Dimerization of the Extracellular Calcium-sensing Receptor (CaR) on the Cell Surface of CaR-transfected HEK293 Cells*

Mei Bai‡, Sunita Trivedi, and Edward M. Brown

From the Endocrine-Hypertension Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) is a G protein-coupled receptor that plays important roles in calcium homeostasis. In this study, we employed epitope tagging, cell-surface biotinylation, and immunoprecipitation techniques to demonstrate that the CaR is expressed mostly in the form of a dimer on the surface of transfected human embryonic kidney (HEK293) cells. Western analysis of cell-surface proteins under nonreducing conditions showed that the CaR exists in several forms with molecular masses greater than 200 kDa. Most of these high molecular mass forms of the receptor could be converted to a single monomeric species at 160 kDa under reducing conditions. This result suggests that the CaR forms dimers or even higher oligomers on the cell surface through intermolecular disulfide bonds that are sensitive to reducing agents. Consistent with this hypothesis, use of a cell-surface cross-linking agent substantially increases the proportion of the putative dimeric CaR at 280 kDa relative to the monomeric form of the receptor at 160 kDa under reducing conditions. Dimerization of the CaR in intact cells was further demonstrated when we co-transfected and co-immunoprecipitated the wild type, full-length receptor and a truncated form of the CaR lacking its cytoplasmic tail. Taken together, we conclude from these results that the functional CaR resides on the cell surface of transfected HEK293 cells in the form of a dimer.

The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) is a G protein-coupled receptor (GPCR) (1). Activation of the CaR by elevated levels of Ca\(^{2+}\) stimulates phospholipase C and raises the cytosolic calcium concentration (Ca\(^{2+}\)). The physiological importance of the CaR in determining the level at which Ca\(^{2+}\) is set in vivo has been documented by the characterization of human syndromes resulting from activating or inactivating mutations of the CaR, which alter the function of parathyroid and kidney so as to produce hypo- or hypercalcemia, respectively (2, 3).

The function and expression of the CaR have been assessed in transiently transfected human embryonic kidney cells (HEK293) (4–9). CaR-transfected HEK293 cells respond to Ca\(^{2+}\) with an EC\(_{50}\) of 4.1 ± 0.1 mM (the effective concentration of Ca\(^{2+}\) producing one-half of the maximal Ca\(^{2+}\) response). The concentration-response curve for the Ca\(^{2+}\)-elicited Ca\(^{2+}\) responses in CaR-transfected HEK293 cells exhibits a relatively high Hill coefficient of about 3. This large Hill coefficient suggests a substantial degree of positive cooperativity in the binding of ligand and/or G protein by the CaR, which may involve intra- or intermolecular interactions (e.g. via formation of homodimers); namely, binding of the first ligand or G protein increases the affinity of the second ligand or G protein. Inter-molecular interactions leading to cooperativity, for example, could result from dimerization of GPCRs (e.g. as for the muscarinic receptor and rhodopsin), which has been suggested as the molecular basis for cooperativity in the binding of their respective agonists (10, 11) or G proteins (12, 13).

Our earlier biochemical studies had shown the presence of considerable amounts of CaR-specific, high molecular mass immunoreactivity by Western analysis, in addition to the putative monomeric form of the receptor, in membrane proteins prepared from both CaR-transfected HEK293 cells and native parathyroid cells (4). These high molecular mass species likely correspond to dimeric and trimeric CaRs. The presence of dimeric forms of the CaR has also recently been described in detergent extracts prepared with the inner medulla of the rat kidney (14).

A structurally related GPCR, mGluR5, forms homodimers via intermolecular disulfide linkages within the amino-terminal extracellular domain. The extracellular domains of mGluR5 and the CaR share 17 cysteines in equivalent positions, raising the possibility that the CaR could also dimerize in this manner (15). In addition, several less structurally related GPCRs have been demonstrated to form homodimers, including the muscarinic receptor (16), β\(_{2}\)-adrenergic receptor (17, 18), glucagon receptor (19), and δ-opioid receptor (20). A non-covalent hydrophobic dimerization motif has been suggested to mediate homodimerization of the β\(_{2}\)-adrenergic receptor (18), which was also present in the CaR. Therefore, the CaR can potentially dimerize through two distinct types of intermolecular interactions, i.e. noncovalent hydrophobic interactions and formation of covalent intermolecular disulfide bonds.

In this report, using molecular and biochemical approaches, we show that the functional CaR normally resides on the cell surface mostly as a homodimer. The unusually high degree of cooperativity in the activation of the CaR by its polycationic agonists suggests that dimerization of the CaR is likely to have functional implications.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis to Produce Tail-truncated Receptors—Site-directed mutagenesis was performed using the approach described by Kunkel (21). The dut-1 ung-1 strain of Escherichia coli, C7236, was transformed with cassette VI of a reconstructed CaR containing unique restriction sites introduced into the wild type human CaR as silent point mutations as described previously (4). Uracil-containing, single-stranded DNA was produced by infecting the cells with the helper
phage, VCSM13. The single-stranded DNA was then annealed to a mutagenesis primer, which contained a stop codon at the desired position and was flanked on both sides by a wild type sequence. The primer was then extended around the entire single-stranded DNA and ligated to generate closed circular heteroduplex DNA. DH5α-competent cells were then used to transform the DNA heteroduplex, and incorporation of the desired mutation was confirmed in all cases by sequencing the entire cassette. Finally, the mutated cassette VIs were cloned into the XhoI and XhoI sites of the reconstructed CaR (4).

**Construction of Flag-tagged CaR**—Cassette III in each of the mutan receptors was replaced with that in the Flag-tagged wild type receptor by double restriction digestion with XhoI and XbaI followed by ligation of the larger restriction fragments containing point mutations and the smaller restriction fragment containing the Flag-tag.

**Transient Expression of CaRs in HEK293 Cells**—The DNA for transfection was prepared using the Midi Plasmid Kit (QIAGEN). LipofectAMINE (Life Technologies, Inc.) was employed as a DNA carrier for transfection (22) according to the manufacturer’s procedures. The HEK293 cells used for transient transfection were provided by NPS Pharmaceuticals, Inc. (Salt Lake City, UT) and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal bovine serum. The DNA-liposome complex was prepared by mixing DNA and LipofectAMINE in Opti-MEM I Reduced Serum Medium (Life Technologies, Inc.) and incubating the mixture at room temperature for 30 min. LipofectAMINE was inactivated by incubating the Opti-MEM I Reduced Serum Medium and added to 90% confluent HEK293 cells plated on 13.5 × 20.1 mm glass coverslips using 0.625 μg of DNA (for measurement of Ca2+), or in 100-mm Petri dishes using 3.75 μg of DNA (for obtaining protein for immunoprecipitation and Western analysis). After 5 h of incubation at 37 °C, equivalent amounts of Opti-MEM I Reduced Serum Medium with 20% fetal bovine serum were added to the medium overlying the transfected cells, and the latter was replaced with fresh Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 24 h after transfection. The expressed Ca2+ -sensing receptor protein was assayed 48 h after the start of transfection. To perform co-expression of two receptors, 0.625 μg each of the two cDNAs were mixed and used to transfect HEK293 cells.

**Biotinylation of Cell-Surface Forms of the CaR and Cross-linking of Multimeric Receptors**—Prior to preparing whole cell lysates, intact HEK293 cells transiently transfected with Flag-tagged CaR were rinsed twice with phosphate-buffered saline and treated with 1 μM ImmunoPure Sulfo-NHS-Biotin (Pierce), a membrandiae biotinylation reagent, at room temperature with constant agitation for 30 min to biotinylate the proteins on the cell surface. The reaction was then quenched by incubating the cells in 0.5 M Tris-HCl, pH 7.5, for 5 min. For cross-linking experiments, we added an appropriate amount of Bis(sulfosuccinimidyl) suberate (BS3), a noncleavable, membrane-impermeable cross-linker, into the labeling solution containing ImmunoPure Sulfo-NHS-Biotin.

**Preparation of Whole Cell Lysates**—The surface-biotinylated and/or cross-linked HEK293 cells were rinsed twice with phosphate-buffered saline, treated with 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 7.4, 2 mM EDTA, 1 mM EGTA, protease inhibitors, including 83 μg/ml aprotinin, 30 μg/ml leupeptin, 1 mg/ml Pefabloc, 50 μg/ml calpain inhibitor, 50 μg/ml benzamid, and 5 μg/ml pepstatin (1 × immunoprecipitation buffer), at room temperature. Insoluble material was removed by centrifuging the cell lysates at 15,000 rpm for 15 min at 4 °C. The supernatants were collected as total cell lysates. The protein concentration was determined using the BCA protein assay (Pierce).

**Immunoprecipitation of Flag-tagged CaR**—First, 5 μg of anti-Flag M2 monoclonal antibody (VWR), 400 μl of H2O, 500 μl of 2 × immunoprecipitation buffer (see above), and 100 μl of total lysate containing approximately 500 μg of protein were added to a microcentrifuge tube. The mixture was incubated at 4 °C for 1 h. Then, 5 μl of an alkaline phosphatase-conjugated, anti-mouse IgG (Sigma) was added to the mixture. The incubation was continued for an additional 30 min at 4 °C. Subsequently, 50 μl of 10% Protein A-agarose (Life Technologies, Inc.) was added to the mixture for an additional 30-min incubation at 4 °C. The Protein A-agarose was washed three times with 1 × immunoprecipitation buffer, and the immunoreactive species were subsequently eluted with SDS-PAGE sample buffer at 65 °C for 30 min. The receptor of interest was detected by Western analysis.

**Western Analysis of the Human CaR Expressed in Whole Cells and on the Cell Surface**—An appropriate amount of immunoprecipitated proteins from CaR-transfected HEK293 cells was subjected to SDS-containing polyacrylamide gel electrophoresis (PAGE) (23) using a linear gradient of polyacrylamide (3–10%). The proteins on the gel were sub-sequently electrotransferred to a nitrocellulose membrane. After blocking with 5% milk, the forms of the receptor present on the cell surface were detected using an avidin-horseradish peroxidase conjugate (Bio-Rad) followed by visualization of the biotinylated bands with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). After removal of the biotinylated bands using washing buffer containing 0.1% Triton X-100, the strips were exposed to a radiographic film for 24 h. After autoradiography, the films were scanned using an imaging system (Amersham Pharmacia Biotech) followed by a secondary, horseradish peroxidase-conjugated, goat anti-rabbit antibody and then an Enhanced Chemiluminescence detection system (Amersham Pharmacia Biotech).

**Measurement of Ca2+ by Fluorometry in Cell Populations**—Coverslips with nearly confluent HEK293 cells previously transfected with the appropriate CaR cDNAs were loaded for 2 h at room temperature with fura-2/AM in 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 0.1% bovine serum albumin, and 0.1% dextrose and were then washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.1% dextrose, and 0.1% bovine serum albumin) at 37 °C for 20 min. The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the bath solution, using a modification of the technique employed previously in this laboratory (14). Extracellular calcium was increased stepwise to give the desired final concentrations with additions of Ca2+ in increments of 1 mM that were followed by 5 mM increments after achieving a level of 5.5 mM Ca2+, and 10 mM increments after reaching a level of 20 mM Ca2+. Excitation monochrometers were centered at 340 nm and 380 nm with emission light collected at 510 ± 40 nm through a wide band emission filter. The 340/380 excitation ratio of emitted light was used to calculate Ca2+, as described previously (24).

**Statistics**—The mean EC50 values for the wild type or truncated receptors determined in response to increasing concentrations of Ca2+ were calculated from the EC50 values for all of the individual experiments and were expressed with the standard error of the mean (S.E.) as the index of dispersion. Comparison of the EC50 values was performed using analysis of variance or Duncan’s multiple comparison test (25)

**RESULTS**

To detect cell-surface expression of the CaR, we introduced the Flag epitope tag into the extracellular domain of CaR. The function as well as the pattern and overall level of expression of the Flag-tagged CaR are identical to those of the wild type receptor, as assessed by high Ca2+-evoked Ca2+ responses and Western analysis, respectively (9). Proteins on the cell surface of HEK293 cells transiently transfected with the Flag-tagged CaR or empty vector were first labeled with membrane-impermeant, Sulfo-NHS-Biotin prior to lysis of the cells. The CaR was then immunoprecipitated with anti-Flag monoclonal antibody and eluted with SDS-sample buffer containing dithiothreitol (DTT) or no DTT. The free thiol groups of the native CaR were prevented from forming nonspecific disulfide bonds during protein preparation by including 100 μM iodoacetamide in the lysis buffer (see below). The immunopurified CaR was first detected with an avidin-horseradish peroxidase conjugate to visualize the forms of the receptor expressed on the cell surface. After removal of the avidin from the blot, the CaR was detected with a polyclonal anti-CaR antiserum (4), 4641, to detect both cell-surface and intracellular forms of the immunopurified receptor.

**Identification of Cell-Surface Forms of the CaR**—On reduced SDS-PAGE, using avidin for detection we identified a major band at 160 kDa (the expected position of the mature monomeric CaR) in the same lane as precipitation with anti-Flag antibody and reduced with DTT (Fig. 1A, lane 2). When we detected with anti-CaR antisera, in contrast, two immunoreactive bands (i.e. a doublet) were observed at the expected positions of the monomeric as well as the putative dimeric and higher multimeric CaRs (Fig. 1B, lane 2). The surface forms of the monomeric receptor corresponded to the upper band of the
of nonspecific disulfide bonds. As shown in Fig. 3, lane 2, inclusion of 100 mM iodoacetamide in the lysis buffer prevented the formation of a smear of CaR immunoreactivity (Fig. 3, lane 1) that otherwise was evident in Western blots performed using the CaR-specific antiserum. Therefore, the high molecular species observed in Figs. 2 and 3, lane 2, are not the artifacts because of formation of nonspecific disulfide bonds.

The CaR Self-dimerizes through Disulfide Bonds—To preserve specific intermolecular disulfide linkages, we surface-biotinylated and immunoprecipitated the CaR, but eluted the immunoprecipitated receptor with SDS-sample buffer lacking DTT. The monomeric CaR observed in Fig. 1A, lane 2, was completely absent when nonreduced conditions were employed (see Fig. 2, lane 2). Instead, we detected two major bands between 200 and 300 kDa with avidin as well as one higher molecular mass species between 300 and 500 kDa for the wild type CaR (Fig. 2, lane 2). In this experiment, the control with DTT in Fig. 2, lane 1, showed a trace amount of a high molecular mass species at 280 kDa (the expected position of the dimeric CaR) in addition to the monomeric species. This 280-kDa band in Fig. 2, lane 1, lined up with the upper band of the doublet observed between 200 and 300 kDa in Fig. 2, lane 2. It is likely that this 280-kDa band in Fig. 2, lanes 1 and 2, represents a more denatured dimeric receptor than the lower band at 250 kDa in Fig. 2, lane 2, if both bands are homodimeric receptors.

To show that the putative dimers of the CaR observed on the cell surface were not artifacts of other types of nonspecific aggregation occurring during protein preparation, we stabilized pre-existing multimeric forms of the receptor on the cell surface by covalently linking them with BS3, a noncleavable, membrane-impermeant cross-linker, while we surface-labeled the cells with Sulfo-NHS-Biotin. The surface-biotinylated and cross-linked cells were then lysed in the presence of iodoacetamide. The Flag-tagged wild type receptor was immunoprecipitated, and the immunoprecipitated CaR was eluted with DTT-containing, SDS-sample buffer. When we increased the concentration of cross-linker, the ratios of dimer to monomer increased progressively (Fig. 4A). Without cross-linker, the monomer is the principal CaR species identified on the cell surface (Fig. 4A, lane 1) while with 5 mM BS3, the dimer becomes the major species visible on the blot (Fig. 4A, lane 4). This result confirms that the dimeric receptor is the principal species present on the cell surface. The reduction in intensities of overall surface labeling by the Sulfo-NHS-Biotin in the presence of BS3 occurs because both reagents form covalent bonds with the same pool of primary amines on the receptor.

When we employed anti-CaR antiserum to detect CaR-immunoreactive proteins to assess sample loading after removal of the avidin, we found that the sample loaded in Fig. 4, lane 1, was somewhat less than others based on the CaR immunoreactivities of the intracellular species, i.e., the lower band at 140 kDa in Fig. 4B, lane 1. Of interest, we found that the cross-linker did not change CaR immunoreactivity at 160 kDa in Fig. 4B, lane 1.
centrations of BS3, a cross-linker, in millimolar, prior to lysing the cells. HEK293 cells were transfected with Flag-tagged CaR. Proteins on the cell surface were treated with both Sulfo-NHS-Biotin and varying concentrations of BS3, a cross-linker, in millimolar, prior to lysing the cells. The CaR was then immunoprecipitated with anti-Flag antibody. The immunopurified protein samples were eluted with DTT-containing SDS buffer and subjected to SDS-PAGE (3–10%). Surface expression of the CaR was detected with avidin (panel A). Both surface and intracellular forms of the CaR were then detected with anti-CaR antibody (4641) after removal of the avidin (panel B).

4B, unlike what we observed for the surface expression of the CaR in Fig. 4A, and all CaR-immunoreactive bands had similar ratios of monomers relative to dimers or oligomers (Fig. 4B). Therefore, the amount of the receptor on the cell surface must be sufficiently small that any changes in surface distribution between the monomeric and oligomeric forms as a result of cell-surface cross-linking does not produce any readily detectable alterations in the ratios of the intensities of the various CaR-immunoreactive bands when detected with anti-CaR antibody.

The Cytoplasmic Tail of the CaR Is Not Required for Dimerization—To examine the role of the CaR cytoplasmic tail in dimerization, several receptors with varying degrees of truncation of their carboxyl-terminal tails were constructed. We introduced stop codons at amino acid positions 863 (located at the beginning of the cytoplasmic tail), 877, and 892; the respective receptors are referred to below as K863Stop, A877Stop, and S892Stop. The truncated receptors, K863Stop (data not shown), similar to a slightly longer receptor terminated at S892Stop was not only active but also had a lower EC50 of 3.2 μM (26), and A877Stop (9), were functionally inactive, whereas S892Stop was only active but also had a lower EC50 of 3.2 ± 0.1 μM (n = 6) for high Ca2+-evoked increases in Ca2+, than did the full-length wild type receptor (4.0 ± 0.2 mM, n = 4).

We then transfected HEK293 cells with the truncated receptors, surface-biotinylated the cells, and immunoprecipitated as above. Western analysis showed that the levels of expression of the truncated receptors were either similar to (K863Stop) or more than (A877Stop and S892Stop) that of the wild type receptor, and all three truncated receptors formed homodimers on the cell surface similar to the full-length, wild type receptor (Fig. 5) under nonreducing conditions. This result raised the possibility that the truncated receptors could form heterodimers with the full-length receptor when the two receptors were co-transfected, if high molecular weight species detected in nonreducing conditions (Fig. 5) were truly homodimers. Therefore, we next undertook studies to prove that the high molecular weight species of the various CaRs observed in Figs. 2 and 5, i.e. the wild type receptor and the truncated receptors, are indeed homodimers.

Co-transfection of Full-length Wild Type and Tail-truncated CaRs Produce Heterodimers in Addition to the Respective Homodimers—To determine whether the full-length and truncated receptors associate, we co-transfected the nontagged truncated receptors with a Flag-tagged full-length receptor or Flag-tagged truncated receptors with a nontagged full-length receptor and immunoprecipitated the tagged receptors. If the nontagged and tagged receptors formed heterodimers, we would be able to co-immunoprecipitate nontagged receptors with tagged receptors. Because monomeric full-length and truncated receptors can be resolved under reducing conditions on SDS-PAGE, we would be able to determine the relative amounts of tagged and nontagged receptors, which were associated during immunoprecipitation prior to elution with SDS-sample buffer containing DTT, on Western blot using avidin as a probe if we surface-biotinylated the co-transfected cells. Subsequently, we would be able to determine the nature of the association, namely dimers or higher oligomers, on nonreduced SDS-PAGE.

Fig. 6A, lanes 2, 4, 6, 9, 11, and 13, shows that surface forms of nontagged receptors in co-transfected cells were co-immunoprecipitated using the anti-Flag antibody, which was resolved from co-transfected tagged receptors on a reduced SDS-PAGE and detected with avidin. As a control, the nontagged receptors isolated from singly transfected cells could not be immunoprecipitated by anti-Flag antibody (Fig. 6A, lanes 1, 10, 12, and 14). On nonreduced PAGE, only oligomers (mostly dimers) were detected, and no monomeric forms of either full-length or truncated receptors were detected on the surface of co-transfected cells (Fig. 6B). As shown in Fig. 6A, the intensity of the Flag-tagged receptor was higher than that of the nontagged receptor, indicating that besides the heterodimers, there were substantial amounts of the homodimers of each of the co-transfected receptors. Of interest, the expression of the full-length receptor was significantly increased in cells co-transfected with S892Stop (Fig. 6A, lane 13) compared with that in cells transfected with the full-length receptor alone (Fig. 6A, lane 8).

DISCUSSION

The cross-linking and co-immunoprecipitation experiments carried out in the present study, together with Western analysis of cell-surface CaR under nonreducing conditions, clearly demonstrate that the CaR resides on the cell surface mostly as a dimer and possibly as higher oligomers but hardly at all as a monomer. Formation of homodimers between cytoplasmic tail-truncated receptors suggests that the cytoplasmic tail is not necessary for homodimerization. Unlike epidermal growth factors, platelet-derived growth factor and fibroblast growth factor...
receptors, which undergo ligand-induced dimerization upon activation (27), the dimerization of the CaR is not agonist-dependent.

Fig. 3 shows that iodoacetamide blocks nonspecific aggregation of the CaR in whole cell lysates. Because iodoacetamide blocks the thiol groups on free cysteines from forming disulfide bonds with other free cysteines in the vicinity, the aggregates formed in the absence of iodoacetamide were most probably because of oxidation of the three cysteines that are present in the cytoplasmic tail of the CaR during the preparation of cellular proteins for gel electrophoresis. In the intact cell, these intracellular cysteines likely remain in a reduced form because of the strongly reducing intracellular environment maintained by the glutathione system (28). Indeed, no such nonspecific aggregation was observed with a mutant CaR in which the entire carboxyl-terminal tail was deleted, even in the absence of iodoacetamide.²

The majority of the surface dimeric form of the CaR and higher oligomers of the receptor observed under nonreducing conditions is readily converted to a single form of the monomeric receptors with a molecular mass of 160 kDa under reducing conditions (Fig. 2). Our earlier biochemical studies had shown that there are three monomeric forms of the CaR in crude membrane preparations of CaR-expressing HEK293 cells with molecular masses of 120, 140, and 160 kDa on reduced SDS-PAGE. These forms of the receptor represent the nonglycosylated CaR and receptors with varying types and extent of glycosylation (4), respectively. The 160-kDa species contains N-linked complex carbohydrates that we refer to as the mature form, whereas the 140-kDa species contains N-linked, high mannose-containing carbohydrates. The exclusive conversion of the surface CaR to a single form of monomeric receptor (160 kDa, Fig. 2, lane 1) suggests that only the CaR N-glycosylated with complex carbohydrate reaches the cell surface. In other words, biosynthetic intermediates, such as the immature forms of the CaR N-glycosylated with high mannose (140 kDa), fail to arrive at the cell surface.

The sensitivity of the dimeric receptor to reducing agents suggests that one type of intermolecular interaction mediating dimerization is intermolecular disulfide bonds. It is likely that the cysteine(s) involved in the intermolecular disulfide bond(s) of the CaR are located in the same general region identified in mGluR5 (15), because mGluR5 and the CaR share the relative positions of 20 cysteines. These shared cysteines may form either intramolecular disulfide bonds that are involved in the correct folding of both the CaR and mGluRs or intermolecular disulfide bonds for dimerization of these two receptors. It has been shown that the cysteine(s) involved in the intermolecular disulfide linkage of dimeric mGluR5 are located within about 17 kDa of the amino terminus (15). In this region, the CaR has six cysteines, including two within the putative signal peptide that are likely cleaved off by signal peptidase during biosynthesis of the receptor. Therefore, one or more of the four cysteines located within 17 kDa of the amino terminus and/or some of the nonconserved cysteines present in CaR may be involved in forming intermolecular disulfide bonds.

Moreover, we noted putative dimers even in the presence of reducing agents when we detected the CaR with either avidin, to detect cell-surface expression (Figs. 2 and 4), or an anti-CaR antiserum (4641) to assess the overall level of CaR protein expression (Figs. 1B and 4B), including both the mature and immature forms. It has been suggested that a noncovalent hydrophobic dimerization motif in the β₂-adrenergic receptor (18) mediates the formation of a functionally important homodimer that is SDS-resistant. Interestingly, this consensus dimerization motif for noncovalent hydrophobic interactions is also present in the CaR within TM5 (LMA[LGF]GYTC[L], note that the conserved amino acids forming the putative intermolecular interface are underlined). Molecular modeling of GPCRs has suggested that TM5 is one of the most membrane-exposed of all the transmembrane segments, likely making this motif in one receptor molecule accessible to the same motif in another receptor molecule (29). Our previous studies showed that P747frameshift (a mutation identified in familial hypocalciuric hypercalcemia syndrome with a single-base deletion as well as a separate transcription in codon 747, which normally encodes proline, thereby resulting in a frameshift and a truncated CaR lacking TM5 and the rest of carboxyl terminus) can no longer form an SDS-resistant dimer in the presence of 100 mM DTT (5). Thus, the CaR’s putative dimerization motif in TM5 could contribute to the formation of dimers of the receptor.

² M. Bai, unpublished observation.
and/or stabilization of dimeric CaR during post-translational processing.

In conclusion, we have demonstrated that the CaR resides on the cell surface as homodimers that likely represent the active form of the receptor and contribute to the high degree of apparent cooperativity observed for the CaR. It is likely that the wild type and mutant CaRs form heterodimers in familial hypocalciuric hypercalcemia patients carrying a normal CaR on one allele and an abnormal CaR on the other. Furthermore, dominant negative phenotypes present in some familial hypocalciuric hypercalcemia patients suggest that formation of heterodimers between wild type and mutant receptors may negatively affect the function of the normal receptor in the heterodimeric complex. For example, a purely random association of the mutant receptor with either wild type CaRs or with other mutant receptors in the heterozygous state in familial hypocalciuric hypercalcemia could potentially reduce the level of the wild type homodimer to approximately one-quarter of that present in normal individuals.

Acknowledgments—We thank Dr. O. Kifor for helpful advice and Drs. J. E. Garrett, I. V. Capuano, A. Paruhar, F. Fuller, R. T. Simin, and K. V. Rogers for providing us with the HEK293 cells and the 4641 antibody.

REFERENCES

1. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993) Nature 366, 575–580
2. Pollak, M. R., Brown, E. M., Chou, Y. H., Hebert, S. C., Marx, S. J., Steinmann, B., Levi, T., Seidman, C. E., and Seidman, J. G. (1993) Cell 75, 1297–1303
3. Pollak, M. R., Brown, E. M., Estep, H. L., McMahan, P. N., Kifor, O., Park, J., Hebert, S. C., Seidman, C. E., and Seidman, J. G. (1994) Nat. Genet. 8, 303–307
4. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H. S., Pollak, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) J. Biol. Chem. 271, 19537–19545
5. Pearce, S. H., Bai, M., Quinn, S. J., Kifor, O., Brown, E. M., and Thakker, R. V. (1996) J. Clin. Invest. 98, 1860–1866
6. Pearce, S. H., Williamson, C., Kifor, O., Bai, M., Coulthard, M. G., Davies, M., Lewis-Barned, N., McCreedie, D., Powell, H., Kendall-Taylor, P., Brown, E. M., and Thakker, R. V. (1996) New Engl. J. Med. 335, 1115–1122
7. Bai, M., Pearce, S. H. Kifor, O., Trivedi, S., Stauffer, U. G., Thakker, R. V., Brown, E. M., and Steinmann, B. (1997) J. Clin. Invest. 99, 88–96
8. De Luca, F., Ray, K., Marcello, E. E., Fan, G. F., Winer, K. K., Gore, P., Spiegel, A. M., and Baron, J. (1997) J. Clin. Endocrinol. Metab. 82, 2710–2715
9. Bai, M., Jancic, N., Trivedi, S., Quinn, S. J., Cole, D. E. C., Brown, E. M., and Hendy, G. N. (1997) J. Clin. Invest. 99, 1917–1925
10. Potter, I. T., Ballesteros, I. A., Bichajian, L. H., Ferrendelli, C. A., Fisher, A., Hanchett, H. E., and Zhang, R. (1991) Mol. Pharmacol. 39, 211–221
11. Hirschberg, B. T., and Schimerlik, M. I. (1994) J. Biol. Chem. 269, 26127–26135
12. Wessling-Resnick, M., and Johnson, G. L. (1987) J. Biol. Chem. 262, 3697–3705
13. Willardson, B. M., Pou, B., Yoshida, T., and Bitensky, M. W. (1993) J. Biol. Chem. 268, 6371–6382
14. Ward, D. T., Brown, E. M., and Harris, H. W. (1998) J. Biol. Chem. 273, 14476–14483
15. Romano, C., Yang, W. L., and O'Malley, K. L. (1996) J. Biol. Chem. 271, 28612–28616
16. Arvisser, S., Amritai, G., and Sokolovsky, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 156–159
17. Venter, J. C., and Fraser, C. M. (1983) Fed. Proc. 42, 273–278
18. Hebert, T. E., Moffett, S., Morello, J. P., Laisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) J. Biol. Chem. 271, 16384–16392
19. Herberg, J. T., Codina, J., Rich, K. A., Rojas, F. J., and Iyengar, R. (1984) J. Biol. Chem. 259, 9285–9294
20. Cvejic, S., and Devi, L. A. (1993) J. Biol. Chem. 268, 26959–26964
21. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
22. Hawley-Nelson, P., Ciccarrone, V., Gebeylehu, G., Jesse, J., and Felgner, P. L. (1993) Focus 13, 73–79
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Fajtova, V. T., Quinn, S. J., and Brown, E. M. (1991) Am. J. Physiol. 261, E151–E158
25. De Luca, F., Ray, K., Fan, G. F., Goldsmith, P. K., and Spiegel, A. M. (1997) J. Biol. Chem. 272, 31135–31140
26. Heldin, C. H. (1995) Cell 80, 213–223
27. Schulz, G. E., and Schirmer, R. H. (1985) Principles of Protein Structure (Cantor, C. R., ed) 1st Ed., pp. 53–55, Springer-Verlag New York Inc., New York
28. Baldwin, J. M. (1993) EMBO J. 12, 1693–1703