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Pro239Ser: A Novel Recessive Mutation of the Pit-1 Gene in Seven Middle Eastern Children with Growth Hormone, Prolactin, and Thyrotropin Deficiency*

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ABSTRACT

Pit-1, a member of the POU-homeo domain protein family, is one of the transcription factors responsible for anterior pituitary development and pituitary-specific gene expression. Here, we describe seven children with GH, PRL, and TSH deficiency from three, reportedly unrelated, Middle Eastern families, harboring a newly recognized Pro->Ser recessive mutation in codon 239 of the Pit-1 gene. The mutated residue is located at the beginning of the second α-helix of the POU-homeodomain and is strictly conserved among all POU proteins. The Pro239Ser mutant binds DNA normally but is unable to stimulate transcription. (*J Clin Endocrinol Metab* **83:** 2079–2083, 1998)

Pit-1/GHF-1 is a pituitary-specific transcription factor required for pituitary development and for expression of several anterior pituitary hormones (1–3). Pit-1 transactivates expression of the genes coding for GH, PRL, and TSH-β (4–8), among others. In addition to its role in cell-specific expression, Pit-1 also plays an essential role in the development of somatotroph, lactotroph, and thyrotroph cells in the anterior pituitary. As a member of the POU family, Pit-1 contains a highly conserved bipartite DNA-binding domain consisting of the POU-homeodomain (60 amino acids), required for low-affinity DNA binding, and of the POU-specific domain (approximately 75 amino acids) responsible for the specificity of DNA binding and for possible interactions with other proteins (9). The major transactivating function of Pit-1 resides in the N-terminal region of the protein, which functions as an independent activation domain when connected to a heterologous DNA-binding domain (1, 10).

Abnormalities in the Pit-1 gene were first observed in Snell and Jackson dwarf mice. Their consequence was a combined pituitary hormone deficiency (CPHD) with hypoplasia of the anterior pituitary and no production of GH, PRL, or TSH (11). A number of CPHD patients are known to have a point mutation in the Pit-1 coding sequence (12–20). Some of these mutations affect the DNA-binding capacity (12, 20) of the Pit-1 protein, others alter its transactivation properties (13–15). From a comparison of their pedigrees, it seems that some mutations in the Pit-1 gene (Arg271Trp and Pro24Leu) lead to a dominant-negative phenotype (13, 15–19); others (Arg172stop, Glu250Stop, Ala158Pro, Arg143Gln), to a recessive phenotype (14, 15, 20).

Here, we report the identification of a novel homozygous Pro239Ser mutation in the Pit-1 gene of seven Middle Eastern hypopituitary children from three reportedly unrelated families. In addition, we present the properties of the mutant Pit-1 protein, with respect to transactivation, DNA binding, and interaction with its wild-type counterpart.

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Materials and Methods

DNA extraction, PCR amplification, and automated direct sequencing

For DNA extraction from blood spots (5 mm^2) on filter paper, we used the ChelexR 100 chelating resin, as described by Walsh and co-workers (21). PCR was used to amplify the six Pit-1 exons independently, using six pairs of oligonucleotides corresponding to the intron/exon boundaries (15). Reactions were carried out as follows: approximately 100 ng genomic DNA, 20 pmol of each primer, 200 μmol/L of each nucleotide, 1 U Dynazyme polymerase, and 1× Dynazyme reaction buffer were mixed in a final vol of 100 μL. Reactions were cycled 30 times for 1 min at 94 C, 1 min at 55 C, and 1 min at 72 C. The amplified products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to evaluate their size, purity, and quantity. Sequencing was performed

directly on the amplified fragments, thus allowing immediate evaluation of heterozygosity.

Site-directed mutagenesis

We used the CHAMELEON double-stranded, site-directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA) to obtain the mutated Pit-1 complementary DNA (cDNA). A plasmid, containing nonmutated human Pit-1 cDNA cloned behind the RSV promoter (pRSVhPit-1) (22), was used as a template.

The oligonucleotide 5′CTCTTGAGAAGAAGATTTATTCTGTTCTCC3′, containing the specific T-to-C substitution in codon 239, on the antisense strand, was employed. Mutated clones were tested by DNA sequencing and restriction analysis.

In vitro transcription/translation reactions

For *in vitro* transcription and translation, both the wild-type and the mutant Pit-1 cDNA were inserted into the *Xba*I-*Xba*I sites of plasmid pSVK3 from Pharmacia LKB Biotechnology (Uppsala, Sweden), which contains a T7 promoter. The *in vitro* reactions were carried out using the coupled transcription/translation system: TnT^a Coupled Reticulocyte Lysate Systems (from Promega, Madison, WI) and [35S]-labeled methionine. Translation products were checked by electrophoresis on 5% polyacrylamide gels, and two major species (31 and 33 kDa) were observed, as expected (data not shown).

Gel-retardation assays

For the binding assays, we used the same amount of *in vitro* translated proteins, as judged by protein gel autoradiography (overnight exposure). The assays were performed in a mixture containing 2 μL translated protein solution, 1 μg poly dIdC, 7 mmol/L HEPES (pH 7.9), 4% glycerol, 4% Ficoll, 100 mmol/L KCl, 1 mmol/L MgCl2, 0.1 mmol/L EDTA, 2 mmol/L dithiothreitol, and 10,000 cpm labeled hPRLP1 oligonucleotide (8), in a final vol of 20 μL. Two microliters of anti-Pit-1 antibodies or control serum was added when necessary. Binding was carried out for 30 min at 4 C (1 h with antibodies), after which samples were loaded on a 5% polyacrylamide gel, in TBE 0.5X, for electrophoresis.

Production of anti-Pit-1 antibodies

A fragment of human Pit-1 cDNA, containing the coding regions for the N-terminus and a small part of the POU-domain (amino acids 1–193), was cloned in the pT7 bacterial expression vector and expressed in the bacterial strain BL21 after addition of 100 mmol/L IPTG and incubation at 37 C for 1 h. The approximately 23-kDa protein was subsequently purified for use as an antigen. The activity of the serum from injected rabbits was tested on Western blots (data not shown) with pituitary-derived cell extracts (GC, GH3), and the expected 31- to 33-kDa doublet bands corresponding to Pit-1 were obtained. We observed no extra bands corresponding to other POU family members, such as Oct-1, proving the high specificity of the antibodies against Pit-1. As expected, no bands appeared in the negative control performed with nonpituitary cell (HeLa) extracts.

Cotransfection assays

Human HeLa cells were plated on 60-mm plates at a density of 5×10^5 cells per plate and transfected with 5 μg of reporter plasmid p(GH1)6XTKCAT (23). The amount of effector plasmid was kept constant by addition of RSV-βgal, where appropriate, as indicated in the figure legends. We used the calcium phosphate copreciptation method for transfection. Cells were harvested 48 h after transfection, and chloramphenicol-acetyl-transferase (CAT) assays were performed as previously described (23). The data shown are averages of at least three independent experiments, with *error bars* indicating the SEM.

Oligonucleotides and enzymes

The oligonucleotides used in this study were from Eurogentec (Seraing, Belgium) and the enzymes from Boehringer, Eurogentec, Gibco-BRL, New England Biolabs, and Pharmacia.

Results

Subjects and endocrine studies

All described patients presented a clinical picture of congenital hypothyroidism and early growth failure and were referred to the King Faisal Specialist Hospital and Research Center. Studies were carried out with the informed consent of all parents.

The seven children were from three, reportedly independent families from the Al-Baha region in the Southwest of Saudi Arabia. In each case, the parents were second-degree relatives (see Fig. 1) and of normal stature for this community. The maternal height ranges from 149.5 to 159.2 cm and the paternal height, from 162.6 to 166.4 cm.

All seven children were GH-, PRL-, and TSH-deficient. In all cases, serum GH concentration was less than 0.3 μg/L, after a clonidine stimulation test or insulin tolerance test; basal PRL concentration in serum was <3 μg/L in either hypothyroid (n = 2) or euthyroid (n = 5) conditions, and thyroid hormone levels were below the reference range for the age, with undetectable serum concentrations of TSH ($<$0.1 mIU/L). In each case, a so-called empty sella turcica was visualized by computed tomography scan.

All children were treated with L-T_4 from early infancy. They also received a GH therapy, which started between the ages of 4 months and 3 yr plus 4 months. The height deficit, after adjustment for midparental height SD increased with advancing age, amounting to -2.9 SD at early start and -6.0 SD at latest start of GH therapy.

Molecular studies

A new point mutation in the Pit-1 gene. The human Pit-1 gene is 17-kb long and composed of six coding exons (24). For each family member analyzed, all six exons of the Pit-1 gene were amplified by PCR and directly sequenced. Sequence analysis revealed only one alteration, a T->C transversion in codon 239, located in exon 6. This alteration leads to the substitution of serine for proline at codon 239. The affected children are all homozygous, the unaffected parents all heterozygous for this mutation.

Pro239Ser mutant is unable to transactivate. To analyze the functional effects of the Pro239Ser mutation, we first used site-directed mutagenesis to produce an identical substitution in human Pit-1 cDNA, borne by the expression vector pRSVhPit-1. We shall refer to the parental plasmid as pRSVPit-1WT and to the plasmid bearing the Pro239Ser mutation as pRSVPit-1M. Cotransfection experiments in HeLa cells were performed using one of the Pit-1 expression vectors with a reporter plasmid, p(GH1)6XTKCAT, bearing six

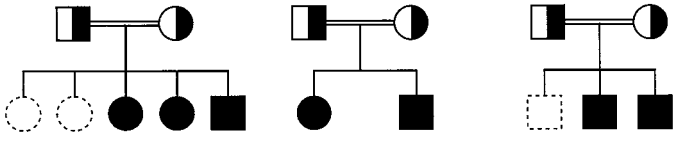

FIG. 1. Pedigrees of the three families with CPHD. *Squares*, Males; *circles*, females; *solid symbols*, individuals who are homozygous for the mutation in the Pit-1 gene; *half-solid symbols*, persons who are heterozygous at this location; *dotted symbols*, phenotypically normal individuals who were not examined.

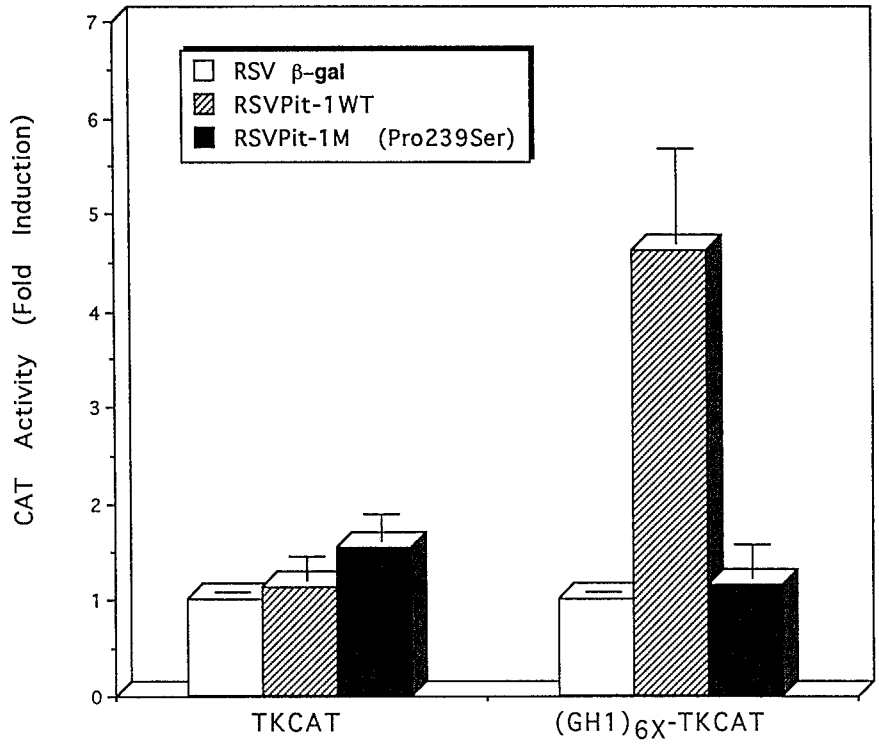

FIG. 2. Transfection of HeLa cells with vectors expressing either wild-type or Pro239Ser mutant Pit-1. The calcium phosphate precipitation technique was used to transfect the cells. We used 5 μg of reporter plasmid and 10 μg of expression vector per plate. CAT assays were performed overnight with 25 μg total cell extract per plate, prepared after a 48-h incubation.

copies of the first Pit-1 binding site of the human GH promoter (hGH1: from −71 to −91, see Ref. 8), inserted upstream from the TK promoter and the CAT reporter gene. As shown in Fig. 2, the mutant Pit-1 was unable to increase transcription of the (GH1)6XTKCAT fusion-gene construct, whereas wild-type Pit-1 stimulates its transcription 5-fold.

The DNA binding properties of the Pit-1 mutant are unaltered. ^{35}S-labeled wild-type and mutant Pit-1 proteins from *in vitro* transcription/translation reactions were used to test the ability of the Pro239Ser mutant to bind DNA in gel retardation assays. Both proteins are of the correct apparent molecular size (33 or 31 kDa, depending on the methionine at which translation is initiated) (data not shown). As shown in Fig. 3, the mutated and wild-type Pit-1 proteins bind with the same affinity to the first Pit-1 DNA-binding site of the human PRL gene (hPRL1P: from −35 to −64, see Ref. 8). The complexes are specific, because they disappear with an excess of cold hPRL1P oligonucleotide but not with an excess of cold, nonspecific DR4 oligonucleotide. The specificity is further confirmed by the fact that the shifted bands disappear in the presence of anti-Pit-1 antibodies but not in the presence of control serum. A nonspecific lower band is observed with unprogrammed reticulocyte lysate.

Coexpression of the Pro239Ser mutant interferes with the wild-type Pit-1 activity. Because the unaffected parents are all heterozygous for the Pro239Ser mutation, we sought to test the ability of the mutant form to interfere with transactivation of reporter gene expression by wild-type Pit-1. To this end, we cotransfected HeLa cells with the p(GH1)6XTKCAT reporter plasmid and either 14 or 28 μg of expression vector, this meaning either pRSVβgal alone (control) or equal amounts of two plasmids: pRSVβgal and pRSVPit-1WT, pRSVβgal and pRSVPit-1M, or pRSVPit-1WT and pRSVPit-1M. As shown in Fig. 4, the CAT activity doubles when the amount of pRSVPit-1WT is increased from 7 μg to 14 μg, whether or

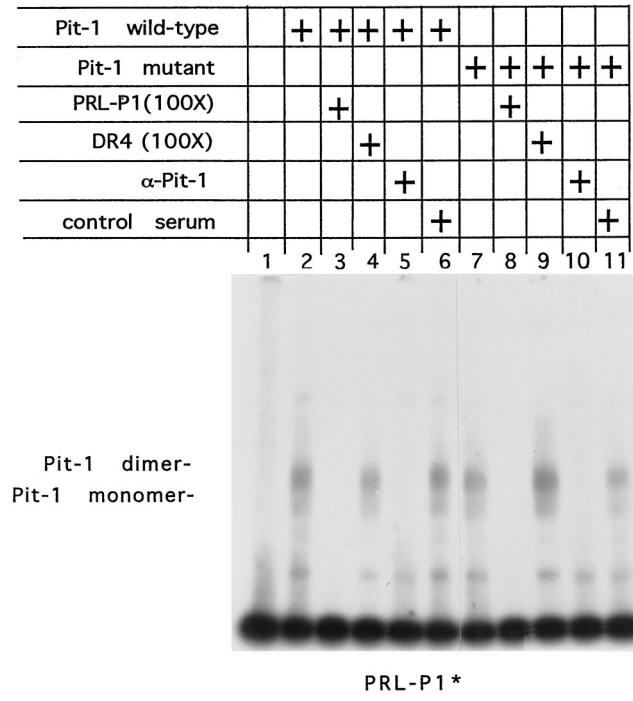

FIG. 3. Binding of the wild-type and Pro239Ser mutant Pit-1 proteins to the hPRL1 Pit-1 binding site. Both *in vitro* translated proteins were tested in gel retardation assays with ^{32}P-labeled hPRLP1 oligonucleotide, corresponding to the first Pit-1 footprint sequence in the proximal human PRL promoter (8).

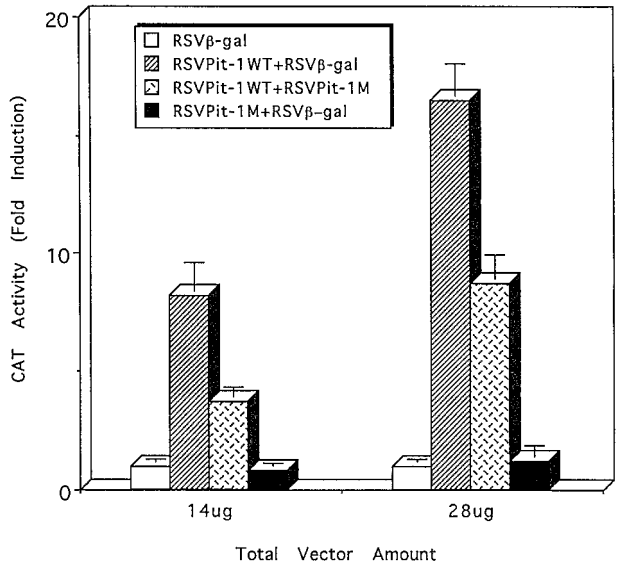

FIG. 4. Transfection of HeLa cells with vectors expressing the wild-type (pRSVPit-1WT) or mutant form of Pit-1 (pRSVPit-1M), introduced alone or simultaneously. In all cases, 5 μg p(GH1)6XTKCAT reporter plasmid was introduced, in addition to the expression vector(s). The total vector amount indicated on the horizontal axis (14 or 28 μg) refers to the total amount of pRSV-derived plasmid; when two such plasmids were introduced simultaneously (*hatched*, *grey*, and *black boxes*), each was present in half the indicated amount.

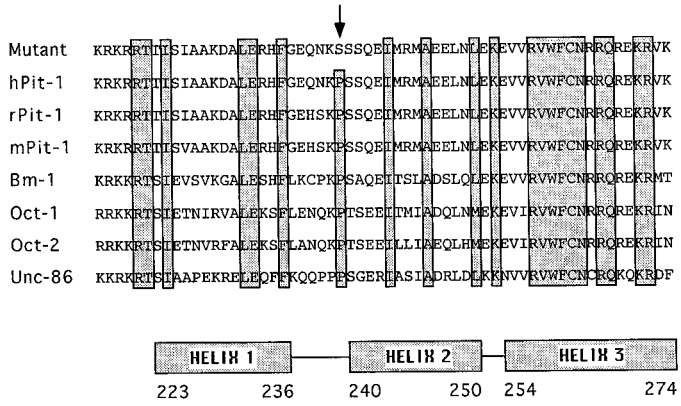

FIG. 5. Alignment of POU protein homeodomains. The species to which each Pit-1 belongs is indicated by an h (human), r (rat), or m (mouse). Amino acids that are identical at any position are *boxed*. The residue mutated in all patients is marked by an *arrow*. Regions that form α-helices, according to the Oct-1 crystallographic structure (21), are also shown by *grey boxes*.

not pRSVPit-1M is also present. We also observe 2-fold lesser transactivation by wild-type Pit-1 at both vector concentrations when the mutated form is expressed. The Pro239Ser form alone is unable to stimulate transcription of the reporter plasmid.

Discussion

We have analyzed the Pit-1 sequence of three apparently independent families in which hypopituitary children are homozygous and phenotypically normal parents are heterozygous for a Pro239Ser mutation.

Proline 239 is strictly conserved among the Pit-1 proteins of several species and among other related POU proteins (Fig. 5), implying that it must play an important role in Pit-1 activity. This residue is located at the beginning of the second α-helix of the homeodomain, which was originally described as the Pit-1 DNA binding domain (3). In gel retardation experiments, we show that the Pro239Ser mutant form of Pit-1 is still able to recognize the first Pit-1 binding site of the human PRL proximal promoter and specifically binds to this sequence with about the same affinity as the wild-type Pit-1. These results are in agreement with the three-dimensional conformation of the POU domain [recently established for Oct-1 (25) and for the Pit-1 POU domain (26) cocrystallized with their respective binding sequence], which seems to indicate that residue 239 is not in direct contact with DNA. In addition, Proline 239 occupies the N-cap position of the helix α2 in the Pit-1 homeodomain. Prolines are frequent at N-cap positions of α-helices, where they play a stabilizing role (27). In our mutant, proline 239 is replaced by a serine, another residue known to have a stabilizing effect on α-helices when in the N-cap position. Thus, one should not expect the stability of the second α-helix of the Pit-1 homeodomain, and hence the conformation of the protein, to be seriously affected by the Pro239Ser mutation. Furthermore, a genetic screen used to define the important residues for Pit-1 DNA-binding, based on loss of function of fusion proteins between the transactivation domain of GCN4 and chemically mutated Pit-1 DNA binding domains, failed to detect the Pro239Ser mutation described here (28). All these considerations support our finding that this Pit-1 variant is capable of binding DNA with the same affinity as the wild-type form.

The results of our cotransfection experiments (Fig. 2) clearly show that the Pro239Ser mutation causes complete loss of transactivation activity. The hypothesis of nonexpression of the mutated protein in HeLa cells must be rejected, because activation by wild-type Pit-1 is partially inhibited when the vector bearing the mutated cDNA is also present (Fig. 4). Without expression of this cDNA, such an effect would be hard to explain.

When pRSVPit-1WT and pRSVPit-1M are present in equal amount (7 or 14 μg each) in cotransfected cells, the transactivation potential of wild-type Pit-1 is reduced by 50% (Fig. 4). These *in vitro* results are in keeping with the clinically observed recessive phenotype of this mutation if we assume that a 50%-reduced level of Pit-1 activity is sufficient to ensure a normal phenotype.

Because Pit-1 forms dimers on DNA through its POU-specific domain, two mechanisms might account for the ability of the mutant to repress wild type Pit-1 activity: competition between mutated and wild-type Pit-1 for DNA binding sites; or formation of inactive heterodimers. The first possibility must be rejected because the reporter plasmid was in excess in our experiments [as shown by the 2-fold increase of CAT activity observed when the amount of vector expressing wild-type Pit-1 was doubled, whether this vector was present alone or in combination with the vector expressing the mutated form (Fig. 4)]. We therefore suggest that the observed inhibitory effect of the Pro239Ser protein is caused by formation of heterodimers that can bind to DNA but cannot stimulate transcription, as is the case with mutant homodimers. This model suggests that the mutation of

Pro239 into a serine abolishes the interaction of Pit-1 with another factor(s) required for transcriptional activation.

In brief, our data show that a single point mutation in codon 239 of the Pit-1 gene, causing the substitution of a serine for a proline, leads to the phenotype of GH, PRL, and TSH deficiency and hypoplasia of the anterior pituitary, when present in both Pit-1 alleles. Because heterozygous individuals are seemingly unaffected, it further seems that a 50%-reduced level of Pit-1 activity is sufficient to ensure a normal phenotype.

The newly recognized, natural, recessive Pit-1 mutation that we describe has been found in only three Middle Eastern families. The relatively frequent and apparently exclusive occurrence of this mutation in a defined geographic region is striking. This occurrence may be based on a so-called founder effect, the three families having a single and common heterozygous ancestor in whom this mutation first arose. Alternatively, we may be in the presence of a hot spot for mutations in the Pit-1 gene within this ethnic community, distinct from the Arg271Trp hot spot that has been described in Caucasians and Mongolians (16).

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