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Mutations Affecting G-Protein Subunit α_{11} in Hypercalcemia and Hypocalcemia

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Abstract

BACKGROUND—Familial hypocalciuric hypercalcemia is a genetically heterogeneous disorder with three variants: types 1, 2, and 3. Type 1 is due to loss-of-function mutations of the calcium-sensing receptor, a guanine nucleotide–binding protein (G-protein)–coupled receptor that signals through the G-protein subunit α_{11} ($G\alpha_{11}$). Type 3 is associated with adaptor-related protein complex 2, sigma 1 subunit (*AP2S1*) mutations, which result in altered calcium-sensing receptor endocytosis. We hypothesized that type 2 is due to mutations effecting $G\alpha_{11}$ loss of function, since $G\alpha_{11}$ is involved in calcium-sensing receptor signaling, and its gene (*GNA11*) and the type 2 locus are colocalized on chromosome 19p13.3. We also postulated that mutations effecting $G\alpha_{11}$ gain of function, like the mutations effecting calcium-sensing receptor gain of function that cause autosomal dominant hypocalcemia type 1, may lead to hypocalcemia.

METHODS—We performed *GNA11* mutational analysis in a kindred with familial hypocalciuric hypercalcemia type 2 and in nine unrelated patients with familial hypocalciuric hypercalcemia who did not have mutations in the gene encoding the calcium-sensing receptor (*CASR*) or *AP2S1*. We also performed this analysis in eight unrelated patients with hypocalcemia who did not have *CASR* mutations. In addition, we studied the effects of *GNA11* mutations on $G\alpha_{11}$ protein structure and calcium-sensing receptor signaling in human embryonic kidney 293 (HEK293) cells.

RESULTS—The kindred with familial hypocalciuric hypercalcemia type 2 had an in-frame deletion of a conserved $G\alpha_{11}$ isoleucine (Ile200del), and one of the nine unrelated patients with familial hypocalciuric hypercalcemia had a missense *GNA11* mutation (Leu135Gln). Missense *GNA11* mutations (Arg181Gln and Phe341Leu) were detected in two unrelated patients with hypocalcemia; they were therefore identified as having autosomal dominant hypocalcemia type 2. All four *GNA11* mutations predicted disrupted protein structures, and assessment on the basis of in vitro expression showed that familial hypocalciuric hypercalcemia type 2–associated mutations decreased the sensitivity of cells expressing calcium-sensing receptors to changes in extracellular calcium concentrations, whereas autosomal dominant hypocalcemia type 2–associated mutations increased cell sensitivity.

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CONCLUSIONS—$G\alpha_{11}$ mutants with loss of function cause familial hypocalciuric hypercalcemia type 2, and $G\alpha_{11}$ mutants with gain of function cause a clinical disorder designated as autosomal dominant hypocalcemia type 2. (Funded by the United Kingdom Medical Research Council and others.)

Familial hypocalciuric hypercalcemia, an autosomal dominant disorder, is characterized by lifelong elevations of serum calcium concentrations with low urinary calcium excretion (mean urinary calcium:creatinine clearance ratio, <0.01) and normal circulating parathyroid hormone concentrations in 80% of patients (see Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[1-3] Patients with familial hypocalciuric hypercalcemia are generally asymptomatic, although pancreatitis or chondrocalcinosis may develop in some affected adults.[4] Familial hypocalciuric hypercalcemia is genetically heterogeneous, with three reported variants. Type 1 is due to loss-of-function mutations of the calcium-sensing receptor (encoded by *CASR*), type 2 is of unknown cause, and type 3 is associated with adaptor-related protein complex 2, sigma 1 subunit (*AP2S1*) mutations, which alter calcium-sensing receptor endocytosis.[2,5] The calcium-sensing receptor is a widely expressed guanine nucleotide–binding protein (G-protein)–coupled receptor that has a pivotal role in extracellular calcium homeostasis as regulated by the parathyroids and kidneys. For example, activation of the calcium-sensing receptor as a result of elevated extra-cellular calcium concentrations causes G-protein–dependent stimulation of phospholipase C activity, through G_q and G_{11}, which results in an accumulation of inositol 1,4,5-trisphosphate and an increase in intracellular calcium concentrations. These changes, in turn, lead to reduced circulating parathyroid hormone concentrations and increased urinary calcium excretion.[4,6]

We hypothesized that since familial hypocalciuric hypercalcemia types 1 and 3 are due to abnormalities of the signaling pathway of the calcium-sensing receptor, type 2 may also involve this pathway and be due to mutation of G-protein subunit α_{11} ($G\alpha_{11}$), which is encoded by *GNA11*, for three reasons. First, $G\alpha_{11}$ is involved in calcium-sensing receptor signaling in parathyroid and renal cells. Second, *GNA11* and the familial hypocalciuric hypercalcemia type 2 locus are colocalized on chromosome 19p13.3. Finally, hypercalcemia develops in mice that have parathyroid-specific deletions encompassing the genes *Gna11* and *Gnaq*, which encode the orthologues of *GNA11* and *GNAQ* (encoding $G\alpha_q$), with increased serum parathyroid hormone concentrations due to parathyroid hyperplasia. This phenotype resembles neonatal severe primary hyperparathyroidism due to biallelic loss-of-function mutations in the calcium-sensing receptor.[1,7] In addition, we hypothesized that $G\alpha_{11}$ mutant proteins with gain of function may lead to hypocalcemia, just as mutations effecting gain of function in the calcium-sensing receptor result in autosomal dominant hypocalcemia type 1, which is associated with low or normal serum parathyroid hormone concentrations.[4,8] In autosomal dominant hypocalcemia type 1, about 50% of patients have mild or asymptomatic hypocalcemia; about 50% have paresthesia, carpopedal spasm, and seizures; about 10% have hypercalciuria with nephrocalcinosis or kidney stones; and more than 35% have ectopic and basal ganglia calcifications (Table S2 in the Supplementary Appendix).[8,9] We performed *GNA11* mutational analysis in a kindred with familial hypocalciuric hypercalcemia type 2[10,11] and in unrelated patients with familial hypocalciuric hypercalcemia who did not have *CASR* or *AP2S1* mutations.[1,5] We also performed this analysis in patients with hypocalcemia who did not have *CASR* mutations.[1] Our hypothesis was that we would detect $G\alpha_{11}$ mutants with loss of function in patients with hypercalcemia and $G\alpha_{11}$ mutants with gain of function in patients with hypocalcemia.

METHODS

PATIENTS

We identified members (10 affected and 8 unaffected) of four generations of a kindred with familial hypocalciuric hypercalcemia type 2 (referred to in other studies as Kindred 11675)[10-12] and 9 unrelated patients with familial hypocalciuric hypercalcemia who did not have *CASR* or *AP2S1* mutations of the coding region and exon–intron boundaries (Table S1 in the Supplementary Appendix). We also identified 8 unrelated patients with hypocalcemia and low or normal serum parathyroid hormone concentrations — findings that were consistent with autosomal dominant hypocalcemia type 1 — who did not have *CASR* mutations of the coding region and exon–intron boundaries (Table 1, and Table S2 in the Supplementary Appendix).[1,5,9,14] Informed consent was obtained from all persons (verbal consent from 82 persons and written consent from 8 persons) with the use of protocols approved by local and national ethics committees.

DNA SEQUENCE ANALYSIS

Leukocyte DNA was used with *GNA11*-specific primers (Table S3 in the Supplementary Appendix) for mutational analysis.[1,13] Sequence changes were assessed for the occurrence of polymorphisms in 55 unrelated persons with normocalcemia and in the exome sequence data from approximately 5400 unrelated persons that were obtained from the Exome Sequencing Project of the National Heart, Lung, and Blood Institute (NHLBI-ESP).[15]

PROTEIN SEQUENCE ALIGNMENTS AND THREE-DIMENSIONAL MODELING OF $G\alpha_{11}$ STRUCTURE

$G\alpha_{11}$ orthologues and paralogues were aligned with the Clustal W program.[16] The crystal structures of $G\alpha_q$,[17,18] which shares 90% identity at the amino acid level with $G\alpha_{11}$, were used to model $G\alpha_{11}$ mutants with the use of the PyMOL Molecular Graphics System (version 1.2r3pre, Schrödinger)[5,17,19-21] (see the Methods section in the Supplementary Appendix).

FUNCTIONAL EXPRESSION OF *GNA11* MUTATIONS

The full-length coding region of *GNA11* was sub-cloned into the bidirectional vector pBI-CMV2 (Clontech), which expresses green fluorescent protein (GFP) and mutations introduced by site-directed mutagenesis.[5] Nonmutant and mutant constructs were transfected into human embryonic kidney 293 (HEK293) cells that stably expressed calcium-sensing receptors.[5] We measured the responses in intracellular calcium concentrations, detected with the use of indo-1 acetoxymethylester, to changes in extracellular calcium concentrations.[1,5] Expression of the calcium-sensing receptor, $G\alpha_{11}$, GFP, and $G\alpha_q$ was confirmed by means of Western blot analysis, immunofluorescence, or both (see the Methods section in the Supplementary Appendix).[1,5]

QUANTITATIVE REVERSE-TRANSCRIPTASE–POLYMERASE-CHAIN-REACTION ANALYSES

Quantitative reverse-transcriptase–polymerase-chain-reaction testing was performed with the use of RNA from parathyroid tumors and normal human tissues (see the Methods section in the Supplementary Appendix).[22]

STATISTICAL ANALYSIS

Statistical analysis for comparison of mutant with nonmutant results was performed with the use of the Mann–Whitney U test or the F-test.[5]

RESULTS

PATIENTS WITH FAMILIAL HYPOCALCIURIC HYPERCALCEMIA

GNA11 Mutations—DNA sequence analysis of the *GNA11* 1077-bp coding region and 12 exon–intron boundaries in a proband from the kindred reported to have familial hypocalciuric hypercalcemia type 2[10-12] identified a heterozygous 3-bp (ATC) deletion at c. 598-600, leading to an in-frame deletion of the Ile200 residue (I200) (Fig. 1A, and Fig. S1A in the Supplementary Appendix). This deletion results in the gain of an *Xmn*I restriction-endonuclease site, which was used to confirm the DNA sequence abnormality (Fig. 1B) and show cosegregation of the deletion with the disease in the 10 affected members from four generations of the kindred (Fig. 1C). *GNA11* mutational analysis in 9 other patients with familial hypocalciuric hypercalcemia (Table 1, and Table S1 in the Supplementary Appendix) revealed, in 1 patient, a heterozygous T→A transversion at c.404 resulting in a Leu135Gln (L135Q) missense substitution, which altered a *Tsp*RI restriction-endonuclease site that was used to confirm the mutation (Fig. S1B and S1C in the Supplementary Appendix). In addition, the absence of these DNA sequence abnormalities in 55 unrelated persons with normocalcemia and in approximately 5400 exomes (from data obtained from the NHLBI-ESP),[15] together with the conservation of I200 and Leu135 (L135) residues in vertebrate $G\alpha_{11}$ subunit orthologues, human paralogues, or both (Fig. S2A in the Supplementary Appendix), indicated that the Ile200del (I200del) and L135Q abnormalities were probably *GNA11* mutations rather than polymorphic variants.

Predicted Effects of $G\alpha_{11}$ Mutant Proteins—The I200 residue is located within a 13-amino-acid region (residues 193 through 205), the length of which is conserved among $G\alpha_{11}$ orthologues and human paralogues (Fig. S2A in the Supplementary Appendix). It links two flexible regions known as switch 1 (αF–β2 loop) and switch 2 (β3–α2 loop and α2 helix) (Fig. 2A, and Fig. S2A and S2B in the Supplementary Appendix). Moreover, the I200 homologue is adjacent to the tetrapeptide β2–β3 loop that forms part of the interface between $G\alpha$ and the G-protein–coupled receptor, and it is considered to have a role in G-protein–coupled receptor–mediated guanosine diphosphate (GDP) release and G-protein activation.[17,18,21,23,24]

An analysis of the predicted effects of the familial hypocalciuric hypercalcemia type 2–associated I200del on the structure of the $G\alpha_{11}$ β2–β3 sheet indicated that it would lead to a loss of five of the six hydrogen bonds (Fig. 2B), thereby disrupting the β2–β3 loop and affecting the switch-region conformational changes, which would limit the transition of the GDP-bound inactive form to the activated guanosine triphosphate (GTP)–bound form (Fig. S2B in the Supplementary Appendix). A similar analysis of the familial hypocalciuric hypercalcemia type 2–associated L135Q mutation, located in the helical domain and close to the αD–αE loop that, together with the flexible switch-3 region, facilitates GDP release[23] (Fig. 2A, and Fig. S2C in the Supplementary Appendix), also indicated a likely loss of $G\alpha_{11}$ function.

Functional Characterization of $G\alpha_{11}$ Mutants—Three-dimensional modeling predicted that the familial hypocalciuric hypercalcemia type 2–associated mutations would probably result in loss of $G\alpha_{11}$ function, and we hypothesized that these mutations would result in decreased sensitivity of cells expressing calcium-sensing receptors to extracellular calcium. To investigate this hypothesis, we used HEK293 cells that stably expressed calcium-sensing receptors, and we transiently transfected them with nonmutated or mutated *GNA11*–pBI-CMV2 expression constructs or with vector containing the GFP reporter gene alone (empty vector). We then assayed the responses of intracellular calcium concentrations to alterations in extracellular calcium concentrations. Expression of calcium-sensing

receptor, $G\alpha_{11}$, $G\alpha_q$, and GFP was detected by means of immunofluorescence, Western blot analysis, or both (Fig. S3A, S3B, and S3C in the Supplementary Appendix). Expression of calcium-sensing receptor and $G\alpha_q$, normalized by comparison with tubulin expression as a loading control, did not differ significantly between cells transfected with nonmutated or mutated *GNA11*–pBI-CMV2 vector and cells transfected with empty vector, whereas expression of $G\alpha_{11}$ was significantly greater in the cells transfected with nonmutated or mutated *GNA11*–pBI-CMV2 vector. This finding is similar to that in human hyperplastic parathyroids, in which endogenous expression of $G\alpha_{11}$ was more than 3 times as high as that of $G\alpha_q$ (Fig. S4 in the Supplementary Appendix).

The familial hypocalciuric hypercalcemia type 2–associated I200del and Q135 $G\alpha_{11}$ mutants resulted in a rightward shift in the concentration–response curves, with significantly higher half-maximal effective concentration (EC_{50}) values (Fig. 2C and 2D); this indicated a decrease in the sensitivity of cells expressing calcium-sensing receptors to alterations in extracellular calcium concentrations and was consistent with the effects of loss-of-function mutations in the calcium-sensing receptor that have been reported in familial hypocalciuric hypercalcemia type 1.[1,2] Moreover, cotransfection of each of the familial hypocalciuric hypercalcemia type 2–associated mutated *GNA11* constructs with a nonmutated construct, to maintain an approximate 1:1 stoichiometric balance, also resulted in rightward shifts in the concentration–response curves, with significantly higher EC_{50} values, which is consistent with a loss of function associated with I200del and Q135 $G\alpha_{11}$ mutants (Fig. S5 and Table S4 in the Supplementary Appendix). Variations in $G\alpha_{11}$ expression, which were approximately 1 to 6 times as high as that in untransfected cells, had no significant effects on the concentration–response curves or on the EC_{50} values (Fig. S6 in the Supplementary Appendix).

To further assess the role of the $\beta 2$–$\beta 3$ loop in $G\alpha_{11}$ function, we generated $\beta 2$–$\beta 3$ mutant constructs with deletions, alanine substitutions, an insertion, and a substitution of Glu197-Asn198-Ile199 of $G\alpha_{11}$ with the Gln197-Ser198-Val199 of $G\alpha_q$ (Fig. S7 and Table S4 in the Supplementary Appendix). Expression of these engineered $\beta 2$–$\beta 3$ loop mutants revealed that alanine substitution of Ile199, Ile200, Leu196, and Glu197, but not Asn198, resulted in significantly higher EC_{50} values, indicating the importance of these residues in forming hydrogen bonds and in maintaining the structural integrity of the $\beta 2$–$\beta 3$ loop (Fig. 2B). In support of these findings, substitution of the $G\alpha_{11}$ Glu197-Asn198-Ile199 with the $G\alpha_q$ Gln197-Ser198-Val199, which permits hydrogen bonding between Val199 and Leu196, did not alter the calcium-sensing receptor EC_{50} value (Fig. S7 in the Supplementary Appendix). Finally, deletion of any one of the loop residues, or an insertion, resulted in a diminished response of the intracellular calcium concentration, whereas the Glu-Asn-Ile197-199Gln-Ser-Val substitution had no effect, findings that indicate the tetrapeptide length requirement of this loop for $G\alpha_{11}$ activation. Thus, the familial hypocalciuric hypercalcemia type 2–associated *GNA11* mutants decreased signal transduction of the calcium-sensing receptor; these findings establish the importance of the $G\alpha_{11}$ $\beta 2$–$\beta 3$ loop.

PATIENTS WITH HYPOCALCEMIA

GNA11 Mutations—*GNA11* mutational analysis in 8 patients with hypocalcemia identified heterozygous sequence abnormalities comprising a G→A transition at c.542 and a C→G transversion at c.1023, which predicted the missense mutations Arg181Gln and Phe-341Leu, respectively, in 2 patients (Fig. 3 and Table 1, and Fig. S8 in the Supplementary Appendix). These DNA sequence abnormalities altered *Pst*I and *Bst*UI restriction-endonuclease sites, which were used to confirm the presence of the mutations (Fig. 3B and 3C, and Fig. S8 in the Supplementary Appendix). In addition, the absence of these DNA sequence abnormalities in 55 unrelated persons with normocalcemia and in

about 5400 exomes (in data obtained from the NHLBI-ESP),[15] together with conservation of Arg181 and Phe341 residues in vertebrate $G\alpha_{11}$ subunit orthologues and human paralogues (Fig. S9A in the Supplementary Appendix), indicated that the Arg181Gln and Phe341Leu abnormalities were probably *GNA11* mutations rather than polymorphic variants. Patients with *GNA11* mutations were therefore designated as having auto-somal dominant hypocalcemia type 2.

Predicted Effects of $G\alpha_{11}$ Mutant Proteins—The mutant Arg181 is located in the αF helix of the helical domain, close to the Arg183 residue in the adjacent linker-2 region (Fig. 4A and 4B), which is important for GTP hydrolysis.[26] The auto-somal dominant hypocalcemia type 2–associated Arg181Gln mutation is predicted to shift the position of Arg183 and disrupt hydrogen bonds between Arg183 and GDP (Fig. 4B, and Fig. S9 in the Supplementary Appendix). The autosomal dominant hypocalcemia type 2–mutated Phe341, located in the GTPase domain, is predicted to disrupt a hydrophobic cluster that also comprises Phe194 and Phe201 in the GTPase domain (Fig. 4A and 4B, and Fig. S9 in the Supplementary Appendix). Both the Arg181Gln mutation and the Phe341Leu mutation probably decrease the stabilization of the GTP hydrolysis transition state by Arg183 and Gln209,[26-30] thereby prolonging the lifetime of the active GTP-bound $G\alpha_{11}$ subunit. We therefore hypothesized that these effects of the autosomal dominant hypocalcemia type 2 $G\alpha_{11}$ mutant proteins would result in an increased sensitivity of calcium-sensing receptor–expressing cells to changes in extracellular calcium concentrations.

Functional Characterization of $G\alpha_{11}$ Mutants—Nonmutated or mutated *GNA11*–pBI-CMV2 constructs or empty vector were expressed in HEK293 cells stably expressing calcium-sensing receptors, and the responses of intracellular calcium concentrations to alterations in extracellular calcium concentrations were assessed. Expression of calcium-sensing receptor, $G\alpha_{11}$, $G\alpha_q$, and GFP was detected by means of immunofluorescence, Western blot analysis, or both, and expressions of calcium-sensing receptor and $G\alpha_q$, when normalized for tubulin, were found to be similar, whereas those of $G\alpha_{11}$ were significantly increased, as compared with transfection with empty vector (Fig. S10 in the Supplementary Appendix). The autosomal dominant hypocalcemia type 2–associated $G\alpha_{11}$ mutants, Gln181 and Leu341, resulted in a leftward shift in the concentration–response curves, with significantly lower EC_{50} values, as compared with nonmutant $G\alpha_{11}$ (Fig. 4C and 4D). Thus, the decreases in the EC_{50} values of the calcium-sensing receptor that are induced by the autosomal dominant hypocalcemia type 2–associated mutants indicate an enhanced sensitivity of cells expressing calcium-sensing receptors to changes in extracellular calcium concentrations; this finding is consistent with the effects of gain-of-function mutations of the calcium-sensing receptor reported in autosomal dominant hypocalcemia type 1.

DISCUSSION

Our study shows that familial hypocalciuric hypercalcemia type 2 is due to mutations that diminish the function of $G\alpha_{11}$, and autosomal dominant hypocalcemia type 2 is due to mutations effecting gain of function of $G\alpha_{11}$. The mutations we describe here appear to be unique human germline disease-causing *GNA11* mutations. Moreover, these germline *GNA11* mutations were detected in more than 10% of patients with familial hypocalciuric hypercalcemia who did not have *CASR* and *AP2S1* mutations and in approximately 25% of patients with autosomal dominant hypocalcemia who did not have *CASR* mutations. Our study also shows a novel form of autosomal dominant hypocalcemia designated as autosomal dominant hypocalcemia type 2. The patients with autosomal dominant hypocalcemia type 2 and germline *GNA11* mutations had clinical features that were similar to those of patients with hypocalcemia who did not have *GNA11* mutations. Moreover, the two patients with autosomal dominant hypocalcemia type 2 were similar to the patients with

autosomal dominant hypocalcemia type 1 in that they had hypocalcemia, which may be asymptomatic or may be associated with neuromuscular irritability.[4] In addition, the patients with autosomal dominant hypocalcemia type 2 did not have hypercalciuria, which is observed in only 10% of patients with autosomal dominant hypocalcemia type 1.[8,9] However, extrapolation from this small number of patients with autosomal dominant hypocalcemia type 2 requires caution, since data are lacking from detailed studies involving larger numbers of patients. Such studies might help to clarify the effects of mutant $G\alpha_{11}$ on renal calcium excretion.

Somatic *GNA11* gain-of-function mutations involving the Arg183 and Gln209 residues have been described in patients with uveal melanomas.[25,31] However, these patients were not reported to have hypocalcemia,[31] and the two patients with autosomal dominant hypocalcemia type 2 who had $G\alpha_{11}$ mutants with gain of function did not have ophthalmologic abnormalities. Such differences in disease manifestations associated with somatic and germline mutations are also observed in other disorders. For example, germline mutations of the α-thalassemia and mental retardation, X-linked (*ATRX*) gene cause the ATR-X syndrome,[32] whereas somatic *ATRX* mutations are detected in about 40% of pancreatic neuroendocrine tumors[33]; germline *KRAS* mutations are detected in approximately 5% of patients with the Noonan syndrome, whereas somatic *KRAS* mutations are commonly detected in human cancers.[34]

In addition, there may be species-specific differences, since an *N*-ethyl-*N*-nitrosourea–induced germline hypermorphic *Gna11* mutation (Ile63Val), which has been described in a mouse model of dark skin (*Dsk7*), has not been reported to have uveal or extracellular calcium abnormalities,[35] and $G\alpha_{11}$-null mice are reported to be normocalcemic.[7] However, $G\alpha_{11}$-null mice with parathyroid-specific deletion of both *Gnaq* alleles are hypercalcemic; this indicates functional redundancy between $G\alpha_{11}$ and $G\alpha_q$ in mouse parathyroids.[7,36] Such functional redundancy is unlikely in human parathyroids, since mutation of one *GNA11* allele resulted in familial hypocalciuric hypercalcemia type 2, and it seems possible that these species-specific differences may stem from altered levels of $G\alpha_{11}$ and $G\alpha_q$ expression in tissues. It is noteworthy that in human parathyroids, $G\alpha_{11}$ expression is higher than that of $G\alpha_q$; these findings are consistent with observations in bovine parathyroids.[37]

Our analysis of structure and function highlighted critical G-protein motifs. G proteins are heterotrimeric complexes that relay signals from G-protein–coupled receptors. Activation of the G-protein–coupled receptor stimulates an exchange of $G\alpha$-bound GDP for GTP, causing dissociation of $G\alpha$ from $G\beta\gamma$ and facilitating interactions with downstream effectors.[23,38] Our results indicate that familial hypocalciuric hypercalcemia type 2–associated mutations probably impair GDP release,[23,24] whereas autosomal dominant hypocalcemia type 2–associated mutations prolong the activated $G\alpha$-GTP state. These results are consistent with the findings from studies of transducin ($G\alpha_t$) and K-ras GTPase mutant proteins.[27,34] Thus, structural analysis of $G\alpha_t$ has shown that the $\beta2$–$\beta3$ loop forms part of the interface between $G\alpha$ and the G-protein–coupled receptor and may facilitate G-protein– coupled receptor–mediated GDP release.[24] In addition, mutations effecting $G\alpha_t$ and K-ras residues that are homologous with the autosomal dominant hypocalcemia type 2–mutated $G\alpha_{11}$ Phe341 have been reported to disrupt the hydrophobic phenylalanine cluster and increase the rate of GDP–GTP exchange, thereby promoting the active GTP-bound form.[27,34] Thus, our studies of disease-causing $G\alpha_{11}$ mutants with loss of function and gain of function provide support for the role of the $\beta2$–$\beta3$ loop and phenylalanine cluster in GDP–GTP exchange and activation of the $G\alpha$ subunit.

In conclusion, our identification of human germline *GNA11* mutations associated with familial hypocalciuric hypercalcemia type 2 and autosomal dominant hypocalcemia type 2

shows the pivotal role of $G\alpha_{11}$ in calcium-sensing receptor–mediated signal transduction and extra-cellular calcium homeostasis in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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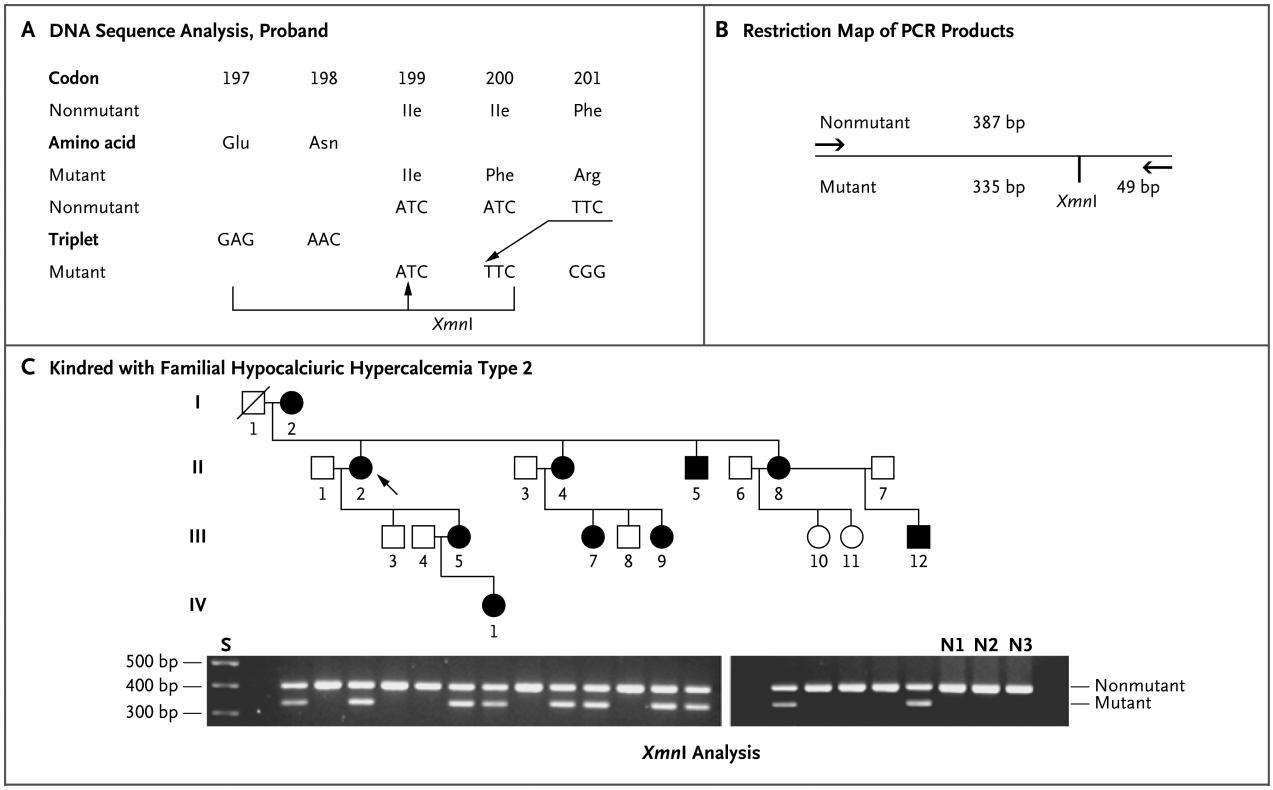

Figure 1. *GNA11* Mutation in a Patient with Familial Hypocalciuric Hypercalcemia Type 2

Panel A shows the predicted outcomes of DNA sequence analysis (Fig. S1 in the Supplementary Appendix) in the proband of the kindred with familial hypocalciuric hypercalcemia type 2 (identified as Kindred 11675 in Table S1 in the Supplementary Appendix), who had a heterozygous 3-bp (ATC) deletion, as compared with a normal unrelated person. The 3-bp deletion leads to an in-frame deletion of the Ile200 residue and gain of an *Xmn*I restriction-endonuclease site (GAACA/TCTTC). Panel B shows the resulting *Xmn*I restriction maps of nonmutant and mutant polymerase-chain-reaction (PCR) products. Panel C shows the use of *Xmn*I to confirm the mutation, which was not present in 110 alleles from 55 unrelated persons with normocalcemia (the findings in 3 of the 55 persons [N1, N2, and N3] are shown), and cosegregation of the Ile200del mutation with disease in the kindred with familial hypocalciuric hypercalcemia type 2 (LOD score, +3.60 at 0% recombination fraction). Each member of the kindred with familial hypocalciuric hypercalcemia type 2 is represented above the corresponding *Xmn*I-digested PCR product and identified with the use of numbers previously reported[10,11]; a sample from Person II.6 was not available. All unaffected persons with normocalcemia were homozygous for nonmutant alleles, whereas affected persons were heterozygous for nonmutant and mutant alleles; these findings are consistent with an autosomal dominant inheritance of familial hypocalciuric hypercalcemia type 2. Squares represent male family members, circles female family members, and black symbols affected family members. S denotes size marker. The proband is indicated by an arrow.

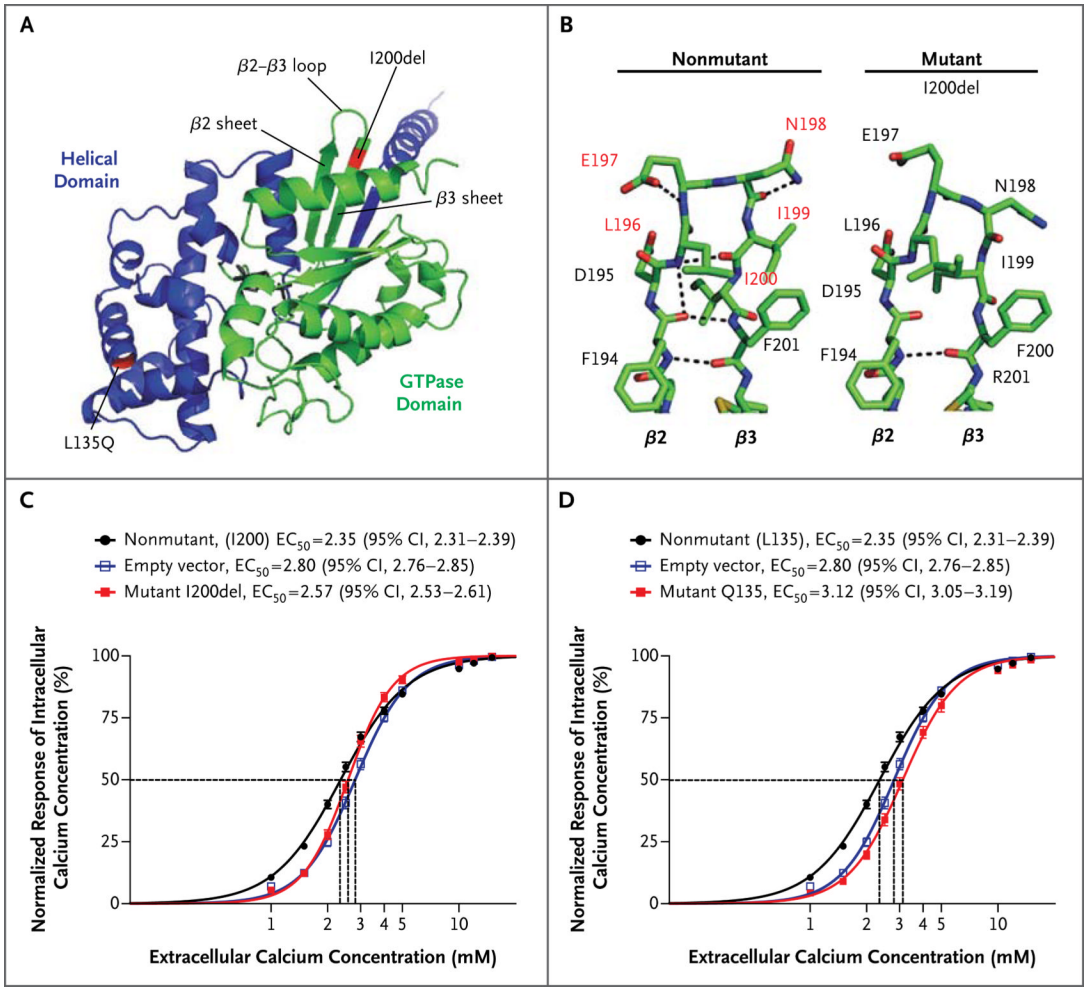

Figure 2. Three-Dimensional Modeling and Functional Characterization of Familial Hypocalciuric Hypercalcemia Type 2–Associated Mutant Gα_{11} Residues

Panel A shows a three-dimensional model of the Gα_{11} helical and GTPase domains, which are the locations of the Leu135 (L135) and Ile200 (I200) residues (red), respectively. Gα_{11} has 90% identity to Gα_q at the amino acid level, and the model is therefore based on the reported three-dimensional structure of Gα_q (Fig. S2 in the Supplementary Appendix).[17,18] I200 is located in the β3 sheet and adjacent to the β2–β3 loop, which is formed by the tetrapeptide comprising Ile(I)199-Asn(N)198-Glu(E)197-Leu(L)196. Panel B shows the β2–β3 loop region of nonmutant and mutant Gα_{11} and the structural effects of the I200del Gα_{11} mutant on hydrogen bonds (broken lines). Residues (red) mutagenized in this study (Fig. S3 and S7 in the Supplementary Appendix). Panels C and D show the responses of intracellular calcium concentrations to changes in extracellular calcium concentrations, in HEK293 cells stably expressing calcium-sensing receptors that were transiently transfected with nonmutant, familial hypocalciuric hypercalcemia type 2– associated mutant (I200del, Gln[Q]135), or empty *GNA11*–pBI-CMV2–green fluorescent protein (GFP) expression vectors (Fig. S3 in the Supplementary Appendix). The intracellular calcium responses to changes in extracellular calcium concentrations were expressed as a percentage of the maximum normalized response and are shown as the mean (±SE) of 8 to 45 assays from 3 to 12 independent transfections. P<0.001 for the comparisons of the mutant vector with the nonmutant and empty vectors. The familial hypocalciuric hypercalcemia type 2–associated mutants (I200del and Q135) led to a rightward shift in the concentration–response curves,

with significantly higher half-maximal effective concentration (EC_{50}) values (i.e., the extracellular calcium concentration required to produce a half-maximal increase in intracellular calcium concentration values) (Table S4 in the Supplementary Appendix), as compared with cells expressing nonmutated *GNA11*–pBI-CMV2-GFP. The familial hypocalciuric hypercalcemia type 2–associated Leu135Gln mutation conferred on the mutant protein a significantly increased EC_{50} as compared with nonmutant $G\alpha_{11}$ or empty vector alone, suggesting a possible dominant-negative effect.

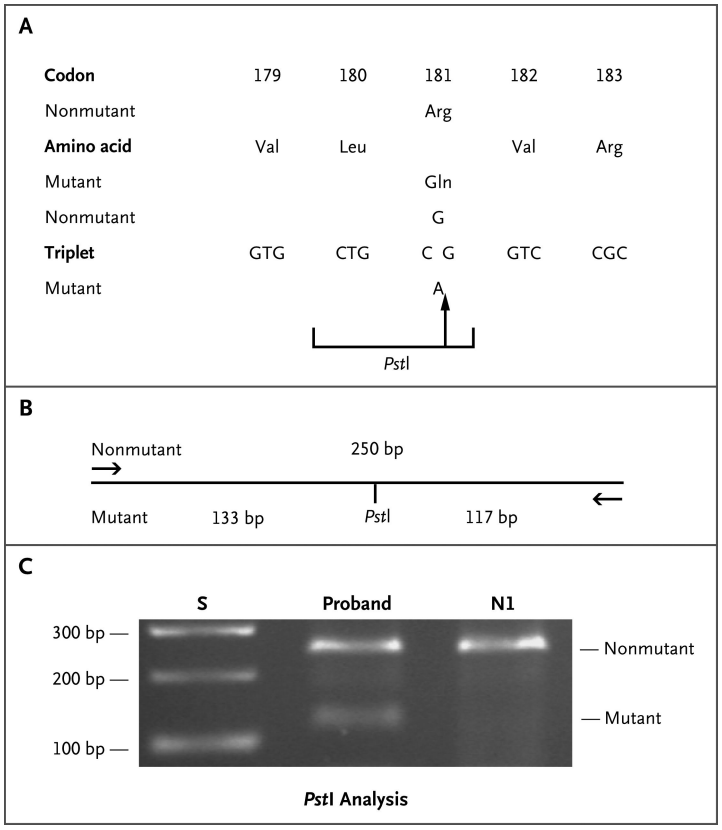

Figure 3. *GNA11* Mutation in a Patient with Autosomal Dominant Hypocalcemia

Panel A shows the predicted outcomes of DNA sequence analysis (Fig. S8 in the Supplementary Appendix) in Patient 3, who is the proband of a family with autosomal dominant hypocalcemia type 2 (Table S2 in the Supplementary Appendix) who had a heterozygous G→A transition at c.542, as compared with a normal unrelated person. The effect of the G→A transition is an alteration at codon 181 from CGG (encoding the nonmutant Arg [R] residue) to CAG (encoding a mutant Gln [Q] residue) and gain of a *Pst*I restriction-endonuclease site (CTGCA/G). Panel B shows the resulting *Pst*I restriction maps of nonmutant and mutant PCR products. Panel C shows the use of *Pst*I to confirm the mutation, which was not present in 110 alleles from 55 unrelated persons with normocalcemia (the results in 1 of the 55 persons [normal control 1, or N1] are shown). Each person is rep resented above the corresponding restriction enzyme–digested PCR products. The unaffected persons with normocalcemia are homozygous for the nonmutant alleles, whereas the affected proband is heterozygous for the nonmutant and mutant alleles; this finding is consistent with an autosomal dominant inheritance of autosomal dominant hypocalcemia type 2. S denotes the size marker.

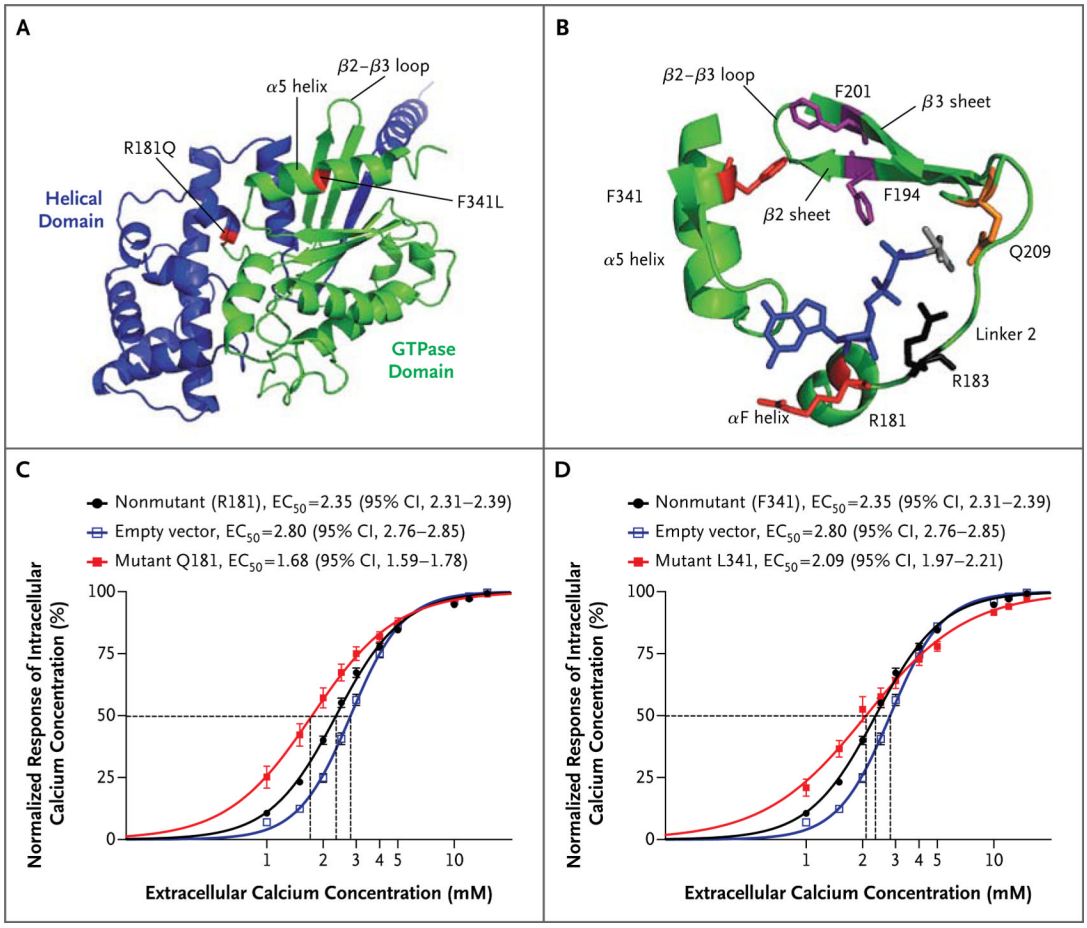

Figure 4. Location of Autosomal Dominant Hypocalcemia Type 2–Associated Gα_{11} Mutants in a Three-Dimensional Model and Effects on the EC_{50} of Calcium-Sensing Receptor–Expressing Cells

Panel A shows a three-dimensional model of the helical and GTPase domains of Gα_{11}. The Arg181 (R181) and Phe341 (F341) residues (red) are located in the helical and GTPase domains, respectively. Panel B shows a three-dimensional model of the guanosine diphosphate (GDP) (blue)–aluminum fluoride (AlF_4) (gray) binding pocket. Shown are the locations of R181 (red); the hydrophobic phenylalanine (F) cluster in which F341 (red), located in the α5 helix, interacts with F194 (magenta) and F201 (magenta), which are on the β2 and β3 sheets, respectively (Fig. S9 in the Supplementary Appendix); and R183 (black) and Q209 (orange), which are involved in somatic activating mutations in uveal melanomas.[25] R181 and R183 form hydrogen bonds with GDP-AlF_4, thereby stabilizing the GTP hydrolysis transition state. Panels C and D show responses of intracellular calcium concentrations to changes in extracellular calcium concentrations, in HEK293 cells stably expressing calcium-sensing receptors that were transiently transfected with nonmutant, autosomal dominant hypocalcemia–associated mutant (Q181 or L341), or empty *GNA11*–pBI-CMV2-GFP expression vectors (Fig. S10 in the Supplementary Appendix). The intracellular calcium responses to changes in extracellular calcium concentrations were expressed as a percentage of the maximum normalized response and are shown as the mean (±SE) value of 8 to 45 assays from 3 to 12 independent transfections. P<0.001 for the comparisons of the mutant vectors with the nonmutant and empty vectors. The autosomal dominant hypocalcemia type 2–associated mutants (Q181 and L341) led to a leftward shift

in the concentration–response curve, with significantly lower EC_{50} values (Table S4 in the Supplementary Appendix).

Table 1

Biochemical Findings in Patients with Familial Hypocalciuric Hypercalcemia Type 2 and Patients with Autosomal Dominant Hypocalcemia Type 2 Who Had *GNA11* Mutations.*

Variable	Normal Range†	Patients with Familial Hypocalciuric Hypercalcemia Type 2‡		Patients with Autosomal Dominant Hypocalcemia Type 2‡	
		Patient 1§	Patient 2§	Patient 3§	Patient 4§
Sex		Male	Female	Female	Female
Family history of hypercalcemia or hypocalcemia		NA	Yes	Yes	Yes
Age at presentation or diagnosis — yr¶		54	45	52	39
Serum measurements					
Calcium — mmol/liter/	2.10–2.50	2.63	2.70	2.06	1.75
Phosphate — mmol/liter	0.70–1.40	0.78	Within normal range	1.09	1.54
Alkaline phosphatase — U/liter	30–130 or 70–330 (in Patient 2)**	74	136	68	38
Magnesium — mmol/liter	0.70–1.05	0.80	>Normal range	0.77	0.76
Creatinine — μmol/liter	54–145	107	71	96	79
Parathyroid hormone	1.3–7.6 pmol/liter (in Patients 1 and 4), <5.0 pmol/liter (in Patient 2), and 10–65 ng/liter (in Patient 3)**	5.0	2.7	50	1.3
25-Hydroxyvitamin D — nmol/liter	>50	NA	47.5	54.0	40.9
Thyrotropin — mU/liter	0.35–5.50	4.05	NA	0.84	1.19
Urinary calcium:creatinine clearance	>0.02	0.003	0.011	0.002	0.012
GNA11 mutation‡		Leu135Gln	Ile200del	Arg181Gln	Phe341Leu

*NA denotes not available.

†Normal ranges are from Pearce et al.[13]

‡In this study, mutational analysis identified *GNA11* mutations (Fig. S1 and S8 in the Supplementary Appendix), and previous mutational analysis of *CASR* and *AP2S1* in the patients with hypercalcemia and mutational analysis of *CASR* in patients with hypocalcemia did not identify any abnormalities of the coding regions or exon–intron boundaries.

§Patient 1 was from Family 13/06 and Patient 2 was the proband from Family 11675[10,11] in Table S1 and Fig. S1 in the Supplementary Appendix. Patient 3 was from Family 03/01 and Patient 4 was from Family 02/03 in Table S2 and Fig. S8 in the Supplementary Appendix. Mean values of serum phosphate and magnesium measurements from affected persons in Family 11675 with familial hypocalciuric hypercalcemia type 2 were reported to be within the normal range or above the normal range.[12]

¶Patients with familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia are frequently asymptomatic, and hence the age at presentation is the same as that at diagnosis; however, if the ages at presentation and diagnosis were not the same, then the age at diagnosis is provided.

/Albumin-adjusted serum calcium values are shown.

**Normal ranges for serum measurements varied according to the assays used and the age of the patients.