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The Second Cytoplasmic Loop of Metabotropic Glutamate Receptor Functions at the Third Loop Position of Rhodopsin[1]

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G protein–coupled receptors identified so far are classified into at least three major families based on their amino acid sequences. For the family of receptors homologous to rhodopsin (family 1), the G protein activation mechanism has been investigated in detail, but much less for the receptors of other families. To functionally compare the G protein activation mechanism between rhodopsin and metabotropic glutamate receptor (mGluR), which belong to distinct families, we prepared a set of bovine rhodopsin mutants whose second or third cytoplasmic loop was replaced with either the second or third loop of Gi/Go- or Gq-coupled mGluR (mGluR6 or mGluR1). Among these mutants, the mutants in which the second or third loop was replaced with the corresponding loop of mGluR exhibited no G protein activation ability. In contrast, the mutant whose third loop was replaced with the second loop of Gi/Go-coupled mGluR6 efficiently activated Gi but not Gt: this activation profile is almost identical with those of the mutant rhodopsins whose third loop was replaced with those of the Gi/Go-coupled receptors in family 1 [Yamashita *et al.* (2000) *J. Biol. Chem.* 275, 34272–34279]. The mutant whose third loop was replaced with the second loop of Gq-coupled mGluR1 partially retained the Gi coupling ability of rhodopsin, which is in contrast to the fact that all the rhodopsin mutants having the third loops of Gq-coupled receptors in family 1 exhibit no detectable Gi activation. These results strongly suggest that the molecular architectures of rhodopsin and mGluR are different, although the G protein activation mechanism involving the cytoplasmic loops is common.

Key words: chimeric mutant, G protein, G protein–coupled receptor, metabotropic glutamate receptor, rhodopsin.

Rhodopsin is the photoreceptive molecule in retinal visual cells and one of the most well-studied G protein-coupled receptors (GPCRs). 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-helical domains connected by three extracellular and three cytoplasmic loops, which are the typical structural motifs of GPCRs (*3–5*). The photoisomerization of the chromophore from 11-*cis*- to all-*trans*-retinal leads to the conformational changes of the opsin that activate retinal G protein (*1*, *2*). Several lines of evidence have revealed that the second, third, and fourth cytoplasmic loops of bovine rhodopsin are involved in the process of activation of G protein transducin (Gt) (*6–13*).

Besides rhodopsin, more than several hundred GPCRs have been identified so far. Comparison of their amino acid sequences has shown that these receptors can be classified into at least three major families (*14*). The first family (family 1) comprises receptors homologous to rhodopsin (called the rhodopsin superfamily), *i.e.* receptors for amines, nucleotides, eicosanoids, peptide hormones and glycoprotein hormones. Receptors homologous to the glucagon receptors and vasoactive intestinal peptide receptors form the second family (family 2). The third family (family 3) contains the metabotropic glutamate receptor (mGluR), the γ-aminobutyric acid, type B receptor (GABA-B receptor), and the Ca^{2+}-sensing receptor. Some structural elements including seven transmembrane domains are common to all three families, but the members of any family exhibit no sequence similarity with those of the other families.

In family 1 receptors, specific regions in the third cytoplasmic loop, especially the N- and C-terminal domains of the loop, have been shown to be crucial for selective G protein activation (*15–19*). In addition, the third loop is reported to induce GDP/GTP exchange on bound G protein based on the finding that the deletion mutant as to the third loop binds to but fails to activate G protein (*8*, *20*). Moreover, the movement of the helical bundles leading to G protein activation has been well discussed (*21–26*). Judging from the results of these studies, the separation of the cytoplasmic

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Abbreviations: DM, dodecyl maltoside; Gi, Gi-type G protein; GPCR, G protein–coupled receptor; Gq, Gq-type G protein; Gt, transducin; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; mGluR, metabotropic glutamate receptor; RhL2/m6L2, the rhodopsin mutant whose second loop (L2) is replaced by the second loop (L2) of mGluR6.

ends of helices III and VI, as rigid bodies, would be essential for formation of the active state, which is supposed to unmask the G protein binding site(s), mainly the third cytoplasmic loop.

As for other families, for mGluR (belonging to family 3), several reports have appeared on the structural determinants of the coupling between the receptor and G protein (*27*, *28*). Analyses of chimeric receptors among several subtypes of mGluRs have indicated that the second loop would have the unique sequence that acts as a determinant of the G protein subtype selectivity, although other cytoplasmic regions could be partly necessary for sufficient G protein selectivity (*27*, *28*). In addition, it has been reported that site-directed mutations in the second loop caused a serious loss of the G protein activation ability (*29*). Recently, a structural change of the extracellular ligand-binding region of mGluR1 was revealed by X-ray crystallography, which emphasizes the functional importance of the dimerization of mGluR and the relocation of dimer interfaces (*30*). However, the molecular mechanism leading to the structural changes of the cytoplasmic regions (especially the second cytoplasmic loop) that are essential for the G protein activation is still unknown.

We previously investigated the roles of cytoplasmic loops in family 1 receptors by preparing rhodopsin mutants whose second or third cytoplasmic loop was replaced with the corresponding loop of the receptors (*31*). The results showed that the third loops of all the Gi/Go-coupled receptors investigated (five species) can function in the rhodopsin molecule as to their G protein subtype selectivity, while those of the Gq-coupled receptors do not function in the molecule. Taken together with the fact that rhodopsin is classified as a receptor that couples with the Gi family of G proteins, these results strengthen the common mechanisms of activation and selection of G proteins by receptors in family 1, although the amino acid sequences are considerably different among receptors. Therefore, it was of interest to determine whether or not the receptors in the other family share common mechanisms with those in family 1 for furthering of our understanding of the G protein activation mechanism by receptors. For this purpose, we have compared the mechanism of G protein activation between rhodopsin and mGluR by preparing bovine rhodopsin mutants having the second or third cytoplasmic loop of mGluR. The mutants in which the second or third loop was replaced with the corresponding loop of Gi/Go-coupled mGluR6 did not activate Gi and Gt, suggesting that the molecular architecture of mGluR6 related to G protein activation is different from that of rhodopsin. In contrast, surprisingly, the mutant whose third loop was replaced with the second loop, the putative G protein recognition site, of mGluR6 efficiently activated Gi but not Gt, indicating that the second loop of mGluR6 can really function at the third loop position of the rhodopsin molecule through a proper conformational change. The activation profile of the mutant is indistinguishable from those of the rhodopsin mutants having the third loops of the receptors in family 1. However, the chimeric mutant in which the third loop of rhodopsin was replaced with the second loop of Gq-coupled mGluR1 showed partial Gi coupling ability. This is in contrast to the fact that all the chimeric mutants having the third loops of Gq-coupled receptors in family 1 showed no Gi activity without exception, indicating that the mechanism of G protein selectivity by mGluRs could be somewhat different from that in the case of receptors in family 1. Based on these results, the G protein activation mechanism of rhodopsin and mGluR is discussed.

MATERIALS AND METHODS

Materials—The hybridoma producing anti-bovine rhodopsin monoclonal antibody 1D4 was generously supplied by Dr. R.S. Molday (University of British Columbia). The cDNA encoding rat Giα1 was kindly provided by Dr. H. Itoh (Tokyo Institute of Technology). Oligonucleotide DNAs and [^{35}S]GTPγS (37 TBq/mmol) were purchased from Amersham Pharmacia Biotech and PerkinElmer Life Sciences, respectively.

Preparation of Rhodopsin Mutants—cDNAs that encode chimeric mutants between bovine rhodopsin and mGluRs, human mGluR1 and mGluR6 were constructed by cassette mutagenesis of the polymerase chain reaction fragments for bovine rhodopsin (*31*). Expression and purification of the wild-type and mutant rhodopsins were carried out according to the methods previously described (*32*). Absorption spectra of the wild-type and mutant rhodopsins in 0.02% dodecyl maltoside (DM) were recorded with a Shimadzu MPS-2000 recording spectrophotometer.

Purification of G Proteins—Purification of transducin from bovine rod outer segments (ROS) was carried out according to the methods previously described (*33*).

The rat Giα1 subunit was expressed in *Escherichia coli* strain BL21 by using Giα1 cDNA constructed in the pQE6 plasmid vector and was purified as described previously (*34*). The purified Giα1 was mixed with an equal amount of the transducin βγ subunit before use in the assays.

G Protein Activation Assays—A radionucleotide filter-binding assay, which measures light-dependent GDP/GTPγS exchange by G proteins, was carried out as described previously (*31*, *35*). All procedures were carried out at 15°C (Gt assay) or 0°C (Gi assay). The assay mixture consisted of 50 mM HEPES (pH 7.0), 140 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.01% DM, 1 μM [^{35}S]GTPγS, and 2 μM (Gt assay) or 4 μM (Gi assay) GDP. Rhodopsin solutions (rhodopsin final concentration: Gt assay, 4 nM; Gi assay, 7 nM) were irradiated with orange light for 30 s or kept in the dark, and then were immediately mixed with a G protein solution (G protein final concentration, 600 nM). After incubation for the selected time in the dark, an aliquot (20 μl) was removed and added to 200 μl of a stop solution [20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM $MgCl_2$, 1 μM GTPγS, and 2 μM (Gt assay) or 4 μM (Gi assay) GDP], and then immediately filtered through a nitrocellulose membrane to trap [^{35}S]GTPγS bound to G proteins. The membrane was washed 4 times with 200 μl of a wash solution [20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM $MgCl_2$] to remove free [^{35}S]GTPγS and then air-dried. The amount of bound [^{35}S]GTPγS was determined by assaying the membrane with a liquid scintillation counter (LS 6000IC, Beckman).

RESULTS AND DISCUSSION

Figure 1A shows the amino acid sequences of the second and third cytoplasmic loops of bovine rhodopsin and mGluRs, Gq-coupled mGluR1 and Gi/Go-coupled mGluR6.

In this study, we designed bovine rhodopsin mutants in which the second or third cytoplasmic loop was replaced with either the second or third loop of two subtypes of mGluRs. We successfully obtained the light-absorbing pigments from the mutant proteins by means of reconstitution with 11-*cis*-retinal. The wild-type (WT) and all the mutant rhodopsins exhibited almost the same absorption maxima at 499 nm (Fig. 1B). All the mutants were expressed at 1/5–1/20 the level of WT and the expression level of each mutant was estimated from the absorbance at 499 nm shown in Fig. 1B. Upon illumination, all the mutants formed the characteristic Meta II intermediates, which exhibited absorption maxima at 380 nm (data not shown). These observations suggest that all the mutants folded properly and changed the conformation of the helical bundles upon absorption of light.

Analysis of the Mutant Rhodopsins Having the Second Loop of mGluR6—So far, eight subtypes of mGluRs have been characterized (*36*, *37*). Among these subtypes, mGluR6 is reported to functionally couple to Gi/Go (*38*), and weakly (~1/18 rate of Go activation) but significantly to Gt in an *in vitro* reconstitution system (*39*). Therefore, we thought that the cytoplasmic domain(s) of mGluR6 could work in the Gt-coupled bovine rhodopsin molecule. We first compared the abilities of Gt and Gi activation between WT and mutant rhodopsins whose second or third cytoplasmic loop was replaced with either the second or third loop of mGluR6 (Fig. 2). It should be noted that we measured the G protein activation rates under the linear correlation between the amount of light-dependently activated G protein and the incubation time after irradiation (*31*). None of the mutants we prepared exhibited detectable G protein activation ability in the dark (data not shown).

The replacement of the second loop of rhodopsin with either the second or third loop of mGluR6 resulted in deletion of the "E(D)RY" motif highly conserved in GPCRs of family 1. Because this motif is essential for rhodopsin to

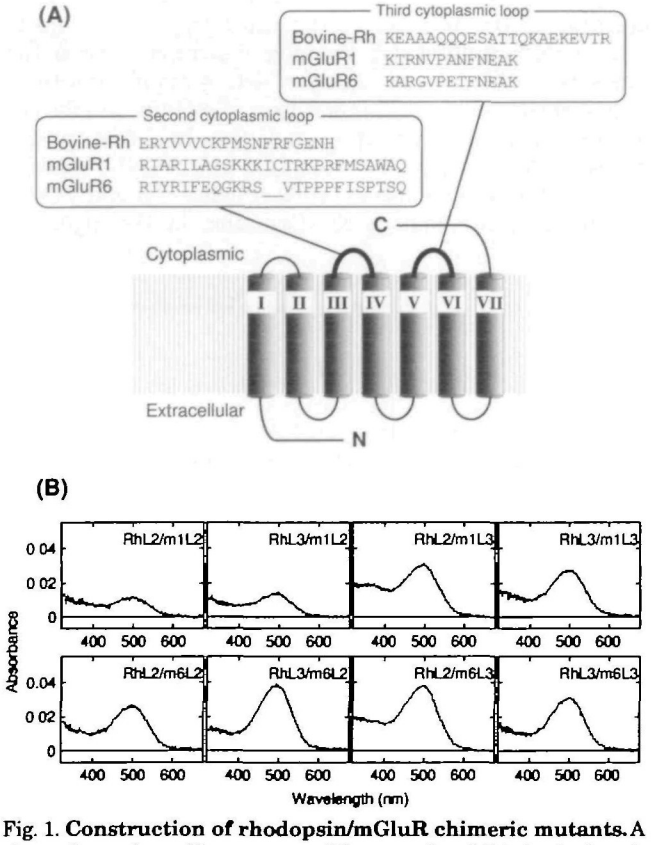

Fig. 1. **Construction of rhodopsin/mGluR chimeric mutants.** A shows the amino acid sequences of the second and third cytoplasmic loops of bovine rhodopsin and human mGluRs. For the sequence comparison of Gq-coupled mGluR1 and Gi/Go-coupled mGluR6 refer to (*27–29*). B shows the absorption spectra of the chimeric mutants. Mutants RhL2/m1L2 and RhL3/m1L2 have the second loop of mGluR1 at the position of the second and third loop of rhodopsin, respectively. Mutants RhL2/m1L3 and RhL3/m1L3 have the third loop of mGluR1 at the position of the second and third loop of rhodopsin, respectively.

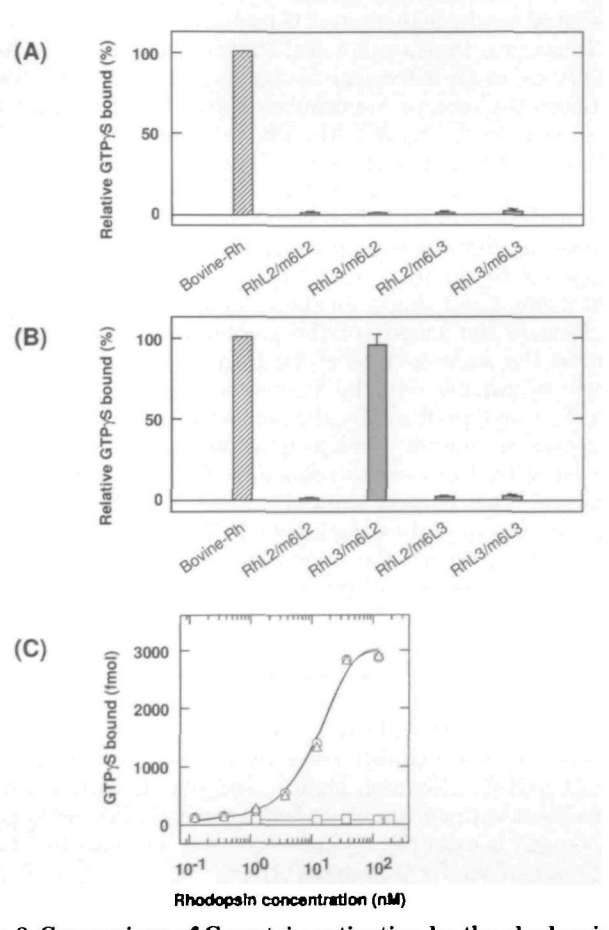

Fig. 2. **Comparison of G protein activation by the rhodopsin/mGluR6 chimeric mutants.** A and B show the relative initial rates of G protein (Gt and Gi, respectively) activation by WT or mutants (RhL2/m6L2, RhL3/m6L2, RhL2/m6L3, and RhL3/m6L3). The amino acid sequences of the cytoplasmic loop regions in rhodopsin and mGluR1 are shown in Fig. 1A. Data are the means ± SD for more than three independent experiments. GTPγS binding activities of the mutants are normalized as to the 293S-expressed WT activity. GTPγS binding assays with the purified mutants and G proteins were carried out as described under "MATERIALS AND METHODS." C shows the dose-response curves for WT, RhL2/m6L2 and RhL3/m6L2-mediated Gi stimulation. Light-dependent GTPγS binding to Gi for 90 s was measured. (Open circles) WT; (open squares) RhL2/m6L2; (open triangles) RhL3/m6L2. The EC_{50} values of WT (11.9 nM) and RhL3/m6L2 (12.5 nM) were obtained from the respective dose-response curves. The data are the means for two independent experiments.

activate G proteins (*8*, *9*, *31*), it was expected that the mutant pigments lost the G protein activation ability. In fact, the mutant having the second loop or third loop of mGluR6 at the position of the second loop (RhL2/m6L2 or RhL2/m6L3) exhibited no activation ability as to Gt and Gi. In addition, the mutant having the third loop of mGluR6 at the third loop position (RhL3/m6L3) exhibited no activation, suggesting that the third loop of mGluR6 has a different role from that of the third loop of rhodopsin, or it could not take on a proper conformation to activate G proteins at the third loop position of rhodopsin. On the other hand, interestingly, the mutant having the second loop of mGluR6 at the third loop position (RhL3/m6L2) efficiently activated Gi in a light-dependent manner, indicating that the second loop of mGluR6 has a role homologous to that of the third loop of rhodopsin and takes on a proper conformation essential for the activation of G proteins.

To confirm that mutant RhL3/m6L2 exhibits equivalent ability as to Gi activation to that of WT, the correlations between the receptor concentration and the activation rate were examined (Fig. 2C). Mutant RhL3/m6L2 (EC_{50} = 12.5 nM) exhibited almost the same Gi activation ability as that of WT (EC_{50} = 11.9 nM). It should be noted that the activation ability as to Gi is indistinguishable from those of the rhodopsin mutants having the third loops of Gi/Go-coupled receptors in family 1 (*31*). On the other hand, mutant RhL2/m6L2 had almost no ability as to Gi.

Because the length of the second loop of mGluR6 is almost the same as that of the third loop of rhodopsin, it might be possible that the functional replacement is due to not the sequence itself but the similar length. Therefore, we prepared a mutant rhodopsin whose third loop was replaced with the reverse sequence of the second loop of mGluR6. This mutant showed a great loss of Gi coupling ability (less than 10% compared to WT, data not shown), which strengthened the importance of not the length but the specific sequence of the second loop of mGluR6. Taken together, we concluded that the second loop of mGluR6 has the capacity to adopt the active conformation at not the second but the third loop position of rhodopsin.

We also prepared a double loop-replaced mutant in which the second and third loops are replaced with the third and second loops of mGluR6, respectively. This mutant showed no Gt and Gi activation ability (data not shown), which is consistent with our previous finding that the second loop of rhodopsin is essential for the rhodopsin molecule to be an activating form for G proteins (*31*).

Analysis of the Mutant Rhodopsins Having the Second Loop of mGluR1—The results described above clearly showed that the second loop of mGluR6 can really function at the third loop position of rhodopsin. However, the mutant exhibited no detectable activation of Gt (Fig. 2), although it has been reported that mGluR6 coupled with Gt weakly but significantly (*39*). This suggests that the second loop of mGluR6 could not fully retain its G protein subtype selectivity when it was introduced into the rhodopsin molecule. Therefore, to further examine the retention of the G protein subtype selectivity, we constructed a chimeric mutant of rhodopsin whose third loop was replaced with the second loop of mGluR1, the Gq-coupled subtype of mGluR, and investigated whether or not the mutant entirely loses the Gi-coupling ability.

Figure 3 shows the experimental results for the four mutants in which the second or third loop of bovine rhodopsin was replaced with the second or third loop of mGluR1. All the mutants except for mutant RhL3/m1L2, having the second loop of mGluR1 at the position of the third loop, exhibited no activation of Gt and Gi (Fig. 3, A and B). The activation efficiency as to Gt of RhL3/m1L2 was also negligible as in the case of the corresponding mutant of mGluR6 (Fig. 3A). However, the mutant exhibited about 25% Gi coupling ability (Fig. 3B), although this is considerably lower than that observed for the corresponding mutant of mGluR6 (RhL3/m6L2). The partial retention of the activity toward Gi is in marked contrast to the results of our previous study, in which the replacement of the third loop of rhodopsin with that of Gq-coupled receptors in family 1 caused the loss of Gi activation without exception (*31*). Taken together with the results for mGluR6, these results suggest that the molecular architecture responsible for the coupling specificity for the G protein subtype in the mGluR system could be somewhat different from that in the rhodopsin system.

Molecular Mechanism of G Protein Activation in mGluR—Although the mechanism of the coupling specificity for the G protein subtype is somewhat different between rhodopsin and mGluR, the present study clearly shows that the structural change in the helical bundle of rhodopsin leads to the active conformation of the second loop of mGluR6 at the position of the third loop of rhodopsin. These results suggest that the molecular mechanism of the exposure of the covered region, which is mainly the third loop of rhodopsin or the second loop of mGluR, in efficient G protein activation would be analogous. In rhodopsin (*21*, *22*, *24*) and other family 1 receptors (*25*, *26*), the relative movement of the cytoplasmic ends of helices III and VI was shown to be prerequisite for formation of the signaling

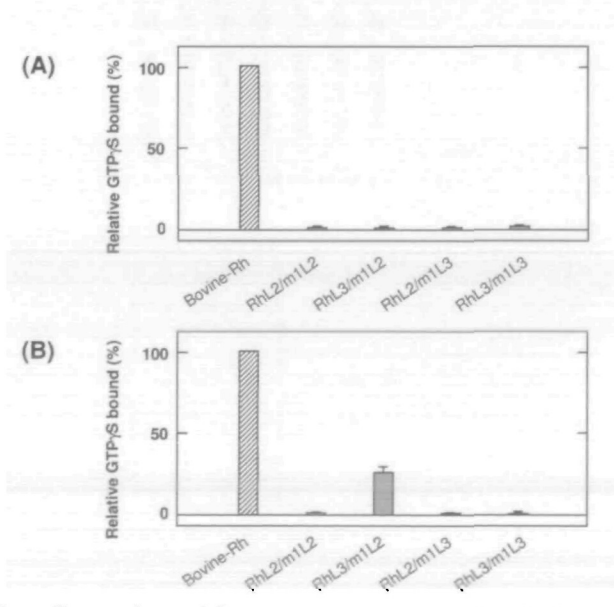

Fig. 3. **Comparison of G protein activation by the rhodopsin/mGluR1 chimeric mutants.** A and B show the relative initial rates of G protein (Gt and Gi, respectively) activation by WT or mutants (RhL2/m1L2, RhL3/m1L2, RhL2/m1L3, and RhL3/m1L3). The amino acid sequences of the cytoplasmic loop regions in rhodopsin and mGluR1 are shown in Fig. 1A. Data are the means ± SD for more than three independent experiments. GTPγS binding activities of the mutants are normalized as to the WT activity.

state (Fig. 4A). However, there have been no reports of direct monitoring of the movements of the transmembrane and cytoplasmic regions in mGluR, although the conformational rearrangements of the extracellular regions coupled with ligand binding have been revealed (*30*). Therefore, speculation is worthwhile as to the movements of the transmembrane and cytoplasmic regions in mGluR on the basis of the results of the present study.

Our results indicated that the second loop of mGluR can function at the third loop position of the rhodopsin molecule. Thus, helices III and IV, which connect to the second loop of mGluR in the mGluR molecule, could change their conformation in analogy to helices V and VI of rhodopsin to uncover the G protein binding region. In rhodopsin, the C-terminal segment of the third loop, which connects to helix VI, is more important for activating G proteins (*13*), so that helix VI should change its conformation relatively more than helix V. Analysis of chimeric mutants of mGluRs showed that the C-terminal segment, rather than the N-terminal one, in the second loop of mGluR is necessary for the G protein coupling (*27*). Overall, it is likely that helix IV, which connects to the C-terminus of the second loop of mGluR, could change its conformation similar to helix VI of rhodopsin (Fig. 4B). Because of the lack of information on the structure of the helical bundle of mGluR at present, we could not exclude the possibility that helix III or other helical regions could change the conformation to uncover the C-terminal region of the second loop in the mGluR molecule. Therefore, it is necessary to determine whether or not the third loop of rhodopsin can function at the second loop position of mGluR, and to directly monitor the conformational change of the helical domains in the mGluR molecule for furthering of our understanding of the G protein activation mechanism in mGluR.

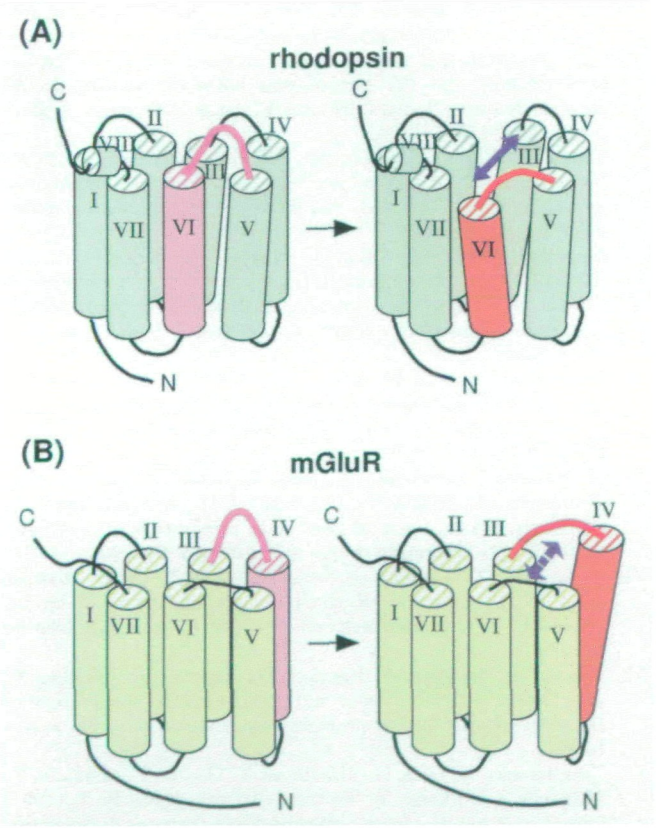

Fig. 4. **Schematic representation of the proposed conformational changes of GPCRs following agonist activation.** A shows the deduced structural changes of helices III and VI in rhodopsin and other family 1 receptors (*21*, *22*, *24–26*). The change in the relative orientation of helices III and VI is supposed to be necessary for G protein activation. B shows a model of the structural change in helix IV of mGluR indicated by the present study. We speculate that the second loop and helix IV of mGluR would have analogous roles to those of the third loop and helix VI of rhodopsin (presented in purple and red), although the possibility of relative movement of helix III has not been entirely excluded (see the text).

A recent crystallographic study revealed that there is an extra helical region (called helix VIII) in the rhodopsin molecule (*5*), which is situated near the C-terminal region of the third loop (Fig. 4A). A site-directed mutagenesis study suggested that the C-terminal region of the G protein α-subunit interacts with helix VIII or nearby helix VII including the NPXXY motif of rhodopsin (*40*, *41*). These results strongly suggest that the role of the third loop in rhodopsin as a determinant of G protein selectivity could be facilitated by helix VII or VIII. As for mGluR, the second loop is reported to cooperate with other cytoplasmic regions, including the extension domain of helix VII, to efficiently control the G protein coupling (*28*). That is, GPCRs have adopted various ways of accomplishing selective G protein coupling through combination of their cytoplasmic domains, although it has been shown that the C-terminal region of the G protein α-subunit plays a critical role in the receptor/G protein interaction in both families 1 (*42*, *43*) and 3 (*44*, *45*) receptors. The distinct mechanisms of G protein selectivity for rhodopsin and mGluR deduced from our data could be caused by the different intramolecular cooperation of the main G protein coupling site with other supporting sites on the basis of the differences in the molecular architecture of transmembrane and cytoplasmic domains. Overall, we conclude that the second loop of mGluR has a role similar to that of the third loop of rhodopsin in G protein activation, while the mechanism of G protein selectivity could be different, probably because of the distinct positions of the G protein recognition sites in these receptors.

As already described, rhodopsin and mGluR exhibit no sequence similarity. From the viewpoint of the molecular evolution of GPCRs, this suggests that they diverged from different ancestral types or diverged at an earlier stage before acquirement of the G protein coupling specificity. If the latter is case, our results suggest that these proteins could have diverged through rearrangement of the helical domains caused by many amino acid mutations or domain shuffling in the course of evolution.

In conclusion, the present study has shown that for both rhodopsin and mGluR, a common mechanism, that is, exposure of the cytoplasmic loops on the helical movement in their adjacent transmembrane domains, would lead to efficient G protein activation irrespective of their positions in each receptor. Thus, analyses of chimeric mutants between receptors of family 1 and other families would provide some insight into the molecular mechanism of G protein activation in other families based on the accumulated evidence for family 1. This would be one of the first steps for directly comparing the G protein activation mechanism among GPCRs in different families. Further chimeric studies involving receptors in not only families 1 and 3 but also family 2 would emphasize the generality of G protein activa-

tion mechanism.

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