O-Glycosylation in *Saccharomyces cerevisiae* is initiated at the endoplasmic reticulum

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The first mannose of O-linked oligomannose chains in *S. cerevisiae* is transferred to Ser/Thr residues via dolichylphosphate mannose. Only this reaction (and not the subsequent reactions requiring GDP-Man) proceeds at the endoplasmic reticulum.

O-Glycosylation *Glycoprotein* *Dolichylphosphate* Saccharomyces cerevisiae
Intracellular transport sec-*mutant*

1. INTRODUCTION

Glycoproteins in the yeast *Saccharomyces cerevisiae* contain N-linked oligo- and polymannose chains and short, O-linked oligosaccharide chains, containing mannose only [1–3]. The biosynthesis of the O-linked oligosaccharides proceeds by the following reaction sequence [4,5]:

$$\text{GDP-Man} + \text{Dol-P} \xrightarrow{Mg^{2+} \text{ or } Mn^{2+}} \text{Dol-P-Man} + \text{GDP} \quad (1)$$

$$\text{Dol-P-Man} + \text{Protein(Ser/Thr)} \xrightarrow{Mg^{2+} \text{ or } Mn^{2+}} \text{Protein(Ser/Thr)-Man} + \text{Dol-P} \quad (2)$$

$$\text{GDP-Man} + \text{Protein(Ser/Thr)-Man} \xrightarrow{Mn^{2+}} \text{Protein(Ser/Thr)-Man-Man}_{1-3} + \text{GDP} \quad (3)$$

So far, this is the only reaction sequence in which a dolichyl intermediate is known to participate in *O*-glycosylation [6,7]. It seems to occur generally in fungal cells [8].

From cell fractionation studies it was concluded that *O*-mannosylation starts at the ER [9,10] possibly as a co-translational event [11]. It was never clear, however, whether the whole oligosaccharide or only part of it was attached to the protein at the ER. Using Schekman's temperature-sensitive sec mutants [12], it will be shown here that only the dolichol-dependent steps, i.e., the transfer of the first mannosyl residue to the protein (reactions (1) and (2) above), proceed at the ER. The sugar chain is extended in the Golgi and no mannosyl residues are transferred at the cytoplasmic membrane, since glycoproteins in secretory vesicles already contain the completed O-linked chains.

2. MATERIALS AND METHODS

The yeast mutants *sec1* and *sec18* which are derived from *S. cerevisiae* X 2180-1A [12], were kindly supplied by Dr Randy Schekman (Berkeley CA). [2-^{3}H]Mannose (spec. act. 13.4 Ci/mmol) was obtained from Amersham.

Cells were grown overnight in 1% yeast extract, 2% bactopeptone and 2% sucrose at 25°C. For labelling with [2-^{3}H]mannose, cells were harvested, washed once with distilled water and transferred to fresh medium containing 0.5% sucrose. After different pre-incubation periods at either 25°C (permissive temperature) or 37°C (restrictive temperature) [2-^{3}H]mannose was added to 25 μCi/ml final conc.

Each experiment was carried out with 2 ml cells with an A_{578} between 1.5 and 2.5; incubation time was 1 h. Cells were then harvested by centrifuga-

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tion, washed twice with 1 ml buffer (50 mM Tris–HCl, pH 7.4), resuspended in 0.5 ml of the same buffer and mechanically broken with glass beads (0.45–0.50 mm) on a Vortex mixer. A pellet and a water-soluble fraction were separated by centrifugation at 48000 × *g* for 1 h.

The pellet was further processed as in [13] and base-labile oligosaccharides were released on incubation with 0.1 M NaOH for 24 h at room temperature. β-Elimination was stopped by passing the solution over Dowex 50 Wx 8/H^+. β-Eliminated oligosaccharides and sugars were separated by paper chromatography in ethyl acetate/butanol/acetic acid/water (3:4:2.5:4, by vol.). To determine the distribution of radioactivity in the various O- and N-linked saccharides, the paper chromatograms were cut into 1-cm strips, whose radioactivity was measured in a liquid scintillation counter.

The water-soluble fraction was lyophilized and treated in the same way as the pellet. Free mannose was not detectable in this fraction. The sum of the radioactivity in these two fractions was taken as the total incorporation of [^{3}H]mannose into glycoprotein.

3. RESULTS AND DISCUSSION

When *sec18* cells were radiolabelled with [2-^{3}H]mannose for 60 min at the permissive temperature and the total polymeric material taken through the β-elimination procedure, the oligosaccharide pattern shown in fig.1A was obtained after paper chromatography. When the labelling was carried out at the non-permissive temperature (37°C) the pattern of the sugars released changed considerably: mannose was now present in by far the highest amount, whereas it was the disaccharide which dominated at 25°C (fig.1A,B).

As shown in table 1, the total incorporation of [2-^{3}H]mannose into *O*-glycosidically linked sugar chains was doubled at the higher temperature, but the increase was exclusively due to the mannosyl residue linked directly to serine/threonine. It amounted to almost 90% of the total O-linked saccharides.

Since the *sec18* mutant is blocked in the transfer of secretory material from the ER to Golgi [12] leading to an accumulation of ER structures at the non-permissive temperature, the obvious conclusion from the results obtained is that only the first mannosyl residue is attached to the protein at the ER. The subsequent ones, which all require GDP-Man as sugar donor, are transferred to the glycoprotein when it has reached a stage in the secretory pathway beyond the ER; i.e., most likely in the Golgi.

However, some disaccharide is synthesized at the non-permissive temperature (table 1). Three possible explanations for this observation are:

(i) Glycoproteins, which had already reached the Golgi before the temperature shift, have their nascent O-linked sugar chain extended when [2-^{3}H]mannose is added.

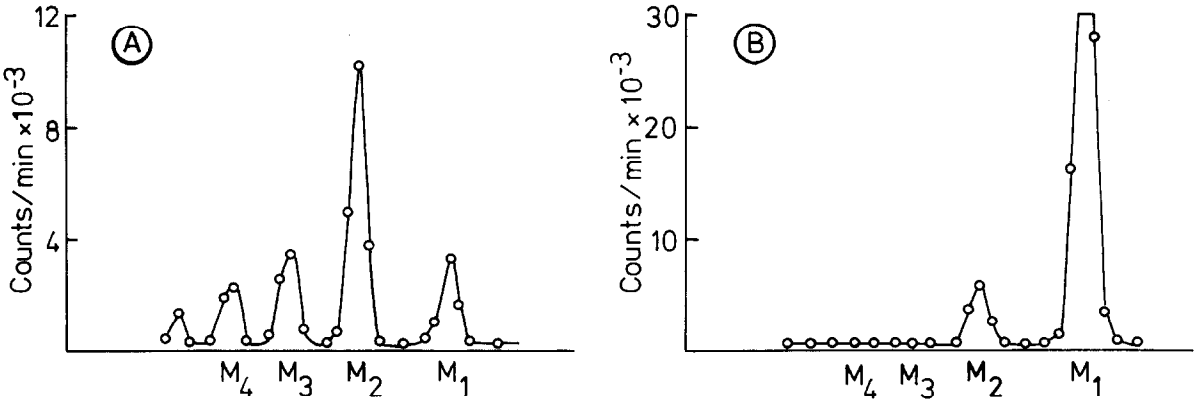

Fig.1. Patterns of β-eliminable saccharides from *sec18* cells radiolabelled with [2-^{3}H]mannose at permissive (A) and non-permissive (B) temperatures. Experimental details are given in section 2: M_1, mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose.

Table 1

Incorporation of [2-^{3}H]mannose into N- and O-linked carbohydrate chains of *sec18* cells at permissive and non-permissive temperatures (cpm × 10^{-2})

Carbohydrate fraction	Incubation temperature	
	25°C	37°C
N-linked oligosaccharides[a]	7543	434
O-linked sugars		
Total	411	1060
M_1	64 (15%)	934 (88%)
M_2	197 (48%)	120 (11%)
M_3	73 (18%)	6 (<1%)
M_4	52 (13%)	0
M_5	25 (6%)	0

[a] Chromatographically immobile radioactivity remaining after β-elimination

Cells, grown at 25°C, were preincubated for 30 min at 25 or 37°C, whereupon [2-^{3}H]mannose was added. Experimental details are given in section 2: M_1, mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; M_5, mannopentaose

(ii) The *sec18* mutant is slightly leaky.
(iii) Some mannoproteins do in fact acquire their second mannose residue at the ER.

The third possibility seems the least likely one. If the first explanation were correct, one would expect the amount of disaccharide formed at the non-permissive temperature to decrease if cells are preincubated at this temperature for longer intervals before they are radiolabelled. This tendency is indeed observed (table 2), although the results were not as clear as expected. Thus, explanations (i) and (ii) given above may both be correct to some extent.

As can be seen from table 1, the amount of *N*-glycosylation strongly decreases at the non-permissive temperature. This was in part expected since the very long outer chain poly-mannose extensions [3] cannot be synthesized at 37°C [14]. The large difference in the extent of radiolabelling, at 37°C, of O-linked carbohydrate chains (increased by >100%) and N-linked ones (decreased to <10%) might, however, also reflect quite different temperature optima for these two processes.

Table 2

Change in the relative amounts of mannose and mannobiose with increasing periods of pre-incubation at the non-permissive temperature

Pre-incubation period (min)	O-linked carbohydrate		
	Total cpm × 10^{-2}	Mannobiose (%)	Mannose (%)
10	355	13.7	86.0
30	384	11.2	88.6
60	537	8.6	91.4

Experimental conditions were as in table 1

Finally, the question was asked whether *O*-mannosylation of mannoproteins is completed within the cell, or whether mannosyl transferases of the cytoplasmic membrane, which have repeatedly been postulated to exist [15,16], contribute to the extension of oligosaccharide chains. Using the *sec1* mutant, which accumulates secretory vesicles in the cytoplasm at the non-permissive temperature [12], an experiment analogous to that of table 1 was carried out. The results obtained are given in table 3. Again the radioactivity in O-linked chains was increased,

Table 3

Incorporation of [2-^{3}H]mannose into N- and O-linked carbohydrate chains of *sec1* cells at permissive and non-permissive temperatures (cpm × 10^{-2})

Carbohydrate fraction	Incubation temperature	
	25°C	37°C
N-linked oligosaccharides	5091	2894
O-linked sugars		
Total	310	519
M_1	31 (10%)	72 (14%)
M_2	183 (59%)	260 (50%)
M_3	44 (14%)	81 (16%)
M_4	29 (9%)	58 (11%)
M_5	22 (7%)	46 (9%)

Cells, grown at 25°C, were pre-incubated for 60 min at 25 or 37°C, whereupon [2-^{3}H]mannose was added. Other conditions as in table 1

whereas that of N-linked ones was inhibited by 43%. The oligosaccharide pattern of the β-eliminable material, however, is the same at both temperatures, indicating that *O*-glycosylation is completed by the time the glycoproteins are packed into secretory vesicles.

The results reported here fully support the earlier notion that *O*-glycosylation in yeast proceeds in part at the ER, most likely co-translationally [9–11]. This differs from events in mammalian cells, where *O*-glycosylation seems to take place exclusively when the corresponding proteins pass through the Golgi, or thereafter [17,18]. This divergence from mammalian cells coincides with old observations that it is only in fungal cells that dolichyl phosphate activated sugars are involved in *O*-glycosylation [4–8]. Here, it has conclusively been shown that only the step that depends on dolichyl phosphate is catalyzed by ER components.

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