Distinct Roles of the Second and Third Cytoplasmic Loops of Bovine Rhodopsin in G Protein Activation*

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In contrast to the extensive studies of light-induced conformational changes in rhodopsin, the cytoplasmic architecture of rhodopsin related to the G protein activation and the selective recognition of G protein subtype is still unclear. Here, we prepared a set of bovine rhodopsin mutants whose cytoplasmic loops were replaced by those of other ligand-binding receptors, and we compared their ability for G protein activation in order to obtain a clue to the roles of the second and third cytoplasmic loops of rhodopsin. The mutants bearing the third loop of four other G coupled receptors belonging to the rhodopsin superfamily showed significant G activation, indicating that the third loop of rhodopsin possibly has a putative site(s) related to the interaction of G protein and that it is simply exchangeable with those of other G coupled receptors. The mutants bearing the second loop of other receptors, however, had little ability for G protein activation, suggesting that the second loop of rhodopsin contains a specific region essential for rhodopsin to be a G protein-activating form. Systematic chimeric and point mutational studies indicate that three amino acids (Glu154, Val138, and Cys140) in the N-terminal region of the second loop of rhodopsin are crucial for efficient G protein activation. These results suggest that the second and third cytoplasmic loops of bovine rhodopsin have distinct roles in G protein activation and subtype specificity.

Rhodopsin is one of the G protein-coupled receptors (GPCRs) that has diverged into a photoreceptive protein in retinal visual cells. It is a membrane protein consisting of a single polypeptide opsin and a chromophore 11-cis-retinal (1, 2). The opsin contains seven transmembrane α-helices, the structural motif typical of the GPCRs (3, 4). Light isomerizes 11-cis-retinal into the all-trans form, which induces the conformational changes of the opsin to activate retinal G protein (1, 2). Several lines of evidence indicate that there are at least three subtypes of rhodopsin, each of which can couple with G (transducin), G or G subtypes of G protein, respectively (5). The advantages of studies on rhodopsin in comparison with those of diffusible-ligand GPCRs are that rhodopsin can be activated synchronously by light even at low temperature and that the chromophore can be utilized as an intrinsic spectroscopic probe to monitor structural changes in the protein. Thus, the light-activated process of rhodopsin has been studied extensively by characterizing the several spectrally distinguished intermediate states in the process at submolecular or atomic resolution (6, 7). Furthermore, the structure of G protein-interacting states of bovine rhodopsin (G coupled rhodopsin) has been investigated by various spectroscopies in combination with the methods of site-directed mutagenesis (8–13), peptide competition (13, 14), and antibody competition (15). These investigations revealed that the second, third, and fourth cytoplasmic loops of bovine rhodopsin are involved in G protein activation and that the relative movements of the helices III and VI are prerequisite for activation of G protein (16–18). Thus, the information obtained from rhodopsin studies provides valuable insight into the activation processes of other GPCRs.

On the other hand, only the G coupled rhodopsins including bovine rhodopsin have so far been expressed successfully in a culture system, although many efforts to express other types of rhodopsin have been tried. This is a great disadvantage to the studies of rhodopsin to elucidate the molecular mechanism of coupling specificity for the G protein subtype; that is, these facts hampered investigation of the mechanism of coupling specificity using chimeric or site-directed mutants, a typical method to elucidate the G protein coupling specificity. Thus, in rhodopsin, the mechanism of coupling specificity has scarcely been investigated, which is in marked contrast to the extensive studies on the mechanism of light-induced conformational changes.

Because the presence of multiple types with different specificity for G protein subtypes is common in GPCRs, the molecular mechanisms of G protein coupling specificity have been widely investigated in several GPCRs. The accumulated evidence suggests that the second and third cytoplasmic loops are the interaction sites with G protein in several GPCRs. Of the two loop domains, the third cytoplasmic loop, especially the N- and C-terminal regions of the loop, has been reported to be related to the selectivity for receptor/G protein coupling in several GPCRs, i.e. muscarinic acetylcholine receptor (19, 20), adrenergic receptor (21), endothelin receptor (22), and vasopressin receptor (23). As for the second cytoplasmic loop, however, several roles have been implicated in different receptors, i.e. complement of the third loop (β-adrenergic receptor) (24), involvement in G protein selectivity independently of or cooperatively with the third loop (α2-adrenergic receptor, endothelin receptor) (21, 22), and a slight effect on efficient G protein

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§ The abbreviations used are: GPCR, G protein-coupled receptor; G, transducin; GTP-S, guanosine 5’-O-(thio)triphosphate; WT, wild type; mAChR1 and mAChR2, muscarinic acetylcholine receptors 1 and 2, respectively; α2BAR and β2AR, adrenergic receptor subtypes α2B and β2AR, respectively; m2L2L3, mutant with second and third loops of mAChR2; mGlur, metabotropic glutamate receptor.
selectivity (m2 muscarinic acetylcholine receptor) (19). Thus, the two loops are suggested to play different roles in G protein activation.

In this study, we have tried to elucidate the roles of the second and third cytoplasmic loops of bovine rhodopsin on G protein activation and/or the coupling specificity. For this purpose, we have constructed rhodopsin mutants whose second and/or third cytoplasmic loops were replaced by the corresponding loops of other ligand-binding GPCRs (muscarinic acetylcholine receptor, adrenergic receptor, prostaglandin receptor, and endothelin receptor) whose selectivities for G protein coupling have been well studied. In other words, we would obtain a clue to the functional difference in the second and third cytoplasmic loops of bovine rhodopsin based on the functional replacements of the cytoplasmic loops of bovine rhodopsin with those of other receptors. The results showed that functional replacements of the second and third cytoplasmic loop of bovine rhodopsin with those of other Gt/Go-coupled GPCRs were successful, whereas those of the second cytoplasmic loop were not, suggesting that each of the loops of bovine rhodopsin exhibits a different role. The loss of function by replacement of the second cytoplasmic loop, which would be a novel observation in the studies of many GPCRs as we know, suggests that this loop contains a region essential for the G protein activation by rhodopsin. Extensive studies using chimeric and site-directed mutagenesis on the second cytoplasmic loop strongly suggest that the N-terminal seven amino acids including the “ERY” triad of rhodopsin are essential for rhodopsin to be a G protein-activating form.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNAs that encode muscarinic acetylcholine receptors were the kind gift of Dr. T. Haga (The University of Tokyo). Adrenergic receptors were from Drs. R. J. Leffkowitz (Duke University) and H. Kurose (The University of Tokyo). Prostaglandin E2 receptors were from Dr. M. Negishi (Kyoto University). Endothelin receptors were from Drs. Y. Furuchi (AGEcNE Research Institute) and T. Masaki (National Cardiovascular Research Institute). The hybridoma producing the anti-bovine rhodopsin monoclonal antibody 1D4 was generously supplied by Dr. R. S. Molday (University of British Columbia). 293S cells were the kind gift of Dr. J. Nathans (The Johns Hopkins University). Oligonucleotide DNAs were purchased from Amersham Pharmacia Biotech. The DNA encoding bovine opsin, which has 30 unique segments was carried out according to methods described previously (25). Opin was expressed in 293S cells and reconstituted to rhodopsin according to Ref. 27.

**Preparation of Rhodopsin Mutants**—The rhodopsin mutant cDNA fragment was constructed by standard polymerase chain reaction mutagenesis techniques using cDNA encoding muscarinic acetylcholine receptor, adrenergic receptor, prostaglandin receptor, and endothelin receptor as templates. The mutant gene was prepared by cassette mutagenesis of the polymerase chain reaction fragment for bovine rhodopsin. Expression and purification of wild type (WT) and mutant rhodopsins were carried out according to methods described previously (25). Opsin was expressed in 293S cells and reconstituted to rhodopsin by mixing the cell suspension with 11-cis-retinal. Rhodopsin was extracted in 1% dodecyl maltoside in buffer P (50 mM HEPES, 140 mM NaCl, pH 6.5) and was purified from the supernatant fraction by immunofinity chromatography. The visible spectra at this stage showed absorption maxima at 499 nm for WT and all of the mutant rhodopsins. Purification of G Proteins—Purification of G from bovine rod outer segments was carried out according to methods described previously (26). Purification of Gt from bovine cerebral cortex was carried out according to Ref. 27.

**G Protein Activation Assays**—A radiolabeled filter binding assay, which measures a light-dependent GDP/GTPγS exchange by G protein, was carried out as described previously (28). All procedures were carried out at 15 °C (Gt assay) and 0 °C (Gt assay). The assay mixture consisted of 50 mM HEPES (pH 7.0), 140 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.01% dodecyl maltoside, 0.1% sodium cholate, 0.8 mg/ml L-a-phosphatidylcholine from egg yolk, 1 μM (G₉ assay) or 0.1 μM (G₉ assay) [35S]GTPγS, and 2 μM (Gt assay) or 100 μM (Gt assay) GDP. Rhodopsin solutions (rhodopsin final concentration in the Gt assay, 4 nM; Gt assay, 40 nM) were irradiated with orange light for 30 s or kept in the dark, and they were mixed immediately with G protein solution (G protein final concentration in the Gt assay, 600 nM; Gt assay, 700 nM). After incubation for the selected time in the dark, an aliquot (20 μl) was removed from the sample into 200 μl of stop solution (Gt assay: 20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl₂, 2 μM GTPγS, and 2 μM GDP; Gt assay: 20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl₂, 0.1 μM GTPγS, and 100 μM GDP), and it was filtered immediately through a nitrocellulose membrane to trap [35S]GTPγS bound to G proteins. The membrane was washed four times with 200 μl of wash solution (20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl₂) to diminish free [35S]GTPγS and then air-dried. The amount of bound [35S]GTPγS was quantitated by assaying the membrane with a liquid scintillation counter (LS 6000IC, Beckman).

**RESULTS**

Fig. 1 shows a comparison of the amino acid sequences of the second and third cytoplasmic loops of several receptors. We designed the bovine rhodopsin mutants whose second and/or third cytoplasmic loops were replaced with those of the receptors. WT and all the mutant rhodopsins possessed almost the same absorption maxima at 499 nm for WT and all of the mutant rhodopsins. Purification of G Proteins—Purification of G from bovine rod outer segments was carried out according to methods described previously (26). Purification of Gt from bovine cerebral cortex was carried out according to Ref. 27.

**Effects of the Replacement of the Third Cytoplasmic Loop on G Protein Activation**—Although bovine rhodopsin couples with Go in the native photoreceptor cells, it exhibits the ability to activate the Gt subtype, but not the Gt subtype, as evidenced by the in vitro studies (28, 29). From this point of view, bovine rhodopsin could be classified as a receptor that couples with the Gt family of G protein. In fact, bovine rhodopsin activates the Gt subtype in an in vitro system (Fig. 2). Fig. 2, A and B, shows the amount of GTPγS-bound G protein as a function of the
incubation time after activating bovine rhodopsin (WT) or the mutants having the third loop of Gq-coupled mAChR2 in the light. Panels C and D, comparison of the initial rates of Gs (panel C) and Gi (panel D) activation by WT and mutants. GTPγS binding assays with purified mutants and G proteins were carried out as described under “Experimental Procedures.” The amino acid sequences of the third cytoplasmic loop region in WT and other receptors are shown in Fig. 1. Data are presented as the means ± S.D. of more than three independent experiments. GTPγS binding activities of the mutants are normalized to WT activity.

explained by the fact that the third loop of bovine rhodopsin has the unique sequence to activate Gs. However, it is possible that the third loops of other receptors could not form the active conformation by interacting with other region(s) of rhodopsin (14, 30, 31). The unique character of Gs such as high affinity for GDP relative to that of Go (32) may be another possibility that accounts for the lower activation of Gs by the mutant rhodopsins.

The mutants having the loop of Gq-coupled (mAChR1 and α1BAR) and Gq-coupled (EP2) receptors had almost no ability to activate Gs. Only one exception was the mutant having the loop of Gq-coupled β2-adrenergic receptor (β2AR). It was reported that the β2AR mainly coupled with Gq but partly also with Gi (33, 34). Therefore, we might detect such a coupling between β2AR and Gi using a chimeric receptor. It should be noted that the activation profile of expressed Gi1 by all the mutants was almost the same as that of Gi (data not shown).

Effects of the Replacement of the Second Cytoplasmic Loop on G Protein Activation—Next, we prepared bovine rhodopsin mutants whose second cytoplasmic loop was replaced by that of various receptors to investigate the effects of the second loop replacement on G protein activation. Like the third loop-replaced mutants, all of the mutants showed a serious decrease in the ability for Gs activation (Fig. 3A). Moreover, these mutants showed the loss of Gs activation (Fig. 3B). In other words, the mutants having the second loop of other receptors hardly activated G proteins regardless of the subtypes.

We then prepared a mutant rhodopsin whose second and third cytoplasmic loops were both replaced by those of mACHR2, one of the Gq-coupled receptors, to examine the possibility that coexistence of the two loops of each receptor is necessary for the efficient activation of G protein. We speculated that the efficiency of Gs activation by the mutant having only the third loop of mACHR2 might be enhanced by adding
the second loop of mACHR2. Contrary to our expectations, however, the mutant bearing the second and third loops of mACHR2 (m2L2L3) exhibited almost no ability for G\(_{\alpha}\) activation (Table I). To examine the possibility that the second loop could function with loops other than the third loop, we prepared additional mutants that have the first loop, the putative fourth loop, and the C-terminal tail of mACHR2, respectively, together with the second loop. We also prepared the mutant rhodopsin having all of the cytoplasmic regions of mACHR2. However, all of the mutants exhibited almost no activity (data not shown), indicating that the second loop replacement in any context resulted in a great loss of G protein activation. These results emphasize the importance of the second loop of bovine rhodopsin for its efficient G protein activation.

The results clearly show the different roles in G protein activation in the second and third loops of bovine rhodopsin; that is, replacement of the second loop caused almost no activation of G proteins, whereas a suitable replacement of the third loop retained the ability for G\(_{\alpha}\) activation. Thus, unlike the third loop, the second loop would contain a crucial and specific amino acid region essential for rhodopsin to be a G protein-activating form. It should be noted that the mutant bearing the second cytoplasmic loop of G\(_{\alpha}\)-coupled chicken red-sensitive cone visual pigment (iodopsin; Fig. 1) activated G\(_{\alpha}\) almost as efficiently as (93%) WT did (data not shown), showing that the second cytoplasmic loop was exchangeable between G\(_{\alpha}\)-coupled visual pigments. Therefore, we next tried to identify specific amino acid residues important for rhodopsin to activate G protein.

**Critical Region(s) of the Second Cytoplasmic Loop of Rhodopsin in Functional Coupling to G Protein**—Comparison of the sequence of the second cytoplasmic loop among several GPCRs in the rhodopsin superfamily shows more than 50% similarity and almost the same numbers of amino acids (20) (Fig. 4A). From our results as described, the mutant having the second loop of iodopsin showed functional coupling to G protein, but the mutants having those of the other receptors did not. The four amino acids (Val138, Cys140, Asn145, and Phe148 in bovine rhodopsin) were supposed to be critical residues for efficient G protein activation because they are conserved between bovine rhodopsin and iodopsin but not among other receptors (Fig. 4B). These results clearly indicate that the N-terminal seven amino acids in the second loop of bovine rhodopsin are the critical region in G protein activation.

In relation to the role of the second loop in forming an active state of rhodopsin, it is of interest to investigate whether or not the second loop of receptors other than the G\(_{\alpha}\)-coupled receptors can function in the rhodopsin molecule if the N-terminal segment of rhodopsin is retained. Thus, we created chimeric mutants between bovine rhodopsin and G\(_{\alpha}\)-coupled mACHR1. Whereas the chimeric mutant having the second loop of mACHR1 showed almost no ability for G\(_{\alpha}\) and G\(_{\beta}\) activation (Fig. 3), replacing the N-terminal segment of the second loop of bovine rhodopsin retained about 50% ability for G\(_{\alpha}\) and G\(_{\beta}\) activation. The extents of ability are almost the same as those in the case of the chimeric mutant (m2L2-3) between rhodopsin and mACHR2. Therefore, we concluded that the N-terminal

### Table I

Comparison of G\(_{\alpha}\) and G\(_{\beta}\) activation rates by the mutants having the cytoplasmic loop(s) of mACHR2

| G\(_{\alpha}\) activation | G\(_{\beta}\) activation |
|------------------------|----------------------|
| WT                     | 100                  |
| m2L2                  | 2                    |
| m2L2                  | 4                    |

* m2L2, m2L3 and m2L2L3 indicate the bovine rhodopsin mutants having the second loop, the third loop and both loops of mACHR2, respectively.

**Fig. 4. Amino acid sequences of the second cytoplasmic loops.**

Panel A, sequence alignment in the second cytoplasmic loop among bovine rhodopsin, iodopsin, and other ligand-binding receptors. Amino acid residues, which are homologous (A=G=P=S=T, I=L=M=V, D=E=N=Q, H=K=R, F=W=Y) among bovine rhodopsin and iodopsin but not among other receptors are boxed in gray. Panel B, amino acid sequences in the second cytoplasmic loop of the rhodopsin mutants which is partly replaced with the corresponding region(s) of mACHR2. The positions where amino acid substitutions are introduced are indicated in bold letters.

**TABLE I**

Comparison of G\(_{\alpha}\) and G\(_{\beta}\) activation rates by the mutants having the cytoplasmic loop(s) of mACHR2

|            | G\(_{\alpha}\) activation | G\(_{\beta}\) activation |
|------------|------------------------|----------------------|
| WT         | 100                    | 100                  |
| m2L2       | 2                      | 1                    |
| m2L2       | 3                      | 135                  |
| m2L2L3     | 4                      | 3                    |

A               Bovine Rh                     
Iodopsin                       
mACHR1   DYFVSTFLRSYAKRFGPR       
mACHR2   DYFCVTVKLTVYKPTMM       
α1BAR     DRYGVVSRQYPTLTVRK       
α2BAR     DRYGAVSALTFKNIKTHR       
β2AR     DRAFTATSSFKQSSRLNK       
EP2       ERLDSTFVTPRNLKRERG       
EP3       ERLADARAKSHKLK       
ETA       DRYVAVSNVPSQIOGLDV   
ETB       DRYVAVSNKLQLOQVFPW   

B          WT                     
m2L2       DYFCVTVKLTVYKPTMM       
m2L2-1     DYFCVTVKLTVYKPTMM       
m2L2-2     DYFCVTVKLTVYKPTMM       
m2L2-3     DYFCVTVKLTVYKPTMM       
m2L2-4     DYFCVTVKLTVYKPTMM       
m2L2-5     DYFCVTVKLTVYKPTMM       
m2L2-6     DYFCVTVKLTVYKPTMM       
m2L2-7     DYFCVTVKLTVYKPTMM       
m2L2-8     DYFCVTVKLTVYKPTMM       
m2L2-9     DYFCVTVKLTVYKPTMM       
m2L2-10    DYFCVTVKLTVYKPTMM       
m2L2-11    DYFCVTVKLTVYKPTMM       
m2L2-12    DYFCVTVKLTVYKPTMM       
m2L2-13    DYFCVTVKLTVYKPTMM       

**Fig. 4. Amino acid sequences of the second cytoplasmic loops.**

Panel A, sequence alignment in the second cytoplasmic loop among bovine rhodopsin, iodopsin, and other ligand-binding receptors. Amino acid residues, which are homologous (A=G=P=S=T, I=L=M=V, D=E=N=Q, H=K=R, F=W=Y) among bovine rhodopsin and iodopsin but not among other receptors are boxed in gray. Panel B, amino acid sequences in the second cytoplasmic loop of the rhodopsin mutants which is partly replaced with the corresponding region(s) of mACHR2. The positions where amino acid substitutions are introduced are indicated in bold letters.
seven amino acids in the second loop of bovine rhodopsin are the critical region in G protein activation rather than in the selective recognition of specific G protein subtypes.

Critical Amino Acid(s) in the N-terminal Region of the Second Cytoplasmic Loop of Rhodopsin in Functional Coupling to G Protein—To investigate further details in the N-terminal region of the second cytoplasmic loop, we compared the amino acid sequences in the seven-amino-acid region between bovine rhodopsin (ERYVVVC146) and mAChR2 (DRYFCVT) (Fig. 6A). In this region, four amino acids are different between bovine rhodopsin (ERYVVVC) and mAChR2 (DRYFCVT). We therefore prepared additional mutants (m2L2-7–13, Fig. 4B) to investigate the effects of the substitutions for the four amino acids on Gt activation. Of the four residues, the substitution of Phe for Val137 was expected to have little influence on G protein activation because the corresponding residue of iodopsin is Phe. In fact, m2L2-7 activated Gt as efficiently as m2L2-3 did (Fig. 6B). The substitutions for the other three amino acids significantly affected the activity for Gt activation (Fig. 6B); that is, the chimeric mutant having the one-third region of mAChR2 showed little ability, whereas stepwise introduction of the residues in the C-terminal two-thirds may be present in rhodopsin molecule, although the detailed interaction manner is not known (see “Discussion”).

**DISCUSSION**

Sequence alignment and comparison of the primary structures among various GPCRs reveal that the third cytoplasmic loop clearly varies in length and amino acid sequence, whereas the second cytoplasmic loop shows high amino acid similarity and almost the same number of amino acids (Fig. 1). At the beginning of the investigation, therefore, we had expected that the replacement of the second loop might retain the G protein activation of rhodopsin, but that of the third loop might affect the activation ability of rhodopsin for various types of G protein. Furthermore, we had expected that several mutants having third loops of a long sequence might fail to be expressed. However, all of the mutants investigated in this study were reasonably expressed in our culture system. In addition, the third loops of Gt-coupled GPCRs functioned in the rhodopsin molecule as did that of the native rhodopsin, whereas none of the second loops functioned in the rhodopsin molecule. These results strongly suggest that the third loop of rhodopsin contains the putative site(s) related to the interaction with G proteins, whereas the second loop of rhodopsin contains amino acid sequences essential for bovine rhodopsin to be an active state. Detailed investigations on the second loop indicate that the loss of function was largely the result of replacing the specific three amino acids (Glu134, Val138, and Cys140) present in the N-terminal region of the second loop of rhodopsin (Fig. 6). Thus, the particular region including these three amino acids in bovine rhodopsin could play a crucial role in efficient G protein activation.

Loss of function induced by the substitutions of Cys for Val138 or of Thr for Cys140 is possibly the result of the change in character of the amino acid residues, whereas substitution of Asp for Glu134 does not alter the character. To examine the effect of the difference in the carboxyl group’s equilibrium constant between glutamic and aspartic acids on G protein activation, we measured the pH dependence of the G protein activation by these mutants. The results showed that in all of the pH range investigated (from pH 5.0 to 9.0), the mutant having aspartic acid exhibited a dramatic decrease in Gt activation compared with that of the mutant having glutamic acid. Furthermore, there was no pH region in which the mutant having aspartic acid showed more activity than the mutant having glutamic acid data not shown). Our data are consistent with the data of Fahmy and Sakmar [35] in which the substitution of Asp for Glu134 causes a decrease in the ability of Gt coupling independently of pH. Therefore, the effects of the three-amino acid substitutions on G protein activation might be caused by the difference in the volumes of the amino acid residues, which changes the original conformation of the N-terminal seven-amino acid region in the active form of bovine rhodopsin.

Although the N-terminal segment of the second loop of rhodopsin is a major component for forming the active state of...
rhodopsin, the subsequent two-thirds region of the loop might have some role in G protein activation. This is because replacement of only the N-terminal segment with that of mAChR2 retained the 20% activity to Gt and Go (Fig. 5). According to peptide inhibition experiments, the synthetic peptide corresponding to the two-thirds region acts as a competitor of rhodopsin-Gt interaction (14), suggesting that the region could be an interaction site with the G protein. Our results indicate that the replacement of this region by that of mAChR2 caused about 50% decrease of activity to Gt and Go, but the remaining activation efficiency relative to that of the WT was almost identical between Gt and Go. Furthermore, the same activation efficiencies remained when the two-thirds region was replaced by that of the Gq-coupled mAChR1. These results suggest that the regions of mAChR1 and mAChR2 may have little roles in interacting with both Gt and Go, but not function in the rhodopsin molecule. In this context, it is noteworthy that the mutations of three amino acid residues involved in the N-terminal segment exhibited different profile of activation efficiency between WT and mutants are shown on the left side of the figures. Data are presented as the means ± S.D. of more than three independent experiments. GTPγS binding activities of the mutants are normalized to m2L2-3 activity.

Site-directed spin labeling studies (36, 37) and disulfide cross-linking studies (16) suggest that the N-terminal segment of the second cytoplasmic loop forms an α-helical extension of the third transmembrane domain. Fig. 7 shows an approximate topological location of the amino acid residues in the N-terminal segment of the second loop. According to this model, the three amino acids (Glu 134, Val 138, and Cys 140), whose replacements prevented G protein activation, are expected to cluster on the same face (temporarily called “Face A”). It should be noted that the three amino acids situated on Face A vary considerably among each receptor group, whereas Arg 135, Tyr 136, and Val 139 on the other face (called “Face B”) are well conserved among almost all of the receptors (Fig. 4A).

The photoisomerization of the chromophore eventually causes the movement of the cytoplasmic ends of helices III and VI in rhodopsin, as a rigid body, away from each other (16–18). The movement is also suggested to play a crucial role in forming the active signaling state of rhodopsin and other GPCRs by experiments using the engineering disulfide bonds (38) or metal ion bridges (39) at appropriate positions in the two helices. The present study strongly suggests that the amino acid residues of Face A, which is connected to helix III of rhodopsin, are essential in forming the active structure of rhodopsin. Thus, it is an intriguing argument that when the conformational change in the chromophore binding domain propagates to the Face A region, Face A could newly interact with a region(s) to form an active conformation of the second and the third cytoplasmic loops.

The replacement of the second loop (including Face A) of rhodopsin by that of mAChR2 impaired both Gt and Go activation even when any cytoplasmic loop or C-terminal tail of mAChR2 was incorporated into rhodopsin together with the
second loop (Table I and data not shown). In addition, these mutants exhibited no activity in the dark. These results indicate that the second loop of mACHr2 could not take proper conformation and/or interaction(s) with other loops when incorporated into the helical bundle of rhodopsin. Thus, the simple explanation may be that the helical alignment of rhodopsin in the resting state is somewhat different from that of mACHr2. Because the chromophore of rhodopsin, 11-cis-retinal, acts as an “inverse agonist” to suppress the ability of rhodopsin to activate the G protein in the dark, it is possible that the binding of chromophore might cause the specific alignment of the helical bundle of rhodopsin different from that of mACHr2, thereby resulting in improper conformation of the second loop of mACHr2 in the rhodopsin molecule even in the activated state. However, the inverse agonist 11-cis-retinal is converted to an agonist all-trans-retinal upon photon absorption, and the resulting helical movement is suggested to be common in several GPCRs (38, 39). Thus, the different helical alignment between rhodopsin and mACHr2 may not explain the loss of function by the replacement of the second loop of rhodopsin with that of mACHr2. Another explanation is that the interaction site of N-terminal segment including Face A is not the other cytoplasmic loop(s) but the region(s) in the transmembrane helices of rhodopsin. Although most of the modeling studies of rhodopsin and other GPCRs suggest that the second loop would interact with other loop regions, this explanation implies that the N-terminal segment has a direct linkage with the conformational change in the helical bundle of rhodopsin.

It should be noted that our results do not exclude the possibility that the N-terminal region including Face A could be the binding site of G protein. In this case, the mutations of this region could result in breaking the direct intermolecular interaction between the second loop of rhodopsin and the G proteins. If so, the N-terminal segment of mACHr2 would not interact with the G proteins because our experiments indicate that its incorporation into the rhodopsin molecule caused a great loss of G and G activities. Because the N-terminal segment contains the “DERY” triad highly conserved among GPCRs, it is unlikely that only the rhodopsin exhibits a unique activation mechanism quite different from those of the other GPCRs. Therefore, we concluded that the N-terminal region of rhodopsin is not the binding site of G protein but the region essential for the formation of active form of rhodopsin.

Our experiments on replacements of the cytoplasmic loops may offer some insight into the molecular evolution of GPCRs. It is general consensus from the molecular phylogenetic analyses that receptors specific to different ligands first diverged from an ancestral type, and then each receptor was diversified individually into several subclasses that showed different specificity for G protein subclasses (40–42). This suggestion raises the possibility that the mechanisms of coupling specificity for G protein subtypes in various GPCRs could be different among respective GPCRs because the coupling specificity had been acquired after the divergence of the receptor groups. The receptors that were subjected to the present study have ligands considerably different in nature and size from each other, namely acetylcholine in muscarinic receptors, catecholamine in adrenergic receptors, prostaglandin in prostaglandin receptors, and the peptide of 21 amino acids in endothelin receptors. Therefore, in addition to the fact of a considerable difference in amino acid sequence, these receptors diversified into different groups in the course of early evolution. Nevertheless, the third cytoplasmic loops of these receptors with G, coupling specificity can function when these are incorporated into the rhodopsin molecule. These results not only support the idea that the third loop could be one of the recognition sites of G protein subtype but also suggest the presence of a common mechanism in the G activation.

We speculated from the present study that the acquisition of at least G, coupling specificity results in a convergent molecular evolution of receptors, and at the time when receptors had acquired their G protein coupling specificity multiple types of G protein had already existed to provide evolutionary pressure to the receptors. In this context, it should be noted that the rhodopsin mutants having the third loop of G-coupled metabotropic glutamate receptors (mGluR) exhibited no activation of both of G and G (data not shown). The mGluR is a member of the family of GPCRs other than the rhodopsin superfamily, and the molecular architecture of mGluR may be different from those of receptors in the rhodopsin superfamily.

On the other hand, the present results clearly show that none of the second cytoplasmic loops can function in the rhodopsin molecule, suggesting that the second loop, especially in the N-terminal segment, is an important element in converting the receptor into a G protein-activating form. Our experiments also indicate that the second loop of rhodopsin did not function in mACHr2, although the third loop activates G (4). A receptor activates G protein by changing its conformation of the cytoplasmic loops through conformational changes in the transmembrane region after ligand binding through the extracellular side of the receptor. Therefore, it is speculated that the second cytoplasmic loop could have some relationship to the divergence of the receptor into different ligand groups, even though it contains almost the same number of amino acid residues.

Rhodopsin is thought to encounter a single type of G protein (G) in vivo, although recent studies suggest the presence of a light-activated G/phospholipase C-β4 cascade in rod photoreceptor cells (43). This is in contrast to receptors expressed in a cell type in which multiple different G protein subtypes exist. The latter class of receptors would be expected to contain elements that can discriminate among multiple G protein subtypes, whereas rhodopsin might not. Although our aim in the present study was to investigate the functional difference between the second and third cytoplasmic loops of rhodopsin, the present study is the first trial to investigate the coupling specificity of various receptors using the rhodopsin molecule as a prototypical receptor. Thus, the generation of the novel type of G protein specificity into rhodopsin by the replacement of the G protein binding regions of other receptors may lead to better understanding of the mechanism of G protein activation and its selectivity in several GPCRs.

* A. Terakita, T. Yamashita, and Y. Shichida, manuscript in preparation.
In conclusion, the present study shows that the second and third cytoplasmic loops of bovine rhodopsin play important and different roles in G protein activation. These two loop domains would have traced different paths in molecular evolution in order to acquire efficient activation of G protein and coupling specificity for the G protein subtype.

Note Added in Proof—After acceptance of this paper, the rhodopsin structure as determined by x-ray crystallography was published (Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745). The N-terminal segment of the second cytoplasmic loop forms a distorted α-helical structure in which Glu 134 and Val 138 are situated toward helices IV and II and Cys 140 is toward helix V. The difference in position of Cys 140 from Glu 134 and Val 138 could account for our experimental data in which the reverse substitutions of D134E and C138V caused recovery of G protein activation more efficiently than that of T140C (Fig. 6).

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