Dimerization of the Calcium-sensing Receptor Occurs within the Extracellular Domain and Is Eliminated by Cys → Ser Mutations at Cys$^{101}$ and Cys$^{236*}$

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Calcium-sensing receptors are present in membranes as dimers that can be reduced to monomers with sulphydryl reagents. All studies were carried out on the human calcium-sensing receptor tagged at the carboxy terminus with green fluorescent protein (hCaR-GFP) to permit identification and localization of expressed proteins. Truncations containing either the extracellular agonist binding domain plus transmembrane helix 1 (ECD/TMH1-GFP) or the transmembrane domain plus the intracellular carboxyl terminus (TMD/carboxyl terminus-GFP) were used to identify the dimerization domain. ECD/TMH1-GFP was a dimer in the absence of reducing reagents, whereas TMD/carboxyl-terminal GFP was a monomer in the absence or presence of reducing agents, suggesting that dimerization occurs via the ECD. To identify the residue(s) involved in dimerization within the ECD, cysteine → serine point mutations were made in residues that are conserved between hCaR and metabotropic glutamate receptors. Mutations at positions 60 and 131 were expressed at levels comparable to wild type in HEK 293 cells, had minimal effects on hCaR function, and did not eliminate dimerization, whereas mutations at positions 101 and 236 greatly decreased receptor expression and resulted in significant amounts of monomer in the absence of reducing agents. The double point mutant hCaR(C101S/C236S)-GFP was expressed more robustly than either C101S or C236S and covalent dimerization was eliminated. hCaR(C101S/C236S)-GFP had a decreased affinity for extracellular Ca$^{2+}$ and produced slower response kinetics upon increases or decreases in agonist concentration. These results suggest that covalent, disulfide bond-mediated dimerization of the calcium-sensing receptor contributes to stabilization of the ECD and to acceleration of the transitions between inactive and active receptor conformations.

Calcium-sensing receptors (CaR)$^1$ couple extracellular Ca$^{2+}$ binding to intracellular Ca$^{2+}$ transients through G$_q$-mediated activation of phosphatidylinositol phospholipase C (1). CaR agonists (Ca$^{2+}$ and other di- and trivalent cations (2, 3), poly-L-arginine (4), spermine (5), and β-amylloid (6)) bind within the large extracellular domain, which has structural homology with a large class of bacterial periplasmic binding proteins selective for ions, amino acids, and sugars (7, 8). CaR is present in cell types involved in organismal Ca$^{2+}$ homeostasis, including the parathyroid, kidney, and bone, as well as neuronal, fibroblast, and epithelial cell types, where its role(s) has yet to be defined (9).

Biochemical studies on heterologously expressed CaR indicate multiple immunoreactive bands on Western blots that reflect both differential glycosylation (10, 11) and higher, disulfide-linked oligomeric complexes (12, 13). Although the physiological relevance of CaR oligomerization is not known, a recent study has demonstrated CaR dimers can be isolated from kidney epithelium (13). Preincubation of kidney epithelial membranes with agonists (Ca$^{2+}$, Mg$^{2+}$, and Gd$^{3+}$) protected CaR dimers from the actions of reducing agents, suggesting the presence of conformationally sensitive disulfide bonds. Additional evidence for CaR dimerization comes from the dominant negative effect observed when CaR bearing an inactivating mutation (R185Q) was coexpressed with wild type receptor in HEK 293 cells (14). mGluR5 receptors have also been shown to exist as covalent dimers that can be converted to monomers by treatment with reducing agents in vitro (15). Glutamate-mediated increases in phosphatidylinositol metabolism in synaptosomes are inhibited by dithiothreitol (16), implying a dependence of mGluR function on dimerization. The region critical to mGluR5 receptor dimerization has been localized to the first 17 kDa of the amino terminus by limited proteolysis (15); this region contains four cysteine residues that are conserved in CaR, mGluRs (17), and some members of the pheromone receptor family (18, 19). Taken together, these studies suggest that the dimerization domain of CaR may be within the extracellular, agonist binding amino-terminal domain.

Dimerization/oligomerization has been noted for many G protein-coupled receptors, including opiate (20), β-adrenergic (21, 22), muscarinic (23, 24), dopamine (25), m5-HT$_{1A}$ (26, 27), substance P (28), C5aaphalotoxin (29), and platelet-activating factor (30) receptors. For some types of receptors, including those for dopamine (31) and muscarine (24, 32, 33), the complex agonist binding isotherms are best explained by oligomerization-induced shifts in agonist affinity. Opiate receptors undergo agonist-mediated monomerization, which is a prelude to receptor sequestration (20). Interestingly, some opiate agonists are not capable of mediating monomerization, and this may account for their longer lasting physiological effects. Dimerization is required for normal functioning of β-adrenergic receptors (34), and in fact, receptor dimerization has been shown to rescue the function of mutant forms of β-adrenergic (35) and...
Cysteine Residues Contributing to CaR Dimerization

angiotensin type II (36) receptors. CaR (12, 13) and the related mGlRs (15) exhibit covalent dimerization, and this may contribute in novel ways to receptor function. In this report, we demonstrate that both CaR and a truncated construct consisting of the extracellular domain plus transmembrane helix 1 exist as dimers in the absence of reducing agents and that disulfide bond reduction causes a shift to the monomeric form of either full-length CaR or the extracellular domain truncation. We have identified cysteine residues that eliminate CaR dimerization by making cysteine to serine point mutations in each of the cysteine residues within the first 17 kDa of the extracellular domain conserved between CaR and the mGlRs, beginning at the amino terminus. We found that the double point mutant hCaR(C101S/C236S)-GFP can be observed on SDS gels as a monomer in the absence of reducing agents. Finally, we demonstrate that hCaR(C101S/C236S)-GFP is functional when heterologously expressed in HEK 293 cells but displays a decreased affinity for agonist and significantly slower kinetics of activation and deactivation.

EXPERIMENTAL PROCEDURES

Materials—Human CaR (hCaR) in pBluescript was obtained from Dr. J. Garrett (NPS Pharmaceuticals, Inc.). The rat β2 adrenergic receptor (β2AR) was obtained from Dr. David Yue (Department of Biomedical Engineering, Johns Hopkins University). GFP fusion proteins were generated using pCDNA3.1 containing GFP in each of three frames (CLONTECH). Restriction enzymes were obtained from Life Technologies, Inc. and New England Biolabs.

Plasmid Construction—All PCRs were performed with Pfu polymerase (Stratagene) using 94, 57, and 72 °C as denaturing, annealing, and elongation temperatures, respectively. All constructs were confirmed by restriction digestion and automated sequencing. hCaR with a carboxy-terminal fusion to EGFP (CLONTECH) was constructed as described previously (37). hCaR with a carboxy-terminal fusion to the FLAG epitope was prepared by replacing EGFP in pEF-BOS with the FLAG construct by inverse PCR (5′ primer, ATATACCTAGGATCCCATACGTTTCTGT; 3′ primer, GTCCTTGTAGTCTGAATTCACTACGTTTCTGT). The ECD/TMH1-GFP construct was prepared by generating a PCR product containing a BamHI site after the first hCaR transmembrane domain (5′ primer, GCCATTACATGTTGATGGCG; 3′ primer, CGCGGATCCCTGCGTGGGTTGGCGCTT); this was cloned into the product of the first cloning step. After amputation, the product with the BamHI site was cloned into XhoI-BamHI and subcloned in frame into pEGFPN3 (CLONTECH). A two-step cloning process was used to generate TM1/2 Raf-GFP. First, a PCR product containing the seven transmembrane domains and the carboxy-terminal domain of hCaR was subcloned into the XhoI-BamHI sites of pEGFPN3 (5′ primer, CGGGCGCTCAGGGAGGATCGAGTTCTGT; 3′ primer, CGGGCATCCACTAGTTTCTCTTTG). In a second step, a PCR product containing the sequence and the GFP signal and bottom border, and washed with a solution containing 140 mM NaCl, 5 mM KCl, 0.55 mM CaCl2, 1 mM MgSO4, 20 mM Hepes, 0.83 mM Na2HPO4, 0.17 mM NaH2PO4, and 25 mM mannose, plus 1 mg/ml bovine serum albumin, pH 7.4. After the loading period, the coverslip was mounted in an imaging chamber (no coverslip). The imaging chamber was filled with a top and bottom buffer, and washed with a solution containing 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl2, 0.5 mM CaCl2, 10 mM Hepes, pH 7.4. The cells were excited at 340/380 nm (emission wavelength 510 nm) at 8-s intervals, and selected regions were recorded on a Universal imaging system based on the MetaFluor software package. Background images at the same gain settings used during a particular experiment were obtained on regions of the coverslip devoid of cells. All solutions were osmolality-matched. Variations in extracellular Ca2+ concentration were produced by isosmolar substitution for NaCl. All experiments were performed at room temperature (22–24 °C). Multiple cells were analyzed from at least three independent transfection experiments (or cell passages for stable cell lines). Data were normalized and averaged as described for each experiment and are presented as the mean ± S.E. Curves were fitted by least squares minimization using the Marquardt-Levenberg algorithm (NFT, Island Products, Galveston, TX).

RESULTS

Dimerization of CaR—Several recent reports suggest that CaR exists in vivo (13) and in heterologously expressed systems (12, 43) in a dimeric form that can be largely converted to the monomer form by treatment with reducing agents such as dithiothreitol, β-mercaptoethanol, or β-mercaptoapropanol. We have further characterized dimerization of human CaR by expressing various constructs (truncations and point mutations) in HEK 293 cells. Fig. 1 illustrates the control constructs studied: human CaR-FLAG (a) to permit antibody detection of hCaR and human CaR-GFP (b). A prominent band estimated at 320–340 kDa was observed in the absence of β-mercaptoethanol for hCaR-FLAG. This was converted to a doublet at 140–160 kDa upon reduction with β-mercaptoethanol. hCaR-GFP presented a greater dispersion of molecular masses in the absence of reducing agents, but upon reduction, there was a prominent doublet at 205–220 kDa (molecular mass of hCaR plus the 27-kDa GFP tag). All samples were exposed to 10 mM iodoacetamide during homogenization to minimize formation of nonspecific disulfide bonds during membrane isolation. Pre-treatment with iodoacetamide reduced the dispersion in the
molecular masses of the oligomeric hCaR complexes and promoted a more complete conversion to the monomer form upon reduction with β-mercaptoethanol, also noted in a recent report (12). Similar conversions of dimer to monomer occur upon reduction of either hCaR-FLAG or hCaR-GFP, suggesting that the GFP tag does not affect the process. All further studies were performed on the GFP-tagged receptor, because it permits rapid screening of expressed clones, cellular localization of mutated receptors, and crude estimates of expression levels to be made prior to functional studies. In previous studies, we have demonstrated that carboxyl-terminal fusion of GFP to hCaR does not alter the functional properties of the receptor (dose/response relationship, desensitization, and subcellular localization) (37). Here, we note that GFP does not alter hCaR dimerization.

Localization of the Dimerization Domain to the ECD—To determine the domain involved in hCaR dimerization, two hCaR truncations were examined, one containing only the ECD plus TMH1 (ECD/TMH1-GFP) and another comprising only the seven transmembrane helices plus the carboxyl terminus (TMD/Cterm-GFP). Both truncations were tagged at their carboxyl termini with GFP and were localized to both intracellular and plasma membranes of transiently transfected HEK 293 cells. Fig. 2 illustrates a representative Western blot. hCaR-GFP (Fig. 2a) and ECD/TMH1-GFP (b) are present predominantly as dimers in the absence of β-mercaptoethanol. Including β-mercaptoethanol in the sample buffer caused a substantial shift to the monomer form of hCaR-GFP and a quantitative shift to the monomer form of ECD/TMH1-GFP. Western blots of membranes from cells stably transfected with TMD/Cterm-GFP (Fig. 2c) revealed that the expressed protein was present in the monomer form in the absence and presence of reducing agents. There was some high molecular mass material evident in the nonreduced sample; this is most likely due to oxidation of cysteines within the cytoplasmic tail during sample preparation and is not prevented by iodoacetamide treatment, as has been noted previously (12). These experiments localize disulfide bond-mediated dimerization in hCaR to the ECD.

Identification of Cys Residues Involved in Intermolecular Disulfide Bond Formation in CaR—Studies on mGluR5 indicated that dimerization was eliminated by cleavage of the first 17 kDa of the amino terminus (15). We therefore began by individually mutating to serine the four cysteine residues of hCaR that are present within the first 17 kDa of the amino terminus (at amino acid positions 60, 101, 131, and 236), which are conserved between CaRs and mGluRs. Whereas single cysteine → serine point mutations at these positions did not eliminate dimerization (Fig. 3, a–d), single point mutations at Cys<sup>101</sup> and Cys<sup>236</sup> did increase the amount of monomer observed in the nonreduced samples. Furthermore, as is apparent in Fig. 3, expression of Cys<sup>101</sup> or Cys<sup>236</sup> was weak compared with a single point mutation at Cys<sup>60</sup> or Cys<sup>131</sup> (all lanes were loaded with equal amounts of membrane protein). We therefore made the double point mutation hCaR(C101S/C236S)-GFP. This mutated receptor expressed more robustly than either of the single point mutations, and was present in the monomer state on Western blots in the absence or presence of reducing agents (Fig. 3e). Direct comparison of C101S, C236S, and C101S/C236S (Fig. 4) at higher protein levels confirms the requirement for both point mutations to eliminate significant hCaR dimerization in the absence of reducing agents.
Noncovalent Dimerization of hCaR(C101S/C236S)-GFP—To ascertain whether hCaR(C101S/C236S)-GFP was correctly folded and processed, we determined its cellular localization by assessing GFP fluorescence (localization was comparable to that of wild type hCaR (37), data not shown), its ability to form noncovalent dimers, and its ability to activate Gαq resulting in increases in intracellular Ca^{2+} (described below). hCaR(C101S/C236S)-FLAG and hCaR(C101S/C236S)-GFP were transiently coexpressed in HEK 293 cells, and membranes were isolated. Immunoprecipitation was performed with either the anti-FLAG or anti-GFP antibodies, and Western blots of the precipitated proteins were probed with the anti-GFP antibody. Illustrated in Fig. 5 are the results of such an experiment. The control lane (Fig. 5a) illustrates membranes from the co-transfected HEK 293 cells (reduced with β-mercaptoethanol) probed with the anti-GFP antibody. Fig. 5b illustrates the results from immunoprecipitations with either the anti-FLAG (F) or anti-GFP (G) antibody. Precipitation with either antibody resulted in the appearance of anti-GFP-reactive protein of the correct molecular weight (monomeric hCaR-GFP) on the Western blot. 

FIG. 5. Noncovalent dimerization of hCaR(C101S/C236S). a. Western blot of 5 μg of protein from a membrane preparation derived from HEK 293 cells co-transfected with hCaR(C101S/C236S)-FLAG and hCaR(C101S/C236S)-GFP. The primary antibody was a polyclonal anti-GFP antibody (CLONTECH) at a 1:2500 dilution. b. Western blot of the results of immunoprecipitation of a membrane preparation derived from HEK 293 cells co-transfected with hCaR(C101S/C236S)-FLAG and hCaR(C101S/C236S)-GFP. F denotes lane loaded with the results of 250 μg of protein precipitated with 15 μg of the anti-FLAG antibody (Sigma). G denotes lane loaded with the results of 250 μg of protein precipitated with 3 μl of the anti-GFP antibody (CLONTECH). In both cases, the primary antibody was the anti-GFP antibody, as in a. c. Western blot of the results of immunoprecipitation of a membrane preparation derived from HEK 293 cells transfected with hCaR(C101S/C236S)-GFP. F and G are as described in b. The primary antibody was anti-GFP, as described in a. d. Western blot of an immunoprecipitation of a membrane preparation from HEK 293 cells transiently transfected with hCaR-FLAG and β2AR-GFP. All other methods were as in b.

Noncovalent Dimerization of hCaR(C101S/C236S)-GFP—In the appearance of anti-GFP-reactive protein of the correct molecular weight (monomeric hCaR-GFP) on the Western blot. Validating the specificity of the anti-FLAG antibody did not result in any anti-GFP-reactive protein on the Western blot, indicating that the noncovalent dimerization of hCaR(C101S/C236S)-GFP involves a noncovalent association. These results suggest that hCaR(C101S/C236S)-FLAG and hCaR(C101S/C236S)-GFP form noncovalent dimers when coexpressed in HEK 293 cells.

Functional Consequences of Cys → Ser Mutations in CaR ECD—The functional consequences of cysteine → serine mutations were examined for those receptor mutations that expressed robustly in HEK 293 cells, namely, C60S, C131S, and C101S/C236S. The dose/response relationships for Ca^{2+}-dependent activation were determined in individual transfected HEK 293 cells using alterations in fura-2 fluorescence as a measure of changes in intracellular Ca^{2+} (Fig. 6). Transfected HEK 293 cells were exposed to sequential applications of increasing concentrations of bath Ca^{2+} (from 2.5 to 30 μM) for periods of 60–90 s (until a steady state response was reached). Ca^{2+} dose/response experiments were performed on hCaR-GFP (Fig. 6a), hCaR(C60S)-GFP (b), hCaR(C131S)-GFP (c), and hCaR(C101S/C236S)-GFP (d). Cells for analysis were chosen so that the average difference in 340/380 ratio was approximately the same for all mutations, to minimize potential differences in kinetics that can arise from large differences in expression levels (44). Fig. 7 illustrates the dose/response relationships calculated from averaged data obtained from at least three independent transfections for each cysteine → serine mutant, assayed as in Fig. 6. The dose/response relationships for Ca^{2+}-dependent activation of hCaR(C60S)-GFP (EC_{50} 2.9 ± 0.19 mM) and hCaR(C131S)-GFP (EC_{50} 4 ± 0.33 mM) were comparable to that of wild type hCaR-GFP (EC_{50} 3.5 ± 0.3 mM). In contrast, the dose/response relationship for Ca^{2+}-dependent activation of hCaR(C101S/C236S)-GFP was linear over the range of Ca^{2+} from 0.5 to 30 mM (Fig. 6). Similar experiments were performed on hCaR(C101S)-GFP and hCaR(C236S)-GFP, but expression levels were extremely low (as corroborated by the Western blots illustrated in Fig. 3), even when stably transfected cell lines were produced. Although sufficient data could not be obtained, a few positive cells for each clone presented behavior qualitatively similar to hCaR(C101S/C236S)-GFP, i.e., a linear response to increases in extracellular Ca^{2+} up to 30 mM.

A second feature unique to hCaR(C101S/C236S)-GFP is a distinct difference in the kinetics of the response to extracellular Ca^{2+}. As can be seen in Fig. 6, the kinetics of hCaR(C101S/C236S)-GFP and hCaR(C131S)-GFP (b and c) are comparable to those of wild type hCaR-GFP (a), with rapid increases in intracellular Ca^{2+} upon exposure to increasing concentrations of extracellular Ca^{2+}. In contrast, the responses of hCaR(C101S/C236S)-GFP were slow (Fig. 6d), and the new steady state level of intracellular Ca^{2+} was not reached for over a minute in each successive Ca^{2+} concentration. Furthermore, the kinetics of washout from 30 to 0.5 mM Ca^{2+} were also slow for hCaR(C101S/C236S)-GFP, taking 81.6 ± 3.5 s for the intracellular Ca^{2+} to drop from the level in 30 mM bath Ca^{2+} to the baseline intracellular Ca^{2+} observed in 0.5 mM bath Ca^{2+}. In contrast, the drop in intracellular Ca^{2+} upon washout of 30 mM bath Ca^{2+} for hCaR-GFP was 38.4 ± 2.7 s, comparable to that observed for hCaR(C131S)-GFP (40 s), whereas the drop in intracellular Ca^{2+} upon extracellular Ca^{2+} washout for hCaR(C60S)-GFP was biphasic, taking a total of 62.4 ± 8.8 s, with 85% of the decrease achieved in 36 ± 3 s.

**DISCUSSION**

Calcium-sensing receptors are observed on Western blots as dimers and/or higher oligomers in the absence of reducing
agents. Treatment with iodoacetamide during membrane isolation minimizes the formation of nonphysiologically relevant disulfide-linked oligomers, although this protection may not be complete, as has been noted in previous studies (12, 13). Despite these potential problems, a prominent dimer band is present on Western blots of nonreduced samples, which is largely converted to monomeric hCaR by treatment with β-mercaptoethanol. Studies with biotinylation and immunoprecipitation of hCaR expressed in HEK 293 cells have suggested that the major form of hCaR on the plasma membrane is a dimer (12). We therefore sought to identify the domain responsible for hCaR dimerization. To confirm suspicions derived from study of mGluRs (15, 39) that suggested that the dimerization domain was localized to the ECD, we expressed truncations of hCaR that contained either the ECD/TMH1-GFP or TMD/Cterm-GFP. The ECD/TMH1-GFP construct was present as a dimer in the absence and monomer in the presence of β-mercaptoethanol, whereas TMD/Cterm-GFP was a monomer in either condition. These results identify the ECD as the locus for covalent dimerization of CaR.

The calcium-sensing receptor sequence contains 19 cysteine residues that are present in comparable positions in mGluRs, 17 of which are in the ECD. Luckily, the suspected covalent dimerization domain for mGluRs was localized to the first 17 kDa of the amino terminus (15), and we thus began by making point mutations (Cys → Ser) in the four conserved cysteines within this region of hCaR. Mutations of the cysteine residues at positions 60 and 131 did not eliminate dimerization of hCaR and had minimal effects on the functional activity of the receptor as assessed by the dose/response relationship and kinetics of the responses to extracellular Ca2+. Mutations of the cysteine residues at positions 101 and 236 decreased the expression of receptor significantly, indicating a potential problem with folding and/or trafficking of the receptor to the plasma membrane. These individual point mutations did not, however, eliminate dimerization of the receptor. When a construct containing both point mutations, C101S/C236S, was expressed, protein levels were significantly increased, and dimerization was eliminated in the absence/presence of reducing agents. It is highly likely, therefore, that these two cysteine residues, at positions 101 and 236, are involved in dimerization of CaR. Alternatively, mutations of cysteine to serine at positions 101 and 236 may affect the conformation of the ECD in a manner that prevents dimerization via other, as yet unidentified cysteines (among the 17 present in the ECD). Here we consider the most parsimonious conclusion, i.e. that Cys101 and Cys236 are directly involved in hCaR dimerization. Our conclusion is based upon several criteria: 1) hCaR(C101S/C236S) is present as a monomer in the absence/presence of reducing agents; 2) expression levels of the single point mutations, hCaR(C101S), and hCaR(C236S) are weak, and monomerization is absent, whereas the double point mutation hCaR(C101S/C236S) expresses more robustly and is present as a monomer in the absence of reduction (these results are reminiscent of what has been observed in mutagenesis studies designed to identified partners in salt bridges in proteins, i.e. elimination of one partner destabilizes the protein, whereas...
elimination of both partners improves protein expression); 3) hCaR(C101S/C236S) is correctly localized to membranes within HEK 293 cells; 4) hCaR(C101S/C236S) exhibits activity equivalent in magnitude to wild type hCaR, albeit with alterations in properties; and, finally, 5) hCaR(C101S/C236S) folds in a manner that maintains noncovalent dimerization of the receptor. Dimerization of many G protein-coupled receptors is mediated by noncovalent interactions among transmembrane domains. In particular, a motif has been identified in the α-adrenergic receptor TM6 that promotes noncovalent dimerization of receptors, which is essential for receptor function (34). This motif is present in TM5 of CaR (12) and mGluRs and may serve to promote noncovalent hCaR dimerization, leading subsequently to disulfide bond formation. Now that the residues contributing to covalent dimerization of CaR have been identified, the contribution(s) of noncovalent interactions to CaR function can be addressed in the hCaR(C101S/C236S)-GFP background.

Studies on hCaR(C101S/C236S)-GFP reveal a significant contribution(s) of disulfide bond-mediated dimerization to normal CaR function. Significantly higher concentrations of Ca\(^{2+}\) are required for modest activation of hCaR(C101S/C236S)-GFP. In fact, increases in the response were linear from 0.5 through 30 mM. The most striking difference in the behavior of hCaR(C101S/C236S)-GFP was the slowing of response kinetics to both increases and decreases in extracellular Ca\(^{2+}\). These results indicate that covalent, disulfide bond-mediated dimerization of hCaR is required for normal agonist-mediated receptor activation. Further support for the importance of dimerization in the function of this class of receptors (including CaR, mGluRs, and GABABRs) comes from recent reports that suggest that GABA\(_B\)Rs are only functional when expressed as heteromeric assemblies of subunits GABA\(_B\)R1 and GABA\(_B\)R2 (40–42).

In conclusion, we have demonstrated that CaR is primarily a disulfide-linked dimer in cell membranes, and we have identified the disulfide bond-mediated dimerization domain as the ECD. Furthermore, we have identified two cysteine residues within the ECD, Cys\(^{101}\) and Cys\(^{236}\) of the human CaR, that mediate covalent dimerization. What remains to be seen is whether the cysteine residues present in comparable positions within the ECD, Cys\(^{101}\) and Cys\(^{236}\) of the human CaR, that mediate covalent dimerization. What remains to be seen is whether the cysteine residues present in comparable positions within the ECD, Cys\(^{101}\) and Cys\(^{236}\) of the human CaR, that mediate covalent dimerization.