RESEARCH COMMUNICATION

Human and mouse *TPIT* gene mutations cause early onset pituitary ACTH deficiency

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Tpit is a highly cell-restricted transcription factor that is required for expression of the pro-opiomelanocortin (POMC) gene and for terminal differentiation of the pituitary corticotroph lineage. Its exclusive expression in pituitary POMC-expressing cells has suggested that its mutation may cause isolated deficiency of pituitary adrenocorticotropin (ACTH). We now show that *Tpit*-deficient mice constitute a model of isolated ACTH deficiency (IAD) that is very similar to human IAD patients carrying *TPIT* gene mutations. Through genetic analysis of a panel of IAD patients, we show that *TPIT* gene mutations are associated at high frequency with early onset IAD, but not with juvenile forms of this deficiency. We identified seven different *TPIT* mutations, including nonsense, missense, point deletion, and a genomic deletion. This work defines congenital early onset IAD as a relatively homogeneous clinical entity caused by recessive transmission of loss-of-function mutations in the *TPIT* gene.

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The *Tpit* transcription factor was first identified as a transcriptional partner of the homeobox factor *Pitx1* acting on the pro-opiomelanocortin (POMC) gene promoter (Lamolet et al. 2001). *Tpit* (*Tbx19*) belongs to the T-box

family of transcription factors named after Brachyury or Tail (T; Papaioannou and Silver 1998; Smith 1999; Papaioannou 2001; Wilson and Conlon 2002). *Tpit* expression is restricted to the two pituitary POMC-expressing lineages in mice, the corticotrophs and melanotrophs (Lamolet et al. 2001). In the corticotroph lineage, interaction between *Pitx1*, *NeuroD1/Beta2*, and *Tpit* is critical for cell-specific transcription of the POMC gene (Poulin et al. 2000; Lamolet et al. 2001) and corticotroph differentiation (Pulichino et al. 2003; B. Lamolet, K. Chu, G. Poulin, F. Guillemot, M.J. Tsai, and J. Drouin, in prep.). *Tpit*-deficient mice revealed that pituitary POMC cells do not reach terminal differentiation (POMC expression) but that precursors of POMC cells still form in absence of this factor (Pulichino et al. 2003). In view of its highly restricted expression, *Tpit* deficiency is likely to only affect pituitary POMC production directly. In mice and humans, POMC is also expressed in nonpituitary tissues, and different biologically active peptides are produced by proteolytic processing of POMC in each expressing tissue (Solomon 1999). Accordingly, mutation of the *POMC* gene itself produced multiple phenotypes (Krude et al. 1998; Yaswen et al. 1999). However, the isolated deficiency of pituitary POMC has not been described in animals, and it is a relatively rare condition in humans. POMC is processed into adrenocorticotropin (ACTH) by pro-convertase 1 (PC1) in anterior pituitary corticotrophs and into α -melanocyte-stimulating hormone (α MSH) by PC2 in rodent intermediate lobe melanotrophs (Seidah et al. 1999). Early development of the human pituitary is grossly similar to rodents, but the human intermediate lobe disappears at the 16th week of gestation (Dubois et al. 1997). Consequently, it does not appear that the pituitary is a major source of α MSH in humans. It is thus likely that in humans, the loss of *TPIT* function would result principally in ACTH deficiency. ACTH regulates metabolic functions through stimulation of glucocorticoid synthesis in the adrenal cortex.

Most human cases of congenital ACTH deficiency involve other deficiencies such as in combined pituitary hormone deficiencies (Cohen and Radovick 2002). Two cases of ACTH deficiency associated with severe obesity and red hair pigmentation have been linked to *POMC* gene anomalies (Krude et al. 1998; Krude and Gruters 2000). One case of ACTH deficiency with gonadotroph deficiency, severe obesity, and glycoregulation anomalies was ascribed to a *PC1* gene mutation (Jackson et al. 1997).

Congenital isolated ACTH deficiency (IAD; i.e., a deficiency of pituitary ACTH without any other hormone deficiency) is very rare. A few cases have been described with onsets from perinatal to early teens (Malpuech et al. 1988; Soo et al. 1994; Kylo et al. 1996). Both the pathophysiology and clinical description of this condition are poorly defined. Although CRH or its receptor gene have been proposed as candidates for this condition (Kylo et al. 1996), *TPIT* is the first gene to exhibit a specificity of expression consistent with IAD (Lamolet et al. 2001). We now report the physiological phenotype of *Tpit* null mice as model of human IAD and provide a genetic analysis of the first series of human congenital IAD patients. These studies define a separate, very homogeneous, and previ-

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ously unrecognized clinical profile of early onset IAD that is associated at high frequency (8/11 cases investigated) with mutations in the *TPIT* gene. The characterization of seven different loss-of-function *TPIT* mutations offers a molecular explanation for this recessive disease.

Results and Discussion

Tpit mutant mice have impaired adrenal function

Pituitary ACTH is the major stimulus for production of adrenal glucocorticoids and this action constitutes an important part of the hypothalamo-pituitary-adrenal axis. Because *Tpit* mutant mice have very few remaining POMC (ACTH)-positive pituitary cells (Pulichino et al. 2003), we measured plasma ACTH levels in these mice. Homozygous ($-/-$) mutant mice have very low, albeit detectable, plasma ACTH levels, whereas wild-type ($+/+$) and heterozygous ($+/-$) mice have similar normal levels (Fig. 1A). The impact of low plasma ACTH on adrenal function was next assessed, and we observed undetectable plasma corticosterone in homozygous mutant, but not in wild-type or heterozygous mice (Fig. 1B). In addition, adrenal glands were found to be hypoplastic, with the most significant loss at the level of the glucocorticoid-producing fasciculata layer (Fig. 1C). These results are clearly in support of a role of POMC-derived peptides in maintenance of adrenal tissue. No other pituitary endocrine dysfunction was suspected in *Tpit* $^{-/-}$ mice, and, in particular, the pituitary gonadal axis appears to function normally, as *Tpit* $^{-/-}$ mice have normal fertility.

In humans, adrenal insufficiency leads to severe hypoglycemia sometimes associated with seizures, reflecting the role of glucocorticoids in regulation of plasma glucose. Basal plasma glucose levels of *Tpit* null mice were found to be significantly higher than wild-type or heterozygous mice, and glycemia dropped significantly lower after fasting in null mice compared with the other groups (Fig. 1D), indicating that the ACTH- and glucocorticoid-deficient *Tpit* null mice are more susceptible to fasting-induced hypoglycemia. In these experiments, one *Tpit* $^{-/-}$ mouse had seizures, and another died during fasting.

It is interesting to note that pigmentation of *Tpit* null mice is deficient (Fig. 1E). Because *Tpit* is not expressed in skin (Pulichino et al. 2003), these results clearly suggest that pituitary POMC-derived peptides, presumably α MSH, are responsible for pigmentation in mice. The results are consistent with the phenotype of POMC $^{-/-}$ mice (Yaswen et al. 1999), but contrasts with humans in which skin pigmentation is not correlated with plasma α MSH, except in pathological conditions (Bertagna 1994). In agreement with this, it was observed that *Tpit*-deficient mice also showed increased water retention in their fur (data not shown), as seen in MC5-R-deficient mice (Chowdhary et al. 1995). This supports the hypothesis that pituitary α MSH and the skin MC5-R receptor are part of a hypothalamic-pituitary-exocrine gland axis.

Early onset IAD patients have TPIT mutations

To define whether a subset of IAD patients might be associated with *TPIT* gene mutations, we investigated the *TPIT* gene sequence in a large panel of 17 patients with a diagnosis of ACTH deficiency. This panel in-

Figure 1. *Tpit* $^{-/-}$ mice are a model of isolated ACTH deficiency (IAD). (A) Plasma ACTH in *Tpit* $^{-/-}$ mice ($n = 7$) is greatly reduced compared with wild-type ($+/+$, $n = 6$) and heterozygous ($+/-$, $n = 6$) mice. (B) Plasma corticosterone is undetectable in *Tpit* $^{-/-}$ ($n = 11$) mice, but normal levels are observed in wild-type ($n = 8$) and heterozygous ($n = 10$) mice. (C) Tissue sections stained with hematoxylin and eosin showing hypoplastic *Tpit* $^{-/-}$ adrenals compared with wild type. (D) Fasting-induced hypoglycemia is greater in *Tpit* $^{-/-}$ ($n = 12$) than in wild-type ($n = 9$) or heterozygous ($n = 12$) mice. Basal glycemia was significantly higher ($p \leq 0.05$) in *Tpit* $^{-/-}$ mice. After a 24-h fast, the glycemia of *Tpit* $^{-/-}$ mice is significantly lower than for other mice ($p \leq 0.001$). Data are means $\pm$ S.E.M. (E) Ventral views of $+/-$ and $-/-$ *Tpit* mice. The mutant has a yellow pigmentation compared with the light gray of $+/-$ or $+/+$ mice.

cluded six patients with juvenile onset (diagnosis at 3.5, 4.5, 10, 15, and 16 yr of age) of disease, and none of them were found to carry *TPIT* gene mutations (data not shown). A panel of 11 patients with neonatal onset of IAD belonging to 9 unrelated families were also investigated for mutations within the coding exons of the *TPIT* gene. Of those, eight patients belonging to six unrelated families were found to have *TPIT* gene mutations (Table 1, Fig. 2A). These patients (four males and four females) were born at term to apparently healthy parents. Two patients were the second children of the sibship, the eldest sibling having died suddenly at birth (families IV and V). Neonatal hypoglycemia was the major reason leading to diagnosis of ACTH deficiency. Three patients suffered from prolonged neonatal cholestatic jaundice. Hepatic needle biopsy in two cases revealed accumulation of biliary pigments in the liver cells and in the bile canaliculi, and also giant cells cholestatic hepatitis. Symptoms of adrenal insufficiency disappeared after cortisol replacement therapy in all cases.

Table 1. Clinical presentation of early onset IAD patients carrying TPIT mutations

Patient	Family	Onset of disease	Hypoglycemia	Prolonged cholestatic jaundice	Basal ACTH ^c (pg/mL)	Basal cortisol ^d (nmol/L)	ACTH/cortisol response to CRH	Cortisol response to ACTH	TPIT mutation
1	I ^a	neonatal	+		<5	35	n.d.	n.d.	R179X
2	II ^{a,b}	neonatal	+	+	13	<20	no	no (acute) yes (repeat)	R286X
3	III ^a	neonatal	+		17	<20	no	no (acute) yes (repeat)	delA
4	III ^a	neonatal	+		<5	<20	n.d.	no (acute)	delA
5	IV ^{a,b}	neonatal	+		<5	55	no	no (acute)	T58A
6	IV ^{a,b}	neonatal	+		<5	25	no	no (acute)	T58A
7	V ^b	neonatal	+	+	18	23	n.d.	no (acute)	S128F, del 5.2 kb
8	VI ^a	neonatal	+	+	<5	<20	n.d.	no (acute)	I171T

^aConsanguinity.^bNeonatal death in another sibling (pts 2 and 7) or related subjects (pts 5 and 6).^cNormal plasma ACTH concentration: 20–60 pg/mL.^dNormal plasma cortisol concentration: 250–600 nmol/L (n.d.) Not determined.

Investigation of pituitary/adrenal function in IAD patients with TPIT mutations (Table 1) showed decreased/undetectable baseline plasma levels of ACTH and cortisol. These patients did not show a cortisol response to

acute injection of ACTH (Table 1), but in two cases (patients 2 and 3), repeated ACTH injection was found to significantly increase cortisol, indicating that adrenal function may be restored upon multiple challenges with

Figure 2. Human TPIT gene mutations in early onset isolated ACTH deficiency (IAD). (A) Summary of different TPIT mutations found in cases of early onset IAD. (B) DNA sequence and pedigree of patients with TPIT mutations. For each family, the black symbol represents the mutant allele indicated at top and revealed by DNA sequencing at bottom. Double horizontal bars in pedigree represent consanguinity. The gray allele in family V has a deletion of exons 3 and 4 as shown in C, and the patient is a compound heterozygote. This patient and her sibling were described previously (Malpuech et al. 1988; Dechelotte et al. 1994). (C) Southern blot analysis of genomic DNA from family V (patient and mother) showing a deletion of exons 3 and 4 in one allele of both subjects. (Lanes 1,2) Control (lane 1) and patient (lane 2) DNA digested with HindIII and hybridized with a probe for exons 3, 4, 5, and 6. A new band of 4.6 kb can be observed (lane 2). (Lanes 3,4,5,6) DNA digested with BamHI and hybridized with an exon 2 probe. In lanes 4 and 6 (patient and mother, respectively), a new band of 3.5 kb is observed compared with controls (lanes 3,5). Schematic representation indicates position of the 5.2-kb deletion in the mutant allele.

ACTH. In contrast, acute or repeated treatment with CRH never elicited a response (Table 1, patients 2, 3, 5 and 6). Investigation at autopsy of the dead sibling of family V revealed bilateral adrenal hypoplasia and normal pituitary morphology with the absence of ACTH immunoreactivity (Dechelotte et al. 1994), which is very similar to the phenotype of *Tpit*^{-/-} mice. There was no other intrinsic pituitary hormone deficiency in these patients, and none of them exhibited pigmentation defects or obesity as observed in patients with *POMC* gene mutations (Krude et al. 1998).

It is noteworthy that all 11 neonatal IAD cases (i.e., both *TPIT* mutations and other three cases) had very similar clinical presentation that included severe plasma ACTH and cortisol deficits, episodes of sudden and severe hypoglycemia sometimes associated with seizures, episodes of prolonged neonatal cholestatic jaundice, and neonatal death when untreated. In summary, *TPIT* gene mutations are relatively frequent (73%) in early onset IAD and define a very homogeneous clinical entity of isolated ACTH deficiency with neonatal onset.

TPIT gene mutations cause loss-of-function and recessive transmission of IAD

Seven different mutations were identified within the coding exons of the *TPIT* gene (Table 1; Fig. 2). We identified two nonsense mutations resulting in a stop codon (families I and II), a 1-bp deletion (family III), three missense mutations (families IV, V, VI), and a large deletion encompassing the third and fourth exons (family V; Fig. 2C). All patients were homozygous for a *TPIT* mutation (Fig. 2B), except one patient who was found to be a compound heterozygote (patient 7). Analysis of parent DNAs indicated that they were all heterozygous carriers of *TPIT* gene mutations, and all were unaffected (Fig. 2B). Similarly, unaffected siblings of IAD patients were either heterozygous for *TPIT* mutations or wild type. This distribution indicates a recessive mode of transmission. All families had some levels of consanguinity except for family V, in which the patient is a compound heterozygote. It is noteworthy that this patient is the sibling of the only well-described early-onset IAD patient for which there is autopsy analyses of pituitary and adrenals (Dechelotte et al. 1994; Malpuech et al. 1988). Patient 7 received an allele with missense mutation S128F from her father, but PCR amplification and sequencing of her mother's DNA did not reveal any DNA sequence changes. Further Southern blot analyses of genomic DNA was required to determine that one maternal *TPIT* allele contained a deletion of ~5.2 kb encompassing exons 3 and 4 (Fig. 2C). In summary, all IAD patients carrying *TPIT* mutations have recessive transmission of mutant alleles, which would be most compatible with loss-of-function mutations.

Mutations in families I, II, and III that result in insertion of stop codons (R179X and R286X) or to read-through and premature stop (delA), are thought to lead to loss-of-function because of nonsense mRNA decay caused by the presence of a stop codon in the penultimate or an earlier exon of the gene (Frischmeyer and Dietz 1999; Hentze and Kulozik 1999). To determine the cause of deficiencies produced by the three missense mutations, these mutations were inserted in plasmid expression vectors and tested by transfection for transcriptional activity and DNA-binding ability. The T58A mu-

tant protein has decreased transcriptional activity with, at most, 10% residual activity in transfection (Fig. 3A). It also has reduced DNA-binding ability (Fig. 3B). The S128F and I171T mutations were found to be completely

Figure 3. *TPIT* missense mutations cause loss-of-function for DNA binding. The activity (A,C,E) and DNA-binding ability (B,D,F) of the three missense mutations identified in *TPIT* patients was assessed. The S128F (C) and I171T (E) mutations are devoid of transcriptional activity, whereas the T58A (A) mutation retains ~10% transcriptional activity when assayed by transient transfection into GH3 cells using a *Tpit*/*Pitx*-dependent reporter plasmid. Data are means $\pm$ S.E.M. of three to five experiments, each performed in duplicate. DNA-binding ability of the three mutant proteins was assessed by gel retardation using in vitro translated proteins and a palindromic consensus binding site of T-box proteins as probe. Synthesis of equivalent amounts of mutant and wild-type proteins was assessed by Western blot (top of each panel). No binding was observed for mutants S128F (D) and I171T (F), whereas slight binding was retained for mutant T58A (B). (G) The position of *TPIT* residues identified in missense IAD patients were modeled onto the crystal structure of the brachyury T-box (Muller and Herrmann 1997).

devoid of activity in transfected cells (Fig. 3C,E) and to have no in vitro DNA-binding activity (Fig. 3D,F). All three missense mutations are located within the T-box (Fig. 2A). Whereas Thr 58 is located close to the DNA-binding cleft (Fig. 3G) and may thus interfere with DNA binding, the position of residues S128 and I171 does not suggest a clear mechanism to prevent DNA interaction, except through conformational changes (Muller and Herrmann 1997). These data clearly suggest that all *TPIT* mutations described in the present work are loss-of-function alleles.

Over the past 10 years, several congenital isolated or combined anterior pituitary deficiencies have been described in humans and animals (Cohen and Radovick 2002). In the absence of treatment, these diseases have irreversible consequences, such as dwarfism, mental retardation, sterility, or neonatal death by acute adrenal insufficiency in the case of corticotroph deficiency. Congenital isolated pituitary hormone deficiencies are currently ascribed to alterations in coding genes for each pituitary hormone, their trophic hypothalamic hormone, or their receptors. For example, mutations of the growth hormone (GH) or GHRH receptor genes have been described in isolated GH deficiency (Argente et al. 2000). In contrast, combined pituitary hormone deficiencies have been ascribed to multiple transcription factor gene mutations, such as those for *Pit-1* (Radovick et al. 1992), *Prop1* (Wu et al. 1998), *Lhx3* (Netchine et al. 2000), *Lhx4* (Machinis et al. 2001), and *Hesx1*(*Rpx*) in septo-optic dysplasia (Dattani et al. 1998). In addition, mutations of the orphan nuclear receptors SF1 and DAX-1 cause pituitary gonadotroph deficiency together with gonadal and adrenal insufficiencies, because they are coexpressed in gonadotrophs, ventromedial hypothalamus, gonads, and adrenals (Ingraham et al. 1994; Ikeda et al. 1996). We have now shown that human and mouse *Tpit* mutations cause IAD, confirming the specificity and key role of *Tpit* in corticotroph differentiation and POMC regulation. We have also described the molecular basis of a clinical entity that was not well characterized previously, congenital early onset IAD. The present study revealed that 8/11 (73%) patients with neonatal IAD carried recessive loss-of-function *TPIT* gene mutations that cause ACTH deficiencies mimicked in *Tpit* mutant mice.

Materials and methods

Mouse studies

The *Tpit*^{-/-} mice are described in Pulichino et al. (2003). Tissue sections were performed as described (Lanctôt et al. 1999). For plasma ACTH and corticosterone measurements (ICN Biomedical, Inc. Diagnostic Division), mice were decapitated and trunk blood was collected onto 10 µL of 60 mg/mL EDTA and centrifuged to collect plasma. For glycemia, tail blood was obtained and measurements were made using the One Touch SureStep blood-monitoring system from Lifescan. Statistical significance was determined using the Scheffe test.

Patients

Patients were included on the basis of isolated ACTH deficiency of unidentified cause. The age at onset of ACTH deficiency was not taken into consideration for inclusion in the study. After informed consent was obtained from parents, DNA samples were collected from 17 patients belonging to 15 unrelated families that originate from 6 different countries.

Plasma ACTH and cortisol concentrations were measured at 0800 h by use of different commercial RIA kits. Plasma ACTH and cortisol responses were studied after a CRH injection test (50 µg) in a subset of

patients (n = 4), with ACTH and cortisol measurements 15, 30, 45, 60, 90, and 120 min after injection. Cortisol response to an intravenous ACTH bolus (250 µg) was assessed with plasma cortisol measurements after 30 and 60 min. To better evaluate adrenal function in a subset of patients (n = 3), cortisol response was also assessed after repetitive intramuscular injections of exogenous ACTH (0.5 mg/m² every 12 h over 3 d). Other anterior pituitary hormone plasma concentrations were measured at baseline and after routine stimulation tests.

Genomic analysis of the *TPIT* gene

All eight exons of the *TPIT* gene were PCR amplified from genomic DNA extracted from the peripheral lymphocytes by use of eight sets of flanking intronic primers for direct sequencing. Amplification was carried out in a 50-µL reaction, using 200 ng genomic DNA with the Vent polymerase (NEB). PCR products were purified on agarose gel using the QIAGEN gel extraction kit. Internal primers were used for sequencing using a CEQ 2000 sequencer from Beckman-Coulter. Primer sequences are available upon request. For Southern blots, 10 µg DNA digested with *Bam*HI or *Hind*III were separated on 1% agarose gels and transferred onto nylon membranes (Pall Corporation). After prehybridization (5 h at 65°C in 4× SET, 0.1% sodium pyrophosphate, 0.2% SDS, 100 µg/mL heparin), hybridization was overnight with 10 × 10⁶ cpm probe labeled by random priming (4× SET, 0.1% sodium pyrophosphate, 0.2% SDS, 500 µg/mL heparin, 10% Dextran sulfate).

Cell culture, transfection, and plasmids

GH3 cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. A total of 250,000 cells were transfected in 12 wells dishes with Lipofectamine (Invitrogen) using 500 ng of reporter plasmid, 0–100 ng of effector plasmid up to a total of 1.5 µg per assay. Cells were harvested 24 h later. *Tpit* reporter and expression plasmids were described (Lamolet et al. 2001). The *Tpit* mutants were generated using the QuickChange mutagenesis kit (Stratagene).

Gel retardation assays and Western blotting

Gel shifts were performed as described (Lamolet et al. 2001) using 5 µL of in vitro-translated protein lysates (wheat germ extract, Promega). Probe used was a palindromic T-box binding consensus site (GATCCAAT TTACACCTAGGTGTGAATT). Western blots were performed as described (Tremblay et al. 1998) with rabbit anti-*Tpit* 1:1000 (Lamolet et al. 2001).

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