Human Ca\textsuperscript{2+} Receptor Extracellular Domain

ANALYSIS OF FUNCTION OF LOBE I LOOP DELETION MUTANTS

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The G protein-coupled Ca\textsuperscript{2+} receptor (CaR) possesses an ~600-residue extracellular domain involved in ligand binding and receptor activation. Based on an alignment of the amino acid sequence of the CaR with that of bacterial periplasmic-binding proteins, the first ~530 residues of the extracellular domain are believed to form a domain resembling a bilobed Venus’s flytrap (VFT). Four insertions in the CaR sequence that do not align with those of bacterial periplasmic-binding proteins correspond to four loops within lobe I of the VFT. We constructed a series of deletion mutants of these four loops and tested their ability to form fully processed CaR as well as their ability to be activated by Ca\textsuperscript{2+}. As many as 21 residues (365–385) of loop III could be deleted without impairing receptor expression or activation. Deletion of portions of either loops I (50–59) or IV (438–445) did not impair receptor expression but significantly reduced Ca\textsuperscript{2+} activation. Deletion of the entire loop II (117–137) abolished receptor expression and function, but the replacement of even a single residue within this deletion mutant led to expression of a monomeric form of the receptor showing increased Ca\textsuperscript{2+} sensitivity but reduced maximal activation. Our results reveal that certain residues within loops I and IV are dispensable in formation of the VFT domain but are critical for Ca\textsuperscript{2+} activation of the receptor. In contrast, the residues in loop II are critical for maintaining the inactive state of the CaR. We discuss these results in light of the recently defined crystal structure of the homologous domain of the type 1 metabotropic glutamate receptor.

The Ca\textsuperscript{2+} receptor (CaR)\textsuperscript{1} plays a central role in the regulation of extracellular calcium ion ([Ca\textsuperscript{2+}]\textsubscript{o}) homeostasis (1). Activation of the CaR leads to stimulation of phospholipase C and resultant increased phosphoinositide (PI) hydrolysis. CaR activation mediates inhibition of parathyroid hormone secretion in parathyroid cells and inhibition of Ca\textsuperscript{2+} reabsorption in the kidney. Inactivating and activating mutations of the CaR cause disorders of [Ca\textsuperscript{2+}]\textsubscript{o} homeostasis: familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia, respectively (2).

The CaR is a member of family 3 of the superfamily of G protein-coupled receptors (3). In addition to amino acid sequence conservation, the CaR shares with other members of family 3, such as the metabotropic glutamate receptors (mGluR), a unique structural feature, namely a large ~600-residue extracellular amino-terminal domain (ECD). The ECD in typical family 3 members consists of an ~530-residue domain, which based on limited homology to bacterial periplasmic-binding proteins has been suggested to resemble a bilobed Venus’s-flytrap (VFT) (4), followed by an ~70-residue cysteine-rich domain (5).

Based on an alignment of the human CaR (hCaR) ECD amino acid sequence with that of the mGluR1 ECD and the *Escherichia coli* leucine/isoleucine/valine bacterial periplasmic-binding protein, we previously created a model of residues 36–513 of the hCaR ECD (see figure 6 in Ref. 6). The model shows a bilobed VFT with lobe I (containing the amino terminus) connected by three strands to lobe II (containing the carboxyl terminus). Four insertions in the hCaR and mGluR1 sequence that could not be aligned with leucine/isoleucine/valine bacterial periplasmic-binding protein could not be modeled, and hence these were shown as unstructured loops (designated I–IV). Loops I–IV were all contained in lobe I in our model. We also showed that the hCaR is an intermolecular disulfide-linked homodimer and identified cysteines 129 and 131, which are located in loop II as the residues involved in covalent dimerization (6).

Recently, Kunishima *et al.* (7) determined the three-dimensional structure of residues 33–522 from the ECD of the rat mGluR1 in two free (unliganded) forms and in a glutamate-bound form. All three forms appear as intermolecular disulfide-linked homodimers with each monomer consisting of a bilobed VFT. The flytrap is closed in the ligand-bound state and either open (free form I) or closed (free form II) in the unliganded state. The sequences corresponding to loops I–IV in our hCaR model are all confirmed to occur in lobe I, and for loops I, III, and IV are shown to be loops connecting regions of secondary structure in the mGluR1 crystal structure. Loop II, which was confirmed to be the site of covalent dimerization (involving the single cysteine 140 in rat mGluR1), appears as a disordered (in the ligand-bound form) or partially disordered (in the free form I) region in the mGluR1 three-dimensional structure.

Given that the VFT structure can be formed in leucine/isoleucine/valine bacterial periplasmic-binding protein without the regions corresponding to loops I–IV, we sought to understand the importance of these loops in the formation of the basic VFT structure. We tested the hypothesis that some or all of the residues comprising loops I–IV are unnecessary for VFT
formation but might be important for activation of the hCaR. To this end, we constructed a series of deletion mutants of loops I–IV of the hCaR (see Fig. 1) and tested their ability to form fully processed receptor protein as well as their ability to be activated by [Ca\textsuperscript{2+}].

MATERIALS AND METHODS

Site-directed Mutagenesis of the hCaR—The hCaR cDNA cloned in the pCR3.1 expression vector was described previously (8) and kindly provided by Dr. Kausik Ray. Site-directed mutagenesis was performed using the QuickChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene, according to manufacturer instructions). Briefly, a pair of complementary primers 35–55 bases long was designed for each deletion mutant, with the sequence to be deleted placed in the middle of the primers (the sequences of all primers are available from the authors upon request). Parental hCaR inserted into pCR3.1 was amplified using Pyrococcus furiosus DNA polymerase with these primers for 16 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the parental DNA with DpnI, the amplified DNA with the deletion incorporated was transformed into E. coli DH-5 strain. The mutations were confirmed by automated DNA sequencing using a dRhodamine Terminator cycle sequencing kit and ABI Prism 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Deletion mutations are designated by a loop number (I, II, III, IV) and a mutant hCaR protein and deletion mutants were detected with an ECL system (Amersham Pharmacia Biotech).

Materials and Methods

Treatment of Detergent-solubilized Whole-cell Extracts with endoglycosidase H—Cell extracts (40 μl) diluted in 40 μl of 50 mM sodium acetate, pH 4.8, were treated with 0.5 milliunits of endoglycosidase H (Boehringer Mannheim) and the specific range of residues deleted (using the numbering of the hCaR amino acid sequence; see Fig. 1A). The residues within the indicated range that were not deleted are indicated in single-letter amino acid code between the numbers indicating the deletion range. The L2Δ117–137 deletion mutant was created by introducing HpaI and Eco47III sites, respectively, before the codon encoding Val367 and after the codon encoding Tyr411 followed by digestion of the resultant mutated sites with both enzymes, and the digestion product was religated to create the deletion mutant. This deletion mutant DNA was then used as template in the next round of mutagenesis to generate the L2Δ118–137, L2Δ117–N137, and L2Δ117–136 deletion mutants. The L3Δ371–385 deletion mutant was created in an analogous way by introducing BstI and HpaI sites, respectively, before the codon encoding Val370 and after the codon encoding Tyr411 followed by digestion and religation. This deletion mutant was used as template in the next round of mutagenesis to generate the L3Δ365–385 deletion mutant. Similarly, the L3Δ338–385 deletion mutant was obtained by introducing the BstI1107.1 and HpaI sites, respectively, before the codon encoding Val370 and after the codon encoding Asn406 in hCaR. The mutant incorporating these sites was digested with both enzymes, and the digestion product was religated to create the deletion mutant. This deletion mutant DNA was then used as template in the next round of mutagenesis to generate the L3Δ359–137 deletion mutant was created by introducing the BstI1107.1 sites before the codons encoding Val370 and Tyr411 followed by digestion with Bst1107.1 and religation. To generate loop 4 deletion mutants, the hCaR was used as template to delete from amino acid 438–445 (L4Δ438–445), 440–444 (L4Δ440–444), 447–453 (L4Δ447–453) and to generate the L4Δ438-NOS-453 deletion mutant. All the DNAs modified were digested, purified with QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) and religated with Rapid DNA ligation kit (Roche Molecular Biochemicals).

Functional Properties of Loop I Deletion Mutant Receptors—Loop I in the hCaR is comprised of the residues 39–67 (Fig. 1), which connect β-sheet “b” to α-helix “A” in the mGluR1 crystal structure. We have shown previously that mutation of either cysteine 60 (within loop I) or cysteine 101 severely impairs the normal expression and function of the hCaR (6). In the mGluR1 crystal structure, the corresponding cysteines are shown to form an intramolecular disulfide that seems critical for receptor expression and function. We reasoned that the deletion of parts of loop I that include cysteine 60 would abolish receptor expression and therefore focused our efforts on the region preceding cysteine 60. We constructed deletion mutants L1Δ50–59 and L1Δ48–59 lacking 10 and 12 amino acids, respectively. These deletion mutants and the wild-type (WT) receptor were transiently transfected into HEK-293 cells, their expression was analyzed by immunoblot, and their ability to respond to [Ca\textsuperscript{2+}]\textsuperscript{2+}, was analyzed by measuring stimulation of PI hydrolysis (Fig. 2). Both the L1Δ48–59 and L1Δ50–59 deletion mutants showed dramatically reduced maximal responses to [Ca\textsuperscript{2+}]\textsuperscript{2+}, and a rightward shift in sensitivity (L1Δ50–59 about 25% of WT maximal response with EC50 values of 5.8 ± 1.2 and 2.53 ± 0.07 μM, respectively). Importantly, the expression of these deletion mutants was at least as good as that of the WT hCaR as seen on immunoblot (Fig. 2, right). Under reducing conditions, the ADD antibody recognizes two major bands of 150 and 100 kDa in both L1Δ50–59 and L1Δ48–59 and L1Δ50–59 receptors. The ~150-kDa band represents the fully processed receptor molecules modified with N-linked and O-linked carbohydrates, and expressed on the cell surface, and the ~130-kDa band represents high mannose-modified intracellular forms of the receptor (6, 12, 13). Both deletion mutant receptors (L1Δ50–59 and L1Δ48–59) were, similar to the WT hCaR, expressed on the cell surface as intermolecular disulfide-linked dimers as revealed by immunoblot under nonreducing conditions (data not shown).
In the alignment of the loop I amino acid sequence of the hCaR with rat mGluR1 (7), six additional residues (42–47) are present in the hCaR. We speculated that these might be deleted without impairing receptor expression or function. Analysis of the L1/H900442–47 deletion mutant, however, showed that its response to \([\text{Ca}^{2+}]_o\) was virtually abolished, and its expression was severely impaired in that only the 130- kDa band corresponding to the intracellular incompletely processed form of the receptor was observed on immunoblot (Fig. 2).

**Functional Properties of Loop II Deletion Mutant Receptors**—Loop II is comprised of residues 117–137 of the hCaR (Fig. 1). This corresponds to a disordered region between helix B and \(\beta\)-sheet “d” in the mGluR1 crystal structure (7). Deletion of this entire region (mutant receptor L2 Δ117–137) almost completely abolished the response to \([\text{Ca}^{2+}]_o\) (maximal response was 14% of the Wt, Fig. 3, left). As shown in the immunoblot in Fig. 3, right, two bands of ~130 and ~118 kDa, distinctly lower than the ~150- and ~130-kDa bands seen with the Wt receptor, were detected under reducing conditions in the lysates of cells expressing the L2 Δ117–137 deletion mutant. Under nonreducing conditions, the Wt receptor shows a doublet above the 213-kDa marker corresponding to the dimeric forms of the ~150- and ~130-kDa bands. In contrast, the L2 Δ117–137 deletion mutant, under nonreducing conditions, shows persistence of immunoreactive bands representing monomeric forms in addition to a higher molecular size band (Fig. 3, right). The persistence of monomers of the L2 Δ117–137 deletion mutant under nonreducing conditions reflects deletion of cysteines 129 and 131, which we have shown to be responsible for the intermolecular disulfide-linked dimerization of the hCaR (6).
higher molecular size bands seen under nonreducing conditions for the mutant receptor may reflect aggregated forms as we have observed previously for other mutant forms of the hCaR (6).

To define the nature of the ~130- and ~118-kDa bands identified by immunoblot under reducing conditions for the L2Δ117–137 deletion mutant, we tested their sensitivity to digestion with Endo H. In contrast with the Wt receptor for which only the lower ~130-kDa band was Endo H-sensitive, both of the deletion mutant immunoreactive bands were digested by Endo H (Fig. 4). Thus both mutant bands represent incompletely processed intracellular forms (6, 9, 12, 13). A faint band (Fig. 4, arrow) resists Endo H digestion and presumably reflects the small proportion of the L2Δ117–137 deletion mutant that is fully processed and expressed at the cell surface. This likely accounts for the small residual activity (Fig. 3, left) of the mutant receptor.

Based on the results obtained with the L2Δ117–137 deletion mutant, we explored the minimal sequence of loop II necessary to produce activity of the hCaR. Three additional loop II deletion mutants were created in which a single amino acid of loop II was replaced within the L2Δ117–137 deletion mutant. These were termed L2Δ118–137 (replacement of Gln117), L2Δ117N-137 (replacement of Asn), and L2Δ117–136 (replacement of Ser). As shown in Fig. 5, left, the resulting mutants had ~40% of the Wt activity as determined by the stimulation of PI hydrolysis. Interestingly, a significant left shift in dose-dependent [Ca\(^{2+}\)]\(_{o}\) response compared with the Wt hCaR was observed. The maximal response to calcium (at 8 mM) when
comparing with the wild type was 39, 44, and 34% for 
L2 Δ118–137, L2 Δ117-N-137, and L2 Δ117–136 deletion mutants, respectively (EC_{50} values for the L2 Δ118–137, L2 Δ117-N-137, and L2 Δ117–136 deleted mutants were 0.518 ± 0.16, 0.779 ± 0.136, and 0.500 ± 0.093 μM, respectively). The expression pattern of these deletion mutants on immunoblot (Fig. 5, right) differed from both Wt and the L2 Δ117–137 deletion mutant. Under reducing conditions, the ~118 kDa detected in the L2 Δ117–137 was clearly diminished in the L2 Δ117–136 deletion mutant and was barely detected in the L2 Δ118–137 and L2 Δ117-N-137 mutants. The faint band just above the ~130 kDa band (Fig. 5, right, arrow) seen for the L2 Δ117–137 deletion mutant was increased in expression in the other three loop II deletion mutants. This band, based on resistance to Endo H, corresponds to the fully processed form of the receptor and persists in monomeric form (arrow) under nonreducing conditions.

**Functional Properties of Loop III Deletion Mutant Receptors**—Loop III is comprised of residues 356–416 of the hCaR and is the longest of the four loops present in lobe I of the VFT domain (Fig. 1). Loop III connects helices L and M in the mGluR1 crystal structure and contains an intramolecular disulfide linking the residues equivalent to cysteines 358 and 395 in the hCaR (7). We have shown previously that mutation of either of these cysteines abolishes expression and activity (6). Not surprisingly then, two deletion mutants we made that each delete one of these cysteines, L3 Δ338–385 and L3 Δ371–410, respectively, abolished activity (Fig. 6, left) and expression of the fully processed form (Fig. 6, right) of the hCaR. Alignment of the sequences corresponding to loop III in the hCaR and in rat mGluR1 shows that the hCaR sequence has an insert of about 21 extra amino acids. To test the functional importance of these residues, we made two further deletion mutants denoted as L3 Δ371–385 and L3 Δ365–385, respectively. As shown in Fig. 6, left, both deletion mutants were nearly comparable in activity to the Wt hCaR, with the L3 Δ365–385 mutant slightly higher in maximal activity than Wt but not left-shifted as with some of the loop II deletion mutants. The expression of the L3 Δ371–385 and L3 Δ365–385 deletion mutants was also similar to that of the Wt receptor on immunoblot except for a small increase in mobility of the first mutant (Fig. 6, right). Both deletion mutants (L3 Δ371–385 and L3 Δ365–385) were expressed at the cell surface as dimers as revealed by immunoblot under nonreducing conditions (data not shown).

**Functional Properties of Loop IV Deletion Mutant Receptors**—Loop IV is the smallest of the four loops and comprises residues 437–449 of the hCaR (Fig. 1). This loop connects helices M and N in the mGluR1 crystal structure and contains an intramolecular disulfide linking cysteine equivalent to 437 and 449 in the hCaR (7). We have shown previously that mutation of either of these cysteines causes a modest reduction in receptor activity (14). Loop IV also contains an insert in the hCaR not present in mGluR1 corresponding to residues 440–444. To test the function of residues in loop IV we made four distinct deletion mutants designated as L4 Δ440–444, L4 Δ438–445, L4 Δ438–NGS–453, and L4 Δ447–453. As shown in Fig. 7, left, all these deletion mutants displayed a dose-dependent response to calcium but with a decrease in maximal activity and right-shift in EC_{50} values. The maximal activity and EC_{50} values, respectively, were 75% and 4.1 ± 0.06 mM for L4 Δ440–444, 50% and 4.9 ± 0.47 mM for L4 Δ438–445, 35% and 4.5 ± 0.07 mM for L4 Δ438–NGS–453, and 25% and 5.45 ± 0.57 mM for L4 Δ447–453, compared with the Wt hCaR (EC_{50} value of 2.47 ± 0.08 mM). The expression levels of these deletion mutants on immunoblot (Fig. 7, right) were either comparable with Wt (L4 Δ440–444 and L4 Δ438–445) or slightly diminished (L4 Δ438–NGS–453 and L4 Δ447–453). All these deletion mutants were expressed at the cell surface as dimers (data not shown).

**DISCUSSION**

The varying consequences of deleting parts of the four loops in lobe I of the hCaR VFT domain reveal clear differences in the functional importance of these parts of the ECD. The deletion of residues 48–59 or 50–59 in loop I drastically reduced receptor activity without significantly impairing receptor expression. This suggests that these residues are not required for VFT formation but that they play an important role in calcium activation of the receptor. One possibility would be that these residues are directly involved in calcium binding. This region contains four acidic amino acids (Asp^{48}, Asp^{50}, Glu^{49}, and Glu^{59}), and acidic amino acids have been speculated to play a role in the low-affinity calcium-binding characteristic of the CaR ECD (15). We noticed, moreover, that Asp^{48}, Asp^{50}, and Glu^{59} correspond to a calcium-binding motif identified in several calcium-binding proteins including troponin C, parvalbumin, and certain integrin repeats (16). To test the possibility that these residues are directly involved in calcium binding, we made single missense mutants (D48A, D50A) as well as a triple missense mutant (D48A, D50A, E59A). Because ligand-binding assays for the CaR have not been developed, we could not assess calcium binding to these or other mutants directly, so instead we tested their response to calcium in the PI assay. All these missense mutants showed expression and calcium activation equivalent to Wt, suggesting that this region in loop I is not involved directly in calcium binding. Instead, we speculate that this portion of loop I is required for a change in conformation that occurs after calcium binding, and that is critical for receptor activation. Consistent with this possibility, the corresponding region in the mGluR1 crystal structure was noted to form part of the dimer interface (7). The extent of the dimer interface involving loop I doubles from three to six of these residues in going from the free to the ligand-bound form.

In contrast to the deletion of loop I residues 48–59, the

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2 G. R. Cruz, unpublished observations.
deletion of residues 42-47 abolished both activation and expression of the fully processed form of the receptor. This was somewhat surprising given that these residues are an "extra" insert in loop I of the hCaR compared with the mGluR sequence but suggests that this region is critical for folding and VFT formation in the CaR. A further indication of the possible functional importance of residues in loop I is that three distinct loss-of-function mutations within this loop, P39A, S53P, and P55L, have been identified in familial hypocalciuric hypercalcemia (2).

Considerable previous evidence suggested that loop II is a critical region for CaR activation. We have shown that when both cysteines 129 and 131 in loop II are mutated, the CaR is expressed as a monomer, when analyzed by immunoblot under denaturing but nonreducing conditions (6). This monomeric form of the CaR is lower in maximal activity than Wt but is left-shifted in its concentration dependence for calcium activation. Similar left shifts in calcium sensitivity are observed for other wild-type hCaR or deletion mutants including L3 Δ371-385, L3 Δ365-385, L3 Δ338-385, and L3 Δ371-410. The methods and format for presentation of results are as described in the Fig. 3 legend.

Deletion of residues 117-137 comprising essentially all of loop II resulted in a drastic reduction in activity. Although the basis for the two bands seen on immunoblots of the L2 Δ118-137 deletion mutant is not completely clear, their sensitivity to Endo H digestion supports the idea that most of the mutant receptors are not fully processed nor do they reach the cell surface. This accounts for the sharp reduction in activity and indicates that loop II is required for the proper folding and processing of the CaR. Replacement of even a single amino acid, specifically Gln117, Asn, or Ser, in this loop II deletion mutant led to a major increase in expression of the fully processed form of the CaR and in response to calcium. In all three cases, although maximal activity was reduced, a left shift in calcium sensitivity was observed, reinforcing the importance of loop II in regulating calcium responsiveness of the CaR. It is highly unlikely that the increase in sensitivity to calcium observed in various loop II mutants reflects a direct role for loop II as a calcium-binding site. Neither the nature of the missense mutations in loop II nor the effect of deleting most of the loop as in the L2 Δ118-137 deletion mutant, for example, is consistent with such a role. Instead, the crystal structure of the mGluR1 receptor (7) offers a possible explanation for the role of loop II in receptor activation. Although most of loop II is disordered in the ligand-bound mGluR1 crystal structure, the first seven residues of loop II undergo a conformational change in the free form to become an α-helical extension of helix B. Helices B and C form the largest surface of the dimer interface and undergo a 70° rotation between the free and ligand-bound forms of the receptor. In the free inactive form of mGluR1, the proximal portion of loop II, in addition to becoming an extension of helix B, becomes part of an extended dimer interface (7). Our present results with the loop II deletion mutants, interpreted in conjunction with the structural information from the crystal structures of the free, inactive, and ligand-bound active forms of mGluR1, suggest that loop II plays a role in maintaining the inactive form of the CaR. We speculate that changes in loop II that still permit folding and trafficking of the receptor to the cell surface lead to increased calcium sensitivity by removing a constraint to receptor activation. Perhaps this is caused by the reduction or abolition of the α-helical extension of helix B, thereby reducing the size of the dimer interface in the free inactive form of the receptor and making it easier to undergo the rotation that characterizes the transition from the inactive to active form of the receptor.

Loop III contains a sequence "insert" in the CaR not found in mGluR. The sequence of this insert is the least conserved within the ECD of the CaR among various species (1), suggesting that it may not be under evolutionary pressure because of a conserved function. Consistent with this possibility, deletion of as many as 21 residues corresponding to this insert in loop III had a minimal effect on either receptor expression or acti-
vation. These results do not of course exclude a functional role of this region in CaR, e.g. interaction with another protein that modulates CaR localization, but they do suggest that both CaR formation and activation can occur without this segment of the VFT.

Loop IV deletion mutants that abolish the presumptive intramolecular disulfide involving cysteines 437 and 449 reduced but did not abolish receptor expression and activation. This is consistent with previous results on missense mutations of the respective cysteines (14) and suggests that this disulfide is important in the folding and formation of the VFT structure. Interestingly, deletion of a five-amino acid insert in the CaR loop IV not found in mGluR did not compromise receptor expression but did cause moderate reduction in receptor activation along with a right shift in calcium sensitivity. Deletion of this insert is unlikely to have abolished the cysteine 437–449 disulfide, because the same disulfide is found in mGluR lacking the insert. Instead, we suggest that the effect of this deletion in loop IV reflects a role for this loop in receptor activation. Although loop IV is not part of the dimer interface in the mGluR1 crystal structure, it appears to be close to loop II and thus may be indirectly involved in receptor activation.

In summary, we have identified sequences within loops I and IV in lobe I of the hCaR VFT that are required for calcium activation, but not expression of the receptor, and sequences within loop II, the deletion of which increases calcium sensitivity of the receptor. A substantial portion of loop III of the receptor can be deleted without affecting expression or activation. These results form the basis for future studies directed at understanding how ligand binding to the ECD of family 3 G protein-coupled receptors leads to receptor activation.

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