Role of Asparagine-linked Oligosaccharides in Protein Folding, Membrane Targeting, and Thyrotropin and Autoantibody Binding of the Human Thyrotropin Receptor*

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The amino-terminal ectodomain of thyrotropin (TSH) receptor (TSHR) is heavily glycosylated with asparagine-linked (N-linked) oligosaccharides. The present studies were designed to evaluate how acquisition and processing of N-linked oligosaccharides play a role in the functional maturation of human TSHR. A glycosylation inhibitor tunicamycin, which inhibits the first step of N-linked glycosylation (acquisition of N-linked oligosaccharides), and a series of mutant Chinese hamster ovary (CHO)-Lec cells defective in the different steps of glycosylation processing were used. Inhibition of acquisition of N-linked oligosaccharides by tunicamycin treatment in CHO cells stably expressing TSHR produced nonglycosylated TSHR, which was totally nonfunctional. In contrast, all of the TSHRs synthesized in mutant CHO-Lec, 2, and 8 cells (mannose-rich, sialic acid-deficient, and galactose-deficient oligosaccharides, respectively) bound TSH and produced cAMP and produced compared with those in TSHR expressed in parental CHO cells (CHO-TSHR; sialylated oligosaccharides). However, Lec1-TSHR and Lec2-TSHR were not efficiently expressed on the cell surface, whereas the expression levels of Lec8-TSHR and CHO-TSHR were essentially identical. All of the TSHRs expressed in CHO-Lec cells cleaved into two subunits. Finally, anti-TSHR autoantibodies from Graves’ patients interacted with all of the TSHRs harboring different oligosaccharides to a similar extent. These data demonstrate that acquisition and processing of N-linked oligosaccharides of TSHR appear to be essential for correct folding in the endoplasmic reticulum and for cell surface targeting in the Golgi apparatus. We also show that complex type carbohydrates are not crucially involved in the interaction of TSHR with TSH and anti-TSHR autoantibodies.

The receptors for glycoprotein hormones (thyrotropin receptor (TSHR), lutropin receptor (LHR), and follitropin receptor (FSH)) are members of a subfamily of G protein-coupled receptors, characterized by an extremely large amino-terminal extracellular domain, which is the high affinity binding site for the respective ligands (1, 2) and is, in the case of TSHR, the primary autoantigen in autoimmune thyroid disease, including Graves’ disease (1). Like many other secreted and cell surface proteins, these receptors undergo a series of posttranslational modifications such as disulfide bonding (1), glycosylation (3–9), palmitoylation (10, 11), and proteolytic cleavage (subunit formation in TSHR) (12–14), many of which appear to play roles in protein maturation and/or intracellular trafficking.

In human TSHR, the receptor ectodomain has asparagine-linked (N-linked) oligosaccharides, which represent 30–40% of its molecular weight (15). Earlier studies with in vitro site-directed mutagenesis suggest that, among six potential N-linked glycosylation sites, the first (amino acid 77) and third (amino acid 113) glycosylation sites are essential for the cell surface expression and function of the full-length TSHR (16). The possibility, however, cannot be excluded in this paper that the amino acid substitutions introduced per se affected receptor function (9). Subsequent studies on the functional role of N-linked oligosaccharides of TSHR have been performed with the truncated form of TSHR ectodomain expressed in mammalian cells and insect cells, yielding controversial data (15, 17, 18). However, it is difficult to interpret these data, because conformational integrity of TSHR protein has not been verified in these studies. Conformationally intact TSHR protein showing high affinity TSH binding has so far been expressed only in mammalian cells as the full-length receptor or the ectodomain fused to a membrane-anchoring peptide (19–21).

The process of N-linked glycosylation of proteins has been well documented (see Fig. 1) (22). Briefly, a dolichol pyrophosphate precursor (Glc3Man9GlcNAc2) is at first transferred to Asn side chain of Asn-X-Ser/Thr consensus sequence for N-linked oligosaccharides in a nascent polypeptide in the endoplasmic reticulum. Processing is initiated by the removal of the three terminal glucose residues and at least one mannose residue in the endoplasmic reticulum, followed by transportation to Golgi apparatus, where mannose residues are further trimmed, and N-acetylgalactosamine, galactose, and sialic acid residues are sequentially added. The newly synthesized glycoproteins then exit the Golgi and are transported to their final destination.

In the present studies, to further clarify the functional roles of N-linked oligosaccharides in human TSHR, we evaluated the functional properties of nonglycosylated TSHR and TSHRs with different types of N-linked oligosaccharides. Nonglycosylated TSHR was produced by treatment of TSHR expressed in...
Chinese hamster ovary (CHO) cells with tunicamycin, and TSHRs with different oligosaccharides were expressed in a series of mutant CHO-Lec cell lines harboring mutations in the distinct steps in carbohydrate processing (see Fig. 1) (29).

**MATERIALS AND METHODS**

**CHO Cell Lines**—Mutant CHO cell lines, CHO-Lec1, 2, and 8, originally established by Dr. Pamela Stanley (23), were obtained from the American Type Culture Collection (Rockville, MD). CHO-Lec1 cells (ATCC CRL-1735) have no detectable N-acetylglucosaminyl-transferase I activity, and proteins expressed carry oligosaccharides bearing mannose-rich intermediates (Man, GlcNAc) at sites normally occupied by complex carbohydrates (Sia, Gal, GlcNAc, Man, GlcNAc) in parental cell line CHO cells (Fig. 1). CHO-Lec2 (ATCC CRL-1736) and -Lec8 (ATCC CRL-1737) cells lack CMP-sialic acid and UDP-galactose transferases and are incapable of transporting CMP-sialic acid and UDP-galactose from the cytosol to the Golgi, thereby producing sialic acid-deficient (Gal, GlcNAc, Man, GlcNAc) and sialic acid- and galactose-deficient (GlcNAc, Man, GlcNAc) oligosaccharides, respectively. These cells were maintained at 5% CO₂ in minimal essential medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Parental CHO cells were grown in Ham’s F-12 medium with 5% fetal calf serum and antibiotics described above.

**Transfection and Cell Culture**—The human full-length TSHR cDNA (24) was ligated into the expression vector pCAGGS (25) to yield pCAG-TSHR. The plasmid was transfected into the cells described above with Lipofectin reagent (Life Technologies, Inc.). The cells were selected with 500 µg/ml G418 (Geneticin; WAKO, Osaka, Japan) and pooled. Surviving clones (12 clones for CHO cells and 24 for CHO-Lec cells) were also isolated with cloning cylinders, and the clonal cell lines expressing the highest levels of the receptor, determined by [125I]TSH binding (see below), were selected.

**Tunicamycin Treatment**—The cells were treated with 5 µg/ml tunicamycin (WAKO) for 3 days. The medium was replaced daily to purge the cells of wild-type TSHR. Tunicamycin inhibits transfer of dolichyl phosphate precursor to Asn in the consensus sequence for N-linked glycosylation (Asn-X-Ser/Thr) (4). Immunoprecipitation of TSHR—Metabolic labeling with [35S]methionine/cysteine (Trans-35S Label; ICN, Irvine, CA), immunoprecipitation of TSHR with mouse anti-TSHR monoclonal antibodies A10 and A11 (26), deglycosylation of TSHR with endoglycosidase H (endo H) and N-glycosidase F, and SDS-polyacrylamide gel electrophoresis were performed as described previously (4, 11, 27). Signals were obtained with a Fujix Bioimaging Analyser BAS-5000 (Fujix, Tokyo, Japan).

**Immunoblot analysis of TSHR—Extraction of the crude cell membrane and immunoblotting were performed as described previously (28) with mouse anti-TSHR monoclonal antibodies A10 and A11 (26).**

**RESULTS**

**Immunoprecipitation and Immunoblot of TSHR in the Clonal Cell Lines Stably Expressing TSHR**—Using clonal cell lines stably expressing the highest levels of receptors, we determined the molecular masses and glycosylation patterns of TSHR expressed in CHO cells treated with and without tunicamycin and in a series of CHO-Lec cells. In immunoprecipitation experiments (2-h pulse followed by 2-h chase), TSHR protein expressed in parental CHO cells (CHO-TSHR) was visualized as an ~95-kDa precursor and an ~120-kDa mature protein (Fig. 2A, lane 1) as previously reported (27). Both decreased in size to ~85 kDa on N-glycosidase F treatment (Fig. 2A, lane 3), a size compatible with that of the core polypeptide chain of TSHR (24) and also identical to that of nonglycosylated TSHR (Fig. 2A, compare with lane 4; see below). The ~95-kDa protein was sensitive to endo H, indicating that this has high mannose type oligosaccharides, whereas the ~120-kDa species possesses complex type oligosaccharides because of its resistance to endo H (Fig. 2A, lane 2).

Tunicamycin treatment completely inhibited synthesis of oligosaccharides to TSHR (Fig. 2A, lanes 4–6). Thus, TSHR was detected as an ~85-kDa single species in CHO cells treated with 5 µg/ml tunicamycin.

With respect to mutant cell lines, CHO-Lec1 cells produced a TSHR that migrated as an ~90-kDa band, slightly smaller than a TSHR with high mannose oligosaccharides (Fig. 2A, compare lanes 1 and 7) and was sensitive to endo H (Fig. 2A, lane 8). This finding is consistent with the fact that proteins synthesized in CHO-Lec1 cells have oligosaccharides of mannose-rich intermediates (Man, GlcNAc), and high mannose type oligosaccharides in the ~95-kDa TSHR in parental CHO cells are presumably Man₇,₇GlcNAc₂,

CHO-Lec8 cells pro...
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Fig. 2. Immunoprecipitation and immunoblot of TSHR expressed in CHO and CHO-Lec cells. A, the cells were metabolically labeled with [35S]methionine/cysteine (2-h pulse and 2-h chase) followed by immunoprecipitation of TSHR with anti-TSHR monoclonal antibodies A10 and A11 (see Materials and Methods). Precipitated samples were left untreated (lanes 1, 4, 7, 10, and 13) or treated with endo H (lanes 2, 5, 8, 11, and 14) or N-glycosidase F (lanes 3, 6, 9, 12, and 15) and subjected to 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions and autoradiography. B, crude membrane preparations (50 μg) of the cells were subjected to 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions. After transfer to a membrane, proteins were probed with A10 and A11. Signals were developed with streptavidin-horseradish peroxidase and N,N-diaminobenzidine/H2O2.

Induced a TSHR migrating as a doublet with molecular masses of ~90 and ~95 kDa (Fig. 2A, lane 10); the ~90-kDa species is likely a TSHR with galactose- and sialic acid-deficient oligosaccharides (GlcNAc2Man5GlcNAc2) because of its resistance to endo H (Fig. 2A, lane 11). In CHO-Lec2 cells, TSHR was visualized as a doublet of ~105 and ~95 kDa (Fig. 2A, lane 13). The ~105-kDa species is likely a TSHR with sialic acid-deficient oligosaccharides (GlcNAc2Man5GlcNAc2).

Although it is well known that TSHR cleaves into two subunits (12–14), TSHR subunits were detectable only after longer chase and N-glycosidase F digestion in our immunoprecipitation (27). Therefore, the subunit collections of TSHRs with different oligosaccharides were analyzed on immunoblotting.

On immunoblotting, the single polypeptide of TSHR, for example, the ~95- and ~120-kDa species in immunoprecipitation of CHO-TSHR, were not visualized. Instead, several nonspecific reactions were observed between 70 and 120 kDa (Fig. 2B). However, the diffuse “A subunit” with the mean molecular mass of ~55 kDa could be clearly detected in CHO-TSHR. The majority of TSHRs expressed on the cell surface probably cleave into two subunits at the steady state levels. A similar diffuse band, slightly smaller than that in CHO-TSHR, was also present in Lec2-TSHR (Fig. 2B, lane 5). The diffuse banding pattern of TSHR A subunit in CHO and CHO-Lec2 cells may be attributable to different degrees of glycosylation of the core polypeptides. These oligosaccharides may contain various outer chain sequences including different sizes of polylactosamine (22). In contrast, TSHR A subunit was observed as a doublet in Lec1-TSHR and particularly in Lec8-TSHR (Fig. 2B, lanes 3 and 4), in which oligosaccharides must be homogeneous. This finding agrees with our recent hypothesis of “two cleavage sites in TSHR” (27). Thus, these data suggest that all of the TSHRs expressed in CHO-Lec cells can cleave into two subunits. Cleavage seems to occur at the cell surface and to be mediated by matrix metalloprotease(s) (31). No cleaved product was observed in tunicamycin-treated, nonglycosylated TSHR (Fig. 2B, lane 2), suggesting that nonglycosylated TSHR may not be expressed on the cell surface.

These data are in agreement with the expected consequences of the mutations represented by the cell lines used.

Effect of Tunicamycin on Expression and Function of TSHR Stably Expressed in CHO Cells—Pooled clones of CHO cells stably expressing TSHR were treated with 5 μg/ml tunicamycin for 3 days and subjected to [125I]TSH binding and TSH-induced cAMP production experiments. As shown in Fig. 2A, 5 μg/ml tunicamycin completely inhibited transfer of dolichol precursor to Asn in the nascent TSHR. Although effect of 3-day treatment with tunicamycin should be evaluated with immunoblot, not immunoprecipitation, immunoblot showed no specific signal as mentioned above. However, this period of treatment has been previously demonstrated to be enough to purge the cells of wild-type recombinant LHR (9). As shown in Fig. 3A, [125I]TSH binding determined with intact cells was of high affinity in CHO-TSH. Untransfected CHO cells also showed nonspecific TSH binding of low affinity. This specific, high affinity TSH binding was largely abolished in CHO-TSHR treated with tunicamycin. Consistent with TSH binding data, little or no TSH-induced cAMP production was observed in tunicamycin-treated CHO-TSH (Fig. 3C). In addition, as shown in Fig. 3B, the solubilized extract from tunicamycin-treated CHO-TSHR also showed no TSH binding, although the solubilized extract from CHO-TSHR cultured in the absence of tunicamycin demonstrated significantly higher TSH binding than untransfected CHO cells. Thus, together with the immunoblot data, nonglycosylated TSHR seems to accumulate intracellularly in an unfolded form, thereby suggesting an essential role for acquisition of oligosaccharides in correct folding and membrane expression of TSHR.
was very similar in all of the cells (Fig. 5 and Table I), thereby suggesting that the apparently lower binding affinity for TSH in CHO-TSHR appears to be related to its higher expression levels as previously reported (32). Therefore, we interpret these data as that TSH binding is likely of high affinity in TSHR expressed in all of the cells.

These data indicate that Lec8-TSHR is correctly folded and fully expressed on the cell surface, and that Lec1-TSHR and Lec2-TSHR accomplish conformational maturation but are trapped intracellularly. However, some of these receptors can reach the cell surface and normally transduce a signal.

**DISCUSSION**

In the present studies, using a glycosylation inhibitor, tunicamycin, and a series of mutant CHO-Lec cells defective in the different steps of glycosylation process, we evaluated how acquisition and modification of oligosaccharides affect the functional maturation of TSHR.

The ability of TSHR to interact with anti-TSHR autoantibodies was examined. In the TBIAb assay shown in Fig. 6A, two Graves' IgG, one with potent TBIAb activity (Graves 1) and the other with moderate activity (Graves 2) in CHO-TSHR, comparably inhibited [¹²⁵I]TSH binding in all of the CHO-Lec cells. TSH activity in two Graves' sera (Fig. 6B, Graves 3 and Graves 4) was also similar in all of the cells. Therefore, receptor recognition by autoantibodies does not involve complex type oligosaccharides in TSHR.

**TABLE I**

| Receptor number/cell | Kᵦ (nM) | EC₅₀ (nM) |
|----------------------|---------|---------|
| CHO                  | 110,400b | 0.85    | 2.0     |
| CHO-Lec1             | 98,700   | 0.50    | 1.9     |
| CHO-Lec8             | 58,000   | 0.32    | 3.8     |
| CHO-Lec2             | 51,800   | 0.24    | 3.4     |

a Determined by Scatchard analysis.
b The mean of two separate experiments, each experiment determined in duplicate.
The medium was determined. The data are mean ± S.E. (n = 4) of two separate experiments determined in duplicate.

FIG. 6. TSAb and TBIAb activities in clonal cell lines of CHO and CHO-Lec cells stably expressing TSHR. A, the solubilized extracts from 100 μg of crude membranes were incubated with [125I]TSH (~15,000 cpm) and a mixture of normal sera or two Graves' sera (Graves 1 and Graves 2) for 1 h at 37 °C, precipitated with PEG, and counted as described under "Materials and Methods." B, the cells were stimulated with 10−8 M bovine TSH, crude IgG fractions from a mixture of normal sera or two Graves' sera (Graves 3 and Graves 4) for 2 h at 37 °C. cAMP released into the medium was determined. The data are mean ± S.E. (n = 4) of two separate experiments determined in duplicate.

treatment is not folded correctly. In contrast, high affinity TSH binding to Lecl-TSHR clearly indicates that TSHR with mannose-rich intermediates (Man₆GlcNAc)₆ is conformationally intact. Therefore, the acquisition of N-linked oligosaccharides appears to allow the nascent, highly unfolded TSHR protein to accomplish conformational maturation in the endoplasmic reticulum. Several roles of addition of N-linked oligosaccharides to nascent proteins have been demonstrated; for example, N-linked oligosaccharides attach to lectin-like molecular chaperons such as calnexin and calreticulin, facilitating correct protein folding, and also play a role in the "quality control" system of the endoplasmic reticulum that ensures selective transporta-tion of the properly folded proteins for the Golgi complex (33).

Therefore, unfolded proteins such as nonglycosylated TSHR mentioned above may be trapped in the endoplasmic reticulum. Impaired cell surface expression in Lecl-TSHR suggests that processing of N-linked oligosaccharides from high mannose type to complex type in the Golgi apparatus appears to participate in cell surface targeting of TSHR. However, efficient cell surface expression of TSHR is not necessarily dependent on completion of complex oligosaccharides, because TSHR with sialic acid- and galactose-deficient oligosaccharides (Lecl-TSHR) is efficiently expressed on the cell surface. Contrarily, TSHR with sialic acid-deficient oligosaccharides (Lecl2-TSHR) is not efficiently expressed on the cell surface. We cannot explain these data at present, although oligosaccharides terminating in galactose residues may somehow interfere with cell surface targeting of TSHR in the Golgi apparatus. Alternatively, lack of sialylation in the trans-Golgi network may affect a role of the trans-Golgi network as an important sorting site for different proteins destined for the apical or basalateral surfaces of cell membranes (34). For any reason, these data are in agreement with the facts that N-linked oligosaccharides generally play critical roles in protein folding and intracellular trafficking in the endoplasmic reticulum and the Golgi apparatus, respectively (33, 35). However, our data on TSHR may not be generalized to other membrane proteins, because (i) the membrane receptor for Newcastle disease virus is reported not to be properly expressed in mouse mammary carcinoma Had-1 cells deficient in UDP-galactose transporter like Lecl8 cells (36), and (ii) the expression levels of rat LHR were decreased by 30–40% of that in CHO cells in all of the CHO-Lec cells.

Our data also indicate that complex type oligosaccharides do not appear to constitute TSH or autoantibody binding sites or to participate in signal transduction, although glycosylation is reported to be related to function in some proteins (37–39). It has recently been reported that the truncated type of TSHR ectodomain, which is not secreted, possesses high mannose type carbohydrates and is incapable of binding to TSH or anti-TSHR autoantibodies in CHO cells (15). However, whether this lack of binding was related to polypeptide misfolding or to the type of oligosaccharides on the protein was unknown in this study. From our results, it is now clear that misfolding is the primary event. Thus, TSHR ectodomain by itself may not be able to accomplish correct folding, thereby being trapped in the endoplasmic reticulum and unable to acquire complex oligosaccharides. Recent studies also suggest that only the TSHR ectodomain fused to a membrane-anchored peptide seems to be correctly folded (19–21).

A nonessential role of galactose and sialic acid in function of TSHR shown in Leclg cells is reminiscent of our previous finding (4, 40) of high affinity TSH binding to TSHR expressed in 293 human embryonal kidney cells. These cells express N-acetylgalactosaminy transferase and N-acetylgalactosaminy-N-acetylglucosaminyl-N-sulfotransferase and produce N-linked oligosaccharides terminating in sulfate in >70% of the chains (41). Of interest is that some glycoproteins synthesized in the thyroid gland have been reported to be sulfated (42, 43). TSHR expressed in the thyroid gland may also be, at least in part, sulfated. Furthermore, N-linked oligosaccharides in bovine pituitary TSH that we used in the present studies are exclusively sulfated, whereas human pituitary TSH terminates both in sulfation and sialylation (44). Biological activity of sulfated TSH has been reported to be more potent than sialylated TSH (45). The carbohydrates, especially the terminal sialic acids, of TSH, have also been demonstrated to attenuate its biological activity (45, 46). In this regard, it will be interesting to compare the relative potency of TSH with different types of oligosaccharides to bind and stimulate TSHR with various oligosaccharides.

In FSH receptor, although removal of N-linked oligosaccharide from the wild-type receptor does not affect FSH binding (5, 7), the deglycosylated receptor produced by in vitro mutagenesis or tunicamycin treatment shows no FSH binding activity (7). Thus, the N-linked oligosaccharides seem to be necessary for the proper folding rather than hormone binding in FSH receptor. However, the role of N-linked oligosaccharides in LHR is controversial; although high affinity human chorionic gonadotropin binding to LHR does not appear to involve the carbohydrates on LHR, it remains unclear whether the oligo-
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saccharides are necessary for the correct folding of the nascent LHR (6, 8, 9).

In conclusion, our data show that acquisition and processing of N-linked oligosaccharides in TSHR are essential for correct folding in the endoplasmic reticulum and for intracellular trafficking in the Golgi apparatus. However, binding of TSH and anti-TSH autoantibodies to TSHR does not appear to involve the oligosaccharides of the receptor.

Acknowledgments—We thank Dr. J. P. Banga (Kings College School of Medicine, London, UK) for providing mouse anti-TSHR monoclonal antibodies and Dr. J. Miyazaki for the expression vector pCAGGS. We are also grateful to Dr. B. Rapoport (Cedars-Sinai Medical Center, Los Angeles, CA) for critical review of the manuscript.

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J. Biol. Chem. 1998, 273:33423-33428.
doi: 10.1074/jbc.273.50.33423

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