Journal of Photoscience (2002), Vol. 9(2), pp. 281–283

Identification of Functional Site of S-Modulin[§]

Shuji Tachibanaki, Kumiko Nanda, Kenji Sasaki, and Koichi Ozaki[#], and Satoru Kawamura[#,*]
Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka, Japan

S-modulin in frog or its bovine homologue, recoverin, is a 26 kDa EF-hand Ca^{2+}-binding protein found in rod photoreceptors. The Ca^{2+}-bound form of S-modulin binds to rhodopsin kinase (Rk) and inhibits its activity. Through this regulation, S-modulin is believed to modulate the light-sensitivity of a rod. In the present study, we tried to identify the interaction site of the Ca^{2+}-bound form of S-modulin to Rk. First, we mapped roughly the interaction regions by using partial peptides of S-modulin. The result suggested that a specific region near the amino terminus is the interaction site of S-modulin. We then identified the essential amino acid residues in this region by using S-modulin mutant proteins: four amino acid residues were suggested to interact with Rk. These residues are located in a small closed pocket in the Ca^{2+}-free, inactive form of S-modulin, but exposed to the surface of the molecules in the Ca^{2+}-bound, active form of S-modulin. Two additional amino acid residues were found to be crucial for the Ca^{2+}-dependent conformational changes of S-modulin. The present study firstly identified the functional site of S-modulin, a member of a neuronal calcium sensor protein family.

Key words: S-modulin, rhodopsin kinase, functional site, calcium, light-sensitivity, rods

INTRODUCTION

In a rod photoreceptor, light activates rhodopsin and the activated rhodopsin (R*) triggers a series of reactions that results in a hyperpolarization of the cell through hydrolysis of cGMP [1-3]. R* is inactivated by phosphorylation by rhodopsin kinase (Rk). The Ca^{2+}-bound form of S-modulin (S-mod/Ca^{2+}) directly binds to Rk and inhibits the kinase activity [4]. R* phosphorylation is the inactivating mechanism of R*, its inhibition probably lengthens the lifetime of R* to increase the hydrolysis of cGMP. With this mechanism, S-modulin is thought to regulate light-sensitivity of a rod in a Ca^{2+}-dependent manner [5]. Since the intracellular Ca^{2+} concentration decreases during background illumination, S-modulin is believed to regulate the light-sensitivity of a rod during light-adaptation.

In this study [6], we tried to identify the interaction site of S-mod/Ca^{2+} to Rk. First we used S-modulin partial peptides to identify roughly the regions necessary for the S-modulin activity: we synthesized the S-modulin partial peptides and examined whether the peptide itself can inhibit R* phosphorylation. If such a peptide is present, it is probably present on the surface of S-mod/Ca^{2+} to interact with Rk. After we successfully identified several of the peptides, we obtained S-modulin mutant proteins expressed in *E. Coli* to search for the essential amino acid residues in these peptides. Our careful analysis led us to conclude that seven amino acids in an S-modulin molecule have essential roles in the interaction with Rk. Most of these residues are present in a small pocket in the Ca^{2+}-unbound (inactive) form of S-modulin but this region is exposed in the Ca^{2+}-bound (active) form. This region seems to be the interaction site of S-modulin to Rk.

*To whom correspondence should be addressed.
E-mail: kawamura@bio.sci.osaka-u.ac.jp
[#]Present address: Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan
[§]The study has been reported in ref. 6.

MATERIALS AND METHODS

Expression and Purification of S-modulin and Mutant Proteins. Site-directed mutagenesis was done as described [7].A DNA fragment encoding each mutant was inserted between the *Nco*I and *Xho*I site of a pET-16b (Novagen) plasmid vector. The sequence of the insert was checked before transfection to *Escherichia coli* (BL21DE3) by using ABI PRISM 310 genetic analyzer (Perkin Elmer). The cell was co-transfected with pBB131, an expression vector of N-myristoyltransferase. S-modulin and its mutant proteins were expressed [8], purified [9] and dissolved in a potassium-gluconate buffer (K-gluc buffer; 115 mM K-gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM EGTA, 10 mM HEPES, pH 7.5). The purified fractions contained 98 %< of S-modulin or its mutant judging from the CBB staining after SDS-PAGE.

The sample was frozen in liquid nitrogen and stored at -80 °C until use.

S-modulin Partial Peptides. Peptides were synthesized and purchased from Sawady Technology, Tokyo.

Isolation of Rod Outer Segment (ROS) Membranes and Phosphorylation Assay. ROS membranes of a frog (*Rana catesbeiana*) were isolated as previously described [9]. All of the manupilations were done in complete darkness with the aid of an infrared image converter (NVR 2015, NEC).

S-modulin, its mutant or a partial peptide of S-modulin was mixed with ROS membranes in complete darkness. Then, the mixture was placed on ice and irradiated with a 100-W tungsten-halogen lamp for 1 min (~100 % bleach), and incubated at room temperature for 9 min so that the reactions in the mixture were quasi-equilibrated. The phosphorylation reaction was done as described [9]. After SDS-PAGE the amount of ^{32}P incorporated into rhodopsin was quantified by using an image analyzer (BAS 2000, Fuji Film).

^{45}Ca-Binding Assay and Measurement of Circular Dichroism Spectrum. Ca^{2+}-binding assay, and measurement of circular dichroism (CD) spectrum were done as described elsewhere [10].

RESULTS AND DISCUSSION

Mapping of the Region(s) Necessary for the interaction of S-modulin to Rk.

By using partial peptides of S-modulin, we first mapped roughly the S-modulin region(s) necessary for the interaction with Rk. The entire amino acid sequence of S-modulin was divided into 11 portions. Each portion containing ~20 amino acid residues was synthesized.

Using each peptide, we examined whether it inhibits rhodopsin phosphorylation by itself. If such a peptide is present, this peptide is probably at the surface of S-mod/Ca^{2+} to interact with Rk. Fig. 1 represents the four peptides that showed the inhibition of rhodopsin phosphorylation. In the Ca2+-free, inactive form of S-modulin (A; 1IKU3 [11]), all of the four peptides (shown in black and dark gray) are positioned separately. However, in the Ca2+-bound, active form of S-modulin (B; 1JSA13 [12]), three of them (shown in black) come close to each other and form a cluster. Because these three peptides by themselves inhibited rhodopsin phosphorylation, it is likely that the cluster formed by the three peptides is a region of S-modulin to interact with Rk. One peptide (shown in dark gray) does not participate in the formation of the cluster.

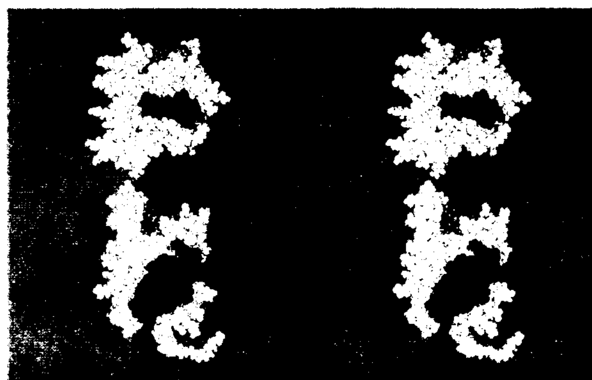

Fig. 1 Map of the peptides inhibiting the Rk activity. Four peptides inhibited the Rk activity. All of the four peptides (shown in black and dark gray) are separated in the Ca^{2+}-free, inactive form of S-modulin but three of them (black) form a cluster in the Ca^{2+}-bound, active form.

The inhibition of rhodopsin phosphorylation by this peptide is probably non-specific.

Amino Acid Residues Responsible for interaction with Rk.

In the amino acid sequences in the three peptides that form a cluster in the Ca^{2+}-bound form of S-modulin, we expressed a mutant S-modulin o identify the amino acid residues responsible for the interaction with Rk. In all of the three peptides, a candidate amino acid residue was determined and substituted by alanine. We measured the effect of each mutant on rhodopsin phosphorylation.

Among fifteen mutant proteins raised, nine of them lost the S-modulin activity (inhibition of rhodopsin phosphorylation), although the extent of the effect was dependent on each mutation. These residues, therefore, are the candidates that are involved in the interaction with Rk

There are many possible mechanisms that account for the loss of the S-modulin activity in a given mutant. We thought three possibilities: (1) loss of the Ca^{2+} binding ability, (2) loss of the ability of the conformational change to the active form of S-modulin, (3) loss of the amino acid residues responsible for the interaction with Rk. We carefully excluded the possibility of (1) by measuring the number of the Ca^{2+} ions bound to a mutant protein, and (2) by measuring the CD spectral changes accompanied by Ca^{2+}-binding.

Interaction Site of S-modulin to Rk.

Fig. 2 summarizes the localization of the amino acid residues that are most probably the site of S-modulin to

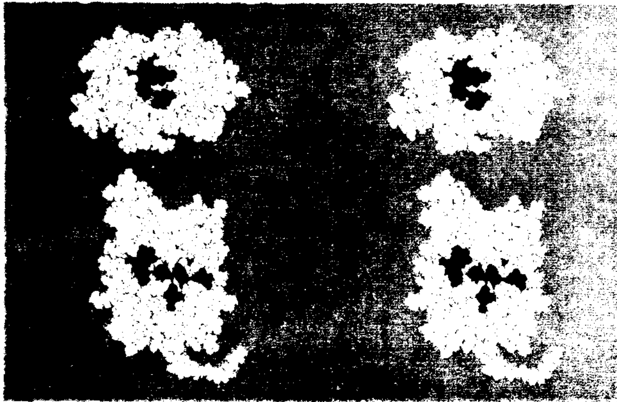

Fig. 2 Map of the amino acid residues responsible for interaction with Rk. A total of seven amino acids (shown in black) are found to be responsible for interaction with Rk.

interact with Rk. These residues are present in a closed pocket in the Ca^{2+}-free form of S-modulin (A), but they appear on the surface around the edge of a groove in the Ca^{2+}-bound form (B).

As far as we have examined, all of the S-modulin family proteins show the S-modulin activity [13]. Therefore, the interaction site identified in this study would be generally found in the S-modulin family proteins.

REFERENCES

1. Kawamura, S. (1995) Phototransduction, excitation and adaptation. In: *Neurobiology of the outer retina.* Ed. by Archer, S., Djamgoz, M. and Vallerga, S., Chapman & Hall, London, pp.105-131.
2. Pugh, E. N. Jr., and Lamb, T. D. (2000) Phototransduction in vertebrate rods and cones: Molecular mechanisms of amplification, recovery and light adaptation. Handbook of Biological Physics. 3, 183-255.
3. Burns, M. E. and Baylor, D.A. (2001) Activation, deactivation, and adaptation in vertebrate photoreceptor cells. *Annu Rev Neurosci.* 24, 779-805.
4. Kawamura, S and Tachibanaki, S. (2002) Chapter 4: S-Modulin. In: *Photoreceptors and Calcium.* Ed. by Baehr, W. and Palczewski, K., Landes Bioscience.
5. Kawamura, S. (1993) Rhodopsin phosphorylation as a mechanism of cGMP phosphodiesterase regulation by S-modulin. *Nature*, 362, 855-857.
6. Tachibanaki, S., Nanda, K., Sasaki, K., Ozaki, K. and Kawamura, S. (2000) Amino acid residues of S-modulin responsible for interaction with rhodopsin kinase. *J. Biol. Chem.*, 275, 3313-3319.
7. Matsuda, S., Hisatomi, O., Ishino, T., Kobayashi, Y., and Tokunaga, F. (1998) The role of calcium-binding sites in S-modulin function. *J. Boil. Chem.* 273, 20223-20227
8. Hisatomi, O., Ishino, T., Matsuda, S., Yamaguchi, K., Kobayashi, Y., Kawamura, S., and Tokunaga, F. (1997) Functional expression and characterization of frog photoreceptor-specific calcium-binding proteins. *Biochem. Biophys. Res. Commun.* 234, 173-177.
9. Sato, N. and Kawamura, S. (1997) Molecular mechanism of S-modulin action: Binding target and effect of ATP. *J. Biochem.* 122, 1139-1145.
10. Miwa, N., Shinmyou, Y. and Kawamura, S. (2001) Calcium-binding of p26olf, an S100-like protein in the frog olfactory epithelium. *Eur. J. Biochem.* 268, 6029-6036.
11. Tanaka, T., Ames, J. B., Harvey, T. S., Stryer, L., and Ikura, M. (1995) Sequestration of the membrane-targeting myristoyl group of recoverin with rhodopsin kinase. *Nature* 376, 444-447.
12. Ames, J. B., Isima, R., Tanaka, T., Gordon, J. I., Stryer, L., and Ikura, M. (1997) Molecular mechanics of calcium-myristoyl switches. *Nature* 389, 198-202.
13. De Castro, E., Nef. S., Fiumelli, H., Lentz, S.E., Kawamura, S. and Nef, P. (1995) Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors. *Biochem. Biophys. Res. Commun.*, 216, 133-140.