Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1.

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Running Title: mutagenesis and proteolysis in the extracellular region of mGluR1
Summary

Previously we produced the whole extracellular region of metabotropic glutamate receptor subtype 1 (mGluR1) in a soluble form. The soluble receptor retained a ligand affinity comparable with that of the full-length membrane-bound receptor and formed a disulfide-linked dimer. Here, we have identified a cysteine residue responsible for the intermolecular disulfide bond and determined domain organization of the extracellular region of mGluR1. A mutant, C140A, was a monomer under nonreduced conditions by SDS-polyacrylamide gel electrophoresis, however, C140A was eluted at the position similar to that of mGluR113, the wild type soluble receptor, by size exclusion column chromatography. Furthermore, C140A bound a ligand, \([^3H]quisqualate\), with an affinity similar to that obtained by mGluR113. Oocytes injected with RNA for full-length mGluR1 containing C140A mutation showed responses to ligands at magnitudes similar to those with wild type full-length RNA. Thus elimination of the disulfide linkage did not perturb the dimer formation and ligand signaling, suggesting that cryptic dimer interface(s) possibly exist in mGluR1. Limited proteolysis of the whole extracellular fragment (residue 33–592) revealed two trypsin sensitive sites, after the residues Arg^{139} and Arg^{521}. A 15 kDa NH$_2$-terminal proteolytic fragment (residue 33–139) was associated with the downstream part after the digestion. Arg^{521} was located before a cysteine-rich stretch preceding the transmembrane region. A new shorter soluble receptor (residue 33–522) lacking the cysteine-rich region was designed based on the protease sensitive boundary. The purified receptor protein gave a $K_d$ value of 58.1 ± 0.84 nM, which is compatible to a reported value of the full-length receptor. The $B_{\text{max}}$ value was 7.06 ± 0.82 nmoles/mg protein. These results indicated that the ligand-binding specificity of mGluR1 is confined to the NH$_2$-terminal 490 amino acid region of the mature protein.
**Introduction**

L-glutamate is a major neurotransmitter of excitatory synapses in mammalian brains. Metabotropic glutamate receptors (mGluRs)\(^1\) are thought to modulate synaptic neurotransmission (1). Eight subtypes of mGluRs and the calcium sensing receptor (CaR) (2) share amino acid sequence homology. mGluRs possess seven transmembrane segments and are considered to be G-protein coupled receptors (GPCRs). mGluRs contain a large extracellular region (~600 amino acids) in contrast to ordinary GPCRs. The NH\(_2\)-terminal extracellular regions have amino acid sequence similarity (3) to that of Leucine-, Isoleucine-, Valine-binding protein (LIVBP) (4), one of the bacterial periplasmic binding proteins. The LIVBP-like region is followed by a cysteine-rich region that precedes the first transmembrane segment. LIVBP-like regions are also found in the GABA\(_B\) receptor (5), the putative pheromone receptor (6, 7). The cysteine-rich region is not shared by the bacterial binding proteins and the GABA\(_B\) receptor. These receptor proteins which contain a large extracellular region similar to that of mGluR have been designated as family 3 GPCR (8). Thus, structural resemblance among the family 3 receptors has been predicted.

Previously we were able to express the whole extracellular region of mGluR1 in a soluble form (9). The receptor protein secreted into the culture medium retained the ligand binding affinity and selectivity comparable with those of the full-length membrane bound receptor. Interestingly the soluble receptor of mGluR1 was a cysteine-linked dimer. Dimer or oligomer forms of other subtypes of mGluRs, mGluR5 (10) and mGluR4 (11), or of CaR (12, 13) have been also reported. However, the precise mechanism of ligand binding to the mGluR is unknown. It is still unclear whether glutamate binds to mGluRs in an analogous way to the Venus’ flytrap model proposed in the bacterial binding proteins (14), whereby the ligand bound to one lobe is trapped to another lobe by the bending motion of the hinge region.
Role(s) of dimerization of mGluR1 in ligand binding and signal transmission remain to be elucidated.

The significance of dimerization in signal transduction of single-span transmembrane receptors for growth factors or cytokines has been studied extensively (15). The main focus is on whether structural details of extracellular regions have an influence on intracellular signaling (16, 17). For GPCRs, hetero- or homo-dimer formation has been reported in a few cases such as some subtypes of dopamine receptors (18), β2-adrenergic receptor (19) and muscarinic acetylcholine receptors (20). Domain swapping among transmembrane segments has been proposed to be a causative factor in self-association of adrenergic receptors (21). Technical difficulties have been accompanied with those studies because of seven hydrophobic transmembrane segments. However, more convincing evidence has recently emerged of dimer formation in GPCRs. A heterodimeric opioid receptor dimer with an affinity and ligand selectivity different from those of the homodimer has been reported (22). Dimerization in the intracellular region is also reported to be essential for functional expression of the GABA<sub>B</sub> heterodimer receptor (23–25). These results suggest a possibility that dimerization of GPCR is not a rare phenomenon but rather a common occurrence.

In this study, with the advantage of purified dimerized material, we have determined a cysteine residue responsible for the intermolecular disulfide bonding by amino acid point mutation. Effects of disruption of the disulfide bond on ligand binding and signaling were examined. The existence of a cryptic dimer interface in mGluR is discussed. In order to delineate ligand binding core of mGluR1, we furthermore defined the domain organization of the extracellular region by proteolysis experiments. The new recombinant virus, designed according to the protease sensitive boundary, produced the shorter soluble receptor lacking the cysteine-rich region. We have determined a ligand-binding constant and a maximal binding value of this soluble receptor that consists solely of the LIVBP-like domain in
comparison with that of the soluble receptor encompassing the whole extracellular domain.
Experimental Procedures

Materials

L-Quisqualic acid was purchased from Tocris Cookson Ltd. (Langford Bristol, UK). L-Glutamic acid monosodium salt was purchased from Nakalai tesque, Inc. (Kyoto, Japan). Linear gradient polyacrylamide gels were MULTIGEL obtained from Daiichi Pure Chemicals (Tokyo, Japan). Ethylene glycol bis(succinimidylsuccinate) (EGS) was obtained from Pierce (Rockford, IL). N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and phenylmethylsulfonyl fluoride (PMSF) were purchased from WAKO Pure Chemical Industries (Osaka, Japan). Type XI trypsin, diphenyl carbamyl chloride treated, from bovine pancreas, was obtained from Sigma Aldrich Fine Chemicals (St. Louis, MO). All other reagents were of analytical grade.

Cell culture and isolation of recombinant viruses

Spodoptera frugiperda (Sf9) cells were propagated in a monolayer at 27 °C in TNM-FH (Grace’s powder medium, 0.3% yeastolate, 0.3% lactalbumin hydrolysate, 0.1% pluronic F-68; Life Technologies, Inc.), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Inc.) or in suspension at 27 °C in IPL-41 (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.3% tryptose phosphate (Life Technologies, Inc.) and 0.1% pluronic F-68. 100 units/ml of penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B were used. Trichoplusia ni BTI-TN-5B1-4 (High Five) cells were cultured in a monolayer at 27 °C in Express Five™ serum-free medium (Life Technologies, Inc.) supplemented with 18 mM L-glutamine. Recombinant baculoviruses were generated by co-transfecting Sf9 insect cells with 1 µg of plasmid DNA and 0.1 µg of BaculoGold Baculovirus DNA (PharMingen) with Lipofectin reagent (Life Technologies, Inc.). After 6 days of incubation, the supernatant was harvested. The virus in
the supernatant was titrated and individual plaques were isolated. Virus stocks were prepared in monolayer or suspension cultures of Sf9 cells. Baculovirus infection was performed as described previously (9). Briefly, after being infected with baculoviruses for soluble receptors, High Five cells were incubated for four or five days.

Construction of transfer vectors for expression of mGluRs in insect cells

Both transfer vectors, pVLmGluR113 and pVLmGluR114, were constructed from pmGluR108 (9). pVLmGluR113 was made as follows. A set of complementary oligonucleotides, HJ110 (5’-

CACAGGCTGTGAGCCCCATTCCTGTCGTTATCTTGAGTGAGTGACATAGAATAG
TGAT-3’) and HJ111 (5’-

CTAGATCACTATTCTATGTCACTCCACTCAAGATAACGGACAGGAATGGGCTCAC
AGCCTGTGAGCT-3’), were annealed and cloned into the SacI/XbaI-digested fragment of pmGluR108. pVLmGluR114 was made as follows. Polymerase chain reaction (PCR) was done with primers TO1 (5’-CATCAATGCCATCTATGCCATGGC-3’) and HJ112 (5’-

TCTAGATTACTAAGATCGTACCATTCCGCTTTTGTTC-3’) using pmGluR108 as a template. TO1 contains an NcoI site. HJ112 contains an XbaI site. The PCR product was digested with NcoI and XbaI and was cloned into the NcoI/XbaI-digested fragment of pmGluR108. Transfer vectors for mutant receptors were made as follows. Mutants in the wild type background are denoted with the number of the residue and amino acid substituted. A BglII-XbaI fragment was cut out of pVLmGluR114 and ligated into BamHI/XbaI-digested pKF19k (TAKARA). With this plasmid as a template, PCR was performed with selection primer and each of primers C67A (5’-GATCTCCCCAGCCTTCTTTC-3’), C109A (5’-

AGAGTGCCAGGGCGAGTTCCCGG-3’) and C140A (5’-

ATCAGGCAGGGCTCGGTCAGC-3’). Following the protocol of Mutan-Super Express Km Kit (TAKARA), the plasmids pKFC67A, pKFC109A and pKF140A, which contain the
mutated sequences, were obtained. 391 bp *NotI-EcoRI* fragments of pKFC67A and pKFC109A and a 390 bp *EcoRI-AatI* fragment were ligated into the *NotI/EcoRI*-digested pVLmGluR113 and *EcoRI/AatI*-digested pVLmGluR113, yielding pVLmGluR113C67A, pVLmGluR113C109A and pVLmGluR113C140A. All the PCR products were fully sequenced. These transfer vectors were used for transfection to insect cells with BaculoGold Baculovirus DNA.

*Immunoblotting*

Essentially the same as described previously (9). Culture medium was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell). mGluRs were identified by using the monoclonal antibody (MAb) mG1Na-1 (9, 26, 27) and anti-(mouse IgG)-conjugated alkaline phosphatase (Promega). Color development was done by a commercial detection kit (Promega).

*Native PAGE*

Samples were analyzed by 2-15% or 4–20% linear gradient polyacrylamide gels not containing SDS in the gel matrix. SDS was not included in either the running buffer or the sample buffer. Gels were silver stained. Molecular weight standards were from Daiichi Pure Chemicals.

*Purification of the soluble receptors*

A conditioned medium of receptor-producing High Five cells was concentrated to 10-50 fold in the presence of protease inhibitor mixtures (1 mM of PMSF, 5 µg/ml of leupeptin, 10 µg/ml of benzamidine, 10 µg/ml of trypsin inhibitor and 1 µg/ml of aprotinin) and washed by 40 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes) (pH 7.5) containing 10% glycerol, 1 M NaCl and the protease inhibitor mixtures. Concentrated material was centrifuged at 15,000 rpm for 20 min, filtrated by a 0.22 µm filter and loaded on an
immunoaffinity column which was conjugated with MAb mG1Na-1 as described previously (9). The column was washed by 30 ml of 10 mM Hepes, pH 7.5, containing 200 mM NaCl, and the bound material was eluted with 100 mM CAPS, pH 11, containing 200 mM NaCl. Eluate was neutralized with 2 M Hepes pH 7.5. Aliquots of the eluate were analyzed by SDS-PAGE followed by immunoblotting. Positively stained fractions were pooled and analyzed by ligand binding assay. Then the pooled fraction was further purified by ion exchange column chromatography. After being diluted five-times with 10 mM Hepes, pH 7.5, the sample was applied on Resource Q (Amersham Pharmacia Biotech) fitted to ÄKTA Explorer 10S (Amersham Pharmacia Biotech) equilibrated with 10 mM Hepes, pH 7.5, and eluted by NaCl at a gradient from 0 to 1.2 M. In order to compare the gel filtration pattern of mGluR113 with that of its cysteine mutant, aliquotes of the immunoaffinity column eluate were loaded on a HiLoad 16/60 Superdex 200pg (Amersham Pharmacia Biotech) equilibrated with 10 mM Hepes, pH 7.5, containing 200 mM NaCl and 10% glycerol, by high performance liquid chromatography (Waters 626LC). The flow rate was 0.5 ml/min and fractions of 5 ml each were collected.

**Ligand binding**

Ligand binding was performed with the polyethylene glycol (PEG) precipitation method as described previously (28). Briefly, 20 nM or 40 nM of [3H]quisqualate (323 GBq/mmol) (a gift from BANYU Tsukuba Research Institute) and soluble receptor samples were mixed in 150 µl of a binding buffer (40 mM Hepes, pH 7.5 containing 2.5 mM CaCl2) at 4 °C for 1 h. After the binding reaction, 6 kDa PEG was added to the sample at the concentration of 15% with 3 mg/ml of γ globulin. Precipitated material was washed twice with 1 ml of 8% 6 kDa PEG and dissolved in 1 ml of water. After addition of 14 ml Scintisol EX-H (WAKO Pure Chemical Industries), the radioactivity was counted in the scintillation counter. Binding data
was analyzed by the software of Prism II (Graphpad Software, San Diego, CA). Saturation binding curves were fitted to a one-site binding model and $K_d$ and $B_{max}$ values were calculated.

**Expression of mGluRs in oocytes and electrophysiology**

A 777 bp SacII-AatI fragment of pKFC140A and a 3.2 kbp AatI-SacII fragment of pmGR1 (29) and a 3.7 kbp SacII-SacII fragment of pmGR1 were ligated. The resulting plasmid, pmGR1C140A, and pmGR1 were used as templates for *in vitro* transcription to yield complementary RNA (cRNAs). The plasmid DNA was linealized by NotI and capped cRNA was synthesized with MEGAscript T7 kit (Ambion, Austin, TX). *Xenopus laevis* oocytes were prepared according to the standard procedure. The follicular cell layer was removed by treatment with Ca$^{2+}$-free ND solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$ and 5 mM Hepes, pH 7.6) containing 100 µg/100 ml collagenase-B (Yakuruto, Tokyo, Japan) for 1.5 h at 18 °C. The oocytes were then injected with 10 ng of cRNAs. The injected oocytes were incubated in ND solution for 1–2 d. A two-electrode voltage clamp system was used to measure glutamate and quisqualate induced chloride ion currents. The membrane potential was held at –60 mV in all measurements. All currents were filtered at 1 kHz, and data were stored and analyzed using MacLab system (Bio-Research-Center, Nagoya, Japan). All measurements were conducted at room temperature.

**Cross-linking procedure**

The cross-linker, EGS, was dissolved at 20 mM in dimethylsulfoxide and diluted into the protein solution to give the final concentration of 1 mM. The reaction proceeding at 25 °C for 30 min was stopped by the addition of Tris-HCl buffer from a 1 M stock solution at pH 7.5 and a further incubation was conducted at 25 °C for 15 min. The cross-linked material was analyzed by 4–20% SDS-PAGE and silver-stained (WAKO Pure Chemical Industries).

**Trypsin digestion and protein sequencing**
Purified protein was digested in 10 mM Hepes, pH 7.5, with different concentrations of trypsin. Reaction was stopped with 0.01 mM PMSF and sample buffer. Aliquot was not heated and was subjected to SDS-PAGE. The gel was electroblotted onto a polyvinylidene difluoride membrane (Trans-Blot Trasfer Medium, Bio-rad), stained with Coomassie Brilliant Blue R250 (PAGE Blue83, Daiichi Pure Chemicals), and destained with 20% methanol. The protein bands were excised and subjected to automated Edman degradation by an Applied Biosystems Procise model 492 protein sequencer (Applied Biosystems).

Low angle rotary shadowing

The purified mGluRs in 50 mM Hepes, pH 7.5, was equilibrated with glycerol (up to 50% (v/v)). Final concentration of the protein was 50 µg/ml each. 50 µl of the sample was sprayed onto mica surface cleaved freshly by using a painter's airbrush (Olympus Model SP-B, f 0.18 mm). Then, the sample on the mica was rapidly brought into a freeze-etching device equipped with a large turbo pump (FR 7000, Hitachi, Mito, Japan), dried for 10 min (room temperature) in vacuum (1x10⁻⁶ Pa) and then cooled down to –100 °C. Subsequently, specimens were rotary shadowed with platinum by an electron gun positioned at an angle of 2.5° to the mica surface and followed by carbon evaporation. Shadowed films were removed from the mica by slowly soaking the mica into water, mounted on copper grids and examined under a JEOL 100CX electron microscope (JEOL Co., Ltd., Tokyo).
Results

Figure 1 shows a schematic view of the transfer vectors, pVLmGluR113, pVLmGluR113C67A, pVLmGluR113C109A, pVLmGluR113C140A and pVLmGluR114, used for making the recombinant viruses for mGluR113, mGluR113C67A, mGluR113C109A, mGluR113C140A and mGluR114. pVLmGluR113 encodes complementary DNA (cDNA) corresponding to the region Met¹–Glu⁵⁹² of the primary amino acid sequence of mGluR1. pVLmGluR114 encodes cDNA corresponding to the region Met¹–Ser⁵²². pmGluR108 that was used in our previous study (9) encoded the amino acid region identical with that of pVLmGluR113 and six histidine codons at its COOH-terminus. In this study, a His tag-sequence of pmGluR108 was omitted in pVLmGluR113. Recombinant viruses for the receptors were prepared as described in “Experimental Procedures”.

mGluR113 encodes the whole extracellular region of mGluR1. Amino acid mutation was introduced at the first three consecutive cysteine residues (Cys67, Cys109 and Cys140). The mutant receptor proteins were designated C67A, C109A and C140A. Concentrated culture medium of insect cells infected with the recombinant viruses for cysteine mutants as well as for mGluR113 was subjected to SDS-PAGE and analyzed by immunoblotting (Figure 2A). In the presence of 20 mM dithiothreitol (DTT) (left pannel), the three mutants were detected as approximate 65 kDa bands. Under nonreduced conditions (right pannel), C67A and C109A was electrophoresed at around 130 kDa bands; however, C140A was electrophoresed much faster with a position corresponding to the monomer band. The sizes of C67A and C109A appeared significantly different from that of mGluR113, suggesting a conformational change. These results clearly showed that alanine mutation at Cys¹⁴⁰ eliminated the intermolecular disulfide bonding.
Next we compared ligand binding capacities between C140A and mGluR113. As concentrated culture medium of insect cells infected with the viruses for C140A showed ligand binding capacity comparable to that of mGluR113 (data not shown), we next purified C140A by an immunoaffinity column as described in “Experimental Procedures”. Although the purified C140A sample showed a band equal to that of mGluR113 under reduced conditions (Figure 2B *left pannel*), C140A migrated much faster than mGluR113 under nonreduced conditions (*center pannel*). Surprisingly, this mutated receptor migrated as a dimer on native-polyacrylamide gels as did mGluR113 (*right pannel*). We also observed a small amount of larger oligomers of C140A. These data indicated that mutation at Cys^{140} eliminated disulfide bonding in the extracellular portion of mGluR1, but the extracellular portion of the C140A receptor folded in a manner that maintained noncovalent dimerization of the receptor molecule. To reinforce this result, C140A was subjected to gel filtration column chromatography in parallel with mGluR113 (Figure 3). Calibration with molecular size markers showed the eluting position of mGluR113 to be around 185 kDa and that of C140A to be around 150 kDa. Both receptors were definitely larger than a monomer and seemed to be a dimer or a larger oligomer.

The ligand binding ability of C140A was quantitively examined by comparing the inhibition of [^{3}H]quisqualate binding to the purified C140A and mGluR113 with unlabeled glutamate and quisqualate (Figure 4A). Both receptors did not make a great difference in dose-response curves of unlabeled glutamate and quisqualate. These data indicated that there was no great difference between mGluR113 and C140A in affinities to glutamate and quisqualate. Then we next examined the importance of the disulfide linkage in signal transmission of mGluR1 using the oocyte expression system (Figure 4B). A messenger RNA (mRNA) which encodes a full-length of mGluR1 whose Cys^{140} was mutated to alanine, was created. Oocytes injected with the mutated mGluR1mRNA as well as the wild type
mGluR1 mRNA were stimulated with ligands, glutamate and quisqualate. The magnitude of
the chloride currents induced by both ligands of oocytes injected with mutant RNA increased
dose-dependently. At each ligand concentration, the current response of mutant mGluR1 was
comparable with that of wild type mGluR1. This result indicated that C140A mutation in a
full-length receptor did not alter the affinity to the ligands nor the ability to couple to
intracellular signaling cascades.

Next in order to understand the domain organization of the mGluR1 extracellular region,
we first determined the NH2-terminal amino acid sequence of soluble receptor protein,
mGluR113, that was generated by removal of a signal peptide and found that mGluR113
began with a residue Ser31. Proteinase digestion was then performed using the purified
mGluR113 (Figures 5A and 5B). Limited proteolysis of mGluR113 by trypsin gave rise to
at least five major bands. Each of these bands (designated as fragments 1–5) were excised
from a polyvinylidene difluoride blot and sequenced at the NH2-terminal end by Edman
degradation. The NH2-terminal amino acid sequence of the 15 kDa band (fragment 4) was
identical with that of mGluR113. NH2-terminal sequencing of the 50 kDa band (fragment 2)
revealed that the digestion site was Arg139 before (C)140 LPDG. Thus the 15 kDa fragment 4
was residues 33–139 and the 50 kDa fragment 2 was residues 140–592. The NH2-terminal
sequence of an 8 kDa band (fragment 5), which was faintly observed by limited digestion
without dithiothreitol (DTT) but was clearly visible in the lanes of samples digested in the
presence of 1 mM DTT, disclosed the other trypsin sensitive site, Arg521 before S522V(C)SE.
Thus the 8 kDa fragment 5 was residues 522-592. A 42 kDa band (fragment 3)
corresponded to residues 140–521. A 57 kDa band (fragment 1) was the intermediate that
resulted from initial digestion at Arg521. The site Arg521 seemed to be more accessible in the
presence of DTT. Although mass spectroscopic analysis or amino acid content calculation is
needed for strict identification of each band, we summarized our interpretation of the results (Figure 5B) and proceeded to the next experiments.

We examined the ligand binding capacity of trypsinized mGluR113. Surprisingly, trypsin digestion, at up to a ratio of 1/10 which is larger than that used in Figure 5, did not abolish ligand binding capacity of mGluR113, as shown in Figure 6A. Trypsinized mGluR113 did show [3H]quisqualate binding comparable to that of undigested mGluR113. In order to know whether or not the 15 kDa NH2-terminal fragment (residue 33–139) is required for ligand binding, the trypsinized sample was loaded on a native polyacrylamide gel that did not contain SDS in the gel matrix (Figure 6B). The trypsinized mGluR113 was electrophoresed at a very close position to that of the undigested mGluR113. To prove that the NH2-terminal fragment is attached to the remaining fragment, we performed a cross-linking experiment (Figure 6C). First mGluR113 was digested by trypsin and aliquot of the reaction mixture was stored in SDS sample buffer. The remaining trypsinized mGluR113 was cross-linked with EGS and analyzed with the non-cross-linked sample by SDS-PAGE under the reduced condition. The proteolytic fragments, 2 and 4, were detected in the lane 2 of trypsinized sample. The cross-linked trypsinized mGluR113 (lane 4) was electrophoresed at a position (asterisk) similar to that of cross-linked undigested mGluR113 (lane 3). These results led us to conclude that the NH2-terminal 15 kDa fragment was associated with the remaining fragment after trypsin cleavage at Arg-Cys bond after residue 139. Thus, on the basis of the trypsin sensitive site, Arg521, we designed a new construct, pVLmGluR114, which encodes cDNA corresponding to the region Met1–Ser522, as shown in Figure 1.

Figure 7A shows the immunoblot of the supernatant of the insect (High Five) cells infected with the recombinant viruses for the new shorter soluble receptor protein, mGluR114, as well as for mGluR113. mGluR114 and mGluR113 were detected by MAb mG1Na-1 as a 57 kDa band and a 65 kDa band under reduced conditions (right panel).
Under nonreduced conditions (left panel), both soluble receptors seemed to be a dimer or oligomer. The levels of expression of the two soluble receptors looked compatible. This result is in contrast to our previous study (9), in which recombinant virus derived from pmGluR103 that encoded cDNA corresponding to the region Met$^1$–Glu$^{492}$ of mGluR1, did not efficiently produce the soluble receptor protein, mGluR103. The COOH-terminal 30 amino acids of mGluR114 may be involved in a secondary structure and contribute to protein stability. Next to purify mGluR114, the 40-fold concentrated medium was applied on an immunoaffinity column as described in “Experimental Procedures”. Then we further purified the material by Resource Q column chromatography. Figure 4B shows Coomasie staining of the purified material of mGluR114 and mGluR113. We also confirmed that the NH$_2$-terminal amino acid sequence of mGluR114 is identical with that of mGluR113 (data not shown).

Using purified mGluR114 as well as mGluR113, a ligand binding study was performed. mGluR114 and mGluR113 showed saturable binding as in Figures 8A and 8B. The $K_d$ value of mGluR114 for quisqualate, which lacks the cysteine-rich region, was 58.1 ± 0.84 nM, similar to the $K_d$ value, 54.1 ± 5.82 nM, of mGluR113. These values are comparable to those reported using full-length membrane bound mGluR1 (30). Thus the LIVBP-like region is sufficient enough to bind ligands. $B_{\text{max}}$ values of mGluR 114 and mGluR 113 were 7.06 ± 0.82 nmole/mg protein and 6.92 ± 0.73 nmole/mg protein, respectively. Right panels are Scatchard plots of the data.

Next we performed rotary shadowing of the soluble receptor proteins, mGluR114 and mGluR113 (Figure 9). Both were globular proteins consisting of two similar components facing each other, consistent with the above finding that they were electrophoresed as dimers in the native gels. Remarkable difference in the shadowing image was not observed between mGluR114 and 113, implying that the LIVBP-like region can fold regardless of the presence or absence of the cysteine-rich region.
Discussion

Previously we have reported dimer formation of soluble forms of mGluR1 expressed in insect cells (9), in which the dimer was disulfide linkaged. In this investigation we have made cysteine to alanine mutants and identified the cysteine residue responsible for the disulfide linkage. One of the mutants, C140A, was electrophoresed in the monomer position under nonreduced conditions by SDS-PAGE. Thus we have demonstrated that Cys$^{140}$ is the responsible residue for the disulfide bond. However, under the native state, namely without SDS in the gel matrix, C140A was electrophoresed at the position similar to that of mGluR113. C140A was also eluted at the position very close to that of mGluR113 by size exclusion chromatography. These data lead us to conclude that the disulfide linkage at Cys$^{140}$ does not play a predominant role in dimer formation of mGluR1. Interestingly, C140A retained the binding capacity to the ligand. Dose-response curves with ligands obtained by purified C140A showed affinities comparable to those of mGluR113. Furthermore, response to the ligands of oocytes injected with RNA corresponding to a full-length form of mGluR1 containing Ala$^{140}$ mutation was similar to that with RNA for the original intact mGluR1. Thus the local environment around the ligand binding site and the signal transmission within the receptor molecule have not been interfered by disruption of the disulfide linkage. We speculate that a putative dimer interface is located apart from Cys$^{140}$.

mGluR1 contains 19 cysteine residues in the extracellular region and these residues are conserved among all eight mGluR subtypes and CaR. CaR contains Cys$^{129}$ in addition to Cys$^{131}$ that exists at the corresponding residue of Cys$^{140}$ of mGluR1. Recently Ray et al. (31) reported that the C129S/C131S double mutant of CaR was detected as monomer by SDS-PAGE, consistent with our results. On the other hand, the C129S/C131S mutant was not co-immunoprecipitated with wild type truncated mutant that contained the whole extracellular region and the first membrane segment of CaR. They speculated that the
intermolecular disulfide bond in CaR plays predominant roles in dimer formation, which was in contrast to our result. Thus roles and formation of the disulfide linkages between mGluR1 and CaR might not be exactly alike. Cysteine residues responsible for intermolecular disulfide dimer or oligomer formation of m3 muscarinic receptor, which is one of the classical GPCRs, have recently been reported (32). Interestingly, a mutant in which the two responsible cysteines located at the 2nd and 3rd extracellular loops were mutated to alanines, lost the intermolecular disulfide linkage but retained the capacity to form non-covalent receptor dimer or multimer.

In this study, we have also succeeded in outlining the domain structure of the extracellular region of mGluR1. A number of Arg and Lys residues are distributed in the extracellular region, nevertheless, trypsin digested mGluR113, which consists of the whole extracellular region of mGluR1, at the site of Arg521. Thus the extracellular region of mGluR1 was subdivided into two domains: an NH2-terminal LIVBP-like region and a cysteine-rich region preceding the first transmembrane segment. We call the former part the ligand binding domain (LBD) and the latter the cysteine-rich domain (CRD) hereafter. Furthermore five major fragments generated by partial tryptic digestion was assigned according to the two trypsin sites.

An extracellular fragment devoid of CRD, mGluR114, was expressed well and showed a $K_d$ value of 58.1 ± 0.84 nM for its ligand, [3H]quisqualate, which is close to that of mGluR113. Both of the soluble receptors, mGluR113 and mGluR 114, showed dimer under nonreduced conditions and monomer under reduced conditions. Thus cysteine 140 responsible for the intermolecular disulfide bond resides within LBD. Because the trypsin-digested mGluR113 electrophoresed at a position similar to that of the undigested one under a native condition, nick introduced by trypsin treatment seems not to interfere with the dimer interface. Interestingly, trypsin-digested mGluR113 as well as mGluR114 (data not shown)
retains ligand binding activity, suggesting that the digestion site, Arg^{139}, is exposed to the surface and resides in a flexible region. The NH_{2}-terminal 15 kDa fragment seems to be associated with the remaining part after trypsin digestion. Therefore we did not make an expression construct that lacked the corresponding cDNA sequence. Thus whether the 15 kDa fragment is required for ligand binding could not be determined. The 15 kDa region might have a role for folding LBD. We speculate that the ligand binding site forms a rather rigid structure.

Provided that two molecules of the ligand, quisqualate, bind to a dimer form of the soluble receptor, the stoichiometry of binding can be calculated to be approximately 39–44% based on the {B_{\text{max}}} values of mGluR113 and mGluR114 for the ligand. Only half of the material might retain binding capacity. Or we may have been unable to measure maximal binding capacity because our PEG precipitation assay is not expected to resolve low affinity site, if any, with a {K_{d}} in the micromolar range. However, if we assume that cooperative binding is operated in the dimer form of the mGluR1 or the two binding sites possess different affinities, this value of stoichiometry would be meaningful. Interestingly, it has been reported that a purified 42 kDa ligand-binding fragment of GluR-D, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, one of glutamate-gated ion channels, showed a {B_{\text{max}}} value of 6–12 nmol/mg, indicating 50% stoichiometry of the theoretical maximum (33).

Model buildings of part of the extracellular region of monomer mGluR1 were proposed on the basis of an atomic structure of a bacterial binding protein that resides in the periplasmic space (34, 3). If the dimerized form is the principal form of active mGluR1, a novel mechanism may be at work in ligand binding and signal transmission in mGluR. Although the cysteine residue(s) responsible for the intermolecular disulfide bonding has been determined, the precise mechanism of the dimer formation has not been explored, and the character of the dimer interface still remains to be elucidated. Whether or not dimer formation
is critical for ligand binding and signal transmission in mGluRs is a topic that should be examined further. Because the cryptic dimer interface which is different from that formed by the intermolecular disulfide bond in mGluR1 is sensitive to SDS detergent, electrostatic or hydrophobic interaction can be suspected to be a driving force for the dimer formation of mGluR1. Intriguingly, a stretch of 24 consecutive uncharged amino acids in the extracellular region of mGluR1 (residues 155 to 178) has been pointed out when the primary amino acid sequence was determined (29).

Whether or not CRD interacts with LBD or the transmembrane region is still unknown. The absence of large differences between the rotary shadowing imagings of mGluR113 and mGluR114 suggests that CRD appears not to perform major roles in dimer formation. Ligand binding within LBD may elicit conformational change of the seven transmembrane segments through CRD. If the structure of CRD is not flexible, magnitude and orientation of the conformational change occurred within LBD, if any, would transmit directly to the seven transmembrane helices. Or CRD may be a flexible stalk and remodel itself upon ligand binding to LBD. Thus elucidation of the structure and function of CRD is an intriguing challenge. Through structural analysis of the ligand binding core of GluR2, Armstrong et al. pointed out a possible interacting site to the allosteric effector or of domain-domain contact through its hydrophobic character (35). CRD in mGluR might be involved in such an interaction, although we do not have any evidence.

In conclusion, Cys\textsuperscript{140} is responsible for the intermolecular disulfide bond of the dimeric mGluR1. C140A mutant remained to be a dimer, suggesting existense of a cryptic dimer interface distinct from the disulfide bond. C140A would be a valuable tool for analysis of the dimer interface. The final answer for the enigmatic dimer interface can be expected to be obtained by the determination of the atomic structure of a soluble form mGluR1. Because of the nine cysteine residues in a 70 amino-acid stretch, mGluR113 preparation might contain
some misfolded material. Thus mGluR114 complexed with ligands may be a good candidate for use in analysis of the tertiary structure of mGluR1.

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Footnotes

The abbreviations used are: mGlur, metabotropic glutamate receptor; CaR, Calcium sensing receptor; GPCR, G-protein coupled receptor; LIVBP, Leucine-, Isoleucine-, Valine-binding protein; EGS, ethylene glycol bis(succinimidylsuccinate); CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MAb, monoclonal antibody; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; PEG, polyethylene glycol; DTT, dithiothreitol; LBD, ligand binding domain; CRD, cysteine rich domain.

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Figure Legends

Figure 1. Schematic diagram of the expression construct of the transfer vectors in insect cells. Full-length wild type mGluR1 was presented as a diagram according to its primary amino acid sequence deduced from mGluR1cDNA (29). The numerical positions of amino acid residues of mGluR1 are indicated. Functional regions are boxed. Inserts of transfer vectors, pVLmGluR113 and pVLmGluR114, used in this study are presented according to the primary sequence of mGluR1. Open triangles indicate cysteine residues within the extracellular region of mGluR1. pVLmGluR113 encodes cDNA corresponding to the entire extracellular region consisting of a signal sequence, an LIVBP-like region and a cysteine-rich region. mGluR113C67A, mGluR113C109A and mGluR113C140A are cysteine to alanine mutants at residues 67, 109 and 140, respectively. pVLmGluR114 is devoid of the sequence corresponding to COOH-terminal 70 amino acids out of pVLmGluR113. NotI-XbaI fragments were ligated into pVL1392.

Figure 2. Effect of cysteine to alanine point mutation on dimerization. A, immunoblotting of culture medium of insect cells infected with the recombinant viruses for mGluR113, C67A, C109A and C140A. Samples were run either in the presence of 20 mM dithiothreitol (DTT+) or under nonreduced conditions (DTT−) on a 7.5% SDS-polyacrylamide gel. Marker proteins are myosin heavy chain (200.0 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68.0 kDa) and ovalbumin (43.0 kDa). B, analysis of purified mGluR113 and C140A by SDS-PAGE and native-PAGE. Purified mGluR113 and C140A (125 ng, respectively) from the immunoaffinity columns were run either in the presence of 20 mM DTT (DTT+) or under nonreduced conditions (DTT−) on 7.5% SDS-polyacrylamide gels. Purified mGluR113 (250 ng) and C140A (750 ng) from the immunoaffinity columns were run on 2–15% native-polyacrylamide gels (Native). Gels were silver stained.
Figure 3. Size exclusion column chromatography of mGluR113 and C140A. 100 µg of mGluR113 and C140A from the immunoaffinity columns were loaded onto Hiload 16/60 Superdex gel filtration columns (Amersham Pharmacia Biotech). The columns were equilibrated and eluted with 10 mM Hepes, pH 7.5, containing 200 mM NaCl and 10% glycerol. 5 ml of each fraction was collected. Aliquots (50 µl) of the indicated gel filtration fractions were subjected to 7.5% SDS-PAGE under reduced conditions with 20 mM DTT, followed by immunoblotting. The vertical arrows indicate the positions of the marker proteins. The molecular masses of the marker proteins were horse spleen ferritin (450 kDa), rabbit muscle aldolase (158 kDa) and bovine serum albumin (68 kDa).

Figure 4. Dose-response curves of glutamate and quisqualate in inhibiting [3H]quisqualate binding to the purified mGluR113 and C140A (A) and electrophysiology of oocytes injected with RNAs for the mutant mGluR1 containing Ala140 mutation and for wild type mGluR1 (B). A, indicated concentrations of quisqualate (right panel) and glutamate (left panel) with 40 nM [3H]quisqualate were incubated with C140A (1.5 µg) and mGluR113 (0.5 µg) from the immunoaffinity column. Closed circles indicate C140A. Open circles indicate mGluR113. Each point shows the mean ± S.E.M. of a representative one of three experiments done in triplicate. The displacement curves were obtained by sigmoidal fitting with Prism II (Graphpad Software, San Diego, CA). B, Xenopus laevis oocytes were injected with 10 ng of in vitro transcribed cRNA as described in “Experimental Procedures”. 24–48 h after the injection, holding potential was set at –60 mV and current was measured with stimulation of ligands, glutamate (left panel) and quisqualate (right panel). Each point shows mean
± S.E.M. of a representative one of two experiments done in quadruplicate. *Closed circles* indicate mGluR1 containing Ala140 mutation. *Open circles* indicate wild type mGluR1.

**Figure 5.** Trypsin digestion (A) and proteolytic cleavage mapping (B) of the soluble mGluR113. *A*, 13.6 µg of immunoaffinity-purified mGluR113 was digested with the indicated ratio (w/w) of trypsin at 25 °C for 1 h, loaded on a 15–25% gradient gel and Coomasie stained. Proteolytic fragments were labelled 1–5. NH₂-terminal sequences of mGluR113 and the five tryptic fragments were determined as described in “Experimental Procedures”. *B*, the five fragments, 1–5, are located relative to the primary amino acid sequence of mGluR1. NH₂-terminal sequences of mGluR113 and the fragments are shown. The vertical arrows indicate the trypsin cleavage sites.

**Figure 6.** Ligand binding (A), native PAGE analysis (B) and chemical cross-linking (C) of trypsinized mGluR113. *A*, 11.3 µg of mGluR113 purified with an immunoaffinity column was digested with 1.1 µg trypsin at 25 °C for 1 h. After addition of 1 mM PMSF for quench, aliquot containing 0.86 µg of trypsin digested mGluR113 were incubated in the binding buffer (40 mM Hepes, pH 7.5 and 2.5 mM CaCl₂) with 20 nM of [³H]quisqualate at 4 °C for 1 h. Undigested mGluR113 was assayed as a control. Nonspecific binding was measured in the presence of 1 mM glutamate. *B*, trypsin-digested mGluR113 was analyzed by native PAGE. 0.75 µg of mGluR113 purified with an immunoaffinity column was digested with 0.075 µg of trypsin at 25 °C for 1 h. After addition of 1 mM PMSF and sample buffer without SDS, the digested sample was loaded onto a 4–20% native gradient gel and silver stained. Control sample treated without trypsin was parallelly electrophoresed. *C*, cross-linking study of trypsinized mGluR113.
Immunoaffinity-purified mGluR113 was digested with 10% (w/w) trypsin at 25 °C for 16 h (lane 2). Half of the reaction mixture was cross-linked with 1 mM EGS (lane 4). 0.75 µg of protein was loaded on each lane of a 4–20% SDS gradient gel under reduced conditions. The undigested samples without cross-linking (lane 1) and with cross-linking (lane 3) underwent with the same procedure parallely. The asterisk indicates cross-linked material.

**Figure 7. Immunoblotting analysis (A) and Coomassie staining of purified mGluR113 and mGluR114 (B).** A, 20 µl of culture medium of High Five cells infected with baculoviruses for mGluR113 and mGluR114 were loaded on 10% SDS-polyacrylamide gels under nonreduced conditions (left panel) and reduced conditions (right panel). Proteins were transferred onto the nitrocellulose membrane and probed with MAb mG1Na-1. B, mGluR113 (4 µg) and mGluR114 (6 µg) purified by Resource Q as described in “Experimental Procedures” were loaded on 10–20% SDS-polyacrylamide gels and Coomassie stained.

**Figure 8. Saturation binding of [³H]quisqualate to mGluR113 and mGluR114.** 0.5 µg of immunoaffinity-purified mGluR113 (A) and mGluR114 (B) were incubated in the binding buffer (40 mM Hepes containing 2.5 mM CaCl₂) with different ligand concentrations of [³H]quisqualate at 4 °C for 1 h. Specific binding was determined by subtraction of nonspecific binding determined in the presence of 1 mM glutamate from total binding. The results were analyzed by Prism II for saturation kinetics (left panels) and for Scatchard analysis (right panels). A representative result from three independent experiments is shown. Each binding was performed in triplicate and is shown as the mean ± S.E.M. A non-linear regression analysis of mGluR113 revealed a $K_d$ of 54.1 ± 5.82 nM and a $B_{max}$ of 6.92 ± 0.73 nmoles/mg protein. mGluR114 showed a $K_d$ of 58.1 ± 0.84 nM and a $B_{max}$ of 7.06 ± 0.82 nmoles/mg protein. These $K_d$ and $B_{max}$ values are means ± S.E.M. (n = 3).
Figure 9. Rotary shadowing image of the soluble mGluRs. Gallery of electron micrographs of soluble mGluR113 (A) and mGluR114 (B).
LIVBP-like region

Transmembrane region

Cytoplasmic region

Signal peptide

pVLmGluR113 (Met\(^1\) Glu\(^{592}\))

pVLmGluR113C67A

pVLmGluR113C109A (Met\(^1\) Glu\(^{592}\))

pVLmGluR113C140A

pVLmGluR114 (Met\(^1\) Ser\(^{522}\))

Figure 1
Figure 2

A

Culture medium

DTT +

DTT -

kDa

200.0

97.4

68.0

43.0

B

Purified receptor

DTT +

kDa

200.0

97.4

68.0

43.0

DTT -

kDa

200.0

97.4

68.0

43.0

Native

kDa

669.0

443.0

139.9
Figure 3
Figure 4

A

B
Figure 5

A

| Trypsin (kDa) | DTT | – | + |
|---------------|-----|---|---|
| 0             | 1/100 | 1/50 | 1/25 |
| 1/200 | 1/100 | 1/50 |
| 220.0        |       |     |     |
| 97.4         |       |     |     |
| 66.0         |       |     |     |
| 46.0         |       |     |     |
| 30.0         |       |     |     |
| 21.5         |       |     |     |
| 14.3         |       |     |     |
| 6.5          |       |     |     |

mGluR113

1

2

3

4

5

B

| Fragment | Estimated size | NH₂-terminal sequence |
|----------|----------------|-----------------------|
| mGluR113 | 65 kDa         | S²³³SQRS              |
| 1        | 57 kDa         | S²³³SQRS              |
| 2        | 50 kDa         | (C)¹⁴⁰LPDG            |
| 3        | 42 kDa         | (C)¹⁴⁰LPDG            |
| 4        | 15 kDa         | S²³³SQRS              |
| 5        | 8 kDa          | S⁵²²V(C)SE            |

Arg¹³⁹

Arg⁵²¹

1 33

592
Figure 7

A

(kDa) mGluR113 mGluR114

nonreduced

reduced

B

(kDa) mGluR113 mGluR114

200.0

97.4

68.0

43.0

29.0

18.4
Figure 8

A

\begin{center}
\begin{tabular}{c}
\begin{tikzpicture}
  \begin{axis}[
    width=0.4\textwidth,
    height=0.3\textwidth,
    xlabel={Ligand Concentration (nM)},
    ylabel={Specific Binding (nmol/mg)},
    xmin=0, xmax=300,
    ymin=0, ymax=7.5,
    \addplot+ [mark=x]
    coordinates {
      (0, 0.5) (50, 2.5) (100, 5.0) (200, 7.5) (300, 7.5)
    };
  \end{axis}
\end{tikzpicture}
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
\begin{tikzpicture}
  \begin{axis}[
    width=0.4\textwidth,
    height=0.3\textwidth,
    xlabel={Specific Binding (nmol/mg)},
    ylabel={Bound/Free},
    xmin=0, xmax=7.5,
    ymin=0, ymax=0.15,
    \addplot+ [mark=x]
    coordinates {
      (0.5, 0.15) (2.5, 0.1) (5.0, 0.05) (7.5, 0.0)
    };
  \end{axis}
\end{tikzpicture}
\end{tabular}
\end{center}

B

\begin{center}
\begin{tabular}{c}
\begin{tikzpicture}
  \begin{axis}[
    width=0.4\textwidth,
    height=0.3\textwidth,
    xlabel={Ligand Concentration (nM)},
    ylabel={Specific Binding (nmol/mg)},
    xmin=0, xmax=300,
    ymin=0, ymax=7.5,
    \addplot+ [mark=x]
    coordinates {
      (0, 5.0) (50, 5.0) (100, 5.0) (200, 5.0) (300, 5.0)
    };
  \end{axis}
\end{tikzpicture}
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
\begin{tikzpicture}
  \begin{axis}[
    width=0.4\textwidth,
    height=0.3\textwidth,
    xlabel={Specific Binding (nmol/mg)},
    ylabel={Bound/Free},
    xmin=0, xmax=7.5,
    ymin=0, ymax=0.19,
    \addplot+ [mark=x]
    coordinates {
      (5.0, 0.19) (7.5, 0.10) (6.0, 0.0) (5.0, 0.0)
    };
  \end{axis}
\end{tikzpicture}
\end{tabular}
\end{center}
Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1
Yuji Tsuji, Yoshimi Shimada, Tomoko Takeshita, Naoko Kajimura, Sayuri Nomura, Naohiro Sekiyama, Jun Otomo, Jiro Usukura, Shigetada Nakanishi and Hisato Jingami

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