Glycosylation of rat NGF receptor ectodomain in the yeast *Saccharomyces cerevisiae*

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Abstract Here we studied the glycosylation of a mammalian protein, the ectodomain of rat nerve growth factor receptor (NGFR), in *Saccharomyces cerevisiae*. NGFR is secreted to the culture medium of *S. cerevisiae* if it is fused to a polypeptide (hsp150Δ) carrier. The hsp150Δ-carrier has 95 serine and threonine residues, which are extensively O-glycosylated. In spite of 41 potential sites, NGFRβ lacked O-glycans, whether fused to the carrier or not. Distortion of the conformation of NGFR, by inhibition of disulfide formation did not promote O-glycosylation, whereas N-glycosylation was enhanced. Thus, the serine and threonine residues of the hsp150Δ-NGFR, fusion protein were highly selectively O-glycosylated.

Key words: Glycosylation; Yeast; Secretion; NGF receptor; Protein folding

1. Introduction

In mammalian cells, O-glycans are initiated in the Golgi by N-acetylgalactosamine, and vary in composition and length [1,2]. Since selected serines and threonines obtain glycans, O-glycosylation must be controlled by specific features of the folded molecules. In yeast, O-glycosylation begins in the endoplasmic reticulum (ER) by transfer of a single mannose residue from dolichol monophosphate to a serine or threonine residue, followed by chain elongation up to pentamannosides in the Golgi [3-7]. Since no consensus sequence for O-glycosylation has been identified, mannosylation of an unfolded polypeptide in the process of translocation across the ER membrane should be less selective than mannosylation of a folded molecule. We have recently shown that the extracellular domain of rat low-affinity nerve growth factor receptor (NGFR, [8]) folded to a ligand-binding conformation and was efficiently secreted to the culture medium of *Saccharomyces cerevisiae*, but only when fused to the C-terminus of the hsp150Δ-carrier [9], an N-terminal fragment of the natural secretory protein hsp150 [10-12]. Here we studied the glycosylation of the NGFRβ portion. Since the hsp150Δ-carrier is extensively O-glycosylated [11], the hsp150Δ-NGFRβ fusion protein provided us with a tool to study selectivity of O-glycosylation in yeast.

2. Materials and methods

2.1. Strains and media

Plasmid propagation was in *Escherichia coli* DH5α, grown in L-broth supplemented with 100 μg/ml of ampicillin. *S. cerevisiae* strains H25 (MATa his3Δ1 trp1Δ0 leu2Δ0, 112 rpl1α ade2Δ1 can1Δ100), H426 (MATa ade2Δ1 ura3Δ1 gal1Δ1, his3Δ1 trp1Δ0, 112 rpl1α ade2Δ1 can1Δ100), H855 strains H25 (MATa his3Δ1 trp1Δ0 ade2Δ1 can1Δ100), H426 (MATa ade2Δ1 ura3Δ1 gal1Δ1, his3Δ1 trp1Δ0, 112 rpl1α ade2Δ1 can1Δ100), and H451 (H25, LEU2::HIS3-10Δ6 Δ6, his3Δ1 trp1Δ0 ade2Δ1 can1Δ100) were grown at 24°C in YPD medium, and strains H845 (H25, pKT4H616) and H660 (MATa ser1Δ1 trp1Δ0, 112 his3Δ1 trp1Δ0, 112 ade2Δ1 can1Δ100) were grown in synthetic complete medium lacking tryptophan.

2.2. Plasmid construction

To construct a cleavage site between the hsp150Δ-carrier and NGFRβ, oligonucleotides 5'-GTACCTATGAGGTTAGGAG and 5'-GTACCTACCATCTTCGAG coding for the recognition site of factor Xa (IEGR) and containing Asp718-compatible ends were ligated to anneal, and were ligated to Asp718-digested pKT4H649 [9]. The new plasmid was named pKT4H616, and the construction verified by sequencing. The HSP150-NGFRβ fragment was detached from pKT4H616 as a 3.3 kb EcoRV-SpeI fragment, the SpeI site was filled in with Klenow polymerase, and the fragment was ligated to the Smal site of pFL26 [13]. Strain H25 was transformed with the resulting plasmid pKT4H612, creating H451.

2.3. Metabolic labeling and immunoprecipitation

Metabolic labeling of cells (2x10^7 cells/400 μl) was with 20 μCi of [35S]Met/Cys (1000 Ci/mmol), and with 100 μCi of [3H]mannose (18 Ci/ mmol) (Amersham, UK). For 35S-labeling, the cells were grown and labeled in synthetic complete medium lacking methionine and cysteine. For 3H-labeling, cell growth was in full (2%) glucose, and labeling was in 0.1% glucose. The expression of SUC-NGFRβ, was induced by 0.1% glucose due to the SUC promoter [9]. Lysis of cells and immunoprecipitations with the polyclonal rabbit antiserum against hsp150 (1:100), anti-NGFR (1:50; raised against the ligand-binding domain of authentic NGFR) and anti-hsp150-NGFRβ (1:50) were as described [14].

2.4. Other methods

Culture medium samples were concentrated using Centricon-30 (Amicon) devices, diluted in 50 mM Tris-HCl, pH 8.3, containing 0.9% NaCl (factor Xa buffer), and digested with factor Xa (Boehringer, Mannheim) at 24°C. Western analysis was performed as before [14]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was in 8% gels, if not otherwise stated. Trichloroacetic acid (14%) precipitation was for 1 h on ice. Cycloheximide, NaNO2, tunicamycin and dithiothreitol were from Sigma, and used at concentrations of 100 μg/ml, 10 mM, 20 μg/ml and 20 mM, respectively. Restriction endonucleases were from Promega, New England Biolabs and Boehringer Mannheim.

3. Results and discussion

3.1. Synthesis and secretion of hsp150Δ-fXαΔ-NGFRβ

The hsp150Δ-carrier has no potential N-glycosylation sites, whereas many of its 95 serines and threonines carry O-glycans containing 2–5 mannose residues [11]. The NGFRβ portion has 41 potential O-glycosylation sites and one N-glycosylation site. To study whether the NGFRβ portion was also O-glyco-
Fig. 1. (A) Hsp150α-ΔXα-NGFRe consists of the hsp150α-carrier (amino acids 1-321) and the whole extracellular domain of mature NGFRe (amino acids 328-550 of the fusion protein), joined by the factor Xα recognition site IEGR plus a GT-linker. The hsp150α-carrier consists of a signal peptide (amino acids 1-18), subunit I (amino acids 19-72) and subunit II, which consists mainly of 11 repeats of a homologous peptide (amino acids 73-321). (B) In hsp150α-NGFRe, the carrier and NGFRe, are joined directly to each other. (C) NGFRe (amino acids 25-246) is preceded by the 22 first amino acids of pre-invertase plus a GT linker. The cysteine residues (arrows) and the N-glycosylation site (N) are indicated.

3. Glycosylation of NGFRe

To study whether the NGFRe portion of hsp150α-ΔXα-NGFRe was O-glycosylated, strain H451 was labeled with [3H]mannose in the presence of TM. The secreted fusion protein was digested with factor Xα as above. SDS-PAGE analysis revealed a single product at the top of the separating gel (Fig. 3B, lanes 2, 3). This was the hsp150α-carrier, as we have shown before by synthesizing it from a truncated HSP150 gene [11]. The carrier barely penetrated an 8% gel, although the molecular mass of the polypeptide is 23.7 kDa. This was due to its extended structure and O-glycosylation [11]. Since

3.2. Release of NGFRe from the carrier

Hsp150α-ΔXα-NGFRe, [35S]Met/Cys-labeled in the presence of TM, was harvested from the culture medium of H451 cells and subjected to factor Xα digestion. The digestes were trichloroacetic acid-precipitated and analyzed in SDS-PAGE. Immunoprecipitation was omitted, since the fusion protein was the major radiolabeled protein in the medium (Fig. 3A, lane 1). With increasing digestion time and enzyme concentrations, products of 48 and 36 kDa appeared at the expense of the intact fusion protein (lanes 2-5). The 48 and 36 kDa products were recognized in Western analysis by anti-hsp150α-NGFRe antisera, but neither of them by anti-hsp150 antisera, whereas the intact fusion protein was recognized by both (not shown). The 48 kDa form most probably was the correct product, NGFRe, because it had the expected size [15]. Moreover, NGFRe expressed in the absence of the hsp150α-carrier by the aid of the invertase signal peptide also migrated like a 48 kDa protein (see below). Cleavage at the factor Xα site apparently made a secondary cleavage site in the NGFRe portion available, since hsp150α-NGFRe lacking the cleavage site (Fig. 1B) was not susceptible to factor Xα (not shown). The secondary cleavage apparently was at the cysteine-free C-terminus of the NGFRe portion, since the 36 kDa product appeared to contain a similar amount of 35S-radioactivity as the intact hsp150α-ΔXα-NGFRe fusion protein (compare lanes 1, 5 in Fig. 3A). Since all methionines, and 24 out of the 25 cysteine residues of the fusion protein were in the NGFRe portion, the released hsp150α-carrier was not visible in the fluorogram. Factor Xα cleavage of N-glycosylated hsp150α-ΔXα-NGFRe gave essentially similar results (not shown).

Fig. 2. Secretion of hsp150α-ΔXα-NGFRe, H451 cells were labeled with [35S]Met/Cys at for 1 h 24°C in the absence (lanes 1-4) or presence of TM (lanes 5-8). Culture medium samples (M) (lanes 1,2,5,6) and lysed cell samples (C) (lanes 3,4,7,8) were immunoprecipitated with anti-hsp150 (lanes 1,3,5,7) or anti-NGFRe antisera (lanes 2,4,6,8), and analyzed by SDS-PAGE and fluorography. Molecular weight markers (kDa) are on the left.
NGFR\(_{\alpha}\) apparently acquires a few O-glycans in mammalian cells, since O-glycanase digestion has been reported to reduce its apparent molecular weight by 2–3 kDa [16,17]. Little information is so far available on O-glycosylation of mammalian proteins in yeast. Selected sites of the Fc\(_{\varepsilon}\) receptor, parathyroid hormone, cell-adhesive lysosome and insulin-like growth factor, unoccupied in the authentic molecules, were O-glycosylated in yeast [18–21], whereas the same sites of granulocyte/macrophage colony-stimulating factor were O-glycosylated in yeast and mammalian cells [22].

### 3.4. Glycosylation of misfolded NGFR\(_{\alpha}\)

Next we studied whether the NGFR\(_{\alpha}\) portion of hsp150\(\alpha\)-NGFR\(_{\alpha}\) was susceptible for O-glycosylation when its conformation was distorted by preventing disulfide formation. We have shown before that treatment of yeast cells with the reducing agent dithiothreitol (DTT) inhibits disulfide formation of newly synthesized proteins leading to their ER retention, without affecting the secretion and glycosylation apparatus of *S. cerevisiae* [14]. Authentic NGFR\(_{\alpha}\) has up to 12 disulfide bonds, and yeast-derived hsp150\(\alpha\)-NGFR\(_{\alpha}\) is also disulfide-bonded [9]. Hsp150\(\alpha\)-NGFR\(_{\alpha}\) (H426) was labeled with \[^{35}\text{S]}\text{Met/Cys}\) in the presence of DTT, followed by chase in the absence of DTT but presence of CHX. The fusion protein molecules released from the DTT block could be immunoprecipitated from the culture medium, and migrated in SDS-PAGE more slowly (Fig. 5, lane 7, 210–260 kDa) than hsp150\(\alpha\)-NGFR\(_{\alpha}\) molecules synthesized and secreted normally in the absence of DTT (lane 5, 160–240 kDa). This suggests that the reduced molecules were more extensively glycosylated than the native ones.

![Diagram](image.png)

**Fig. 3.** Digestion of hsp150\(\alpha\)-DX\(_{\alpha}\)-NGFR\(_{\alpha}\) with factor Xa. (A) Strain H451 was preincubated with TM for 10 min, and labeled with \[^{35}\text{S]}\text{Met/Cys}\) in the presence of TM at 37°C for 60 min. In (B) the labeling was with \[^{3}H\text{mannose}\) for 90 min. The culture medium samples were subjected to factor Xa digests as indicated. The digests were trichloroacetic acid-precipitated and analyzed by SDS-PAGE (7.5–15% gel) and fluorography. The arrowheads between the panels indicate the hsp150\(\alpha\)-carrier, intact hsp150\(\alpha\)-DX\(_{\alpha}\)-NGFR\(_{\alpha}\) of 180 kDa, and the products of 46 and 38 kDa. The arrows indicate the border of the stacking and separating gels. Molecular weight markers are on the left.

**Fig. 4.** Incorporation of \[^{3}H\text{mannose}\) into NGFR\(_{\alpha}\). (A) Strain H487 (NGFR\(_{\alpha}\)) was labeled for 90 min at 37°C with \[^{35}\text{S]}\text{Met/Cys}\) (lanes 1,2), or with \[^{3}H\text{mannose}\) (lanes 3,4), in the absence (lanes 1,3) or presence of TM (lanes 2,4). (B) Strain H640 (NGFR\(_{\alpha}\)/sec18) was labeled, after a pre-incubation for 10 min at 37°C, with \[^{35}\text{S]}\text{Met/Cys}\) for 90 min at 37°C in the absence (lane 1) or presence of TM (lane 2), as in lanes 1,2 of panel (A). (C) Strain H538 (hsp150\(\alpha\)-NGFR\(_{\alpha}\)/sec18) was labeled with \[^{35}\text{S]}\text{Met/Cys}\) (lane 1) or \[^{3}H\text{mannose}\) (lane 2) as in lanes 1,3 of panel (A). The cells were lysed and immunoprecipitated with anti-hsp150\(\alpha\)-NGFR\(_{\alpha}\), (A,B) or anti-hsp150 antisem (C), and the proteins separated by SDS-PAGE (12% gel in panels A,B, and 8% gel in panel C) and visualized by fluorography. Molecular weight markers are on the left.
Glycosylation was increased on the reduced molecules, hsp150α-NGFR, was labeled with [35S]Met/Cys in the presence of DTT and TM. DTT was removed and the label chased with CHX in the presence of TM. In the absence of N-glycosylation, hsp150α-NGFR, released from the DTT block migrated in SDS-PAGE (Fig. 5, lane 3) like native hsp150α-NGFR, secreted in the absence of DTT (lane 1). This suggests that N-glycosylation, but not O-glycosylation, was more efficient on the reduced hsp150α-NGFR, molecules. We could not differentiate whether increased N-glycosylation was due to more frequent utilization of the single N-glycosylation site, or to enhanced elongation of the glycan in the Golgi. Increase in N-glycosylation of insufficiently used sites on misfolded tissue-type plasminogen activator has been detected in mammalian cells [23].

Clearly, glycosylation of serines and threonines is highly selective in *S. cerevisiae*. In case it occurs strictly co-translationally, it must be controlled by the amino acid sequence, whereas in the case of a post-translational event, other features of the folded molecule regulate it. The two portions of hsp150α-NGFR, are very different: the carrier appears not to adopt any regular secondary structure [11], whereas NGFRα is likely to fold to an elongated end-to-end assembly of four domains [24]. This would suggest post-translational, rather than co-translational O-glycosylation.

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**References**