Thyrotropin (TSH) is a member of the glycoprotein hormone family, which includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). These are heterodimers composed of two noncovalently linked subunits, α and β. We have recently converted the hTSH heterodimer to a biologically active single chain (hTSHbCTPa) by fusing the common α-subunit to the C-terminal end of the hTSH β-subunit in the presence of a ω-30-amino acid peptide from hCG β (CTP) as a linker. The hTSHbCTPa single chain was used to investigate the role of the N-linked oligosaccharides of α- and β-subunits in the secretion and function of hTSH. Using overlapping PCR mutagenesis, two deglycosylated variants were prepared: one lacking both oligosaccharide chains on the α-subunit (hTSHβ-CTPa1+2) and the other lacking the oligosaccharide chain on the β-subunit (hTSHβ-CTPα(deg)). The single chain variants were expressed in CHO cells and were secreted into the medium. hTSH variants lacking the oligosaccharide chains were less potent than hTSHbCTPa wild-type with respect to cAMP formation and thyroid hormone secretion in cultured human thyroid follicles. Both deglycosylated variants competed with hTSH in a dose-dependent manner. The hTSHbCTPa1+2 variant blocked cAMP formation and thyroid hormone secretion stimulated by hTSH as well as by the antibody, thyroid-stimulating immunoglobulins, responsible for the most common cause of hyperthyroidism, Graves disease. Thus, this variant behaves as a potential antagonist, offering a novel therapeutic strategy in the treatment of thyrotoxicosis caused by Graves’ disease and TSH-secreting pituitary adenoma.

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§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, Carmel Medical Center, 7 Michal St. 7, Haifa 34362, Israel. Tel.: 972-4-8250407; Fax: 972-4-8343023; E-mail: fares@actcom.co.il.

The abbreviations used are: TSH, thyrotropin; FSH, follitropin; LH, lutropin; hCG, human chorionic gonadotropin; CHO, Chinese hamster ovary cells; TSI, thyroid-stimulating immunoglobulins; CTP, C-terminal peptide, T3, triiodothyronine; PCR, polymerase chain reaction.

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Enzymes used in the construction of DNA vectors and constructs were purchased from New England BioLabs (Beverly, MA). Oligonucleotides used for chimeric construction were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). hTSH was a gift from the National Hormone and Pituitary Distribution Program, NIDDK, National Institutes of Health, and hTSH (MRC Research standard B, 65/122) was a gift from the National Institute for Biological Standards and Control, London, UK.

Construction of hTSH Single Chain Variants—The hTSH single chains were constructed using overlapping PCR mutagenesis, as described previously (7, 24). For construction of the hTSHβ/CTPα12 gene, we used the TSHβ/CTPα12 fragment containing the N-linked glycosylation sites on Asn23 and Asn28 of the α-subunit and at Asn25 of the β-subunit. hTSHβ/CTPα12 was used as template DNA, and three PCR reactions were performed for generating the TSHβ/CTPα12 deg coding sequence. The first reaction included primers 1 and 5, the second reaction included primers 6 and 4, and the final reaction included primers 1 and 4, which resulted in the former fragments containing the mutation in position 23 of the β-subunit (Fig. 1A).

Construction of Expression Vectors—The eukaryotic expression vector pMT/HA-a was used as template DNA, and three PCR reactions were performed for generating the TSHβ/CTPα12 or TSHβ/CTPα12 deg chimeric genes were inserted at the BamHI/SalI cloning site of pMT/HA and used for transfection.

DNA Transfection and Clone Selection—Chinese hamster ovary (CHO) cells (wild-type and/or ldlD), were transfected with pMT/SHβ/ CTPα12, pMT/hTSHβ/CTPα12, or pMT/hTSHβ/CTPα12 deg vectors, according to the calcium phosphate precipitation method (4). Cells were selected for insertion of the plasmid DNA by growth in culture medium containing 0.25 mg/ml of the neomycin analog, G418. Transfected colonies resistant to G418 were harvested and screened for the expression of hTSH variants by metabolic labeling of the cells and immunoprecipitation.

Cell Culture—CHO cells were maintained in Medium 1 (Ham’s F-12 medium supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (2 mM) containing 5% fetal calf serum), at 37 °C in a humidified 5% CO2 incubator. Transfected clones were maintained in the above culture medium supplemented with 0.25 mg/ml active G418 (Medium II). For hormone incorporation, cells secreting hTSH variants were plated and grown to confluence in T-75 flasks. Cells were washed twice with serum-free medium and 12 ml of Medium II (Medium I without fetal calf serum) were added. Medium was collected every 24 h, and concentrated using centrifugation and concentrated using centriprep concentrators (Amicon, Corp., Danvers, MA). Concentrations of hTSH variants were determined by hTSH immunoradiometric assay and a double antibody radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). In addition, medium from nontransfected CHO cells was collected as described above and used as control.

Metabolic Labeling—On day 0, cells were plated into 12-well dishes (350,000 cells/well) in 1 ml of Medium I. For continuous labeling experiments, cells were washed twice with cysteine/methionine-free Medium IV (Medium I supplemented with 5% dialyzed calf serum) and labeled for 5 h in 1 ml of cysteine/methionine-free Medium IV containing 50 μCi/ml [35S]cysteine/methionine mix. For pulse-chase labeling, the cells were washed twice and preincubated for 1.5 h with cysteine-free Medium IV, followed by a 20-min pulse-labeling in cysteine/methionine-free Medium IV containing 100 μCi/ml [35S]cysteine/methionine. Pulse-chase experiments using ID1 cells were performed in the presence or absence of 10 μM galactose or 100 μM N-acetyl galactosamine. The labeled cells were then washed twice with Medium IV containing 1 mM unlabeled cysteine/methionine and incubated in this chase medium for the indicated time. Media and cell lysates were prepared, immunoprecipitated using monoclonal antisera against the α-subunit and resolved on 15% SDS-polyacrylamide gels as described previously (27).

Tunicamycin Treatment—Cells were plated into 12-well dishes in 1 ml of Medium I. On the second day, medium was changed with medium containing 10 μg/ml tunicamycin. Cells were incubated at 37 °C for 1.5 h. At the end of the incubation time, the medium was exchanged with Medium II containing 2 μg/ml tunicamycin and 50 μCi/ml of [35S]cysteine/methionine mix. Further analysis proceeded with metabolic labeling as described above.

In Vitro Bioassay—The bioactivity of hTSH variants was determined by measuring their ability to stimulate cAMP formation and T3 secretion from cultured thyroid follicles which had previously been infected (17, 18). Essentially, human thyroid cells were prepared from colloid tissue obtained at thyroidectomy from patients with benign nodules. 200 × 10^6 plated onto 24-well microtiter plates and incubated with 0.5 ml of serum-free medium (DCCM-I, which contains insulin (1 μg/ml), and antibiotics), in the presence or absence of the hTSH variants and cultured for 7 days at 37 °C, in an atmosphere of 5% CO2 in a water
saturated incubator. For T₃ measurements, potassium iodide (0.1 μM) was added to the medium at the start of the culture period. For cAMP measurements, 1-methyl-3-isobutylxanthine (0.5 mM), which inhibits cAMP degradation, was added to the medium. At the end of the culture period, the cAMP and T₃ secreted into the medium (concentrations remaining in the cells were negligible) were measured by radioimmunoassay as described previously (17).

Statistical Analysis—Each experiment was repeated at least three times, and results are presented as the mean ± S.E. of at least three replicate determinations. Statistical analysis of the data was performed using Student’s t test and analysis of variance. p < 0.05 was considered significant.

RESULTS

Oligonucleotide-directed mutagenesis was chosen to examine the functional importance of N-linked oligosaccharides in hTSH single chain (hTSHβ-CTPa) bioactivity. The hTSHβ-CTPa contains three N-linked glycosylation sites: two on the α- and one on the β-subunit (Fig. 1B). Mutagenesis of the Asn in the Asn-X-Thr/Ser recognition sequence for Asn-linked glycosylation is sufficient to prevent transfer of the carbohydrate to the protein (4). The coding sequence of Asn (5′-AAC-3′) at positions 52 and 78 of the α-subunit was converted to the coding sequence for Asp (5′-GAC-3′). This variant was deglycosylated only on the α-subunit (pM₂₆hTSHβ-CTPaα₁₋₂). To construct deglycosylated variants on the α- and β-subunits (hTSHβ-CTPaα(deg)), the coding sequence for Asn (5′-AAC-3′) on the β-subunit of the hTSHβ-CTPaα₁₋₂ variant was converted to the coding sequence for Asp (5′-GAC-3′) (Fig. 1B). The coding sequence of hTSHβ-CTPa variants was sequenced to verify the mutations and the absence of other sequence alterations.

Stable clonal cell lines expressing hTSHβ-CTPa, hTSHβ-CTPaα₁₋₂, and hTSHβ-CTPaα(deg) were selected. Cells were labeled in the presence of [35S]methionine/cysteine mix for 7 h, media and lysates were immunoprecipitated with polyclonal human anti-α antisera, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis. Intracellular (lysate) forms of hTSHβ-CTPa wild-type and its mutants migrated faster than corresponding extracellular (medium) forms (Fig. 2A). This is because of the differences in terminal processing of the N-linked oligosaccharides and the addition of the O-linked oligosaccharides prior to secretion. The secreted mutant forms migrated faster than wild-type (19). This is because of their lower content of oligosaccharide chains. To confirm this assumption, CHO cells were treated with tunicamycin. Because tunicamycin prevents the addition of N-linked oligosaccharides to the protein, a difference in mobility is expected between proteins secreted from cells treated with tunicamycin compared with those untreated. The results showed that hTSHβ-CTPa and hTSHβ-CTPaα₁₋₂ variants secreted from cells treated with tunicamycin gave, as expected, the same mobility as hTSHβ-CTPaα(deg) secreted from treated and untreated cells (Fig. 2B). This indicates that N-linked oligosaccharides are present in the α- and β-subunits of hTSHβ-CTPa as well as in the β-subunit of hTSHβ-CTPaα₁₋₂ and absent in the hTSHβ-CTPaα(deg) variant.

The secretion kinetics of hTSH variants was determined by pulse-chase analysis and immunoprecipitation with anti-α antisera (Fig. 3). Whereas the hTSHβ-CTPa (Fig. 3A) and hTSHβ-CTPaα₁₋₂ (Fig. 3B) were secreted efficiently with a similar t₅₀ of ~2 h, the secretion rate of hTSHβ-CTPaα(deg) (Fig. 3C) was significantly slower with a t₅₀ of ~17 h. These differences were not because of the O-linked oligosaccharides associated with the CTP, because similar results were detected using LDL cells (results not shown), which had a reversible defect in the synthesis of O-linked oligosaccharides (20). The data thus indicate that conversion of hTSH into a single peptide chain together with CTP as a linker allows the heterodimeric-like configuration of the mutated α- and β-subunits, as shown by

secretion of the protein into the medium.

The biological activity of hTSH variants was examined by their ability to stimulate cAMP formation and T₃ secretion from cultured human thyroid follicles. Treatment of the cells with increasing concentrations (1–100 microunits/ml) of hTSH single chain resulted in a dose-dependent increase in cAMP formation (Fig. 4A) and triiodothyronine (T₃) secretion (Fig. 4B). The maximal effect on cAMP formation and T₃ secretion was seen at concentrations of 50 and 5 microunits/ml, respectively.

Compared with hTSHβ-CTPa, the maximal effect of hTSHβ-CTPaα₁₋₂ or hTSHβ-CTPaα(deg) on cAMP formation in thyroid cells was about 8 and 23% (p < 0.001), respectively (Fig. 4A). Similarly, the maximal effect on T₃ secretion was 64% (p < 0.001) after exposure to hTSHβ-CTPaα(deg) and to undetectable amounts after exposure to hTSHβ-CTPaα₁₋₂ (Fig. 4B).

In competition experiments, cells were grown in the presence of submaximal concentrations of normal hTSH (50 microunits/ml) or of hTSH (0.75 milliunits/ml) and different concentrations (20–200 microunits/ml) of deglycosylated variants (Fig. 5). Both deglycosylated variants competed with hTSH in a dose-dependent manner. The cAMP levels induced by submaximal doses of hTSH were decreased in the presence of 200 microunits/ml of hTSHβ-CTPαα₀₋₁ (IC₅₀ = 50 microunits/ml) or hTSHβ-CTPα(deg) (IC₅₀ = 158 microunits/ml) by 87 and 66% (p < 0.001), respectively (Fig. 5A). Similarly, the T₃ levels induced by submaximal doses of hTSH were decreased in the presence of 200 microunits/ml of hTSHβ-CTPαα₀₋₁ (IC₅₀ = 33 microunits/ml) or hTSHβ-CTPα(deg) (IC₅₀ = 135 microunits/ml) by 92 and 87% (p < 0.001), respectively (Fig. 5B). The cAMP formation and T₃ secretion induced by submaximal doses of hTSH were decreased in 40% (p < 0.001, IC₅₀ = 2.5 microunits/ml) (Fig. 6A) and 55% (p < 0.001, IC₅₀ = 42 microunits/ml, Fig. 6B) in the presence of 100 microunits/ml of hTSHβ-CTPαα₀₋₁, respectively. 200 microunits/ml of the variant hTSHβ-CTPαα₀₋₁ reduced cAMP formation by 90% (p < 0.001, IC₅₀ = 58 microunits/ml, Fig. 6A) and completely blocked (p < 0.001, IC₅₀ = 15 microunits/ml)
DISCUSSION

The present study indicates that deglycosylated variants of hTSH single chains are expressed and secreted from CHO cells. These variants are less potent than wild-type hTSH with regard to cAMP formation and \( T_3 \) secretion in cultured human thyroid follicles. Moreover, hTSH\( \beta \)-CTP\( \alpha \) (deg) reduced the biological activity of hTSH or hTSI, whereas hTSH\( \beta \)-CTP\( \alpha \) significantly blocked the activity of hTSH and hTSI.

The Asn-linked oligosaccharides have been implicated in several physiologic functions such as maintenance of intracellular stability, secretion, biological activity, and modulation of the plasma half-life (1, 3). Site-directed mutagenesis has become an effective method to study the role of individual carbohydrate chains on multiglycosylated proteins. However, site-directed mutagenesis may affect assembly of heterodimer subunits. It has been reported that mutating the hTSH\( \beta \)-subunit significantly reduced TSH dimer formation (14). Other studies indicate that loss of oligosaccharides from the \( \alpha \)-subunit reduced assembly and/or stability of hCG (4). To bypass the problem of dimerization of deglycosylated subunits, the subunits \( \alpha \) and \( \beta \) were genetically fused in a single chain hormone. Single chains of hCG (7), hFSH (8), and hTSH (11, 12) retained a biologically active conformation similar to that of the heterodimer (11, 12). Therefore, fusion of \( \alpha \)- and \( \beta \)-subunits in a single chain bypasses the assembly of the subunits, which is a rate-limiting step for hormone secretion and bioactivity. It is apparent that despite the single chain structure, correct conformation of the subunits occurs, and the single chains have a normal biological activity (11, 12).

For studying the role of N-linked oligosaccharides on hTSH function, we used the single chain of hTSH that contains the CTP as a linker between \( \alpha \)- and \( \beta \)-subunits (11). Previous studies indicated that fusing the CTP to the C-terminal end of hFSH\( \beta \) (13, 21), to the N-terminal of hCG\( \alpha \) (15), or to hTSH\( \beta \) (22) does not affect the assembly, secretion, and signal transduction of the respective dimers compared with the wild type.

Fig. 3. Kinetics of hTSH variants secretion from CHO cells. Cells expressing hTSH\( \beta \)-CTP\( \alpha \) (A), hTSH\( \beta \)-CTP\( \alpha_{1+2} \) (B), and hTSH\( \beta \)-CTP\( \alpha \) (deg) (C) were pulse-labeled with 100 \( \mu \)Ci/ml of \([35S]\)cysteine for 20 min and chased for the indicated times (h). Lysate and medium samples were immunoprecipitated with antiserum against the \( \alpha \)-subunit and subjected to SDS-polyacrylamide gel electrophoresis. The positions of the molecular mass markers are indicated in kDa.

Fig. 4. In vitro biological activity of hTSH variants. cAMP formation (A) and \( T_3 \) secretion (B) were measured after exposure of human thyroid follicles for 7 days at 37 \( ^\circ \)C to different concentrations of hTSH variants. The cAMP and \( T_3 \) concentrations in the medium were assayed by radioimmunoassay. Each point represents the mean \( \pm \) S.E. of triplicate culture wells.
In addition, it was reported that CTP and associated O-linked oligosaccharides in hCG are not important for receptor binding or in vitro signal transduction but are critical for in vivo biological response (23). Moreover, it has been shown that the secretion of the hFSH single chain increased in the presence of CTP as a linker between the subunits (8). The fact that deglycosylated variants of hTSH are secreted efficiently from CHO and ldlD cells (ldlD cells have a reversible defect in synthesizing oligosaccharide chains, data not shown) indicates that N-linked and O-linked oligosaccharides are not vital for the secretion of the hTSH single chain. Therefore, the signal for the secretion of the hormone exists in the single chains itself.

The bioactivity of the variants was examined in an in vitro system of human thyroid follicles cultured in suspension, under serum-free conditions, in which the follicular three-dimensional structure is retained (17, 18). This bioassay has several advantages over the current methods used for testing thyroid biological activity. First, it allows the measurement of T3 secretion, the physiologically relevant hormonal end-point response, which is very seldom measured when testing for thyroid biological activity. Second, the cells are of human origin, which is important in view of wide species variation in thyroid response to TSH agonists. The results indicated that deletion of the N-linked oligosaccharides from the single chain diminished biological activity. However, a difference in bioactivity between the variants was apparent. hTSHβ-CTPα(deg) was more potent than hTSHβ-CTPα112, and this may be related to the difference in conformation of the variants.

The competition experiments indicated that hTSHβ-CTPα112 markedly blocked cAMP formation and T3 secretion induced by hTSH and hTSI. Therefore, this variant can be considered as a potential antagonist to both hTSH as well as hTSI. It is worth noting that we tested, to the best of our knowledge for the first time, not only TSH but TSI as well, i.e. the factor responsible for the most common cause of hyperthyroidism, Graves’ disease, thus substantiating considerably the clinical implications of our study. Therefore, the hTSHβ-CTPα112 variant, behaves as a potential antagonist that may offer a novel therapeutic strategy of thyrotoxicosis because of Graves’ disease and TSH-secreting pituitary adenoma. The existence of CTP as a linker between the subunits in the hTSHβ-CTPα112 may prevent rapid degradation in vivo and increase their half-life in the circulation. The therapeutic efficacy of this analog needs to be established in in vivo studies and clinical trials.

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