Expression of Human Thyrotropin in Cell Lines with Different Glycosylation Patterns Combined with Mutagenesis of Specific Glycosylation Sites

CHARACTERIZATION OF A NOVEL ROLE FOR THE OLIGOSACCHARIDES IN THE IN VITRO AND IN VIVO BIOACTIVITY*

(Received for publication, June 7, 1995, and in revised form, September 6, 1995)

Mathis Grossmann†, Mariusz W. Szukulinski, Joseph E. Tropea, Leonora A. Bishop‡, N. Rao Thotakura, Peter R. Schofield§, and Bruce D. Weintraub

From the Molecular and Cellular Endocrinology Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-1758 and the Garvan Institute of Medical Research, Sydney, Australia 2000

We used a novel approach to study the role of the Asn-linked oligosaccharides for human thyrotropin (hTSH) activity. Mutagenesis of Asn (N) within individual glycosylation recognition sequences to Gln (Q) was combined with expression of wild type and mutant hTSH in cell lines with different glycosylation patterns. The in vitro activity of hTSH lacking the Asn\(^{\text{52}}\) oligosaccharide (αQ52/TSH\(\beta\)) expressed in CHO-K1 cells (sialylated oligosaccharides) was increased 6-fold compared with wild type, whereas the activities of αQ78/TSH\(\beta\) and α/TSH\(\beta\)NQ23 were increased 2-3-fold. Deletion of the Asn\(^{\text{52}}\) oligosaccharide also increased the thyrotropic activity of human chorionic gonadotropin, in contrast to previous findings at its native receptor. In the in vitro activity of wild type hTSH expressed in CHO-LEC2 cells (sialic acid-deficient oligosaccharides), CHO-LEC1 cells (Man\(_3\)GlcNAc\(_2\) intermediates), and 293 cells (sulfated oligosaccharides) was 5-8-fold higher than of wild type from CHO-K1 cells. In contrast to CHO-K1 cells, there was no difference in the activity between wild type and selectively deglycosylated mutants expressed in these cell lines. Thus, in hTSH, the oligosaccharide at Asn\(^{\text{52}}\) and, specifically, its terminal sialic acid residues attenuate in vitro activity, in contrast to the previously reported stimulatory role of this chain for human chorionic gonadotropin and human folliculin activity. The increased thyrotropic activity of αQ52/CG\(\beta\) suggests that receptor-related mechanisms may be responsible for these differences among the glycoprotein hormones. Despite their increased in vitro activity, αQ52/TSH\(\beta\), and αQ78/TSH\(\beta\) from CHO-K1 cells had a faster serum disappearance rate and decreased effect on T\(_4\) production in mice. These findings highlight the importance of individual oligosaccharides in maintaining circulatory half-life and hence in vivo activity of hTSH.

Thyrotropin (thyroid-stimulating hormone, TSH)\(^1\) is a member of the glycoprotein hormone family, which also includes chorionic gonadotropin (CG), lutropin (luteinizing hormone, LH), and follitropin (follicle-stimulating hormone, FSH). These hormones are structurally related heterodimers consisting of a common \(\alpha\) subunit and a distinct \(\beta\) subunit that confers the biological specificity for each hormone (1). The common \(\alpha\) subunit bears two N-linked oligosaccharides, and the \(\beta\) subunit bears either one (in TSH and LH), or two (in CG and FSH) (2, 3). The oligosaccharides, which represent 18–35% of total weight (2–4), have been shown to play a role in the proper folding, assembly, secretion, metabolic clearance, and biological activity of these hormones (for recent review, see Refs. 3 and 4). In the case of hCG and hFSH, enzymatic or chemical deglycosylation led to a decrease or loss of cAMP production and steroidogenesis, while high affinity binding was maintained (3–5). Sairam et al. (6) reported that the carbohydrates of the common \(\alpha\) subunit rather than those of the \(\beta\) subunits were important for the activity of these hormones. Using site-directed mutagenesis, Matzuk et al. (7) identified the oligosaccharide at position 52 of the \(\alpha\) subunit to be critical for the in vitro bioactivity of hCG. Subsequently, this oligosaccharide was shown to be similarly important for the stimulatory activity of hFSH (8, 9).

In contrast to hCG and hFSH, the roles of the individual oligosaccharides for the activity of TSH or the more closely related LH are not known. Due to the limited availability of purified pituitary human TSH (phTSH), studies on the role of the carbohydrates for TSH have mostly used pituitary bovine TSH. Similar to the findings for the gonadotropins, these studies have shown by chemical or enzymatic deglycosylation that the oligosaccharides, and particularly those of the \(\alpha\) subunit, were important for activities in vitro of bovine TSH (10, 11). The few studies on phTSH have yielded conflicting results and the role of carbohydrates for hTSH action remained controversial (12, 13). The recent availability of recombinant human TSH (rhTSH) (14) allowed further investigation of the role of the carbohydrates in the action of hTSH. rhTSH expressed in CHO-K1 cells contains N-linked oligosaccharides which terminate with Sia\(_{2–3}\)Gal\(_{\text{a2–3}}\)Gal\(_{\text{a1–4}}\)GlcNAc\(_{2}\)–2Man\(_3\) (14–16). By comparison, phTSH, which physiologically occurs in a variety of glycosylation isoforms, terminates both in SO\(_4\)\(^{-}\)sialic acid-deficient oligosaccharides, CHO, chinese hamster ovary cell line; FRTL-5, Fischer rat thyroid cell line; 293, 293 human embryonic kidney cell line; T\(_3\), 3,5,3\(^{-}\)-triiodo-L-thyronine; T\(_4\), 3,5,3,5\(^{-}\)-tetraiodo-L-thyronine.
Role of Oligosaccharides for hTSH

4GalNAcβ1–4GalNAcβ1–2Manα2 and Siaα2–3Galβ1–4GlcNAcβ1–2Manα2 as described by Green and Baenziger (2). Interestingly, enzymatic removal of terminal sialic acid residues of rhTSH increased the in vitro bioactivity of the hormone (17) similar to findings for recombinant bovine LH (15), but unlike for hCG, in which sequential deglycosylation resulted in a stepwise reduction of activity (18). These findings suggested that the role of the oligosaccharides may be different for hTSH compared with hCG and hFSH.

In the present study we have used site-directed mutagenesis to study the role of individual oligosaccharides of hTSH by selectively inhibiting their cotranslational attachment. We combined site-directed mutagenesis with the expression of the selectively deglycosylated hTSH mutants in different cell lines producing hormones with distinct carbohydrate patterns (19–21), using our recently developed and optimized transient transfection protocol (22). This novel approach allowed us to identify unique roles for individual oligosaccharides and their terminal sialic acid residues for the in vitro as well as in vivo activity of hTSH.

EXPERIMENTAL PROCEDURES

Materials—The following materials were generous gifts: pAXNe-ΔrXia, an expression plasmid encoding the common α subunit from Pacific Biotechnology Ltd. (Sydney, Australia); hCGi cDNA in pcDNA I neo (Invitrogen) from Dr. T. H. Ji (Laramie, WY); CHO cells stably transfected with hTSH receptor from Dr. J. Vassart, Belgium; FRTL-5 cells expressing the endogenous rat TSH receptor from Dr. L. D. Kahn, Interthyr Research Foundation (Baltimore, MD); oX antibody from Dr. J. L. Vaitukaitis, National Institutes of Health (Bethesda, MD); polyclonal antibodies NIADDK-anti-hTSH-3 and NIDDK-anti-hTSH-IC-2 from Dr. A. F. Parlow, Pituitary Hormones & Antisera Center, Harbor-UCLA Medical Center (Torrance, CA); rhTSH, expressed from stably transfected CHO cell line (clone P09) from Dr. G. Vassart, Genzyme Corp. (Framingham, MA). Cell culture media and reagents were purchased from Life Technologies, Inc. 125I-cAMP (specific activity, 40–60 μCi/μg) was from Hazleton (Vienna, VA); polymerase chain reaction reagents were from Boehringer Mannheim and New England Biotabs (Beverly, MA); neuraminidase attached to beaded agarose was from Sigma, immobilized Limax flavus agglutinin was from EY Laboratories (San Mateo, CA), and recombinant N-glycanase was from Genzyme Corp. CHO-K1, CHO-LEC2, CHO-LEC1, and 293 human embryonic kidney cells (293 cells) were obtained from ATCC (Rockville, MD). CHO-LEC2 and CHO-LEC1 cells were deposited there by Dr. P. Stanley, Albert Einstein College of Medicine, New York, NY (20, 21, 23).

Site-directed Mutagenesis—Mutagenesis of the full-length human hTSH α cDNA in the bacteriophage M13 mp18 was performed using M13-based site-directed mutagenesis as described previously (8). After subcloning the wild type and mutant α constructs into pAXNeOExa, the resulting constructs were confirmed by DNA sequencing. Mutagenesis of the hTSH β subunit (24) was accomplished by the polymerase chain reaction-based megaprimmer method (25). Briefly, a primer encompassing the desired mutation (AAC to CAA, TSH to Gln) was used to amplify a 196-base pair 5′ fragment of the gene, and this fragment (the megaprimmer) was used in a second polymerase chain reaction together with a 3′ primer to amplify the entire gene. After subcloning into the plBCMV expression vector (24), the entire polymerase chain reaction product was sequenced to verify the mutation and to rule out any undesired polymerase errors.

Transient Expression—CHO-K1 cells were maintained as described (22), CHO-LEC cell lines 2 and 1 were grown in α-modified minimum essential medium supplemented with 5% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and glucose (4 mM). 293 cells were grown in Dulbecco’s minimum essential medium containing 10% fetal calf serum supplemented as above. Cells were transiently cotransfected in 60-mm culture dishes with wild type or mutant pAXNeα/bXia, wild type or mutant pLCMVβ/βa/gene, or pcDNAI/neohCGi using a liposome formulation (Lipofectamine reagent, Life Technologies, Inc.) in a protocol modified according to the manufacturer’s instructions (22). After culture in CHO-serum-free medium (Life Technologies, Inc.) for 48 h, supernatants, including control medium from mock transfections using the expression plasmids without gene inserts, were harvested, concentrated with Centriprep 10 concentrators (Amicon, Beverly, MA), and stored at −20 °C to prevent neuraminidase digestion.

Immunassays—Wild type and mutant hTSHs were quantified using four different hTSH immunoassays. Two third-generation assays utilizing different monoclonal antibodies (Nichols Institute, San Juan Capistrano, CA, and ICN, Costa Mesa, CA) were used, following the manufacturers’ instructions. Further, wild type and mutant hTSHs were measured with two different polyclonal antibodies by radiomunoassay using polyclonal antibodies NIADDK-anti-hTSH-3 and NIDDK-anti-hTSH-IC-2 with rhTSH (Genzyme Corp.) (14) as the standard. Intracellular immunoreactivity in the cell lysates was determined after four freeze-thaw cycles on a methanol/dry ice mix and in a 37 °C water bath, respectively. Wild type and mutant hCG was measured with a specific third-generation immunoassay without cross-reactivity to other hormones (Nichols Institute).

Cell Filtration—Conditioned media were chromatographed on a Superdex 75-HPLC column (Pharmacia Biotech Inc.), and eluted at a flow rate of 0.3 ml/min in phosphate-buffered saline (pH 7.4), using hCG as an internal standard. hTSH and hCG immunoreactivities were monitored by immunoassay (Nichols Institute).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Conditioned media were concentrated, fractionated on ConA-Sepharose (Pharmacia), reconstituted, and denatured by boiling in 0.25% SDS, 0.5% β-mercaptoethanol. After digestion with N-glycanase, samples were resolved on 14% Tris/glycine polyacrylamide gels, transferred to nitrocellulose membranes, and incubated overnight with a rabbit antibody directed against the hTSH α subunit. Antibody-antibody complexes were subsequently visualized by chemiluminescence using a horseradish peroxidase-coupled anti-rabbit IgG and a luminol substrate (Boehringer Mannheim).

Enzymatic Deglycosylation—Concentrated conditioned media were incubated with 250 microunits of neuraminidase/10 mg of total protein in 100 mM sodium acetate, pH 5.0, for 12 h at room temperature, followed by 1 h at 37 °C. After separation of the agarse-beaded neuraminidase by spinning in a microcentrifuge, media were washed, concentrated, and reassedayed for hTSH immunoreactivity. Similarly, N-glycanase digestion was performed for 20 h at 37 °C with 12.5 units/10 mg of protein in 50 mM sodium phosphate, pH 7.6.

cAMP Production—Confluent CHO cells stably expressing the rhTSH receptor (P09) (26) and FRTL-5 cells expressing the endogenous rat TSH receptor (27) were incubated in 96-well tissue culture plates for 2 h at 37 °C, 5% CO2, with serial dilutions of wild type and mutant hTSH or hCG as well as control medium from mock transfections. The amount of cAMP released into the medium was assayed by radiomunoassay (22).

Growth Assay in FRTL-5 Cells—Cells were grown with wild type hTSH, hCG/β2TSH/β2, or concentrated mock medium for 48 h. 24 h after adding 1 μCi of [3H]thymidine (DuPont, Wilmington, DE), cells were washed and solubilized, and [3H]thymidine uptake was determined as described (16).

Radioimmunoassay of hTSH—The receptor-binding activity of wild type and mutant hTSHs was determined by their ability to displace 125I from a solubilized recombinant membrane hormone receptor preparation (Krus) as described previously (11).

In Vivo Bioassay—The in vivo bioactivity of various mutants was determined using a bioassay recently developed and characterized in our laboratory (28). Briefly, male albino swiss Crl:CF-1 mice were given 3 μg/ml of T4 (Sigma) in their drinking water for five to six days to suppress endogenous TSH secretion. Wild type and mutant hTSH as well as mock-concentrated medium at equal volumes was injected intraperitoneally, and blood samples for determination of T4 (T4, Kit, Nichols Institute) and hTSH values were obtained from the orbital sinus 6 h later.

Serum Disappearance Rate—The serum disappearance rate of wild type hTSH and selected mutants was determined in the rat by injecting 200–300 ng of the recombinant hormones intravenously and then measuring serum hTSH concentrations from 1–120 min. Experimental details of this procedure are given elsewhere (16).

RESULTS

hTSH bears three N-linked oligosaccharide chains at positions Asn52 and Asn78 of the α subunit and at Asn23 of the β subunit (1–3). To prevent the cotranslational attachment of individual oligosaccharides to the hTSH molecule, we disrupted the respective glycosylation recognition sequences NXT(T/S), widely considered to be an absolute requirement for glycosylation to occur. In the α subunit (individually and in a composite mutation) and in the TSH β subunit we mutated Asn
Role of Oligosaccharides for hTSH

Secreted and intracellular hTSH immunoreactivity

|                | Secreted ng/dish | Intracellular ng/dish | % total |
|----------------|------------------|-----------------------|--------|
| Wild type      | 228.5 ± 19.0     | 10.8 ± 0.6            | 95.5   |
| αQ52/TSHβ      | 86.6 ± 8.0       | 3.3 ± 0.3             | 96.3   |
| αQ78/TSHβ      | 48.2 ± 4.8       | 2.9 ± 0.3             | 94.3   |
| αTSH/Q23       | 21.5 ± 1.8       | 0.8 ± 0.1             | 96.4   |
| αQ52.Q78/TSHβ  | 0.68 ± 0.23      | 0.02 ± 0.01           | 97.1   |
| αQ52/TSHβQ23   | 5.0 ± 1.1        | 0.25 ± 0.06           | 95.2   |
| αQ78/TSHβQ23   | 5.2 ± 0.9        | 0.19 ± 0.7            | 96.4   |
| αQ52.Q78/TSHβQ23 | <0.01       | <0.01                | ND     |

hTSH Expression—By cotransfected wild type and mutant human α and TSHβ subunits in various combinations into CHO-K1 cells, we generated the following recombinant proteins: αQ52/TSHβ, αQ78/TSHβ, αQ52.Q78/TSHβ, αTSHβQ23, αQ52/TSHβQ23, and αQ78/TSHβQ23. Elimination of the carbohydrate at a single site reduced expression 2.5-10-fold compared with wild type, and deletion of more than one site decreased levels even further (Table I). The concomitant decreases of intracellular and secreted immunoreactivity suggest that these reductions were not due to intracellular retention of the hTSH mutants. In fact, >90% of total hTSH immunoreactivity was detected in the medium in all cases, indicating efficient secretion of the mutants. This is in accord with the proposed role of the oligosaccharide chains in early cotranslational events in promoting proper folding and maintaining the intracellular stability of TSH (4, 29). Interestingly, we could not detect any significant production of completely deglycosylated hTSH (αQ52.Q78/TSHβQ23). This is in agreement with previous findings on hFSH (8) and emphasizes the essential role of carbohydrates in the biosynthesis of the glycoprotein hormones. Similar reductions of the mutants tested were also observed in 293, CHO-LEC2, and CHO-LEC1 cells (data not shown), indicating that these reductions were independent of differences in the overall carbohydrate pattern.

In Vitro Bioactivity: CAMP Induction—Next, we assessed the ability of wild type and mutant hTSH expressed in CHO-K1 cells that produce highly sialylated carbohydrate chains (15, 16) to stimulate cAMP production in CHO cells stably transfected with the rhTSH receptor, JP09 (26). Whereas the maximal CAMP stimulatory activity of the mutants was unchanged, there was a highly significant (p < 0.001) 6-fold, 2-fold, and 3-fold lower EC50 of mutants αQ52/TSHβ, αQ78/TSHβ, and αTSHβQ23 compared with wild type (Fig. 2, Table II). Further, αQ52/TSHβ was significantly more active than αQ78/TSHβ and αTSHβQ23 (p < 0.001). Due to the dramatically reduced expression levels, it was not possible to obtain full dose-response levels for mutants lacking more than one carbohydrate chain. However, there was no significant CAMP stimulation of mutant αQ52.Q78/TSHβ at 0.5 ng/ml, whereas wild type hTSH at the same concentration induced CAMP 3-fold over baseline levels, indicating that the presence of at least one oligosaccharide of the α subunit is required to activate the TSH receptor. This is in accord with previous findings on pituitary bovine TSH, which suggested that enzymatic deglycosylation of the α subunit led to a decrease of bioactivity (11). At 2 ng/ml, αQ52/TSHβQ23 was twice as active as wild type hTSH (p = 0.02, n = 3), whereas there was no difference between wild type hTSH and αQ78/TSHβQ23.

In FRTL-5 cells expressing endogenous rat TSH receptor (27), we observed, relative to wild type (EC50 = 12.6 ± 2.1
ng/ml), a similar 6-fold decrease in the EC50 of αQ52/TSHβ
(EC50 = 2.0 ± 0.7 ng/ml, p < 0.001) (Fig. 3).

We next expressed mutants αQ52/TSHβ, αQ78/TSHβ, and α/TSHβQ23 in 293 cells. In contrast to CHO-K1 cells, 293 cells express N-acetylgalactosaminyl-transfase and GalNAcβ1,4GlcNAcβ1,2Manα4-sulfotransferase and produce oligosaccharides terminating in >70% in sulfated N-linked carbohydrate moieties (19). Since we previously found that 293 cells produce small amounts of free α subunit (29), we initially cotransfected the hTSHβ minigene alone to assess whether significant amounts of wild type hTSH would be produced. This, however, was not the case, indicating that significant contamination of the mutants with wild type hTSH did not occur. Wild type hTSH, expressed in 293 cells had increased cAMP inducing activity, evidenced by a 5.6-fold left shift in the EC50 compared with wild type expressed in CHO-K1 cells (Fig. 4, Table II) (p < 0.001). However, in relation to the increased wild type activity, there was no difference in activity between wild type and the selectively deglycosylated hTSH mutants (Fig. 4, Table II).

To further understand which carbohydrate components may be associated with the increase of bioactivity upon expressing the hTSH mutants in CHO-K1 cells, we also transiently transfected CHO-LEC2 and CHO-LEC1 cells. These are CHO glycosylation mutant cell lines selected from the wild type CHO-K1 cell line for resistance to toxic plant lectins (23). CHO-LEC2 cells have a defect in the CMP-sialic acid translocation into the Golgi resulting in the synthesis of glycoproteins with a > 90%

Fig. 2. cAMP induction in CHO cells expressing the rhTSH receptor (J P09) by the hTSH mutants produced in CHO-K1 cells. Increasing concentrations of wild type or mutant hTSH were incubated with J P09 cells, and the cAMP concentration in the resulting supernatants was assayed by radioimmunoassay. The amount of cAMP released from the cells in the presence of concentrated medium from mock-transfected cells was not different from base-line levels (buffer only). A representative experiment, repeated at least twice, is shown. Values from triplicate determinations are depicted as mean ± S.E.

TABLE II

| Cell type     | CHO-K1  | 293   | CHO-LEC 2 | CHO-LEC 1 |
|---------------|---------|-------|-----------|-----------|
|               | EC50 (ng/ml) | (n)   | EC50 (ng/ml) | (n)   | EC50 (ng/ml) | (n)   | EC50 (ng/ml) | (n)   |
| Wild type     | 5.6 ± 1.0 | (8)   | 1.0 ± 0.3 | (4)   | 0.7 ± 0.3 | (3)   | 0.8 ± 0.5 | (3)   |
| αQ52/TSHβ     | 0.9 ± 0.2* | (6)   | 0.7 ± 0.4 | (4)   | 0.5 ± 0.4 | (3)   | ND         |        |
| αQ78/TSHβ     | 2.6 ± 0.5* | (4)   | 0.7 ± 0.2 | (3)   | 0.6 ± 0.5 | (3)   | ND         |        |
| α/TSHβQ23     | 2.2 ± 0.4* | (5)   | 0.8 ± 0.4 | (3)   | 0.7 ± 0.2 | (3)   | ND         |        |
with recent findings of Yoshimura et al. (31), who, using purified hCG in the same system, showed that 50 μg/ml was required for significant cAMP production. In contrast, there was a dose-dependent significant stimulation with α52/hCG in the same concentration range, whereas deletion of the oligosaccharide at Asn78, tested in a concentration up to 6 μg/ml, did not increase the thyrotropic activity of hCG.

In Vivo Bioactivity—Injection of wild type hTSH, α52/TSHβ, and α78/TSHβ from CHO-K1 cells into T3-suppressed mice led to a dose-dependent stimulation of T4 production that was significant compared with mock at 100 ng/mouse (p < 0.05) (Fig. 10). Wild type hTSH was significantly (p < 0.05) more potent than either α52/TSHβ or α78/TSHβ. Determination of hTSH levels in the serum of these mice indicated that, compared with wild type hTSH, the relative amounts of injected α52/TSHβ and α78/TSHβ remaining were decreased by 60–70%.

Serum Disappearance Rate—To further establish whether site-specific deletion of the α-linked oligosaccharides may lead to a reduced circulatory half-life, we tested the serum disappearance rate of these mutants from CHO-K1 cells after intravenous injection into rats. Whereas the serum disappearance rate of α52/TSHβ was slightly increased (p = 0.057), α78/TSHβ was cleared significantly faster than wild type hTSH (p < 0.05) (Fig. 11). These differences were not observed in the first 15 min after intravenous injection but only after this initial phase, in keeping with previous findings that the carbo-
hydrates are not important for the initial phase of tissue
distribution but are involved in hepatorenal clearance
mechanisms (16, 17).

DISCUSSION

The glycoprotein hormones hTSH, hCG, hLH, and hFSH
share a common signal transduction pathway involving inter-
action with a hormone-specific G protein-coupled receptor and
the generation of cAMP as the initial event in the signal trans-
duction cascade (3, 4). Previous studies on hCG (7) and hFSH
(8, 9) had indicated a site-specific requirement of the oligosac-
charide at Asn\(^{52}\) for their \(\text{in vitro}\) activity, but similar studies
on hTSH and the more closely related hLH have not been
performed. Moreover, previous studies using recombinant DNA
techniques to study oligosaccharides in glycoprotein hormones
had been limited to using either site-directed mutagenesis of
glycosylation attachment sites or glycosylation mutant cell
lines to synthesize these hormones. Therefore, in a novel ap-
proach to study the role of carbohydrates for hTSH, we com-
bined site-specific deletion of individual oligosaccharides with

![Fig. 7. Inhibition of \(^{125}\text{I}\)-bTSH binding by wild type and \(\alpha\)Q52/TSH\(\beta\) expressed in CHO-K1, CHO-LEC2, and 293 cells. Increasing
doses of wild type or mutant hTSH were incubated with porcine mem-
branes in the presence of a constant amount of \(^{125}\text{I}\)-bTSH. \(^{125}\text{I}\)-bTSH
bound to membranes was precipitated and quantitated in a \(\gamma\) counter,
and radioactivity precipitated in the presence of concentrated medium
from mock transfections was defined as 100%. Values are shown as
mean \(\pm\) S.E. of triplicate determinations. Experiments were repeated
two times. Binding of wild type and \(\alpha\)Q52/TSH\(\beta\) expressed in CHO-
LEC1 was not different from that expressed in CHO-LEC2 cells.]

![Fig. 8. Inhibition of \(^{125}\text{I}\)-bTSH binding by the hTSH mutants expressed in CHO-K1 cells. The binding assay was performed as
described in the legend to Fig. 7. Values of a representative experiment,
performed at least three times, are shown as mean \(\pm\) S.E. of triplicate
determinations.]

![Fig. 9. Thyrotropic activity of CHO-K1-expressed, site-specifically \(\alpha\)-deglycosylated hCG in J PO9 cells. For experimental
details, see the legend to Fig. 2 and “Experimental Procedures.” Values of
a representative experiment, performed at least three times, are de-
picted as mean \(\pm\) S.E. of triplicate determinations.]

![Fig. 10. In vivo activity of site-specifically hTSH mutants from
CHO-K1 cells in T\(_3\)-suppressed mice. T\(_4\) values of nonsuppressed
mice ranged from 6 to 8 \(\mu\)g/ml. Mock injection (n = 3) did not signifi-
cantly increase T\(_4\) values of suppressed mice (control, n = 3). For each
concentration, 100, 200, and 400 ng, a total number of five mice were
injected intraperitoneally with wild type as well as with each hTSH
mutant. After 6 h, blood was obtained from the orbital sinus for T\(_4\)
determinations. Data were compiled from individual injections. *, \(p <
0.05\) compared with an equal dose of wild type. The expression of recombinant hormones in a variety of cell
lines that differentially process the carbohydrate moieties.

In this study we provide evidence for a different role of the
oligosaccharides for hTSH bioactivity, distinct from their role
for hCG and hFSH. Site-specific disruption of each of the three
individual carbohydrate attachment sites led to an increase in
the \(\text{in vitro}\) activity of hTSH expressed in CHO-K1 cells pro-
ducing sialylated carbohydrates (14–16). Our findings using a
sialic acid-binding lectin (30) as well as enzymatic desialylation
show that the hTSH transiently expressed in CHO-K1 cells
was indeed sialylated. Further, its in vitro bioactivity as well as
its circulatory half-life was indistinguishable from that of
rhTSH (Genzyme Corp.),\(^2\) which contains 1.8–2.2 sialic acid
residues/chain (14, 16). Interestingly, this increase of \(\text{in vitro}\) activity upon site-specific deglycosylation was most pro-
nounced upon deletion of the carbohydrate at Asn\(^{52}\). In con-
trast, this Asn\(^{52}\) oligosaccharide had previously been shown to
be essential for receptor stimulation of hCG and hFSH, which
were also expressed in CHO-K1 cells (7–9). The increase of

\(^2\) M. Grossmann, M. W. Szkudlinski, J. E. Tropea, and B. D. Weintraub, unpublished observations.
Fig. 11. Serum disappearance rate of hTSH mutants from CHO-K1 cells in male rats. After bolus injection of 200–300 ng of wild type or mutant hTSH into the femoral vein, blood for hTSH determinations was obtained over 120 min at equal time points. An IRMA without cross-reactivity to rat TSH (Nichols Institute), was used. Immunoreactivity was expressed as percentage remaining, and serum concentration at 0 min was defined as 100%. Wild type, n = 4; αQ52/TSHβ, n = 4; αQ78/TSHβ, n = 3. Data were compiled from individual experiments.

cAMP-inducing activity in our study occurred to a similar degree with the recombinant hTSH receptor and the endogenous rat receptor, indicating that these effects were neither system-dependent nor species-specific. Furthermore, in addition to the immediate induction of cAMP synthesis, site-specific deglycosylation of hTSH also affected long-term effects, including DNA synthesis by and growth of target cells in a similar fashion.

Wild type hTSH expressed in 293, CHO-LEC2, or CHO-LEC1 cells had a higher in vitro activity than wild type from CHO-K1 cells, and this increased activity was not further augmented by site-specific deglycosylation. CHO-LEC mutant cell lines lack a defined glycosylation activity and accumulate specifically truncated carbohydrates typical of intermediates in the biosynthetic pathway (23). In contrast to CHO-K1 cells, which produce terminally sialylated oligosaccharides, Golgi vesicle membranes from CHO-LEC2 cells translocate CMP-sialic acid at only 2% of the rate of vesicles from CHO-K1 cells and thus produce carbohydrates with a greater than 90% decrease in sialic acid content (20). CHO-LEC1 cells lack N-acetylgalactosaminyl-transferase I and hence terminate synthesis by and growth of target cells in a similar fashion.

The carbohydrate moieties at Asn

*degree than those at the Asn

*report (33) showed that the activity of wild type hCG expressed in these CHO glycosylation mutant cells was lower compared with hCG from CHO-K1 cells. The same group reported that the in vitro activity of hFSH did not change upon expression in these cell lines (33). These studies, however, did not assess the effects of site-specific deglycosylation on the activity of hCG or hFSH expressed in these cells. Taken together, these findings indicate that the terminal sialic acid residues can differentially modulate glycoprotein hormone activity in a hormone-dependent manner.

Our study has further demonstrated a unique role of the carbohydrate at Asn

Specific deglycosylation of rhTSH and recombinant bovine LH (15) had previously been reported that the weak thyrotropic activity of hCG (for review, see Ref. 39), which has recently been linked to a direct interaction with the rhTSH receptor (33, 40, 41), increased upon desialylation (42), in contrast to a decrease at its native receptor (18). When we tested the thyrotropic activity of CHO-K1-expressed hCG site-specifically deglycosylated at the α subunit, we observed an increased activity of αQ52/hCG but not of αQ78/hCG. This leads to the conclusion that the deletion of the oligosaccharide at Asn−52 can have opposite effects on hCG activity, depending on the receptor with which the hormone interacts. Therefore, these findings point to the intriguing possibility that the observed differences in the role of individual oligosaccharides for glycoprotein hormone action may be related, at least in part, to differences in glycoprotein hormone receptor structure and/or to receptor-dependent differences in receptor-ligand interaction.

The carbohydrate moieties are known to be important for the circulatory half-life and hence the in vivo bioactivity of the glycoprotein hormones (for review, see Ref. 43). However, the role of individual oligosaccharides for the in vivo activity of hTSH has not, to our knowledge, been investigated previously. Since the availability of a hTSH superagonist may have potential clinical applications (44), we were interested to determine whether the increased in vitro activity of sialylated αQ52/TSHβ from CHO-K1 cells could be maintained in vivo, despite the known protective effect of terminal sialic acid for the plasma half-life of rhTSH (16). However, we found a significant decrease in the in vivo activity of αQ52/TSHβ at the highest dose tested, as well as a slightly increased serum disappearance rate. By comparison, the significantly greater relative loss in the in vivo activity of the mutant αQ78/TSHβ was correlated with a significant increase in its serum disappearance rate. This significantly greater decrease in circulatory half-life upon
deletion of the oligosaccharide at Asn
1. Pierce, J. G., and Parsons, T. F. (1981) Annu. Rev. Biochem. 50, 465–495
2. Green, E. D., and Baenziger, J. U. (1988) J. Biol. Chem. 263, 25–35
3. Thotakura, N. R., and Bilié, D. L. (1995) Glycobiology 5, 3–10
4. Magner, J. A. (1990) Endocr. Rev. 11, 345–385
5. Kalyan, N. K., and Bilié, O. P. (1983) J. Biol. Chem. 258, 67–71
6. Sairam, M. R., and Bhargavi, G. N. (1985) Science 223, 65–67
7. Matzuk, M. M., Keene, J. L., and Boime, I. (1988) J. Biol. Chem. 263, 2409–2414
8. Bishop, L. A., Robertson, D. M., Cahir, N., and Schofield, P. R. (1994) Mol. Endocrinol. 8, 722–731
9. Flack, M. R., Froehlich, J., Bennet, A. P., Anasti, J., and Nisula, B. C. (1994) J. Biol. Chem. 269, 14015–14020
10. Thotakura, N. R., Kubota, K., Tramontano, D., Ingbar, S. H., and Keutmann, H. T. (1987) Endocrinology 120, 345–352
11. Thotakura, N. R., Desai, R. K., Szkudlinski, M. W., and Weintraub, B. D. (1992) Endocrinology 131, 82–88
12. Berman, M. I., Thomas, C. G., Manjunath, P., Sairam, M. R., and Nayfeh, S. N. (1985) Biochem. Biophys. Res. Commun. 133, 680–687
13. Nissim, M., Lee, K.-O., Pettick, P. A., Dahlberg, P. A., and Weintraub, B. D. (1989) Endocrinology 129, 1298–1305
14. Cole, E. S., Lee, K., Lauziere, K., Kelton, C., Chappell, S., Weintraub, B. D., Ferrara, D., Peterson, P., Bernasconi, R., Edmonds, T., Richards, S., Dickrell, L., Kleemann, J. J., McPherson, J. M., and Pratt, B. M. (1993) Bio/Technology 11, 1014–1024
15. Smith, P. L., Kaezeli, D., Nilsson, J., and Baenziger, J. U. (1990) J. Biol. Chem. 265, 874–881
16. Szkudlinski, M. W., Thotakura, N. R., Bucci, I., Joshi, L. R., Tsai, A., East-Palmer, J., Shiloach, J., and Weintraub, B. D. (1993) Endocrinology 133, 1490–1503
17. Thotakura, N. R., Szkudlinski, M. W., and Weintraub, B. D. (1994) Glycobiology 4, 525–533
18. Moviey, W. R., Bahl, O. P., and Marz, L. (1975) J. Biol. Chem. 250, 9163–9169
19. Smith, P. L., Skelton, T. P., Fiete, D., Dharmesh, S. M., Beranek, M. A., MacPhail, L., Broze, G. J. J., and Baenziger, J. U. (1992) J. Biol. Chem. 267, 19140–19146
20. Deutcher, S. L., Nuywayk, N., Stanley, P., Briles, E. E. B., and Hirschberg, C. (1984) Cell 39, 295–299
21. Stanley, P., Narasimhan, S., Simovitch, L., and Schachter, H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3423–3327
22. Grossmann, M., Szkudlinski, V., Nigro, M., Kraiem, Z. J., I. Tropea, E. J. H. T., and Weintraub, B. D. (1995) Mol. Endocrinol. 9, 948–958
23. Stanley, P. (1992) Glycobiology 2, 99–107
24. Joshi, L. R., Murata, Y., Wondisford, F. E., Szkudlinski, M. W., Desai, R., and Weintraub, B. D. (1995) Endocrinology 136, 8339–8348
25. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
26. Costagliola, S., Swillens, S., Niccoli, P., Dumont, J. E., Vassart, G., and Goldt, G. (1992) J. Cell Biochem. 49, 1540–1544
27. Ambs-Impiomato, F. S., Parks, L. A. M., and Coon, H. G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3455–3459
28. East-Palmer, J., Szkudlinski, M. W., Lee, J., Thotakura, N. R., and Weintraub, B. D. (1995) Thyroid 5, 55–59
29. Lash, R. W., Desai, R. K., Zimmermann, C. A., Flack, M. R., Yoshida, T., Wondisford, F. E., and Weintraub, B. D. (1992) J. Clin. Endocrinol. Invest. 15, 225–233
30. Miller, R. L., Collawn, J. F., Jr., and Fish, W. W. (1982) J. Biol. Chem. 257, 7574–7580
31. Yoshimura, N., Hershman, J. D., Pang, X.-P., Berg, L., and Pekary, E. (1993) J. Clin. Endocrinol. Metab. 77, 1009–1013
32. Keene, J. L., Matzuk, M. M., and Boime, I. (1989) Mol. Endocrinol. 3, 2011–2017
33. Galway, A. B., Husew, A. J. W., Keene, J. L., Yamato, M., Fauser, B. C. J. M., and Boime, I. (1990) Endocrinology 127, 93–100
34. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosenblit, N., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (1989) Science 245, 494–499
35. Ji, J., and Ji, T. H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 4396–4400
36. Lapthorn, A. J., Harris, D. C., Littiejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, H. W., Saad, N. W. (1994) Nature 369, 455–463
37. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure 2, 545–558
38. Strickland, T. W., and Putt, D. (1982) Endocrinology 111, 95–100
39. Mann, K., and Hoermann, R. (1993) J. Clin. Endocrinol. Invest. 16, 378–384
40. Toner, Y. H., Huber, G. K., and Davies, T. F. (1992) J. Clin. Endocrinol. Metab. 74, 1477–1479
41. Hoermann, R., Broecker, M., Grossmann, M., Mann, K., and Derwael, M. (1994) J. Clin. Endocrinol. Metab. 78, 933–938
42. Hoermann, R., Keutmann, H. T., and Amirim, A. M. (1991) Endocrinology 128, 1129–1135
43. Dickman, K. (1991) Cell 67, 1029–1032
44. Meier, C. A., Braaverman L. E., Ebner, S. A., Veronikis, I., Daniels, G. H., Ross, D. S., Derasa, D. J., Davies, T. F., Valentine, M., DeGroot, L. J., Curran, P., McCullin, K., Reynolds, J., Robbins, J., and Weintraub, B. D. (1994) J. Clin. Endocrinol. Metab. 78, 188–196
45. Bishop, L. A., Nguyen, T. V., and Schofield, P. R. (1995) Endocrinology 136, 2635–2640
46. Fares, F. A., Suganuma, N., Nishimori, K., LaPolt, P. S., Husew, A. J. W., and Boime, I. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4304–4308
47. Stockell Hartree, A., and Renwick, A. C. G. (1992) Biochem. J. 287, 665–679
Expression of Human Thyrotropin in Cell Lines with Different Glycosylation Patterns Combined with Mutagenesis of Specific Glycosylation Sites: CHARACTERIZATION OF A NOVEL ROLE FOR THE OLIGOSACCHARIDES IN THE IN VITRO AND IN VIVO BIOACTIVITY

Mathis Grossmann, Mariusz W. Szkudlinski, Joseph E. Tropea, Leonora A. Bishop, N. Rao Thotakura, Peter R. Schofield and Bruce D. Weintraub

J. Biol. Chem. 1995, 270:29378-29385.
doi: 10.1074/jbc.270.49.29378

Access the most updated version of this article at http://www.jbc.org/content/270/49/29378

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 14 of which can be accessed free at http://www.jbc.org/content/270/49/29378.full.html#ref-list-1