Impaired cotranslational processing of the calcium-sensing receptor due to signal peptide missense mutations in familial hypocalciuric hypercalcemia

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Received March 3, 2005; Revised April 19, 2005; Accepted April 29, 2005

The CASR, a cell surface glycoprotein expressed in parathyroid gland and kidney, is critical for maintaining extracellular calcium homeostasis. The inherited disorders, familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT), are caused by inactivating mutations in the CASR gene. The CASR has an N-terminal, 19 amino acid signal peptide that is predicted to direct the nascent polypeptide chain, as it emerges from the ribosome, into the endoplasmic reticulum (ER). Here, we report the functional characterization of three CASR mutations identified in hypercalcemic/hyperparathyroid patients. The mutations, L11S, L13P and T14A, lie within the signal peptide hydrophobic core. When transiently transfected into kidney cells, L11S and L13P mutants demonstrated reduced intracellular and plasma membrane expression and signaling to the mitogen-activated protein kinase pathway in response to extracellular calcium relative to wild-type CASR and the T14A mutant. All mutant CASR RNAs translated into protein normally. In cotranslational processing assays, which test the functionality of the signal peptide in the early secretory pathway, the wild-type CASR and mutant T14A nascent polypeptides were targeted to microsomal vesicles, representing the ER, translocated into the vesicular lumen and underwent core N-glycosylation. In contrast, the L11S and L13P mutants failed to be inserted in the microsomes and undergo glycosylation. This is the first study examining the function of the CASR signal sequence and reveals that both L11S and L13P mutants are markedly impaired with respect to cotranslational processing, accounting for the observed parathyroid dysfunction.

INTRODUCTION

The calcium-sensing receptor (CASR), a cell surface G protein-coupled receptor, is expressed abundantly in the parathyroid gland and the kidney tubule (1). The CASR is responsive to small changes in blood calcium concentrations. Cation binding couples to intracellular signaling pathways that modify parathyroid hormone (PTH) secretion or renal cation handling (2). In this way, the CASR plays a critical role in orchestrating mineral ion homeostasis. The essential function that the CASR plays in maintaining blood calcium levels within a narrow normal range has been emphasized by the finding that inherited abnormalities of the CASR gene can cause either hypercalcemia or hypocalcemia depending upon whether they are inactivating or activating, respectively (3–5).

Loss of function mutations of the CASR occur in familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT). FHH (also known as familial
benign hypercalcemia) is an autosomal dominant disorder characterized by modest elevation of the serum calcium concentration (that is generally asymptomatic) and PTH levels that are not suppressed by the hypercalcemia and are inappropriately normal or, in some cases, frankly elevated (6–10). Relative hypocalciuria reflected by a low calcium-to-creatinine clearance ratio occurs in the majority of cases (11,12), although some members of FHH kindreds have hypercalciuria and/or nephrocalcinosis (10,13–15). The inheritance of a single copy of a mutated CASR gene causes FHH, whereas homozygous or compound heterozygous individuals who inherit two inactive gene copies usually present with more marked symptoms, often as NSHPT with marked hypercalciemia, skeletal demineralization and parathyroid hyperplasia (16–18).

At the present time, approximately 50 unique inactivating mutations have been identified in patients with FHH/NSHPT, with missense mutations being by far the most common type (19). The mutations are clustered in the N-terminal portion of the extracellular domain, predominantly in a part that may constitute the calcium-binding site, and in the transmembrane domain. The clinical importance of identifying hypercalciemic individuals as having CASR mutations lies in distinguishing them from patients having primary hyperparathyroidism (12,20). For the majority of FHH patients, there is little benefit to be gained from parathyroidectomy, as the renal CASR defect is not rectified by such treatment (21,22). From a more basic standpoint, analysis of CASR mutants in vitro has led to important insights into how the receptor functions (23,24).

The human CASR mRNA encodes a protein of 1078 amino acids (25). The first 19 amino acids (26) encode a signal peptide followed by a 593 amino acid extracellular domain (ECD), a 250 amino acid transmembrane domain and a 216 amino acid intracellular tail. The N-terminal hydrophobic signal peptide is characteristic of proteins destined for residence in the plasma membrane (like the CASR) or for secretion. The signal peptide targets the nascent chain of a preprotein to, and translocates it into, the endoplasmic reticulum (ER) (27,28). The signal peptide of the CASR has not been studied with respect to cotranslational processing.

To date, very few human inherited disorders have been ascribed to signal peptide mutations. Those that have been described include familial isolated hypoparathyroidism (29,30), familial central diabetes insipidus (31), coagulation factor X deficiency (32), Crigler–Najjar type II (33) and thyroxine-binding globulin deficiency (34).

In the present report, we describe the identification of missense mutations in the hydrophobic core of the CASR signal peptide of two patients presenting with hypercalcemia. We conducted a functional analysis of CASR cDNAs encoding these mutations, as well as another mutation described previously (35), with respect to cellular and plasma membrane expression, cell signaling and the ability of the mutant mRNAs to direct translation and the signal peptides to mediate the cotranslational processing of the CASR preprotein. This is the first report of disease-causing mutations in the signal peptide of a member of the GPCR superfamily.

RESULTS
Case 1
A 43-year-old male was diagnosed with primary hyperparathyroidism (HPT) with elevated serum ionized calcium [1.5 mmol/l (normal, 1.15–1.28)] and parathyroid hormone (PTH) [122 pg/ml (normal, 10–65)]. The patient underwent two neck explorations, during one of which a hyperplastic left lower parathyroid gland was removed. However, hypercalcemia persisted post-operatively. The patient has a history of recurrent nephrolithiasis (24 h urinary calcium, 299 mg). There was a history of psychiatric disturbance with bipolar affective disorder and mild schizophrenia for more than 20 years. Family history includes two siblings with hypercalcemia, a 23-year-old daughter with IgA nephropathy, kidney stones and hypercalcemia and a 22-year-old son who is normal. At the first visit to NIH, at 50 years of age, the serum biochemistries of the proband were as follows: total calcium, 2.7 mmol/l; ionized calcium, 1.59 mmol/l; creatinine 1.1 mg/dl. Urinary calcium excretion was elevated at 8.37 mmol/24 h with urinary creatine excretion 1.58 g/24 h, and a calcium-to-creatinine clearance ratio of 0.022. Parathyroid tumor imaging studies were consistent with right lower parathyroid gland localization. At the third neck exploration, a hypercellular right lower parathyroid gland was excised. Intraoperatively, serum PTH fell from 93 to 18 pg/ml. Upon discharge from hospital, the patient’s serum biochemistries were as follows: total calcium, 2.35 mmol/l (normal, 2.05–2.50); albumin, 3.3 g/dl (normal, 3.7–4.7); ionized calcium, 1.45 mmol/l (normal, 1.17–1.31); PTH, 28.4 pg/ml (normal 10–65).

Case 2
A 37-year-old female was diagnosed with HPT after presentation with bone pain and fatigue. After the first neck exploration and right superior parathyroid adenoma removal, HPT persisted. At a subsequent neck exploration, at 40 years of age, at NIH, a left inferior parathyroid adenoma was removed, and the patient became normocalcemic. The patient’s 24 h urine calcium-to-creatinine ratio was 0.005 and both parents were normocalcemic.

Case 3
The full clinical details of this case have been reported previously (35). In brief, the parents of the proband, their daughter, were consanguineous and both had mild asymptomatic hypercalciemia and relative hypocalciuria. The patient presented at 9 years of age with periorbicular headache, nausea and vomiting, and cranial computed tomography revealed calcifications in the falx cerebri and tentorium cerebelli. Laboratory tests showed serum total calcium of 3.53 mmol/l (normal, 2.0–2.55), ionized calcium of 2.1 mmol/l (normal, 1.12–1.32 mmol/l) and PTH up to 110 pg/ml (normal, 11–62), and urinary calcium-to-creatinine ratio of 0.0047 (normal >0.02). At parathyroidectomy, four enlarged hyperplastic parathyroid glands were removed. Following surgery, serum total calcium, ionized calcium and PTH levels decreased into the normal range. Mutational
analysis showed that the proband was homozygous and both parents heterozygous for a missense mutation (L13P) encoded by exon 2 of the CASR gene.

Identification of CASR mutations

Case 1. Direct sequence analysis of PCR-amplified CASR exons identified a heterozygous mutation (L11S, TTG → TCG) at the N-terminus of the CASR encoded by exon 2 of the gene in the proband (Fig. 1A). This change did not alter a restriction site; however, a modified forward primer (Fig. 1B) in combination with the change in the proband’s DNA introduced an Esp31 site present in wild-type DNA (Fig. 1C). This change was not found in 100 CASR gene alleles from 50 unrelated normal individuals.

Case 2. Direct sequence analysis of PCR-amplified CASR exons revealed a heterozygous mutation (T14A, ACC → GCC) in the proband (Fig. 1D). This mutation destroyed an HphI site present in normal DNA (Fig. 1E), and this provided a convenient test to confirm the mutation in the proband (Fig. 1F).

Transfected L11S and L13P mutants demonstrate markedly reduced expression in HEK293 cells

We first examined whether the mutant CASRs were expressed normally and demonstrated similar molecular species as wild-type CASR. By site-directed mutagenesis, c-Myc-tagged L11S, L13P and T14A mutants were created, and were transiently transfected into HEK293 cells. Cells were also transfected with either the c-Myc-tagged wild-type (positive control) or empty vector (negative control). Western blot analysis was conducted with an antibody against the c-Myc
epitope tag. The CASR exists in both monomeric and dimeric forms: the monomeric unglycosylated species is 120 kDa, the core glycosylated (immature) species is 140 kDa and the mature, fully glycosylated species is 160 kDa (36,37). For the wild-type, the predominant monomeric species observed (Fig. 2) was the 140 kDa form, with the 160 kDa form present in lesser amount. The T14A mutant demonstrated an expression level and pattern of molecular species identical to that of the wild-type (Fig. 2). However, although monomeric and dimeric species were present in the L11S and L13P mutant-transfected cells, the expression levels were much reduced. It is worth noting that the 120 kDa species, not apparent in the wild-type-transfected cells, could be observed in the L11S and L13P mutant-transfected cells (Fig. 2). The HEK293 cell transfection and western blot analysis showed that the expression of the L11S and L13P mutants was markedly defective when compared with the wild-type and T14A receptors. When equal amounts of wild-type and either L11S or L13P mutant CASR cDNAs were transiently coexpressed, in each case, the pattern of monomeric and dimeric species was not different from that with wild-type alone (data not shown). Therefore, the mutant CASRs do not have a negative effect on the later processing of the wild-type CASR.

Transfected L11S and L13P mutants demonstrate no or little cell surface expression, respectively, in HEK293 cells

To examine whether the CASR mutants were expressed on the cell surface, fluorescence immunocytochemistry was performed on HEK293 cells transiently transfected with c-Myc-tagged wild-type and mutant CASR cDNAs. The analysis was performed in (i) nonpermeabilized cells to detect cell surface staining only, indicating (if present) appropriate receptor maturation and trafficking to the plasma membrane, and (ii) permeabilized cells to assess the amount of receptor present intracellularly and undergoing maturation and trafficking to the plasma membrane. Cells mock-transfected or transfected with untagged CASR DNA showed no specific staining with the c-Myc antibody (data not shown). Strong staining was present at the cell surface of nonpermeabilized HEK293 cells transfected with c-Myc-tagged wild-type receptor or the T14A mutant (Fig. 3A). Permeabilization of such cells revealed further intracellular perinuclear staining associated with the ER and Golgi apparatus (Fig. 3B). In contrast, nonpermeabilized cells that had been transfected with either the L11S or L13P mutant demonstrated either no or very little cell surface staining, respectively (Fig. 3A). Likewise, permeabilized cells transfected with these mutants demonstrated markedly reduced staining relative to those transfected with the wild-type receptor (Fig. 3B). The HEK293 cell transfection and fluorescent immunocytochemistry analysis showed that although the T14A mutant was expressed at the cell surface as well as wild-type, the L11S and L13P mutants were markedly deficient in terms of intracellular and cell surface expression.

Transfected L11S and L13P mutants demonstrate impaired cell signaling in response to increases in extracellular calcium concentration in HEK293 cells

The ability of mutant receptors to respond to increases in extracellular calcium relative to the wild-type receptor was assessed by means of a trans-reporting system that measures the activity of Elk-1, an ETS domain transcription factor targeted by mitogen-activated protein kinase (MAPK) pathways. The wild-type and T14A CASR cDNAs, when transiently expressed in HEK293 cells, showed a half-maximal response ($EC_{50}$) of $3.8 \pm 0.16$ mm (mean $\pm$ SE; Fig. 4A) to increasing extracellular calcium concentrations. In contrast, the L11S and L13P mutants were unresponsive even to an extracellular calcium concentration of 15 mM (Fig. 4A). These results show that the T14A mutant is as effective as the wild-type in triggering cell signaling pathways in response to increasing extracellular calcium concentrations. However, the L11S and L13P mutants lacked the ability to signal in this way, most likely because of the markedly reduced levels of expression noted in the western blot and immunocytochemical analyses. When equal amounts of wild-type and either L11S or L13P mutant CASR cDNAs were transiently coexpressed, in each case, the MAPK assay dose–response curves were not different from those of the wild-type alone (Fig. 4B). Therefore, the mutant CASRs do not have a negative impact on the wild-type CASR.
Mutants L11S, L13P and T14A lie within the hydrophobic core of the CASR signal peptide

The CASR has an N-terminal signal peptide that would be predicted to direct the nascent polypeptide chain into the ER. The CASR signal sequence has been little studied. Goldsmith et al. (26) found that HEK293 cells stably expressing CASR (1–596) (the extracellular domain) secreted into the culture medium a protein that began with tyrosine (+20 of the preprotein) indicating that signal peptide cleavage had occurred after alanine (+19). Signal peptides are heterogeneous but have three essential conserved regions: the N-terminal n-region; the hydrophobic core, the h-region, critical for co-translational processing; and the C-terminal, c-region that ends at the signal peptide cleavage site. The N-terminal 25 amino acids of the CASR were examined by the SignalP neural networks (NN) and hidden Markov models (HMM) programs. The SignalP-HMM program (eukaryotes) predicts a signal peptide (probability 0.999) and not a signal anchor (probability 0.000) with cleavage site probability, 0.958 between position 19 and 20. The CASR signal peptide is predicted to have an n-region from +1 to +5, an h-region from +6 to +14, and a c-region from +15 to +19 (Fig. 5A and B). Insight into whether particular amino acids in a signal sequence are consistent with signal peptide function can be gained with the SignalP-NN program. Analysis of putative signal sequences in this way yields the s-score, which reports the signal peptide prediction for every single amino acid position, with high scores indicating that the particular amino acid is part of a functioning signal peptide. The c-score is the cleavage site score, with the cleavage site position number indicating the first residue in the mature protein. The y-score is a derivative of the c-score combined with the s-score, resulting in a better cleavage site prediction. For the CASR wild-type sequence all amino acids (+1 to +19) are >50% for the s-score (Fig. 6A); however, for the L11S (Fig. 6B) and L13P (Fig. 6C) mutants the s-score falls on or below the 50% cutoff in two places. The T14A mutant s-score plot is more similar to that of wild-type than the plots of the other two mutants, touching the 50% cutoff at one point (Fig. 6D).

In vitro translation of all CASR mutants is normal

To assess whether any of the mutants were abnormal with respect to the ability of their mRNAs to be translated, in vitro transcription/translation with plasmid DNA templates encoding the wild-type and mutant CASRs and [35S]methionine was carried out. For all these mutants, L11S, L13P and T14A, a 120 kDa translation product was generated identical in size and amount to the wild-type receptor (Fig. 7A). Therefore, the nucleotide changes of the CASR mutants do not lead to any alteration in their mRNA translation ability.

The L11S and L13P mutants do not undergo proper core glycosylation

We next used canine pancreatic microsomal membranes to study the cotranslational and initial post-translational processing of the wild-type CASR and mutants. In vitro translation of the mRNA of interest in the presence of the microsomal membranes allows an examination of processing events such as translocation and core glycosylation. Glycosylation of a subset of the several N-linked sites in the CASR’s
The extracellular domain is required for expression of the receptor at the cell surface (38). The CASR is a large protein of 1078 amino acids (120 kDa) and its core glycosylated form is 140 kDa. To facilitate the analysis, we prepared using in vitro transcription, RNA encoding CASR (1–296) by digesting CASR cDNA with SfiI, which uniquely cleaves codon 297. The resulting RNA encodes a 33 kDa protein and retains four potential N-linked glycosylation sites. When the truncated wild-type RNA was translated, a 33 kDa product was observed (Fig. 7B). In the presence of canine microsomal membranes, the amount of the 33 kDa (unglycosylated) product or generation of the 49 kDa (core glycosylated) product (Fig. 7B). These data show that while the T14A mutant is processed like wild-type, neither L11S nor L13P undergo core glycosylation in vitro.

The L11S and L13P mutants do not translocate into the ER in vitro

To determine the location of nascent chains within the microsomal vesicles, a protease protection assay was carried out. The truncated CASR RNAs were translated in the presence of microsomal membranes and proteinase K was added in the absence or presence of Triton X-100. Protein products that have translocated to the lumen of the microsomal vesicles are protected from digestion with the proteolytic enzyme. For the wild-type CASR, in the presence of canine microsomal membranes, the 49 kDa processed product was protected against proteolysis with proteinase K that is unable to enter the vesicles (Fig. 7C). The specificity of the protection by the microsomal membranes was shown by addition of Triton X-100, which permeabilizes the membranes allowing access to the protease. Upon addition of the detergent, all CASR proteins were completely degraded (Fig. 7C). A similar pattern to wild-type was observed when the T14A mutant RNA was used in the proteinase protection assay (data not shown). However, for both L11S and L13P mutants addition of proteinase K alone was sufficient to degrade the CASR protein. These results indicate that the T14A mutant is translocated normally into the microsomal vesicle lumen, but that both the L11S and L13P mutants are defective in this in vitro translocation assay.

DISCUSSION

Molecular genetic analysis is useful in identifying patients with FHH (and NSHPT) caused by loss of function mutations in the CASR gene relative to other forms of hypercalcemia and hyperparathyroidism. The characteristic findings in FHH individuals are mild hypercalcemia, a serum PTH level within the normal range (but inappropriately elevated given the hypercalcemia and relative hypocalciuria). However, some patients with FHH present with frankly elevated serum PTH levels and/or hypercalciciuria and are often diagnosed with primary hyperparathyroidism (21). Some kindreds with familial isolated hyperparathyroidism (FIHP) due to inactivating mutations in the CASR gene may have the index case present as primary hyperparathyroidism, with other hypercalcemic relatives exhibiting the more benign FHH phenotype (39,40). In those cases, it may well be that other interacting factors, either genetic or environmental, were important in
modifying the phenotype. These types of argument could well apply to Case 1 of the present study in which the presentation was of primary hyperparathyroidism with frankly elevated serum PTH levels and urinary calcium excretion that was not low. This patient was documented to harbor an inactivating \( \text{CASR} \) gene mutation, as was Case 3, in which the calcium-to-creatinine clearance ratio was very low, and the appreciation of the hypercalcemia and hyperparathyroidism was made at a later stage than normal for typical neonatal hyperparathyroidism due to homozygous \( \text{CASR} \) gene loss of function. There was no evidence of severe musculoskeletal deficits. For Case 2, in which a second parathyroidectomy was successful in the attainment of normocalcemia, the only mutation identified in the \( \text{CASR} \) gene was not associated with any abnormalities when a functional analysis of the mutant \( \text{CASR} \) was carried out. Hence, despite the finding of a low calcium-to-creatinine ratio in the patient the involvement of the \( \text{CASR} \) gene has not been proved. The sequence substitution found probably represents a very uncommon (benign) polymorphism.

Functional characterization of the effects of the \( \text{CASR} \) mutations was done by transfecting \( \text{CASR} \) cDNAs, wild-type and those engineered to contain particular mutations, into human kidney cells. The L11S and L13P mutants were much less well expressed than either the wild-type or T14A mutant receptors, as assessed by immunoblot analysis. Despite the reduced levels of expression, the relative amounts of the immature (140 kDa) and mature (160 kDa) monomeric forms and aggregates (>280 kDa), likely to be dimers, were normal. The L11S mutant was not expressed at the cell surface, as evaluated by immunofluorescence and confocal microscopy, and cell surface staining of the L13P was much less than that of the wild-type or T14A mutant. Consistent with the marked deficit in cellular and cell surface expression, the L11S and L13P mutants were virtually without activity with respect to MAPK responses to increasing extracellular calcium concentrations. The T14A mutant had identical responsiveness to the wild-type.

None of the mutants were abnormal with respect to the ability of their mRNAs to be translated. In vitro transcription/translation of plasmid DNA templates yielded a product identical in size (120 kDa) and amount to wild-type. Therefore, the nucleotide changes did not affect mRNA translation ability.

To further assess the consequences of the mutations (that are clustered within the \( \text{CASR} \) signal peptide sequence), the proteins produced by cell-free translation of wild-type and mutant cRNAs were used in assays that reconstitute the initial steps of the secretory pathway. Processing events such as translocation and core glycosylation can be examined by conducting the experiments in the presence of microsomal membranes. It was demonstrated that the wild-type signal peptide of the \( \text{CASR} \) is competent to direct the nascent poly peptide to the ER and through the membrane to the cisternal space. Once this had occurred, core glycosylation took place. The T14A mutant was just as effective as wild-type

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**Figure 5.** Signal peptide of the \( \text{CASR} \). The probability that the N-terminal region of the \( \text{CASR} \) constitutes a signal peptide was examined with the SignalP-HMM program (version 3.0) as described in Materials and Methods. (A) A highly significant probability score was obtained and the positions of the n-, h- and c-regions were predicted in addition to a signal peptide cleavage site between amino acids 19 and 20. (B) A summary of the SignalP predictions. The \( \text{CASR} \) mutations in the hydrophobic core are arrowed.
in directing translocation and undergoing core glycosylation. However, both L11S and L13P mutants were deficient in the ability to attain entry to the ER and be core glycosylated.

Signal peptides range in size from 16 to 30 amino acids and are extremely heterogeneous with respect to their primary sequences. However, three essential, conserved features are found. The N-terminal region of five to eight amino acids is hydrophilic due to the presence of positively charged basic residues. The five amino acid n-region of the mammalian CASR signal peptide is atypical in not possessing a basic amino acid like arginine or lysine (Figs 5B and 8). Possibly, when there are no charges to fix the position of the n-region in the ER membrane, it may be able to move more freely and bind productively with the signal peptidase. A hydrophobic core of seven to 15 amino acids in signal peptides is critical for the cotranslational processing of the protein. The n-region of the CASR signal peptide is nine amino acids long and two to four (depending upon species) leucine residues contribute to its hydrophobicity (Figs 5B and 8). A comparison of CASR sequences across species reveals that the leucine corresponding to the human CASR L11S mutation characterized in the present study is absolutely conserved attesting to its importance (Fig. 8). With respect to the leucine corresponding to the human L13P mutation, though present in some species, it is found to be substituted in others by phenylalanine or serine. Leucine residues are still well represented in other parts of the hydrophobic core (Fig. 8). With respect to the threonine corresponding to the human T14A mutation, it can be noted that this residue is substituted in several species, and in some of these by an alanine residue (Fig. 8). Therefore, it is not surprising that the CASR T14A mutant was found to function as wild-type in all respects tested. The polar C-terminal region of approximately six amino acids contains the signal peptide cleavage site. In this region positions −1 and −3 are usually occupied by small neutral residues that can fit into the active site of the cleavage enzyme. The c-region of the CASR signal peptide has five amino acids with residue −1 being either alanine or threonine (or in one case glycine) (Fig. 8). Alanine is commonly found at position −1 in eukaryotic signal sequences (41). A tyrosine residue is invariant at position +1 of CASR across species; this is rarely found at this position in eukaryotic signal sequences (41).

In summary, we have demonstrated that missense mutations within the hydrophobic core of the signal peptide of the CASR and in hypercalcemic patients show marked functional deficits. Both the L11S and L13P mutations result in markedly impaired cotranslational processing of CASR, accounting for parathyroid dysfunction, thus emphasizing the key role of the signal peptide in the proper targeting of this preprotein.

**MATERIALS AND METHODS**

**Subjects**

All subjects gave informed consent for the study that was approved by the Institutional Review Board (IRB) of the National Institutes of Health and the Ethics Committee of the Royal Victoria Hospital.
Sequence analysis of the CASR gene

Patient leukocyte DNA was isolated using standard methods. Protein-coding exons 2–7 of the CASR gene were amplified as described previously (37). Gel purified PCR products were directly sequenced.

Site-directed mutagenesis

The Quik Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used. For each mutation, the primers were complementary with the mutant sequence placed in the middle. The primers were annealed to the template c-Myc-tagged human CASR cDNA in pcDNA3.1 (42), and 12 rounds of extension were performed with Pfu Turbo DNA polymerase, followed by digestion of the template with DpnI enzyme. The reaction was used to transform Escherichia coli strain XL1-Blue and colonies were screened by restriction enzyme digestion for the presence of the mutation. The correctness of all constructs was confirmed by sequencing.

Transient transfection of human CASR cDNA

Human embryonic kidney (HEK293) cells (provided by NPS Pharmaceuticals, Inc., Salt Lake City, UT, USA) were cultured in 100 mm culture dishes and transfected with human CASR cDNA (8 μg) using PolyFect transfection reagent. Forty-eight hours after transfection, cells were harvested for total cellular protein extraction and western blot analysis of total cell extracts was performed with the c-Myc 9E10 mouse monoclonal antibody. All the experiments were repeated at least three times and membranes were stripped and re-probed with β-tubulin mouse monoclonal antibody as a loading control.

Fluorescence immunocytochemistry and confocal microscopy

HEK293 cells were grown on 22 mm² coverslips in six-well plates (250 000 cells/well), and transiently transfected with either c-Myc-tagged wild-type or mutant CASR cDNA (2 μg) (43). Forty-eight hours after transfections, the phosphate-buffered saline (PBS)-washed cells were fixed.
in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min, if required. Washed cells were incubated in 10% goat serum for 1 h and then incubated with 9E10 c-Myc mouse monoclonal antibody at a 1:100 dilution for 3 h at room temperature. Washed cells were incubated for 1 h with a goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Molecular Probes, Inc., Eugene, OR, USA). Slides were mounted with mount medium and dried overnight at room temperature. Confocal images of labeled cells were acquired with a Zeiss LSM 510 META laser-scanning microscope (Carl Zeiss, Jena, Germany) using a 60× oil immersion lens. FITC fluorescence was visualized using a singletrack mode with laser excitation (488 nm) and emission (LP 505) filter sets.

**MAPK assay**

MAPK assays were done as described (44). Briefly, a trans-reporting system (Stratagene) was used to measure the activity of Elk-1, an ETS domain transcription factor targeted by MAPK pathways. HEK293 cells were transiently cotransfected with wild-type (0.5 μg) or mutant (0.5 μg) CASR expression vectors [or wild-type and mutant receptor (0.25 μg of each)] plus Elk-1 reporter constructs. The following day, cells were serum-starved in DMEM containing 0.5 mM CaCl₂ for 8 h and cultured in various concentrations of CaCl₂ ranging from 0.25 to 15 mM for 16 h. The cells were washed in PBS and lysed in lysis buffer on ice. Luciferase activity was measured using 45 μl cell lysate and D-luciferin using Fluostar Optima (BMG Labtech). Luciferase activity was normalized to β-galactosidase.

**In vitro transcription**

In vitro transcription reactions containing 0.5 μg of in vitro transcribed RNA, 17.5 μl of rabbit reticulocyte lysate (Promega), 25 μM methionine-free amino acids and 20 μCi [³⁵S]methionine were carried out according to the manufacturer’s instructions (Promega). Nuclease-treated canine pancreatic microsomal membranes (Promega) were added as indicated. Reactions were incubated at 30°C for 1 h and the translation products were analyzed by gradient (4–20%) SDS–PAGE.

**Protease protection assay**

In an ice-water bath, 20 μl of the translation reactions were chilled (0°C) and CaCl₂ was added to 10 mM. To the translation reactions, 10 μg/ml of proteinase K was added in the presence or absence of 1% Triton X-100. Reactions were incubated at 0°C for 30 min. The reactions were stopped by addition of complete protease inhibitor and immediately transferred to SDS–PAGE loading buffer and boiled for 5 min.

**Digestion of CASR with endoglycosidase H**

Twenty micrograms of protein were first denatured at 37°C for 15 min in the presence of 0.05% SDS and 50 mM 2-mercaptoethanol (10× denaturing buffer) and then incubated with or without 2 μl of endoglycosidase H in a buffer containing 70 mM sodium acetate, pH 5.2 and 0.8% Triton X-100 at 37°C for 1 h.
Signal peptide cleavage site prediction by signal IP server

CASP sequences from all species for which the sequences are known and sequences (human or rodent) of other members of family C GPCRs were analyzed with the SignalP World Wide Web prediction server version 3.0. The method incorporates a prediction of cleavage sites (c-score) and a signal peptide/non-signal peptide prediction (s-score) based on a combination of several artificial neural networks and hidden Markov models (http://www.cbs.dtu.dk/services/SignalP/) (46,47).

Sequence alignment by CLUSTAL W

Sequences predicted by SignalP were aligned with Clustal W in FASTA format (http://www.ebi.ac.uk/clustalw/).

ACKNOWLEDGEMENTS

We thank all family members for their participation, Irina Mosesova for technical assistance, Drs Sunita K. Agarwal and Carmen Mateo for patient DNA samples, Dr Stephane A. Laporte and Delphine Fessart for facilitating the confocal microscopy studies and Dr Hugh P.J. Bennett for critical review of the manuscript. This work was supported by Canadian Institutes of Health Research (CIHR) Grant MOP-57730 and a Kidney Foundation of Canada grant (to G.N.H.). S.P. was the recipient of a studentship from the McGill University Hospital Center Research Institute and a scholarship from the CIHR Strategic Training Program in Skeletal Health.

Conflict of Interest statement. None declared.

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