THE SPECIFIC SITE OF TUNICAMYCIN INHIBITION IN THE FORMATION OF DOLICHOL-BOUND *N*-ACETYLGLUCOSAMINE DERIVATIVES

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1. Introduction

Tunicamycin, a *N*-acetylglucosamine containing antibiotic from *Streptomyces lysosuperificus* was originally isolated on the basis of its antiviral activity [1,2]. Subsequent work demonstrated that tunicamycin selectively interferes with glycoprotein biosynthesis. This has been shown for the suppression of virus membrane glycoproteins [3,4]. In *Saccharomyces cerevisiae* the formation of the external mannoproteins invertase, acid phosphatase and cell wall mannan as well as that of the vacuolar carboxypeptidase Y is stopped in the presence of tunicamycin [5,6]. In contrast to this the synthesis of non-glycosylated proteins or wall polysaccharides is not influenced [5,6].

There are several lines of evidence indicating that tunicamycin exerts its effect by blocking the attachment of the carbohydrate residue to the protein [3,4,7]. Polyprenol-bound sugar phosphates function as intermediates in glycosyl transfer [8,9] and it has indeed been observed that the transfer of *N*-acetylglucosamine to dolichylphosphate is sensitive to tunicamycin as examined in microsomal fractions from calf liver and chick embryo [10,11]. The question, however, has been left unanswered so far, whether the transfer of only the first or also the second Glc*N*Ac as well as further glycosyl groups to dolichol is prevented by tunicamycin.

A membrane fraction from *S. cerevisiae* catalyzes in vitro the following reaction sequence, which is supposed to be involved in the formation of mannoproteins [12]

1. UDP-Glc*N*Ac + Dol-P ⟶ Dol-PP-Glc*N*Ac + UMP
2. UDP-Glc*N*Ac + Dol-PP-Glc*N*Ac ⟶ Dol-PP(Glc*N*Ac)$_2$ + UDP
3. GDP-Man + Dol-PP-(Glc*N*Ac)$_2$ ⟶ Dol-PP-(Glc*N*Ac)$_2$ -Man + GDP.

It will be shown that tunicamycin inhibits the transfer of Glc*N*Ac to dolichol phosphate (reaction 1) also in yeast. In addition it will be demonstrated that neither reaction 2 or 3 at all sensitive to the antibiotic.

Abbreviations: Dol-PP-Glc*N*Ac, dolichyl diphosphate *N*-acetylglucosamine; Dol-PP-(Glc*N*Ac)$_2$, dolichyl diphosphate di-*N*-acetylchitobiose

2. Materials and method

The membrane fraction was prepared from *S. cerevisiae* (strain 66.24, Fleischman Laboratories) as previously described [13]. Transfer of *N*-acetylglucosamine from UDP-Glc*N*Ac to Dol-P has been tested in the following way: 5 μg Dol-P was mixed with 2 μmol MgEDTA and dried under nitrogen. 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.17% Triton X-100, 4.76 μM [^{14}C]UDP-Glc*N*Ac (spec.act. 300 Ci/mol, Amersham) and membrane preparation (0.06 mg protein) were added to a total volume of 0.07 ml. Incubations were carried out at 23°C for various intervals. Labelled lipids were extracted and washed as described [14].

Thin layer chromatography was performed on

Silica Gel G (Merck) in the solvent system chloroform/methanol/water (65:25:4). Labelled components were located by radiochromatography with Agfa Gevaert Osray T4 film. Radioactive spots were scraped off the plates and counted by liquid scintillation in dioxane.

3. Results and discussion

Yeast membranes incubated with [^{14}C]UDP-Glc*N*Ac incorporate radioactivity into a lipophilic fraction, which separated into four compounds upon TLC (fig.1). Only peaks III and IV can be stimulated by the addition of exogenous Dol-P to the reaction mixture and have previously been identified as Dol-PP-Glc*N*Ac and Dol-PP-(Glc*N*Ac)$_2$, respectively, [12]. Peak I and peak II (the latter is difficult to separate from peak III) have not been identified up to now. Their nature, however, seems to be different from that of a polyprenol derivative, since mild alkaline saponification (0.1 N NaOH, 37°C, 15 min), which brings about deacylation of glyceroglycolipids converted more than 90% of the radioactivity water-soluble.

The effect of tunicamycin on the formation of the radioactive glycolipids is shown in fig.1 and 2. The antibiotic completely inhibited the formation of Dol-PP-Glc*N*Ac and Dol-PP(Glc*N*Ac)$_2$, but did not affect the synthesis of both the other radioactive compounds peak I and II.

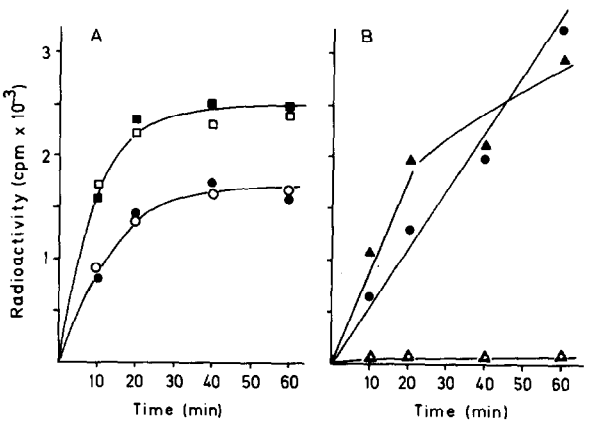

Fig.2. Time course of incorporation of [*N-acetyl*-^{14}C]-glucosamine from [UDP-^{14}C]Glc*N*Ac into glycolipids. A: peak I (●,○), peak II (■,□); B: peak III corresponding to Dol-PP-Glc*N*Ac (●,○), peak IV corresponding to Dol-PP-Glc*N*Ac (▲,△). Closed circles without, open circles with tunicamycin (7 μg/ml). Experiments were carried out as described under Materials and methods.

The dependence of the inhibition of Glc*N*Ac-transfer on the concentration to tunicamycin was determined after an incubation for 15 min (fig.3).

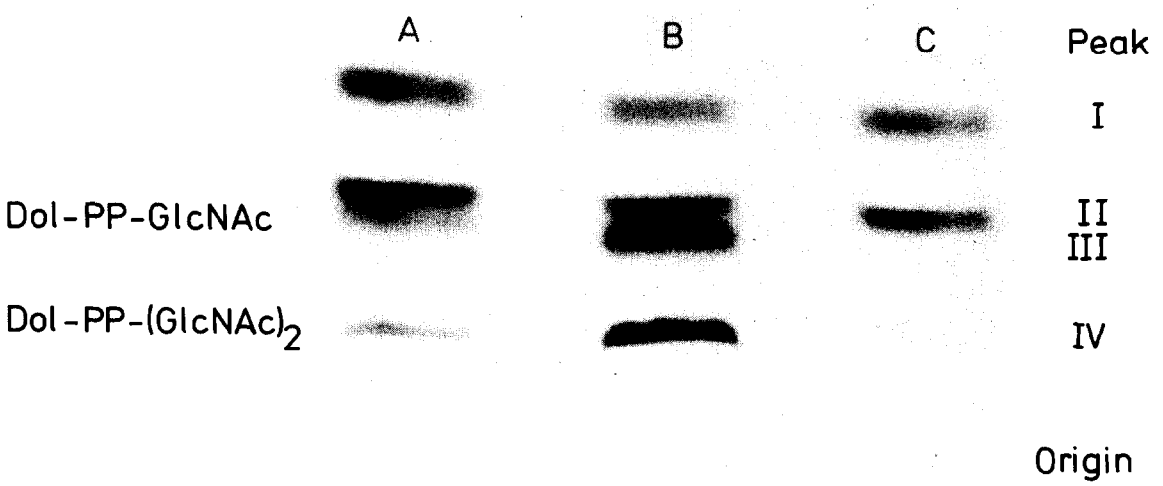

Fig.1. Thin layer chromatography of [*N-acetyl*-^{14}C]glucosamine lipid synthesized from [UDP-^{14}C]Glc*N*Ac. Incubations were carried out as described in Materials and methods in the absence (A) or in the presence (B,C) of exogenous Dol-P. Experiment C contained in addition tunicamycin (7 μg/ml).

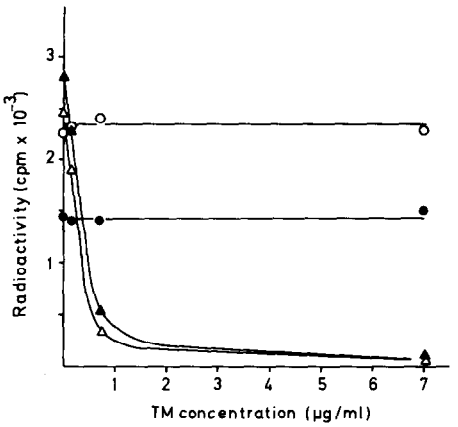

Fig.3. Dose response of tunicamycin on the formation of [^{14}C]Glc*N*Ac labelled lipids. Incubations were carried out under standard conditions with various amounts of tunicamycin: peak I (○–○), peak II (●–●), Dol-PP-Glc*N*Ac (△–△), Dol-PP-(Glc*N*Ac)$_2$ (▲–▲).

The antibiotic is very potent 50%; inhibition is achieved at a concentration of about 0.3 μg/ml.

It has previously been demonstrated [12] that Dol-PP-Glc*N*Ac is the precursor for the formation of Dol-PP-(Glc*N*Ac)$_2$. From the data obtained so far, it cannot be decided, therefore, whether tunicamycin blocks the formation of peak IV directly or only on account of a shortage of its precursor Dol-PP-Glc*N*Ac. The following experiment was carried out, to decide between these two possibilities. Reincubation of the isolated radioactive glycolipid fraction (containing peak I–IV) with unlabelled UDP-Glc*N*Ac led to a decrease in radioactive Dol-PP-Glc*N*Ac and a corresponding increase in the chitobiose containing lipid. The increase in radioactivity in peak IV in the presence of tunicamycin (expt. C) indicates that the antibiotic does not interfere with the transfer of the second Glc*N*Ac to Dol-PP-Glc*N*Ac. When GDP-Man is present in addition to UDP-Glc*N*Ac, Dol-PP-(Glc*N*Ac)$_2$ acts as acceptor for the formation of Dol-PP-(Glc*N*Ac)$_2$-Man (expt. D). Also this mannosyl transfer is not affected by tunicamycin (expt. E).

The results presented in this paper show that in yeast, too, tunicamycin is a very potent inhibitor for the biosynthesis of dolichyl diphosphate-bound Glc*N*Ac derivatives. Moreover, the inhibitory action of tunicamycin seems to be restricted exclusively to the transfer of that Glc*N*Ac moiety which is directly linked to the lipid component, whereas further glycosyl transfer reactions leading to lipid-linked oligosaccharides are not affected. The specificity of the antibiotic is also indicated by the fact that the synthesis of peak I and II is not influenced. In addition no effect of tunicamycin has been observed for the synthesis of polyprenyl phosphate-linked mannose and galactose [10,11], whereas Glc*N*Ac-transfer to undecaprenyl phosphate in *Bacillus subtilis* again is inhibited by the antibiotic [15].

As indicated by the observations of Kuo and Lampen [5], tunicamycin will be a valuable tool in

Table 1
Influence of tunicamycin on the formation of Dol-PP-di-*N*-acetylchitobiose and dolichyl diphosphate-bound oligosaccharide

Expt.	Incubation conditions	Radioactivity (cpm) found in[a]		
		Dol-PP-Glc*N*Ac	Dol-PP-(Glc*N*Ac)$_2$	Dol-PP-(Glc*N*Ac)$_2$-Man
A	^{14}C-Labelled glycolipid (control)	1710	5400	0
B	+ UDP-Glc*N*Ac	180	7020	0
C	+ UDP-Glc*N*Ac + tunicamycin	216	6966	0
D	+ UDP-Glc*N*Ac + GDP-Man	405	180	6300
E	+ UDP-Glc*N*Ac + GDP-Man + tunicamycin	387	207	6120

[a]Peak I and II are not listed; their radioactivity remained completely unchanged during all experiments

^{14}C-Labelled glycolipid fraction (containing peak I–IV) was synthesized in a scaled up reaction mixture according to Materials and methods, extracted and used for the following experiments. ^{14}C-Labelled glycolipid (14 000 cpm) was dried under a stream of nitrogen and the following components were added in a final volume of 0.07 ml: 28 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 0.17% Triton X-100, 0.06 mg protein (particulate fraction), 1 mM UDP-Glc*N*Ac (expt. B–E), 1 mM GDP-Man (expt. D,E) and where indicated tunicamycin (7 μg/ml). After incubation for 120 min the lipids were extracted again, washed and analyzed by TLC.

studies of secretion and the possible involvement of glycosylation reactions in this process. Knowing the very specific site of action of tunicamycin should be helpful when applying the antibiotic.

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