The Second Intracellular Loop of Metabotropic Glutamate Receptors Recognizes C Termini of G-protein α-Subunits*

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Heptahedral receptor coupling selectivity to G-proteins is controlled by a large contact area that involves several portions of the receptor and each subunit of the G-protein. In the G-protein α subunit, the C-terminal 5 residues, the N terminus, and the αN–β1 and α4–α5 loops play important roles. On the receptor side, both the second and third (i2 and i3) intracellular loops as well as the C-terminal tail probably contact these different regions of the G-protein. It is now accepted that the C terminus of the α subunit binds in a cavity formed by the i2 and i3 loops. Among the various G-protein-coupled receptors (GPCRs), class III receptors that include metabotropic glutamate (mGlu) receptors greatly differ from the rhodopsin-like GPCRs, but the contact zone between the receptors and the G-protein is less understood. The C terminus of the α subunit has been shown to play a pivotal role in the selective recognition of class III GPCRs. Indeed, the mGlu2 and mGlu4 and -8 receptors can discriminate between α subunits that differ at the level of their C-terminal end only (such as Gq6 and Gq7). Here, we examine the role of the i2 loop of mGluRs in the selective recognition of this region of the α subunit. To that aim, we analyzed the coupling properties of mGlu2 and mGlu4 or -8 receptors and chimeras containing the i2 loop of the converse receptor to G-protein α subunits that only differ by their C termini (Gq6, Gq7, and their point mutants). Our data demonstrate that the central portion of the i2 loop is responsible for the selective recognition of the C-terminal end of the α subunit, especially the residue on position −4. These data are consistent with the proposal that the C-terminal end of the G-protein α subunit interacts with residues in a cavity formed by the i2 and i3 loops in class III GPCRs, as reported for class I GPCRs.

G-protein-coupled-receptors (GPCRs) typically modulate specific signaling pathways depending on the subset of G-proteins activated. This requires positive interaction of certain parts of the GPCR with portions of the heterotrimeric G-protein. Recent progress has been made in defining the structure of G-proteins and functional studies using site-directed mutagenesis of receptors and/or G-proteins. This progress has provided increasingly more specific and precise descriptions of the contact sites between these proteins (1). From the G-protein α, β, and γ subunits, probably both the Gα and the βγ dimer interact with the receptors. The Gα subunit has a decisive role in discriminating between different receptor subtypes (2–4) and also between different functional states of the receptor (5, 6). Several regions along the sequence of the Gα-proteins are involved in the selective coupling to GPCRs (1, 7, 8). The best characterized region is the extreme C terminus where residues at positions −3 and −4 are decisive for coupling Gα proteins to some receptors (1, 9–13). Residue −4 in the Gα family of G-proteins is the cysteine residue, which is ADP-ribosylated by pertussis toxin, a covalent modification that prevents the G-protein from interacting with the receptor. In the Gβγ family, the residue −4 must be a bulky aromatic residue (14), or alternatively, this Tyr residue may be phosphorylated (15) to efficiently couple to the phospholipase C-activating receptors. Conformational changes in the C-terminal structure upon coupling to the receptor may play a role in the activation process. Other regions of the α subunit determining coupling selectivity are the extreme N terminus (16) and the region between the α4 and α5 helices, which includes the L9 loop and β6 sheet (17). This latter region is involved in the coupling selectivity, probably by interacting directly with either class I receptors (rhodopsin-like family) during activation (8, 18–21) or class III GPCRs.

On the receptor side, several intracellular portions are involved in the selective coupling to G-proteins. These have been extensively studied in class I GPCRs and include both the i2 and i3 loops, as well as the amphipathic helix, called H8 (and referred to previously as the i4 loop) and the C-terminal tail of the receptor (3, 22). For example, studies using the splice variants of prostanoid receptors pointed to the C terminus to be one region that discriminates between G-proteins (23). A study that employed chimeras between muscarinic and β-adrenergic receptors pointed to the i2 and i3 loops to control coupling selectivity (24). Within the class III GPCRs, although most of these regions have been shown to control coupling efficacy (25–27), only the i2 loop has been found to play a pivotal role in coupling selectivity (25, 28, 29).

GABAγ, γ-aminobutyric acid, type B; mGlu, metabotropic glutamate; mGluR, mGlu receptors; i2 or i3 loop, second or third intracellular loop; HER293 cells, human embryonic kidney cells; IP, inositol phosphates; ELISA, enzyme-linked immunosorbert assay; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline.

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‡ The abbreviations used are: GPCR, G-protein-coupled receptor;
Surprisingly, several regions of both the G-protein α subunit and class III receptors have been identified as being involved in coupling selectivity. However, which part of the receptor recognizes a specific part of the G-protein coupling selectivity. The present study was aimed at identifying the specific region of the G-protein α subunit that is recognized by the i2 loop of mGluRs. Our data revealed that this i2 loop, and more precisely the central part of it, discriminates between G-protein α subunits that differ at the level of their extreme C terminus, especially at position −4. This is consistent with what has been proposed for class I rhodopsin-like receptors and what has been proposed for mGlu2, mGlu4, and mGlu8 receptors.

EXPERIMENTAL PROCEDURES

Materials—Chemicals including glutamate were obtained from Sigma, unless otherwise indicated. Serum, culture media, and other solutions used for cell culture were from Invitrogen. The plasmids (Invitrogen) containing 0.4 mM glucose, 20 mM Hepes, pH 7.4) and incubated with 1 ml of Hepes-buffered saline containing 1 unit of GPT/ml and 2 mM pyruvate for 1 h. After washing once with Hepes-buffered saline, LiCl was added to a final concentration of 10 mM. The agonist was applied 5 min later and left for 30 min at 37 °C. Replacing the incubation medium with 0.5 ml of perchloric acid (5%) stopped the reaction, and the clusters were kept on ice for 30 min. Supernatants were recovered, and the IPs were purified on Dowex columns (31). Total radioactivity remaining in the membranes fraction was counted after treatment with 10% Triton X-100, 1× NaOH for 30 min and used as standard. Results are expressed as the percentage of IP produced over the radioactivity present in the membranes. The dose-response curves were fitted according to the equation $y = \left( y_{\text{max}} - y_{\text{min}} \right) \frac{1}{1 + \left( x/EC_{50} \right)^n} + y_{\text{min}}$ using the Kaleidagraph program (Abelbeck Software, Reading, PA).

Construction of Chimeric mGlu Receptors—To be able to analyze the expression and correct plasma membrane targeting of the wild-type and chimeric receptors studied, mGlu2, mGlu4, and mGlu8 receptors were first epitope-tagged at their N-terminal end. For the insertion of either an HA or c-Myc epitope at the N-terminus, mGlu2/8α i2, mGlu8 i2, mGlu8-1st (mGlu8 with the N-terminal portion of the central zone of mGlu2), mGlu8 i2), mGlu8-1st (mGlu8 with the N-terminal portion of the central zone of mGlu2) and mGlu8/2i2 (mGlu8 with the i2-loop sequence of mGlu2), mGlu8-1st (mGlu8 with the N-terminal portion of the central zone of mGlu2) and mGlu8/2i2 (mGlu8 with the i2-loop sequence of mGlu2), mGlu2-1st (mGlu2 with the N-terminal portion of the central zone of mGlu2) and mGlu8-1st (mGlu8 with the N-terminal portion of the central zone of mGlu2) and mGlu8-VTA and mGlu8-AQR for the mGlu2 protein.

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FIG. 1. Differential coupling of Gαq, Gαz, and their point mutants to mGlu2, mGlu4, and mGlu8 receptors. A, the 5 extreme C-terminal amino acid (C-term AA) residues of G-proteins used in this study. Gαq, Gαz, and their reciprocal point mutants on the position −4 were co-expressed with the mGlu receptors. The coupling was measured as inositol phosphate accumulation (see “Experimental Procedures”). Activation of corresponding G-protein α subunits is expressed as: +, greater than 3-fold stimulation of IP formation; +, 2-3-fold stimulation; 0, no significant increase in IP formation upon glutamate stimulation. As shown in B, basal (white bars) and 1 mM glutamate-induced (black bars) IP formations were determined in HEK 293 cells co-expressing the mGlu8 receptor with the G-protein α subunits Gαq, Gαz, and GαqCl. Data represent the radioactivity in the IP fraction divided by the total radioactivity in the membranes and are means ± S.E. of at least three experiments performed in triplicate.
and mGlu8 receptors in which the VTA/AQR sequences were swapped. The correct replacement of the loop was checked by double strand DNA sequencing.

Quantification of Cell Surface Receptors Using ELISA—Determination of expression level of surface receptors was performed using an ELISA assay adapted from Ref. 34. Twenty-four h after transfection (10^6 cells), cells were fixed with 4% paraformaldehyde and then blocked with PBS + 5% fetal bovine serum. After 30 min of reaction with primary antibody (monoclonal anti-HA clone 3F10 (Roche Applied Science) at 0.5 μg/ml) in the same buffer, the goat anti-rat antibody (Jackson ImmunoResearch Laboratories) diluted 1/1000 in PBS-gelatin. The incubation was performed in the dark for 1.5 h. The preparations were then washed three times 10 min with PBS-gelatin and fixed.

**FIG. 2.** Sequence alignment of the second intracellular loops (i2) of group II and group III mGlu receptors. A, amino acid sequences of the predicted second intracellular loops of the rat and Drosophila melanogaster (Dro) receptors. The residues in black boxes are those that differ substantially between the members of the two groups. Underlined residues at the central portions of the i2 loops are those that were swapped resulting in the chimeric receptors mGlu2/8i2 and mGlu8/2i2. The entire i2 sequences were swapped in the case of mGlu2/4i2 and mGlu4/2i2 receptors. nt, nucleotides. B, schematic representations of the receptors and their chimeras used in this study. The circles are extracellular domains followed by a square representing the heptahedral domain with the i2 loops (semicircles).

**FIG. 3.** mGlu2/4i2 has the same coupling selectivity toward G\textsubscript{o,q}o and G\textsubscript{o,q}z as the mGlu4 receptor. As shown in a, mGlu2, mGlu4, and mGlu2/4i2 are expressed at a similar level at the surface of transfected cells as revealed by an ELISA assay using an HA antibody. Basal represents the signal obtained with mGlu2 transfected cells using the secondary antibody only. Mock represents the signal obtained with mock-transfected cells using both the HA and secondary antibodies. Values are means ± S.E. of triplicate determinations from a typical experiment out of three and correspond to the raw values obtained with the luminometer. b, basal (white bars) and 1 mM glutamate-induced (black bars) IP formations were determined in HEK-293 cells co-expressing the mGlu2, mGlu4, or mGlu2/4i2 receptor and the G-protein α subunits G\textsubscript{o,q}o or G\textsubscript{o,q}z. Data represent the percentage of radioactivity in the IP fraction as compared with the total radioactivity in the membranes and are means ± S.E. of at least three experiments performed in triplicate.

**RESULTS**

Differential Coupling of G\textsubscript{o,q}o and G\textsubscript{o,q}z and Their –4 Point Mutants to mGlu4, mGlu8, and mGlu2 Receptors—Many G\textsubscript{o,q}o-coupled GPCRs have been shown to activate chimeric G-proteins corresponding to G\textsubscript{o,q}o in which the C-terminal 5 residues are replaced by those of either G\textsubscript{o,q}z, G\textsubscript{o,q}z, or G\textsubscript{o,q}o. Since these chimeric G-proteins activate phospholipase C-β, the effective coupling between the receptor and the chimeric G-protein can easily be assessed by measuring IP accumulation after receptor activation.

Group II (mGlu2 and -3) and group III (mGlu4, -6, -7, and -8) mGlu receptors couple to both G\textsubscript{o,q}o and G\textsubscript{o,q}z. G\textsubscript{o,q}o is sufficient to allow coupling to mGlu2 (12). As a positive
control, all these various G-protein α subunits can be efficiently activated by either mGlu4 (12) or mGlu8 receptors (Fig. 1).

Sequence Differences of the Intracellular Loops of mGlu2, mGlu4, and mGlu8 Receptors—Analysis of the intracellular portions of these receptors revealed highly conserved and short intracellular loops 1 and 3. Within the i1 loop, a single residue is different between group II and group III mGluRs, and in the i3 loop, only two positions are clearly different (see Ref. 35). In contrast, the i2 loop is more variable (Fig. 2A), and the C-terminal tail is the less conserved region. We therefore decided to examine the role of the i2 loop in the specific recognition of the C-terminal tail of the G-protein α-subunit, and more specifically, residue −4. To that aim, we constructed a series of chimeric receptors in which the i2 loop or a portion of it has been swapped between mGlu2 and either mGlu4 or mGlu8 receptors (Fig. 2B). These constructs also include a c-Myc or HA epitope at the N-terminal end, just after the signal peptide. We previously reported that the presence of such an epitope at an equivalent position in mGlu5 (32, 39), mGlu7 (33, 40), or GABAA (41, 42) receptors affect neither their functional expression nor their pharmacology.

FIG. 4. Glutamate-induced IP formation in cells co-expressing mGlu2, mGlu4 or mGlu2/4i2 receptors with either Gqo or Gqz proteins. As shown in A, cells co-expressing mGlu2 receptor with Gqo (open circles) or Gqz (closed squares) were stimulated with increasing concentrations of glutamate. B, same as in A with mGlu4 receptor. C, Same as in A with the chimerical mGlu4/2i2 receptor. Data represent the radioactivity in the IP fraction divided by the total radioactivity in the membranes. Values are means ± S.E. of three independent experiments performed in triplicate and are expressed as the percentage of the IP production measured under basal conditions with cells expressing the indicated receptor with Gqo.

FIG. 5. mGlu2/4i2 receptors co-expressed with Gqo is activated by mGlu2 selective agonists. Cells co-expressing mGlu2/4i2 receptor with Gqo were treated with various compounds acting on mGluRs. The general mGlu receptor agonist glutamate, the group II selective agonist LY354740 and 2R,4R-aminopyrrolydine-2,4-dicarboxylic acid (2R,4R-APDC) and the group III agonist DL-AP4 were used at increasing concentrations. Values are means ± S.E. of triplicate determinations from one typical experiment and are expressed as the percentage of the IP production measured under basal conditions.

FIG. 6. Immunocytochemistry showing surface expression of the receptors and their chimeras. Extracellular N-terminal tags were detected using corresponding antibodies on non-permeabilized transfected HEK 293 cells. The surface expression was visualized using secondary antibodies labeled by fluorescence. Background was compared using cells transfected with irrelevant cDNAs (Mock).

Sequence within the i2 Loop Recognizes the Extreme C Terminal of Ga Subunit—To examine the role of the i2 loop of mGlu receptors in the selective recognition of the C-terminal tail of the G-protein α subunit, the coupling of mGlu2/4i2 to Gqo and Gqz was first examined. As shown in Fig. 3a, these recep-
FIG. 7. mGlu2/8i2 and mGlu8/2i2 have the same coupling selectivity toward Gqo and Gqz, as the mGlu8 and mGlu2 receptors, respectively. As shown in a, mGlu2, mGlu8, mGlu2/8i2, and mGlu8/2i2 are expressed at a similar level at the surface of transfected cells as revealed by an ELISA assay using an HA antibody. Basal represents the signal obtained with mGlu2 transfected cells using the secondary antibody only. Mock represents the signal obtained with mock-transfected cells using both the HA and secondary antibodies. Values are means ± S.E. of triplicate determinations from one typical experiment out of three and correspond to the raw values obtained with the luminometer. As shown in b, basal (white bars) and 1 mM glutamate-induced (black bars) IP formations were determined in HEK 293 cells co-expressing the mGlu2/8i2 or mGlu8/2i2 receptor and the G-protein α subunits Gqo, Gqz, GqoCI, or GqzIC. Data represent the percentage of radioactivity in the IP fraction as compared with the total radioactivity in the membranes and are means ± S.E. of three experiments performed in triplicate.

TABLE I

EC<sub>50</sub> values (μM) for glutamate determined on cells expressing the indicated receptor and G-protein α subunit.

|       | mGlu2/8i2 | mGlu2/8i2 | mGlu8 | mGlu8/2i2 |
|-------|-----------|-----------|-------|-----------|
| Gqo   | 7.6 ± 1.5 | 13.4 ± 3.2| 11.9 ± 2.8 | 9.1 ± 1.9 |
| Gqz   | NE<sup>a</sup> | 27.9 ± 9.5 | ND<sup>b</sup> | NE<sup>ab</sup> |
| GqoCI | NE<sup>a</sup> | 22.9 ± 5.1 | ND<sup>b</sup> | NE<sup>ab</sup> |
| GqzIC | 11.8 ± 8.8 | 7.7 ± 2.3 | 8.3 ± 3.0 | 3.1 ± 2.7 |

<sup>a</sup>Values are taken from Ref. 12.
<sup>b</sup>NE, no effect.
<sup>ab</sup>N.D. not determined.

These data highlight the i2 loop of mGlu4 as being the structure that enables coupling to G<sub>qz</sub>. Unfortunately, we were unable to obtain large enough responses in cells expressing the converse chimera, mGlu4/2i2, with any of the tested G-proteins.

We decided to pursue our analysis using mGlu8 receptor because we always obtained robust and reproducible responses with this receptor that shares the same G-protein coupling selectivity with mGlu4 (36) (Fig. 1). To this aim, the chimeric receptor subunits mGlu2/8i2 and mGlu8/2i2, in which only the central portion of the i2 loop has been swapped, were tested for their ability to activate G<sub>qo</sub> and G<sub>qz</sub>. As depicted in Fig. 2, the central portion of the i2 loop contains most of the residues that differ between group II and group III mGluRs. When expressed in HEK 293 cells, these constructs were correctly targeted to the cell surface (Fig. 6) and found at the same density (Fig. 7a). As shown in Fig. 7b, the mGlu2/8i2 couples well to G<sub>qz</sub>, like the wild-type mGlu8 (Fig. 1B), whereas the mGlu8/2i2 coupling to this G-protein is largely reduced as compared with that obtained with the wild-type mGlu8. The low effective stimulation of the IP formation in cells expressing mGlu2/8i2 and G<sub>qz</sub> challenged with 1 mM glutamate was not due to a large right shift of the glutamate dose-response curve. As shown in Table I and Fig. 8, the EC<sub>50</sub> value measured in these cells was in the 10 μM range, only three times higher than that measured with G<sub>qo</sub>.
The Central Portion of the i2 Loop Recognizes the /H11002 4 Residue of the G H9251 Subunit—The coupling of mGlu2/8i2 and mGlu8/2i2 to GqoCI and GqzIC was tested to analyze the role of the central portion of the i2 loop in the selective recognition of the /H11002 4 residue. As shown in Figs. 7b and 8, GqoCI behaves like Gqz and GqzIC behaves like Gqo, demonstrating the pivotal role of the central region of the mGlu8 i2 loop (EQGKRSVTAPR) in the recognition of the −4 Ile of Gz C-terminal end or the role of the central portion of the mGlu2 i2 loop (GGAREGAQRPK) to prevent interaction with the −4 Ile-containing C termini.

The VSA/AQR Segment Plays an Important Role in the Recognition of the C Terminus of the Ga Subunit—As illustrated in Fig. 2, there are two tripeptides within the central portion of the i2 loop of group II and group III mGluRs that share no similarity. To identify the specific role of these two regions in the selective coupling to the Gz C terminus, these regions were swapped individually between mGlu2 and mGlu8 receptors. First, we verified that under our experimental conditions, all these mutant receptors were expressed at a similar level at the cell surface (Fig. 9). When co-expressed with the chimeric Ga-subunits, mGlu2-1st and mGlu8-1st were found to activate the same G-proteins as the wild-type mGlu2 and mGlu8, respectively. Interestingly, replacing the AQR motif of mGlu2 with VTA is sufficient to allow the resulting mutant mGlu2-VTA to activate Gqz (Fig. 10). The reverse chimera mGlu8-AQR still significantly activate Gz, but to a lower extent than the wild-type mGlu8 (Fig. 10).

DISCUSSION

Previous studies have identified that the 5 C-terminal residues of the G-protein α-subunit play important roles in determining the coupling not only to class I (3, 4) but also to class III GPCRs (12, 35). However, other regions of the G-protein α-subunit also play a role in the selective interaction to class III GPCRs (30). On the receptor side, the second intracellular loop of the class III GPCRs has been reported to be the main determinant controlling G-protein coupling selectivity (25–28), although in this case also, other regions such as the C-terminal tail are involved. The present study demonstrates the pivotal role played by the i2 loop, and especially the central portion of it, in the selective recognition of the extreme C-terminal end of the α-subunit, and more precisely, residue −4.

The crystal structure of both a seven-transmembrane core domain receptor (rhodopsin) (43) and several states of different G-proteins have been solved (44). Several regions of both receptors and G-proteins have also been shown to be part of the contact area between these two proteins (3, 4, 45). However, the
precise positioning of the G-protein on the receptor remains to be elucidated. Although there is growing evidence indicating that GPCRs may function as dimers, it is still not known whether both seven-transmembrane core domain proteins contact a unique G-protein (45, 46) or whether both seven-transmembrane core domain proteins contact a single G-protein, as recently suggested for rhodopsin (47) and shown for the LTB4 receptor (48). The crystal structure of a receptor-G-protein complex will be necessary to clarify this important issue. However, in the meantime, other approaches may help propose models of this interaction. Using biochemical methods, as well as specific cross-linkers, the i3 loop of rhodopsin was shown to be in close proximity to both C-and N-terminal parts of transducin (49, 50). Mutagenesis experiments aimed at identifying the regions involved in the selectivity of muscarinic receptor coupling to G-proteins also identified the i3 loop as well as the i2 loop as being involved in the recognition of the 5 C-terminal residues of the α-subunit (11, 51). According to these and other data, the C-terminal peptide of the α-subunit has been proposed to interact in a cavity formed by the i2 and i3 loop of the class I GPCRs (3, 4).

When compared with the GPCRs from the other classes, class III GPCRs are unique, not only because of their large N-terminal domain containing the agonist binding site but also because of their very short (30 residues at most for i2) and highly conserved intracellular loops. Among these, i3 is the most conserved within all class III GPCRs, and i2 is the most variable, consistent with this loop playing a critical role in G-protein coupling selectivity. This is in contrast to the class I GPCRs in which the i3 loop is often the longest and more variable loop, whereas i2 contains the highly conserved DRY motif. Interestingly, it has recently been reported that the i3 loop of rhodopsin can be replaced by the i2 loop of a class III GPCR (mGlur6) without preventing G-protein coupling (29). In contrast, no coupling could be observed if the i3 loop is replaced by the i3 loop of mGlur6. This suggests that class III and class I GPCRs do not contact G-proteins the same way, although both classes of receptors recognize the same determinants of the G-protein α-subunit. However, our data indicate that the C-terminal end of the G-protein α-subunit is recognized by the i2 loop, consistent with the possibility that this part of the α-subunit also interacts in a cavity lined by the i2 and i3 loops in class III GPCRs.

In class I muscarinic receptors, the C-terminal end of the i3 loop that corresponds to the intracellular portion of transmembrane helix 6 (TM6) has been shown to play a major role in the selective recognition of the C-terminal end of the α-subunit (11, 37). The equivalent region of the class III GPCRs is also likely to be involved in an α-helix that prolongs TM6 in the intracellular side of the receptor. However, the region corresponds to the NFNEAKX motif conserved in all class III GPCRs (52) and is therefore unlikely involved in G-protein coupling selectivity. In a three-dimensional model of the heptahelical domain of the mGlur receptors (generated based on the crystal structure of rhodopsin, Dr. Gilles Labesse, Montpellier, France), the residues GGA (mGlur2) and EQQ (mGlur4 or -8) (Fig. 2) are just at the bottom of TM3 and in very close proximity to the bottom of TM6, facing a putative binding pocket formed by i2 and i3. However, they are not directly facing the NFNEAKX motif but are located deeper in the cytoplasm, an observation that would not be consistent with the C-terminal end of the Gα-subunit being in close proximity to the NFNEAKX motif. In agreement with this proposal, swapping this region between mGlur2 and mGlur8 did not change their specificity of interaction with the G-proteins tested (Fig. 11). Indeed, in our three-dimensional model, the residue of the i2 loop that is different between mGlur2 and mGlur8 and closer to this motif, is the Ala-658 (mGlur2) or His-674 (mGlur8) that is also not responsible for the recognition of the −4 residue of the α-subunit, according to our data. In agreement with this finding, this residue is not directed toward TM6 but rather toward the lipids. Another tripeptide segment that mostly differs between group II and group III mGlur receptors (AQR/VTST/AA/?) aligns with a segment of rhodopsin, the structure of which has not been solved. Accordingly, it cannot be introduced with confidence in our three-dimensional model. This is unfortunate because our data point to this short segment as playing an important role in the recognition of the C terminus of the Go subunit. Indeed, swapping this tripeptide between mGlur2 and mGlur8 allows the resulting mGlur2-VTA to activate Gqα, and decreases the efficacy of mGlur8-AQR to activate this G-protein (Fig. 11). However, the AQR motif did not suppress coupling to G1, when mGlur8 i2 loop environment. This may result from a different position of one of the three side chains of this tripeptide when in the i2 loop of mGlur2 rather than in that of mGlur8. Although we did not analyze further the role of the individual residues, it is likely that the Arg residue with its positive charge does not tolerate the binding of a C-terminal tail with an Ile at position −4, whereas replacing this Arg by an Ala in mGlur8 is compatible with the C-terminal end of Gq. The side chain of Arg is long, and its conformation largely depends on its environment. This can result in the long hydrophobic side chain being more exposed and ready to better accept the Ile-4 of the G-protein. As such, a role for this residue fits with our observation that the AQR sequence within the i2 loop of mGlur8 is still compatible with the activation of Gqα, although with a lower efficacy. More work using either modeling studies or structural analysis of the intracellular loops of mGlurS will be required to solve this issue.

Taken together, the present data are in agreement with the possible interaction of the C-terminal tail of the G-protein α-subunit in a cavity lined by i2 and i3 loops of class III GPCRs. However, other possibilities exist, but more data will be necessary to demonstrate that both class I and class II receptors couple similarly to G-proteins.

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REFERENCES
1. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
2. Savarre, T. M., and Fraser, C. M. (1992) Biochem. J. 283, 1–19
3. Bourne, H. R. (1997) Curr. Opin. Cell Biol. 9, 134–142
4. Weiss, J. (1997) J. Biol. Chem. 272, 314–354
5. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Nature 390, 88–91
6. Spengler, D., Waecher, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journet, L. (1993) Nature 365, 176–175
7. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7507–7511
8. Ourrut, R., Herzmark, P., Chi, P., Garcia, P. D., Lichtarge, O., Kingsley, C., and Bourne, H. (1997) Science 275, 381–384
9. Conklin, B. R., Farfel, Z., Lustig, R. D., Julius, D., and Bourne, H. R. (1993) Nature 363, 274–276
10. Conklin, B. R., Herzmark, P., Ishida, S., Voyer-Vasenetkaya, T. A., Sun, Y., Farfel, Z., and Bourne, H. (1996) Mol. Pharmacol. 50, 585–590
11. Kostienia, E., Gomeza, J., Lerche, C., and Wess, J. (1997) J. Biol. Chem. 272, 20675–20681
12. Bilodeau, J. S., Mary, S., Perrey, J., De Calle, C., Brabet, I., Bockaert, J., and Pin, J.-P. (1998) J. Biol. Chem. 273, 25765–25769
13. Bahia, D. S., Wise, A., Fanelli, F., Lee, M., Rees, S., and Milligan, G. (1998) Biochemistry 37, 11555–11562
14. Liu, S., Carrillo, J. J., Pediani, J. D., and Milligan, G. (2002) J. Biol. Chem. 277, 25707–25714
15. Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K., and Yamamoto, T. (1997) Science 276, 1878–1881
16. Kostienia, E., Zeng, F., and Wess, J. (1998) J. Biol. Chem. 273, 17886–17892
17. Neel, J. P., Hamm, H. E., and Sigler, P. B. (1993) Nature 366, 654–663
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18. Mazzoni, M. R., and Hamm, H. E. (1996) *J. Biol. Chem.* 271, 30034–30040
19. Bae, H., Anderson, K., Flood, L. A., Skiba, N. P., Hamm, H. E., and Graber, S. G. (1997) *J. Biol. Chem.* 272, 32071–32077
20. Hamm, H. E., Deretic, D., Aresu1, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) *Science* 241, 852–835
21. Bockaert, J., and Pin, J.-P. (1999) *EMBO J.* 18, 1723–1729
22. Wess, J. (1998) *Pharmacol. Ther.* 80, 231–264
23. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) *Nature* 365, 166–170
24. Francesconi, A., and Duvoisin, R. M. (1998) *J. Biol. Chem.* 273, 5615–5624
25. Chang, W., Chen, T. H., Pratt, S., and Shoback, D. (2000) *J. Biol. Chem.* 275, 19955–19963
26. Ango, F., Albani-Torregrossa, S., Joly, C., Robbe, D., Michel, J.-M., Pin, J.-P., Bockaert, J., and Fagni, L. (1999) *Neuropharmacology* 38, 793–803
27. Perroy, J., Gutierrez, G., Coulon, V., Bockaert, J., Pin, J.-P., and Fagni, L. (1998) *Mol. Pharmacol.* 53, 778–786
28. Sprang, S. R. (1997) *Annu. Rev. Biochem.* 66, 639–678
29. Hamm, H. E. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4819–4821
30. Bouvier, M. (2001) *Nat. Rev. Neurosci.* 2, 274–286
31. Berridge, M. J., and Irvine, R. F. (1984) *Nature* 312, 315–321
32. Ango, F., Albani-Torregrossa, S., Joly, C., Robbe, D., Michel, J.-M., Pin, J.-P., Bockaert, J., and Fagni, L. (1999) *Neuropharmacology* 38, 793–803
33. Perroy, J., Gutierrez, G., Coulon, V., Bockaert, J., and Fagni, L. (2000) *J. Neurosci.* 20, 7896–7904
34. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* 22, 537–548
35. Parmentier, M. L., Joly, C., Restituito, S., Bockaert, J., and Pin, J.-P. (1996) *Mol. Pharmacol.* 50, 923–930
36. Gomeza, J., Mary, S., Brabet, I., Parmentier, M.-L., Restituito, S., Bockaert, J., and Pin, J.-P. (1998) *Mol. Pharmacol.* 53, 778–786
37. Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11642–11646
38. Ango, F., Albani-Torregrossa, S., Joly, C., Robbe, D., Michel, J.-M., Pin, J.-P., Bockaert, J., and Fagni, L. (1999) *Neuropharmacology* 38, 793–803
39. Perroy, J., Gutierrez, G., Coulon, V., Bockaert, J., Pin, J.-P., and Fagni, L. (1998) *Mol. Pharmacol.* 53, 778–786
40. Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11642–11646
41. Fourgeaud, L., Bessis, A.-S., Rossignol, F., Pin, J.-P., Olivo, J.-C., and Hémar, A. (2003) *J. Biol. Chem.* 278, 12222–12230
42. Pagano, A., Rovelli, G., Mesbacher, J., Lehmann, T., Duthey, B., Stauffer, D., Rustig, D., Schulzer, V., Meigel, I., Lampert, C., Stein, T., Prézeau, L., Blahos, J., Pin, J.-P., Froesti, W., Kuhn, R., Reif, J., Kasprianski, K., and Béttler, B. (2001) *J. Neurosci.* 21, 1189–1202
43. Galvez, T., Duthey, B., Knaieff, J., Blahos, J., Rovelli, G., Béttler, B., Prézeau, L., and Pin, J.-P. (2001) *EMBO J.* 20, 2152–2159
44. 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
45. Sprang, S. R. (1997) *Annu. Rev. Biochem.* 66, 639–678
46. Bouvier, M. (2001) *Nat. Rev. Neurosci.* 2, 274–286
47. Liang, Y., Fotiadis, D., Filipsek, S., Superstein, D. A., Palczewski, K., and Engel, A. (2003) *J. Biol. Chem.* 278, 21655–21662
48. Baneres, J.-L., and Parello, J. (2003) *J. Mol. Biol.* 329, 815–829
49. Cai, K., Itoh, Y., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4877–4882
50. Dek, Y., Cai, K., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4883–4887
51. Kostieni, E., Conklin, B. R., and Wess, J. (1997) *Biochemistry* 36, 1487–1495
52. Pin, J.-P., Galvez, T., and Prezeau, L. (2003) *Pharmacol. Ther.* 98, 325–354