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Amino Acid Residues in GRK1/GRK7 Responsible for Interaction with S-Modulin/Recoverin†

Aya Torisawa[1], Daisuke Arinobu[1], Shuji Tachibanaki[1,2] and Satoru Kawamura*[1,2]

[1]Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan
[2]Department of Biology, Graduate School of Science, Osaka University, Osaka, Japan

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ABSTRACT

GRK1 is a visual pigment kinase in rods and is essential for inactivation of light-activated rhodopsin. The GRK1 activity is inhibited by binding of the Ca^{2+}-bound form of S-modulin/recoverin. We previously identified the S-modulin/recoverin site to interact with GRK1. In the present study, we identified its counterpart in GRK1. We synthesized 29 of GRK1 or GRK7 partial peptides that cover the entire sequence of GRK1/GRK7, and examined whether these peptides inhibit S-modulin/recoverin activity most probably by preoccupying the binding site for GRK1. The inhibition was the greatest with the N-terminal peptide (p1, aa 3–23 in GRK7). On mutation of each of eight amino acid residues highly conserved in the p1 region of more than 10 orthologs, the inhibition was significantly reduced in the mutation of Leu^6, Asn^{12} and Tyr^{15}. We further examined the binding of the peptides, including mutated ones, to S-modulin/recoverin with a resonance mirror biosensor. The binding correlated well with the degree of the inhibition by a peptide. The inhibition, therefore, seemed to be due to a direct binding of the kinase peptide to the binding site of active S-modulin/recoverin. A GRK1 region close to its C-terminus also seemed to be the binding site for S-modulin/recoverin.

INTRODUCTION

On light absorption, visual pigment becomes activated and triggers a series of reactions in the phototransduction cascade to hydrolyze cGMP. The reduction in the cGMP concentration induces closure of a cGMP-gated cation channel, resulting in membrane hyperpolarization. After cessation of light, all of the reactions in the cascade need to be inactivated to restore the dark state. Inactivation of activated visual pigment is achieved first by phosphorylation of visual pigment with G-protein-coupled receptor kinase (GRK1 in rods and GRK7 in cones in many animals) and then arrestin binds to the phosphorylated pigment to completely shut off its activity (1,2).

Photoreceptors not only respond to the on and the off of the light stimulus but also adapt to environmental light conditions by changing the Ca^{2+} concentration in the outer segment. S-modulin in frog (3,4), or its bovine ortholog, recoverin (5,6), is an EF-hand Ca^{2+}-binding protein that regulates the phosphorylation of visual pigments in a Ca^{2+}-dependent manner. At high Ca^{2+} concentrations in the dark, the Ca^{2+}-bound form of S-modulin/recoverin directly binds to GRK1 to inhibit the kinase activity (7–9). With this inhibition, it was expected that S-modulin/recoverin prolongs the lifetime of activated visual pigment and therefore prolongs the duration of a photoresponse under dark-adapted conditions. Recent studies on recoverin-knock out mice showed that it is actually the case (10,11). When the cell is light adapted and the Ca^{2+} concentration is decreased, S-modulin/recoverin is expected not to inhibit the visual pigment phosphorylation so that the photoresponse recovery time course will be speeded up. As this type of facilitation is found in recoverin-knock out mice even when rods are dark-adapted, it is highly probable that S-modulin/recoverin is involved in this type of regulation.

In our previous study (12), we identified several S-modulin/recoverin amino acid residues that are responsible for the interaction with GRK1. Based on the 3D structure of recoverin (13,14), it was found that these amino acid residues reside at the edge of a groove that is exposed to the surface of the recoverin molecule when it binds two Ca^{2+}. The 3D structure of GRK1 is not known, and therefore, the structural basis of the interaction of GRK1 to S-modulin/recoverin cannot be predicted. In the present study, therefore, we searched for the interaction site in the kinase, the counterpart of the binding site of S-modulin/recoverin. For this purpose, we synthesized partial peptides of GRK1 or those of GRK7 depending on the solubility of each peptide, and measured the effect of a partial peptide of GRK1/GRK7 on S-modulin/recoverin activity. If a kinase peptide inhibits S-modulin/recoverin activity, the peptide is thought to preoccupy the binding site of the active, Ca^{2+}-bound form of S-modulin/recoverin to hamper the effect of S-modulin/recoverin on the kinase. In addition, the binding of a peptide to the Ca^{2+}-bound form of S-modulin/recoverin was quantified directly with a resonance mirror biosensor (IAsys). Our study showed that in GRK1/GRK7, there are several regions that interact with S-modulin/recoverin, and that in the N-terminal region, Leu^6 (L6), Asn^{12} (N12) and Tyr^{15} (Y15) are involved in the interaction. Furthermore, a region close to the GRK1 C-terminus seems to be involved in the binding to S-modulin/recoverin.

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*Corresponding author email: kawamura@fbs.osaka-u.ac.jp (Satoru Kawamura)

MATERIALS AND METHODS

Expression and purification of S-modulin. Recombinant myristoylated S-modulin was used in the present study. It was overproduced in *Escherichia coli* and purified with a phenyl Sepharose column as described previously (19). Purified S-modulin was suspended in a potassium gluconate buffer (K-gluc buffer; 115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM EGTA, 10 mM HEPES, 1 mM dithiothreitol, pH 7.5).

Preparation of rod membranes. Rod membranes of a frog (*Rana catesbeiana*) were prepared according to the method of Tachibanaki *et al*. (12). The animals (frog and carp; see below) were cared for in accordance with our institutional guidelines. Rod membranes were suspended in K-gulc buffer so that the concentration of rhodopsin was 50 μM. The rod membranes were stored at −80°C until use. All of these procedures were carried out in complete darkness with the aid of an infrared image converter (NVR 2015; NEC, Tokyo, Japan).

Preparation of cone membranes. Cones were purified from carp (*Cyprinus carpio*) according to the method of Tachibanaki *et al*. (15,16). Cone membranes were prepared from purified carp cones. The purified cones were frozen, freeze-thawed and washed twice with K-gluc buffer. The thawed membranes were further washed to completely remove s26 (cone S-modulin; [17]) with an EGTA buffer (115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 1 mM dithiothreitol, pH 7.5). The membranes were frozen and freeze-thawed. This wash was repeated twice, and finally the membranes were suspended in K-gluc buffer. An aliquot of the washed membranes was used to quantify the content of each type of cone visual pigment in the cone membranes. The cone membranes were stored at −80°C until use. All of these manipulations were performed in complete darkness with the aid of an infrared image converter (NVR 2015; NEC).

Phosphorylation assay. A peptide was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 5 mM, and then an equal volume of a peptide buffer solution (20 mM HEPES, 240 mM KCl, 40 mM $MgCl_2$, 0.004% Tween 80, pH 7.5) was added to obtain a peptide solution. A reaction mixture was prepared by mixing 5 μL of a peptide solution, 2.5 μL of an S-modulin solution containing 50 μM S-modulin dissolved in K-gluc buffer, 2.5 μL of a Ca^{2+} buffer solution (see below) and 5 μL of rod membranes. The reaction mixture was irradiated with white light on ice for 1 min to fully activate rhodopsin. After it was left at room temperature for 8 min, an ATP solution (10 μL) containing 0.25 mM [γ-^{32}P] ATP, 1.25 mM GTP and 40 mM $MgCl_2$ dissolved in K-gluc buffer was added to 15 μL of the reaction mixture to initiate the phosphorylation reaction. The final concentrations of the chemicals were: 0.5 mM peptide, 5 μM S-modulin, 10 μM rhodopsin, 0.002% (wt/vol) Tween 80, 10% (vol/vol) DMSO, 20 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (24 MBq μmol^{-1}) and 0.5 mM GTP. The Mg^{2+} concentration used was 20 mM, because in the peptide binding measurement (see below), this high concentration of Mg^{2+} was necessary to measure the effect of Ca^{2+} (18). Ca^{2+} concentrations were buffered at a low concentration (30 nM; 80 μM $CaCl_2$ plus 560 μM EGTA) or at a high concentration (120 μM; 320 μM $CaCl_2$ plus 200 μM EGTA) by adding a Ca^{2+}-buffer solution containing 0.1 mM $CaCl_2$ and 4.2 mM EGTA, or that containing 2.5 mM $CaCl_2$ and 0.6 mM EGTA, respectively, in a HEPES buffer (10 mM HEPES 120 mM KCl, 20 mM $MgCl_2$, 0.002% Tween 80, pH 7.5). The phosphorylation reaction was performed using endogenous GRK1 in the frog rod membranes. At 2 min after the addition of the ATP solution to the reaction mixture, the reaction was terminated by the addition of 150 μL of 10% (vol/vol) trichloroacetic acid. After centrifugation (20 000 ***g***, 10 min), the precipitate was subjected to SDS-PAGE, and the amount of ^{32}P incorporated into the rhodopsin band was quantified by using an image analyzer (BAS 2500; Fuji Film, Tokyo, Japan). Phosphorylation assays in cone membranes were performed similarly according to the method reported previously (15,16).

Peptide binding measurement. Ca^{2+}-dependent binding of a partial peptide of GRK1/GRK7 to S-modulin was monitored by a resonant mirror biosensor (IAsys; Affinity Sensors, Cambridge, UK). S-modulin was immobilized to carboxymethylated dextran (CMD) on the surface of an IAsys cuvette (S-modulin cuvette) according to the manufacturer's protocol. Succinimide ester-activated CMD was linked to a thiol group of S-modulin through a linker, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL). In previous studies (19,20), recoverin was linked similarly to CMD through C39, the only cystein residue in recoverin. In S-modulin, however, there are two cystein residues. The 3D structure of recoverin, the bovine ortholog of frog S-modulin, indicates that C39 is exposed to the surface of the molecule and that S107, which is replaced by C107 in S-modulin, is also present at the surface, although the latter seems to be surrounded by other residues. It is possible that either of the two cystein residues was linked to CMD in our present study, but both C39 and S107 (C107 in S-modulin) are located at the opposite side of the molecule with respect to the reported binding site of S-modulin/recoverin to GRK1 (12). For this reason, we believe that the linkage by the linker molecule probably does not affect the binding of a GRK1/GRK7 peptide to the binding site in an S-modulin molecule.

For the measurement of a Ca^{2+}-dependent binding of a peptide to S-modulin, we first added 75 μL of a low Ca^{2+} solution (10 mM HEPES, 120 mM KCl, 20 mM $MgCl_2$, 0.002% Tween 80, 100 μM EGTA, pH 7.5) to the S-modulin cuvette and after stabilization of the baseline, we added 5 μL of a peptide solution containing 1 mM peptide in the HEPES buffer. The binding signal was monitored for 2–3 min. On addition of a peptide, the signal increased most probably because of the increase in the refractive index introduced by the peptide. The cuvette was then washed twice with the low Ca^{2+} solution. The cuvette was then equilibrated with 75 μL of a high Ca^{2+} solution (10 mM HEPES, 120 mM KCl, 20 mM $MgCl_2$, 0.002% Tween 80, 100 μM $CaCl_2$, pH 7.5), and then 5 μL of a peptide solution was added. When the binding level was higher in the high Ca^{2+} solution, we regarded it a binding of that peptide to the binding site of S-modulin (see Results and Discussion). All measurements were performed at 25°C.

Peptides. GRK1/GRK7 peptides were from GeneDesign (Ibaraki, Osaka, Japan), Sawady Technology (Tokyo, Japan) or GenScript (Scotch Plains, NJ). The purity of a peptide was > 75%.

RESULTS AND DISCUSSION

Survey of the site in GRK1 interacting with the Ca^{2+}-bound form of S-modulin

GRK1 binds to the Ca^{2+}-bound form of S-modulin most probably at the site identified previously in S-modulin (12). Using partial peptides of GRK1, we tried to identify the counterpart in GRK1. A GRK1 partial peptide that has a site to interact with S-modulin probably preoccupies the binding site in S-modulin and reduces or inhibits S-modulin activity.

The entire amino acid sequence of bovine GRK1 was divided into 29 peptides (Fig. 1a, peptide 1–peptide 29; p1–p29), each consisting of approximately 20 amino acid residues. Some of the peptides (p1, p7 and p8) were very hydrophobic so that the synthesis of such a peptide seemed to be difficult. Because the visual pigment kinase in carp cones (carp GRK7) is also inhibited by S-modulin at high Ca^{2+} concentrations (Fig. 1b), the binding site of GRK7 to the Ca^{2+}-bound form of S-modulin would be similar to that of GRK1. In the case of carp GRK7, the hydrophobicity is lower in p1, p7 and p8, and thus the synthesis seemed to be easier. Therefore, we used the sequence in carp GRK7 to obtain these peptides. In GRK1, the N-terminus cystein in carp GRK7 is not present, and therefore, in most of the studies, we used p1 that lacked the N-terminus cystein.

First, we excluded peptides that nonspecifically inhibited GRK1 activity. We measured rhodopsin phosphorylation in frog rod membranes in the presence of each peptide at low Ca^{2+} concentrations. As shown in Fig. 2, phosphorylation was severely inhibited by some of the peptides. We set a criterion level at 60% to decide which peptides we should continue to survey, and examined 19 peptides that exceeded this level (see Fig. 3). Accordingly, we did not study further the

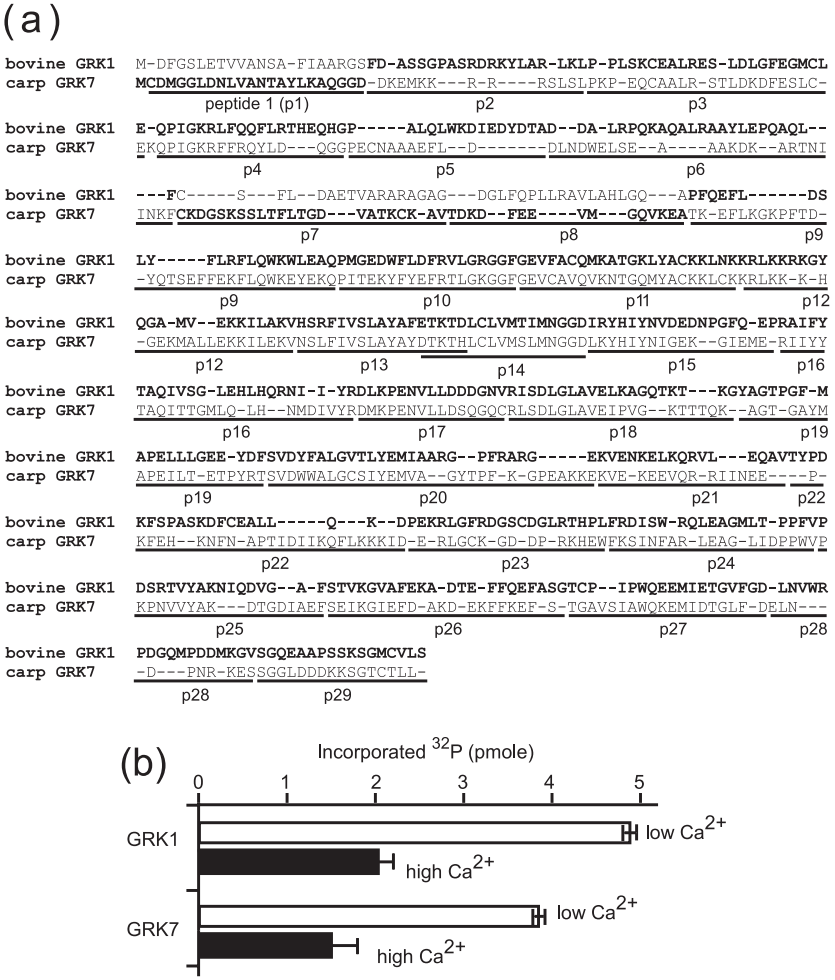

Figure 1. Partial peptides of GRK1/GRK7. (a) The amino acid sequence of bovine GRK1 and that of carp GRK7 were divided into 29 portions, each of which contained about 20 amino acid residues. Peptide 1 (p1) through peptide 29 (p29) were synthesized mostly based on the sequence of bovine GRK1, but in some of the peptides (p1, p7 and p8), we used the sequence of carp GRK7 (see text). Amino acid residues in the synthesized peptides are indicated in bold. (b) Inhibition of frog GRK1 and carp GRK7 by frog S-modulin. Visual pigment phosphorylation was measured at low (10–30 nM, open bar) and high Ca^{2+} (100–200 μM, filled bar) concentrations in the presence of 5 μM frog S-modulin. The result is shown as a mean ± standard deviation ($n = 3$ for both GRK1 and GRK7).

peptides that inhibited the phosphorylation below this level (p4, p7, p9, p10, p11, p13, p14, p16, p26 and p27).

To study whether a peptide inhibits S-modulin activity, we defined the S-modulin activity as follows. S-modulin inhibits rhodopsin phosphorylation at high Ca^{2+} concentrations (Fig. 3a). S-modulin activity was defined as the extent of inhibition of rhodopsin phosphorylation—the reduction in phosphorylation observed under high Ca^{2+} conditions was divided by the total amount of phosphorylation obtained under low Ca^{2+} conditions (*i.e.* the maximum GRK1 activity). However, the maximum GRK1 activity varied among the peptides examined (Fig. 2). Therefore, to quantify the effect of a peptide, we always performed control measurements in the absence of a peptide, and the relative S-modulin activity in the presence of a peptide was expressed as percent of the control. For example, in the result shown in Fig. 3a, the S-modulin activity in the absence of a peptide (no peptide) was calculated to be 65% (at a high Ca^{2+} concentration, rhodopsin phosphorylation was 35% of that at a low Ca^{2+} concentration). In the presence of p1, although the maximum phosphorylation level was decreased, the S-modulin activity was inhibited to 16% (at a high Ca^{2+} concentration, the phosphorylation level was 84% of that at a low Ca^{2+} concentration). From these values, the relative S-modulin activity in the presence of p1 was calculated to be 25% (*i.e.* 16%/65%).

Figure 3b shows that most of the peptides were not effective, but some of them did inhibit S-modulin activity. Among them, p1 was most effective—it reduced S-modulin activity to 25% of the control. Other peptides such as p3, p24 and p25 were moderately effective. These results suggest that p1 (aa 3–23 in carp GRK7), p3 (aa 42–64 in bovine GRK1), p24 (452–470) and p25 (471–487) bind to the binding site of the Ca^{2+}-bound form of S-modulin. Because the inhibition was the greatest in the presence of p1, we further analyzed which amino acid residues in p1 are responsible for the inhibition of S-modulin activity.

Identification of the amino acid residues in p1 responsible for the inhibition of S-modulin activity

S-modulin has been shown to inhibit visual pigment kinase not only in rods of frog (4), bovine (6) or carp (D. Arinobu,

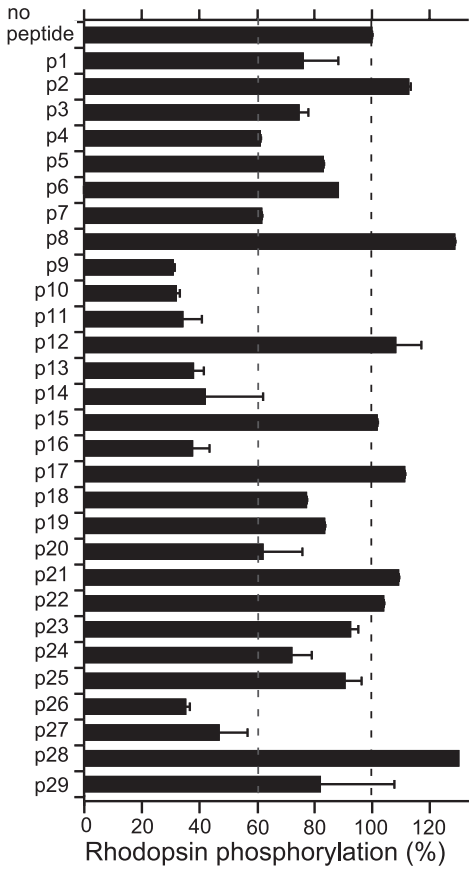

Figure 2. Effects of partial peptides on rhodopsin phosphorylation. Partial peptides, p1–p29 shown in Fig. 1a, were added to frog rod membranes at a low Ca^{2+} concentration and rhodopsin phosphorylation was measured. The phosphorylation level without the addition of a peptide was set to 100% and the phosphorylation in the presence of a peptide is expressed as a relative value to this control. Error bars represent standard deviations (n = 5 for p1; n = 3 for p3, p24, p25 and p29; and n = 1 for the others; triplicate determinations were repeated independently n times). The dotted line at the 60% level indicates a criterion level (see text). The final concentration of each peptide was 0.5 mM throughout this study.

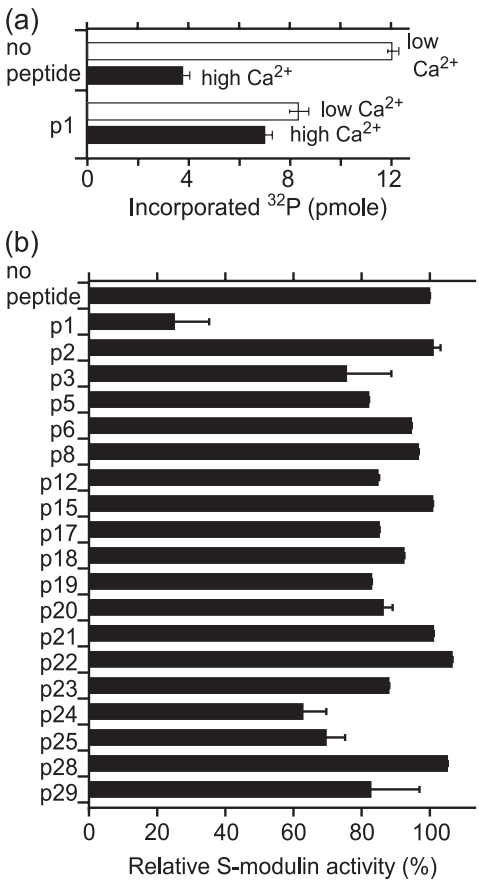

Figure 3. Inhibition of S-modulin activity by partial peptides. (a) Control phosphorylation levels in the presence of S-modulin without the addition of a peptide (no peptide) or with p1 (p1) at a low (open bar) and a high (filled bar) Ca^{2+} concentration. Relative S-modulin activity was defined based on these measurements (see text). (b) Relative S-modulin activity determined in the presence of a peptide indicated. The activity without the addition of a peptide was set to 100%. Result is shown as a mean ± standard deviation (n = 5 for p1 and n = 3 for the others; triplicate determinations were repeated independently n times). The final concentration of each peptide was 0.5 mM throughout this study.

S. Tachibanaki and S. Kawamura, unpublished), but also in carp cones (Fig. 1b). From these results, it is highly possible that the amino acid residues in GRK1/GRK7 responsible for the interaction with S-modulin are conserved in the GRK1/GRK7 protein family. Based on this idea, we selected eight highly conserved amino acid residues in p1 (Fig. 4a, boxed), and mutated each of them to alanine or threonine. Using these single-mutated peptides, we found that mutations of leucine at the position of 6 to alanine (L6A), asparagine at 12 to alanine (N12A) and tyrosine at 15 to alanine (Y15A) introduced the loss of the effect of p1 (Fig. 4b). The result indicated that these amino acid residues in p1 are important for the interaction with S-modulin. The effect was large in L6A and Y15A mutation. Other mutations did not affect the activity of p1 significantly (Fig. 4b).

Two or three of these three amino acids were then mutated at once to examine whether all of the three amino acids act together (L6A/N12A, N12A/Y15A, L6A/Y15A and L6A/N12A/Y15A mutations). The result showed that in most of the double mutations, the effect was larger than that observed with the peptide of a single mutation. In the peptide of triple mutation, the activity of p1 was lost almost completely. The result, therefore, indicated that L6, N12 and Y15 in p1 act together to inhibit S-modulin activity.

In p24 and p25, we tried to determine the amino acid residues responsible for the inhibition of S-modulin activity by using mutant peptides similar to the study of p1. However, the effects of p24 and p25 were not as large as that of p1 (see Fig. 3b), hence the effect of a mutation was not clearly detected. We, therefore, did not study p24 and p25 further.

Binding of effective peptides to the Ca^{2+}-bound form of S-modulin

The above studies suggest that the three amino acid residues in p1 are essential for the binding of GRK1/GRK7 to the Ca^{2+}-bound form of S-modulin. We then directly measured the binding of a peptide to the Ca^{2+}-bound form of S-modulin by using a resonant mirror biosensor (IAsys). S-modulin

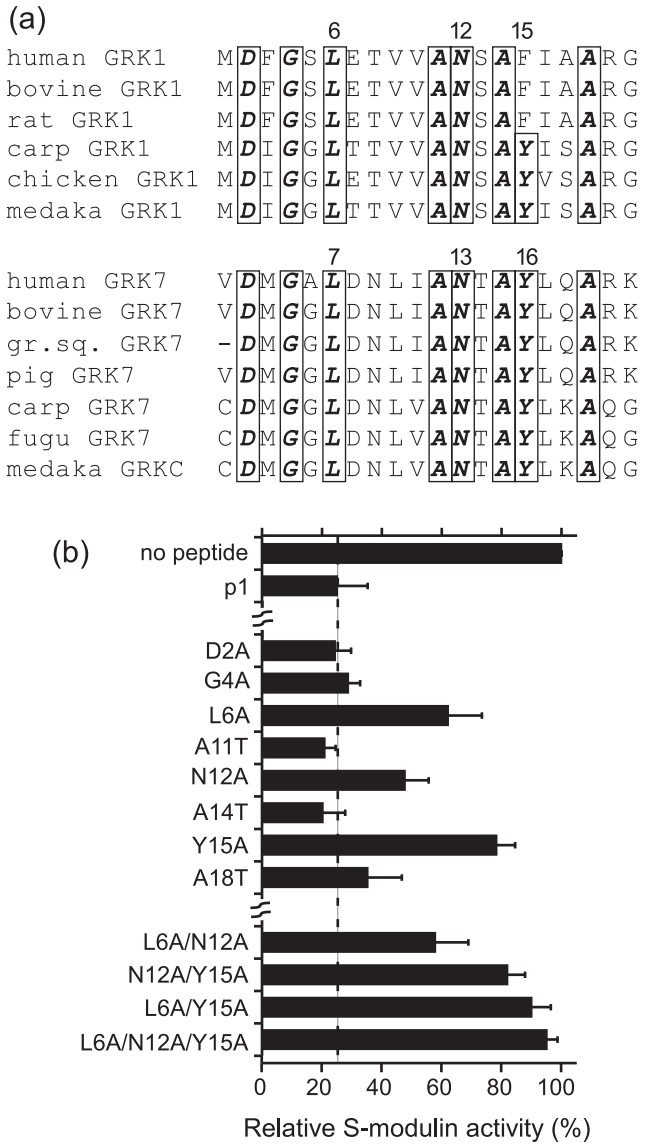

Figure 4. Inhibition of S-modulin activity by mutated peptides of p1. (a) The sequences of p1 region of GRK1 and GRK7 in various animal species. gd.sq. = ground squirrel. Eight amino acid residues (boxed) are highly conserved in this protein family. (b) Relative S-modulin activity was determined in the presence of a mutated peptide indicated. The activity without the addition of a peptide was set to 100%. Result is shown as a mean ± standard deviation (n = 5 for p1 and n = 3 for the others; triplicate determinations were repeated independently n times).

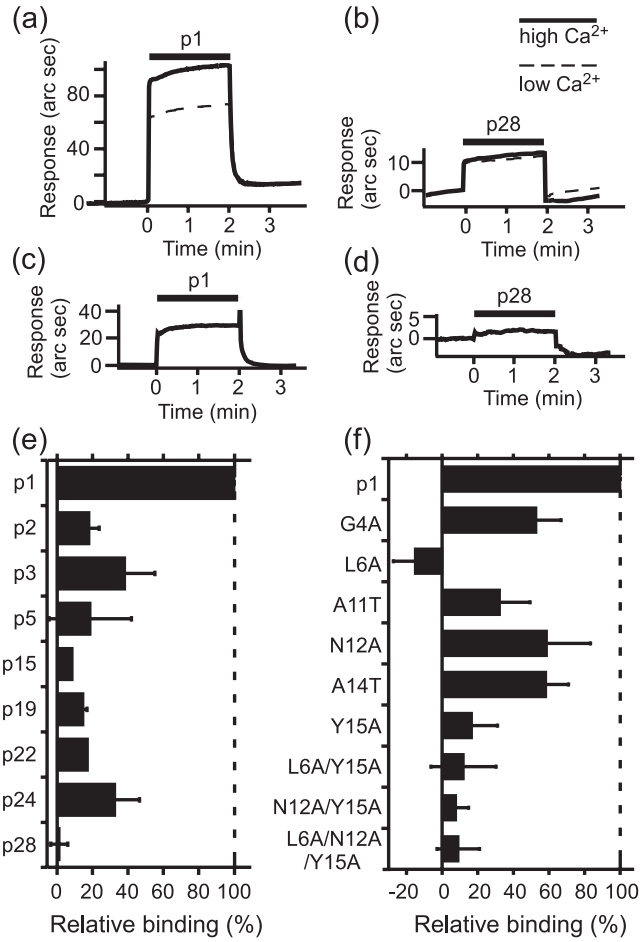

Figure 5. Binding of peptides to the Ca^{2+}-bound form of S-modulin. (a, b) Binding of p1 (a) and p28 (b) to immobilized S-modulin was monitored by a resonant mirror biosensor at a low (broken line) and a high (solid line) Ca^{2+} concentration. At time 0, a peptide solution was added to an S-modulin cuvette. At 2 min after the addition, the peptide was washed out. (c, d) Ca^{2+}-dependent binding of p1 (c) and p28 (d) to S-modulin was determined by subtracting the signal obtained at the low Ca^{2+} concentration from that at the high Ca^{2+} concentration in (a) and (b), respectively. (e, f) The increase in the response signal was converted to a relative value proportional to the number of molecules, and the value for p1 was set to 100%. The amount of binding of each peptide was expressed as a relative value to this control. The results for the wild-type peptide (p1, p2 and so on) are shown in (e) (n = 4 for p3, p5 and p24; n = 3 for p2; n = 2 for p19 and p28; n = 1 for p15 and p22), and those for mutant peptides of p1 are shown in (f) (n = 3–6). Result is shown as a mean ± standard deviation. The final concentration of each peptide was 62.5 μM throughout this study.

molecules were immobilized on the carboxymethylated surface of an IAsys cuvette. As a control, a peptide solution was added at a low Ca^{2+} concentration (Fig. 5a, broken line). With the addition of a solution of a peptide at time 0 (in Fig. 5a, the peptide was p1), the resonance signal increased due to the increase in the refractive index of the solution. After wash of the peptide at 2 min, the signal returned to baseline. The same procedure was repeated but at a high Ca^{2+} concentration (solid line in Fig. 5a). When p1 was added at a high Ca^{2+} concentration, the signal increased rapidly and stayed at a constant level. The plateau level of the signal was higher at the high Ca^{2+} concentration, which indicated that p1 bound to the Ca^{2+}-bound form of S-modulin. At 2 min after the addition of p1, the cuvette was washed with the high Ca^{2+} solution. Because the wash of the cuvette was carried out manually, the timing of the wash varied slightly in each measurement (see below).

Similar measurements were performed using 18 other GRK1/GRK7 partial peptides that showed higher scores exceeding the initial criterion level shown in Fig. 2. In sharp contrast to p1, many of the peptides did not show the binding to the Ca^{2+}-bound form of S-modulin. Such an example is shown in Fig. 5b. No significant differences were observed at a high and a low Ca^{2+} concentration in the case of p28.

The Ca^{2+}-dependent binding of a peptide was determined by subtracting the signal at a low Ca^{2+} concentration from that at a high Ca^{2+} concentration (Fig. 5c,d). (Because the timing of the wash was not controlled precisely, artifactual increase or decrease of a signal was obtained in this subtraction. However, these signals should be neglected.) In the measurement of a binding of a peptide, we always used p1 as a control. Because the response amplitude is a linear function of the mass at the sensor surface, the response amplitude was divided by the molecular mass of the peptide to calculate the relative number of molecules that bound to S-modulin. The binding of each peptide was expressed as percent of that of p1 (relative binding in Fig. 5e,f). Note that with this way of subtraction (Fig. 5c,d), we always observed Ca^{2+}-dependent binding of a peptide to the Ca^{2+}-bound form of S-modulin.

As shown in Fig. 5e, the binding was largest in p1. The result, together with the highest inhibition of p1 on S-modulin activity, strongly suggested that p1 inhibits S-modulin activity by binding directly to the binding site of S-modulin. Although the effect was small, p24 and possibly p3 may also act similarly because these peptides moderately inhibited S-modulin activity (Fig. 3) and moderately bound to the Ca^{2+}-bound form of S-modulin (Fig. 5e). Because 10 (p6, p8, p12, p17, p18, p20, p21, p23, p25 and p29) out of 19 peptides (Fig. 2) did not dissolve in the HEPES buffer well, we could not measure the binding of these peptides.

Similar measurements were performed in the presence of p1-mutant peptides (Fig. 5f). The peptides that showed a reduced effect of p1, namely L6A, N12A, Y15A and their double and triple mutants, showed decreased binding to the Ca^{2+}-bound form of S-modulin compared with the control, p1. Among them, L6 seemed to be essential for the binding of p1, because the peptides including L6A all showed very severe loss of binding.

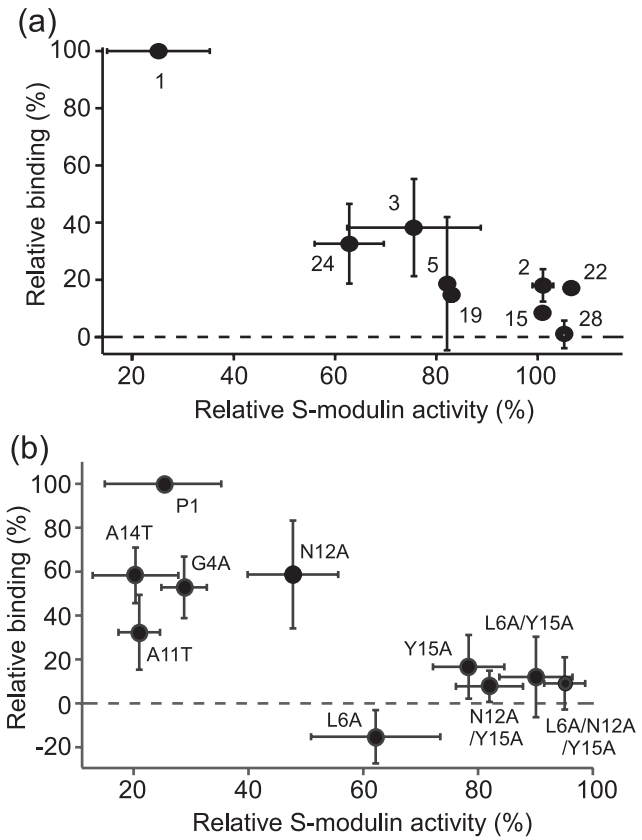

Figure 6. Correlation of the effects of peptides on the inhibition of S-modulin activity and binding to S-modulin. The amount of binding of each peptide to the Ca^{2+}-bound form of S-modulin was plotted against the relative S-modulin activity measured in the presence of the peptide. The amount of the binding of p1 was set to 100% in the ordinate, and the relative S-modulin activity in the absence of a peptide was set to 100% (abscissa). The results are shown for partial peptides (a) and for mutated peptides of p1 (b).

Correlation between the inhibition of S-modulin activity and binding to the Ca^{2+}-bound form of S-modulin

To examine whether the inhibition of S-modulin activity caused by a peptide correlates with its binding to the Ca^{2+}-bound form of S-modulin, we plotted the relative binding of a peptide against the relative S-modulin activity measured in the presence of the peptide (Fig. 6). For GRK1/GRK7 partial peptides, the binding and inhibition of S-modulin activity showed a good correlation overall (Fig. 6a).

Similarly, the relative binding of a peptide was plotted against the relative S-modulin activity for the mutant peptides of p1 (Fig. 6b). Again, overall, a good correlation seems to be present except for several mutations such as G4A, L6A, A11T and A14T. In N12A and Y15A mutants, the relative binding and inhibition of S-modulin activity relations could be plotted on a straight line, which suggests that N12 and Y15 are involved in the binding of p1 to the binding pocket of the Ca^{2+}-bound form of S-modulin. In G4A, A11T and A14T mutants, S-modulin activity was severely inhibited to an extent similar to that attained by p1, although the binding was reduced. In the L6A mutant, the binding ability was almost lost while it inhibited S-modulin activity slightly. It seems that the inhibition mechanisms of S-modulin activity by these peptides are not so simple. It may be the case that, depending on each mutant peptide, these peptides have some additional nonspecific inhibitory effects on S-modulin activity.

Additive effect of p1 and p24

So far, the inhibition of S-modulin activity by GRK1/GRK7 partial peptides seemed to be brought about by the direct binding of a peptide to the binding site in the S-modulin molecule (Fig. 6a). We then examined whether the effect of each peptide was additive. If this were the case, we can expect that each peptide acts on a different site in an S-modulin molecule. We used p1 and p24 to explore this possibility, because they were the most effective and the second-most effective peptides in the inhibition of S-modulin activity (Fig. 3).

S-modulin activity was inhibited by p1 to approximately 25% of the total activity, and by p24 to approximately 62% (Fig. 3). When the inhibition by these peptides is additive, we can expect that the inhibition in the presence of both peptides would be 16% (25% × 62%). In the presence of these peptides, S-modulin activity was inhibited to 17.7 ± 10% (n = 3) in agreement with our prediction.

Using a resonant mirror biosensor, we examined whether the bindings of p1 and p24 are additive. To test this possibility,

it was necessary to saturate the binding site of one peptide before adding the other peptide. The dose dependency was measured for p1 and p24, and the resonance signal was found to be saturated at approximately 0.6 mM for both peptides. Figure 7a shows the Ca^{2+}-dependent binding signal under the condition that p1 and p24 of saturating concentrations are present. Even after the addition of saturating concentration of p1, addition of p24 increased the binding signal (solid line). The addition of p28, which neither showed inhibition on the S-modulin activity (Fig. 3) nor binding to S-modulin (Fig. 5), did not increase the signal (broken line in Fig. 7a). Even when the order of the addition of p1 and p24 was reversed, the signal increased on addition of the second peptide. It should be noted that we always measured a Ca^{2+}-dependent signal (see Fig. 5a,c, for example) and therefore, Fig. 7a shows the Ca^{2+}-dependent binding of p24 in the presence of p1. The binding signal of p24 in the absence of p1 (Fig. 7b) was similar to that in the presence of p1 (Fig. 7a). The result, therefore, clearly indicates that the binding of p1 and that of p24 are independent, so that the two peptides bind different regions of the Ca^{2+}-bound form of S-modulin.

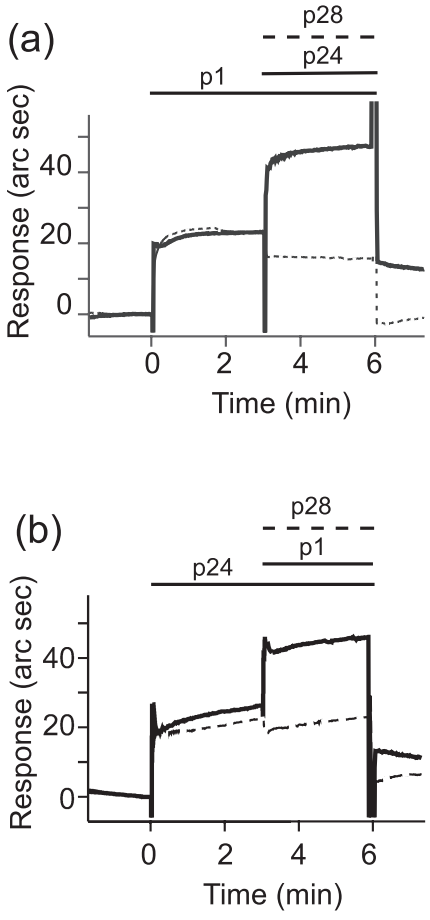

Figure 7. Independent binding of p1 and p24 to the Ca^{2+}-bound form of S-modulin. (a) p1 was added to the S-modulin cuvette at its saturating concentration and then either p24 (solid line) or p28 as a control (broken line) was added. The concentrations of the peptides at the end of the measurement were: p1, 0.66 mM; p24, 0.54 mM; p28, 0.54 mM. (b) Similar measurements as in (a), but the order of the addition of p1 and p24 was reversed. The final concentrations of the peptides were: p1, 0.66 mM; p24, 0.54 mM; p28, 0.26 mM.

Inhibition mechanism of visual pigment phosphorylation by S-modulin

In the present study, we found that several GRK1/GRK7 partial peptides inhibit S-modulin activity. Among them, the N-terminal region, p1 (aa 3–23 in carp GRK7), showed the greatest effect (Fig. 3) and showed firm binding to S-modulin (Fig. 5e). An N-terminal region of GRK1 (aa 17–34 in bovine) corresponding to p1 in this study has been shown to interact with activated rhodopsin (21). Our result, therefore, suggests that the Ca^{2+}-bound form of S-modulin/recoverin binds to the N-terminus region of GRK1 and thereby competitively inhibits the binding of GRK1 to activated rhodopsin.

Previous work has shown that a C-terminal conserved region of GRK1 (aa 456–544 in bovine) also interacts with activated rhodopsin (22). In the present study, this region corresponds to p24–p29. Our studies showed that p24 moderately inhibits S-modulin activity (Fig. 3) and binds to this molecule (Fig. 5e). The result, therefore, also suggests that S-modulin binds to the p24 region of GRK1 (aa 452–470 in bovine) to hamper the access of GRK1 to light-activated rhodopsin.

The binding site of S-modulin/recoverin to GRK1 has been shown to localize at the edge of a groove that is exposed to the surface of S-modulin/recoverin upon Ca^{2+}-binding (12). The effective peptides identified in the present study most probably bind to this region. Because the inhibitory effect and the binding were most pronounced with p1, it is highly possible that p1 fits into this groove. Studies by Higgins *et al.* (23) and Ames *et al.* (24) support this view. With an NMR study, Ames *et al.* showed that the N-terminus amino acids (residues 1–25 in bovine GRK1) form an amphipathic helix and the hydrophobic face interacts with an exposed hydrophobic groove on the surface of recoverin (24). L6 and Y15, which we identified as

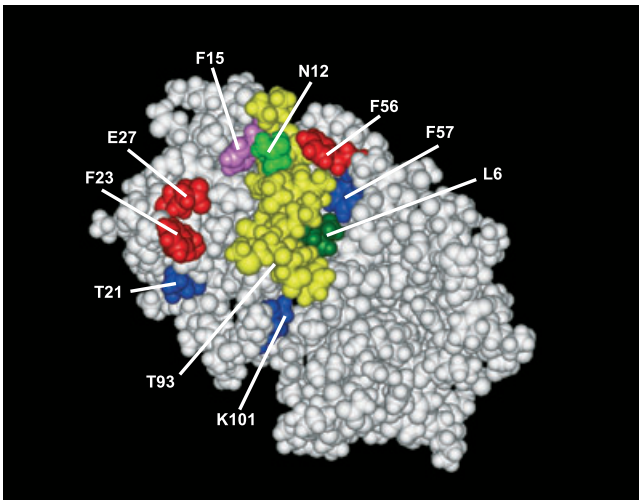

Figure 8. Interaction of an N-terminal peptide of GRK1 with recoverin. The N-terminal peptide of GRK1 colored yellow (aa 1–25 in bovine) binds to a hydrophobic groove of the surface of recoverin (RK structure 3 in 2i94_pdb [24]). The important amino acid residues determined in the present study are shown in dark green (L6), light green (N12) and pink (F15 in bovine GRK1 or Y15 in carp GRK7). The important amino acid residues of S-modulin/recoverin to interact with GRK1 reported previously (12) are indicated in red (essential amino acids) and blue (possible amino acids for the interaction). T93 is under the GRK1 N-terminal peptide and is not seen.

important residues for the interaction with S-modulin, fit well to the hydrophobic groove of S-modulin/recoverin (Fig. 8). On the other hand, N12 is at the hydrophilic face of the amphipathic helix and does not seem to affect the binding of the N-terminus region directly. One possibility could be a difference in the ternary structure between the wildtype peptide (p1) and the mutant N12A peptide.

The essential and possible amino acid residues responsible for the binding of S-modulin to GRK1 were found to be T21, F23, E27, F56, F57, T93 and K101 (12). Among these residues, F56, F57, T93 and K101 are located in the hydrophobic groove of recoverin (Fig. 8), and therefore are probably responsible for the binding to the p1 region of GRK1. However, residues T21, F23 and E27 are away from the binding site (Fig. 8). It is possible that these three residues form an interacting site with the GRK1 region corresponding to p3 (aa 42–64 in bovine), which we did not study in detail in this study, and/or p24 (aa 452–470 in bovine) that interacts with S-modulin (Figs. 3 and 7).

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