The 612-residue extracellular domain of the human Ca²⁺ receptor (hCaR) has been speculated to consist of a Venus’s-flytrap domain (VFT) and a cysteine-rich domain. We studied the function of the hCaR Cys-rich domain by using mutagenesis and chimera approaches. A chimeric hCaR with the sequence from residues 540–601 replaced by the corresponding sequence from the Fugu CaR remained fully functional. Another chimeric hCaR with the same region of sequence replaced by the corresponding sequence from metabolotropic glutamate receptor subtype 1 (mGluR1) still was activated by extracellular Ca²⁺ ([Ca²⁺]o), but its function was severely compromised. Chimeric receptors with the hCaR VFT and mGluR1 seven-transmembrane domain plus C-tail domain retained good response to [Ca²⁺]o, whether the Cys-rich domain was from hCaR or from mGluR1. Mutant hCaR with the Cys-rich domain deleted failed to respond to [Ca²⁺]o, although it was expressed at the cell surface and capable of dimerization. Our results indicate that the hCaR Cys-rich domain plays a critical role in signal transmission from VFT to seven-transmembrane domain. This domain tolerates a significant degree of amino acid substitution and may not be directly involved in the binding of [Ca²⁺]o.

The Ca²⁺ receptor (CaR) plays a central role in the regulation of [Ca²⁺]o, homeostasis (for reviews, see Refs. 1 and 2). Activation of CaR by elevated levels of [Ca²⁺]o stimulates phospholipase C via the Gq subfamily of G-proteins, resulting in the increase of both phosphoinositide (PI) hydrolysis and the concentration of cytosolic calcium [Ca²⁺]i. The CaR mediates the inhibitory actions of [Ca²⁺]o on parathyroid hormone secretion by the parathyroid gland and on Ca²⁺ reabsorption by the kidney. It may also be involved in diverse cellular responses to [Ca²⁺]o within microenvironments in other organs such as brain, skin, bone, and intestine (2). The physiological importance of the CaR has been documented by the characterization of gain-of-function and loss-of-function mutations of the CaR resulting in disorders of calcium homeostasis such as autosomal dominant hypocalcemia (3) and familial hypocalciuric hypercalcemia (4).

Homologous CaR genes have been identified from bovine (5), human (6), rat (7), rabbit (8), chicken (9), and most recently puffer fish Fugu rubripes (10) with high amino acid sequence homology. CaR is a member of family 3 of the G-protein-coupled receptor (GPCR) superfamily. Family 3 also includes metabotropic glutamate receptors (11), γ-amino butyric acid type B receptor (GABA_B) (12), putative pheromone receptors (13), and putative taste receptors (14). Their distinctively large extracellular domains (ECDs) have limited but significant homology in amino acid sequences among them and also have limited homology to bacterial periplasmic binding proteins such as the Escherichia coli leucine/isoleucine/valine-binding protein (15–17). Three-dimensional structures of the bacterial periplasmic binding proteins have been determined by x-ray crystallography (15) and show bilobed “Venus’s-flytrap”-like structures. ECDs for metabotropic glutamate receptor subtype 1 (mGluR1), GABA_B1, and human CaR (hCaR) have been modeled as Venus’s-flytrap (VFT) structures (15, 17, 18). It was hypothesized that the closing of the two lobes after binding of ligands triggers the transmission of signal from ECD to the receptor’s cytoplasmic signaling loops. For hCaR, a model of the VFT domain begins at amino acid Gly306 and ends at amino acid Val313 (17) (Fig. 1A). The majority of naturally occurring activating and inactivating mutations identified in patients with autosomal dominant hypocalcemia and familial hypocalciuric hypercalcemia cluster in this region. This region also contains multiple aspartates and glutamates possibly involved in low affinity calcium binding.

Between VFT domain and 7 TM domain, a Cys-rich domain with nine highly conserved cysteines in a closely spaced (about 60-amino acid-long) sequence is present in all of the members of family 3 except GABA_B1 (Fig. 1B). Aside from the nine cysteines, the majority of other residues in this region are not conserved in family 3 members. It is speculated that multiple intramolecular disulfide bonds are formed within this region, which may give rise to a separate and tightly packed domain (Cys-rich domain) in ECD. A recent report (19) showed that truncated forms of mGluR1 receptor containing ECD without the Cys-rich domain did not express well and showed no significant ligand binding, while other truncated receptors containing whole ECD with Cys-rich domain expressed well and bound the ligand. It was speculated that mGluR1 Cys-rich domain might impose a structural constraint on the receptor. Other than this, little is known about the function of this Cys-rich domain.

Previous results from our laboratory showed that substitution of any of the nine cysteines in this domain in hCaR by
serine causes loss of receptor function (20). Mutants failed to express at the cell surface, possibly due to misfolding or incomplete processing of the receptor. C582Y, a naturally occurring inactivating mutation of the hCaR, was identified in a subject with neonatal severe primary hyperparathyroidism (21), indicating the structural importance of one of these nine cysteines. In contrast to VFT domain, very few naturally occurring mutations, only C582Y (21) and G549R (22), have been identified so far in the hCaR Cys-rich domain.

Given the lack of information on the function of the Cys-rich domain and the dearth of naturally occurring mutations in this domain, we set out to address two questions. Is the Cys-rich domain required for activation of hCaR by [Ca2+]o? If required, is the specific hCaR sequence of the Cys-rich domain (other than the nine highly conserved cysteines) critical? To address these questions, we constructed a series of chimeric hCaRs as well as a Cys-rich deletion mutant hCaR and analyzed their expression and function.

MATERIALS AND METHODS

Site-directed Mutagenesis of the hCaR—The hCaR cDNA cloned in the pcR3.1 expression vector (23) and was kindly provided by Dr. Kausik Ray. Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), according to the manufacturer’s instructions. The mutagenic oligonucleotide primer pair for introducing a Phe→Arg mutation in hCaR was 5′-GGGTCTCCAGGGATTGTCCCAGGGCTTGGCAATGCAGGAGGTGTGG-3′ and 5′-GCACTGTCGTGAGGGTTCTCCAGGGAGGTGTGG-3′ and coding sequence for five alanines in the N-terminal region of the receptor. C582Y, a naturally occurring mutation in hCaR was 5′-GGGGCTTTGGCAATGCAGGAGGTGTGG-3′. The primer pair for introducing the Glu602→Ile603→Glu604→Pro→Val→Arg mutation in hCaR was 5′-CCACACTCTCGATTCGAGGCGGATCTCTCAGGTTCTGCTGTGAGGACGAC-3′ and 5′-GCTCTCGAGCAAGACAGAAACCGTGAGGCTCCGTCTGGAAGAGGTGTGG-3′. Parental hCaR DNA in pcR3.1 vector was amplified using Pfu Turbo DNA polymerase with these primers for 16 cycles in a DNA thermal cycler (Perkin-Elmer). After digestion of the parental DNA with DpnI for 1 h, the amplified DNA with incorporated nucleotide substitution was transformed into E. coli (XL-1 blue or DH-5α strain). The mutations were confirmed by automated DNA sequencing using a dRhodamine Terminator Cycle Sequencing Kit and ABI prism-373A DNA sequencing (PE Applied Biosystems, Foster City, CA).

Oligonucleotide Primers Used in Polymerase Chain Reaction Amplification and Linker Adaptor Synthesis—Sense primer 5′-GATCCCGCCGCTCTAAACTCGCCAGAGATTGTTG-3′ and antisense primer 5′-GACGTCGTGACGCCTAGAAGGAAGGTGTTG-3′ were used to amplify Fugu CaR Cys-rich sequence with incorporation of SacII and BsiWI sites at its 5′- and 3′-end, respectively. Sense primer 5′-GATCCCGCCGCTCTAAACTCGCCAGAGATTGTTG-3′ and antisense primer 5′-GACGTCGTGACGCCTAGAAGGAAGGTGTTG-3′ were used to amplify mGlur1 Cys-rich sequence with incorporation of SacII and BsiWI sites at its 5′- and 3′-end, respectively. mGlur1 CDNA cloned in pcDNA1 expression vector was kindly provided by Dr. Robert Wenthold. Sense primer 5′-GACGTCGTGACGCCTAGAAGGAAGGTGTTG-3′ and antisense primer 5′-GATCTCGAGTACATTTACAAGGGTGAGGTGTTG-3′ were used to amplify mGlur1 7TM plus C-tail with incorporation of BsiWI and XhoI sites at its 5′- and 3′-end, respectively. Primer pairs 5′-GATCCCGCCGCTCTAAACTCGCCAGAGATTGTTG-3′ and 5′-GACGTCGTGACGCCTAGAAGGAAGGTGTTG-3′ were annealed to form a linker adaptor with SacII and BsiWI sites at its 5′- and 3′-end, respectively, and coding sequence for five alanines in between. All of the polymerase chain reaction products and the synthetic fragment were digested by unique restriction enzymes and subsequently cloned into hCaR receptor at matching sites, as explained under “Results.”

Transient Transfection of Wild Type and Mutant Receptors in HEK-293 Cells—In our previous study, we used 13 μM of plasmid DNA for single plasmid transfection or 6.5 μg of DNA of each of two different plasmids in co-transfection in each 75-cm2 flask of HEK-293 cells. DNA was diluted in serum-free DMEM (BioFluids Inc., Rockville, MD) and mixed with diluted Lipofectamine (Life Technologies, Inc.), and the mixture was incubated at room temperature for 30 min. The DNA-Lipofectamine complex was further diluted in 6 ml of serum-free DMEM and was added to 100 μl of confluently HEK-293 cells plated in 75-cm2 flasks. After 6 h of incubation, 15 ml of complete DMEM containing 10% fetal bovine serum (BioFluids) was added. 24 h after transfection, transfected cells were split and cultured in complete DMEM.

Bioinjection of Cell Surface CaR—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). Briefly, adherent cells were washed once with ice-cold phosphate-buffered saline and treated with 50 μg/ml Biotin-7-NHS in vitamin buffer (50 mM sodium borate, 150 mM NaCl) for 15 min at room temperature to biotinylate cell surface proteins. The reaction was stopped by adding 50 mM NH4Cl and incubated for 15 min on ice. The cells were washed twice with ice-cold phosphate-buffered saline and solubilized with 1 ml of lysis buffer per well containing 1% Triton X-100, 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, freshly added 50 mM iodoacetamide (Sigma), and freshly added protease inhibitor mixture (Roche Molecular Biochemicals).

Immuno precipitation of Wild Type, Mutant, and Chimeric hCaR Receptors—600 μg of total protein of the whole cell lysate was incubated with either 5 μg of 7F8 mouse monoclonal hCaR-specific antibody (raised against the purified hCaR ECD) or 5 μg of affinity-purified rabbit polyclonal hCaR-specific antibody GGD (raised against a synthetic peptide corresponding to amino acids 1037–1050 of the hCaR) for 1–2 h at 4 °C. Subsequently, 25 μl of protein A/G-agarose (for 7F8) or protein A-agarose (for GGD) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the incubation was continued for an additional 1 h. The precipitates were washed three times with lysis buffer containing 0.1% SDS, and the immunoreactive proteins bound on agarose were eluted in 100 μl of 1× sample buffer containing either no β-mercaptoethanol or 300 mM β-mercaptoethanol at room temperature for 5 min. 50 μl of sample was loaded per lane, and immunoblotting was performed as described below.

Immunoblotting Analysis with Detergent-solubilized Whole Cell Extracts—Confluent cells in 75-cm2 flasks or six-well plates were rinsed with ice-cold phosphate-buffered saline and scraped on ice in lysis buffer containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100, freshly added 50 mM iodoacetamide (Sigma), and freshly added protease inhibitors mixture (Roche Molecular Biochemicals). The protein content in each sample was determined by the modified Bradford method (Bio-Rad), and protein samples were separated by SDS-PAGE gel. The proteins on the gel were electrotransferred onto nitrocellulose membrane and incubated with 0.1 μg/ml of protein A-purified mouse monoclonal anti-hCaR antibody (raised against a synthetic peptide corresponding to residues 214–235 of hCaR protein). Subsequently, the membrane was incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:5000. The hCaR protein was detected with an ECL system (Amerham Pharmacia Biotech). Biotinylated proteins were detected using peroxidase-conjugated streptavidin (Roche Molecular Biochemicals) followed by visualization of the biotinylated bands using the same ECL kit from Amerham Pharmacia Biotech. Densitometry of the Western blot was performed by using the NIH Image software (National Institutes of Health Corp.) and results were expressed as ratio of mutant to wild-type (WT) hCaR. Note that only the density of upper band of CaR immunoreceptors on the blot was analyzed, since this was previously shown to correspond to cell surface expression (23).

PI Hydrolysis Assay—The PI hydrolysis assay has been described previously (23). Briefly, 24 h after transfection, transfected cells from a confluent 75-cm2 flask were split. Typically, one-eighth of cells were plated in a six-well plate, and whole confluent plates were prepared 48 h post-transfection for Western blot assay. Remaining cells were placed in two 12-well plates in complete DMEM containing 3.0 μM of [3H]myoinositol (NEN Life Science Products) and cultured for another 24 h. Culture medium was replaced by 1× PI buffer (120 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl2, 20 mM LiCl in 25 mM Pipes buffer, pH 7.2) and incubated for 1 h at 37 °C. After removal of PI buffer, cells were incubated for an additional 1 h with different concentrations of Ca2+ in PI buffer. The reactions were terminated by the 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, and radioactivity for each sample was counted with liquid scintillator counter. Graphs of concentration dependence for stimulation of phosphoinositide hydrolysis by [Ca2+]o, for each transfection were drawn by using GraphPad Prism version 2.0 software. Each value on a curve is the mean of duplicate determinations. Graphs shown in this paper were representative ones from at least three independent experiments.
RESULTS

Assay of Chimeric hCaRs with Cys-rich Region Replaced by Corresponding Fugu CaR and mGluR1 Cys-rich Sequence—

The Cys-rich sequence alignment (Fig. 1B) shows that although the overall amino acid sequence homology among members in family 3 of GPCRs is low, all nine cysteines in the Cys-rich region are strictly conserved in the hCaR, Fugu CaR, mGluR1, putative pheromone receptors, and putative taste receptors but not in GABA_R1, which completely lacks these cysteines. To test if the hCaR Cys-rich domain is tolerant of amino acid changes in residues other than the conserved cysteines, we constructed hCaR/Fugu CaR and hCaR/mGluR1 Cys-rich domain chimeras, taking advantage of the fact that all nine cysteines in Cys-rich domain are strictly conserved among hCaR, Fugu CaR, and mGluR1, while multiple noncysteine amino acid differences exist in this domain.

To facilitate making the chimeras, we introduced unique restriction sites for SacII and BsiWI to flank the nine cysteines in hCaR. Site-directed mutagenesis was applied to mutate Phe_539 to arginine with incorporation of a SacII restriction site, which was followed by a second round of mutation changing amino acids Glu_602-Ile_603-Glu_604 to BsiWI site, which gave rise to a mutant hCaR termed hCaR/SB. Note that in addition to the incorporated new restriction sites, the amino acid substitutions in hCaR/SB change hCaR residues Phe_539 and Glu_602-Ile_603-Glu_604 to the corresponding residues of mGluR1. We transfected hCaR/SB DNA into HEK-293 cells and analyzed mutant and wild type receptor expression on immunoblots stained with anti-hCaR monoclonal antibody ADD to detect total hCaR immunoreactive species. Fig. 2 shows, under reducing conditions, ADD antibody detected two major bands in size of about 130 and 150 kDa for both WT hCaR and hCaR/SB at comparable expression levels. Previous studies have shown that the monomeric 150-kDa band represents hCaR forms expressed at the cell surface and modified with N-linked, complex carbohydrates; the 130-kDa band represents high mannose-modified forms, trapped intracellularly and sensitive to Endo-H digestion (23–25). We tested whether the mutant receptor is capable of signal transduction by using the intact cell [Ca^{2+}]_{o}-stimulated PI hydrolysis assay. Fig. 2 shows that hCaR/SB's maximal responses to [Ca^{2+}]_{o} and EC_{50} were identical to those of WT hCaR, indicating that the four amino acid changes did not change the receptor's response to [Ca^{2+}]_{o}.

We amplified by polymerase chain reaction the corresponding Fugu CaR and mGluR1 Cys-rich sequence using primers with the same mutations for SacII site and BsiWI site at their 5'- and 3'-end, respectively, and cloned into hCaR/SB to replace the hCaR Cys-rich sequence. In the hCaR/Fugu Cys-rich chimera (Ca-Fugu-Ca; note that in this and other chimeras we designate the source of the VFT/Cys-rich/7 TM plus C-tail domains), 20 of 53 non-cysteine amino acids were substituted in the Cys-rich domain (Fig. 1B). Subsequent transient expression and PI hydrolysis assay showed that the chimera was well expressed in HEK-293 cells and was fully functional if compared with WT hCaR (Fig. 3).
In hCaR/mGluR1 Cys-rich chimera (Ca-Glu-Ca), 40 of 53 non-cysteine amino acids were substituted in the same region (Fig. 1B). Transient expression in HEK-293 cells shows this chimera’s mobility was altered on a SDS-PAGE gel, and it was expressed at a lower level than that of WT hCaR when an equal amount of cDNAs was used for transfection (Fig. 4; also see Figs. 5 and 8). Densitometry analysis of blots in Figs. 4, 5, and 8 showed the protein expression ratios of Ca-Glu-Ca/WT hCaR were 0.80, 0.71, and 0.52). PI assay shows that the chimera’s function was severely compromised, but it was still partially functional at high concentrations of $[\text{Ca}^{2+}]_o$. Its EC$_{50}$ was significantly right-shifted from 4 mM to more than 10 mM. As an indirect test of the folding and function of VFT domain and 7 TM domain of this chimera, we superimposed L125P (a naturally occurring gain of function mutation in the VFT domain of the hCaR) (26) and F788C (a naturally occurring gain of function mutation in the fifth transmembrane domain of the hCaR) (27) mutations individually onto this chimera and found that both these activation mutations significantly left-shifted Ca-Glu-Ca’s $[\text{Ca}^{2+}]_o$ response curve (Fig. 4). Thus, some of the 24 amino acids that are conserved between hCaR and Fugu CaR but changed in mGluR1 appear to be critical for the normal function of the hCaR Cys-rich domain. Inspection of this sequence showed that among those 24 amino acids, there is one postulated glycosylation site (Asn$^{541}$) (25) and two acidic residues (Glu$^{566}$ and Asp$^{587}$) that could be involved in low affinity calcium binding. We individually changed back the glycosylation site and two acidic residues by site-directed mutagenesis but found that none of these changes were able to restore the chimera’s response to $[\text{Ca}^{2+}]_o$ toward WT hCaR (data not shown).

Assay of Chimeras with hCaR Cys-rich and mGluR1 Cys-rich in the Context of hCaR Venus’s-Flytrap and mGluR1 Transmembrane Domain—Since WT mGluR1 and Ca-Fugu-Ca function well in transfected HEK-293 cells but Ca-Glu-Ca functioned poorly, it is likely that the mGluR1 Cys-rich domain has some important sequence differences from those of hCaR and Fugu CaR, which make it unable to interact well enough with the hCaR’s VFT domain and/or 7 TM domain to reach optimal response to $[\text{Ca}^{2+}]_o$. To compare the cooperation among the three components: VFT, Cys-rich region, and 7 TM, we constructed two more chimeras, by use of the hCaR/SB plasmid and the same polymerase chain reaction approach. In one chimera, the entire hCaR ECD was linked to mGluR1 7 TM at the junction of the BsiWI site in hCaR/SB (termed Ca-Ca-Glu), and in the other, the hCaR VFT domain was linked to mGluR1 Cys-rich domain and 7 TM at the junction of the SacII site in hCaR/SB (termed Ca-Glu-Glu). Transient expression assay shows both chimeric receptors were well expressed with altered mobility on SDS-PAGE (Fig. 5). The slower mobility of Ca-Ca-Glu and Ca-Glu-Glu compared with WT hCaR appear to be critical for the normal function of the hCaR Cys-rich domain. Inspection of this sequence showed that among those 24 amino acids, there is one postulated glycosylation site (Asn$^{541}$) (25) and two acidic residues (Glu$^{566}$ and Asp$^{587}$) that could be involved in low affinity calcium binding. We individually changed back the glycosylation site and two acidic residues by site-directed mutagenesis but found that none of these changes were able to restore the chimera’s response to $[\text{Ca}^{2+}]_o$ toward WT hCaR (data not shown).

![Fig. 2](image1.png)

**Fig. 2.** Concentration dependence for $[\text{Ca}^{2+}]_o$ stimulation of PI hydrolysis (left) and immunoblot of CaR (right) in transiently transfected HEK-293 cells expressing WT hCaR, vector, and mutant hCaR with SacII and BsiWI unique sites (hCaR/SB). Transfection, PI assay, SDS-PAGE at reducing conditions (with β-mercaptoethanol added in sample buffer), and immunoblot with anti-hCaR monoclonal ADD were performed as described under “Materials and Methods.” Molecular mass standards are indicated at the right of the blots. Left, results of PI assay are expressed as percentage of maximal response (WT hCaR at 8 mM). Right, immunoblot of whole cell lysate of transfected HEK-293 cells. The immunoblots shown here and in Figs. 3–7 were done using cells from the same transfection as the cells used for the PI hydrolysis assay.

![Fig. 3](image2.png)

**Fig. 3.** Concentration dependence for $[\text{Ca}^{2+}]_o$ stimulation of PI hydrolysis (left) and immunoblot of CaR (right) in transiently transfected HEK-293 cells expressing WT hCaR and chimeric hCaR with Cys-rich sequence replaced by corresponding Fugu CaR Cys-rich sequence (Ca-Fugu-Ca). Methods and format for presentation of results are as in the legend to Fig. 2.
when it is linked to the mGluR1 Cys-rich domain together with mGluR1 7 TM. The lower response to \([\text{Ca}^{2+}]_{o}\) by the Ca-Glu-Ca chimera could be accounted for by its lower expression levels if compared with WT hCaR, Ca-Ca-Glu, and Ca-Glu-Glu. To adjust the expression levels, we adjusted the amount of DNAs used in transfection of HEK-293 cells so that Ca-Glu-Ca was expressed at comparable levels with WT hCaR and other chimeras. Fig. 6 shows that the maximal response of Ca-Glu-Ca increases toward WT hCaR when the protein expression ratio of Ca-Glu-Ca/WT hCaR is 1.09 as determined by densitometry; however, its EC_{50} value was still right-shifted (>10 mM). Hence, the functional difference between Ca-Glu-Ca and other chimeras was not simply due to different protein expression levels.

**Construction and Functional Study of hCaR Cys-rich Deletion Mutant**—In light of the fact that GABA-B1 receptor, a member of family 3 of GPCRs, lacks a Cys-rich region, we constructed a hCaR Cys-rich deletion mutant and studied its function. We cloned a synthetic adapter, which consists of SacII and BsiWI sites at its 5' - and 3'-end, respectively, and five alanines in between, into hCaR/SB SacII and BsiWI sites so as to remove the Cys-rich region from hCaR. Transient expression assay showed this Cys-rich deletion mutant was expressed at a lower level than WT hCaR and migrated faster on SDS-PAGE due to the decrease in protein size (Fig. 7). PI assay showed this Cys-rich deletion mutant receptor has a correctly folded 7 TM domain and is able to express at the cell surface.

To document cell surface expression of this Cys-rich deletion mutant hCaR, we labeled the transfected HEK-293 cells with membrane-impermeant 6-biotinyl-\(\varepsilon\)-aminocaproic acid-N-hydroxysuccinimide ester prior to lysing the cells. The cell lysate was immunoprecipitated with receptor-specific 7F8 monoclonal antibody and eluted with gel loading sample buffer containing \(\beta\)-mercaptoethanol as reducing agent. Immunoprecipitates were run on SDS-PAGE and analyzed on immunoblots stained with streptavidin-peroxidase to detect biotinylated cell surface proteins. Fig. 8A shows that Cys-rich deletion mutant was expressed at the cell surface.

Immunoblot with monoclonal antibody ADD in nonreducing conditions showed that the Cys-rich deletion mutant formed homodimers (Fig. 8B). To assess further the ability of the Cys-rich deletion mutant to form dimers, the mutant and TM1 mutant hCaR were coexpressed in HEK-293 cells, and coinmunoprecipitation was performed with GGD antibody, which was raised against a synthetic peptide corresponding to amino acids 1037–1050 near the C terminus of the hCaR. TM1, which has intact ECD and first transmembrane domain of hCaR but is truncated at lysine 644 in the first intracellular loop, cannot be recognized by GGD, while it can be recognized by ADD, whose epitope is within ECD. Fig. 8B shows that Cys-rich deletion mutant retained the ability to form heterodimer with TM1, indicating that its dimerization interface in the VFT domain remained intact.

**DISCUSSION**

Each member of family 3 of the GPCRs has a distinctively large ECD, a part of which has a sequence homologous to bacterial periplasmic binding proteins (15). VFT models based
on the crystal structure of E. coli leucine/isoleucine/valine binding protein have been postulated for this part of the structure in the ECDs of hCaR, mGluR1, and GABAB1. Naturally occurring mutations identified in subjects with autosomal dominant hypocalcemia and familial hypocalciuric hypercalcemia cluster in the VFT, suggesting that VFT function is very sensitive to amino acid changes. It is noteworthy that all members of this family except GABAB1 have an additional Cys-rich domain with nine highly conserved cysteines. Other proteins having multiple cysteines in a short region feature multiple intramolecular disulfide bonds giving rise to a separate and compact domain (29, 30). At this point, little is known about the structure and function of the Cys-rich domain in family 3 members. Our previous study by site-directed mutagenesis showed that each of the nine cysteines in hCaR Cys-rich domain is critical for the receptor’s function. Meanwhile, only two naturally occurring mutations have been identified so far in this domain. To gain further insight into the function and sequence specificity of the Cys-rich domain, we constructed and studied a series of mutants and chimeras.

Our results show that the hCaR Cys-rich domain is tolerant of multiple amino acid changes. Replacing 20 of 53 non-cysteine amino acids in hCaR Cys-rich domain by the corresponding Fugu CaR sequence (Ca-Fugu-Ca) did not affect the receptor’s function. However, more amino acid substitution, such as replacing 40 of 53 non-cysteine amino acids in hCaR Cys-rich domain by the corresponding mGluR1 sequence (Ca-Glu-Ca), impaired the receptor’s function, indicating that the function of the Cys-rich domain does have some sequence-specific requirements. The comparison of function of Ca-Glu-Ca with the Ca-Ca-Glu and Ca-Glu-Glu chimeras further confirmed this. Both Ca-Ca-Glu and Ca-Glu-Glu showed a Ca2+ response with EC50 of about 4 mM, indicating that chimeric receptors with a Cys-rich domain from the same receptor as either the VFT or 7 TM have much better function than a chimera with a Cys-rich domain different from both VFT and 7 TM (Ca-Glu-Ca). Among the three domains (VFT, Cys-rich, and 7 TM), the Cys-rich domain may need at least one of the other domains to be from the same receptor in order to retain optimal function. Additional chimeras involving other family 3 members need to be tested to see if this is common among all members of this family.

Ca-Glu-Ca, a chimera with the entire hCaR ECD linked to the mGluR1 7 TM, retains responsiveness to [Ca2+]0, confirming a recent report that a hCaR/mGluR1 7 TM chimera with the junction site different from our chimera was able to respond to [Ca2+]0 when expressed in injected Xenopus oocytes (31). This was taken as evidence that hCaR ligand specificity resides in the ECD and not the 7 TM domain. Our observation that Ca-Glu-Glu, a chimera with the mGluR1 VFT domain replaced by the hCaR VFT domain, functions comparably with Ca-Ca-Glu, strongly suggests that the hCaR Cys-rich domain is not directly involved in [Ca2+]0 binding, leaving the VFT portion of the ECD as the likely site of [Ca2+]0 binding.

Our results with the Cys-rich domain deletion mutant show that the Cys-rich domain is not essential for at least some degree of cell surface expression and dimerization of the hCaR. For the mGluR1, the Cys-rich domain may play a role in the correct folding of the ECD. Okamoto et al. (19) reported that truncated forms of mGluR1 receptor containing ECD without Cys-rich domain did not express well and showed no significant ligand binding, while other truncated receptors containing the
whole ECD with Cys-rich domain expressed well and bound ligand. They speculated that mGluR1 Cys-rich domain might impose a structural constraint on the receptor. Our cell surface expression assay shows that 7F8 monoclonal antibody, which was raised against purified hCaR ECD and recognizes native hCaR but not denatured hCaR, efficiently bound the mutant with deletion of the Cys-rich domain (Ca-/Ca) expressed at the cell surface. Our dimerization assay also shows that the Ca-/Ca VFT is capable of heterodimerizing with the intact ECD of the TM1 mutant. These results suggest indirectly that the Cys-rich domain is not absolutely required for folding of the VFT domain and 7 TM domain of the hCaR, but the overall level of expression of the Ca-/Ca mutant was reduced, suggesting that the Cys-rich domain is required for optimal hCaR expression.

While the Cys-rich domain may not be critical for hCaR cell surface expression, the presence of this region is critical for normal receptor function. Deletion of the Cys-rich domain (Ca-/Ca) abolished the receptor's response to [Ca^{2+}]), although the deletion mutant showed detectable cell-surface expression and dimerization. Superimposing a constitutively activating mutation, A843E, onto the seventh transmembrane domain allowed the Ca-/Ca deletion mutant to stimulate PI hydrolysis, indicating the ability of the mutant's 7 TM plus C-tail to activate G protein. In contrast, superimposing L125P (a left-shifting mutation speculated to enhance the binding of [Ca^{2+}]) to the VFT domain) failed to restore the function of Ca-/Ca’s, indicating that the Cys-rich domain plays a pivotal role in signal transmission from VFT domain to 7 TM domain. On the other hand, the Ca^{2+} response of the Ca-Glu-Ca chimera was significantly activated by either L125P or F788C (a left-shifting mutation in the 7 TM domain). The contrasting effects of L125P in the Ca-Glu-Ca chimera (activating) versus in the Cys-rich deletion mutant (no effect) show that even a functionally impaired Cys-rich domain is capable of transmitting signals from a mutationally activated VFT to 7 TM domain.

One question that emerges from our results is: how does the GABA_{B1}R1, another family 3 GPCR, function without a Cys-rich domain? While we have no clear answer to this question, we note that GABA_{B1}R1 has other structural features that distinguish it from other members of the family. Unlike hCaR or mGluR1, which have been characterized to form intermolecular disulfide linked homodimers involving the ECD, GABA_{B1}R1 forms heterodimers with GABA_{B2}R2 involving coiled-coil interaction in the C-tail (32, 33). The mechanism for signal transfer from VFT to 7 TM in GABA_{B1}R1 probably differs from that in other members of family 3.

In summary, our results provide clear evidence for an important role of the Cys-rich domain in signal transfer from VFT to 7 TM of the hCaR and for sequence specificity in communication between the VFT, Cys-rich, and 7 TM domains among family 3 members of GPCRs. Further work is now needed to define the structure (e.g., intramolecular disulfides) of this domain and to delineate the specific residues that confer specificity in communicating with corresponding VFT and 7 TM domains.

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