Identification of the Cysteine Residues in the Amino-terminal Extracellular Domain of the Human Ca\(^{2+}\) Receptor Critical for Dimerization

IMPLICATIONS FOR FUNCTION OF MONOMERIC Ca\(^{2+}\) RECEPTOR*

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We analyzed the effect of substituting serine for each of the 19 cysteine residues within the amino-terminal extracellular domain of the human Ca\(^{2+}\) receptor on cell surface expression and receptor dimerization. C129S, C131S, C437S, C449S, and C482S were similar to wild type receptor; the other 14 cysteine to serine mutants were retained intracellularly. Four of these, C60S, C1018S, C3588S and C3958S, were unable to dimerize. A C129S/C131S double mutant failed to dimerize but was unique in that the monomeric form expressed at the cell surface. Substitution of a cysteine for serine 132 within the C129S/C131S mutant restored receptor dimerization. Mutation of residues Cys-129, Cys-131, and Ser-132, singly and in various combinations caused a left shift in Ca\(^{2+}\) response compared with wild type receptor. These results identify cysteines 129 and 131 as critical in formation of intermolecular disulfide bond(s) responsible for receptor dimerization. In a “venus flytrap” model of the receptor extracellular domain, Cys-129 and Cys-131 are located within a region protruding from one lobe of the flytrap. We suggest that this region represents a dimer interface for the receptor and that mutation of residues within the interface causes important changes in Ca\(^{2+}\) response of the receptor.

The Ca\(^{2+}\) receptor (CaR)\(^1\) regulates extracellular calcium ion ([Ca\(^{2+}\)]\(_o\)) homeostasis by controlling the rate of parathyroid hormone secretion from the parathyroid gland and the rate of calcium reabsorption by the kidney (1). [Ca\(^{2+}\)]\(_o\), activates the CaR, leading to activation of phospholipase C\(\beta\) via the G\(_q\) subfamily of G-proteins; this increases phosphoinositide (PI) hydrolysis and causes release of Ca\(^{2+}\) from intracellular stores (2). Recent evidence suggests that the CaR is also involved in diverse cellular responses to extracellular Ca\(^{2+}\) within microenvironments in other organs such as brain, skin, bone, and intestine (3).

The CaR is a member of the superfamily of G-protein-coupled receptors (GPCR) and belongs to the subfamily (family 3 (4)) that includes metabotropic glutamate receptors (mGluR) (5), putative pheromone receptors in the vomeronasal organ (VNR) (6–8), putative taste receptors (TR) (9), and GABA\(_B\) receptors (10). Family 3 GPCR are characterized by a large (>600 residues in the CaR) extracellular amino-terminal domain (ECD) thought to be structurally related to the bilobed (“venus flytrap”) structure of bacterial periplasmic binding proteins (10–12).

Recently, it has been shown that both the mGluRs (13–15) and the CaR (12, 16–19) are expressed at the cell surface as intermolecular disulfide-linked dimers. For mGluR1 (14), mGluR4 (15), and the CaR (12), 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. However, the cysteine(s) forming intermolecular disulfide bond(s) in the CaR or mGluR ECD have not yet been identified. Proteolysis of the mGluR5 receptor localized cysteine(s) critical for dimer formation to the first 17 kDa of the ECD (13). This region contains three cysteines conserved in all mGluRs and in the CaR. The human CaR (hCaR) ECD contains a total of 19 cysteines (20) all of which are highly conserved in bovine (2), rat (21, 22), and rabbit (23) CaRs, and all but cysteine 482 (hCaR sequence numbering) are conserved in the chicken CaR (24). We showed previously that individual cysteine → serine mutations of 14 of these 19 cysteines (all but cysteines 129, 131, 437, 449, and 482) abolish or drastically reduce receptor cell surface expression and/or function, likely by causing misfolding and improper processing of the receptor (18). In the present study, we performed a detailed analysis of ECD cysteine responsible for dimer formation. We found that mutation of both cysteines 129 and 131, but not mutation of either alone, blocks dimer formation. Unlike other ECD cysteine mutations, however, mutation of both cysteine 129 and 131 results in a monomeric form of the hCaR expressed at the cell surface and with unique functional properties.

**MATERIALS AND METHODS**

Site-directed Mutagenesis of the hCaR—Site-directed mutagenesis was performed on hCaR cDNA in the pCR3.1 vector using a commercial kit (QuickChange\textsuperscript{TM} site-directed mutagenesis kit, Stratagene Inc., La Jolla, CA). The hCaR cDNA is a secreted protein, exists as a disulfide-linked dimer, suggesting that one or more cysteines in the ECD is involved in receptor dimer formation. However, the cysteine(s) forming intermolecular disulfide bond(s) in the CaR or mGluR ECD have not yet been identified. Proteolysis of the mGluR5 receptor localized cysteine(s) critical for dimer formation to the first 17 kDa of the ECD (13). This region contains three cysteines conserved in all mGluRs and in the CaR. The human CaR (hCaR) ECD contains a total of 19 cysteines (20) all of which are highly conserved in bovine (2), rat (21, 22), and rabbit (23) CaRs, and all but cysteine 482 (hCaR sequence numbering) are conserved in the chicken CaR (24). We showed previously that individual cysteine → serine mutations of 14 of these 19 cysteines (all but cysteines 129, 131, 437, 449, and 482) abolish or drastically reduce receptor cell surface expression and/or function, likely by causing misfolding and improper processing of the receptor (18). In the present study, we performed a detailed analysis of ECD cysteine responsible for dimer formation. We found that mutation of both cysteines 129 and 131, but not mutation of either alone, blocks dimer formation. Unlike other ECD cysteine mutations, however, mutation of both cysteine 129 and 131 results in a monomeric form of the hCaR expressed at the cell surface and with unique functional properties.

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Jolla, CA), according to the manufacturer's instructions. Briefly, a pair of complementary primers with 25–35 bases was designed for each mutagenesis, and the mutation to change cysteine to serine or alanine was placed in the middle of the primers. Parental hCaR inserted in pCR3.1 was amplified using Pyrococcus furiosus DNA polymerase with these primers and cloned into a T7 promoter vector in a DNA cloning kit in competent DH-5α E. coli (Stratagene, CA). After digestion of the parental DNA with DpnI, the amplified DNA with the nucleotide substitution incorporated was transformed into Escherichia coli (DH-5α strain). The mutations were confirmed by automated DNA sequencing using a Taq DyeDeoxy Terminator Cycle Sequencing kit and ABI prism-377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). In the 19 single cysteine to serine substitutions used in this study were described earlier (18). The C129S and S132C mutants and the Cys→ Ala mutants including C60A, C101A, C358A, and C395A were newly generated. The C129S/C131S and C129A/C131A double mutants and the C129S/C131S/S132C triple mutant were created by changing cysteine at a given site to the desired amino acid and using this mutant DNA as template in the next round of mutagenesis. A truncation mutant containing the ECD and the first transmembrane domain (henceforth termed TM1) was generated by introducing a stop codon in the first intracellular domain of the WT hCaR clone at amino acid position lysine 644. For all the newly generated mutants, we confirmed that two independent clones of the same mutant receptor cDNA showed identical properties.

Treatment of Wild Type and Mutant Receptors in HEK-293 Cells—For transfection, a given amount of the plasmid DNA was diluted in Dulbecco's modified Eagle's medium (DMEM) (BioFluids Inc., Rockville, MD), mixed with diluted LipofectAMINE, and the mixture was incubated at room temperature for 30 min. The DNA-LipofectAMINE complex was further diluted in serum-free DMEM, and 8–15 μg of DNA was added to 80–90% confluent HEK-293 cells plated in 75-cm² flasks. For 6-well plates, transfections were performed using 2 μg of DNA for each well for single cysteine to serine substitutions. After incubation, the intact cells were incubated and washed in PBS to prevent nonspecific disulfide bond formation during protein extraction. The cells were then lysed with ice-cold phosphate-buffered saline (PBS) and treated with 50 μg/ml Biotin-7-NHS in biotinylation 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 for 15 min on ice. The cells were washed twice with ice-cold PBS and solubilized with 1 ml of buffer B per well containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA with freshly added protease inhibitor mixture.

Immunoprecipitation of hCaR Receptors—300 μl (approximately 600 μg of total protein) of the whole cell lysate prepared by scraping cells from 6-well plates in buffer B as described above was further diluted with 300 μl of buffer B and incubated with either 5 μl of 7F8 mouse monoclonal hCaR-specific antibody (made against the purified hCaR ECD (12); 1 mg/ml stock) or 7 μl of affinity purified rabbit polyclonal hCaR-specific antibody GGD (made against a synthetic peptide corresponding to amino acids 1037–1050 of the hCaR protein; 1 mg/ml stock) for 1–2 h at 4 °C. Subsequently, 25 μl of protein A/G (for 7F8) or protein A (for GGD)-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the incubation was continued for an additional 1–2 h. The protein A/G- or A-agarose was washed three times with buffer B containing 0.5% SDS, and the immunoreactive proteins were eluted in 120 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 Analyses with Detergent-solubilized Whole Cell Extracts—Confluent cells in 75-cm² or 6-well plates were rinsed with ice-cold PBS and scraped on ice 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 freshly added protease inhibitors mixture. The protein content of each sample was determined by the modified Bradford method (Bio-Rad), and 40–60 μg of protein per lane was separated on 5% SDS-PAGE. The proteins on the gel were electrotransferred to nitrocellulose membrane and incubated with 0.1 μg/ml protein A-purified mouse monoclonal anti-hCaR antibody ADD (raised against a synthetic peptide corresponding to residues 214–235 of hCaR protein (25)). 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 1:10,000. The hCaR protein was detected with an enhanced chemiluminescence system (Amersham Pharmacia Biotech Corp.). Biotinylated proteins were detected using peroxidase-conjugated streptavidin followed by visualization of the biotinylated bands using BM chemiluminescence kit (Roche Molecular Biochemicals).

Results

RESULTS

Intermembrane Disulfide Bridge Involves ECD of the hCaR—To determine the role of the ECD in dimer formation of the membrane-bound hCaR, a mutant construct (TM1) containing the whole ECD and first transmembrane domain of hCaR and truncated at lysine 644 in the first intracellular loop was prepared and transiently expressed in HEK-293 cells. Cell surface proteins were labeled with membrane-impermeant Biotin-7-NHS prior to lysing the cells as described before (33). To prevent nonspecific disulfide bond formation during protein extraction, the intact cells were incubated and washed in PBS containing 50 mM iodoacetamide, and 10 mM iodoacetamide was included in the lysis buffer. Both the wild type hCaR and TM1 were then immunoprecipitated with receptor-specific 7F8 monoclonal antibody and eluted with gel loading sample buffer either containing β-mercaptoethanol as reducing agent or with no β-mercaptoethanol. Immunoprecipitates were run on SDS-PAGE gel and analyzed on immunoblots stained either with streptavidin to detect biotinylated cell surface proteins or with anti-hCaR monoclonal antibody ADD to detect total hCaR immunoreactive species. As shown in Fig. 1 (ADD blot, 1st lane), under nonreducing conditions, ADD antibody detected two major dimeric bands of hCaR—260–300 kDa in size; ~150- and 130-kDa monomeric forms appeared only after reducing the samples (ADD blot, 3rd lane). Previous studies have shown that the monomeric ~150-
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Fig. 1. Determination of cell surface expression of WT hCaR and TM1 mutant. HEK-293 cells were transfected with either WT hCaR or with TM1 mutant, and cell surface proteins were labeled with Biotin-7-NHS as described under "Materials and Methods." The cell lysate was immunoprecipitated with anti-hCaR 7F8 monoclonal antibody. Immunoreactive proteins eluted with SDS-PAGE loading sample buffer containing either β-mercaptoethanol (reducing, R) or no β-mercaptoethanol (non-reducing, NR) were separated by SDS-PAGE. All forms of the WT and TM1 mutant receptors were detected with anti-hCaR ADD monoclonal antibody (blast labeled ADD). Biotinylated forms of WT and TM1 mutant receptors were detected with peroxidase-conjugated streptavidin (labeled Biotin-Strep) in a duplicate blot of the same samples. The positions of molecular mass standards are indicated on the right.

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 (26, 33, 34). In accord with this, streptavidin identified only the upper 150-kDa monomeric form under reducing conditions; under nonreducing conditions, only the upper dimeric form is stained by streptavidin indicating that only the upper dimeric form is expressed at the cell surface (Fig. 1, Biotin-Strep blot, 1st and 3rd lanes). Similarly, under nonreducing conditions (ADD blot, 2nd lane), ADD antibody identified two TM1 mutant dimeric bands of ~160–180 kDa. These forms were largely reduced to two bands of ~85 and 95 kDa (ADD blot, 4th lane). Streptavidin detected only the upper form of the non-reduced or reduced TM1 bands (Biotin-Strep blot, 2nd and 4th lanes) showing that only the upper form of TM1 is expressed at the cell surface, as with the wild type receptor. These results indicate that TM1 is capable of forming a dimer expressed at the cell surface like the wild type hCaR but lacking the full seven transmembrane domain does not stimulate IP hydrolysis in response to [Ca²⁺]₀ (data not shown). The ability of TM1 to form dimers, together with our previous observation that the secreted, purified ECD of the hCaR is a disulfide-linked dimer (12), indicates that the determinants including the cysteines important for dimer formation are present in the ECD of the hCaR.

Screening of Single Cysteine Mutants for Their Ability to Form Homodimers or Heterodimers with TM1—We previously generated single Cys → Ser mutants of all 19 cysteines in the hCaR ECD (18). We now coexpressed each of these Cys → Ser mutants and the wild type hCaR with the TM1 mutant and tested for their ability to heterodimerize with TM1 in a coinmunoprecipitation assay. A polyclonal antibody "GGD" made against a peptide from the carboxy-terminal tail region of the hCaR is able to immunoprecipitate full-length wild type hCaR and full-length Cys → Ser mutants but not TM1 because TM1 lacks the GGD epitope. After immunoprecipitation with GGD antibody, samples were run on SDS-PAGE under reducing conditions and immunoblotting performed with ADD antibody whose epitope within the ECD is contained in both TM1 and full-length forms of hCaR. GGD antibody fails to immunoprecipitate TM1 when it is transfected by itself, as no immunoreactivity is detected on ADD immunoblots of such immunoprecipitates (data not shown). Fig. 2A shows the results for wild type and seven of these mutants. When coexpressed with wild type hCaR, TM1 immunoreactivity is detected with ADD on blots of the immunoprecipitate. Both the 95- and 85-kDa bands of TM1 were detected under reducing conditions as were the 150- and 130-kDa forms of the wild type hCaR (Fig. 2A, 1st lane). These results indicate that TM1 and wild type hCaR heterodimerize, allowing TM1 to be coprecipitated with wild type hCaR by GGD antibody. We suggest that the upper forms of TM1 and wild type detected on ADD blot reflect heterodimers at the cell surface and the lower forms, heterodimers of the respective incompletely processed, intracellular forms of TM1 and wild type hCaR. Similarly, with C129S and C131S mutants, both 95- and 85-kDa forms of TM1 were coimmunoprecipitated along with 150- and 130-kDa forms of the C129S and C131S mutant receptors. C236S mutant expressed primarily as the 130-kDa form and coimmunoprecipitated mainly with the lower 85-kDa form of TM1. A small amount of the upper 95-kDa TM1 monomeric band was detected corresponding to the faint 150-kDa band detected for C236S (Fig. 2A, 6th lane). In contrast, C60S, C101S, C358S, and C395S failed to immunoprecipitate the 95-kDa form of the TM1 mutant and little if any of the 85-kDa form. Each of these mutants was expressed primarily as the incompletely processed, 130-kDa form.

The Cys → Ser mutants were further analyzed by determining their homodimerization patterns on ADD immunoblots run under nonreducing conditions. Cells were treated with iodoacetamide as described under "Materials and Methods" to prevent aggregates forming secondary to nonspecific disulfide bond formation. Fig. 2B shows that C60S, C101S, C358S, and C395S mutant receptors remained mostly as a 130-kDa monomeric form and showed very little or no dimeric forms. In contrast, wild type hCaR, C129S, and C131S mutant receptors formed two homodimeric bands with little or no monomeric forms visible on immunoblot. C236S mutant receptor showed a strong dimeric band with mobility differing from either wild type hCaR, C129S, or C131S mutant receptors. Taken together, the results suggest that substituting serine (or alanine; data not shown) for cysteines 60, 101, 358, or 395 may directly or indirectly block dimerization, whereas serine substitution for either Cys-129 or Cys-131 has minimal effect on dimer formation. Substitution of serine for cysteine 236 does not block dimer formation, but the conformation of the C236S dimeric forms based on different mobility on SDS-PAGE appear to differ from those of wild type hCaR. Of the other Cys → Ser mutants, C447S, C449S, and C482S showed essentially the same pattern of hetero- and homodimerization as the wild type hCaR. The C542S, C546S, C561S, C562S, C565S, C568S, C582S, C585S, and C595S mutant receptors showed similar heterodimerization patterns with TM1 as the C236S mutant; their homodimerization patterns also resembled C236S with variable degrees of dimer mobility differences from wild type hCaR on non-reducing SDS-PAGE (data not shown).

Cys → Ser Mutation of Both Cys-129 and Cys-131 Blocks Functional Dimer Formation and Generates a Monomeric Form of hCaR Expressed at the Cell Surface—The first 17 kDa of the mGluR5 was shown to be critical for dimer formation (13). The mGluRs all have three conserved cysteines within this region. Two correspond to Cys-60 and Cys-101 of the hCaR, but the hCaR has two cysteines, 129 and 131, in the position corresponding to the third mGluR-conserved cysteine (20). This led us to examine the possibility that the lack of effect of serine substitution for either Cys-129 or Cys-131 on hCaR dimer cell aggregates forming secondary to nonspecific disulfide bond formation. Fig. 2B shows that C60S, C101S, C358S, and C395S mutant receptors remained mostly as a 130-kDa monomeric form and showed very little or no dimeric forms. In contrast, wild type hCaR, C129S, and C131S mutant receptors formed two homodimeric bands with little or no monomeric forms visible on immunoblot. C236S mutant receptor showed a strong dimeric band with mobility differing from either wild type hCaR, C129S, or C131S mutant receptors. Taken together, the results suggest that substituting serine (or alanine; data not shown) for cysteines 60, 101, 358, or 395 may directly or indirectly block dimerization, whereas serine substitution for either Cys-129 or Cys-131 has minimal effect on dimer formation. Substitution of serine for cysteine 236 does not block dimer formation, but the conformation of the C236S dimeric forms based on different mobility on SDS-PAGE appear to differ from those of wild type hCaR. Of the other Cys → Ser mutants, C447S, C449S, and C482S showed essentially the same pattern of hetero- and homodimerization as the wild type hCaR. The C542S, C546S, C561S, C562S, C565S, C568S, C582S, C585S, and C595S mutant receptors showed similar heterodimerization patterns with TM1 as the C236S mutant; their homodimerization patterns also resembled C236S with variable degrees of dimer mobility differences from wild type hCaR on non-reducing SDS-PAGE (data not shown).

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Materials and Methods. Immunoreactive proteins were eluted with SDS-PAGE sample buffer containing anti-hCaR polyclonal antibody GGD performed as described under “Materials and Methods.” Immunoactive proteins were eluted with SDS-PAGE sample buffer containing β-mercaptoethanol (R, reducing) and separated on 5% SDS-PAGE. Immuno blot was developed with anti-hCaR monoclonal antibody ADD. This blot is representative of several similar experiments. B, immunoblot analysis under non-reducing (NR) condition to detect homodimeric expression patterns of the WT, C60S, C101S, C129S, C131S, C236S, C358S, and C395S mutant receptors. Whole cell extracts obtained from HEK-293 cells transiently transfected with wild type hCaR or different cysteine mutants were fractionated on 5% SDS-PAGE under non-reducing condition. Immunoblotting was performed with anti-hCaR monoclonal antibody ADD. Molecular mass standards are indicated at the right of the blots.

formation could be due to the ability of the remaining, nearby cysteine to substitute in a putative intermolecular disulfide bond for that mutated to serine. We therefore created a C129S/C131S double mutant and compared its expression and dimer formation pattern with wild type, C129S, and C131S mutant receptors. As seen in Fig. 3A (ADD blot), under nonreducing conditions, a pair of immunoreactive bands in the >200-kDa size range is detected for the C129S/C131S double mutant as for the wild type and C129S and C131S mutants. The mobility of these bands, however, differs from that of the two major dimeric bands of the wild type hCaR or C129S or C131S mutant receptors. Moreover, whereas wild type hCaR showed no monomeric forms and C129S and C131S showed only a small amount of the intracellular 130-kDa monomeric form under nonreducing conditions, the C129S/C131S double mutant generated significant amounts of both 150- and 130-kDa monomeric forms. Streptavidin blot under nonreducing conditions (Fig. 3A, Biotin-Strep) showed that the wild type hCaR, C129S, and C131S mutants are expressed at the cell surface as the ~300-kDa upper dimer form. In contrast, the C129S/C131S double mutant was expressed at the cell surface only as the monomeric 150-kDa form. Importantly, neither of the apparent dimeric forms detected for the C129S/C131S double mutant with ADD appear to be expressed at the cell surface as judged by lack of streptavidin staining.

This suggested that the dimeric forms detected for the C129S/C131S double mutant could represent an aggregate of improperly processed forms that do not reach the cell surface. To determine further the biochemical identity of the ~300-kDa dimeric band of the C129S/C131S double mutant, we tested for sensitivity to Endo-H digestion to distinguish between the fully processed hCaR forms that are modified with complex carbohydrates (Endo-H-resistant) and high mannose-modified forms (Endo-H-sensitive) that have not trafficked from the endoplasmic reticulum to the Golgi (26, 33, 34). As shown in Fig. 3B, for samples run under nonreducing conditions, digestion with Endo-H caused a decrease in the size of the lower dimeric form of the wild type hCaR, and the upper form remained mostly resistant to Endo-H digestion. For the C129S/C131S double mutant, however, the ~300-kDa dimer and 130-kDa monomeric forms were sensitive to Endo-H digestion, whereas the 150-kDa form was resistant. When these samples are analyzed under reducing conditions, the upper, monomeric 150-kDa band of both the wild type hCaR and C129S/C131S double mutant is Endo-H-resistant, whereas the respective 130-kDa monomeric forms are Endo-H-sensitive. These data strongly suggest that the ~300-kDa band of the C129S/C131S double mutant represents an aggregate that remains intracellularly trapped.

To assess further the ability of the C129S/C131S double mutant to form dimers, the double mutant and TM1 mutant receptors were coexpressed in HEK-293 cells, and immunoprecipitation was performed with GGD antibody as described above. As shown in Fig. 3C, as for wild type hCaR, both the 150- and 130-kDa monomeric forms of the C129S/C131S double mutant were detected by ADD after GGD immunoprecipitation. Unlike for the wild type hCaR, however, the 95-kDa form of TM1 fails to coprecipitate with the double mutant receptor, and only a small amount of the lower monomeric 85-kDa form of TM1 coprecipitates. This result indicates that despite the fact that the monomeric 150-kDa form of the C129S/C131S double mutant is fully processed and resistant to Endo-H digestion (Fig. 3B) and expressed at the cell surface (Fig. 3A), it is incapable of forming a heterodimer with the fully processed 95-kDa form of TM1. The small amount of the 85-kDa intracellular form of TM1 that does coprecipitate with the C129S/C131S double mutant presumably reflects interaction (aggregation?) with the form of the double mutant that remains intracellularly trapped and is seen as an ~300-kDa band under nonreducing conditions. A C129A/C131A double mutant showed the same changes seen with the C129S/C131S double mutant (data not shown) indicating that loss of cysteine rather than specifically serine substitution was responsible for the changes observed.

The inability of the C129S/C131S double mutant to dimerize compared with the relatively unimpaired ability of either C129S or C131S mutants to dimerize suggested that a cysteine residue in either position is sufficient to form an intermolecular disulfide bond critical for dimerization. To explore further the requirements for CaR dimerization, we constructed a triple mutant receptor, C129S/C131S/S132C, in which the serine normally found at amino acid position 132 is changed to a cysteine in the context of the C129S/C131S double mutant. Fig. 4 shows that under nonreducing conditions, ADD antibody detects two
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**Fig. 3.** A, determination of cell surface expression of the C129S/C131S double mutant. HEK-293 cells were transfected with WT hCaR, C129S, or C131S single cysteine mutants or C129S/C131S double cysteine mutant, and cell surface proteins were labeled with Biotin-7-NHS. The cell lysate was immunoprecipitated with anti-hCaR monoclonal antibody 7F8; immunoreactive proteins were eluted with loading sample buffer containing no β-mercaptoethanol (non-reducing, NR) and subjected to SDS-PAGE. All CaR forms were detected with anti-hCaR monoclonal antibody ADD (blot labeled ADD). Biotinylated forms of WT and mutant receptors were detected with peroxidase-conjugated streptavidin (blot labeled Biotin-Strap) in a duplicate blot of the same samples. B, enzymatic deglycosylation studies to determine Endo-H sensitivity of dimeric/monomeric forms of the WT and C129S/C131S double mutant receptors. 40 μg of whole cell extracts of cells transfected with wild type (WT) or C129S/C131S double mutant hCaR cDNAs were incubated without (−) or with (+) Endo-H for 2 h at 37 °C as described under “Materials and Methods.” The extracts after digestion were mixed with sample buffer either containing (R) or not containing (NR) 300 mM β-mercaptoethanol and subjected to 5% SDS-PAGE. After transfer to nitrocellulose membranes immunoblot was developed with monoclonal antibody ADD. C, cotransfection of the C129S/C131S double mutant receptor or WT receptor with TM1 mutant receptor followed by immunoprecipitation to detect heterodimerization. HEK-293 cells were cotransfected with C129S/C131S double mutant or WT hCaR and TM1 mutant. Whole cell extracts were immunoprecipitated with anti-hCaR polyclonal antibody GGD. Immunoreactive proteins were eluted and separated in sample buffer containing 300 mM β-mercaptoethanol (R, reducing) or 5% SDS-PAGE. Immunoblot was detected with anti-hCaR monoclonal antibody ADD. The positions of molecular mass standards are shown on the right side of each figure.

**Fig. 4.** Determination of cell surface expression pattern of the C129S/C131S/S132C triple mutant receptor. HEK-293 cells were transiently transfected with wild type hCaR or C129S/C131S/S132C mutant receptor DNA, and cell surface proteins were biotinylated. Whole cell extracts were immunoprecipitated with monoclonal antibody 7F8 and then fractionated on 5% SDS-PAGE under non-reducing conditions (NR). Total hCaR immunoreactivity was detected by immunoblot with ADD antibody and cell surface-expressed forms by streptavidin blot. Molecular mass standards are indicated at the right of the blots.

major dimeric bands of the C129S/C131S/S132C triple mutant receptor like the wild type hCaR, and both triple mutant receptor and wild type show little or no monomeric forms in contrast to the C129S/C131S double mutant which generates significant amounts of both the 150- and 130-kDa monomeric forms. The streptavidin blot (Fig. 4) also shows that like the wild type hCaR, the upper dimeric form of the C129S/C131S/S132C triple mutant is expressed at the cell surface. This contrasts with the C129S/C131S double mutant which shows the 150-kDa monomer form but not the dimeric form expressed at the cell surface.

**Function of Cysteine Mutants in PI Hydrolysis Assay**—Because the monomeric form of the C129S/C131S double mutant reaches the cell surface, we tested whether the mutant receptor is capable of signal transduction using the intact cell [Ca2+]o-stimulated PI hydrolysis assay. Since we sought to compare the signaling properties of the single cysteine mutants, C129S and C131S, and the C129S/C131S double mutant at similar expression levels as the wild type receptor, we first determined the levels of cell surface expression for each receptor by whole cell enzyme-linked immunosassay after transfecting HEK-293 cells with equal amounts of plasmid DNA. We found that for a given amount of plasmid DNA transfected, the C129S/C131S double mutant showed a reduced cell surface expression compared with wild type hCaR or other mutants used in this study (data not shown). To assess comparable levels of expression, we varied the amount of plasmid DNA used for transfection, and we achieved comparable levels of expression by transfecting HEK-293 cells with 12 μg of C129S/C131S double mutant receptor DNA and 8 μg of wild type hCaR or other mutant hCaR DNA. Intact cell enzyme-linked assay (Fig. 5, lower inset) showed that cell surface expression was comparable for wild type and mutant receptors transfected with these DNA amounts. Streptavidin blot (Fig. 5, upper inset) confirmed that all the mutants expressed at the cell surface as dimers except for the monomeric C129S/C131S double mutant. We then compared the PI hydrolysis response to [Ca2+]o of all the mutant receptors, including an S132C mutant, with the wild type hCaR (Fig. 5). All the mutant receptors showed a significant left shift in dose-dependent [Ca2+]o response compared with the wild type hCaR, but the C129S/C131S double mutant was most significantly left-shifted. EC50 values (mean ± S.E.; n = 3–6) averaged: WT, 3.8 ± 0.2 mM; C129S, 2.1 ± 0.3; C131S, 1.6 ± 0.1;
C129S/C131S, 1.0 ± 0.2; C129S/C131S/S132C, 2.2 ± 0.2; S132C, 1.9 ± 0.1.

**DISCUSSION**

The major reduction in size observed on SDS-PAGE following disulfide reduction for both the CaR and mGluRs suggests that these receptors are dimers linked by one or more intermolecular disulfide bonds (13, 16–18). The cysteine(s) involved in the relevant intermolecular disulfide bond(s) are localized to the ECD of the CaR and mGluRs (12, 14, 15), and in mGluR5 are localized within the amino-terminal 17 kDa of the ECD (13). To identify the specific cysteine(s) in the CaR ECD involved in intermolecular disulfide linkage, we tested the ability of a series of cysteine mutants to homodimerize and to heterodimerize with a truncation mutant, TM1.

TM1, truncated within the first intracellular loop, was found to express well at the cell surface, in contrast to mutants truncated within the second or third intracellular loops which we previously showed fail to be processed normally and fail to reach the cell surface (26). This difference suggests that the number of transmembrane domains in a given CaR construct (one and seven for TM1 and wild type, respectively; three and five for second and third intracellular loop truncation mutants, respectively) is critical for folding and normal processing. TM1 also was able to homodimerize, in agreement with recently reported results for a similar truncation mutant (19), and to heterodimerize with wild type CaR. The dimerization ability of TM1 is further evidence for the importance of the ECD in CaR dimer formation.

Only C60S, C101S, C358S, and C395S of the single cysteine to serine mutants we tested showed substantial reduction in homodimerization and in heterodimerization with TM1. Because these cysteine mutant receptors are expressed primarily as incompletely processed 130-kDa monomers, it is possible that mutation of these cysteines blocks dimerization by causing misfolding of the protein rather than because such cysteines are directly involved in intermolecular disulfide-linked dimer formation. Our results are similar to those recently reported in another study (19) for C131S (similar to wild type) and C101S (reduced total expression and reduced ability to dimerize) but differ importantly for several other cysteine mutants. Unlike our results, that study reported that the C60S was similar in all respects to wild type and that the C236S mutant was largely unable to dimerize. These authors also reported that a C101S/C236S double mutant was well expressed and appeared exclusively as a monomer. Since we did not study a C101S/C236S double mutant, we cannot comment on the results with that mutant. We are unable to explain the differences seen for mutants such as C60S and C236S examined in both studies except that the other study, unlike ours, involved green fluorescent protein-tagged CaR constructs and did not directly assess cell surface expression as we did with the biotinylation method.

Given the similarity between the CaR and mGluRs and the evidence for mGluR5 that the first 17 kDa of the ECD is the region critical for dimerization, we decided to test the effect of substituting serine for both Cys-129 and Cys-131. The resultant double mutant failed to dimerize, like C60S, C101S, C358S and C395S mutants, but unlike these, C129S/C131S formed a...
Identification of Cys Critical for hCaR Dimerization

Fig. 6. A, alignment of a portion of the amino acid sequence of the CaR ECD and mGluR1 ECD with that of the LIVBP. The entire 344-residue LIVBP sequence is shown aligned with a portion of the sequence of the rat mGluR1 ECD and the human CaR ECD. Amino acid numbering on the right refers to the full-length LIVBP, mGluR1 and CaR. Identical amino acid residues are shown in bold. The alignment places Gly-36 of the hCaR at residue Glu-1 of LIVBP and ends with Val-513 of the hCaR. Four insertions in the mGluR1 and CaR sequences that do not align with LIVBP are labeled I–IV. In the largest of these insertions, III, the hCaR sequence from Phe-347 to Ser-403 has been omitted. The secondary structure (arrows, β sheet; cylinders, α helix; thin lines, turns and loops) is superimposed above the alignment and is based on the three-dimensional structure of LIVBP. B, venus flytrap model of the three-dimensional structure of part of the ECD of the hCaR monomer. A ribbon diagram of the hCaR ECD model is shown with α helices in red, β sheets in yellow, and loops and turns in cyan. N represents the amino-terminal amino acid.
monomer that was normally processed (Endo-H resistance) and expressed at the cell surface (biotinylation experiment). These results strongly suggest that mutation of both Cys-129 and Cys-131 does not block dimerization by causing misfolding and abnormal processing of the receptor but rather by disrupting intermolecular disulfide linkage. The results do not allow us to distinguish between three possibilities as follows: (a) dimer formation involves intermolecular disulfide linkage between both Cys-129 and Cys-131 on respective monomers; mutation of either cysteine fails to block dimer formation because the intermolecular disulfide bond between the remaining cysteines is sufficient to maintain dimerization; (b and c) dimer formation normally involves a single intermolecular disulfide bond between either Cys-129 or Cys-131 and their counterparts on the other monomer; mutation of either cysteine fails to block dimer formation either because that cysteine is ordinarily uninvolved in intermolecular disulfide linkage or because the nearby, unmutated cysteine substitutes for the mutated one in disulfide bond formation. It is interesting in this respect that creation of a cysteine at adjacent position 132, normally a serine, restores normal dimer formation to the monomeric C129S/C131S mutant. This suggests that residues 129, 131, and 132 are all located within a putative dimer interface that permits intermolecular disulfide bond formation.

Mutations at these positions, singly or in combination, cause a significant left shift in receptor sensitivity (Fig. 5). The EC_{50} for [Ca^{2+}]_o is reduced by a factor of 2 compared with wild type in each of the mutants except for the C129S/C131S double mutant whose EC_{50} is reduced nearly 4-fold. In our previous study of individual C129S and C131S mutants (18), we failed to discern a clear left shift in comparison with wild type in part because we did not measure response at several [Ca^{2+}]_o concentrations <2.0 mM. The left shift in sensitivity seen with the mutations is not due to lack of dimer formation since only the C129S/C131S double mutant fails to dimerize. Interestingly, five mutations in the hCaR (A116T, N118K, L122P, E127A, and F128L) shown to cause a left shift in receptor sensitivity and identified in subjects with the disease autosomal dominant hypocalcemia (3) are located close to Cys-129 and Cys-131 in the putative dimer interface. None of these mutations disrupted dimer formation (data not shown). The ability of so many different missense mutations involving residues between 116 and 132 of the hCaR to enhance sensitivity to [Ca^{2+}]_o suggests that this region is involved in some critical but as yet undefined way in receptor activation. The more profound increase in sensitivity seen with the monomeric C129S/C131S double mutant may be a result of an inability to form the dimer. Note also (Fig. 5) that despite its enhanced sensitivity to [Ca^{2+}]_o, the C129S/C131S double mutant fails to reach wild type levels of activation even at the highest concentrations tested.

A recent study (35) provided functional evidence for the importance of CaR dimer formation in that coexpression of inactive but cell surface-expressed mutants led to heterodimer formation and some reconstitution of activity. The present results show that dimer formation is not essential for CaR function per se but may be essential for normal activation. The differences in activity between wild type dimer and the C129S/C131S mutant monomer lead us to speculate that dimer formation may constrain activation by low [Ca^{2+}]_o concentrations. Missense mutations at a number of residues within the dimer interface reduce this constraint resulting in receptor activation at “inappropriately” low agonist concentrations, as observed in subjects with autosomal dominant hypocalcemia. Complete loss of dimer formation in the C129S/C131S mutant causes an even more extreme left shift in receptor sensitivity but appears to compromise the maximum level of signal transduction achieved.

It is instructive to consider the present results in the context of a model of the three-dimensional structure of the hCaR ECD (Fig. 6). This model is based on the original alignment of mGluRs with bacterial periplasmic binding proteins by O’Har et al. (11), a reasonable extrapolation given the high sequence identity between CaR and mGluRs (2, 20). The alignment (Fig. 6A) places glycine 36 of the hCaR at residue 1 of LIVBP and ends with valine 513 of the hCaR. There are four insertions in both the CaR and the mGluR sequence (labeled I–IV in Fig. 6) that cannot be aligned with the LIVBP, and hence their structure cannot be modeled. The model shows the hCaR ECD monomer as a bilobed, venus flytrap-like structure with three strands connecting the two globular lobes each of which consists of β strands flanked by α helices (Fig. 6B). The four insertions are all contained in the amino-terminal lobe 1 as in the mGluR model (11).

The carboxyl terminus in the model corresponds to a part of the ECD just before the cysteine-rich region that contains 9 of the 19 cysteines in the CaR ECD. Interestingly, the GABA_b receptor ECD that completely lacks the cysteine-rich region found in other GPCR family 3 members including CaR, mGluRs, TR, and VNR has recently also been modeled as a venus flytrap-like structure, consistent with the idea that the cysteine-rich region represents a separate domain of the ECD (10). Mutation of any of the nine cysteines of the hCaR cysteine-rich region did not block dimer formation but led to intracellularly trapped proteins that were incompletely processed. We speculate that mutation of these cysteines likely causes misfolding of the receptor, perhaps by disrupting intramolecular disulfide bond formation, but this occurs only after dimers involving the venus flytrap-like portion of the ECD have already been formed.

Of the remaining 10 cysteines within the ECD, only Cys-236 is located within the carboxyl-terminal lobe 2 of the flytrap model (Fig. 6B). Mutation of this residue does not impair dimer formation but causes expression of an intracellularly trapped, presumptively misfolded protein. On the basis of the model, in which Cys-236 is remote from other cysteines in the venus flytrap structure, it appears unlikely that Cys-236 is involved in intramolecular disulfide bond formation with other cysteines in the venus flytrap. We cannot, however, exclude its involvement in formation of a disulfide with one of the nine cysteines of the cysteine-rich region. Thus misfolding caused by mutation of Cys-236 could either be due to disruption of an intramolecular disulfide or to a more subtle effect of substitution of serine for this cysteine.

In contrast to Cys-236, Cys-60, Cys-101, Cys-358, Cys-395, Cys-437, Cys-449, and Cys-482 are all located within the amino-terminal lobe 1 of the flytrap. Mutation of the first four of these residues does block dimer formation. We speculate that this is due to misfolding of lobe 1 and prevention of formation of the dimer interface region (region II in the model) that normally protrudes from this lobe. These residues may be involved in intramolecular disulfide bonds critical for achieving
the correct tertiary structure of lobe 1, but verification of this speculation awaits direct biochemical evidence. In the model (Fig. 6B), Cys-60 and Cys-101 are in close proximity suggesting that they may indeed form a disulfide. Whether Cys-358 and Cys-395 are likewise in close proximity cannot be predicted from the model since both of these cysteines are located in the large insertion III which cannot be modeled. Interestingly, mutation of any of the other three cysteines located in lobe 1 (Fig. 6B), Cys-437, Cys-449, and Cys-482, neither disrupts dimerization nor normal processing of the receptor. This suggests that none of these cysteines is critical in terms of disulfide bond formation.

The putative dimer interface containing cysteines 129 and 131 corresponds to region II in the model (Fig. 6B). Because this insertion cannot be modeled on the basis of the three-dimensional structure of LIVBP, we cannot predict accurately the location of these two cysteines in relation to the rest of the protein. Indirect evidence, however, suggests that they are located at the surface of the protein, since we have shown previously that the intervening residue, Asn-130, is glycylated (33). This region, in addition to containing the cysteine(s) involved in intermolecular disulfide bond formation, may be involved in specific recognition of homodimerization partners. Alignment of this region in CaR and mGluR shows substantial sequence diversity, far greater in particular than in the regions of the ECD encompassed in the venus flytrap model. The mGluR5 was shown to homodimerize but not to heterodimerize with mGluR1 (13), reflecting the importance of specific recognition sequences in addition to the conserved cysteine necessary for dimerization. If our model is correct, we predict that chimeras of a given mGluR containing region II of a different mGluR may be able to heterodimerize with that other mGluR. We cannot, of course, exclude the involvement of additional parts of the ECD, for example region IV, in forming the dimer interface.

An alignment of the ECD of family 3 GPCR shows that all mGluR subtypes have a single cysteine corresponding to cysteines 129 and 131 of the hCaR. Our data would suggest that mutation of this single cysteine would disrupt dimer formation and result in a cell surface-expressed monomer. Several VNTR (6) also have a single cysteine in this position and are therefore predicted to be dimers. TR, lacking this cysteine, may either be monomers or may have another basis for dimer formation. The GABA<sub>B</sub> receptor is interesting in this respect in that it lacks not only this cysteine but others conserved in the ECD of family 3 GPCR. Indeed, a recent modeling and mutagenesis study has identified, ligand binding to the ECD has not been demonstrated for the CaR. Only very indirect evidence (36, 37) and analogy to mGluRs and bacterial periplasmic binding proteins suggests binding within the ECD.

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