A Circulating, Biologically Inactive Thyrotropin Caused by a Mutation in the Beta Subunit Gene

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Abstract

Mutation of a critical carboxy-terminal cysteine residue (C105V) in the thyrotropin-β (TSH-β) subunit gene was found in two related families with central hypothyroidism. Affected patients had low thyroid hormone levels and radioactive iodine uptake in the thyroid gland associated with measurable serum TSH. Thyrotropin-releasing hormone–stimulated TSH secretion did not increase thyroid hormone production in these patients as compared to their unaffected siblings, suggesting that the mutant TSH was biologically inactive in vivo. Recombinant TSH harboring this mutation was confirmed to be biologically inactive in an in vitro bioassay. Based on crystallographic structure of chorionic gonadotropin, a disulfide bond between C19 and C105 in the TSH-β subunit is predicted to form the “buckle” of a “seat belt” that surrounds the common α subunit and maintains the conformation and bioactivity of the hormone. This natural mutation of the TSH-β subunit confirms the importance of the seat belt in the family of pituitary and placental glycoprotein hormones. (J. Clin. Invest. 1996. 97:1250–1256.)

Key words: thyrotropin β subunit • central hypothyroidism • gene mutation • pituitary

Introduction

The etiologies of anterior pituitary hormone deficiency are numerous, and include vascular, neoplastic, immunologic, developmental, and genetic abnormalities of the pituitary and thalamus. β subunit gene mutations of hormones within the pituitary glycoprotein family, comprised of lutetizing hormone (LH), follicle-stimulating hormone (FSH), follicle-stimulating hormone- LH, lutetizing hormone; RAIU, radioactive iodine uptake; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.
using PCR (12). The strategy and oligonucleotide primers used in the PCR reaction are shown in Fig. 2A. Both the 5′ (A, 5′ TTATTACAGCTTTAAAATAATGTTTC 3′) and 3′ primers (B, 5′ GGCAAGCAGTTATTTACAAATGCAAATTATA 3′) contained HindIII restriction sites (underlined) to facilitate their cloning into pGEM4Z (Promega Biotech, Madison, WI). Successful PCR amplification resulted in a 900-bp DNA fragment, containing both the second and third exons and second intron. This PCR was performed on patients IV.15, IV.16, V.2, V.3, V.4, V.5, and V.6; six to ten different subclones of the PCR product from each patient were sequenced using the chain termination method of Sanger et al. (13). Allele-specific PCR was used to confirm a mutation in codon 105 of the human TSH-β gene (14). Fig. 2A illustrates the primers used in this method. Two separate reactions were performed on each genomic DNA sample. The same 5′ primer (C, 5′ TTATCCAGGATATCATG-GCAAC 3′) was used in both reactions, but the 3′ primer contained either a normal (D, 5′ TTCTGAGGTTTGGTACAG 3′) or mutant 105 (E, 5′ TTCTGAGGTTTGGTGACCA 3′) codon; codon 105 is underlined. A 235-bp fragment should only be obtained with the mutant 3′ primer (E) when at least one allele contains a codon 105 mutation.

Expression of recombinant TSH in mammalian cell culture. To determine the functional properties of the mutant TSH, we synthesized wild-type and mutant TSH in a mammalian expression system. The Rous sarcoma virus promoter (pRSV) was placed upstream of a human TSH-β minigene containing both coding exons or a human common subunit cDNA in the vector, pREP-9, (Invitrogen Corp., San Diego, CA) driven by the cytomegalovirus promoter in pcDNA1 and a human common α subunit promoter fragment (~346 bp) fused to the luciferase reporter gene (manuscript in preparation). When exposed to TSH, adenylate cyclase is activated via the G protein–coupled TSH receptor in a concentration-dependent manner and luciferase activity increases to a maximum of sixfold. The sensitivity of this assay is 3 mU/liter, and maximal activity is observed at ~500 mU/liter of recombinant human TSH. No significant cross-reactivity to a human LH preparation is observed at 4,000-fold hormone excess, confirming this assay’s specificity. Recombinant TSH preparations were progressively diluted and assayed at each dilution in triplicate. Immunoreactivity of TSH was determined at each dilution with a sensitive and specific TSH chemiluminescent immunoassay. The wild-type and three mutant TSH-β expression constructs were used in these studies. The carboxy-terminus of the TSH-β subunit was truncated after either the tyrosine residue at position 112 (Y112) or after the cysteine residue at position 105 (C105) by introducing a stop codon (TAG) in the appropriate location. The natural mutation (C105V) is a frameshift deletion in codon 105 resulting in a 9 amino acid nonhomologous extension. Expression constructs were transfected into a human kidney cell line secreting free α subunit (293 cells) using previously described methods (16), and pools of neo-mycin-resistant clones (G418, Gibco BRL, Gaithersburg, MD) were expanded. Transfection efficiency was monitored by cotransfecting a construct containing the herpes thymidine kinase promoter fused to the human growth hormone gene (pTKGH) (17).

In vitro bioassay of recombinant TSH. Bioassay of recombinant TSH was performed in a stable 293 cell line expressing the human TSH receptor cDNA (gift of B. Rapport, University of California, San Francisco, CA) driven by the cytomegalovirus promoter in pcDNA1 and a human common α subunit promoter fragment (~346 bp) fused to the luciferase reporter gene (manuscript in preparation). When exposed to TSH, adenylate cyclase is activated via the G protein–coupled TSH receptor in a concentration-dependent manner and luciferase activity increases to a maximum of sixfold. The sensitivity of this assay is 3 mU/liter, and maximal activity is observed at ~500 mU/liter of recombinant human TSH. No significant cross-reactivity to a human LH preparation is observed at 4,000-fold hormone excess, confirming this assay’s specificity. Recombinant TSH preparations were progressively diluted and assayed at each dilution in triplicate. Immunoreactivity of TSH was determined at each dilution with a sensitive and specific TSH chemiluminescent immunoassay.

Table I. Serum Hormone Levels and Radioactive Iodine Uptakes in a Kindred with a TSH-β Subunit Gene Mutation

<table>
<thead>
<tr>
<th>Family Member</th>
<th>A</th>
<th>T3</th>
<th>T4</th>
<th>Basal</th>
<th>TRH-Stim</th>
<th>RAU</th>
<th>TSH-Stim</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>yr</td>
<td>μg/dl</td>
<td>mg/dl</td>
<td>mU/liter</td>
<td>% in 24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-15</td>
<td>37</td>
<td>10.8</td>
<td>100</td>
<td>1.0</td>
<td>6.4</td>
<td>26.9</td>
<td>ND</td>
</tr>
<tr>
<td>IV-16</td>
<td>47</td>
<td>8.5</td>
<td>119</td>
<td>1.0</td>
<td>5.0</td>
<td>19.8</td>
<td>ND</td>
</tr>
<tr>
<td>IV-22</td>
<td>38</td>
<td>5.0</td>
<td>190</td>
<td>1.3</td>
<td>7.1</td>
<td>24.1</td>
<td>ND</td>
</tr>
<tr>
<td>IV-23</td>
<td>34</td>
<td>9.0</td>
<td>180</td>
<td>2.9</td>
<td>19.3</td>
<td>33.3</td>
<td>ND</td>
</tr>
<tr>
<td>V-2</td>
<td>17</td>
<td>9.5</td>
<td>132</td>
<td>2.0</td>
<td>7.6</td>
<td>16.5</td>
<td>ND</td>
</tr>
<tr>
<td>V-3</td>
<td>15</td>
<td>0.5</td>
<td>45</td>
<td>0.8</td>
<td>1.7</td>
<td>0.9</td>
<td>5.8</td>
</tr>
<tr>
<td>V-4</td>
<td>13</td>
<td>0.5</td>
<td>35</td>
<td>0.02</td>
<td>0.8</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>V-5</td>
<td>10</td>
<td>7.1</td>
<td>121</td>
<td>3.0</td>
<td>15.0</td>
<td>32.2</td>
<td>ND</td>
</tr>
<tr>
<td>V-6</td>
<td>6</td>
<td>0.5</td>
<td>25</td>
<td>0.3</td>
<td>0.9</td>
<td>1.4</td>
<td>4.2</td>
</tr>
<tr>
<td>V-8</td>
<td>14</td>
<td>0.5</td>
<td>79</td>
<td>0.1</td>
<td>1.6</td>
<td>1.0</td>
<td>9.2</td>
</tr>
<tr>
<td>V-9</td>
<td>2</td>
<td>9.7</td>
<td>180</td>
<td>2.6</td>
<td>19.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Normal range</td>
<td>5–12</td>
<td>80–200</td>
<td>0.4–5.0</td>
<td>5–20</td>
<td>12–35</td>
<td>18–48</td>
<td></td>
</tr>
</tbody>
</table>

* Peak TSH after administration of 200 μg i.v. TRH. † 131I uptake after bovine TSH administration, 10 U i.m. every day for 3 d. ‡ Measurements of thyroid function after withdrawal of thyroid hormone treatment for at least 4 wk. To convert T3 and T4 to SI units multiply by 12.87 (nM) or .015 (nM), respectively. Affected family members are shown in bold type; carriers of the mutation are underlined. ND, not determined; Stim., stimulation.
say (Nicholas Institute, San Juan Capistrano, CA). The sensitivity of this assay is 0.01 mU/liter for recombinant TSH synthesized in tissue culture medium. The in vitro biological activity of mutant TSH was determined from a standard curve for wild-type recombinant TSH and a biologic to immunologic ratio (B/I) was determined.

**Results**

Congenital hypothyroidism without goiter was diagnosed in three members of Family A and one member of Family B. Affected patients (V.3, V.4, V.6, and V.8) all had low serum thyroid hormone levels and inappropriately low or normal serum TSH levels (Table I). Serum TSH increased in all affected patients in response to TRH treatment, although peak values were significantly lower than expected for hypothyroid individuals. Basal RAIU was also low in these patients but increased in response to bovine TSH treatment. Thyroid scans before and after bTSH administration confirmed the presence of functional thyroid tissue in the neck in patients V.3, V.4, V.6, and V.8.

Fig. 3 A demonstrates that three of the four affected patients (V.3, V.6, and V.8) had elevated serum α subunit levels when compared to patients with primary hypothyroidism whose α subunit levels should be < 2.5 μg/liter (arrowhead). Note that α subunit levels increased 2- to 3.4-fold in response to TRH stimulation in all affected patients. As expected, two normal individuals (C.1 and C.2) had low α subunit levels, which increased ~ 30% with TRH stimulation. The serum α subunit/TSH molar ratio was also markedly elevated in all affected patients as compared to controls (Fig. 3 B), indicating that free α subunit was secreted in high amount in these patients (18, 19). Moreover, this ratio increased in patients, but not controls, after TRH stimulation, suggesting that the thyrotroph was the source of the free α subunit in affected patients.

Since a partial deficiency of TSH with preservation of other pituitary hormone function was observed in affected patients, we investigated TSH-β gene structure using PCR. Primers complementary to the 3′ end of the first intron (A) and the 3′ end of exon 3 (B) were synthesized (Fig. 2 A) (13). Exon 2

![Figure 2. Genetic analysis of a kindred with central hypothyroidism caused by a TSH-β gene mutation. (A) The location of oligonucleotide primers (arrowheads and accompanying letters) used in the PCR are illustrated here above a schematic representation of the TSH-β gene. Coding sequences are depicted by open boxes, whereas 5′ and 3′ untranslated sequences are shown with shaded boxes. Intronic and flanking DNA is illustrated with a thin line. (B) A DNA sequences of normal and mutant TSH-β DNA fragments, obtained from a PCR using primer A and B, are shown. A 1-bp frameshift deletion (T, underlined) was found in codon 105 converting a cystine to a valine residue, and yielding an additional 8- amino acid nonhomologous peptide extension on the mutant protein. (C). Allele-specific PCR in selected patients from this kindred. Wild-type and mutant TSH-β gene PCRs were performed on genomic DNA from family members using the C and D primer pair and the C and E primer pair, respectively. A PCR product (arrowhead) indicates the presence of the allele.](https://doi.org/10.1172/JCI118540)
a loss of a cysteine residue at position 105, and the addition of a nonhomologous 9 amino acid extension (partial predicted peptide sequence shown). Cysteine 105 is 1 of 12 cysteine residues conserved among the pituitary and placental glycoprotein β subunits contained in follicle-stimulating hormone, LH, and TSH, and CG (18).

To confirm the C105V mutation and to determine carriers of this mutation in Family A and B, allele-specific PCR was performed (12). Fig. 2C shows the results of this analysis. The predicted 235-bp product was observed in the mutant but not wild-type amplification of affected patients V.3, V.4, V.6, and V.8, indicating that both TSH-β alleles contained this mutation. The predicted 236-bp product was observed in only the wild-type amplification of unaffected family members IV.16, V.1, and V.5, indicating that these patients do not carry the C105V mutation. A PCR product was observed in both the wild-type and mutant amplifications of unaffected family members IV.15, IV.22, IV.23, V.2, and V.9, indicating that they are carriers of the C105V mutation. Curiously, the father (IV.16) of Family A does not carry the C105V mutation. Analysis of nine independent dinucleotide repeat microsatellite loci on different chromosomes confirmed that IV.16 was the biological father of V.2, V.3, V.4, V.5, and V.6 with a 99.99% probability (data not shown). Since the mother (IV.15) is heterozygous for the 105 mutation, the father’s germ line must contain either a deleted TSH-β allele (affecting individuals are compound heterozygotes) or a mutant TSH-β allele not found in leukocyte genomic DNA.

To determine the functional properties of TSH, we synthesized wild type and mutant TSH in a mammalian expression system. Rous sarcoma expression constructs containing either the common α or TSH-β subunits and a neomycin gene driven by the thymidine kinase promoter were transfected into a human kidney cell line (293), and pools of neomycin-resistant clones were expanded. Transfection efficiency was similar between wild-type and mutant TSH-β transfections as measure by growth hormone levels in cell culture medium (data not shown). Medium of cell cultures transfected with the α construct and either the wild-type TSH-β, mutant TSH-β, or “empty vector” construct was assayed for immunoreactive TSH. Immunoreactive TSH in medium from either the human α subunit and TSH, and CG (18).

Figure 3. Free α subunit and α subunit/TSH molar ratios in control patients and patients with a TSH-β gene mutation. (A) Free α subunit was measured before (time 0), 15, and 30 min after TRH administration in control patients (C.1 and C.2) and affected patients (V.3, V.4, V.6, and V.8). Affected patients were withdrawn from thyroid hormone replacement for at least 4 wk before TRH testing. The arrowhead marks the maximal free α subunit observed in patients with primary hypothyroidism. (B) Molar ratio of simultaneously determined free α subunit and TSH levels in control and affected patients.

contains the leader peptide and first 34 amino acids of the mature peptide. Exon 3 encodes amino acids 35 through 118 of the mature peptide. Successful PCR amplification resulted in a band of ~900 bp. PCR products were cloned and individual subclones were subjected to DNA sequencing. Both coding exons from an unaffected child (V.5), one parent (IV.16), and control DNA had a normal DNA sequence (Fig. 2B). Approximately half of the clones from the mother and patient V.2 and all of the clones from affected children (V.3, V.4, and V.6) had a single base deletion in codon 105, resulting in a shift in the peptide reading frame (Fig. 2B). This mutation resulted in

Table I: Genotypes from Family A and B

<table>
<thead>
<tr>
<th>Family</th>
<th>Genotype</th>
<th>Affected Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C105V/−</td>
<td>V.2, V.3, V.4, V.6</td>
</tr>
<tr>
<td>B</td>
<td>C105V/C105V</td>
<td>V.8, V.9</td>
</tr>
</tbody>
</table>

Data from the family, however, suggest that the mutant TSH is also biologically inactive. Twin sisters from Family B had TRH testing performed, and their serum TSH and T₄ was measured. As shown in Table II, patient V.9, a carrier of the mutation, had a normal increase in serum TSH and T₄ after TRH administration. In contrast her affected twin sister (V.8) had an impaired TSH and T₄ response to TRH. This patient has functional thyroid tissue (Table I) as indicated by a signifi-
In contrast to the C105 truncation, substitutes a valine for a critical cysteine residue responsible for TSH subunit conformation and interaction (see below).

### Discussion

Congenital hypothyroidism in this kindred is associated with a frameshift deletion in codon 105 of the TSH-β subunit gene C105V. Cysteine 105 is one of 12 cysteines conserved among all dimeric pituitary and placental glycoprotein hormone β subunits. Data from chemical modification of the TSH-β subunit peptide suggest that cysteine 105 forms a disulfide bond with cysteine 19 during the folding of this subunit (22). Recent crystallographic structure of CG indicates that this bond forms the “buckle” of a “seat belt” that holds the α subunit in place (23). Based on this structure, a model of TSH can be developed where a seat belt from the β subunit surrounds the α subunit and holds it in place with a buckle formed between C19 and C105 (Fig. 5). Thus, the C105V mutation would be predicted to affect the subunit conformation, and either enhance TSH-β subunit intracellular degradation or diminish its ability to interact with the α subunit. Data from in vitro expression of this mutant confirm this prediction. TSH expression from cell cultures transfected with the C105V mutant β subunit was significantly reduced relative to cell cultures transfected with the wild-type β subunit.

#### Table II. Serum Hormone Levels after a TRH Stimulation Test in Sisters from Family B

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TSH (mU/liter)</th>
<th>T3 (ng/dl)</th>
<th>TSH (mU/liter)</th>
<th>T3 (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.11</td>
<td>79</td>
<td>2.64</td>
<td>180</td>
</tr>
<tr>
<td>30</td>
<td>1.62</td>
<td>81</td>
<td>19.81</td>
<td>211</td>
</tr>
<tr>
<td>60</td>
<td>1.52</td>
<td>89</td>
<td>16.62</td>
<td>212</td>
</tr>
<tr>
<td>120</td>
<td>0.14</td>
<td>79</td>
<td>12.04</td>
<td>242</td>
</tr>
<tr>
<td>180</td>
<td>0.09</td>
<td>81</td>
<td>9.77</td>
<td>280</td>
</tr>
</tbody>
</table>

To confirm that this mutant TSH was biologically inactive, and TSH containing carboxy-terminal truncations of the TSH-β subunit gene were tested in a sensitive bioassay which measures the ability of occupied TSH receptor to stimulate the common α subunit gene activity via increases in cAMP production (20, 21). Fig. 4 demonstrates the ratios of the biologic to immunologic activity (B/I) for wild-type and mutant recombinant TSH preparations. TSH containing either carboxy-terminal truncation of the TSH-β subunit (Y112 or C105) displayed an increased B/I relative to wild-type TSH. These data indicate that the carboxy-terminal 13 amino acids of the TSH-β subunit are not important for TSH biological activity as measured in this assay. In contrast, the C105V mutant TSH from these patients displayed a much lower ratio, indicating that it had significantly reduced bioactivity. The C105V mutant, in contrast to the C105 truncation, substitutes a valine for a critical cysteine residue responsible for TSH subunit conformation and interaction (see below).

#### Figure 4. Ratios of biologic to immunologic activity (B/I) of wild-type and mutant recombinant TSH measured in an in vitro bioassay. A human α subunit RSV expression vector was transfected with either a wild-type TSH-β (Wt), a TSH-β subunit truncated after tyrosine 112 (Y112), a TSH-β subunit truncated after cysteine 105 (C105), or a frameshift mutation of codon 105 (C105V). RSV expression vector in 293 cells and recombinant TSH assay was synthesized. Biologic activity of recombinant TSH was tested on 293 cells containing human TSH receptors and a human common α subunit gene linked to the luciferase reporter gene. A standard curve of luciferase activity relative to immunoreactive wild-type TSH was generated and the biologic activity of mutant TSH was compared to this curve. Data are the mean ± SD of the ratios.

#### Figure 5. Representation of TSH structure based on the crystal structure of HCG. This is a possible model of TSH structure based on crystallographic structure of HCG. The α subunit and β subunits of TSH are indicated by a black or shaded structure. The location of a carbohydrate chain is indicated by a Y. The seat belt of the β subunit is formed from residues located near the COOH terminus and loops around the α subunit and forms a bond with C19 near the NH2-terminus of the β subunit. The location of the cystine bond between C19 and C105 is indicated by the line.
Impaired TSH subunit combination was also demonstrated by Hayashizaki et al. for a mutation in the CAGYC region of the TSH-β subunit gene (G29R) (7). The CAGYC region is named for its one letter amino acid codes, is located near NH₂ terminus of all pituitary and placental glycoprotein β subunits, and is necessary for α-β subunit combination (22). Affected family members with the G29R mutation had no measurable TSH in the serum even after TRH stimulation in the hypothyroid state. TSH levels in affected patients with the C105V mutation, however, were low-normal in the hypothyroid state and increased in response to TRH stimulation. Synthesis of TSH, therefore, is completely abolished with the G29R mutation but only partially impaired with the C105V mutation. As such, this is the first report of a genetic abnormality in the TSH-β subunit gene where immunoreactive mutant TSH is measurable in the serum.

Clinical data in this family also indicate that an impairment in TSH subunit combination exists. Fig. 3 demonstrates elevated basal and TRH-stimulated glycoprotein-free α subunit levels in affected family members. Since the origin of TRH-stimulated α subunit is the thyrotroph, in the absence of a pituitary adenoma (19), these data indicate the lack of efficient TSH-β subunit combination in affected patients. This is reflected in low serum TSH levels in affected family members, which are 10- to 100-fold lower than expected for hypothyroid individuals (Table I). As noted in Table I, however, affected children did have a 3- to 15-fold rise in serum TSH levels after TRH stimulation, confirming that some TSH subunit combination must occur with the mutant TSH-β subunit molecule.

In addition to impaired secretion of mutant TSH in affected family members, the mutant TSH has reduced biological activity. Two separate lines of evidence support this conclusion. First, when hypothyroidism was induced in affected family members by stopping their hormone replacement, mutant TSH serum levels increased to a low-normal range, but radioactive iodine uptake was negligible (Table I). For example, the father of Family A (IV.16) had a serum TSH of 1.0 mU/liter and a 24 h RAIU of 19.8%, but his daughter (V.3) with a similar TSH serum levels increased to a low-normal range, but radioactive iodine uptake was negligible (Table I). For example, the father of Family A (IV.16) had a serum TSH of 1.0 mU/liter and a 24 h RAIU of 19.8%, but his daughter (V.3) with a similar TSH level increased 15-fold (Table II). Of course, the impaired ARAU response to mutant TSH molecule may be due to chronic TSH deficiency in affected family members. Bovine TSH administration, however, increased RAIU in the thyroid bed of affected individuals, V.3, V.4, V.6, and V.8 indicating functional thyroid tissue. Second, recombinant TSH harboring the C105V mutation in the TSH-β subunit had reduced bioactivity compared to wild-type TSH. The B/I could be even lower for the C105V mutant TSH, since the immunonoassay may underestimate the amount of mutant TSH due to epitope loss. In contrast, the artificial carboxy-terminal truncations of the TSH-β subunit demonstrate that the mutation of cysteine at position 105 and not the absence of the remaining 13 amino acids is responsible for the impaired bioactivity of mutant TSH in this family.

Measurement of TSH is a valuable tool for evaluating thyroid function in individuals and in the differential diagnosis of thyroid disorders. This kindred, however, points out the difficulties of using a TSH measurement alone to evaluate thyroid disorders in the absence of a goiter. For example, if TSH was used as the primary screen for hypothyroidism in this kindred, hypothyroidism may have been missed, since TSH levels were normal or low in affected individuals. Moreover, since TSH levels were normal in many of the affected patients, central hypothyroidism might not have been considered in the differential diagnosis unless thyroid hormone levels were also determined. Unlike previous reports of unmeasurable TSH due to TSH-β subunit gene mutations (7, 8), a circulating form of mutant TSH exists in these patients, suggesting that other biologically inactive forms of TSH due to TSH-β subunit gene mutations remain to be discovered.

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References


