Metabotropic glutamate receptor 1 (mGluR1) expresses at the cell surface as disulfide-linked dimers and can be reduced to monomers with sulfhydryl reagents. To identify the dimerization domain, we transiently expressed in HEK-293 cells a truncated version of mGluR1 (RhodC-R1) devoid of the extracellular domain (ECD). RhodC-R1 was a monomer in the absence or presence of the reducing agents, suggesting that dimerization occurs via the ECD. To identify cysteine residues involved in dimerization within the ECD, cysteine to serine point mutations were made at three cysteines within the amino-terminal half of the ECD. A mutation at positions Cys-67, Cys-109, and Cys-140 all resulted in significant amounts of monomers in the absence of reducing agents. The monomeric C67S and C109S mutants were not properly glycosylated, failed to reach the cell surface, and showed no glutamate response, indicating that these mutant receptors were improperly folded and/or processed and thus retained intracellularly. In contrast, the monomeric C140S mutant was properly glycosylated, processed, and expressed at the cell surface. Phosphoinositide hydrolysis assay showed that the glutamate response of the C140S mutant receptor was similar to the wild type receptor. Substitution of a cysteine for Ser-129, Lys-134, Asp-143, and Thr-146 on the C140S mutant background restored receptor dimerization. Taken together, the results suggest that Cys-140 contributes to intermolecular disulfide-linked dimerization of mGluR1.

The metabotropic glutamate receptors (mGluRs) are a family of neurotransmitter receptors that mediate a variety of physiological functions in the central nervous system (1). The mGluRs are members of the superfamily of G-protein-coupled receptors (GPCRs) and belong to the subfamily (family 3) of mGluRs are members of the superfamily of G-protein-coupled receptors (GPCRs) and belong to the subfamily (family 3 (2)) of mGluRs and other family 3 GPCRs are characterized by a very large (approximately 600 residues) extracellular amino-terminal domain (ECD). The mGluR1 ECD is the glutamate binding domain and believed to be structurally related to the bilobed “venus flytrap” structure of bacterial periplasmic binding proteins (9, 10). Recently, it has been shown that both the mGluR1 (11) and the Ca²⁺ receptor (12, 13) are expressed at the cell surface as intermolecular disulfide-linked dimers. For mGluR1 (10), mGluR4 (14), and Ca²⁺ receptor (15), the ECD of each receptor, purified as a secreted protein, exists as a disulfide-linked dimer, suggesting that one or more cysteines in the ECD is involved in receptor dimer formation. The rat mGluR subtype 1α (described as mGluR1 from now on) ECD contains a total of 19 cysteines (16) of which several are highly conserved in other mGluRs, Ca²⁺ receptor, V2Rs, and T1Rs. Proteolysis of the mGluR5 receptor localized cysteine(s) critical for dimer formation to the first 17 kDa of the ECD (17). This region contains three cysteines conserved in all mGluRs, and we mutated these cysteines of the mGluR1 and investigated the role of these cysteine mutants in receptor dimerization and function. This study led to the identification of a conserved Cys-140 as critical for disulfide-linked dimerization of mGluR1, and functional study indicated that, like the wild type receptor, C140S mutant receptor is also capable of intracellular signaling via Gq-phospholipase C pathway.

MATERIALS AND METHODS

Construction of a Rhodopsin Epitope-tagged Truncation Mutant of mGluR1—Using Turbo Pfu DNA polymerase (Stratagene Inc.), a polymerase chain reaction (PCR) was performed to add 20 amino acid residues (MNQTEGPNFYVPSNKTGV) corresponding to the amino terminus of bovine rhodopsin to the mGluR1 before amino acid residue 584. The 1.8-kilobase PCR product was subcloned to the pCR3.1 expression vector (Invitrogen) as a HindIII-XhoI fragment. The entire nucleotide sequence of the PCR product was confirmed by using a rhodopsin terminase cycle sequencing reaction kit and ABI prizm-377 DNA sequencer (Applied Biosystems). The constructed truncation mutant, designated as RhodC-R1, was devoid of 1–583 amino-terminal ECD of the mGluR1 but included 20 amino acids of the amino terminus rhodopsin tag and the amino acid residues 584–1199 of mGluR1.

Site-directed Mutagenesis of the mGluR1—mGluR1 cDNA was cloned in pcR3.1 expression vector (Invitrogen) as a BamHI-NolI fragment. Site-directed mutagenesis was performed using a commercial kit (QuikChange site-directed mutagenesis kit, Stratagene) as described by Ray et al. (18). Three cysteine point mutants, C67S, C109S, and C140S were created by changing cysteine at a given site to serine. Seven Cys-scanning mutants, S119C, S126C, S129C, K134C, D143C, T146C, and T152C were created in a second round of mutagenesis by using C140S mutant plasmid DNA as a template. The mutations were con-
firmed by automated DNA sequencing using a dRhodamine terminator cycle sequencing reaction kit and the ABI prism-377 DNA sequencer (Applied Biosystems).

**Transient Transfection of Wild Type and Mutant Receptors in HEK-293 Cells**—Receptor plasmid DNAs were prepared by a maxi-plasmid preparation kit (Qiagen) and were transiently transfection in HEK-293 cells using Lipofectamine (Life Technologies) as described previously (19). Protein extraction for immunoblotting or biontination-immuno-precipitation experiments was performed 48 h after transfection.

**Immunoblotting Analyses with Detergent-solubilized Crude or Whole Cell Extracts**—Crude membrane extracts were prepared as described previously by Ray et al. (19). Briefly, confluent cells in 75-cm² flasks or 6-well plates were rinsed with ice-cold phosphate-buffered saline and scraped on ice in buffer A (5 mM Tris (pH 7.2), 2 mM EDTA), containing 10 mM iodoacetamide with freshly added Complete protease inhibitors mixture (Roche Molecular Biochemicals). The cells were forced through a 22-gauge needle five to eight times, and the lystate was spun in a TLA-45 centrifuge at 45,000 rpm for 30 min at 4 °C to collect a crude membrane pellet. The pellet was resuspended in buffer B containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100 with 10 mM iodoacetamide and freshly added protease inhibitors mixture. Whole cell extracts were prepared by solubilizing cells directly in buffer B containing 1% Triton-100 as described earlier (15). The protein content of each sample was determined by the modified Bradford method (Bio-Rad) and 20–30 μg of protein per lane was separated on 5% gels by SDS-PAGE. Electrotransferred proteins to nitrocellulose membranes were incubated with monoclonal anti-mGlur1 antibody (Transduction laboratories, catalog # M72620) at a dilution of 1: 10,000 or anti-mGlur1 polyclonal antibody at a dilution of 1:500 (Chemicon, catalog # AB1553). Subsequently, the nitrocellulose membrane was incubated with a secondary goat anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (Kierkegaard and Perry Laboratories) at a dilution of 1: 5000, respectively. The mGlur1 bands were detected with an Enhanced Chemiluminescence system (ECL) (Amersham Corp). Biotinylated protein bands were detected using peroxidase-conjugated streptavidin-POD following by visualization of the biotinylated bands using a BM chemiluminescence kit (Roche Molecular Biochemicals).

**Biotin-labeling of The Cell Surface mGlur1**—48 h after transfection, cell surface proteins of the intact HEK-293 cells were labeled with membrane-impermeant Biotin-7-NHS using the cellular labeling kit (Roche Molecular Biochemicals) as described earlier (19). Briefly, intact cells were labeled with 50 μg/ml Biotin-7-NHS in biontination buffer (50 mM sodium borate, 150 mM NaCl) to biotinylate cell surface proteins. The reaction was stopped by adding 50 mM NH₄Cl for 15 min on ice. The cells were washed with phosphate-buffered saline and solubilized with lysis buffer B.

**Immunoprecipitation of mGlur1**—300 μl (approximately 600 μg of total protein) of the whole cell lysate of biotin-labeled cells was further diluted with 300 μl of buffer B and incubated with 5 μl of mouse monoclonal mGlur1-specific (made against the carboxyl-terminal peptide and the amino acids 1042–1160) or mouse anti-mGlur1 polyclonal antibodies (Transduction laboratories, Lexington, KY) for 1–2 h at 4 °C. Subsequently, 25 μl of Protein A/G-agarose (Santa Cruz Biotechnologies) was added, and the incubation was continued for an additional 2 h. The Protein A/G-agarose was washed three times with buffer B containing 0.5% SDS, and the immunoreactive proteins were eluted in sample buffer containing either no β-mercaptoethanol or 300 mM β-mercaptoethanol. Samples were analyzed by SDS-PAGE, and immunoblotting was performed as described before.

**Glycosidase Treatment of Detergent-solubilized Extracts**—For cleavage with PNGase-F or Endo-H (Roche Molecular Biochemicals), whole cell extracts (30 μl) were diluted in 20 μl of 50 mM sodium acetate (pH 4.8). Samples were incubated with 0.5 million units of Endo-H or 1.0 unit of PNGase-F for 2 h at 37 °C.

**Phosphoinositide Hydrolysis Assay**—Phosphoinositide (PhI) hydrolysis assay has been described previously (18). Briefly, 24 h after transfection, transfected cells from a confluent 75-cm² flask was replated in 24-well plates in medium containing 3.0 μCi/ml of [3H]myoinositol (PerkinElmer Life Sciences) in complete Dulbecco’s modified Eagle’s medium containing no glucose for another 24 h, followed by 1-h pretreatment with 0.1 μg/ml Phl buffer (120 mM NaCl, 0.5 mM CaCl₂, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 20 mM LiCl in 25 mM Pipes buffer, pH 7.2). After removal of Phl buffer, cells were incubated for an additional 30 min with different concentrations of ligand in Phl buffer. The reactions were terminated by addition of 1 ml of acid-methanol (1:1000, v/v) per well. Total inositol phosphates were purified by chromatography on Dowex 1-X8 columns.

**RESULTS**

**mGlur1 Is a Cell Surface-expressed Disulfide-linked Dimer**—To determine the domain(s) involved in mGlur1 dimerization, we sought to construct a truncated form of the mGlur1 lacking the ECD. The amino terminus of this truncated mutant (RhodC-R1) consisted of the first 20 amino acid residues of rhodopsin followed by the remainder of the mGlur1 beginning with residue 584. The rhodopsin N terminus tag was added, because it has been shown to enhance proper processing and cell surface expression of several GPCRs (20, 21). Next, the wild type mGlur1 and RhodC-R1 cDNAs were transiently transfected in HEK-293 cells, and after 48 h, whole cell extracts were prepared. To prevent non-specific disulfide bond formation during protein extraction, 10 mM iodoacetamide was included in the lysis buffer. On an immunoblot, as shown in Fig. 1A (left), under non-reducing conditions, a mGlur1-specific monoclonal antibody made against a carboxyl-terminal epitope detected two major bands of the wild type mGlur1 around ~260- to 270-kDa molecular mass range and two fainter 130- and 135-kDa bands. The same four immunoreactive bands were also recognized by a mGlur1-specific polyclonal antibody (Fig. 1B) and not detected in vector-transfected cells (data not shown). The intensity of the lower two 130- and 135-kDa bands varied from no immunoreactivity to faint immunoreactivity between different immunoblotting experiments. After reduction with β-mercaptoethanol, a majority of the wild type mGlur1 ~260- to 270-kDa bands were reduced to a broad monomeric ~133-kDa band, and a small portion remained as SDS-resistant dimeric aggregates. The RhodC-R1 mutant expression pattern revealed a major ~90-kDa immunoreactive band, and the protein was expressed as a monomer, because the presence or absence of β-mercaptoethanol did not shift this band (Fig. 1A, right).

To further determine whether the dimeric mGlur1 and monomeric RhodC-R1 forms were expressed at the cell surface, cell surface proteins were labeled with membrane impermeant Biotin-7-NHS prior to lysing the cells. The wild type mGlur1 and the RhodC-R1 were then immunoprecipitated with anti-mGlur1 monoclonal antibody and eluted with gel loading sample buffer containing no β-mercaptoethanol. Immunoprecipitates were analyzed by SDS-PAGE, and immunoblots were stained either with streptavidin-POD to detect biotinylated cell surface proteins or with a mGlur1 polyclonal antibody to detect all the immunoreactive bands. As shown in Fig. 1B, under non-reducing conditions, streptavidin-POD detected the upper ~260- to 270-kDa dimeric bands but no visible 130- or 135-kDa monomeric bands of the mGlur1. Similarly, streptavidin-POD detected the 90-kDa band of the RhodC-R1 mutant. A duplicate blot of these samples with anti-mGlur1 polyclonal antibody detected all the immunoreactive bands of the wild type mGlur1 and RhodC-R1.

mGlur1 ECD contains four potential asparagine-linked glycosylation sites (Asn-Xaa-Ser/Thr) and is shown to undergo glycosylation (11), and the RhodC-R1 mutant contains two potential asparagine-linked glycosylation sites within the rhodopsin tag sequence. Therefore, to determine further the biochemical identity of the ~260- to 270-kDa dimeric bands of the wild type and the 90-kDa RhodC-R1 mutant receptors, we conducted deglycosylation experiments with two glycosidase enzymes, PNGase-F and Endo-H. The PNGase-F enzyme cleaves all asparagine-linked carbohydrates (both intermediate high mannose forms and fully processed complex carbohydrate forms) from glycoproteins (22). Sensitivity to Endo-H digestion distinguishes between the fully processed mGlur1 forms that are modified with complex carbohydrates (Endo-H-resistant) and intermediate high mannose modified forms (Endo-H-sen-
prepared from HEK-293 cells transiently transfected with either wild type (WT-R1) or RhodC-R1 cDNAs. 20 μg of protein were loaded per lane with sample buffer containing no β-mercaptoethanol (−) or 300 mM β-mercaptoethanol (+) and fractionated on a 5% gel by SDS-PAGE (A). For the biotin-streptavidin labeling experiment (B), cell surface proteins were labeled with Biotin-7-NHS as described under “Materials and Methods.” The cell lysate was immunoprecipitated with an anti-mGluR1 monoclonal antibody (Transduction Laboratories), and proteins eluted with sample buffer containing no β-mercaptoethanol (−) were separated by SDS-PAGE. WT-R1 and RhodC-R1 mutant receptor bands were detected with anti-mGluR1 polyclonal antibody purchased from Chemicon (blot labeled Anti-mGluR1). These same bands of the WT-R1 and RhodC-R1 mutant receptors were detected with peroxidase-conjugated streptavidin (labeled Strep-POD) in a separate blot of the same samples. C, enzymatic deglycosylation studies with PNGase-F (second lane from left for WT-R1 or RhodC-R1 blot) and Endo-H (third lane from left for WT-R1 or RhodC-R1 blot) to identify asparagine-linked glycosylation states of WT-R1 and RhodC-R1 mutant receptors. 30 μl of whole cell extract of each sample was incubated without (−) or with PNGase-F (+) or with Endo-H (+) for 2 h at 37 °C. The reaction was stopped by adding sample buffer, and digested samples were analyzed under non-reducing condition by immunoblotting with anti-mGluR1 monoclonal antibody. The positions of molecular weight standards are indicated on the left for blots shown in A, B, and C. Similar results were seen in additional experiments with independent transfections and immunoblots.

![Fig. 1. Determination of cell surface expression of wild type mGluR1 and RhodC-R1 mutant receptors.](image)

**FIG. 1.** Determination of cell surface expression of wild type mGluR1 and RhodC-R1 mutant receptors. Whole cell extracts were prepared from HEK-293 cells transiently transfected with either wild type (WT-R1) or RhodC-R1 cDNAs. 20 μg of protein were loaded per lane with sample buffer containing no β-mercaptoethanol (−) or 300 mM β-mercaptoethanol (+) and fractionated on a 5% gel by SDS-PAGE (A). For the biotin-streptavidin labeling experiment (B), cell surface proteins were labeled with Biotin-7-NHS as described under “Materials and Methods.” The cell lysate was immunoprecipitated with an anti-mGluR1 monoclonal antibody (Transduction Laboratories), and proteins eluted with sample buffer containing no β-mercaptoethanol (−) were separated by SDS-PAGE. WT-R1 and RhodC-R1 mutant receptor bands were detected with anti-mGluR1 polyclonal antibody purchased from Chemicon (blot labeled Anti-mGluR1). These same bands of the WT-R1 and RhodC-R1 mutant receptors were detected with peroxidase-conjugated streptavidin (labeled Strep-POD) in a separate blot of the same samples. C, enzymatic deglycosylation studies with PNGase-F (second lane from left for WT-R1 or RhodC-R1 blot) and Endo-H (third lane from left for WT-R1 or RhodC-R1 blot) to identify asparagine-linked glycosylation states of WT-R1 and RhodC-R1 mutant receptors. 30 μl of whole cell extract of each sample was incubated without (−) or with PNGase-F (+) or with Endo-H (+) for 2 h at 37 °C. The reaction was stopped by adding sample buffer, and digested samples were analyzed under non-reducing condition by immunoblotting with anti-mGluR1 monoclonal antibody. The positions of molecular weight standards are indicated on the left for blots shown in A, B, and C. Similar results were seen in additional experiments with independent transfections and immunoblots.

**Screening of Single Cysteine Mutants for Their Ability to Form Homodimers—**We generated single C → S mutants of three cysteines at positions 67, 109, and 140 in the mGluR1 ECD. The C → S mutants were further analyzed by determining their dimerization patterns on immunoblots run under non-reducing conditions. Transiently transfected cells expressing these mutant receptors were treated with iodoacetamide to prevent aggregates forming secondary to non-specific disulfide bond formation prior to crude membrane preparation. The membrane extracts were run on immunoblots, and immunoreactive bands were detected with an anti-mGluR1 monoclonal antibody. As seen in Fig. 2A, the wild type mGluR1 receptor expressed as two dimeric bands with little or no monomeric forms visible on immunoblot. C67S and C109S mutant receptors expressed predominantly as two 130- and 135-kDa monomeric forms, and one or two fainter upper dimeric band corresponding to the upper ~260- to 270-kDa dimeric bands of the wild type mGluR1 were sometimes visible. These fainter dimeric bands were more visible when samples run on gels by SDS-PAGE were enriched as crude membrane extracts or as immunoprecipitated samples but not routinely seen in whole cell extracts prepared from C67S- and C109S-transfected cells.

![Fig. 2. Immunoblot analysis under non-reducing and reducing conditions to detect homodimeric expression patterns of the wild type mGluR1, C67S, C109S, and C140S mutant receptors.](image)

**FIG. 2.** Immunoblot analysis under non-reducing and reducing conditions to detect homodimeric expression patterns of the wild type mGluR1, C67S, C109S, and C140S mutant receptors. Crude membrane extracts obtained from HEK-293 cells transiently transfected with wild type mGluR1 (WT-R1) or different cysteine mutants were fractionated on a 5% gel by SDS-PAGE under non-reducing condition without β-mercaptoethanol (A) or under reducing condition with 300 mM β-mercaptoethanol (B) in sample buffer. Immunoblotting was performed with anti-mGluR1 monoclonal antibody. Molecular weight standards are indicated at the left side of the blots. Each blot shown here is representative in terms of sizes and expression levels of the wild type and mutant receptors in two independent transfections and three separate immunoblots.

Interestingly, C140S mutant receptor expressed as a broad ~140-kDa monomeric band that ran at slightly higher molecular mass than the 130- and 135-kDa monomeric bands of C67S and C109S mutants and also generated a ~300-kDa dimeric band. This dimeric band of the C140S mutant ran higher than the dimeric bands of the wild type mGluR1 or C67S or C109S mutant receptors. The intensities of the immunore-
active dimeric bands relative to the monomeric bands for each mutant receptor and wild type receptor were then measured by densitometric scanning (Table I). The data showed that C67S, C109S, and C140S mutant receptors expressed mostly as monomeric forms and generated a small amount of dimeric forms. In contrast, the wild type receptor expressed predominantly as dimers and generated a small amount of monomers. After reduction (Fig. 2B), a majority of the dimeric ~260- to 270-kDa bands of the wild type receptor were reduced to a broad monomeric ~133-kDa band and a small portion remained as SDS-resistant dimeric aggregates, whereas the dimeric bands generated by C67S, C109S, and C140S mutant receptors remained mostly as SDS-resistant dimeric aggregates. Also, the 140-kDa monomeric band of the C140S mutant showed a small shift of molecular mass to a 133-kDa band like the wild type receptor. Taken together, the results suggest that substituting serine for cysteines 67, 109, and 140 directly or indirectly blocks dimerization.

C → S Mutation of Cys-140 Generates a Monomeric Form of mGluR1 Expressed at the Cell Surface—Because point mutations of three cysteine residues in mGluR1 generated monomeric receptors, we wanted to distinguish between properly processed cell surface versus intracellularly trapped monomeric forms. Cell surface proteins of the HEK-293 cells transiently transfected with wild type, C67S, C109S, and C140S mutant receptors were labeled with membrane impermeant Biotin-7-NHS. The whole cell extracts were immunoprecipitated with anti-mGluR1 monoclonal antibody, ran under non-reduced condition, and analyzed on immunoblots stained either with streptavidin-POD to detect biotinylated cell surface proteins or with anti-mGluR1 polyclonal antibody to detect total mGluR1 immunoreactive species. As seen in Fig. 3A, streptavidin-POD detected only the dimeric ~260- to 270-kDa bands of the wild type mGluR1 and a fainter dimeric band of C67S and C109S mutants but no monomeric 130- to 135-kDa bands of these receptors. In contrast, streptavidin detected both the dimeric 300-kDa and monomeric 140-kDa bands of the C140S mutant receptor. A duplicate blot of the samples with anti-mGluR1 polyclonal antibody detected both the cell surface and the intracellular forms of the wild type and mutant receptors (Fig. 3A, anti-mGluR1 blot). To further determine the biochemical identity of the expressed bands of the C67S, C109S, and C140S mutant receptors, we tested for sensitivity to Endo-H digestion. Whole cell extracts prepared from HEK-293 cells transiently transfected with wild type, C67S, C109S, and C140S cDNAs were digested with Endo-H and analyzed by SDS-PAGE under non-reducing conditions. As seen in Fig. 3B, digestion with Endo-H caused no decrease in the sizes of the dimeric ~260- to 270-kDa bands of the wild type mGluR1, which remained mostly resistant to Endo-H digestion. Whereas, both the lower 130- and 135-kDa bands of the wild type, C67S, and C109S mutant receptors showed sensitivity to Endo-H digestion with downward mobility shifts, indicating that these are intracellularly trapped, high mannose receptor forms. For the C140S mutant, however, both the 300- and 140-kDa bands were largely resistant to Endo-H digestion, and only a small fraction of both bands showed mobility shift after Endo-H digestion as shown by arrows in Fig. 3B (right side). These results suggest that, of the different monomeric forms generated by C67S, C109S, or C140S mutants, only the 140-kDa monomeric form of C140S mutant receptor is expressed at the cell surface.

Mapping a Dimeric Interface Region Encompassing Cys-140—The ability of the monomeric forms of the C140S mutant to express at the cell surface suggested that the Cys-140 residue is sufficient to form an intermolecular disulfide bond critical for dimerization. To further confirm this notion, we introduced ectopic cysteines at different positions on C140S mutant

### Table I

| Transfection | Dimeric bands | Monomeric bands |
|--------------|---------------|----------------|
| Wild type mGluR1 | 1.85 ± 0.04 | 0.15 ± 0.06 |
| C67S | 0.25 ± 0.05 | 1.36 ± 0.04 |
| C109S | 0.20 ± 0.06 | 1.42 ± 0.04 |
| C140S | 0.36 ± 0.04 | 1.30 ± 0.03 |

**Fig. 3. Determination of cell surface expression patterns of the C67S, C109S, and C140S mutant receptors.** A, HEK-293 cells were transfected with WT mGluR1 (WT-R1), C67S, C109S, or C140S cysteine mutants, and cell surface proteins were labeled with Biotin-7-NHS. The whole cell lysates were immunoprecipitated with anti-mGluR1 monoclonal antibody; immunoreactive proteins were eluted with loading sample buffer containing no β-mercaptoethanol and subjected to SDS-PAGE. All mGluR1 forms were detected with anti-mGluR1 polyclonal antibody (blot labeled Anti-mGluR1). Biotinylated forms of WT-R1 and mutant receptors were detected with peroxidase-conjugated streptavidin (blot labeled Strep-POD) in a duplicate blot of the same samples. B, enzymatic deglycosylation studies to determine Endo-H sensitivity of dimeric/monomeric forms of the WT-R1 and mutant receptors. 20 μg of whole cell extract of cells transfected with WT-R1 or mutant mGluR1 cDNAs was incubated without (−) or with (+) Endo-H for 2 h at 37 °C as described under "Materials and Methods." The extracts after digestion were subjected to SDS-PAGE under non-reducing conditions with sample buffer containing no β-mercaptoethanol. After transfer to nitrocellulose membranes, immunoblot was developed with anti-mGluR1 monoclonal antibody. Arrows on the right show the location of Endo-H-sensitive bands. The positions of molecular weight standards are shown on the left side of each blot. The experiments were performed twice with similar results.
Several similar experiments. Molecular weight bands of the C140S mutant. Molecular weight standards are T146C Cys-scanning mutants and the location of the missing 140-kDa location of the rescued dimeric band of S129C, K134C, D143C, and T146C Cys-scanning mutants and the location of the missing 140-kDa monomeric band of the C140S mutant. Molecular weight standards are indicated at the left side of the blot. The blot is a representative of several similar experiments.

Because the monomeric form of the C140S mutant reached the band, probably corresponding to the 130- to 135-kDa unprocessed, cell surface monomers in the presence or absence of reducing agents. This identified the ECD as the locus for covalent-linked dimerization of mGluR1. Within this ECD, the suspected covalent-linked dimerization domain was localized by proteolysis of mGluR5 ECD within the amino-terminal 17 kDa of the ECD (17). Thus, we began to analyze three cysteine mutations within this region of mGluR1. Mutations of the cysteine residues at positions 67, 109, and 140 generated a substantial amount of monomers, making it difficult to confirm whether all these cysteines are directly involved in disulfide-linked dimerization. Further studies showed that monomers generated by mutations of the cysteine residues at positions 67 and 109 lead to primarily intracellularly trapped, incompletely processed receptors, indicating a potential problem with folding, processing, or trafficking of these receptors to the cell surface. In contrary, mutation at Cys-140 generated monomers that were processed normally and expressed at the cell surface. Thus, it is conceivable that mutation at Cys-67 and Cys-109 may affect the conformation of the ECD in a manner that prevents dimerization, whereas it is highly likely that the cell surface-expressed, properly processed (thus properly folded) monomeric receptor forms generated by mutation of Cys-140 is a direct consequence of disruption of an intermolecular disulfide linkage. Surprisingly, all three cysteine mutations also generated some residual dimeric receptor forms along with the monomeric forms. It is unclear how these dimeric forms are generated and expressed at the cell surface, but some of these processes could represent "artifacts" of mutagenesis and/or an overexpression problem in the heterologous cell system. Reduction with β-mercaptoethanol showed that much of these high molecular weight bands do not get reduced like the wild type dimers and mostly remain as SDS-resistant receptor forms, which may have resulted from aggregation of overexpressing proteins. However, we cannot conclude from this study whether mutations at these cysteines fail to completely block dimer formation, either because another cysteine ordinarily uninvolved in intermolecular disulfide linkage forms an "illegiti-

**DISCUSSION**

To confirm observations derived from study of mGluRs (10, 11) that suggested that the dimerization domain is localized to the ECD, we expressed a truncation mutant receptor (RhodC-R1) that contained the seven transmembrane domains and carboxyl-terminal tail of the mGluR1 but lacked the ECD. As expected, the RhodC-R1 mutant receptor expressed as properly processed, cell surface monomers in the presence or absence of reducing agents. This identified the ECD as the locus for covalent-linked dimerization of mGluR1. Each 75-cm² flask of HEK-293 cells was transfected with 8 μg of wild type or 12 μg of C140S plasmid DNA. After 24 h, cells were replated in 24-well plates containing 3.0 μCi/ml [3H]myoinositol for PhI assay as described under "Materials and Methods." Data (mean ± S.E.) are from one of three similar experiments, all of which were performed in triplicate. When not shown, S.E. values fall within the symbols. Results (cpm of labeled total inositol phosphates generated) are expressed as a percentage of the response at 200 μM L-glutamate of the wild type mGluR1.

**FIG. 4. Cysteine-scanning mutagenesis study using S119C, S126C, S129C, K134C, D143C, T146C, and T142C mutants created on C140S mutant background.** Crude membrane preparations (20 μg of each sample per lane) from HEK-293 cells transiently transfected with wild type mGluR1 (WT-R1) or other mutant receptor cDNAs were fractionated by SDS-PAGE under non-reducing conditions. Total mGluR1 immunoreactivity was detected by immunoblotting with anti-mGluR1 monoclonal antibody. Two arrows on the right indicate the location of the rescued dimeric band of S129C, K134C, D143C, and T146C Cys-scanning mutants and the location of the missing 140-kDa monomeric band of the C140S mutant. Molecular weight standards are indicated at the left side of the blot. The blot is a representative of several similar experiments.

**FIG. 5. Concentration dependence for L-glutamate stimulation of PhI hydrolysis in transiently transfected HEK-293 cells expressing wild type (WT) mGluR1 and C140S mutant receptors.** Each 75-cm² flask of HEK-293 cells was transfected with 8 μg of wild type or 12 μg of C140S plasmid DNA. After 24 h, cells were replated in 24-well plates containing 3.0 μCi/ml [3H]myoinositol for PhI assay as described under "Materials and Methods." Data (mean ± S.E.) are from one of three similar experiments, all of which were performed in triplicate. When not shown, S.E. values fall within the symbols. Results (cpm of labeled total inositol phosphates generated) are expressed as a percentage of the response at 200 μM L-glutamate of the wild type mGluR1.
Cysteine Residue Critical for mGluR1 Dimerization

We report that the consensus Cys-140 residue is critical for mGluR1 dimerization. This residue is conserved in all known group 1 mGluRs (1, 2). The Cys-140 residue is critical for correct folding of the C-terminal extracellular domain (ECD) of the mGluR1 receptor. We hypothesize that a lobe-1 intermolecular disulfide bond is formed between the Cys-140 residues of two monomeric mGluR1 receptors. This disulfide bond is critical for the formation of functional dimers of mGluR1.

METHODS

We employed a variety of experimental approaches to study the role of Cys-140 in the formation of mGluR1 dimers. These included mutation of Cys-140 to serine (C140S) and analysis of the effects of this mutation on receptor function.

RESULTS

1. The C140S mutant receptor showed a decrease in the glutamate response compared to the wild type receptor.

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