Identification and Dissection of Ca\(^{2+}\)-binding Sites in the Extracellular Domain of Ca\(^{2+}\)-sensing Receptor*

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Ca\(^{2+}\)-sensing receptors (CaSRs) represent a class of receptors that respond to changes in the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) and activate multiple signaling pathways. A major barrier to advancing our understanding of the role of Ca\(^{2+}\) in regulating CaSRs is the lack of adequate information about their Ca\(^{2+}\)-binding locations, which is largely hindered by the lack of a solved three-dimensional structure and rapid off rates due to low Ca\(^{2+}\)-binding affinities. In this paper, we have reported the identification of three potential Ca\(^{2+}\)-binding sites in a modeled CaSR structure using computational algorithms based on the geometric description and surface electrostatic potentials. Mutation of the predicted ligand residues in the full-length CaSR caused abnormal responses to [Ca\(^{2+}\)]\(_{o}\) similar to those observed with naturally occurring activating or inactivating mutations of the CaR, supporting the essential role of these predicted Ca\(^{2+}\)-binding sites in the sensing capability of the CaSR. In addition, to probe the intrinsic Ca\(^{2+}\)-binding properties of the predicted sequences, we engineered two predicted continuous Ca\(^{2+}\)-binding sequences individually into a scaffold protein provided by a non-Ca\(^{2+}\)-binding protein, CD2. We report herein the estimation of the metal-binding affinities of these predicted sites in the CaSR by monitoring aromatic-sensitized Tb\(^{3+}\) fluorescence energy transfer. Removing the predicted Ca\(^{2+}\)-binding ligands resulted in the loss of or significantly weakened cation binding. The potential Ca\(^{2+}\)-binding residues were shown to be involved in Ca\(^{2+}\)-binding by high resolution NMR and site-directed mutagenesis, further validating our prediction of Ca\(^{2+}\)-binding sites within the extracellular domain of the CaSR.

Temporal and spatial changes of the Ca\(^{2+}\) concentration in the extra- and intracellular environments of cells affect the regulation of numerous cellular processes by modulating the activity of Ca\(^{2+}\) receptors and/or Ca\(^{2+}\)-binding proteins (1–3). Multiple Ca\(^{2+}\)-binding proteins with differing affinities have been identified in a variety of cellular compartments in all eukaryotic cells (4–6). Ca\(^{2+}\)-binding proteins have Ca\(^{2+}\) affinities that vary by 10\(^5\)–fold or more depending upon their locations and functions (7, 8). Intracellularly, Ca\(^{2+}\)-binding trigger proteins, such as calmodulin with its conserved EF-hand Ca\(^{2+}\)-binding sites, have Ca\(^{2+}\)-binding affinities in the submicromolar range (9). They respond to changes in the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) and regulate numerous cellular events and processes (10, 11). Extracellularly, Ca\(^{2+}\) also functions as a first messenger to direct numerous intracellular functions (12–14). The Ca\(^{2+}\)-sensing receptor (CaSR)\(^{4}\) that was initially cloned from the parathyroid gland (15) is a sensor of the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) that transforms the [Ca\(^{2+}\)]\(_{o}\) stimulus into a variety of intracellular responses to regulate multiple signaling pathways (including activation of phospholipases C, A\(_2\), and D) and inhibition of cAMP formation (16, 17). This receptor, along with the metabotropic glutamate receptors (mGluRs), γ-aminobutyric acid type B receptors, and receptors for pheromones, amino acids, and sweeteners, belongs to family C of the G protein-coupled receptor superfamily. Most members in this family have the capacity to sense [Ca\(^{2+}\)]\(_{i}\) (18, 19). More than 100 mutations and polymorphisms have been identified in the CaSR that either inactivate (have a reduced sensitivity to [Ca\(^{2+}\)]\(_{o}\)) or activate (have an enhanced sensitivity to [Ca\(^{2+}\)]\(_{o}\)) the receptor and that cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism (in the former) and autosomal dominant hypoparathyroidism (in the latter case) (20, 21).

The CaSR and other members of family C of the G protein-coupled receptor consist of a large extracellular domain (ECD), a transmembrane domain with seven transmembrane segments, and an intracellular C-tail segment (22, 23). Based on the functional responses at the cellular level, the ECD regions have been proposed to contain the major Ca\(^{2+}\)-binding sites and to respond to [Ca\(^{2+}\)]\(_{o}\) for both the mGluRs and CaSRs. The Hill coefficient suggests that 3–5 Ca\(^{2+}\) ions bind cooperatively to the CaSR (22, 24). In addition to Ca\(^{2+}\), the CaSR also responds to other ions, such as Mg\(^{2+}\), Ca\(^{2+}\)-sensing receptor; mGluR, metabotropic glutamate receptor; ECD, extracellular domain; ESI-MS, electrospray ionization mass spectrometry; PIPES, 1,4-piperazinediethanesulfonic acid; FRET, fluorescence energy transfer; HSQC, heteronuclear single quantum correlation; CaM, calmodulin.

* The abbreviations used are: CaSR, Ca\(^{2+}\)-sensing receptor; mGluR, metabotropic glutamate receptor; ECD, extracellular domain; ESI-MS, electrospray ionization mass spectrometry; PIPES, 1,4-piperazinediethanesulfonic acid; FRET, fluorescence energy transfer; HSQC, heteronuclear single quantum correlation; CaM, calmodulin.

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to Mg\(^{2+}\) (25), polyamines, and amino acids, such as L-Phe. Similar to the capacities of the mGluRs to respond to both L-Glu and high [Ca\(^{2+}\)]\(_{o}\), the Ca\(^{2+}\)-induced activation of the CaSR is potentiated by L-amino acids, particularly aromatic amino acids (26).

Progress in understanding the mechanism mediating [Ca\(^{2+}\)]\(_{i}\) regulation is largely hampered by a lack of knowledge regarding the Ca\(^{2+}\)-binding sites in the CaSR. To date, the identity of the Ca\(^{2+}\)-binding sites in the CaSR and related G protein-coupled receptors still remains unknown. X-ray structural determination by Kunishima et al. (27) reveals that the ECD of mGluR1 contains a Venus flytrap module. Key residues involved in glutamate binding were located at the interface between the two lobes (LB1 and LB2). However, no bound Ca\(^{2+}\) has been observed in these structures with or without the ligand glutamate (28). Major challenges in probing Ca\(^{2+}\)-binding sites in this class of receptors include the difficulties in crystallization, the rapid off rates because of the low affinity of the Ca\(^{2+}\) binding and the existence of multiple conformations that are in equilibrium with one another (29). Indeed, successful crystallization of the CaSR ECD has not been reported despite a decade or more of effort directed to this end. Furthermore, methods for direct measurement of Ca\(^{2+}\) binding to the CaSR have not yet been established (30, 31), possibly because of its large size, multiple binding sites, and conformational flexibility.

In this study, to reveal the potential Ca\(^{2+}\)-sensing locations in the CaSR, we first created model structures of the CaSR based on its structural homology to mGluR1. We then predicted several possible Ca\(^{2+}\)-binding sites using our established computational algorithms based on the common structural properties of Ca\(^{2+}\)-binding sites in proteins. We have shown that removing the predicted ligand residues in the full-length CaSR results in significant changes in the intracellular responses to [Ca\(^{2+}\)]\(_{i}\). Two predicted continuous Ca\(^{2+}\)-binding sites (e.g., sites with ligand-binding sites within short continuous primary sequences (<30 residues)) were further validated by inserting the sequences individually into a non-Ca\(^{2+}\)-binding host protein, CD2. The resulting engineered proteins (designated CD2-CaSR1 and CD2-CaSR2) possess the ability to bind Ca\(^{2+}\) and Ln\(^{3+}\), in which the predicted Ca\(^{2+}\)-binding residues were shown to be involved in Ca\(^{2+}\)/Ln\(^{3+}\) binding by high resolution NMR and site-directed mutagenesis, further validating our prediction of Ca\(^{2+}\)-binding sites within the ECD of the CaSR.

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To Mg\(^{2+}\) (25), polyamines, and amino acids, such as L-Phe. Similar to the capacities of the mGluRs to respond to both L-Glu and high [Ca\(^{2+}\)]\(_{o}\), the Ca\(^{2+}\)-induced activation of the CaSR is potentiated by L-amino acids, particularly aromatic amino acids (26).

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**MATERIALS AND METHODS**

**Computational Prediction of Ca\(^{2+}\)-binding Sites from a Model Structure**—A sequence alignment of the ECD region (1–540 residues) of the human CaSR and mouse mGluR was carried out using the ClustalW program (32). Structural modeling of the CaSR was performed using SWISS-MODEL (33, 34) and MODELLER software (35) based on the structures of mGluR1 (Protein Data Bank [PDB] codes 1EWT (27) and 1ISR (28)). Thereafter, the putative Ca\(^{2+}\)-binding sites in the CaSR were predicted using the program Metalfinder as described previously (36). Asp was used as the anchor ligand, because it is the most frequently used amino acid residue in Ca\(^{2+}\)-binding sites. The oxygen atoms from backbone carbonyls and the side chains of Asp, Glu, Asn, Gln, Ser, Thr, and Tyr were used as potential Ca\(^{2+}\) ligand residues. In the computational calculation, an increased Ca-O distance (1.0–4.0 Å) and O-Ca-O angles (±60°) were used to compensate for the uncertainties in the model structures (37–39). The resulting potential sites identified by the algorithm were scored and ranked based on the ligands. Those that used all native, existing residues of the CaSR sequence as the ligands received the highest scores. The predicted sites that required mutations to similar residue types received higher scores than others. Similar residue types refer to the following pairs: Asn-Asp, Gln-Glu, Asp-Glu, and Ser-Thr. In contrast, the requirement of mutating non-Ca\(^{2+}\)-binding ligands suggests that the location does not bind Ca\(^{2+}\) in the native CaSR. Finally, the electrostatic potentials were calculated using the program DelPhi (40, 41), and the model structures with the hydrogen atoms were built in by SYBYL. For the DelPhi calculations, interior and exterior dielectric constants of 2 and 80, respectively, were used. The salt concentration was 0.2 M, and the linear solution of the Poisson Boltzmann equation was imposed until convergence was reached. The geometrically predicted locations with 2–4 negatively charged ligands, strong negative surface potential, and functionally necessary residues were more likely to bind Ca\(^{2+}\). The model structures of CD2 variants with grafted potential Ca\(^{2+}\)-binding sites of the CaSR were generated by SWISSMODEL (33, 34).

**Measurement of [Ca\(^{2+}\)]\(_{i}\) in the Cell Population by Fluorimetry**—The [Ca\(^{2+}\)]\(_{i}\) responses of wild type and mutant CaSRs were assessed as described by Bai et al. (42). In brief, the HEK293 cells transfected with the CaSR or its mutant cDNAs were loaded with Fura-2/AM. The remaining extracellular Fura-2/AM was washed out before the cells were transferred to a fluorescence cuvette. The emission at 510 nm was measured with excitation at 340 and 380 nm under varying [Ca\(^{2+}\)]\(_{o}\) (0.5–20.5 mM). The ratio of the fluorescence intensities was used to derive [Ca\(^{2+}\)]\(_{i}\). All measurements were carried out in triplicate.

**Protein Engineering, Expression, and Purification**—The CaSR sequences Gly\(^{222–235}\) (GIEKFREEAAERDI) and Gly\(^{383–398}\) (GHEESGDRFNSSTAFRPLCTGDENI) were individually inserted between Ser\(^{52}\) and Gly\(^{57}\) of CD2 in the plasmid pGEX-2T (denoted as CD2-CaSR1 and CD2-CaSR2, respectively) by PCR using an established protocol (63). Two mutants, CD2-CaSR1-E228A/E229A and CD2-CaSR2-E378A/E379A, were also made by site-directed mutagenesis. Three glycines at the N terminus and two at the C terminus of the inserted sequences were thereafter inserted using a similar procedure (denoted as CD2-CaSR1-5G and CD2-CaSR2-5G). The mutants of the engineered proteins were produced using standard PCR methods. All sequences were verified by automated sequencing on an ABI PRISM-377 DNA sequencer (Applied Biosystems) at the Advanced Biotechnology Core Facilities of Georgia State University.

The proteins were expressed as glutathione S-transferase fusion protein using Escherichia coli BL21 (DE3) cells in LB medium with 100 mg/liter of ampicillin at 37 °C. For \(^{15}\)N isotopic labeling, \(^{15}\)NH\(_4\)Cl was supplemented as the sole source of nitrogen in the minimal medium. Isopropyl-\(\beta\)-D-thiogalacto-
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pyranoside at 100 μM was added when the A_{280} reached 0.6 to induce protein expression for another 3–4 h. The cells were centrifuged at 5000 × g for 30 min. The purification procedures followed the protocols for glutathione-S-transferase fusion protein purification using glutathione-Sepharose 4B beads (GE Healthcare). The glutathione-S-transferase tag of the proteins was cleaved on the beads by thrombin. The eluted engineered CD2 variants were further purified using Superdex 75 and Hitrap SP columns (GE Healthcare). The purified proteins were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry at the Advanced Biotechnology Core Facilities of Georgia State University. The protein concentrations were determined using an Advanced Biotechnology Core Facilities of Georgia State University.

The Ca^{2+} competition data were first analyzed to derive the apparent dissociation constant by Equation 1. By assuming that the sample is saturated with Tb^{3+} at the starting point of the competition, the Ca^{2+}-binding affinity is further obtained by using the equation,

\[ K_{d, Ca} = \frac{K_{app} \times K_{d, Tb}}{K_{d, Tb} + [Tb]} \]  

(Eq. 2)

where \( K_{d, Ca} \) and \( K_{d, Tb} \) are the dissociation constants of Ca^{2+} and Tb^{3+}, respectively. \( K_{app} \) is the apparent dissociation constant.

**RESULTS**

**Prediction of Ca^{2+}-binding Sites in the CaSR**—The ECD of the CaSR contains the majority of the Ca^{2+}-sensing determinants, and glycosylation does not play a significant role in the activation of the receptor by high [Ca^{2+}]. Both mGlURs and CaSR respond to high [Ca^{2+}], and are potentiated (activated) by l-amino acids (26). Their ECDs share ~30% sequence identity and a highly similar arrangement of secondary structural elements to that of mGlUR. Therefore, we performed sequence alignment of the ECD region (1–540 residues) of the human CaSR and mouse mGlUR, and then model structures of the ECD of the CaSR were created based on three structures of mGlUR1, the ligand-free form (PDB code 1EWK), the Glu- and Mg^{2+}-bound forms (PDB code 1EWK (27), and the Glu- and Gd^{3+}-bound form (PDB code 1ISR) (28). Fig. 1 shows the model structure of the ECD of the CaSR with the Venus flytrap structure. Two subdomains, termed as lobe 1 (N terminus) and lobe 2 (C terminus), are linked by several loops. The structure is very similar to the model structures previously reported by Bai (22) and Silve et al. (44). This similarity of the models from different groups and methods strengthens the assumption that the model structures accurately represent the true structures of the CaSR.

We then predicted potential Ca^{2+}-binding pockets in the modeled CaSR structure using our developed computational algorithms (“Materials and Methods”). Fig. 1 and Table 1 show three predicted potential Ca^{2+}-binding pockets (sites 1–3) in the modeled CaSR after the evaluation of their local geometric properties and electrostatic potentials. site 1 is located in lobe 2.
It contains five glutamate residues in a nine-residue sequence (Glu\textsuperscript{224}–Glu\textsuperscript{232}). Three positive residues (Lys\textsuperscript{225}, Arg\textsuperscript{227}, and Arg\textsuperscript{233}) and one aspartate residue (Asp\textsuperscript{234}) are either in or follow this sequence closely. Site 2, located in lobe 1, includes all of the predicted ligand residues in a 22-residue sequence (Glu\textsuperscript{378}–Glu\textsuperscript{399}), which are Glu\textsuperscript{378}, Glu\textsuperscript{379}, Thr\textsuperscript{396}, Asp\textsuperscript{398}, and Glu\textsuperscript{399}. Predicted sites 1 and 2 do not have extensive interactions with other parts of the protein. Site 3 is formed by residues Ser\textsuperscript{147}, Ser\textsuperscript{170}, Asp\textsuperscript{190}, Tyr\textsuperscript{218}, and Glu\textsuperscript{297}.
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Asp\(^{190}\), Tyr\(^{218}\), and Glu\(^{297}\), which are located in the crevice between the two lobes. All three sites are located at flexible loops or helical regions and are largely exposed on the surface of the protein. These geometrically predicted sites overlap with the negatively charged “hot spots” predicted by the electrostatic potential (Fig. 1).

Effect of Mutation of Putative Ca\(^{2+}\)-binding Ligand Residues on the Biological Function of the CaSR—The CaSR responds to elevated levels of [Ca\(^{2+}\)]\(_i\) by activating phospholipase C, which leads to the production of inositol 1,4,5-trisphosphate and further results in transient increases in the cytosolic Ca\(^{2+}\) concentration. To investigate the role of the proposed Ca\(^{2+}\)-binding sites in the biological function of the CaSR, we created several mutations in the predicted charged ligand residues in the full-length CaSR. Specifically, we used site-directed mutagenesis to mutate the following residues: E297I in predicted site 3 located at the crevice, E224I and E228I/E229I in site CaSR1 located in lobe 2, and E378I/E379I and E398I/E399I in site CaSR2 located in lobe 1 (Tables 1 and 2). The wild type CaSR and the respective mutants were expressed in HEK293 cells using previously reported methods (45). The effects of the mutations on the biological function of the CaSR are summarized in Table 2 and Fig. 2. As shown in Fig. 2, removal of putatively charged ligand residues at all three predicted Ca\(^{2+}\)-binding sites led to significant alterations in either maximal Ca\(^{2+}\) response and/or sensitivity to [Ca\(^{2+}\)]\(_i\) of the CaSR. E224I and the double mutant E228I/E229I at predicted site 1 decreased the maximal Ca\(^{2+}\) response by 35–38% with a slight left shift in sensitivity to [Ca\(^{2+}\)]\(_i\) (EC\(_{50}\) = 2.5 ± 0.3 and 2.8 ± 0.2 mM, respectively, versus 3.1 ± 0.2 mM for the wild type CaSR, n = 3, p < 0.05). The mutation E398I/E399I in the predicted site 2 also resulted in a 37% decrease in the maximal intracellular Ca\(^{2+}\) response. This mutant, however, exhibited a right-shifted EC\(_{50}\) of 4.4 ± 0.2 mM (p < 0.01). Interestingly, the E378I/E379I mutant at the same predicted site resulted in a 23% enhancement in the maximal response and a left-shifted EC\(_{50}\) (EC\(_{50}\) = 2.2 ± 0.1 mM, n = 3, p < 0.01). Mutation E297I in site 3 significantly impaired the sensitivity to the [Ca\(^{2+}\)]\(_i\) response with an EC\(_{50}\) of 9.6 ± 0.2 mM.

Our functional characterization of the putative ligand residues in the predicted Ca\(^{2+}\)-binding sites of the CaSR were consistent with many mutations around the proposed Ca\(^{2+}\)-binding sites, which are associated with clinical syndromes (autosomal dominant hypoparathyroidism and familial hypocalciuric hypercalcemia) due to either a decrease or an increase in the sensitivity of the respective receptors to [Ca\(^{2+}\)]\(_i\). By monitoring the intracellular Ca\(^{2+}\) response of HEK293 cells transfected with the wild type and mutant receptors, mutations of Ser\(^{147}\), Ser\(^{170}\), Asp\(^{190}\), Tyr\(^{218}\), and E297K were shown to largely impair the activation of the human CaSR (45–47). Recently, Silve \textit{et al.} (44) have shown that the missense mutations, E297K and Y218S, significantly reduce the maximum Ca\(^{2+}\)-induced [\(^{3}H\)IP response. They postulate that the residues Ser\(^{170}\), Asp\(^{190}\), Glu\(^{193}\), Ser\(^{296}\), and Glu\(^{297}\) are critical for the CaSR Ca\(^{2+}\) bind-

### TABLE 2

Summary of the maximal response and EC\(_{50}\) values of CaSR and its mutants

| Predicted site | Mutation       | Maximal response | Ca\(^{2+}\), EC\(_{50}\) |
|---------------|----------------|------------------|-------------------------|
| Site 1        | Wild type      | 100              | 3.1 ± 0.2               |
|               | E224I          | 62 ± 1           | 2.5 ± 0.3\(^a\)         |
|               | E228I/E229I    | 62 ± 6\(^a\)     | 2.8 ± 0.2               |
| Site 2        | E378I/E379I    | 123 ± 6\(^a\)    | 2.2 ± 0.1\(^a\)         |
|               | E398I/E399I    | 63 ± 12\(^a\)    | 4.4 ± 0.2\(^a\)         |
| Site 3        | E297I          | 87 ± 6           | 9.6 ± 0.2\(^a\)         |

\(^a\)p < 0.01.  
\(^b\)p < 0.05.

![FIGURE 2. Effect of mutations in charged residues of the predicted Ca\(^{2+}\)-binding sites on the responsiveness of the CaSR by monitoring intracellular Ca\(^{2+}\) using Fura-2. Functional characterization of the wild type CaSRs and CaSRs with mutations in site 1 (A), site 2 (B), or site 3 (C) were performed using HEK293 cells transfected with the wild type and CaSR mutated in charged residues and are expressed as the normalized Ca\(^{2+}\) responses.](Image)
ing and downstream biological functions, which is in excellent agreement with our prediction. Although these findings are not based on direct measurement of Ca\(^{2+}\) binding, they provide strong experimental evidence to support our model structures as well as the prediction of Ca\(^{2+}\)-binding sites in the ECD of the CaSR.

Engineering Proteins by Grafting the Predicted Sequences into CD2—Investigation of the site-specific Ca\(^{2+}\)-binding properties of the CaSR is one important step toward fully understanding the mechanism underlying its Ca\(^{2+}\)-modulated functions. Our laboratory had previously established a grafting approach to investigate the site-specific metal-binding properties of calmodulin (CaM) by inserting the individual EF-loops of CaM into a host frame, CD2 (48). Because predicted Ca\(^{2+}\)-binding sites 1 and 2 of the CaSR have contiguous stretches of amino acids, similar to the Ca\(^{2+}\)-binding motifs in CaM, we extended the grafting approach to probe the site-specific Ca\(^{2+}\)-binding affinity of these two sites. The continuous sequences were inserted into CD2 at position 52 between the strands C\(^{\prime}\) and D, because our previous studies had shown that this position tolerates the insertion of EF-hand motifs from CaM. The distances between the two termini of the inserted Ca\(^{2+}\)-binding sites in the model structures of the CaSR were within 15 Å. Accordingly, a total of 5–6 glycine linkers is sufficient to enable the grafted motifs to retain a native metal-binding conformation (48), and two more variants were thus engineered with 3 and 2 flanking Gly residues at the N and C termini of the CaSR sequence. Fig. 3 shows the modeled structure of CD2 with grafted Ca\(^{2+}\)-binding sites from the CaSR. Trp\(^{32}\) and Tyr\(^{76}\) in the host proteins are \(\sim\)12 Å distant from the grafted Ca\(^{2+}\)-binding sites.

**FIGURE 3.** Modeled structure of CD2 with grafted Ca\(^{2+}\)-binding sites in the CaSR. Trp\(^{32}\) and Tyr\(^{76}\) in the host protein are \(\sim\)12 Å away from the grafted Ca\(^{2+}\)-binding sites.

**FIGURE 4.** The secondary structures of wild type CD2 and engineered proteins. A, far UV CD spectra of 15 \(\mu\)M wild type CD2, CD2-CaSR1, CD2-CaSR1-5G, CD2-CaSR2, CD2-CaSR2–5G in 10 mM Tris, pH 7.4. B, Trp fluorescence spectra of wild type CD2 and the engineered proteins with grafted Ca\(^{2+}\)-binding sites. C, comparison of chemical shifts of the \(\alpha\) and backbone amide protons between wild type CD2 and the engineered protein CD2-CaSR1 with grafted Ca\(^{2+}\)-binding sites from the CaSR.
bound to the sites, providing a spectroscopic method to monitor the metal-binding process.

The Conformation of Scaffold Protein Is Not Altered after Grafting Ca^{2+}-Binding Sites—To ensure that the grafted Ca^{2+}-binding site has no major interaction with the host protein and that the host protein has not changed its native conformation, we carried out conformational analyses using various methods. As shown in Fig. 4A, the far UV CD spectra of all four engineered proteins shows a trough at 216 nm, indicating a typical β-sheet secondary structure, as in the wild type CD2. The deeper negative molar ellipticity in the short wavelength region compared with that of CD2 was consistent with the addition of loop/less structured sequences of the CaSR. In addition, the Trp fluorescence spectrum of wild type CD2 overlaps that of the engineered proteins, both of which exhibit two peaks at 314 and 335 nm, suggesting that the Trp environment remained unchanged after engineering of the protein (Fig. 4B). Furthermore, the majority of the NMR chemical shifts of the host frame in the engineered variants were not significantly different from those of CD2 (Fig. 4C), suggesting that the scaffold protein was minimally perturbed after the insertion of the foreign sequences. All of these results imply that the host scaffold protein retained its native structure, thereby ensuring a minimal contribution to the metal binding of the inserted sequences.

The Grafted Sequences from CaSR Bind Cations—The Ca^{2+}-binding capability of the inserted sequences was first revealed by NMR. Upon the addition of 10 mM Ca^{2+} under high salt conditions (150 mM KCl), several resonances from the inserted sequences had altered resonances as shown in the HSQC spectra (Fig. 5). For example, Ca^{2+}-binding resulted in chemical shift changes of at least two of the peaks arising from glutamates in CD2-CaSR1—5G (Fig. 5A). The addition of Mn^{2+} led to the disappearance of resonances from the inserted sequences and the CD2 host residues in proximity due to the line-broadening effect caused by the Mn^{2+} that substituted for the Ca^{2+} (data not shown). In contrast, wild type CD2 did not exhibit any detectable changes under identical conditions. Furthermore, the addition of La^{3+} to the CD2-CaSR2 led to significant changes of, for example, at least two resonances originating from the grafted sequence as well as Gly53 of CD2 (Fig. 5C). More convincingly, after substituting two proposed Ca^{2+}-binding ligand residues within site 1 (Glu^{208}/Glu^{229}) or site 2 (Glu^{778}/Glu^{799}) with alanines, we could not detect such significant chemical shift changes upon the addition of metal ions (Figs. 5B and D), further supporting the involvement of these residues in the chelation of Ca^{2+} within each predicted metal-binding site. Mutagenesis studies on these putative Ca^{2+}-binding ligands have also been shown to affect the biological function of CaSR (Fig. 2). Moreover, the mass peaks corresponding to the formation of 1:1 Ca^{2+}-protein or Tb^{3+}-protein complexes were observed in ESI-MS spectra in the presence of excess metal ions (Fig. 6). These results suggest that the inserted sequences from the CaSR have the capacity to bind Ca^{2+} and its trivalent analogs.

The addition of Tb^{3+} into the engineered proteins (Fig. 7A) or vice versa resulted in large increases of Tb^{3+} fluorescence at 545 nm because of Trp-sensitized Tb^{3+}-fluorescent resonance energy transfer (Tb^{3+}-FRET) (36, 49), which was not observed for wild type CD2. The addition of Ca^{2+} into the Tb^{3+}-protein mixture decreased the Tb^{3+} signal because of competition (Fig. 7B). The Tb^{3+}- and Ca^{2+}-binding affinities were derived from the Tb^{3+} fluorescence change and the metal competition, respectively. For CD2-CaSR1, the Tb^{3+}- and Ca^{2+}-binding dissociation constants obtained were 35 ± 3 and 890 ± 80 μM at low salt concentrations, respectively. At high salt concentrations (e.g. ~150 mM NaCl), as in the extracellular environment within which the extracellular domain of the native CaSR normally resides, the Ca^{2+}-binding affinity decreased at least
10-fold (Table 3). For CD2-CaSR2, the Tb\(^{3+}\)/H\(^{11001}\) and Ca\(^{2+}\)/H\(^{11001}\) dissociation constants were 25 ± 2 \(\mu\)M and 4.3 ± 0.8 mM at low and 98 ± 7 \(\mu\)M and 18.6 ± 0.5 mM at high salt concentrations, respectively. The relatively stronger affinity for Tb\(^{3+}\) than for Ca\(^{2+}\) is consistent with other natural Ca\(^{2+}\)-binding proteins and is partly because of the electrostatic nature of the metal binding (50). Further, the variants with or without flanking glycine linkers exhibit no significant differences in their metal-binding affinities, suggesting that the grafted Ca\(^{2+}\)-binding sites are flexible and less sensitive to the host protein.

Site-directed mutagenesis that removed the predicted charged ligand residues resulted in a dramatic decrease of the Tb\(^{3+}\)-FRET signal, suggesting attenuated metal-binding affinities. For example, the Tb\(^{3+}\) fluorescence enhancements of both mutants E378A/E379A and D398A/E399A are 70% lower than that of CD2-CaSR2. The signal of the mutant E231A/E232A is also 30% lower than that of CD2-CaSR. These results are consistent with our functional study of the mutations (Fig. 2 and Table 2) and further validate our prediction of Ca\(^{2+}\)-binding sites in the ECD of the CaSR.

**DISCUSSION**

**Predicting Ca\(^{2+}\) Binding in the CaSR and the Effect of Mutations on the Function of the CaSR—**Ca\(^{2+}\) bound to proteins is predominantly chelated with oxygens with an average coordination of 6 – 7 (51 – 53). The binding of Ca\(^{2+}\) to multiple sites in a highly cooperative manner facilitates the response of a protein to small Ca\(^{2+}\) concentration changes (54 – 56), such as the binding of four Ca\(^{2+}\) ions to CaM. The Hill coefficient suggests that 3 – 5 Ca\(^{2+}\) ions bind cooperatively to the CaSR. However, the Ca\(^{2+}\)-binding sites have not been identified.

Our laboratory has shown that naturally evolved Ca\(^{2+}\)-binding sites can be identified and novel Ca\(^{2+}\)-binding proteins can be designed using the pentagonal bipyramidal geometry with the side chains of the aforementioned residues and main chain carbonyls as potential ligands (53). In this study, taking advantage of the previously determined structures of mGLuR1 and the sequence homology of the CaSR to mGluR1, we predicted several Ca\(^{2+}\)-binding sites in the modeled structure of the CaSR by applying our well developed computational algorithms. These sites possess high electrostatic potential and are located in the negatively charged environments (Fig. 1), which are preferred for Ca\(^{2+}\) binding (36). In addition, these putative Ca\(^{2+}\)-binding sites are located in the flexible locations either in the crevices between two lobes (site 3) and/or at loop and exposed regions. This is consistent with a survey that revealed that almost all of the known Ca\(^{2+}\)-binding sites are located in or adjacent to the flexible structures of their respective proteins, such as loop regions, turns, or the ends of \(\alpha\)-helices and \(\beta\)-sheets (57).

Our model structure and predicted Ca\(^{2+}\)-binding sites, especially site 3, are consistent with the reported functional studies. The x-ray structure of mGluR1 complexed with its ligand, glutamate, reveals that residues Ser\(^{164}\), Ser\(^{165}\), Thr\(^{188}\), Tyr\(^{218}\), and Glu\(^{297}\) in the protein form hydrogen bonds with the \(\alpha\)-carboxyl group of the glutamate (58). These residues correspond to Ser\(^{147}\), Ser\(^{170}\), Asp\(^{190}\), Tyr\(^{218}\), and Glu\(^{297}\) in the CaSR, respectively. Two mutations, Y218S and E297K, markedly reduce the

![Figure 6. ESI-MS spectra of CD2-CaSR1 with metal ions.](image)

The binding of Ca\(^{2+}\) (A) or Tb\(^{3+}\) (B) led to the emergence of additional peaks with molecular mass differences of +38 and +156, respectively.
responsiveness and sensitivity of the receptor to \([\text{Ca}^{2+}]_o\) (24). Furthermore, mutations around these predicted locations have been shown to alter the \([\text{Ca}^{2+}]_o\) response of cells expressing the respective mutant receptors, supporting our prediction. For example, T151M in autosomal dominant hypoparathyroidism and R185Q in familial hypocalciuric hypercalcemia are adjacent to \([\text{Ca}^{2+}]_o\) ligand residues in site 3. Additional mutations that alter the CaSR responses, such as Y218S/C, E297K, and R220S and R221S, are also either at or near the predicted ligand residues of site 3 (59, 60). In addition, Zhang et al. (24) have demonstrated that Ser170 is critical for functional modulation of the CaSR by L-Phe, in that the S170A mutation led to no significant change in the EC\(_{50}\) value of the protein for \([\text{Ca}^{2+}]_o\) but a 1.6-fold increase in its response at 50 mM \([\text{Ca}^{2+}]_o\). Recently, Mun et al. (61) further showed that the double mutation T145A/S170T selectively disables L-amino acid sensing but does not change the \([\text{Ca}^{2+}]_o\)-sensing capability of the CaSR, thereby suggesting that the two processes can be dissociated and that the L-amino acid is mainly bound near Ser170.

The predicted \([\text{Ca}^{2+}]_o\)-binding site in the crevice is likely to play a central role in modulating the function of the CaSR by \([\text{Ca}^{2+}]_o\)-induced conformational changes. Previous studies have shown that mutations in this site inactivate the protein (44). The mutations E297I and D215I provide additional support for this hypothesis. The glutamate-binding site in mGluR was also within the crevice of the protein, suggesting that the hinge that connects the two lobes directly responds to the stimuli-inducing receptor activation in the G protein-coupled receptor family C proteins.

The mutations outside of the crevice demonstrate the complexity of the regulatory mechanism of the CaSR. Removal of charged residues in both lobe 1 (E224I and E228I/E229I) and lobe 2 (E398I/E399I) decreased the maximal response levels but resulted in oppositely directed changes in the sensitivity of the receptor to \([\text{Ca}^{2+}]_o\). Perhaps the maximal responses and the sensitivity were determined by different locations of the protein via distinct mechanisms. In addition, loss of the charges on these residues could alter the interactions of these amino acids with charged residues elsewhere on the protein, potentially favoring conformations that either activate or inactivate the protein, depending on local geometrical factors. Because both mutations resulted in significantly weakened metal binding in the CD2-CaSR variants, the results suggest that \([\text{Ca}^{2+}]_o\) binding at the predicted locations is necessary for maintaining the full decrease of Tb\(^{3+}\) fluorescence due to competitive binding of metal ions in CD2-CaSR1 that was preincubated with Tb\(^{3+}\). C, comparison of relative Tb\(^{3+}\) signal changes of wild type CD2-CaSR2 and its charged mutants.

**TABLE 3**

| Variant        | Tb\(^{3+}\) | Ca\(^{2+}\) |
|----------------|-------------|-------------|
|                | 10 mM KCl   | 150 mM NaCl|
|                | 10 mM KCl   | 150 mM NaCl|
| CD2-CaSR1      | 35 ± 3      | 144 ± 5     |
| CD2-CaSR1-5G   | 7 ± 1       | 94 ± 7      |
| CD2-CaSR2      | 25 ± 2      | 98 ± 7      |
| CD2-CaSR2-5G   | 19 ± 2      | 80 ± 3      |

**FIGURE 7.** Probing metal binding with aromatic residue-sensitized Tb\(^{3+}\)-FRET. A, the enhancement of Tb\(^{3+}\) fluorescence of CD2-CaSR1 at 545 nm as a function of titrated Tb\(^{3+}\). A 1:1 binding mode was assumed for data fitting. B, Ca\(^{2+}\) competition assay. The addition of Ca\(^{2+}\) led to a decrease of Tb\(^{3+}\) fluorescence due to competitive binding of metal ions in CD2-CaSR1 that was preincubated with Tb\(^{3+}\). C, comparison of relative Tb\(^{3+}\) signal changes of wild type CD2-CaSR2 and its charged mutants.
function of the CaSR. Therefore, Ca$^{2+}$ acts as both a stimulus and a regulator for the CaSR. The potential Ca$^{2+}$ binding to Glu$^{399}$/Glu$^{399}$ might be crucial, because removal of these two charges causes a dual effect by decreasing both the maximal activity and the sensitivity to [Ca$^{2+}$]$_o$.

**Grafting Approach for Probing Ca$^{2+}$-binding Capability**—To overcome the limitations of solely investigating the Ca$^{2+}$-binding sites in native proteins, we established a grafting approach to dissect their site-specific properties. This approach has been used previously in the investigation of single EF-hand motifs in CaM (62, 63). CD2 has been shown to be a suitable host system, because it retains its native structure after insertion of the foreign sequences, both in the presence and absence of Ca$^{2+}$ ions, so that the influence of the host protein on the inserted sites is minimized. The difficulties inherent in directly confirming the predicted Ca$^{2+}$-binding ligands due to the multiple binding and conformational changes of the CaSR prompted us to investigate this issue indirectly using the grafting approach. In CD2-CaSR variants, additional residues and glycine linkers are at both termini of the predicted sequences to provide the conformational freedom needed for the formation of their native structures. In addition, the host protein retains the native structure of CD2, as indicated by CD, fluorescence, and NMR studies with and without cations. The addition of cations induced chemical shift changes of the resonances arising from the inserted sequences but not those from the host proteins, indicating that the binding is independent of the host protein and occurs at the inserted sequences.

The capability of the two predicted Ca$^{2+}$-binding sites of the CaSR grafted into CD2 to bind Ca$^{2+}$ and Ln$^{3+}$ validated our computational identifications. In the CaSR model structures, the predicted sequences are in flexible loop regions. In CD2-CaSR variants, these sequences are also in flexible loops indicated by the far-UV CD spectra. The variants with or without glycine linkers exhibited similar Tb$^{3+}$ and Ca$^{2+}$-binding affinities, which also implies that these Ca$^{2+}$-binding sites are highly flexible, and the contribution of the host protein frame to the metal binding is less likely to be significant. The probed site-specific Ca$^{2+}$-binding affinities at 150 mM NaCl for CD2-CaSR1 and CD2-CaSR2 are 4.2 ± 0.3 and 18.6 ± 0.5 mM, respectively. These affinities are weaker than the EC$_{50}$ value of the CaSR (Tables 2 and 3), which can be explained by several possible factors. First, the interaction of coupled metal-binding sites and cooperativity are likely to contribute to the overall sensing capacity of the intact CaSR to respond to [Ca$^{2+}$]$_o$. This is similar to the contribution of the cooperativity observed for CaM. This cooperativity could be the result of direct site-to-site interactions or through Ca$^{2+}$-induced conformational change. The cooperativity is not necessarily positive in all cases. In fact, the opposite effects of the mutations at predicted sites 1 and 2 on the sensitivity of the protein to [Ca$^{2+}$]$_o$ indicate that Ca$^{2+}$ binding at various locations within the CaR ECD has diverse influences on the subsequent Ca$^{2+}$ binding to other sites. If Ca$^{2+}$ effectively cross-linked and neutralized negative charges in two different parts of the molecule (one of which was one of our binding sites), for example, which then caused the lobes to close, then loss of one of those negative charges could reduce the repulsion between the negative charges that was present in the absence of calcium and favor activation of the receptor at lower extracellular calcium. Second, other factors, such as dimer formation and the protein environment (e.g., hydrogen bonding and salt bridges), may also play a part in the affinity differences between the isolated sites and the full protein. In the engineered proteins, the host protein has minimal effects on metal binding. However, in the native CaSR, the surrounding residues will surely influence the electrostatic potentials at the Ca$^{2+}$-binding sites, in a Ca$^{2+}$-dependent or -independent manner. Third, it is possible that there are additional Ca$^{2+}$ ligands for the sites that have not been identified in our prediction. Our work has opened up the opportunity for investigating the mechanism(s) underlying Ca$^{2+}$-modulated function by determining Ca$^{2+}$-binding properties in CaSRs at local levels.

In conclusion, several Ca$^{2+}$-binding sites have been predicted in the model structure of the CaSR. Two continuous predicted sites have been grafted into the host protein CD2. The metal-binding studies on the engineered proteins using various spectroscopic methods validate the computational predictions and demonstrate the Ca$^{2+}$-binding capability of the predicted sequences. Mutations that remove the predicted charged Ca$^{2+}$ ligand residues lead to weakened metal binding in the engineered proteins and abnormal responses to [Ca$^{2+}$]$_o$, in the full-length CaSR, supporting the predictions and indicating the importance of Ca$^{2+}$ binding in regulating CaSR functions.

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