Mutations of the GREAT gene cause cryptorchidism

Ivan P. Gorlov1, Aparna Kamat1, Natalia V. Bogatcheva1, Eric Jones2, Dolores J. Lamb2,3, Anne Truong1, Colin E. Bishop1,4, Ken McElreavey5 and Alexander I. Agoulnik1,*

1Department of Obstetrics and Gynecology, 2Scott Department of Urology, 3Department of Molecular and Cellular Biology and 4Human and Molecular Genetics Department, Baylor College of Medicine, Houston, TX 77030, USA and 5Reproduction, Fertility and Populations, Institut Pasteur, 75724, Paris Cedex 15, France

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In humans, failure of testicular descent (cryptorchidism) is one of the most frequent congenital malformations, affecting 1–3% of newborn boys. The clinical consequences of this abnormality are infertility in adulthood and a significantly increased risk of testicular malignancy. Recently, we described a mouse transgene insertional mutation, crsp, causing high intraabdominal cryptorchidism in homozygous males. A candidate gene Great (G-protein-coupled receptor affecting testis descent), was identified within the transgene integration site. Great encodes a seven-transmembrane receptor with a close similarity to the glycoprotein hormone receptors. The Great gene is highly expressed in the gubernaculum, the ligament that controls testicular movement during development, and therefore may be responsible for mediating hormonal signals that affect testicular descent. Here we show that genetic targeting of the Great gene in mice causes infertile bilateral intraabdominal cryptorchidism. The mutant gubernaculae fail to differentiate, indicating that the Great gene controls their development. Mutation screening of the human GREAT gene was performed using DHPLC analysis of the genomic DNA from 60 cryptorchid patients. Nucleotide variations in GREAT cDNA were found in both the patient and the control populations. A unique missense mutation (T222P) in the ectodomain of the GREAT receptor was identified in one of the patients. This mutant receptor fails to respond to ligand stimulation, implicating the GREAT gene in the etiology in some cases of cryptorchidism in humans.

INTRODUCTION

A distinctive manifestation of male dimorphism in many animals is a scrotal position of the gonads. During development the testes descend through a complex, multistage process whereby the embryonic gonads migrate from their initial abdominal position to the scrotum. Failure of this process causes undescended testes or cryptorchidism, which in humans represents one of the most common birth defects (1,2). The clinical consequences of this abnormality are infertility in adulthood and a significantly increased risk of testicular malignancy (3,4).

Testicular descent in mammals consists of two major stages: transabdominal and inguinoscrotal descent (1). During both stages, a crucial role has been attributed to the genital mesentery connecting the gonads and genital ducts to the abdominal wall (1,2,5). Specifically, two ligaments direct movements of the testis. The caudal genital ligament, or gubernaculum, undergoes intensive differentiation in males, whereas the cranial suspensory ligament (CSL) gradually regresses (1,5). During the first transabdominal stage [between 10 and 15 weeks of gestation in human embryos and between 15.5 and 17.5 d.p.c (days post coitum) in mice], the testes remain close to the future inguinal region during enlargement of the abdominal cavity. In contrast, in the female, the ovary moves relatively more cranially. During the inguinoscrotal phase of migration, which ends at birth in humans, the testes move from the inguinal region to the scrotum. This occurs in parallel with a shortening of the gubernacular cord, outgrowth of the gubernacular bulb and eversion of the cremaster muscle. In mice, the inguinoscrotal phase occurs within 2–3 weeks after birth (1).

A critical role in testicular descent has been attributed to hormones (6,7). Fetal testicular testosterone appears to play a key role in the prevention of the outgrowth of the cranial gonadal ligaments. Mice lacking androgen receptors (Tfm) have testicular feminization, with intraabdominal testes situated at the level of the bladder neck and no eversion of the scrotal sac (8,9). Tfm male mice, as well as male rats treated prenatally with antiandrogens, retain the cranial suspensory ligament (8,10). The connection between cryptorchidism and hormones is also manifested in congenital disorders that cause hypogonadism or androgen resistance, such as functional prepubertal castrate syndrome, Noonan syndrome, Klinefelter
syndrome, Reifenstein syndrome and hypogonadotropic eunuchoidism (11). Mutation of the testicular hormone Insl3 (insulin-like factor 3, also called Rfl, relaxin-like factor) in the mouse results in bilateral intraabdominal cryptorchidism, due to impaired development of the gubernaculum (12,13). Insl3 is expressed in pre- and postnatal Leydig cells in the testis and at reduced levels in postnatal thecal cells of the ovary (14,15). It has been suggested that Insl3 may act as an inducer of the growth and differentiation of the gubernaculum, thereby mediating transabdominal testicular descent (13). The receptor for Insl3 remains unknown.

Recently, we described a mouse transgene insertional mutation, crsp, causing high intraabdominal cryptorchidism in homozygous males (16). The integration was accompanied by a 550 kb deletion in the proximal part of chromosome 5, affecting several genes in the region. Direct sequencing of the affected region led to the identification of a new gene, named Great (16). The gene encodes a novel G-protein-coupled receptor (GPCR) and belongs to the same family of GPCRs as three glycoprotein hormone receptors (FSHR, LHR and TSHR). Expression of Great is restricted to the testis, brain and skeletal muscles, with the highest level of expression in the gubernaculum, making this gene an excellent candidate for the mutant phenotype.

In the present work, we show that genetic targeting of the Great gene in mice causes infertility secondary to bilateral intraabdominal cryptorchidism. The gubernaculae of the mutant males fail to differentiate, indicating that the Great gene controls their development. Furthermore, we have cloned the human GREAT gene and screened genomic DNAs from 60 cryptorchid patients for mutations within the gene. Several nucleotide substitutions were found in GREAT cDNA, in both the patient and the control populations. A unique missense mutation (T222P) in the ectodomain of the GREAT receptor has been identified in one of the patients. We established that relaxin, recently identified as a ligand for the GREAT receptor (17), fails to stimulate cAMP production in cells transfected with the mutant receptor.

RESULTS

Genetic targeting of the Great gene in mice results in high intraabdominal cryptorchidism

To assess the role of the Great gene in the cryptorchid phenotype of the crsp mutation, we have produced animals with a mutant allele of the gene. We have targeted Great in ES cells using insertional-type constructs (18). Targeting of the gene resulted in the duplication of exons 12–16 and insertion of the 10 kb vector DNA into the 16th intron (Fig. 1). Heterozygous Great<sup>ko/+</sup> males and females showed a normal phenotype. Crosses of the heterozygous carriers with the original crsp mice produced crsp/Great<sup>ko</sup> double heterozygotes. Intercrossing between heterozygous Great<sup>ko/+</sup> animals resulted in the production of Great<sup>ko</sup>/Great<sup>ko</sup> homozygotes. Analysis of the testicular phenotype in 14 Great<sup>ko</sup>/Great<sup>ko</sup> males and >50 crsp/Great<sup>ko</sup> males reveals that these animals exhibit the same high intraabdominal cryptorchidism observed in the original crsp mutation (Fig. 2A). All tested heterozygous males had a fertile wild-type phenotype with a normal scrotal position of the testes. The size of the adult mutant testes is significantly decreased. At 60 days, the average testis weight of the wild-type and heterozygous Great<sup>ko</sup>/+ animals was 84.5 ± 5.3 mg (n = 8) and the crsp/Great<sup>ko</sup> testis weight was 31.0 ± 1.1 mg (n = 6) (63% reduction). Furthermore, histological examination reveals progressive degeneration of the spermatocytes, with absence of spermatids and mature sperm (Fig. 2B). As in original crsp mutants, gubernacular development in Great knockout males is distinctively altered. The mutant mice have an extended thread-like gubernaculum, and the inguinal canal fails to form. Other urogenital structures in the mutant males are normal, including the seminal vesicles, prostate and external genitalia. Mutant males exhibit normal mounting and copulatory behavior. Vaginal plugs are present in females after copulation with mutant males, indicating that the males produce an ejaculate.

Analysis of Great gene expression at the RNA level by RT–PCR revealed the presence of a specific RNA transcript in the crsp/Great<sup>ko</sup> mutant animals. We detected Great mRNA using a primer pair derived from exons 9 and 16 (before vector insertion) and primers from exons 17–18 (after vector insertion) (Fig. 2C) (16). RT–PCR with primers from exons 2 and 18 failed to amplify the expected 2 kb cDNA fragment from crsp/Great<sup>ko</sup> brain RNA (Fig. 2C), however, indicating inclusion of the backbone of the targeting vector DNA into the cDNA transcript (18) and the absence of properly processed Great cDNA.

The first stage of testicular descent (the transabdominal stage) occurs in mice between 15.5 and 17.5 d.p.c (1). We analyzed expression of the Great gene by northern blot hybridization in mouse embryos at 7–18 d.p.c. Expression of the Great gene was detected as early as in 7 d.p.c. embryos, with stable expression on 11 d.p.c. and thereafter (Fig. 3). Four different bands have been detected on the northern blot, apparently representing several alternatively spliced transcripts (Fig. 3). In RNA isolated from mouse or human brain, the main transcript is ~4.4 kb; in testis, it is ~1.6 kb (data not shown).

Cloning of the human GREAT gene

The human GREAT cDNA was predicted through comparisons of the mouse cDNA with corresponding human genomic sequence from chromosome 13q12–13 upstream of the BRCA2 region. Subsequently, full-length cDNA has been isolated by RT–PCR from a human gubernaculum RNA sample (GenBank accession no. AF453828). Comparison of the human and mouse cDNA sequences revealed that the human open reading frame starts 51 bp 5’ to the first mouse ATG codon. This leads to the additional 17 amino acids at the N-terminal end of the human GREAT product. Overall identity of the mouse and human GREAT genes is 82% at nucleotide and amino acid level. The human gene has the same exon–intron structure as its mouse counterpart. Phylogenetic analysis of the mouse and human GREAT receptors shows that they belong to the LGR group of receptors, with the highest homology to the LGR7 receptor (19). Hsu et al. (17) named the human GREAT gene LGR8 to indicate its close homology to the other members of the LGR group. GREAT is a third
member of the leucine-rich repeat (LRR) subgroup, with a hallmark LDL-receptor domain at the N-terminal region of the protein (19).

**Mutation analysis of the GREAT gene in cryptorchid patients**

Cryptorchidism is a common human congenital abnormality with a multifactorial etiology that likely reflects the involvement of endocrine, environmental and hereditary factors. Eighteen exons of the GREAT gene have been identified and subjected to mutation analysis using a recently developed high-throughput denaturing high-performance liquid chromatography (DHPLC) approach (20) and direct sequencing. We performed a mutation screen of the human GREAT gene in 61 cases of idiopathic unilateral or bilateral cryptorchidism. The results of the mutation analysis are shown in Figure 4. Two silent mutations were observed in exon 12, in both cryptorchid (40 out of 61) and normal men. These are the A/G transversions at nucleotide positions 957 and 993 (position 1 is taken as the first A of the figure).

**Figure 1.** Generation of mice with a mutant allele of the Great gene. (A) An insertional targeting construct (top) was prepared through isolation of the phage containing Great exons 12–16 from the 5’hprt genomic library (18), converting phage into a plasmid and creating a gap with NheI reductase. Neo, neomycin resistance cassette; 5’hprt, 5’ half of the hprt; Ty, tyrosinase minigene; the loxP site is shown as a thick arrow, the Great genomic fragment is in bold, and black and white boxes represent Great exons. The wild-type chromosome is in the middle. After homologous integration (bottom) of the targeting construct, the NheI gap is repaired, the backbone of the targeting vector is inserted into the chromosome, and the genomic fragment included into targeting vector is duplicated (exons in black). To verify homologous integration, we have used PCR analysis of the embryonic stem (ES) cell DNA with one primer designed from the vector backbone and the other designed from the genomic sequence deleted from the targeting construct (K/o primers). Also indicated are the positions of the primers used to assess presence of Great transcripts in the RNA isolated from Great−/crsp males (RT–PCR primers). (B) Identification of the ES recombinant clones with a mutation of the Great gene. The mutant allele (+/−) contains both K/o primers, providing successful amplification of the 2 kb DNA fragment.
Three different haplotypes were detected: A957 and A993, A957 and G993, and G957 and G993. In four cryptorchid men, an A/G transversion was observed at nucleotide position 1810 in the fifth transmembrane domain. This nucleic acid substitution is predicted to result in a conservative amino acid change of an isoleucine to a valine residue (I604V). The same sequence variant was detected in 2 out of 30 controls. Thus this sequence variant is most likely a polymorphic allele not associated with a cryptorchid phenotype.

In one of the patients of European origin, we identified a unique mutation in exon 8 (Fig. 4B). The patient had bilateral cryptorchidism, with the gonads located in the inguinal canal at the external ring. The mutation resulted in an A-to-C nucleotide change at position 664, and was in heterozygous condition. This nucleotide change causes a missense substitution T222P. Analysis of the 192 control samples (162 from the same geographical area as the mutant carrier) did not reveal a sequence variation in this position. Importantly, no other variations in 18 exons of the GREAT gene of this patient have been detected. Direct sequencing of the 350 bp fragment upstream of the ATG codon also did not reveal any variations.

Signal transduction by wild-type, I604, and T222P variants of GREAT

It was shown recently that relaxin, a hormone important for the growth and remodeling of reproductive and other tissues during pregnancy is capable of activating the GREAT receptor through an adenosine 3',5'-monophosphate (cAMP)-dependent pathway (17). To investigate the significance of the amino acid substitution in the mutant receptor, we have analyzed relaxin-induced signal transduction in cells transfected with a wild-type and a mutant cDNA. The cAMP concentration was determined in the transfected cells treated with increasing amounts of relaxin. As shown in Figure 5, treatment of cells transfected with a wild-type or mutant I604V GREAT cDNA with relaxin resulted in dose-dependent increases in cAMP production. In contrast, cAMP levels in cells transfected with a mutant T222P GREAT showed little response in cAMP production.

In the next experiment, we have co-transfected wild-type receptor cDNA with either T222P mutant or an empty vector DNA to study possible interactions between wild-type and mutant polypeptides. As shown in Figure 6, relaxin stimulation causes the same increase in cAMP production in both cases, indicating that the non-functional mutant receptor does not affect the signaling properties of the wild-type protein coexpressed in cells.
have normal fertility and litter size, and a normal development of mammary glands (N. Bogatcheva and A.I. Agoulnik, unpublished data), in contrast to the relaxin or InsI3 mutants (13, 21, 22). It is possible that LGR7, which is closely related to the GREAT GPCR receptor (17, 19), may be responsible for the signaling of relaxin and/or InsI3 in females.

We have cloned the human GREAT gene and performed a mutation analysis in 61 cryptorchid patients. Several polymorphic sites have been identified within the GREAT gene, and are present in both the control and the patient population. We detected one functionally deleterious mutant allele of the GREAT gene among 61 patients with the diagnosis of clinical cryptorchidism. Notably, only half of the patients (26 out of 49) had bilateral cryptorchidism, including mutant carriers. A more expanded analysis is obviously needed to estimate the significance of GREAT mutations in the occurrence of human disease. The primary analysis using the DHPLC approach may result in some underestimation of the frequency of mutations. The reported sensitivity of the DHPLC screening of unknown mutations varies, and depends strongly on the presence of a high-melting-point DNA domain embedded in a low-melting-point sequence (20). Neither the DHPLC approach nor direct sequencing of the PCR fragments can detect partial deletions (if the breakpoint of the deletion lies outside of the amplicons) or complete deletions of one of the alleles.

Two polymorphic sites have been found in the 12th exon of the GREAT gene. Both substitutions are synonymous—they do not alter the amino acid sequence. In some patients, as well as in control samples, we found a nucleotide substitution in the predicted fifth transmembrane domain (I604V). Interestingly, the corresponding mouse sequence also contains a valine. These results imply that the predicted amino acid substitution is evolutionarily more conserved, present in the general population and probably not associated with the abnormal phenotype.

Direct measurements of the relaxin-mediated stimulation of this variant did not reveal differences in comparison with the wild-type protein.

A unique T222P mutation found in one of the patients with bilateral cryptorchidism is located in the fourth LRR of the receptor ectodomain. LRRs, which are among the most common module found in the extracellular regions of proteins, consist of alternatively spaced α helices and β strands that are positioned parallel to each other. In ectodomains of glycoprotein hormone receptors, numerous LRRs form a horseshoe-shaped structure (23). It is generally believed that this structure is essential for glycoprotein hormone binding (24, 25). Although neither relaxin nor INSⅢ belong to the family of glycoprotein hormones, we suggested that a mutation in one of the LRRs could impair the binding of the receptor with a ligand and/or downstream transmission of the signal. Indeed, we have shown that the mutant receptor is not able to mediate relaxin-induced cAMP production.

The analysis of the secondary structure of the protein region surrounding the mutation site in the receptor predicted destabilization of the α helices. PHD (profile network prediction Heidelberg, http://www.ebi.ac.uk/~rost/predictprotein/) analysis showed that the mutant receptor has higher structural flexibility in the region surrounding proline 222 in the fourth LRR compared with the same region in the wild-type protein (threonine 222). The T222P substitution can therefore cause a
deterioration in relaxin–GREAT signal transduction, either through impairing ligand binding or through modification of the relaxin-induced conformational changes essential for G-protein activation. Thus, mutations in the GREAT receptor can be responsible for the cryptorchid phenotype by directly affecting the proposed INSL3 signaling during development.

The other possibility is that the mutant receptor is not properly expressed or localized in the cells. As, in the experiments described in this study, we did not monitor cell membrane expression of the transfected receptor, we cannot formally exclude such a scenario. Nevertheless, the facts that mutation occurred in the middle of the extracellular part of the receptor, and similar mutants of other glycoprotein hormone receptors are expressed correctly, indicate that there is a functional defect of the mutant receptor. Regardless of the specific mechanisms, T222P mutation is functionally inactive.

The question arises as to why the human patient heterozygous for T222P mutation has bilateral cryptorchidism whereas crsp/+ or Great+/+ heterozygous mice show no testicular descent abnormalities. Several possible explanations can be put forward. Obviously, there is a clear difference between deletion mutations in the mouse and the missense mutation in the human. In the latter case, both alleles are apparently expressed in heterozygotes, producing mutant and wild-type receptor. It is interesting to note that the abnormality in patient is less severe than in the mouse mutants, indicating that there is some degree of receptor signaling. One explanation is that the mutant receptor might bind the ligand, competing with the wild-type receptor and thus compromising the response to hormone stimulation. The mutant receptor may also irreversibly sequester G proteins and stoichiometrically inhibit G signalling in a dominant-negative manner, as shown previously for α1B-adrenergic GPCR (26). However, the cotransfection experiments with wild-type and mutant receptors did not support this scenario. At least in vitro, under the conditions used in our experiments, there was no interaction or suppression of wild-type receptor signaling. The other reason could be a relatively higher concentration of the cognate ligand in mice than in humans during the prenatal period, or a higher affinity of the mouse receptor for the hormone sufficient to mediate testicular descent even in the presence of only half the amount of receptor. Lastly, we cannot exclude the possibility that the patient with this mutation was in fact compound heterozygous for an additional mutation, located in another exon of the gene. Further analysis of the properties of the mutant and wild-type receptors are now underway to clarify the mechanism of ligand–receptor interaction.

MATERIALS AND METHODS

Gene targeting and production of mice with Great mutation

We isolated a genomic λ phage clone containing exons 12–16 of the Great gene from a 5′ HPRT library (18) kindly provided by A. Bradley. An insertional targeting vector was constructed by conversion of the λ phage into a plasmid, gapping the genomic insert with Nhel, followed by linearization of the vector with the same restriction enzyme. We electroporated the targeting construct DNA into AB2.1 ES cells and selected recombinant clones with G418 as described previously (18). Analysis of the DNA from ES clones was performed by PCR, with K/o primers derived from the vector backbone and from the Nhel-deleted
zygous females to produce mice heterozygous for the targeted allele. GREAT PCR of the tail DNA. Intercrossing of the di-heterozygotes.

Functional analysis of mutant GREAT receptors. Porcine relaxin stimulates dose-dependent cAMP production in transfected HEK 293T cells expressing wild-type (circle) and mutant I604V (upward-pointing triangles) GREAT. In contrast, relaxin stimulation of cells expressing mutant T222P (downward-pointing triangles) GREAT has little effect on cAMP production. Relaxin does not affect cAMP production in cells transfected with an empty vector (squares). cAMP production was normalized based on the efficiency of transfection estimated by the activity of secreted alkaline phosphatase produced by the co-transfected pAP-tag5 plasmid. Intracellular cAMP was measured in duplicate by a specific enzyme immunoassay.

RT–PCR analysis of gene expression

Total RNA from mouse and human tissues was extracted with the TRIzol reagent (Life Technologies, Rockville, MD). First-strand cDNA was synthesized using the oligo(dT) primer and RETROscript kit (Ambion, Austin, TX). The following primers were used for analysis of Great expression: (Receptor2F) 5'-AGAAGAGCAGAATAGCAGT-3' and (insert-reverse) 5'-ACCGCTCAGGGTCAACT-3'. During homologous integration, the gap in the targeting vector is repaired and the PCR produces a 2 kb fragment. We injected three selected independent clones into C57BL/6J blastocysts and reimplanted these into pseudopregnant female mice using standard procedures. Chimeric males were bred to C57BL/6J females to produce mice heterozygous for the targeted allele. Great<sup>+/−</sup> heterozygous males were bred to crsp/crsp homozygous females to produce Great<sup>−/−</sup>/crsp di-heterozygotes. The presence of the Great<sup>−/−</sup> mutant allele was detected by PCR of the tail DNA. Intercrossing of the Great<sup>−/−</sup>/crsp heterozygotes produced homozygous animals which have been identified by long-range PCR (Roche Biochemical) with primers outside the genomic fragment used in the targeting vector: (forward) 5'-CATGGTGAGAACCAGCT-3' and (reverse) 5'-GCAATCCAAAGCTCTAGC-3'. PCR conditions were denaturation at 94°C for 2 min, 10 cycles of 92°C for 10 s, 55°C for 30 s and 72°C for 15 min, followed by 25 cycles of 92°C for 10 s, 55°C for 30 s and 72°C for 15 min + 20 s per cycle, with a final extension at 72°C for 5 min. Presence of the 16 kb fragment indicates the presence of the wild-type allele.

The animal studies were approved by the Baylor College of Medicine Institutional Committee on animal care.

Northern blot analysis

Northern blots with 2 μg of poly(A)<sup>+</sup> RNA isolated from embryos of different age were used (Clontech Labs, Palo Alto, CA). 1.3 kb 3′-end cDNA of the GREAT gene was labeled by random priming (Stratagene, La Jolla, CA) and hybridized overnight in PerfectHyb Plus hybridization buffer (Sigma, USA) at 65°C. Blots were washed under highly stringent conditions at 68°C three times for 30 min with 0.1 × SSC: 0.1% SDS and exposed to Kodak X-Omat film with one intensifying screen at −80°C.

Histopathological analysis

Dissected testes were fixed in Bouin’s solution (Sigma), embedded in paraffin, sectioned, and stained with hematoxylin–eosin according to standard protocols. Photomicrographs were taken with an AxioLab microscope equipped with an automatic camera (Karl Zeiss, Germany).

Cloning of the human GREAT gene

Eighteen exons of the human GREAT gene were identified through BLAST analysis of the human genomic DNA with the mouse cDNA sequence. Primers flanking both sides of the predicted open reading frame (ORF) were designed: [5′-untranslateral...
region (UTR) 5'-TCAATTGCTGTAAACCTATGATTG-3' and (3-UTR) 5'-CTTGCCGTTGGTAAAGATGAA-3'. These primers were used to amplify a cDNA with 2265 bp GREAT ORF. Both strands of the fragment were sequenced to confirm the predicted splice sites/exons of the gene.

Patient and control DNA samples

A total of 61 cases of idiopathic bilateral or unilateral cryptorchidism were used for the study. Twenty cases of European origin were obtained from French clinics and 41 samples of mixed origin from the Urology Department of Baylor College of Medicine. A total of 193 men of known fertility and absence of a clinical history of cryptorchidism were used as a control. Among them, 62 samples were from France, 100 from Germany and 31 from the USA. The population studies were approved by the authors’ Institutional Review Boards.

DHPLC mutation analysis and sequencing

Mutation analysis was performed on an automated WAVE Nucleic Acid Fragment Analysis System according to conditions recommended by the manufacturer (Transgenomic, Omaha, NE). Using available genomic sequence information, we have designed 18 pairs of primers for amplification of each exon and flanking intron sequence of the human GREAT gene (Table 1). PCR was performed in 25 μl volume using ampliTaq (Perkin Elmer, Branchbury, NJ), and a 5 μl aliquot was analyzed on agarose gel. Prior to DHPLC analysis, 15 μl (containing 100–300 ng of DNA) of the experimental sample was mixed with 5 μl of the control amplicon. PCR products were then denatured at 95°C for 5 min and gradually cooled down to room temperature at 1°C/min decrements. Gradient parameters were determined based on size and G/C content of the amplicons. DHPLC conditions for successful resolution of heteroduplex formation have been established using the DNA melt software of the WAVEMaker software (Transgenomic). The column temperature was calculated using software supplied with the WAVE system. The predicted melting profiles for 6 exons of the gene required runs at two temperatures, the remaining 12 exons were analyzed at one temperature (Table 1). Chromatograms of the elution profiles obtained from the experimental samples were compared with those of the controls. All samples showing deviation from the wild-type profile were subject to direct sequencing. PCR fragments were separated on an agarose gel and purified.

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using Ultrafree-DA spin columns (Millipore, Bedford, MA). Both strands of the DNA fragment were sequenced by the dye terminator method on an automated 373 DNA sequencing machine using the same primers as for PCR.

Expression of GREAT in mammalian cells

Full-length wild-type GREAT cDNA was amplified by RT–PCR from human gubernaculum RNA with 5'-UTR and 3'-UTR primers. The resulting 2.3 kb cDNA was subcloned into the eukaryotic cell expression vector PC3.1 (Invitrogen, San Diego, CA). Efficient targeting of the receptor to the cell surface was provided by an internal GREAT signal peptide encoded by the first 36 amino acids of the cDNA sequence (16). The plasmids were purified using the Concert Midi-prep plasmid preparation kit (Life Technologies, Rockville, MD). The sequence of the construct was verified by sequencing of both DNA strands using gene-specific and vector-derived primers.

To produce targeted mutations in the GREAT cDNA, we used the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). The resulting cDNA sequence was verified by sequencing of both DNA strands. A 1 kb cDNA EcoRI/HindIII fragment from the wild-type plasmid was substituted with the same fragment from the mutated plasmid; junction sites were verified by sequencing. This final plasmid, containing mutant GREAT cDNA, was used for further experiments.

293T cells derived from human embryonic kidney (HEK) fibroblasts were maintained in DMEM supplemented with 10% FBS, 1 mM glutamine, and an antibiotic/antimycotic mixture (all from Life Technologies, Rockville, MD). For the cAMP determination experiment, cells were seeded in 24-well plates one to two days prior to transfection. The cells were transfected at 80% of confluency with 500 ng/well of plasmid of interest and 25 ng/well of reporter plasmid (pAP–tag5, GeneHunter Corporation, Nashville, TN) in OptiMEM without antibiotic and 250 ng/well of each plasmid (wild-type plus vector, or wild-type plus mutant). After 24 h, the efficiency of transfection was estimated by the activity of secreted alkaline phosphatase in the media using pNPP as a substrate (Sigma). The cells were stimulated with 0.25 mM IBMX (Sigma) for 30 min, then harvested by aspiration and centrifuged. The pellet was washed briefly with PBS and extracted with cAMP extraction buffer (Amersham Pharmacia Biotech) for 40 min. Aliquots of supernatant were used in an enzyme immunoassay (Amersham Pharmacia Biotech) for the cAMP determination. The cAMP concentrations in each well were measured in duplicate. All experiments were repeated three times using cells from independent transfections.

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Note added in proof

After submission of the manuscript it was reported that chemically synthesized INSL3 peptide could activate GREAT/LGR8 receptor (Kumagai et al., J. Biol. Chem., in press. Published online July 11, 2002 as Manuscript C200398200).

REFERENCES


