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 ($NGFR_e$), in *Saccharomyces cerevisiae*. $NGFR_e$ 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 were extensively *O*-glycosylated. In spite of 41 potential sites, $NGFR_e$ lacked *O*-glycans, whether fused to the carrier or not. Distortion of the conformation of $NGFR_e$ by inhibition of disulfide formation did not promote *O*-glycosylation, whereas *N*-glycosylation was enhanced. Thus, the serine and threonine residues of the hsp150Δ-$NGFR_e$ 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 ($NGFR_e$) [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 $NGFR_e$ portion. Since the hsp150Δ-carrier is extensively *O*-glycosylated [11], the hsp150Δ-$NGFR_e$ fusion protein provided us with a tool to study selectivity of *O*-glycosylation in yeast.

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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 H23 (*Matα hsp150::URA3 his3-11,15 leu2-3, 112 trp1-1 ade2-1 can1-100*), H426 (*MATα ade2-101 suc2Δ9 gal2, hsp150::URA3 LEU2::HSP150Δ-NGFR$_e$*), H538 (*Matα sec18-1 trp1-289 hsp150::URA3 his$^-$ LEU2::HSP150Δ-NGFR$_e$*) and H451 (H23, *LEU2::HSP150ΔfX$_a$-NGFR$_e$*) were grown at 24°C in YPD medium, and strains H487 (H23, *[pKTH4616]*) and H640 (*Matα sec18-1 trp1-289 leu2-1,112 his$^-$ hsp150::URA3 [pKTH4616]*) [9] in synthetic complete medium lacking tryptophan.

2.2. Plasmid construction

To construct a cleavage site between the hsp150Δ-carrier and $NGFR_e$, oligonucleotides 5′-GTACCATCGAAGGTAGAG and 5′-GTACCTCTACCTTCGATG coding for the recognition site of factor X_a (IEGR), and containing Asp718-compatible ends, were allowed to anneal, and were ligated to Asp718-digested pKTH4594 [9]. The new plasmid was named pKTH4610, and the construction verified by sequencing. The *HSP150Δ-NGFR$_e$* fragment was detached from pKTH4610 as a 3.3 kb *Eco*RV-*Spe*I fragment, the *Spe*I site was filled in with Klenow polymerase, and the fragment was ligated to the *Sma*I site of pFL26 [13]. Strain H23 was transformed with the resulting plasmid pKTH4612, creating H451.

2.3. Metabolic labeling and immunoprecipitation

Metabolic labeling of cells (2×10^8 cells/400 μl) was with 20 μCi of [^{35}S]Met/Cys (1000 Ci/mmol), or with 100 μCi of [^{3}H]mannose (18 Ci/mmol) (Amersham, UK). For ^{35}S-labeling, the cells were grown and labeled in synthetic complete medium lacking methionine and cysteine. For ^{3}H-labeling, cell growth was in full (2%) glucose, and labeling was in 0.1% glucose. The expression of SUC-$NGFR_e$ was induced by 0.1% glucose due to the *SUC* promoter [9]. Lysis of cells and immunoprecipitations with the polyclonal rabbit antisera anti-hsp150 (1:100), anti-NGFR (1:50; raised against the ligand-binding domain of authentic NGFR) and anti-hsp150Δ-$NGFR_e$ (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 X_a buffer), and digested with factor X_a (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, NaN_3, 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-fX$_a$-NGFR$_e$

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 $NGFR_e$ portion has 41 potential *O*-glycosylation sites and one *N*-glycosylation site. To study whether the $NGFR_e$ portion was also *O*-glyco-

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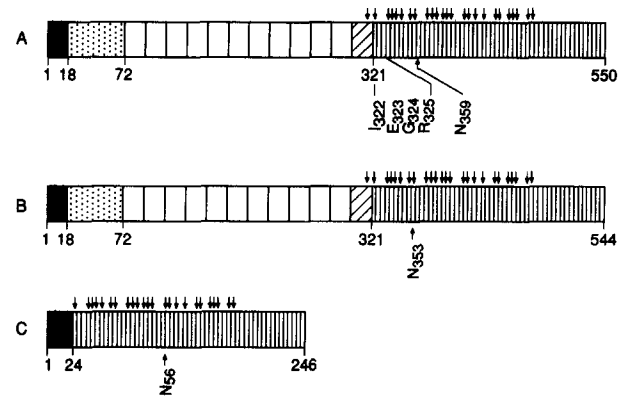

Fig. 1. (A) Hsp150Δ-fX$_a$-NGFR$_e$ consists of the hsp150Δ-carrier (amino acids 1–321) and the whole extracellular domain of mature NGFR$_e$ (amino acids 328–550 of the fusion protein), joined by the factor X$_a$ recognition site IEGR plus a GT-linker. The hsp150Δ-carrier consists of a signal peptide (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 hsp150Δ-NGFR$_e$ the carrier and NGFR$_e$ are joined directly to each other. (C) NGFR$_e$ (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.

sylated, we needed to release it from the carrier. For this purpose, a DNA fragment encoding the proteolytic cleavage site for the blood clotting factor X$_a$ was engineered between the DNA fragments coding for the carrier and NGFR$_e$ (Fig. 1A). The fusion gene was targetted to the *leu2* locus of *S. cerevisiae* to create strain H451. The cells were labeled with [^{35}S]Met/Cys, followed by chase with cycloheximide (CHX). Anti-hsp150 antiserum precipitated heterogenous hsp150Δ-fX$_a$-NGFR$_e$ molecules from the culture medium (Fig. 2, lane 1), whereas only a little was immunoprecipitated from the cell lysate (lane 3). The secreted (lane 2) and cell-associated (lane 4) protein was recognized by anti-NGFR$_e$ antiserum raised against authentic NGFR, although poorly. When labeling was performed in the presence of tunicamycin (TM) to inhibit *N*-glycosylation, similar results were obtained, except that secreted hsp150Δ-NGFR$_e$ was more homogenous and migrated like a 180 kDa protein (Fig. 2, lanes 5–8). A 130 kDa protein, the ER form (see below), was detected in the cell lysate (lane 7). Thus, hsp150Δ-fX$_a$-NGFR$_e$ was secreted nearly as efficiently in the absence of *N*-glycosylation, as in its presence. Antiserum raised against hsp150Δ-NGFR$_e$ [9] recognized NGFR$_e$ much better than anti-NGFR$_e$, and was used henceforth.

3.2. Release of NGFR$_e$ from the carrier

Hsp150Δ-fX$_a$-NGFR$_e$, [^{35}S]Met/Cys-labeled in the presence of TM, was harvested from the culture medium of H451 cells and subjected to factor X$_a$ digestion. The digests 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-hsp150Δ-NGFR$_e$ antiserum, but neither of them by anti-hsp150 antiserum, whereas the intact fusion protein was recognized by both (not shown). The 48 kDa form most probably was the correct product, NGFR$_e$, because it had the expected size [15]. Moreover, NGFR$_e$ expressed in the absence of the hsp150Δ-carrier by the aid of the invertase signal peptide also migrated like a 48 kDa protein (see below). Cleavage at the factor X$_a$ site apparently made a secondary cleavage site in the NGFR$_e$ portion available, since hsp150Δ-NGFR lacking the cleavage site (Fig. 1B) was not susceptible to factor X$_a$ (not shown). The secondary cleavage apparently was at the cysteine-free C-terminus of the NGFR$_e$ portion, since the 36 kDa product appeared to contain a similar amount of ^{35}S-radioactivity as the intact hsp150Δ-fX$_a$-NGFR$_e$ 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 NGFR$_e$ portion, the released hsp150Δ-carrier was not visible in the fluorogram. Factor X$_a$ cleavage of *N*-glycosylated hsp150Δ-fX$_a$-NGFR$_e$ gave essentially similar results (not shown).

3.3. Glycosylation of NGFR$_e$

To study whether the NGFR$_e$ portion of hsp150Δ-fX$_a$-NGFR$_e$ was *O*-glycosylated, strain H451 was labeled with [^{3}H]mannose in the presence of TM. The secreted fusion protein was digested with factor X$_a$ as above. SDS-PAGE analysis revealed a single product at the top of the separating gel (Fig. 3B, lanes 2,3). This was the hsp150Δ-carrier, as we have shown before by synthesizing it from a truncated *HSP150Δ* 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

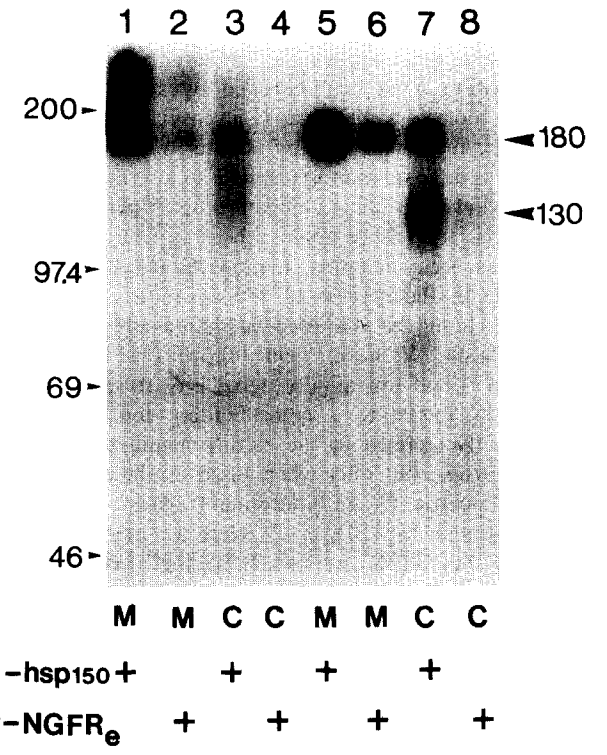

Fig. 2. Secretion of hsp150Δ-fX$_a$-NGFR$_e$. H451 cells were labeled with [^{35}S]Met/Cys at for 1 h 24°C in the absence (lanes 1–4) or presence of TM (lanes 5–8). Culture medium samples (M) (lanes 1,2,5,6) and lysed cell samples (C) (lanes 3,4,7,8) were immunoprecipitated with anti-hsp150 (lanes 1,3,5,7) or anti-NGFR$_e$ antiserum (lanes 2,4,6,8), and analyzed by SDS-PAGE and fluorography. Molecular weight markers (kDa) are on the left.

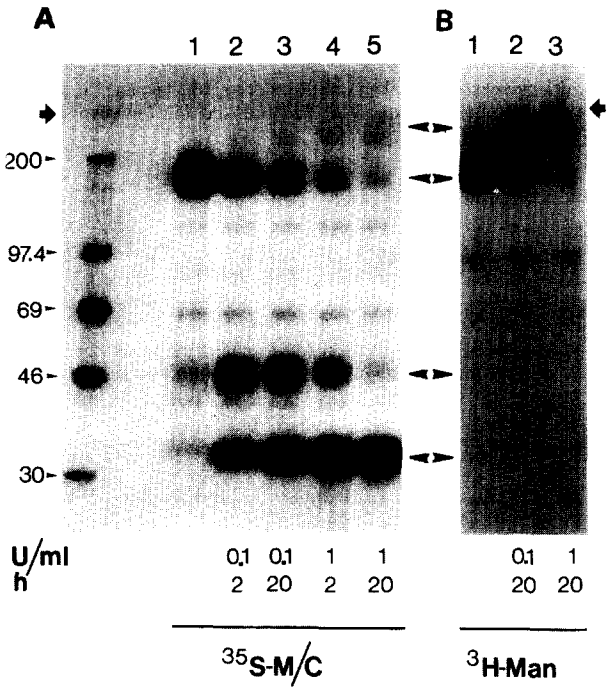

Fig. 3. Digestion of hsp150-fX$_a$-NGFR$_e$ with factor X$_a$. (A) Strain H451 was preincubated with TM for 10 min, and labeled with [^{35}S]Met/Cys in the presence of TM at 37°C for 60 min. In (B) the labeling was with [^{3}H]mannose for 90 min. The culture medium samples were subjected to factor X$_a$ digestions 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 hsp150Δ-carrier, intact hsp150Δ-fX$_a$-NGFR$_e$ 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.

no [^{3}H]mannose-labeled 48 or 36 kDa products were detected, the NGFR$_e$ portion had very few if any *O*-glycans, in spite of numerous potential sites, and extensive *O*-glycosylation of the carrier fragment.

NGFR$_e$ was then synthesized without the hsp150Δ-carrier using the invertase signal peptide (Fig. 1C). NGFR$_e$ (strain H487) was [^{35}S]Met/Cys-labeled in the absence (Fig. 4A, lane 1) or presence of TM (lane 2). Parallel cells were [^{3}H]mannose-labeled in the absence (lane 3) or presence of TM (lane 4). Immunoprecipitation with anti-hsp150Δ-NGFR$_e$ antiserum and SDS-PAGE analysis revealed, as shown before [9], that [^{35}S]NGFR$_e$ migrated as a 48 kDa protein when *N*-glycosylated (lane 1) and like a 45 kDa protein in the absence of *N*-glycosylation (lane 2). When retained in the ER at 37°C due to a *sec18* block (strain H640), NGFR$_e$ also migrated as a 48 kDa protein in the absence of TM (Fig. 4B, lane 1) and like a 45 kDa protein in the presence of TM (lane 2). [^{3}H]Mannose was incorporated into *N*-glycosylated NGFR$_e$ in strain H487 (Fig. 4A, lane 3), whereas [^{3}H]mannosylation was negligible in the presence of TM (lane 4). Since the [^{3}H]mannose labeling of NGFR$_e$ was due to the 9 or so mannose residues of the single primary *N*-glycan, NGFR$_e$ apparently lacked *O*-glycans. Hsp150Δ-NGFR$_e$ (Fig. 1B) served as a control: lane 1 (Fig. 4C) shows [^{35}S]Met/Cys-labeled hsp150Δ-NGFR$_e$, retained at 37°C in the ER in strain H538 (*sec18*) and migrating like a 130 kDa protein. A considerable amount of [^{3}H]mannose was incorporated into it because of extensive *O*-glycosylation (lane 2).

NGFR$_e$ 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 lysozyme 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$_e$

Next we studied whether the NGFR$_e$ portion of hsp150Δ-NGFR$_e$ 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$_e$ has up to 12 disulfide bonds, and yeast-derived hsp150Δ-NGFR$_e$ is also disulfide-bonded [9]. Hsp150Δ-NGFR$_e$ (H426) was labeled with [^{35}S]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 hsp150Δ-NGFR$_e$ 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. To differentiate whether *N*- or *O*-glyco-

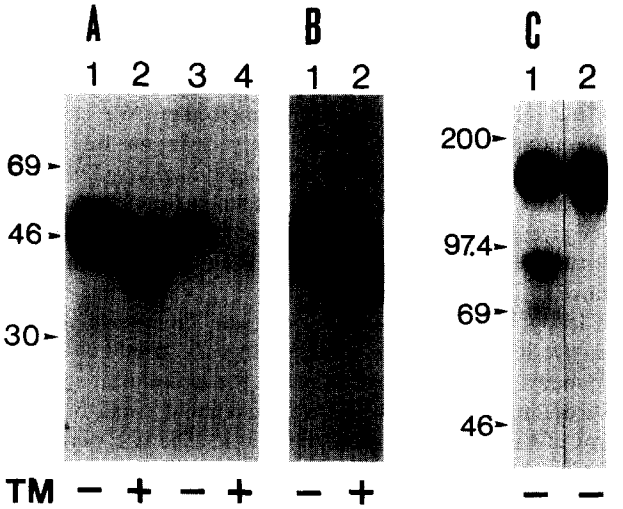

Fig. 4. Incorporation of [^{3}H]mannose into NGFR$_e$. (A) Strain H487 (NGFR$_e$) was labeled for 90 min at 37°C with [^{35}S]Met/Cys (lanes 1,2), or with [^{3}H]mannose (lanes 3,4), in the absence (lanes 1,3) or presence of TM (lanes 2,4). (B) Strain H640 (NGFR$_e$/*sec18*) was labeled, after a pre-incubation for 10 min at 37°C, with [^{35}S]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 (hsp150Δ-NGFR$_e$/*sec18*) was labeled with [^{35}S]Met/Cys (lane 1) or [^{3}H]mannose (lane 2) as in lanes 1,3 of panel (A). The cells were lyzed and immunoprecipitated with anti-hsp150Δ-NGFR$_e$ (A,B) or anti-hsp150 antiserum (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.

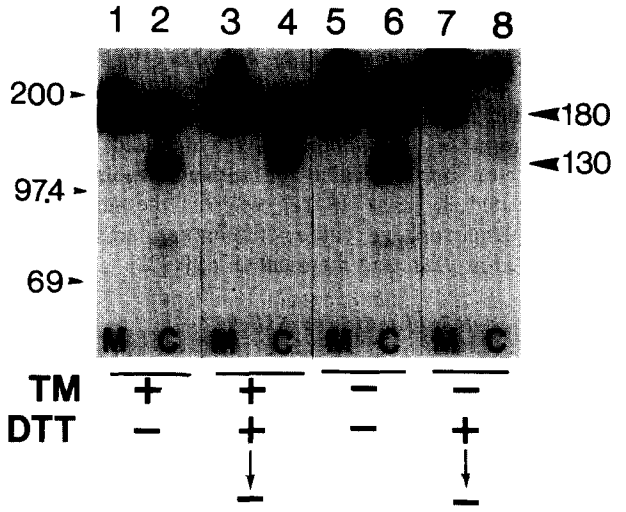

Fig. 5. Glycosylation of reduced hsp150Δ-NGFR$_e$. Strain H426 (hsp150Δ-NGFR$_e$) was labeled for 1 h at 24°C in the presence of TM with [^{35}S]Met/Cys (lanes 1,2). A parallel sample was labeled similarly, but in the presence of DTT, whereafter DTT was washed away and the sample chased with CHX in the presence of TM at 24°C for 60 min (lanes 3,4). A third sample was labeled as in lanes 1,2, but in the absence of TM (lanes 5,6). A fourth sample was labeled and chased as in lanes 3,4, but in the absence of TM (lanes 7,8). The culture media (M) and lyzed cells (C) were subjected to immunoprecipitation with anti-hsp150 antiserum, and the proteins were analyzed by SDS-PAGE and fluorography. Molecular weight markers are on the left.

sylation was increased on the reduced molecules, hsp150Δ-NGFR$_e$ was labeled with [^{35}S]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, hsp150Δ-NGFR$_e$ released from the DTT block migrated in SDS-PAGE (Fig. 5, lane 3) like native hsp150Δ-NGFR$_e$ secreted in the absence of DTT (lane 1). This suggests that *N*-glycosylation, but not *O*-glycosylation, was more efficient on the reduced hsp150Δ-NGFR$_e$ 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 inefficiently 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-translocationally, it must be controlled by the amino acid sequence, whereas in the case of a post-translocational event, other features of the folded molecule regulate it. The two portions of hsp150Δ-NGFR$_e$ are very different: the carrier appears not to adopt any regular secondary structure [11], whereas NGFR$_e$ 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

[1] Johnson, D.C. and Spear, P.G. (1983) Cell 32, 987–997.
[2] Abeijon, C. and Hirschberg, C.B. (1987) J. Biol. Chem. 262, 4153–4159.
[3] Haselbeck,A. and Tanner, W. (1983) FEBS Lett. 158, 335–338.
[4] Strahl-Bolsinger, S., Immervoll, T., Deutzmann, R. and Tanner, W. (1993) Proc. Natl. Acad. Sci. USA 90, 8164–8168.
[5] Lussier, M., Gentzsch, M., Sdicu, A-M., Bussey, H. and Tanner, W. (1995) J. Biol. Chem. 270, 2770–2775.
[6] Immervoll, T., Gentzsch, M. and Tanner, W. (1995) Yeast 11, 1345–1351.
[7] Häusler, A., Ballou, L., Ballou, C.E. and Robbins, P.W. (1992) Proc. Natl. Acad. Sci. USA 89, 6846–6850.
[8] Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A. and Shooter, E.M. (1987) Nature 325, 593–597.
[9] Simonen, M., Vihinen, H., Jämsä, E., Arumäe, U., Kalkkinen, N and Makarow, M. (1996) Yeast (in press).
[10] Russo, P., Kalkkinen, N., Sareneva, H., Paakkola, J. and Makarow, M. (1992) Proc. Natl. Acad. Sci. USA 89, 3671–3675.
[11] Jämsä, E., Holkeri, H., Vihinen, H., Wikström, M., Simonen, M., Walse, B., Kalkkinen, N., Paakkola, J. and Makarow, M. (1995) Yeast 11, 1381–1391.
[12] Simonen, M., Jämsä, E. and Makarow, M. (1994) J. Biol. Chem. 269, 13887–13892.
[13] Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L. and Lacroute, F. (1991) Yeast 7, 609–615.
[14] Jämsä, E., Simonen, M. and Makarow, M. (1994) Yeast 10, 355–370.
[15] Welcher, A.A., Bitler, C.M., Radeke, M.J. and Shooter, E.M. (1991) Proc. Natl. Acad. Sci. USA 88, 159–163.
[16] Grob, P.M., Ross, A.H., Koprowski, H. and Bothwell, M. (1985) J. Biol. Chem. 260, 8044–8049.
[17] Zupan, A.A., Osborn, P.A., Smith, C.E., Siegel, N.R., Leimgruber, R.M. and Johnson, E.M. (1989) J. Biol. Chem. 264, 11714–11720.
[18] Kalsner, I., Schneider, F.-J., Geyer, R., Ahorn, H. and Maurer-Fogy, I. (1992) Glycoconj. J. 9, 209–216.
[19] Olstad, O.K., Reppe, S., Gabrielsen, O.S., Hartmanis, M., Blingsmo, O.R., Gautvik, V.T., Haflan, A.K., Christensen, T.B., Oyen, T.B. and Gautvik, K.M. (1992) Eur. J. Biochem. 205, 311–319.
[20] Yamada, T., Uyeda, A., Takao, T., Shimonishi, Y., Matsushima, M. and Kikuchi, M. (1995) Eur. J. Biochem 230, 965–970.
[21] Gellerfors, P., Axelsson, K., Helander, A., Johansson, S., Kenne, L., Lindquist, S., Pavlu, B., Skottner, A. and Fryklund, L. (1989) J. Biol. Chem. 264, 11444–11449.
[22] Ernst, J.F., Mermod, J.-J. and Richman, L.H. (1992) Eur. J. Biochem. 203, 663–667.
[23] Allen, S., Naim, H.Y. and Bulleid, N (1995) J. Biol. Chem. 270, 4749–4804.
[24] Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H.-R. and Lesslauer, W. (1993) Cell 73, 431–445.