Many membrane receptors are made of a ligand binding domain and an effector domain mediating intracellular signaling. This is the case for the metabotropic glutamate-like G-protein-coupled receptors. How ligand binding leads to the active conformation of the effector domain in such receptors is largely unknown. Here, we used an evolutionary trace analysis and mutagenesis to identify critical residues involved in the allosteric coupling between the Venus flytrap ligand binding domain (VFT) and the heptahelical G-protein activating domain of the metabotropic glutamate-like receptors. We have shown that a conserved interdomain disulfide bridge is required for this allosteric interaction. Taking into account that these receptors are homodimers, this finding provides important new information explaining how the different conformations of the dimer of VFT lead to different signaling of such dimeric receptors.

Many cell surface receptors are composed of multiple domains, one being involved in ligand recognition and another in signal transduction. Understanding how ligand binding leads to the activation of the effector domain is an important issue. Recently, models have been proposed for the coupling between ligand binding and channel opening in the GABAA and nicotinic receptors (1–3). These models took advantage of the known structure of the binding domain and were based on both modeling and mutagenesis studies performed on full-length receptors. However, for most membrane receptors, the molecular mechanism involved in the allosteric coupling between binding and effector domains is poorly understood.

Among the large family of G-protein-coupled receptors (GPCRs), the metabotropic glutamate (mGlu)-like receptors are such allosteric multidomain proteins. This receptor class (class C) includes the receptors for the two main neurotransmitters, glutamate and GABA, as well as the Ca2+-sensing, some taste (T1R), and pheromone (V2R) receptors (4). These GPCRs possess an extracellular Venus flytrap domain (VFT) where agonists bind and a heptahelical transmembrane domain (HD) common to all GPCRs and responsible for G-protein activation. For most of these receptors, except the GABA receptor, a cysteine-rich domain (CRD) linked these two domains. Structural studies as well as functional analysis of receptor mutants indicated that the bilateral VFT adopts two major conformations, an inactive open conformation stabilized by competitive antagonists, and an active closed conformation stabilized by agonists (5–8). Expression studies of receptors deleted of both the VFT and the CRD also indicated that the HD can fold alone in a unit oscillating between various active and inactive conformations stabilized by specific synthetic ligands (9, 10).

Biochemical and structural data recently helped in understanding how VFT closure resulting from agonist binding may stabilize the active conformation of the HD. Indeed, class C GPCRs are dimers, either homodimers like the mGlu and Ca2+-sensing receptors or heterodimers like the GABA and T1R receptors, and this appears necessary for allosteric coupling between the VFT and the HD. As revealed by x-ray studies, isolated VFTs form dimers, and the stabilization of at least one VFT in a closed state with agonist is associated with a major difference in the relative orientation of these domains (5). Accordingly, this major conformational change in the VFT dimer has been proposed to affect the general organization of the dimer of HDs leading to their activation (Fig. 1A). However,
In the present study, we hypothesized that key residues of the VFT involved in the functional coupling between the VFT and HD domains in these receptors must be conserved during evolution. Using an evolutionary trace analysis associated with mutagenesis and functional studies, we identified a conserved disulfide bridge that cross-links the VFT with the CRD of the mGlu-like receptors and that is crucial for the intramolecular receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Coldan[^1H][LY341495 ((2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) were purchased from Tocris Cookson (Bristol, UK). 4-MPPTS (LY487379; 2,2,2-trifluoro-N-[4-(2-methoxyphenoxy) phenyl]-N-(3-pyridinylmethyl)-ethanesulfonamide) was a gift from ADDEX Pharmaceuticals (Geneva, Switzerland). CP-PHA (N-[4-chloro-2-[(1,3-dioxo1,3-dihydro-2H-isoindol-2-yl)methyl] phenyl]-2-hydroxybenzamide) was a gift from Dr. J. Conn (Vanderbilt University Medical Center, Nashville, TN). Human thrombin was obtained from CalBiochem.

**Construction of mGlu2 and mGlu5 Mutants**—The plasmids encoding the wild-type mGlu2 or mGlu5 tagged with the hemagglutinin (HA) or c-Myc epitope inserted just after the signal peptide, under the control of a cytomegalovirus promoter, were described previously (11, 12). mGlu2 and mGlu5 single mutants and the construct mGlu2THR-WT were generated using QuickChange mutagenesis protocol (Stratagene, La Jolla, CA). mGlu2THR-WT results in the addition of the amino acid sequence GLVPRGSGG after residue Gly-493 of mGlu2 (thrombin recognition site underlined).

**Cell Culture and Transfection**—Human embryonic kidney 293 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected by electroporation as described elsewhere (11). Ten

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*FIGURE 1. Evolutionary trace analysis of the class C receptor VFTs.* **A,** structural model of dimeric mGlu-like receptors (class C GPCRs) in the resting and active state. Ribbon view of the crystal structure of the resting (left, PDB accession number 1EWT) and fully active (right, accession number 1ISR) state of the mGlu1 VFT dimer, and apposition of two membrane (effector) domains according to rhodopsin structure. The yellow subunit is in the front; the blue subunit is in the back. **B,** evolutionary conservation of residues at the surface of mGlu VFTs visualized on Lobe 1 (top) and Lobe 2 (bottom) of mGlu1 VFT crystal structure. Conservation scores are according to a color scale from variable (blue) to conserved (purple) residues. The blue arrows highlight the dimerization interface; the yellow one highlights the conserved area located at the bottom of Lobe 2. **C,** evolutionary conservation of residues of the VFT of all mGlu-like receptors, visualized on the dimerization face as in panel B. The patch of conserved residues at the bottom of Lobe 2 remains highly conserved (yellow arrow). **D,** electrostatic surface representation (negative, red; neutral, white; positive, blue) of the VFT faces. The green ribbons correspond to the helixes of the associated subunit illustrating the dimerization interface.
million cells were transfected with plasmid DNA containing mGlu2-WT (2 μg) or mutants (6 μg), mGlu5-WT (0.6 μg) or mutant (3 μg) and completed to a total amount of 10 μg of plasmid DNA with pRK5 empty vector.

**Inositol Phosphate and Intracellular Calcium Measurements**—Measurement of IP accumulation and calcium signal in human embryonic kidney 293 transfected cells was performed in 96-well microplates as previously described (9).

**Cell Surface Quantification by ELISA**—Experiments were conducted as described (13). c-Myc-tagged constructs were detected with mouse anti-c-Myc 9E10 ascites supernatant and goat anti-mouse antibodies coupled to horseradish peroxidase (Amersham Biosciences) at 0.25 μg/ml.

**Ligand Binding Assay**—Ligand binding assay on intact human embryonic kidney 293 cells was performed as previously described using 2 nM [3H]LY341495 (14). Radioligand was displaced by increased concentrations of either cold LY341495 or glutamate, in the absence or presence of positive allosteric modulator LY487379 at 10 μM. The curves were fitted according to the equation:

\[
y = \frac{y_{\text{max}} - y_{\text{min}}}{1 + (\frac{IC_{50}}{IC_{50}^{\text{alt}}})^n} + y_{\text{min}}
\]

where the IC<sub>50</sub> is the concentration of the compound that inhibits 50% of bound radioligand and n is the Hill coefficient.

**Time-resolved FRET Measurements**—Time-resolved FRET experiments were conducted as described (13, 15). This methodology is based on the non-radiative energy transfer between rare earth cryptates such as europium (Eu<sup>3+</sup>) cryptates and acceptor fluorophores such as AlexaFluor<sup>647</sup> (Molecular Probes). Briefly, COS-7 cells expressing the HA-tagged mGlu2 were incubated with 1 nM of two different monoclonal anti-HA (12CA5) antibodies carrying either Eu<sup>3+</sup>-cryptate pyridine bipyridine or AlexaFluor<sup>647</sup> (provided by Cis Bio Interna-

**Thrombin Digestion**—For Western blot analysis, 20 h after transfection human embryonic kidney 293 cells were washed with phosphate-buffered saline (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free) and harvested. The membranes were prepared as previously described (7). For each sample, 50 μg of total proteins were incubated in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl in the absence or presence of 25 units of human thrombin. After 4 h of digestion at 37 °C, each sample was incubated with 6 × loading buffer (pH 8.0) in the absence or presence of 10 mM dithiothreitol (DTT) and was subjected to SDS-PAGE by using 6% polyacrylamide gels, containing 6% urea for the samples treated with DTT. Proteins were transferred to nitrocellulose membrane (Hybond-C; Amersham Biosciences), probed with anti-HA rabbit polyclonal antibody (dilution 1/400; Zymed Laboratories Inc., San Francisco, CA) and then with anti-rabbit antibody horseradish peroxidase-conjugated (Amersham Biosciences) at 0.25 μg/ml, and visualized by chemiluminescence (West Pico; Pierce, Rockford, IL).
For ELISA analysis, 20 h after transfection COS-7 cells in 96-well microplates were incubated with Tris-Krebs buffer, pH 7.4, containing 0.1% glucose and 0.1% bovine serum albumin as previously described (13), supplemented with 1 μg/ml brefeldin A, in the absence or presence of thrombin (0.5 unit/well) for 4 h at 37 °C. After digestion with thrombin, cells were washed with phosphate-buffered saline and incubated in Tris-Krebs buffer, pH 8.0, containing 0.1% glucose and 0.1% bovine serum albumin in the absence or presence of 10 mM DTT for 30 min at 37 °C. After DTT treatment, cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde, and an ELISA was performed.

Molecular Modeling—A homology model of mGlu1 CRD was generated using the crystal structure of Protein Data Bank accession number 1EXT as template. Models were manually refined with ViTO (16) using the sequence alignment of the mGlu-like CRD. Final models were built using Modeler 7.0 (17) and evaluated using dynamic evolutionary trace as implemented in ViTO.

RESULTS

Evolutionarily Conserved Areas in mGlu VFT—An evolutionary trace analysis of the surface of class C receptor VFTs was performed after aligning a number of sequences of mGlu, Ca^2+ sensing, fish olfactory, taste T1R, and pheromone V2R receptors, including those from nematodes, insects, birds, fishes, and mammals (see supplemental Figs. S1 and S2). The alignment generated with ClustalW was submitted to the ConSurf website server (Consurf.tau.ac.il) (18). The x-ray structure of the mGlu1 VFT (PDB accession number 1EWK:B) (5) was used to visualize the conservation score of each residue (Fig. 1, B and C). These scores were calculated by taking into account the phylogenetic relationships among the sequences and the similarity between the amino acids in the alignment.

A first analysis using the mGlu sequences only (62 sequences) revealed three highly conserved areas at the surface of the mGlu VFT, with most residues displaying a score higher than 8 within a range of 1 to 9 (Fig. 1B). The two larger areas are located on one side of the VFT, one on Lobe 1 and the other on Lobe 2. These two areas correspond to the dimerization interface as identified in the x-ray structures. The third conserved patch of residues is located at the bottom of Lobe 2 and is made of residues that do not make contact with the other protomer in the dimeric VFT structure. A second analysis conducted using the sequences of all mGlu-like receptors (125 sequences) shows the area at the bottom of Lobe 2 is the most conserved one, better conserved than the dimerization interface (Fig. 1C). This cluster includes Tyr-226, Cys-254, and Ala-256 in mGlu1. Electrostatic surface analysis shows this patch of conserved residues forms an apolar surface in mGlu1 (Fig. 1D), consistent with this area being buried in an interface contact with another domain of the receptor subunit.

Conserved Cys-234 in Lobe 2 of mGlu2 Is Crucial for Agonist-induced Receptor Activation—We suspected that these residues are involved in the allosteric coupling between the VFT and the HD. In agreement with this proposal, the mutation of Cys-234 into Ala, Ser, Met, or Glu in mGlu2 (equivalent to
Cys-234 of mGlu2) (Fig. 2A) completely abolishes agonist-induced responses (Fig. 2B). This clearly results from a loss of function of these receptors because they were correctly expressed at the cell surface as revealed by ELISA performed on intact cells (Fig. 2C). This loss of function does not also result from the absence of agonist binding on the mutated receptors, as verified by glutamate displacement of the antagonist [3H]LY341495 binding (Fig. 2D). Glutamate and LY341495 affinities on Cys-234 mutants are similar to those measured on the wild-type mGlu2 (Fig. 2D and data not shown), indicating that Cys-234 mutation prevents agonists from activating the receptor. However, mutation of Tyr-206 (equivalent to Tyr-226 of mGlu1) into Ala or Glu strongly impairs cell surface expression of the receptor, but it does not prevent agonist-induced activation (data not shown). All together, these results show that the highly conserved Cys in Lobe 2 plays a crucial role in receptor activation; meanwhile, the conserved Tyr likely has a structural role only.

Cys-234 Mutation in mGlu2 Uncouples the HD from the VFT—To examine whether the HD of the mGlu2 Cys-234 mutants could still activate G-proteins, we tested the effect of the positive allosteric modulator (PAM) 4-MPPTS (19, 20) (Fig. 3A). PAMs of mGlu receptors are known to bind in the HD, to further stabilize the agonist-induced active state, and to increase agonist but not antagonist affinity (21). As observed for all mGlu receptor PAMs, 4-MPPTS displays no or little agonist activity on the full-length mGlu2 receptor (Fig. 3B and C). In contrast, 4-MPPTS directly activated the Cys-234 mutants, like mGlu2 receptor deleted of its entire extracellular domain. This demonstrates that the HD of the mutated receptor retains its ability to reach an active state and stimulate G-proteins. This also shows that the inactive form of the VFT no longer prevents PAMs from acting as agonists in the Cys-234 mutants. As expected for an uncoupling between the VFT and the HD, the left shift of the glutamate displacement curve of [3H]LY341495 binding by 4-MPPTS on the wild-type mGlu2 receptor is not observed for the Cys-234 mutants (Fig. 4). All together, these results demonstrate the conserved Cys-234 plays a crucial role in the allosteric coupling between the VFT and the HD in mGlu-like receptors.

Cys-234 Mutation Does Not Prevent Receptor Dimerization—We verified that the loss of function resulting from the Cys mutation is not due to the impairment of dimer formation at the cell surface. FRET experiments were conducted on intact cells using anti-HA antibodies conjugated with either the energy donor fluorophore, europium cryptate pyridine bipyridine, or the fluorophore acceptor, AlexaFluor647 (Fig. 5A). Such an approach enables the detection of receptor dimers at the cell surface only (15). As shown in Fig. 5B, when cells expressing the N-terminal HA-tagged version of the wild-type or Cys mutant mGlu2, large and similar FRET signals were measured. No such signal was observed between HA-GABAB1 subunits co-expressed with untagged GABAB2 despite a similar expression of the GABAB heterodimer (Fig. 5B, inset).

Cys-234 of mGlu2 VFT Is Involved in a Disulfide Bridge with the Other Part of the Receptor—To test whether the VFT is cross-linked to the rest of the molecule (CRD plus HD) via a disulfide bridge, we examined whether the VFT can remain...
covalently linked to the other part of the receptor under non-reducing conditions after cleavage of a unique thrombin site (THR) introduced between the VFT and the CRD of mGlu2 (mGlu2THR) (Fig. 6A). Introduction of the thrombin site does not impair receptor targeting to the cell surface (data not shown) or normal functioning of the receptor (Fig. 6B). Western blot analysis revealed that the HA-tagged mGlu2 VFT remains covalently linked to the rest of the receptor after cleavage of the thrombin site under control conditions, but not after reduction with DTT (Fig. 6C). In contrast, the VFT can be cleaved of the rest of the receptor by thrombin in the C234A mutant containing the thrombin site, indicating that Cys-234 is involved in a covalent linkage between the VFT and another domain of the protein. When using this mGlu2THR-C234A mutant, thrombin cleavage releases a dimer of VFTs, as expected because of the known disulfide bridge that cross-links the VFTs in the dimer (Fig. 6A). VFT monomer of this mutant receptor can only be observed after DTT treatment.

We further demonstrated that Cys-234 cross-links the VFT to the rest of the mGlu2 receptor in the cell surface proteins. We examined whether or not the HA-tagged VFT could be released from the cell surface after cleavage with thrombin. ELISA analysis showed that the VFT can be cleaved of the cell surface under non-reducing conditions in the mGlu2THR-C234A mutant only, but not in the wild-type mGlu2 or in mGlu2THR that still contains Cys-234 (Fig. 6D). In contrast, after DTT treatment, the HA-tagged VFT of both mGlu2THR-C234A and mGlu2THR can be released after thrombin.

Cys-234 of mGlu2 VFT is involved in a disulfide bridge with the CRD domain. A, release of HA-tagged VFT by thrombin in mGlu2THR where the nine conserved Cys in CRD were changed into Ala in mGlu2THR and in wild-type mGlu2 expressed at the cell surface as in Fig. 6D. Values are means ± S.E. of triplicates from a typical experiment. B, pictogram of the dimeric HA-tagged mGlu2THR where the disulfide bond between Cys-234 and the third conserved Cys in CRD (Cys-518) is depicted.

Cys-518 of mGlu2 CRD Is the Partner of Cys-234—We employed the above approach in an attempt to identify the possible partner of Cys-234 in mGlu2. Within the HD, only two conserved Cys can be identified that correspond to those highly conserved in nearly all GPCRs and involved in a disulfide bridge between the top of TM3 and the extracellular loop 2 (22, 23). The CRD is composed of nine highly conserved Cys, such that at least one of these cannot be involved in an intra-CRD disulfide bridge and may possibly be linked to the VFT. We mutated any of the nine Cys of the CRD into

FIGURE 7. Cys-234 of mGlu2 VFT is involved in a disulfide bridge with the CRD domain. A, release of HA-tagged VFT by thrombin in mGlu2THR where the nine conserved Cys in CRD were changed into Ala in mGlu2THR and in wild-type mGlu2 expressed at the cell surface as in Fig. 6D. Values are means ± S.E. of triplicates from a typical experiment. B, pictogram of the dimeric HA-tagged mGlu2THR where the disulfide bond between Cys-234 and the third conserved Cys in CRD (Cys-518) is depicted.

FIGURE 8. Effect of the mutation C518A on the mGlu2 activation. A, IP accumulation by the indicated mGlu2 wild-type and mutants after glutamate stimulation. B, intracellular Ca²⁺ response mediated by the mGlu2 wild type and mutants upon glutamate or PAM (4-MPPTS) stimulation. Values are means ± S.E. of triplicates from a typical experiment.
Ala in the mGlu2 THR, and the release of HA-tagged VFT by thrombin at the cell surface was measured as previously (Fig. 7A). All of these mutants were correctly expressed at the cell surface, whereas their glutamate-induced stimulation were abolished (data not shown). Under non-reducing conditions, HA-tagged VFT was released only for the Cys-518 mutant, suggesting Cys-518 is the partner Cys involved in the disulfide bond with Cys-234. Under reducing conditions, HA-tagged VFT was released efficiently for all mGlu2 THR CRD mutants, indicating thrombin digestions were efficient for all Cys mutants.

If Cys-518 is involved in a disulfide bridge formation with Cys-234, then one expects that a similar functional phenotype will be observed for both C234A and C518A mutants. As expected, mutation of Cys-518 into Ala resulted in a drastic decrease in receptor activation by glutamate, although in that case a small Glu-mediated response could still be seen (Fig. 8A). This may well result from the different environments of Cys-234 and Cys-518. Moreover, and as observed with the Cys-234 mutants, the mGlu2 PAM acts as an agonist, being able to activate alone the C518A mutant (Fig. 8B). This further indicates that the HD of the C518A mutant is functional and that the VFT no longer exerts its inhibitory action.

Similar Role of the Conserved Cys in mGlu5—We verified the conserved Cys in Lobe 2 is crucial for the activation of another class C GPCR, by changing the Cys corresponding to Cys-234 in mGlu2 in the mGlu5 receptor. Mutation of Cys-240 into Glu (C240E) in mGlu5 almost suppresses agonist stimulation of the receptor (Fig. 9A); meanwhile, the mutant is correctly expressed at the cell surface as revealed by ELISA (Fig. 9B). Because of the absence of high affinity radioligand, the affinity of agonist on the mutant could not be measured. However, we showed the loss of function does not result from an inactive HD because the mutant C240E displays a constitutive activity and it is activated by CPPHA (24), a PAM specific of mGlu5 (Fig. 9C). The presence of a disulfide bridge linking this Cys residue of mGlu5 with the rest of the receptor was also shown, taking advantage of a thrombin
cleavage site inserted between the VFT and the CRD of this receptor (data not shown).

**DISCUSSION**

In the present study, we identified a residue that is crucial for the coupling between the ligand binding domain (VFT) and the effector domain of mGlu-like GPCRs. We showed that a cysteine that is part of a conserved patch in the VFT is required for any known allosteric interaction between the VFT and the HD: 1) activation of the HD upon ligand binding in the VFT, 2) prevention of PAM activation of the HD by the empty VFT, and 3) increased agonist affinity in the VFT by PAM binding in the HD. Our data demonstrate that this Cys is involved in a disulfide bridge that cross-links the VFT to the third conserved Cys of the CRD.

We propose that the complex between VFT and CRD via the conserved patch in the Lobe 2 of the VFT is important for the intramolecular receptor signaling. CRD is composed of nine highly conserved Cys, and it is known to be important for signal transduction in mGlu-like receptors (25), although the role of each Cys was not clear (26). Intermolecular disulfide bridge in a mGlu dimer involved a single conserved Cys in Lobe 1 (27, 28), suggesting the absence of disulfide bond between both CRDs in a dimeric receptor. A recent model for the CRD, based on low sequence similarity with an extracellular domain of the tumor necrosis factor receptor, identified three free Cys, one of these being possibly involved in the cross-link with the VFT (29). Our data are not consistent with this model. We showed the third highly conserved Cys of the CRD (Cys-518 in mGlu2) cross-links the VFT, whereas it makes an intra-CRD disulfide bridge in the former model. Thanks to our identification of the disulfide cross-link between Cys-234 in the VFT and Cys-518 in the CRD, we can propose a three-dimensional model of the CRD in which the eight other Cys make intradomain disulfide bridges (Fig. 10). Further studies would be necessary to test this model and to evaluate the importance of each Cys of the CRD for receptor activation.

Cysteine of the conserved patch in the VFTs could control the relative movement of the two HDs during activation as supported by FRET studies (30), and it could explain the different signaling states of the receptor (31). The x-ray structure of the dimer of mGlu1 VFTs solved in the absence of ligand (5), with bound agonist (5, 6), or antagonist (6) led to important observations for the understanding of mGlu-like receptor activation. As shown in Fig. 11, agonist binding in at least one VFT leads to a major change in the relative position of the VFTs. Crystal structures with bound glutamate revealed two different conformations, an "Active-closed-closed" (Acc) where both VFTs are closed, and an "Active-closed-open" conformation (Aco), when only one VFT is closed (Fig. 11). The addition of a cation like Gd$^{3+}$ appears necessary to stabilize the Acc state. Recent data show that only the Acc state leads to full activation of $G_q$, whereas the Aco state leads both to $G_q$ coupling and to partial $G_\text{Gq}$ activation (31, 32). Such functional differences between the Acc and Aco states can be explained by the different relative positions of the Cys-254 (Cys-234 in mGlu2) at the bottom of the mGlu1 VFTs in the resting (Roo), Aco, and Acc states; meanwhile, the distance between the C-terminal ends of the VFTs is identical in Aco and Acc states (63 Å).

Taken together, these data are consistent with the proposal that a relative positioning of the two HDs, in addition to the conformation of each HD in a GPCR dimer, is likely important in controlling its activity as well as its signaling. Whether this is specific to class C GPCRs or is also valid for class A rhodopsin-like GPCRs remains an important issue. It is worth noting that such a change during activation of the dimer interface has been recently reported for the D2 dopamine receptor (33).
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