Expression, Purification, and Biochemical Characterization of the Amino-terminal Extracellular Domain of the Human Calcium Receptor*

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We purified the extracellular domain (ECD) of the human calcium receptor (hCaR) from the medium of HEK-293 cells stably transfected with a hCaR cDNA containing an isoleucine 599 nonsense mutation. A combination of lectin, anion exchange, and gel permeation chromatography yielded milligram quantities of >95% pure protein from 15 liters of starting culture medium. The purified ECD ran as an ~78-kDa protein on SDS-polyacrylamide gel electrophoresis and was found to be a disulfide-linked dimer. Its NH2-terminal sequence, carbohydrate content, and CD spectrum were defined. Tryptic proteolysis studies showed two major sites accessible to cleavage. These studies provide new insights into the structure of the hCaR ECD. Availability of purified ECD protein should permit further structural studies to help define the mechanism of Ca2+ activation of this G protein-coupled receptor.

The calcium receptor (CaR) is a unique member of the G protein-coupled receptor superfamily expressed in parathyroid and kidney cells where it has been shown to play a critical role in extracellular calcium homeostasis (1). The CaR is also expressed in a variety of other sites such as the central nervous system where it may serve functions beyond systemic calcium homeostasis (2). In amino acid sequence and presumed topographic structure, the CaR is most closely related to a distinct subset of G protein-coupled receptors that includes the metabotropic glutamate receptors (mGluRs) (3, 4) and a multigene family of putative vomeronasal pheromone receptors (5–7). In addition to the seven transmembrane domain characteristic of all G protein-coupled receptors, the CaR and other members of its subset contain a large, ~600-residue, amino-terminal extracellular domain (ECD) that is heavily glycosylated and contains a large number of highly conserved cysteines (8).

Based on limited amino acid homology to bacterial periplasmic binding proteins, it has been suggested that the ECD of the mGluRs (9) and CaR (10) has a bi-lobed, “venus flytrap”-like structure; however, there is very little structural or biochemical information available for the CaR or mGluR ECDs. Using tunicamycin, we have previously shown that N-linked glycosylation of the CaR is essential for its expression at the cell surface (11), but the nature and sites of glycosylation have not been defined. Both the intact mGluRs (12) and the CaR (13–15) have been shown to be disulfide-linked dimers; for the mGluRs, it was found that intermolecular disulfide linkage of the ECD accounts for dimer formation (12, 16), but the role of the ECD in dimerization of the CaR has not been defined. To obtain biochemical and structural information, we developed methods for large scale culture of a cell line stably expressing a mutant form of the human CaR (hCaR) that results in secretion of the ECD into the medium. We report here the purification of the secreted hCaR ECD and results of biochemical analysis, including amino-terminal sequence, carbohydrate content, dimeric structure, and accessibility to tryptic digestion.

MATERIALS AND METHODS

Stable Transfection of HEK-293 Cells with a Mutant hCaR cDNA—A mutant form of the full-length human parathyroid CaR cDNA (17) in which cysteine 598 was changed to serine and isoleucine 599 was changed to a stop codon (see Fig. 1) was subcloned into NotI/HindIII-digested pCEP4 expression vector (Invitrogen, San Diego, CA). The mutant hCaR cDNA was transfected into HEK-293 cells with calcium phosphate, and 200 μg/ml hygromycin was used to select stable transfecants. Resistant colonies were subcloned and screened for hCaR expression by a solution hybridization assay. Clone 32 used in this study was chosen based on high levels of ECD secretion as determined by immunoblot analysis of cell culture media. Cells were routinely cultured in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin and streptomycin, and 200 μg/ml hygromycin at 37 °C in a 5% CO2 environment.

Large Scale Cell Culture for Production of ECD—ECD-secreting clone 32 HEK-293 cells were immobilized on cellulose discs in a packed bed configuration using a 2.2-liter Celligen Plus bioreactor (New Brunswick Scientific, Edison, NJ) with a vertical mixing impeller assembly and internal basket. Production consisted of a propagation stage of approximately 140 h with perfusion of serum-containing media. When a cell density of 2 × 10^9 was reached, the second stage was initiated by perfusion with serum-free media for 400 h at a flow rate of 4–6 liters/day while maintaining the residual glucose concentration at 1 g/liter. Perfusate was pumped directly on 100-ml bed volume Q-Sepharose fast flow column (Amersham Pharmacia Biotech) equilibrated with phosphate-buffered saline (PBS). Protein was eluted with a 1 M NaCl step elution or 0–1 M NaCl gradient in 0.01 M phosphate buffer, pH 7.4. ECD-containing fractions were determined by immunoblotting with a monoclonal antibody, ADD, specific for peptide 214–235 of hCaR (18). The purified ECD was expressed in a variety of other sites such as the central nervous system where it may serve functions beyond systemic calcium homeostasis (1).
**Purified Calcium Receptor Extracellular Domain**

**Fig. 1. Schematic representation of the human calcium receptor.** The amino acids are shown for the amino-terminal ECD, and the remainder of the receptor is indicated schematically. The arrows indicate a cysteine to serine mutation at residue 598 and isoleucine to stop codon mutation at residue 599 in the mutant form of the receptor engineered to lead to secretion of the ECD into the cell culture medium. The gap between residue 19, alanine, and residue 20, tyrosine, indicates the site of signal peptide cleavage as determined by amino acid sequencing of the purified ECD. Putative glycosylation sites are marked by branches, and cysteine residues in the ECD are darkened. Underlined and labeled regions with amino acids in **boldface** type represent peptide sequences used to generate peptide-specific rabbit polyclonal antibodies.

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first run over a 25-ml bed volume RCA-1 lectin agarose column (EY Laboratories Inc., San Mateo, CA) followed by step elution with 0.2 M lactose in PBS. Lectin affinity-purified proteins were further purified by FPLC anion exchange chromatography using a 1-ml bed volume Mono Q column (Amersham Pharmacia Biotech) with elution at 1 ml/min and a gradient of 0–1 M NaCl in 0.02 mM phosphate buffer, pH 7.4. ECD-containing fractions from the anion exchange step were concentrated by centrifugal ultrafiltration using Centricon YM-50 units (Amicon Inc., Beverly, MA) before HPLC gel permeation chromatography using a 7.8-mm × 30-cm TSK-3000 column ( Tosohaas, Montgomeryville, PA) run at 0.5 ml/min in PBS. Protein concentrations were determined by the Bradford dye binding method (Bio-Rad).

**Amino Acid Sequence Analysis**—Amino-terminal amino acid sequence analysis was performed with a model 477A protein sequencer coupled to a model 120A PTH analyzer (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s program NORMAL-1. Reversed phase analysis of the PTH-derivatives was done using a Brownlee PHT-C-18 column (2.1 × 220 mm).

**Carbohydrate Analysis**—Samples of ECD proteins were analyzed by high performance anionic exchange chromatography on a Dionex system (Dionex Corp., Sunnyvale, CA) equipped with a pulsed amperometric detector PAD2 and a pellicular PA1, 4 mm × 7.8-mm TSK-3000 column (Tosohaas, Montgomeryville, PA) according to the manufacturer’s program NORMAL-1. Reversed phase analysis of the PTH-derivatives was done using a Brownlee PHT-C-18 column (2.1 × 220 mm).

**Circular Dichroism Analysis**—Circular dichroic spectra were measured on a Jasco J-500C spectrophotometer attached to a DP500N data processor at 25 °C. Protein concentrations were 100 μg/ml in 0.1 M phosphate buffer, pH 7.0. Spectra were digitized and analyzed as described previously (19). Measured ellipticities were converted into mean residue ellipticity using Eq. 1.

\[
\theta = \frac{\text{Deflection in millidegrees} \times 10 \times \text{mean residue weight}}{\text{conc.} \ (\text{mg/ml}) \times \text{pathlength} \ (\text{cm}) \times 100}
\]

(Eq. 1)

A value of 110 was used for the mean residue weight. Spectra were analyzed in terms of secondary structures using the CONTIN program (20).

**Polyclonal Peptide-specific Antibody Production**—Five peptides, GP, LSN, ADD, LRG, and DGE, corresponding to sequences within the ECD of the hCaR (see Fig. 1) were synthesized and used for production of affinity-purified rabbit polyclonal antibodies as described previously (21). Three additional peptide-specific rabbit polyclonal antibodies, FF20-10, FF20-11, and FF20-7, representing other regions of the hCaR ECD (see Fig. 1) were produced as described previously (22).

**Tryptic Digestion of the ECD**—Samples of ECD that were to be denatured before tryptic digestion were first heated for 10 min at 100 °C in 0.1% SDS. Digestion of denatured and undenatured ECD samples was performed in 25 mM Hepes buffer, pH 8.0, for varying times at 37 °C using a trypsin:protein ratio of 1:100 (w/w). The reaction was terminated by adding an equal volume of SDS-PAGE loading buffer (23).

**SDS-PAGE and Immunoblotting**—Proteins were separated on 10% SDS-polyacrylamide gels as described (23). For visualization of bands, gels were either stained with Coomassie Blue dye or used for immunoblotting as described previously (11, 18). Briefly, after overnight transfer to nitrocellulose membranes at 150 mA constant current, membranes were blocked in 5% horse serum, 0.05 M Tris, pH 8.0, 0.5 M NaCl containing 0.1% Tween 20 (TBST) for 1 h. Blots were incubated overnight in horse serum-TBST with first antibodies at a concentration of 2 μg/ml for the antipeptide monoclonal antibody, ADD, and 5 μg/ml for affinity-purified rabbit polyclonal antipeptide antibodies. After washing three times for 20 min with TBST, blots were incubated for 2 h with 1/2,000 dilutions of peroxidase-conjugated goat anti-mouse antibodies (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) or per-
oxidase-conjugated sheep anti-rabbit antibodies (The Binding Site, San Diego, CA) in horse serum-TBST. Membranes were again washed twice for 10 min with TBST and once with Tris-buffered saline for 10 min and then developed with 4-chloronaphthol as substrate.

**RESULTS AND DISCUSSION**

HEK-293 cells were stably transfected with an episomal vector containing a mutant form of the hCaR cDNA engineered to lead to secretion of the ECD into the culture medium (see Fig. 1). A single clone (clone 32) was selected by immunoblot analysis of culture medium for high level secretion of the ECD, and this clone was adapted to large scale culture conditions in a bioreactor for production and purification of the ECD. Analysis of the amino acid sequence of the ECD using the GCG program (Genetics Computer Group, Madison, WI) indicated a protein with a calculated pI of 4.95 and a net of 25 negative charges. The sequence contains 11 putative glycosylation sites, and in the context of the expressed intact receptor, the ECD is known to be heavily glycosylated (8, 11, 24). Using a lectin affinity chromatography screening kit (EY Laboratories Inc.) it was determined that RCA-1 lectin quantitatively removed ECD protein from cell culture medium (data not shown). Our purification protocol was based on this property and the presumed relatively acidic nature of the ECD.

For a typical isolation, 15 liters of bioreactor perfusate containing 84 μg/ml protein were pumped over a 100-ml volume column of Fast Q-Sepharose anion exchanger. Step elution with 1 M NaCl, 0.1 M phosphate buffer, pH 7.4, and localization of ECD-containing fractions by immunoblotting resulted in a pooled volume of 450 ml containing 0.56 mg/ml protein, which was run over a 25-ml bed volume column of RCA-1 lectin agarose affinity beads. Step elution with 0.2 M lactose in PBS gave 9.5 ml of solution containing 0.53 mg/ml protein.

The affinity-purified lactose eluate from the lectin column was then subjected to FPLC Mono Q anion exchange chroma-
tography (Fig. 2). As seen by immunoblot (panel B) and Coomassie Blue staining (panel C) of representative fractions, a broad elution pattern of ECD protein begins at 0.22 M NaCl and ends at about 0.65 M NaCl, with a peak of ECD protein eluting in fraction 38 at a concentration of 0.32 M NaCl. This elution profile is suggestive of the existence of several tightly bound charged forms of the ECD protein that could reflect heterogeneity of carbohydrate content, specifically sialic acid (see below). Fractions 33–42 were pooled (16 ml of solution containing 0.153 mg/ml of protein) and then concentrated before HPLC TSK-3000 gel permeation chromatography (Fig. 3). The ECD protein elutes as a single, well defined peak at fraction 17, which corresponds to the approximate elution volume seen for a bovine IgG immunoglobulin calibration standard with a molecular mass of 154 kDa (data not shown) and suggests that the ECD may be a dimer.

Coomassie Blue staining (Fig. 3, panel C) of SDS-reducing electrophoretic gels of the TSK-3000 chromatographic fractions revealed two bands of protein, a major diffuse band between the 64- and 98-kDa markers with an apparent molecular mass of ~78 kDa and a minor band at ~48 kDa. The more sensitive immunoblot (Fig. 3, panel B) also shows a major diffuse band at 78 kDa and a minor band at 48 kDa as well as two minor bands at 160–170 kDa, and a faint band at 52 kDa. Those extra bands, which are not seen by Coomassie Blue staining, represent very minor amounts of protein. Based on immunoblots with region-specific antibodies (see below), we believe that the faster migrating bands at 48 and 52 kDa represent proteolytically clipped fragments of the ECD. The 160–170-kDa bands may represent trace amounts of undissociated dimeric ECD protein (see Fig. 4).

A typical purification yielded 1.33 mg of protein (representing 0.105% of the starting amount of bioreactor perfusate protein) at >95% purity based on visual inspection of Coomassie Blue-stained gels (Fig. 3C, lane 17). The pooled TSK-3000 fractions containing purified ECD were used for further biochemical characterization. ECD protein migrated in reducing electrophoresis gels with an apparent molecular mass of 78 kDa, which is much greater than the predicted molecular mass of 67,071 Da for the sequence of amino acids between residues 1–598. NH₂-terminal amino acid sequencing of the purified protein gave the following sequence: YGPDQRAQKKGDIILGGLFP, corresponding to residues 20–39 of the hCaR (Fig. 1) and indicating that signal peptide cleavage occurs after alanine 19. This agrees well with an algorithm for predicting signal
sequence cleavage sites (25) that shows alanine very frequently in the −1 position and tyrosine, previously thought to be the site of cleavage in the bovine CaR (8), never in the −1 position. The calculated molecular mass of the ECD protein minus the signal peptide residues and with the serine for cysteine substitution is 64,886 Da.

The difference in apparent molecular weight on SDS-PAGE versus calculated molecular weight, the diffuse nature of the ECD band on SDS-PAGE, the presence of multiple presumptive N-glycosylation sites in the ECD, and our ability to isolate the ECD by lectin affinity chromatography are all consistent with its being glycosylated. Table I shows the carbohydrate composition of several ECD preparations determined as described under “Materials and Methods.” Summation of the molecular weights of the determined species of carbohydrates indicates that a range of 8.2 to 37% of the mass of the ECD protein is carbohydrate. Addition of the carbohydrate masses to the mass of the predicted amino acid sequence results in a calculated molecular mass of the ECD protein minus the signal peptide residues and with the serine for cysteine substitution of 64,886 Da.

Circular dichroism measurements of the purified ECD were performed and a typical CD spectrum is shown in Fig. 5. The CD spectrum of the purified ECD was unchanged by addition of β-mercaptoethanol. This is similar to the behavior of the purified mGluR1 ECD and is consistent with the possibility that dimerization of the intact CaR occurs secondary to intermolecular disulfide linkage of its ECD. The minor 48-kDa band, which we have interpreted as arising from action of unknown protease during the isolation procedure, is seen on immunoblot (Fig. 4) only under reducing conditions suggesting that it is kept associated to the rest of the ECD protein by disulfide bonds.

Circular dichroism measurements of the purified ECD were performed and a typical CD spectrum is shown in Fig. 5. The CD spectrum of the purified ECD was unchanged by addition of EDTA, neomycin, gadolinium chloride, or terbium chloride at concentrations up to 100 μM. Analysis of all spectra indicated an α-helix content of 41–56% and a β-sheet content of 7–19%.

The purified ECD was subjected to trypsin proteolysis with and without prior denaturation. The denatured ECD was rapidly degraded by trypsin yielding small fragments visualized on immunoblot with the ADD antibody (Fig. 6, left). In contrast, the non-denatured ECD was not surprisingly less susceptible to trypptic digestion with persistence of intact ECD even after 40 min of digestion (Fig. 6, right). Of greater interest was the rapid appearance of an ~50-kDa band with trypptic digestion of the non-denatured ECD. This trypptic fragment persisted even after 180 min of digestion, the longest time tested. The non-denatured ECD was then digested with trypsin for 30 or 90 min and the digests analyzed by immunoblot with a panel of polyclonal antibodies raised against synthetic peptides (see Fig. 1) ranging from the amino to the carboxyl terminus of the ECD (Fig. 7). Although the pattern of smaller trypptic fragments varied with each antibody, the six antibodies going from NH₂ terminus (GP) to residue 358 (FP20-7) showed the identical ~50-kDa trypptic fragment after both 30 and 90 min of digestion.

The LRG and DGE antibodies, in contrast, did not stain this band (labeled 1 in the figure); instead, LRG stained two diffuse, lower molecular weight bands (labeled 2 and 3 in the figure), whereas DGE stained only band 2. We interpret these results to indicate that the native ECD possesses relatively few sites accessible to trypsin, despite the many potential basic
cleavage sites. The two major sites of cleavage are defined by the pattern of reactivity of the region-specific peptide antibodies. The site generating bands 1 and 2 is localized between the epitopes for FF20-07 and LRG. Band 1 is then an NH₂-terminal fragment encompassing epitopes of GP through FF20-07, and band 2 is a COOH-terminal fragment encompassing the LRG and DGE epitopes. Band 3 is generated by tryptic cleavage at the other highly accessible site, which is just proximal to the DGE epitope based on the loss of DGE reactivity, retention of LRG reactivity, and modest reduction in size of band 3.

It is interesting to relate these tryptic cleavage sites to a speculative model of the CaR ECD. A sequence alignment based on limited homology between bacterial periplasmic binding proteins (PBPs) and mGluR ECDs (9) can be extended to the CaR given its relatively high degree of homology to the mGluRs (8, 17). The model predicts that like PBP, the mGluR and CaR ECDs are venus flytrap-like structures with two lobes each consisting of α-helix and β-sheet folds connected by a hinge region of three strands (9). In this model, regions of mGluR or CaR ECD that align with PBP are assigned secondary structure based on the x-ray crystallography-determined structure of PBP, whereas insertions in the mGluR or CaR ECD that do not align with PBP are left as loops of undefined secondary structure. All four such insertions in the CaR ECD model cluster in one of the two putative flytrap lobes. The tryptic cleavage site generating bands 1 and 2 is localized to one of these four insertions, consistent with the possibility that this in fact represents a surface-exposed loop of the protein. The site that generates band 3 is localized to a presumptive “stalk” that would tether the flytrap to the first transmembrane domain in the intact receptor. We speculate that this stalk is left freely accessible to tryptic cleavage in the secreted ECD.

For the mGluR1 ECD, the availability of high affinity agonists and antagonists permitted the demonstration that the ECD alone is capable of high affinity ligand binding (16). As yet, it has not been possible to measure directly Ca²⁺ binding to either the intact CaR or its ECD. One report showed that Ca²⁺ induces dimer formation of CaR solubilized from purified renal endosomes (13), but another report failed to see Ca²⁺-induced dimer formation in CaR expressed in transfected HEK-293 cells (14). Consistent with the latter report, we failed to see evidence of divalent cation-induced dimerization of the purified hCaR ECD (data not shown). As another indirect measure of Ca²⁺ binding to the hCaR ECD, we tested the effect of Ca²⁺ on the rate of tryptic digestion of the ECD. Although there was a suggestion that Ca²⁺ slowed the rate of digestion (data not shown), effects observed were small and not consistently observed. Failure to detect changes in CD spectrum with addition of cation agonists such as neomycin and gadolinium chloride does not exclude binding of such agonists to the ECD. In fact, the venus flytrap model predicts no change in secondary structure and therefore CD spectrum with ligand binding, but rather a rotation of ~45° of one lobe relative to the other (9). Although it has been speculated that the ECD is the site of Ca²⁺ binding in the CaR (8), at this point we are unable to demonstrate this with the purified ECD.

In summary, we have shown that the hCaR ECD can be purified in milligram amounts, defined the site of signal peptide cleavage, and shown that it is a glycosylated, disulfide-linked dimer with a folded structure that contains only two highly accessible tryptic cleavage sites. Availability of the purified ECD should permit further studies to define the actual sites of glycosylation, to identify intermolecular disulfides involved in dimer formation, and intramolecular disulfides critical for tertiary structure, and ultimately, the three dimensional structure. This information will be critical to understanding how Ca²⁺ leads to receptor activation.

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