Amino Acid Mutagenesis of the Ligand Binding Site and the Dimer Interface of the Metabotropic Glutamate Receptor 1

IDENTIFICATION OF CRUCIAL RESIDUES FOR SETTING THE ACTIVATED STATE*

Received for publication, October 8, 2002, and in revised form, November 18, 2002
Published, JBC Papers in Press, November 19, 2002, DOI 10.1074/jbc.M210278200

Toshihiro Sato‡, Yoshimi Shimada‡, Naoko Nagasawa‡, Shigetada Nakanishi§,
and Hisato Jingami¶

From the ‡Department of Molecular Biology, Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita-City,
Osaka 565-0874, Japan and the §Department of Biological Sciences, Kyoto University Faculty of Medicine, Yoshida,
Sakyo-ku, Kyoto 606-8501, Japan

Previously, we determined the crystal structures of the dimeric ligand binding region of the metabotropic glutamate receptor subtype 1. Each protomer binds l-glutamate within the crevice between the LB1 and LB2 domains. We proposed that the two different conformations of the dimer interface between the two LB1 domains define the activated and resting states of the receptor protein. In this study, the residues in the ligand-binding site and the dimer interface were mutated, and the effects were analyzed in the full-length and truncated soluble receptor forms. The variations in the ligand binding activities of the purified truncated receptors are comparable with those of the full-length form. The mutated full-length receptors were also analyzed by inositol phosphate production and Ca2+ response. The magnitude of the ligand binding capacities and the amplitude of the intracellular signaling were almost correlated. Alanine substitutions of four residues, Thr188, Asp208, Tyr236, and Asp318, which interact with the α-amino group of glutamate in the crystal, abolished their responses both to glutamate and quisqualate. The mutations of the Tyr74, Arg78, and Gly293 residues, which interact with the γ-carboxyl group of glutamate, lost their responsiveness to glutamate but not to quisqualate. Furthermore, a mutant receptor containing alanine instead of isoleucine at position 120 located within an α helix constituting the dimer interface showed no intracellular response to ligand stimulation. The results demonstrate the crucial role of the dimer interface in receptor activation.

Glutamate is a major neurotransmitter in excitatory neurons in the central nervous system. Glutamate released into the synaptic space is recognized by two distinct receptors, glutamate-gated ion channels and metabotropic glutamate receptors (mGluRs)1, 2. The mGluRs consist of eight subtypes (mGluR1 to -8), which couple with a variety of effector systems, including inositol phosphate pathway, adenylyl cyclase, ion channels, etc. The mGluRs are considered to modulate synaptic neurotransmission and thus to play roles in memory, learning, and brain disorders such as epilepsy and neurodegenerative diseases.

The mGluR consists of three regions: a large extracellular region, a seven-transmembrane-spanning region, and an intracellular region. Previously, we determined the crystal structures of the extracellular ligand-binding region (LBR) of mGluR1 (3). In combination with biochemical studies (4, 5), the mGluR1-LBR (m1-LBR) was found to be a homodimer consisting of two protomers. Each protomer consists of an LB1 domain and an LB2 domain. The glutamate-binding structure is a dimer composed of closed and open protomers, which differ in the relative orientation of the LB1 and LB2 domains. Without glutamate, two crystal forms of m1-LBR were obtained; one form exists as an open-open dimer, and the other is an open-closed form. The two main functioning sites were then elucidated: the ligand-recognition site and the LB1 dimer interface. In the ligand binding site, glutamate interacts mainly with 13 amino acid residues from the LB1 and LB2 domains of the protomer. We proposed that the ligand-binding domain of mGluR1 is in dynamic equilibrium between the activated state and the resting state, which are defined mainly by the different dimer interface conformations of the three crystal forms. An antagonist binding crystal structure, which was recently solved (6), maintained the resting dimer conformation, supporting our proposal.

Recently, numerous dimer formation studies of G-protein-coupled receptors have suggested a potential level of receptor complexity and diversity (7). However, the precise structural basis of the dimer formation has not been established. In this context, mGluR1, which is considered to be a type C G-protein-coupled receptor (8), is unique in that its extracellular dimer interface has been defined structurally, as described above, and hence provides an opportunity to investigate the functional role of dimerization.

Here, in order to elucidate the significance and the contribution of the individual amino acid residues in ligand binding and the correlation with intracellular events, such as inositol phosphate (IP) formation and subsequent Ca2+ responses, we have analyzed the functions of mutant receptors with amino acid mutations of the ligand-binding residues. Furthermore, we also mutated the residues within the helices forming the LB1 dimer interface and analyzed the effects of the mutations. One of the dimer interface mutants lost the Ca2+ response to ligand stimulus, indicating the critical role of dimer interface relocation in receptor activation.

* This work was supported by a research grant endorsed by the New Energy Technology Development Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶ To whom correspondence should be addressed. Tel.: 81-6-6872-8214; Fax: 81-6-6872-8219; E-mail: jingami@beri.or.jp.
† The abbreviations used are: mGluR, metabotropic glutamate receptor; m1-LBR, mGluR1-LBR; LBR, ligand-binding region; IP, inositol phosphate; HEK, human embryonic kidney; BSS, balanced salt solution; PBS, phosphate-buffered saline; cRNA, complementary RNA.

Published, JBC Papers in Press, November 19, 2002, DOI 10.1074/jbc.M210278200

4314 This paper is available on line at http://www.jbc.org

Downloaded from http://jbc.org by guest on April 26, 2016
EXPERIMENTAL PROCEDURES

Materials—L-Quisqualic acid was purchased from Tocris Cookson Ltd. (Langford Bristol, UK). L-Glutamic acid monosodium salt was purchased from Nacalai Tesque (Kyoto, Japan). LipofectAMINE 2000 and oligonucleotide primers were purchased from Invitrogen. Q/F/C filters were purchased from Millipore Corp. (Bedford, MA). The AG-1-X resin (100−200 mesh formate form) and the Poly-Prep chromatography columns for anion exchange chromatography were obtained from Bio-Rad. All other reagents were used of molecular or analytical grade.

Construction of Expression Vectors and Oligonucleotide Mutagenesis—The pcDNAmGluR114His plasmid, which is an expression vector for His6-tagged m-LBR was made as follows. A PCR was done with the primers T01 and HJ113 (4), using pVLMmGluR113 (5) as a template. The PCR product was digested with NcoI and XbaI and was cloned into the NcoI/XbaI-digested fragment of pmGluR108 (4), resulting in pVLMmGluR114His. The NotI/XbaI fragment of pVLMmGluR114His was subcloned into pcDNA3.1 (+) (Invitrogen). pcDNAmGluR114, an expression vector for the full-length mGluR1, was made by subcloning the NotI/XbaI fragment of pmGluR102 (4) into pcDNA3.1 (+). For the construction of the single amino acid Y74A, R78A, S164A, S165A, S166A, S188A, T188A, D208A, Y236A, E292A, C393A, T394A, and D318A mutants, the first PCR was performed with mutagenic primers and TS36 (5′-TGTGATGCCGACCTGTCATGCCAGCCTGAAG-3′). The second PCR product was digested with EcoRI and SfiI and was subcloned in place of the corresponding wild-type fragments of pcDNAmGluR114His and pcDNAmGluR1. For the construction of the D208A, Y236A, E292A, C393A, and K409A mutants, the first PCR was done with mutagenic primers and TS36 (5′-CCTGCTGCCGCTTGTGGGC-3′) with pmGluR114His as the template, and the second PCR product was digested with EcoRI and SfiI and was subcloned in place of the corresponding wild-type fragments of pcDNAmGluR114His and pcDNAmGluR1. All of the PCR products mentioned above were confirmed by sequencing.

Ligand Binding Assay—For m-LBRs, ligand binding was performed with the polyethylene glycol precipitation method, as described previously (10). Briefly, 20 nm [3H]quisqualate (999 GBq/mmol) (Amersham Biosciences) and m-LBR samples were mixed in 150 μl of binding buffer (40 mM HEPES (pH 7.5), containing 2.5 mM CaCl2) at 37 °C for 1 h. After the binding reaction, 6-kDa polyethylene glycol was added to the sample to a concentration of 15% with 3 mg/ml bovine serum albumin and was incubated for 1 h at room temperature with anti-His antibodies (Qiagen Inc., Valencia, CA), or the monoclonal antibody mG1Na-1 (4, 9), which was produced against the extracellular region of mGluR1. The membrane was washed and then incubated with a goat anti-mouse IgG conjugated with alkaline phosphatase. Color development was done with a commercial detection kit (Promega).

免疫印迹分析—对于m-LBRs，用聚乙二醇沉淀法测量了配体结合。简言之，将20 nm [3H] quisqualate（999 GBq/mmol）（Amersham Biosciences）和m-LBR样品混合在150 μl的结合缓冲液（40 mM HEPES, pH 7.5）中，含有2.5 mM CaCl2）在37 °C下孵育1 h。在结合反应后，加入6-kDa的聚乙二醇，使样品的浓度达到15%与3 mg/ml牛血清白蛋白，并与抗His抗体（Qiagen Inc., Valencia, CA）或单克隆抗体mG1Na-1（4, 9），该抗体是针对mGluR1的胞外区域的产物。膜被洗涤，然后用 goat anti-mouse IgG与碱性磷酸酶偶联。最后，通过商业检测试剂盒（Promega）检测颜色生成。
Determination of IP Accumulation—HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were plated at 2 × 10^3/well of a poly-L-lysine-coated 24-well plate, and 1 µg of DNA was transfected using 2 µl LipofectAMINE 2000 for 4 h. After transfection, the medium was exchanged with isotoisol-free Dulbecco's modified Eagle's medium (ICN Biomedicals Inc., Aurora, OH) supplemented with 10% dialyzed fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 µM/ctl [H]myo-inositol (95.0 Ci/mmol) (Amersham Biosciences), and the cultures were incubated at 37 °C for 16–18 h. The cells were then washed with HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl\(_2\), 0.1% glucose, 20 mM HEPES (pH 7.4)) and were incubated for 30 min with 2 mM pyruvate and 2 units/ml glutamic-pyruvic transaminase (Sigma). After washes with HEPES-buffered saline, the cells were incubated with HEPES-buffered saline containing the agonists and 10 mM LiCl at 37 °C for 1 h. The reactions were stopped by adding an equal volume of ice-cold 40 mM formic acid, and then the mixture was incubated for 30 min at 4 °C. Cell extracts were loaded onto 1-ml packed volume columns of AG-1-X8 anion exchange gel resin. After loading, the columns were washed with 2 ml of 40 mM NH\(_4\)OH (pH 9.0) and 2 ml of 40 mM ammonium formate, and the bound material was eluted with 3 ml of 2 M ammonium formate, 0.1 M formic acid. This procedure collects inositol mono-, bis- and trisphosphates (11). Results are expressed as the ratio of the radioactivity collected in the IP fraction over the radioactivity recovered from the solubilized cellular membranes.

Expression of mGluRs in Oocytes and Electrophysiology—The 1330-bp Not\(_1\)-Sfi fragment of the mutant pcDNAmGluR1s and the 6.3-kb Not\(_1\)-Sfi fragment of pmGR1 (12) were ligated. The resulting plasmids (mutants) were used as templates for in vitro transcription to yield complementatory RNA (cRNA). The plasmid DNA was linearized by NotI, and capped cRNA was synthesized with the MEGAscript T7 kit (Ambion, Austin, TX). Xenopus laevis oocytes were prepared, injected with 10 ng of cRNAs, and incubated for 1–2 days. Current measurements were conducted as previously described (5). Measurement of [Ca\(^{2+}\)]—HEK 293 cells were seeded on sterile poly-lysine-coated coverslips (Asahi Techno Glass Corp, Tokyo, Japan) in a 12-well tissue culture plate (1.6 × 10^5/well), and were incubated overnight at night at 37 °C. The cells were then transfected with 1.6 µg of either pcDNAmGluR1 or mutant pcDNAmGluR1s and 0.4 µg of pDsRed2-N1 (Clontech) with 5 µl of LipofectAMINE 2000. After 24 h, the cells were loaded for 30–60 min with Fura-2/AM (5 µM) dissolved in balanced salt solution (BSS), containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.9 mM MgCl\(_2\), and 10 mM HEPES (pH 7.4), postincubated for 30–60 min in BSS at 37 °C, and maintained in BSS until the assay. About 80% of the cells in the field of view expressed mGluR1, as judged by immunocytochemistry, and almost all of them expressed DsRed2 under these conditions. No significant difference was observed in terms of the [Ca\(^{2+}\)] response with and without pDsRed2 co-transfection (data not shown). Therefore, the fluorescence of DsRed2 through 499 nm was used to detect the transfected-rich region. The coverslips were mounted in a glass flow chamber with a flow rate of 2 ml/min at 22 °C. The cells were challenged with 100 µM glutamate or 10 µM quisqualate in BSS for 1 min and then were washed free of agonist. 100 µM of carbachol in BSS was perfused for 1 min at the end of each assay in order to confirm the integrity of the IP,/Ca\(^{2+}\) pathway. The chamber was mounted on a Nikon inverted stage microscope. The output from an intensified charge-coupled camera was digitized and stored by a computerized imaging system, Argus 50 (Hamamatsu Photonics, K.K., Hamamatsu, Japan).

RESULTS

We constructed mutant receptors containing point mutations in the ligand binding residues, as determined by our crystallographic study (Fig. 1). We have designed two types of receptors: one type is the soluble form of the receptor, including the N terminal to Glu522, just before the start residue of the cysteine-rich sequence ahead of the membrane-spanning domain, and the other is the full-length membrane-bound form of the receptor.

Fig. 2A shows the immunoblot analysis of the mutant proteins expressed in the culture medium of the HEK 293 cells transfected with plasmids encoding the mutant soluble form of the receptors. The mutants sS164A, sS186A, sG293A, and sK409A (where the small letter “s” represents the soluble form) were expressed at levels similar to that of the nonmutated soluble form of mGluR1 (sWT) (formerly designated as mGluR114 (5) with a His tag). The partially purified receptors expressed in the culture medium of the HEK 293 cells were expressed well. These soluble forms were detected in the culture medium at 5% normal goat serum for 1 h at room temperature without permeabilization. They were expressed, fixed with 4% paraformaldehyde in PBS for 5–10 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS containing 5% normal goat serum for 1 h. They were immunoreacted with the secondary antibody, fluorescein anti-mouse IgG (H + L) (Vector Laboratories, Burlingame, CA) and were visualized using a Carl Zeiss confocal microscope, model LSM510.

[Ca\(^{2+}\)], values were calculated from the ratios of sequential 340/380-nm excitation image pairs taken every 5 s by comparison with a standard curve constructed with a Fura-2 calcium imaging calibration kit (Molecular Probes, Inc., Eugene, OR). The results are expressed as [Ca\(^{2+}\)] above the basal level, subtracted from the peak [Ca\(^{2+}\)]. Data analysis was performed using the Prism 3.0 software.
Site-directed Mutagenesis in mGluR1

The inhibition of \(^{[3]H}\)quisqualate binding to the mutant m1-LBRs and mGluR1s by gluta-mate was examined. Mutant mGluR1s were also tested in the saturation binding assay.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>IC(_50) of glutamate for (^{[3]H})quisqualate binding</th>
<th>K_D of quisqualate (mGluR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1-LBR</td>
<td>(\mu M)</td>
<td>nm</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.2 ± 0.3</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>S164A</td>
<td>0.7 ± 0.3</td>
<td>6.3 ± 2.0</td>
</tr>
<tr>
<td>S186A</td>
<td>1.3 ± 0.2</td>
<td>7.9 ± 2.5</td>
</tr>
<tr>
<td>R323A</td>
<td>9.1 ± 1.1</td>
<td>14.0 ± 5.4</td>
</tr>
<tr>
<td>K409A</td>
<td>15.6 ± 0.4</td>
<td>27.5 ± 7.1</td>
</tr>
</tbody>
</table>

with that of the wild type soluble form receptor, as shown in Fig. 2C and Table II. On the other hand, the sR323A and sK409A mutants showed 1-order of magnitude higher values.

Next, the mutant full-length receptors, which possess an amino acid point mutation, were constructed, and their expression and ligand binding capacities were examined. An immunoblot analysis of membrane preparations derived from the cells transfected with plasmids encoding the mutant receptors and the wild type mGluR1 (WT) is shown in Fig. 3A. Although the R78A, T188A, and Y236A mutants were not detected in their soluble forms, their full-length form mutants were detected in the immunoblot. The D208A mutant was hardly detected. Although the ligand binding values obtained with the membrane preparations in Fig. 3B were 2–3 orders of magnitude less than those of the soluble forms in the medium (Fig. 2B) (see pmol versus fmol in the units at the ordinate), the variations in the expression levels among the mutants seem to parallel those of the soluble forms of the mutants. Again, the S164A, S186A, R323A, and K409A mutants showed significant binding values. Furthermore, the glutamate IC\(_{50}\) values for these mutants are strikingly comparable with those for the soluble form mutants, as shown in Fig. 3C and Table II. These data are compatible and support our previous experiment involving the functional extraction of the ligand binding region without the membrane region (4).

In order to examine the effects of the amino acid mutations in the ligand binding core on intracellular signaling, the enhancement of the IP concentration evoked by glutamate and quisqualate stimuli was investigated in HEK 293 cells (Fig. 4). 1 mM glutamate stimulation of the cells expressing the mutant mGluR1s, S164A, S186A, R323A, and K409A mutants, evoked IP responses comparable with that of the wild type mGluR1. The responses to the quisqualate stimulation were almost parallel to those of the glutamate stimulation. The S165A and E292A mutants showed an intermediate level response with quisqualate. Although a lower glutamate response level was observed with the Y236A mutant, the response with quisqualate was almost lost. Interestingly, in the Y74A and R78A mutants, the response to glutamate was undetectable; however, a significant response to quisqualate was observed. The amplitude of the quisqualate response in the G293A mutant was very small but appears to be significant. No response to either ligand was detected with the T188A, D208A, and D318A mutants, which hardly expressed the soluble form and exhibited weak ligand binding capacities in the full-length forms. In the wild type mGluR1, the IP level before ligand dose-response curves in inhibiting \(^{[3]H}\)quisqualate binding to the mutant m1-LBRs. The indicated concentrations of glutamate and 20 nM \(^{[3]H}\)quisqualate were incubated with the purified m1-LBR and mutant m1-LBRs. Each point shows the mean ± S.E. of a representative of three experiments done in triplicate. The displacement curves were obtained by sigmoidal fitting with the Prism 3 software.
stimulation (white bar) was significantly higher than that in the cells transfected with the vector plasmid alone (mock).

Since the so-called basal level of IP production by unliganded mGluR1 expression, also observed in Ref. 15, was lost in the mutants S165A and E292A, which showed reduced ligand coupling, it seemed that the “basal level” required agonist-like conformational changes. In these mutants, movement of the LB1-LB2 interdomain orientation angle might be restricted at the basal state, and thus the dimer interface relocation, possibly related to closing of at least one protomer, might be hampered.

Alterations of the intracellular Ca\(^{2+}\) concentrations, which are a downstream effect of IP production, were examined, as shown in Fig. 5A. Glutamate elicited an enhancement of the intracellular Ca\(^{2+}\) concentration in cells expressing the S164A, S165A, S186A, E292A, R323A, and K409A mutants as well as the mGluR1 (WT) and the control mutant, S166A. These data are comparable with the increment of the IP concentration in these mutants. Thus, the intermediate level of IP enhancement in the S165A and E292A mutants seems to be significant. These data indicate the different sensitivities between the ligand binding assay and the intracellular Ca\(^{2+}\) measurement.

The T188A, D208A, Y236A, and D318A mutants did not show any Ca\(^{2+}\) response. In the Y74A, R78A, and G293A mutants, only quisqualate showed a Ca\(^{2+}\) response. The Ca\(^{2+}\) responses in the Y74A and R78A mutants seemed to be comparable with their IP responses.

We also examined the membrane currents in oocyte expression systems, as shown in Fig. 5B. After the injection of crnas corresponding to the mGluR1 and its mutants, ligands were

---

**Fig. 3.** Biochemical and pharmacological characterizations of the wild-type and mutant mGluR1s. A, immunoblotting analysis of mutant mGluR1s. Cells were transfected with vector alone (mock), pcDNAmGluR1 (WT), and mutant pcDNAmGluR1s. Membrane fractions (40 \(\mu\)g) of HEK 293 cells transfected with each plasmid were loaded on a 4–20% gradient SDS-polyacrylamide gel. The marker proteins are myosin heavy chain (212.0 kDa), β-galactosidase (122.0 kDa), bovine serum albumin (83.0 kDa), ovalbumin (51.8 kDa), carbonic anhydrase (36.2 kDa), and soybean trypsin inhibitor (29.9 kDa). Proteins were transferred onto a nitrocellulose membrane and were probed with the monoclonal antibody, mG1Na-1.

B, [\(^{3}H\)]quisqualate binding to mutant mGluR1s. Membrane fractions (100 \(\mu\)g) of HEK 293 cells transfected with plasmids encoding the wild-type and mutant mGluR1s were incubated in binding buffer (40 mM Hepes, pH 7.5, containing 2.5 mM CaCl\(_2\)) with 20 nM [\(^{3}H\)]quisqualate on ice for 1 h. The reaction mixtures were aspirated onto GF/C filters, and the material remaining on the filters was counted with a scintillation counter. Nonspecific binding was measured in the presence of 1 mM glutamate. Each binding shows the mean ± S.E. of at least three experiments done in triplicate.

C, dose-response curve of inhibiting [\(^{3}H\)]quisqualate binding to the wild-type and mutant mGluR1s. The indicated concentrations of glutamate and 20 nM [\(^{3}H\)]quisqualate were incubated with membrane fractions of HEK 293 cells transfected with plasmids for the wild-type and mutant mGluR1s. Each point shows the mean ± S.E. of a representative of three experiments done in triplicate.

---

**Fig. 4.** Ligand-induced inositol phosphate production in cells expressing the wild-type and mutant mGluR1s. Cells were transfected with control vector alone, pcDNAmGluR1, and mutant pcDNAmGluR1s. Transfected cells were labeled for 17 h with [\(^{3}H\)]myo-inositol and were stimulated with 1 mM glutamate or 0.1 mM quisqualate in the presence of 10 mM LiCl. Inositol phosphates were extracted and separated by anion exchange chromatography, as described under “Experimental Procedures.” Results are expressed as the ratio of [\(^{3}H\)]IPs accumulated over total radioactivity in the membrane fraction and represent the means ± S.E. of at least three independent experiments performed in duplicate or triplicate.
Site-directed Mutagenesis in mGluR1

In our previous structural studies, the LB1 dimer interface, which mainly consists of helices B and C, is crucial for setting and selecting the activation state in the ligand binding region. Therefore, we mutated the five main amino acid residues in the interface and analyzed their properties. The Leu116 and Ile120 residues are on the B helix, whereas Leu174, Leu177, and Phe178 are on the C helix. Single, double (Leu116/Leu174), and triple (Leu116/Leu177/Phe178) mutants were made as described under “Experimental Procedures.” Results are expressed as Ca2+ concentrations above the basal level. Data are means ± S.E. for greater than 14 individual cells from at least two independent experiments. B, electrophysiology of oocytes injected with RNAs for the wild-type and mutant mGluR1s related to the ligand interaction. X. laevis oocytes were injected with 10 ng of in vitro transcribed cRNA as described under “Experimental Procedures.” Between 24 and 48 h after the injection, the holding potential was set at 60 mV, and the current was measured with the ligand stimulation, either glutamate or quisqualate. Each point shows the mean ± S.E. of a representative of two experiments done in triplicate.

Applied to the oocytes, and their membrane potentials were measured. The characteristic variation in the amplitude response was somewhat similar to that in the intracellular Ca2+ response. The minor differences were that both ligands elicited membrane currents in Y236A, and quisqualate caused a small response in D318A.

In our previous structural studies, the LB1 dimer interface mutants. A, intracellular Ca2+ response to glutamate or quisqualate in cells transfected with plasmids encoding ligand-binding site mutants as well as the wild type (WT) were examined. Either 100 μM glutamate or 10 μM quisqualate in BSS was used in a 1-min perfusion. The peak and basal intracellular Ca2+ concentrations were determined, as described under “Experimental Procedures.” Results are expressed as Ca2+ concentrations above the basal level. Data are means ± S.E. for greater than 14 individual cells from at least two independent experiments. B, electrophysiology of oocytes injected with RNAs for the wild-type and mutant mGluR1s related to the ligand interaction. X. laevis oocytes were injected with 10 ng of in vitro transcribed cRNA as described under “Experimental Procedures.” Between 24 and 48 h after the injection, the holding potential was set at 60 mV, and the current was measured with the ligand stimulation, either glutamate or quisqualate. Each point shows the mean ± S.E. of a representative of two experiments done in triplicate.

The membrane currents induced by the increased intracellular Ca2+ concentration were measured by an oocyte expression system. Again, the I120A mutant showed no membrane current change, despite the maintenance of the current change in other mutants (data not shown). The cell surface expression of these dimer interface mutants was probed immunocytochemically, as shown in Fig. 8. The mGluR1-1 monoclonal antibody detected the mutants, including I120A, as well as the wild type mGluR1 under the nonpermeabilized condition. Thus, the I120A mutant appears to lose the signaling function, despite the maintenance of its ligand binding capability. We obtained an ~350 nM Kd value of [3H]quisqualate for the I120A mutant, which ruled out the possibility that the elimination of the signaling capacity in the I120A mutant was due to a loss of ligand affinity.

Applied to the oocytes, and their membrane potentials were measured. The characteristic variation in the amplitude response was somewhat similar to that in the intracellular Ca2+ response. The minor differences were that both ligands elicited membrane currents in Y236A, and quisqualate caused a small response in D318A.

In our previous structural studies, the LB1 dimer interface mutants. A, intracellular Ca2+ response to glutamate or quisqualate in cells transfected with plasmids encoding ligand-binding site mutants as well as the wild type (WT) were examined. Either 100 μM glutamate or 10 μM quisqualate in BSS was used in a 1-min perfusion. The peak and basal intracellular Ca2+ concentrations were determined, as described under “Experimental Procedures.” Results are expressed as Ca2+ concentrations above the basal level. Data are means ± S.E. for greater than 14 individual cells from at least two independent experiments. B, electrophysiology of oocytes injected with RNAs for the wild-type and mutant mGluR1s related to the ligand interaction. X. laevis oocytes were injected with 10 ng of in vitro transcribed cRNA as described under “Experimental Procedures.” Between 24 and 48 h after the injection, the holding potential was set at 60 mV, and the current was measured with the ligand stimulation, either glutamate or quisqualate. Each point shows the mean ± S.E. of a representative of two experiments done in triplicate.

The membrane currents induced by the increased intracellular Ca2+ concentration were measured by an oocyte expression system. Again, the I120A mutant showed no membrane current change, despite the maintenance of the current change in other mutants (data not shown). The cell surface expression of these dimer interface mutants was probed immunocytochemically, as shown in Fig. 8. The mGluR1-1 monoclonal antibody detected the mutants, including I120A, as well as the wild type mGluR1 under the nonpermeabilized condition. Thus, the I120A mutant appears to lose the signaling function, despite the maintenance of its ligand binding capability. We obtained an ~350 nM Kd value of [3H]quisqualate for the I120A mutant, which ruled out the possibility that the elimination of the signaling capacity in the I120A mutant was due to a loss of ligand affinity.

Applied to the oocytes, and their membrane potentials were measured. The characteristic variation in the amplitude response was somewhat similar to that in the intracellular Ca2+ response. The minor differences were that both ligands elicited membrane currents in Y236A, and quisqualate caused a small response in D318A.

In our previous structural studies, the LB1 dimer interface mutants. A, intracellular Ca2+ response to glutamate or quisqualate in cells transfected with plasmids encoding ligand-binding site mutants as well as the wild type (WT) were examined. Either 100 μM glutamate or 10 μM quisqualate in BSS was used in a 1-min perfusion. The peak and basal intracellular Ca2+ concentrations were determined, as described under “Experimental Procedures.” Results are expressed as Ca2+ concentrations above the basal level. Data are means ± S.E. for greater than 14 individual cells from at least two independent experiments. B, electrophysiology of oocytes injected with RNAs for the wild-type and mutant mGluR1s related to the ligand interaction. X. laevis oocytes were injected with 10 ng of in vitro transcribed cRNA as described under “Experimental Procedures.” Between 24 and 48 h after the injection, the holding potential was set at 60 mV, and the current was measured with the ligand stimulation, either glutamate or quisqualate. Each point shows the mean ± S.E. of a representative of two experiments done in triplicate.

The membrane currents induced by the increased intracellular Ca2+ concentration were measured by an oocyte expression system. Again, the I120A mutant showed no membrane current change, despite the maintenance of the current change in other mutants (data not shown). The cell surface expression of these dimer interface mutants was probed immunocytochemically, as shown in Fig. 8. The mGluR1-1 monoclonal antibody detected the mutants, including I120A, as well as the wild type mGluR1 under the nonpermeabilized condition. Thus, the I120A mutant appears to lose the signaling function, despite the maintenance of its ligand binding capability. We obtained an ~350 nM Kd value of [3H]quisqualate for the I120A mutant, which ruled out the possibility that the elimination of the signaling capacity in the I120A mutant was due to a loss of ligand affinity.
mM quisqualate in the presence of 10 mM LiCl. Inositol phosphates were fraction and represent the means/H11006 described under extracted and separated by anion exchange chromatography, as de-
tions of intracellular Ca2+ were determined, as described under “Experimental Procedures.” Results are expressed as the ratio of [3H]myo-inositol and were stimulated with 1 mM glutamate or 0.1 mM quisqualate in the presence of 10 mM LiCl. Inositol phosphates were extracted and separated by anion exchange chromatography, as de-
scription under “Experimental Procedures.” Results are expressed as values above the basal concentration. Data are means ± S.E. of at least three independent experiments performed in duplicate or triplicate. B, cells were trans-
fected with pcDNAmGluR1 and mutant receptors. Glutamate (100 μM in BSS) was used for a 1-min challenge. The peak and basal concentra-
tions of intracellular Ca2+ were determined, as described under “Ex-
perimental Procedures.” Results are expressed as values above the basal concentration. Data are means ± S.E. for 10–17 cells of two independent experiments.

DISCUSSION

Based on the crystal structure of the ligand-binding region of mGluR1, we performed an amino acid mutagenesis of two groups of amino acid residues, one series of residues interacting with the ligand, glutamate, and the other series of residues residing in the dimer interface. We prepared mutants of the full-length receptor and the truncated receptor containing only the ligand-binding region. The relationship between ligand-binding and intracellular signaling in the mutant receptors was extensively examined along with the wild type receptor.

Among the 14 full-length mutants, the S164A, S186A, R323A, and K409A mutants were expressed well and showed ligand binding capacities comparable with that of the wild type. These mutants elicited normal IP responses and intracellular calcium increments. The S165A and E292A mutants did not show remarkable binding, but their intracellular responses to both ligands were clear, indicating that the intracellular signaling assay is more sensitive than the present ligand binding assay. Interestingly, the Y74A, R78A, and G293A mutants lost their responses to glutamate but maintained their responsive-
ness to quisqualate. Jensen et al. (16) reported that the potency of glutamate is much lower than that of quisqualate when Arg78 is mutated to leucine. The T188A, D208A, Y236A, and D318A mutants lost their responses to both ligands. The side chains of these four residues, which are conserved among all mGlur subtypes, interact with the α-amino group of glutamate. The manner of the α-amino group interaction with the hydroxyl group of Thr188 and the carboxylate of Asp318 is a hydrogen bond, whereas the interactions with Asp208 and Tyr236 are a salt bridge and a cation–π interaction, respectively. The 4-fold increase of the $K_d$ value of quisqualate in the S186A mutant (Table II) may be caused by an abrogation of the interaction between the backbone carbonyl oxygen of Ser186 with the amino group. As the expressions of the T188A, D208A, and Y236A mutants in the soluble form were unsuccessful, these residues may also be important for the folding of the receptor protein. Intriguingly, water molecules are not involved in the recognition of the α-amino group, implying the importance of the side chain orientations of the four residues. This is in contrast to the adaptable recognition of the γ-carboxyl group through the water molecules. The binding mode and the envi-
ronment of the agonists, including glutamate, in the binding core of the AMPA receptor (17) suggest that the interactions with the α-amino group are conserved among the several ago-

We used labeled quisqualate as a ligand in our binding assay. The displacement experiment of labeled binding quis-
qualate by an excess of unlabeled glutamate not quisqualate was performed to assess the specific binding. Quisqualate differs from glutamate by the presence of 3,5-dioxo-1,2,4-oxadia-
zolidine, as shown in Fig. 1A. In the crystal, the γ-carboxyl group of glutamate interacts with Tyr74, Arg78, Gly293, Arg323, and Lys409. Although the crystal structure complexed with quisqualate has not been solved, the interactions of quisqualate

Fig. 7. Glutamate-induced inositol phosphate production and Ca2+ response in cells expressing the dimer interface mutants. A, cells were transfected with control vector (mock), pcDNAmGluR1 (WT), and mutant receptors. Transfected cells were labeled for 17 h with [3H]myo-inositol and were stimulated with 1 mM glutamate or 0.1 mM quisqualate. B, cells were transfected with pcDNAmGluR1 and mutant receptors. Glutamate (100 μM in BSS) was used for a 1-min challenge. The peak and basal concentra-
tions of intracellular Ca2+ were determined, as described under “Ex-
perimental Procedures.” Results are expressed as values above the basal concentration. Data are means ± S.E. of at least three independent experiments performed in duplicate or triplicate. B, cells were transfected with pcDNAmGluR1 and mutant receptors. Glutamate (100 μM in BSS) was used for a 1-min challenge. The peak and basal concentra-
tions of intracellular Ca2+ were determined, as described under “Ex-
perimental Procedures.” Results are expressed as values above the basal concentration. Data are means ± S.E. for 10–17 cells of two independent experiments.

Fig. 8. Cell surface expression of the dimer interface mutants. HER 293 cells were transfected with cDNAs encoding the five single mutants and each of the double (positions 116 and 120) and triple (positions 174, 177, and 178) mutants in mGluR1. After 24 h, the cells were directly stained with the mG1Na-1 monoclonal antibody under the nonpermeabilized condition.
with these residues may be different, due to the presence of the ring. Quisqualate may utilize distinct binding residues, which may cause its higher affinity as compared with that of glutamate. This may reflect the loss of glutamate signaling and the preservation of quisqualate signaling in the mutants Y74A, R78A, and O293A. The backbone amide nitrogen of Gly293 interacts with the γ-carboxyl oxygen of the ligand glutamate through a water molecule. According to the modeling analysis, the methyl moiety of alanine, which replaces Gly293, collides with neighboring residues, Arg213 and Gly319, disturbing the main chain and hindering the glutamate binding. The lower Kd values of quisqualate in the R323A and K409A mutants (Table II) may also reflect the idea that the removal of their side chains causes stronger quisqualate binding and thus produces higher IC50 values of glutamate than those in the wild type.

Based on our crystal structure of mGlur1 and molecular modeling, mutagenesis experiments have been performed on the Group II and III mGlurRs. For mGlR4, mutations of the amino acid residues possibly corresponding to Arg78, Thr184, Gly203, and Lys409 of mGlur1 abolished the ligand binding (L-AP4) (18). For the mGlur2 mutants, mutations of the residues corresponding to Tyr174, Tyr236, and Asp318 abolished the ligand binding (LY34740) (19). Those data are consistent with the present data. In addition, alanine mutations of Ser159 in mGlur4 and Ser145 in mGlur2, which correspond to Ser165 in mGlur1, abolished the ligand binding and functional responses. The S165A mutation did not completely eliminate the glutamate- and quisqualate-induced signaling in the present study, which is consistent with the report of O’Hara et al. (20) that the mutant retained the functional activity, despite its reduced affinity. Thus, Ser165 in mGlur1 does not appear to be as critical for functional expression as the four residues mentioned above. However, notably, the basal activity measured by the intracellular IP level was lost in the S165A mutant. The inefficient expression of this mutant in the truncated version, in addition to the reported key roles of the corresponding residues in related receptors such as the calcium-sensing receptor (21) and GABA-B receptor (22), suggests us that the residue is important for the ligand binding domain fold. Since the main chain nitrogen of Ser165 is hydrogen-bonded with the α-carboxyl oxygen of glutamate, a similar main chain interaction may remain after the alanine replacement. Thus, the corresponding residues in the other subtypes may interact with glutamate in somewhat different manners.

In previous structural studies, we showed that mGlur1 is a homodimer containing the dimer interface, which consists of the B and C helices in each of the two LB1 domains. The construction of the dimer interface defines the two states of the ligand binding domain, the activated state and the resting state, which are supposed to be in dynamic equilibrium. The recent structural analysis of the antagonist binding crystal reinforces our proposal. Thus, to clarify the significance of the LB1 interface in the signaling into the intracellular environment, the dimer interface was analyzed. The ligand binding capacities of these mutants were markedly decreased as compared with that of the wild type. Out of the five single amino acid mutants in the hydrophobic interface, four point mutants responded to the glutamate stimulus; however, I120A lost the intracellular responses. Namely, the increments of both the IP concentration and the intracellular Ca2+ were abolished. Although our ligand binding assay is not as sensitive as the signaling assay, the four other point mutants showed clear intracellular responses, despite their lower levels of ligand binding than that of I120A mutant. Notably, the rotation axis of the dimer interface passes in the close vicinity of Ile120. The alanine replacement of Ile120 may hinder the ability of the dimer interface to set the activated state, even upon ligand binding, although the small reduction of the ligand affinity might contribute to the signal loss to some extent. The structural determination of this mutant is intriguing. These results demonstrate that the LB1 interface performs the critical role of a gateway for determining the initial activation status of the receptor molecule and also provides an indispensable structural backbone for the dimer formation. Thus, the dimer interface may provide a new target for the modification of signal transduction in mGlurRs. The dominant-negative character of the mutant is under investigation.

In conclusion, we performed a systematic site-directed mutagenesis study of mGlur1, focusing on the ligand binding site and the dimer interface, which were revealed by the crystal structure. The mutant analyses of the ligand binding site disclosed the critical residues that directly bind to the α-amino group of glutamate. The alanine replacement of isoleucine 120, located close to the relocation axis of the dimer interface, abolished the Ca2+ response, indicating the crucial role of the dimer interface relocation for the initial activation of mGlur1.

Acknowledgments—We thank Dr. Dai Watanabe and Dr. Jun Kitano for assistance with Ca2+ response measurements and the immunocytochemistry, respectively. We also thank Dr. Jun Otomo and Dr. Tomoko Takekishi for the current measurements by oocyte expression. We are grateful to Dr. Daisuke Tsuchiya and Dr. Kosuke Morikawa for Fig. 1 and critical reading of the manuscript.

REFERENCES
