsin (n = 3) and 68.4 ± 3.8% of S-modulin (n = 4). The molar abundance of S-modulin to rhodopsin was 7.0 ± 1.1% (n = 3), and it was almost 10 times higher than our previous estimate (4). The overall molar abundance among rhodopsin, s26, and S-modulin was 100.7:5:1.

Amino Acid Sequence of s26—Our preliminary study showed that the N terminus of s26 is blocked. For this reason, partial amino acid sequences were first determined in the proteolyzed fragments of s26. Purified s26 was digested with lysyl endopeptidase, and the peptide fragments were isolated by reversed phase HPLC (peaks a–f in Fig. 2). The amino acid sequences of the six peptides were analyzed by a protein sequence analyzer (insets a–f at the top of Fig. 2). The result showed that the partial amino acid sequence of s26 is similar but definitely different from that of S-modulin, which showed that s26 is a protein distinct from S-modulin.

The amino acid sequence data of the six peptides were compared with those of S-modulin. Based on this comparison, PCR amplification of a partial cDNA clone of s26 was attained between two regions, one determined in peptide a (large dots in Fig. 3) and the other having consensus amino acid sequence.
cDNA of s26. A primer RVF1 (Fig. 3, broken arrow *) was used for determination of the 3'-end of s26. Degenerate oligonucleotides corresponding to these regions were synthesized and used as the primers. The PCR product was then used for the search of cDNAs encoding s26. The results, however, showed that our frog retinal cDNA library did not contain the full-length cDNA of s26. Then, RACE-PCR method was applied to determine the 5'-end of the cDNA of s26. A primer RFV1 (Fig. 3, broken arrow indicated by *) was used for determination of the 3'-end region and RVR1 (broken arrow indicated by **) of the 5' region (see "Experimental Procedures").

Fig. 3 shows the nucleic acid and the deduced amino acid sequences of s26. The first ATG is followed by an open reading frame of 588 bases and fulfills the Kozak criteria for the initiation signal in eukaryote (27). As this ATG being the translational initiation codon, this cDNA encodes 196 amino acids.

As underlined in Fig. 3, the deduced amino acid sequence in the cDNAs contained all the sequences found in the six peptide fragments of s26 shown in Fig. 2. In addition, the calculated molecular mass was 22,818, which is close to the molecular mass of s26 estimated by our SDS-PAGE. We, therefore, concluded that these cloned cDNAs encode s26.

In Fig. 4, the amino acid sequence of s26 was compared with those of S-modulin (7) and visinin (13). Similarly as in S-modulin and visinin, s26 contains three putative EF hand structures (Fig. 4, dotted lines) However, EF1 might be defective because of the cysteine residue in this structure, and EF2 and EF3 are probably the actual Ca$^{2+}$-binding sites (28). The N-terminal region of s26 has a consensus N-terminal myristoylation site (GXXXXS; broken line) (29); therefore, the terminal methionine would be cleaved, and the N-terminal glycine is modified by lipids. This idea is consistent with our finding that the N terminus of s26 is resistant to Edman degradation. Even though both s26 and S-modulin are found in frog retina, the amino acid sequence of s26 shows only 67% identity to S-modulin but 77% identity to visinin, a Ca$^{2+}$-binding protein reported to be present in chicken cones (Table I). The evolutionary distances calculated from the sequence data showed that s26 is a closer member to visinin than S-modulin. We also tried to obtain an anti-S-modulin antibody against a synthetic peptide of the corresponding region of S-modulin to examine the localization of S-modulin. However, the attempt failed. As an alternative, we obtained a polyclonal anti-S-modulin antibody raised against S-modulin whole protein. This antibody recognized both S-modulin and s26 (data not shown); for this reason, the S-modulin antibody was preadsorbed by s26 before use. This partially purified s26 antibody recognized S-modulin but not s26 on our Western blot (Fig. 6, middle three lanes).

Localization of s26 in frog retina was detected by fluorescence attached to the secondary antibody. Immunoreactivity of anti-s26 antibody was present in the photoreceptor layer in frog retina but not in other layers (Fig. 7). A magnified picture indicated that cones are immunoreactive but rods are not (Fig. 7B). The result, therefore, showed that s26 is expressed in cone photoreceptors. The immunoreactivity of s26 distributed uniformly within a cell. By comparing a transmission image with a fluorescence image, it was found that most of the immunoreactive cells had oil droplets and the cells having oil droplets were mostly immunoreactive.

Inhibition of Rhodopsin Phosphorylation by s26—From the similarity of the amino acid sequence, it was expected that s26 has a similar activity as S-modulin. Fig. 8 shows the autoradiogram of $^{32}$P incorporated into the rhodopsin band of our SDS-PAGE gel. As expected, s26 inhibited the rhodopsin phosphorylation at a high Ca$^{2+}$ concentration (10 μM) as S-modulin and recoverin did. The extent of inhibition by s26 was close to those of S-modulin and recoverin; inhibitions were 85% (S-modulin), 77% (recoverin), and 80% (s26).
DISCUSSION

In the present study, s26 in frog retina was purified (Fig. 1). The cDNAs of s26 were cloned, and the deduced amino acid sequence showed a higher homology to visinin (Figs. 4 and 5, Table I). Immunohistochemical analysis showed that the immunoreactivity of anti-s26 antibody was found in cones (Fig. 7). The purified s26 inhibited phosphorylation of rhodopsin at high Ca\(^{2+}\) concentrations as S-modulin does (Fig. 8). These results showed that s26 is frog visinin and is a cone homologue of S-modulin in frog retina. In addition, the immunoreactivity against anti-S-modulin antibody was found in rods (Fig. 7).

The rod-and-cone cell-type specific expression of S-modulin homologue—The present study showed that s26 is present in cones and S-modulin in rods in frog retina. This is the first demonstration that a different set of the S-modulin homologues is expressed in the same retina in a cell-type specific manner. The rod-and-cone cell-type specific expression is generally observed in the proteins involved in the phototransduction. For example, besides visual pigments, arrestin (30, 31), transducin (32, 33), cGMP phosphodiesterase (34, 35), and cGMP-gated channel (36) are expressed in a cell-type specific manner. In this sense, the result in the present study is consistent with previous studies. In mammalian retina, however, cone homologues of S-modulin have not been found. Namely, immunoreactivity of anti-recoverin antibody was found in both rods and cones in human, monkey, bovine and rat retinas (5, 15–17). Furthermore, immunoreactivity against an antibody specific to chicken visinin has not been found in human and bovine retinas (16), and an attempt to isolate visinin from bovine retina was unsuccessful (16). In mammals, therefore, recoverin might be expressed exceptionally in both rods and cones. However, the cone homologue may have not been detected because the cone homologue has high homology to recoverin and differs significantly from chicken visinin in its amino acid sequence.

The present study suggests that cell-type specific expression of S-modulin homologue is also present among cone cells in frog retina. In frog retina, three kinds of cone cells are present (37): single cones and double cones consisting of principal and accessory member. Single cones and the principal member of the

![Fig. 4. Alignment of amino acid sequences of S-modulin, s26, and visinin. A colon shows identical amino acid and dotted lines EF hand structures. Broken line (Gly-2 to Ser-6) shows a consensus N-terminal myristoylation site, and a line (Gln-174 to Met-183) shows the sequence used for generation of anti-s26 antibody.](http://www.jbc.org/)

| S-modulin | Recoverin | Visinin | s26 |
|-----------|-----------|---------|-----|
| %         | %         | %       | %   |
| S-modulin | 100       | 83      | 61  |
| Recoverin | 100       | 59      | 77  |
| Visinin   | 100       | 77      | 100 |

![Fig. 5. A phylogenetic tree of S-modulin protein family. According to the reported sequences of the proteins shown, evolutionary distances of the sequences were calculated. The sequences used were those of visinin (13), S-modulin (7), bovine recoverin (5), human recoverin (43), and mouse recoverin (44). Bar indicates 10% replacement of an amino acid per site (k = 0.1; see "Experimental Procedures").](http://www.jbc.org/)

![Fig. 6. Western blot analysis of antibodies raised against an s26 peptide and S-modulin molecule. Purified s26 (s26), S-modulin (S-mod.), and retinal homogenate (retina homog.) were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The transferred proteins were stained with Coomassie Brilliant Blue (left three lanes) or subjected to Western blot analysis by the anti-s26 (middle three lanes) and the anti-S-modulin antibodies (right three lanes). Immunoreactivity was detected by the ABC method and visualized with horseradish peroxidase-diaminobenzidine reaction.](http://www.jbc.org/)
double cone have oil droplets, but the accessory member does not. In the present study, most of the cones immunoreactive to anti-s26 antibody possessed oil droplets and inversely, cones having oil droplets were mostly immunoreactive to s26 antibody (see "Results"). In addition, we have not seen the immunostaining in the cells sitting attached to each other. These observations suggest that s26 is present in both single cones and the principal member of the double cone but not in the accessory member. In agreement with this view, microspectrophotometry has shown that the absorption maximum of the visual pigment in single cones is the same as that in the principal member but differs from that in the accessory member (38). The above consideration, therefore, suggests that another S-modulin-like protein is present in the accessory member of the double cone in frog retina.

In frog retina, another type of rod photoreceptor, green rods are present. In the present study, we did not observe rods immunoreactive to anti-s26 antibody possessed oil droplets and inversely, cones having oil droplets were mostly immunoreactive to s26 antibody (see "Results"). In addition, we have not seen the immunostaining in the cells sitting attached to each other. These observations suggest that s26 is present in both single cones and the principal member of the double cone but not in the accessory member. In agreement with this view, microspectrophotometry has shown that the absorption maximum of the visual pigment in single cones is the same as that in the principal member but differs from that in the accessory member (38). The above consideration, therefore, suggests that another S-modulin-like protein is present in the accessory member of the double cone in frog retina.

Function of s26—As shown in the present study (Fig. 8), s26 inhibits the phosphorylation reaction of rhodopsin at high Ca²⁺ concentrations. This observation is consistent with the previous finding that visinin and recoverin have the same effect on the prolongation of a photoresponse in gecko rods (14). S-modulin in frog rods has been postulated to be a Ca²⁺-dependent regulator of rhodopsin phosphorylation (6). Similarly, as S-modulin does, s26 would regulate the phosphorylation reaction of cone visual pigments in a Ca²⁺-dependent manner.

Two Peaks in Mono Q Column Chromatography—In the purification by the Mono Q column (Fig. 1C), s26 was purified in two peaks. Native recoverin can be separated into three peaks on reversed phase HPLC (39), probably because of the difference in the lipid (40) attached to the N-terminal glycine (41). In the Mono Q column chromatography, recoverin showed at least two peaks in its elution profile similarly as s26, and each of the two peaks showed three peaks on reversed phase HPLC. This result suggests that the separation of s26 and recoverin by the Mono Q column is not due to the difference in the lipid modification. The reason for the separation by the Mono Q column has yet to be determined.

Distribution of s26 and S-modulin in a Cell—According to the postulated function of s26 and S-modulin, these proteins are expected to be present mainly in the outer segment. However, it does not seem to be the case for the two lines of evidence. One is that immunoreactivities against s26 and S-modulin antibodies distributed throughout the cell (Fig. 7). This observation agrees with the previous studies done by others (5, 15, 16). However, this result does not always mean that these proteins distribute throughout the cell under in situ condition. Since S-modulin and s26 are essentially soluble, these proteins may diffuse from the outer segment to other parts of the cell during fixation. This possibility, however, is excluded by the second line of the evidence that the molar ratio of s26 to S-modulin is much higher than that of their corresponding visual pigments. The molar ratio of cone to rod visual pigment could be an indicative of the volume ratio of their outer segments. For this reason, if s26 and S-modulin are present exclusively in the outer segment, the molar ratio of s26/S-modulin should be similar as that of cone/rod visual pigment. According to the microspectrophotometric measurement in the retina of a frog (Rana pipiens), the molar ratio of cone visual pigment to that of rod visual pigment (rhodopsin) is ~0.2/1 (38), while s26/S-modulin ratio was ~7/10 in the present study (see "Results"). The population of cones is almost equal to that of rods in frog retina (38), and the sizes of the inner segment and cell body of a cone are not so different from those of a rod (rough estimation from electron micrographs in Ref. 37). For this reason, the ratio of s26/S-modulin (~7/10 in this study) could be explained by uniform spatial distribution of s26 and S-modulin in cones and rods, respectively.

The uniform distribution of these proteins may mean that these proteins not only inhibit phosphorylation of visual pigment but also have some other function(s) in other part of the cell. Alternately, the uniform distribution may just reflect that these proteins are essentially soluble. Further studies are required to solve this issue.

In Situ S-modulin Concentration—In our original isolation of S-modulin (4), the molar ratio of S-modulin to rhodopsin was...
allowing us to use a confocal microscope.

Acknowledgments—We thank Drs. Usukura and Obata at Nagoya University and Dr. Oghara at Osaka University for their help in the immunohistochemical work, and Prof. Kaneko at Keio University for

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~0.7/100, which gave a value of ~40 μM as the intracellular concentration of S-modulin. Later, we isolated recoverin from bovine retina (7) and obtained a recoverin/rod opsin ratio of ~9/100. This ratio was about 10 times higher than our previous estimate in frog retina and is close to the ratio obtained in the present study (~7/100; see “Results”). This difference in the yield of S-modulin and recoverin could be explained by the difference in the isolation procedure. In our original isolation, we used rod outer segments purified with sucrose flotation, and therefore S-modulin obtained was probably from the outer segment. In the latter study together with the present study, we did not purify the outer segments. Since S-modulin homologue seems to distribute uniformly in a cell as discussed above, it is likely that the homologue present in the part other than the outer segment was collected in the later studies. From the molar ratio of 7/100, we obtained a value of 140 μM as the intracellular concentration of S-modulin in a rod under the following assumptions. First, the total volume of the outer segment and rhodopsin is present at 3 mM concentration in the total volume of the outer segment (42). Second, S-modulin is present in the extracellular space (about 1/2 of the total outer segment volume; Ref. 42) and in the inner segment, cell body, and terminal. The estimated value of 140 μM is slightly higher than that obtained previously in purified rod outer segments (~40 μM), but the two values agree broadly.
Photoreceptor Protein s26, a Cone Homologue of S-modulin in Frog Retina
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J. Biol. Chem. 1996, 271:21359-21364.
doi: 10.1074/jbc.271.35.21359

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