Role of complex asparagine-linked oligosaccharides in the expression of a functional thyrotropin receptor

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To evaluate the functional role of complex asparagine-linked oligosaccharides of the human thyrotropin receptor (TSHR), a Chinese hamster ovary cell line (JP09) and a K562 cell line (K562-TSHR) expressing this receptor were treated with deoxymannojirimycin (dMM), a mannosidase I inhibitor. dMM blocks the formation of complex-type structures and leads to the formation of high-mannose-type structures. Treatment of cells with dMM led to a decrease in the number of thyrotropin (TSH)-binding sites at the cell surface. Detection of the TSHR at the cell surface using a monoclonal antibody directed against the A subunit showed that this decrease was not due to a decrease in the number of TSHRs expressed at the cell surface. However the recognition of TSHR by a monoclonal antibody directed against the C peptide was greatly decreased. On immunoblotting, after deglycosylation using peptide N-glycanase F, the A subunit was

visualized as a doublet (36 and 41 kDa). In control cells the species of higher molecular mass was more abundant whereas after dMM treatment the species of lower molecular mass became more abundant. This difference in molecular mass between the two peptides is compatible with the removal of the C peptide. In conclusion, the results show that inhibition of complex-type structure formation leads to (i) an incapacity for TSHR to bind TSH, without affecting its intracellular transport and (ii) an increase of TSHR susceptibility to proteases that remove the C peptide. We then hypothesized that removal of the C peptide could contribute to the formation of a non-functional TSHR.

Key words: deoxymannojirimycin, N-glycan, intracellular transport, TSH receptor, tunicamycin.

INTRODUCTION

The biological action of thyrotropin (TSH) is mediated by its interaction with a specific receptor localized at the basolateral membrane of the thyroid cells. The TSH receptor (TSHR) has been studied extensively (for review see [1,2]). Cloning of the human TSHR revealed a single reading frame coding for 764 amino acids. It has a large extracellular domain and seven transmembrane segments, a characteristic of G-protein-coupled receptors (see Figure 1). After synthesis and intracellular transport, the receptor is cleaved at the cell surface, and the structure of the TSHR then consists of an extracellular subunit (A) and a membrane-spanning subunit (B) [3,4]. This intracellular cleavage does not occur at a single site but can occur at two different sites, called sites 1 and 2. A peptide segment (peptide C) can also be removed, either by a cleavage at the two different sites [5] or by being progressively clipped out by a series of cleavage reactions from the B subunit after cleavage at site 1 [6,7]. The human TSHR has six potential N-glycosylation sites. Mutation analysis of these six sites showed that glycosylation at Asn-77 and Asn-113 is important for high-affinity TSH binding and cAMP response [8]. In this work the authors did not determine whether the decrease in binding activity resulted from a loss of receptor at the cell surface and/or from a decreased affinity. On the other hand the expression of the human TSHR extracellular domain in insect cells leads to the production of receptors that are not

Figure 1 Schematic representation of a TSHR with glycosylation sites (▲), and regions of cleavage sites 1 and 2

properly glycosylated and that have a low affinity for TSH [9–11]. Nagayama et al. [12] investigated the role of the N-glycans of the TSHR and concluded that (i) inhibition of

Abbreviations used: TSH, thyrotropin; TSHR, TSH receptor; Tu, tunicamycin; dMM, deoxymannojirimycin; TBS, Tris-buffered saline; PNGase F, peptide N-glycanase F; CHO, Chinese hamster ovary; mAb, monoclonal antibody.

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N-glycosylation by tunicamycin (Tu) leads to synthesis of a non-functional receptor which is unable to reach the cell surface, and (ii) in cells unable to synthesize complex-type structures (Lec 1) or sialylated complex-type structures (Lec 2), TSHR was not efficiently expressed at the cell surface. However in cells synthesizing asialo-agalacto-complex-type structures (Lec 8) TSHR was normally expressed at the cell surface.

In the present paper we re-investigate the role of complex-type structures in TSHR behaviour and show that TSHRs bearing high-mannose-type structures are transported to the cell surface, where they are more accessible to proteolytic cleavage than TSHRs bearing complex-type structures, and are unable to bind TSH.

EXPERIMENTAL

Materials

JP09, a Chinese hamster ovary (CHO) cell line expressing the TSHR, and the K562-TSHR cell line have been described previously [13,14]. Highly purified bovine TSH (iodination grade) was from UCB (Brussels, Belgium), and bovine TSH used for displacement in Scatchard analysis was from Sigma (St Louis, MO, U.S.A.). Monoclonal antibodies (mAbs) BA8 [14], A10 [15] and 3G4 [16] have been described previously. Secondary antibodies coupled to FITC were from Calbiochem (La Jolla, CA, U.S.A.). PVDF membranes (Immobilon P) were from Millipore (Milford, MA, U.S.A.). Deoximannojirimycin (dMM), Tu and peptide N-glycanase F (PNGase F) were from Roche Molecular Biochemical.

TSH-binding assays

Highly purified TSH was radio-iodinated to a specific activity of $\approx 90 \mu\text{Ci}/\mu\text{g}$ by the chloramine-T method [17] followed by Sephadex G-100 chromatography. Cells in 24-well plates (JP09) or 0.25×10^6 cells in suspension (K562-TSHR) were incubated for 2 h at 36°C with 30000 c.p.m. of ^{125}I -TSH and increasing concentrations of unlabelled TSH (10^{-11} to 10^{-6} M) in 0.25 ml of modified Hanks buffer, without NaCl and supplemented with 0.25 % BSA and 280 mM sucrose. At the end of the incubation times the cells were rinsed rapidly three times with 0.5 ml of modified Hanks buffer, and then solubilized in 0.5 ml of 1 M NaOH. Radioactivity was measured in a gamma counter. The binding affinity and the maximal binding capacity were calculated with the Ligand program [18].

Immunoblotting of TSHR

After centrifugation (200 g, 5 min) the K562-TSHR cells were resuspended in 1 ml of 10 mM Tris/HCl supplemented with protease inhibitors (Complete, Roche Molecular Biochemical), and cells were disrupted using a Potter-Elvehjem homogenizer. The suspension was centrifuged for 10 min at 125 g, the pellet was discarded, and the supernatant was centrifuged for 10 min at 10000 g. The membrane pellet was resuspended in Laemmli sample buffer [19] with or without 2-mercaptoethanol and heated for 30 min at 45°C . The samples (corresponding to 2×10^6 cells) were run on 7.5 % PAGE gels. After Western blotting on a PVDF membrane, non-specific sites were blocked with 3 % non-fat milk powder in Tris-buffered saline (TBS) containing 0.1 % Tween 20. Membranes were incubated for 2 h at room temperature with mAb A10 (1:1000 dilution) in TBS supplemented with 0.1 % Tween 20 and 0.3 % non-fat milk powder. After being washed, the membranes were incubated for 2 h at room tem-

perature with gold-conjugated goat anti-mouse IgG (British BioCell, Cardiff, U.K.) in TBS with 0.1 % Tween 20 and 0.3 % non-fat milk powder. After four washes in the same medium without IgG and two washes with TBS the signal was developed using silver enhancing kit (British BioCell).

Enzymic deglycosylation

We adopted the following methods after doing assays to optimize TSHR deglycosylation. The membrane pellet was prepared as described for immunoblotting then resuspended in the buffer used for incubation with PNGase F (50 mM Tris/HCl, pH 8.5/1 % Triton X-100/0.1 % SDS/1.6 % 2-mercaptoethanol). The samples were incubated for 16 h at 37°C with 1 unit of PNGase F. Controls were treated using the same conditions as the corresponding assays, except that the PNGase F was omitted. The samples were then prepared for analysis by SDS/PAGE in denaturing conditions.

Pronase and drug treatments

K562-TSHR cells were preincubated for 8 h at 37°C with and without cycloheximide (30 $\mu\text{g}/\text{ml}$), Tu (30 $\mu\text{g}/\text{ml}$) or dMM (400 $\mu\text{g}/\text{ml}$). The cells were then centrifuged (200 g, 7 min), washed once with PBS, and resuspended in a Pronase solution (protease type XIV, 5 $\mu\text{g}/\text{ml}$) for 5 min at 37°C . Protease-inhibitor cocktail (40 μl) was then added. In some cases after centrifugation (200 g, 7 min), the cells were resuspended in RPMI 1640 medium supplemented with 10 % fetal bovine serum, with and without cycloheximide, Tu or dMM, and incubated again for 16 h at 37°C .

Fluorescence analysis

K562-TSHR cells ($\approx 1 \times 10^6$) were washed twice in PBS, resuspended in 1 ml of PBS containing 0.1 % BSA, and cooled for 5 min at 4°C . After centrifugation (200 g, 7 min) cells were incubated with antibodies diluted (1:25 for BA8 and 3G4) in PBS supplemented with 0.1 % BSA for 1 h at 4°C . Then cells were washed four times in PBS/0.1 % BSA and incubated for 45 min at 4°C under gentle agitation with FITC-labelled anti-mouse IgG diluted 1:50 in 30 μl of PBS/0.1 % BSA. After being washed with PBS the cells were resuspended in 100 μl of PBS and analysed on a fluorescence microplate reader (Fluostar, Toucan, France).

RESULTS

Effect of dMM on TSH binding

To study the role of complex asparagine-linked oligosaccharides in the expression of a constitutively active form of the TSHR, we used two cell lines expressing this receptor: a CHO cell line (JP09) and a K562 cell line (K562-TSHR) [13,14]. We tested the capacity of these cells to bind TSH in the absence or presence of a drug acting on complex-type structure formation, dMM, which inhibits mannosidase I and leads to the formation of high-mannose-type structures instead of complex-type structures [20]. After treatment of JP09 cells or K562-TSHR cells with dMM (400 $\mu\text{g}/\text{ml}$) for 72 h the B_{max} values obtained were decreased by 55 and 43 % respectively; in contrast, the K_d values were unchanged in both cases (Figure 2 and Table 1). Inhibition of complex-type structure formation then led to impairment of TSH binding at the cell-surface level.

Figure 2 Competition binding assays of ^{125}I -TSH on JP09 (A) or K562-TSHR (B) cells treated for 16 h with (●) or without (○) dMM

Cells in 24-well plates (JP09) or 0.25×10^6 cells in suspension (K562-TSHR) were incubated with trace concentrations of ^{125}I -TSH and increasing concentrations of unlabelled bovine TSH (bTSH) in 0.25 ml of modified Hanks buffer. After a 2 h incubation at room temperature the cells were rinsed rapidly three times and then the cells were solubilized in 0.5 ml of 1 M NaOH. The radioactivity was measured in a gamma counter. Results are means $\pm$ S.D. from three different experiments.

The importance of glycosylation for trafficking of TSHRs to the cell surface

The decrease in the number of TSH-binding sites at the cell surface in the presence of dMM may reflect the absence of TSHRs on the cell surface, resulting from an inhibition of TSHR trafficking, or it may reflect the presence of a TSHR unable to bind TSH. To determine which was involved, we checked the presence on the cell surface of TSHRs using anti-TSHR antibodies. For these studies the cell line expressing the greater number of TSHRs (K562-TSHR) and two different antibodies directed against TSHRs were used.

In mammalian cells the rate of acquisition of complex-type structures (and then of trafficking to the cell surface) of TSHR is relatively low [21]. In order to obtain better results in this study we had to eliminate most of the pre-existing receptors bearing complex-type structures. To achieve this we preincubated cells for

Table 1 Dissociation constants (K_d), receptor density (B_{max}) and numbers of TSHRs/cell in JP09 and K562 cells treated with dMM

Results are means $\pm$ S.D. from three different experiments.

Treatment	K_d (nM)	B_{max} (fmol/ 10^6 cells)	No. of TSHRs/cell
JP09 cells	4.0 ± 0.7	148 ± 24	87000
JP09 cells treated with dMM	6.7 ± 1	66 ± 10	39000
K562-TSHR cells	1.6 ± 0.3	590 ± 88	355000
K562-TSHR cells treated with dMM	2.3 ± 0.3	340 ± 41	205000

Figure 3 Immunoblotting of the TSHR with mAb A10

Lane A, K562-TSHR cells; lane B, K562-TSHR cells treated with Pronase (5 $\mu\text{g}/\text{ml}$); lane C, K562-TSHR cells treated with Pronase and then cultured for 16 h in a standard medium.

8 h with dMM, and the residual receptors bearing complex-type structures at the cell surface were then eliminated by Pronase treatment (see the Experimental section). For this treatment, preliminary experiments were conducted to determine the experimental conditions leading to a cell surface devoid of TSHRs without affecting cell viability and impermeability to erythrosine. The results of this treatment were verified by immunoblotting using mAb A10 (Figure 3). As expected (see [1,4,21]) in the control, cells expressed TSHRs bearing high-mannose-type structures (97 kDa band) and complex-type structures (115 kDa band; Figure 3, lane A). Treatments of the cell extracts with endoglycosidase H and PNGase F confirmed this assumption (results not shown).

Treatment of the cells with Pronase led to disappearance of the TSHRs bearing complex-type structures (Figure 3, lane B). After this treatment, if cells were cultured for 16 h in a standard medium, TSHR-bearing complex-type structures were again apparent (Figure 3, lane C). In order to control the effect of dMM, cells were preincubated for 8 h with or without dMM, treated with Pronase to eliminate TSHR from the cell surface, and incubated further for 16 h with dMM. Upon performing the immunoblotting procedure after dMM treatment, only TSHRs bearing high-mannose-type structures were recovered (Figure 4, lane C), whereas in the control cells, TSHRs were found to bear high-mannose-type and complex-type structures (Figure 4, lane B). This indicates that the action of the drug was complete. This was confirmed by performing deglycosylation using endoglycosidase H (results not shown). On the other hand, we checked the level of TSHR synthesis in culture with dMM. For this purpose, cells were incubated for 8 h with or without dMM and then radiolabelled for 1 h with $[^{35}\text{S}]\text{Met}$. After cell extraction, TSHR was immunoprecipitated with mAb A10 and analysed by

Figure 4 Immunoblotting of TSHR with mAb A10 after treatment with dMM

Lane A, K562-TSHR cells treated by Pronase (5 µg/ml); lane B, K562-TSHR cells treated by Pronase and then cultured for 16 h at 37 °C in a standard medium; lane C, K562-TSHR cells preincubated for 8 h at 37 °C with dMM (400 µg/ml), treated with Pronase and then incubated again for 16 h at 37 °C with dMM.

SDS/PAGE. The bands corresponding to the TSHR were quantified using a PhosphorImager. Under these conditions no difference in TSHR synthesis was observed between the control and dMM treated cells (results not shown).

In order to quantify the receptor present at the cell surface fluorescence analysis was carried out under the same conditions: preincubation with or without cycloheximide, Tu or dMM, Pronase treatment, and then a 16 h incubation with or without drugs. Under the conditions used here, cycloheximide and Tu inhibited the incorporation of [³⁵S]Met and [³H]mannose, respectively, by 90–95%. Two mAbs able to recognize the TSHR were used, BA8 and 3G4. mAb BA8 recognized a three-dimensional structure in the A subunit and mAb 3G4 recognized an epitope (VFEEQ, 336–341) localized in the C peptide. Negative controls were done using (i) non-transfected K562 cells with mAb BA8 or 3G4 and the secondary antibodies coupled to FITC, and (ii) K562-TSHR cells and the secondary antibodies coupled to FITC.

Antibodies BA8 and 3G4 showed a significant rate of binding to K562-TSHR as compared with the control non-transfected K562 cells and the K562-TSHR cells incubated with only the secondary antibodies (results not shown). These control values (5–15% of binding observed for K562 cells in presence of BA8 or 3G4 antibodies) were subtracted from the results obtained with mAbs and K562-TSHR cells. After Tu treatment, the fluorescence decreased significantly (54% with mAb BA8 and 86% with mAb 3G4), reflecting a great decrease in the density of TSHRs at the cell-surface level (Figure 5). Similar results were obtained with cycloheximide (Figure 5). It should be mentioned, however, that for some unknown reason the decrease obtained with the mAb BA8 after treatment with Tu and cycloheximide was not as large as that obtained with the mAb 3G4. In the presence of dMM, K562-TSHR cells reacted with the mAb BA8 in the same way as the control cells (Figure 5A), which indicated that the formation of complex-type structures was not essential to the intracellular trafficking of TSHRs to the cell surface; whereas in the case of mAb 3G4 a 37% decrease in the fluorescence was observed between the control cells and the dMM-treated cells (Figure 5B). This confirms that (i) Tu inhibits the traffic of TSHRs to the cell surface, (ii) inhibition of complex-type structure formation does not inhibit the traffic of TSHR (because of the recognition by mAb BA8) and (iii) in the presence of dMM the mAb 3G4 loses its capacity to recognize the TSHR. The difference between the mAb BA8 and 3G4 is that mAb BA8 recognized a sequence in the A subunit while 3G4 recognized a

Figure 5 Binding analysis of mAb BA8 (A) or mAb 3G4 (B) to K562-TSHR cells after treatment with Tu, cycloheximide or dMM

Cells were treated with Pronase and incubated with or without drug as described in Figure 4. K562-TSHR cells without drug (open bars); K562-TSHR cells treated with cycloheximide (stippled bars), Tu (black bars) or dMM (hatched bars). Control data obtained using K562-TSHR cells incubated with the secondary antibodies were similar to those obtained with K562 cells incubated with anti-TSHR antibodies and secondary antibodies (results not shown). Results are means $\pm$ S.D. from four different experiments.

Figure 6 Immunoblotting analysis of native and deglycosylated TSHRs

Treatment of cells was performed as described in Figure 4 and then cell extracts were incubated in the presence or absence of PNGase F. Analysis by immunoblotting was done after denaturation by SDS and with reduction by 2-mercaptoethanol. Lanes 1 and 3, control cells; lanes 2 and 4, dMM-treated cells; lanes 3 and 4, cell extracts treated with PNGase F. The experiment was repeated three times with similar results.

sequence in the C peptide. It is known that this part of the molecule can be removed by proteases [5,6].

One explanation of the results was that the TSHR bearing high-mannose-type structure was more sensitive to proteolytic

degradation at the cell surface. To verify this hypothesis cell extracts obtained from control cells or from dMM-treated cells were analysed after reduction by immunoblotting using mAb A10 (Figure 6). After deglycosylation with PNGase F, the A subunit appears in the control cells as a doublet (36 and 41 kDa) with considerable predominance of the higher-molecular-mass species, whereas after dMM treatment the band of lower molecular mass was much more intense. The difference in molecular mass between the two peptides is compatible with the removal of the C peptide and could explain the absence of recognition of the TSHR by the mAb 3G4.

DISCUSSION

Our aim was to elucidate the functional role of complex asparagine-linked oligosaccharide in relation to TSHR function. In a previous work Russo et al. [8] expressed, in CHO cells, recombinant TSHRs mutated at the six potential glycosylation sites and showed that mutations at Asn-77 and at Asn-113 led to a decrease in TSH binding at the cell surface. But they did not determine if this decrease resulted from a loss of receptor at the cell surface or from a lower affinity. More recently Nagayama et al. [12] using Tu and different mutant CHO cells defective in different steps of glycosylation processing concluded that (i) inhibition of N-glycosylation by Tu leads to blocking of intracellular trafficking of TSHR, and (ii) mutant CHO cells unable to form complex-type structures (Lec 1 cells) or complex-type structures bearing sialic acid (Lec 2 cells) do not efficiently express the TSHR on their cell surface.

In the present work we used Tu and dMM that respectively lead to the formation of non-glycosylated protein and high-mannose-type structure. We showed that inhibition of N-glycosylation by Tu leads to the absence of TSHRs at the cell surface. Glycosylation is then essential for its intracellular transport. Unglycosylated, the TSHR was probably improperly folded, and its transport blocked by the 'quality control' that prevents exit out of the endoplasmic reticulum, and the protein was then degraded by the ubiquitin proteasome pathway [22]. By contrast, dMM leads to a decrease in the number of TSH-binding sites expressed at the cell-surface, but fluorescence experiments using mAb BA8 showed that TSHRs bearing high-mannose-type structures were transported efficiently to the cell surface (Figure 5). This conclusion is the opposite of that reached by Nagayama et al. [12]. These authors used three mutant cell lines, Lec 1, Lec 2 and Lec 8 cells, which synthesized high-mannose-type structures, asialo-complex-type structures and asialo-agalacto-complex-type structures, respectively. In Lec 1 and Lec 2 the intracellular TSHRs were able to bind to TSH but this led to a great decrease in the TSH binding at the cell surface. The authors conclude that in these cells TSHRs were not expressed at the cell surface. Use of dMM is comparable with the result obtained with Lec 1 cells. Therefore, concerning the TSH binding, we agree with the results obtained by Nagayama et al. [12]. But comparison of their results with those we obtained using fluorescence analysis suggest that in Lec 1 and Lec 2 cells TSHRs were expressed at the cell surface but were unable to bind TSH.

The next question is: what are the modifications of the receptor at the cell surface leading to its inability to bind TSH? We showed that recognition by the mAb 3G4 is greatly decreased in the presence of dMM. This mAb recognizes an epitope localized in the C peptide, which can be removed by proteases [5,6]. Furthermore, immunoblotting of the deglycosylated A subunit showed a decrease in the molecular mass of the peptide chain when cells were treated with dMM. This strongly suggests a

higher susceptibility to proteases of the TSHR at the cell-surface. From these results we can conclude that formation of complex-type structures is not essential for cell-surface expression of the TSHR but that complex-type structures are essential to protect the TSHR from proteolytic degradation leading to removal of the C peptide. However, this conclusion is difficult to understand if site 1 is the first site of cleavage, because the peptide C should be linked to the B subunit and its removal could not be accounted for by a decrease in the molecular mass of the A subunit. The possibility that site 2 might sometimes be the first cleavage site has been suggested previously [2,23], and it was demonstrated recently that this cleavage process could lead to the formation of a 'big' A subunit [24]. However, if site 1 is the first cleavage site it is possible that the differences in the molecular masses of the A subunits may have been due to other proteolytic cleavage processes.

From our results it is tempting to assume that deletion of the C subunit leads to formation of a non-functional receptor. However it has been reported that a TSHR in which this region (residues 317–366) was deleted was able to bind TSH [25,26]. In these experiments, the TSHR did not undergo cleavage at site 1 but was able to be cleaved at site 2 [26]. In this case, the three-dimensional structure was perhaps slightly different from that of the native TSHR both before and after C peptide removal. Consequently it cannot be totally assumed that inhibition of TSHR binding is directly related to the C peptide release. On the other hand it is not known if a TSHR bearing high-mannose-type structures is able to bind TSH and it is difficult to answer this point. In a normal cell, the TSHRs bearing complex-type structures, which are present at the cell surface, co-exist with the TSHRs from the endoplasmic reticulum that bear high-mannose-type structures. In cells treated with dMM, TSHRs bear only high-mannose-type structures, but under these conditions, the receptor present at the cell surface is unable to bind to TSH. All these facts make it difficult to obtain a preparation with only TSHRs bearing high-mannose-type structures without any cleavage, which is required to be able to answer this question. In addition, other post-translational modifications might occur during the maturation of the receptor.

It can therefore be concluded from the results obtained by Nagayama et al. [12] and ourselves that (i) N-glycosylation of TSHRs is essential for the correct folding of TSHRs and then for its intracellular trafficking, (ii) formation of sialylated complex-type structures is not necessary for their intracellular transport but sialic acid certainly protects the receptor from proteolytic degradation leading to removal of the C peptide, and (iii) TSHRs bearing non-sialylated structures lose their capacity to bind TSH at the cell surface due to this proteolysis or some other modification.

S. S.-F. was supported by the Association pour le Développement des Recherches Biologiques et Médicales.

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Received 28 June 2000/7 November 2000; accepted 8 December 2000