Biosynthesis of glycoproteins in Candida albicans: activity of dolichol phosphate mannose synthase and protein mannosylation in a mixed membrane fraction

Blanca L. Arroyo-Flores, Carlos Calvo-Méndez, Arturo Flores-Carreón and Everardo López-Romero

A mixed membrane fraction (MMF) was isolated from yeast cells of Candida albicans with the ability to synthesize dolichol phosphate mannose (Dol-P-Man) from GDP-Man and dolichol phosphate (Dol-P) and transfer the sugar to proteins. Temperature of incubation (20-37 °C) did not affect the synthesis of Dol-P-Man but protein mannosylation occurred better at physiological temperatures (28 °C and 37 °C). Most of the sugar (87-93%) in the mannoproteins was O-linked as judged by its release by β-elimination. Mannose was identified as the sole product after this treatment. Following incubation of MMF with the sugar donor, parallel levels of Dol-P-Man and mannosylated proteins were detected up to 30 min. Thereafter, Dol-P-Man levels reached a steady value whereas mannoproteins rapidly accumulated. Lipid-linked oligosaccharides were also detected in incubation mixtures, though in much lower amounts than those of Dol-P-Man or mannoproteins. Dol-P-Man synthase activity increased proportionally in response to increasing concentrations of either of the two enzyme substrates. A \( K_m \) value of 0.36 \( \mu \)M for GDP-Man was calculated. MMF failed to use exogenous Dol-P-Man for protein glycosylation. Specific inhibition of Dol-P-Man synthesis with amphomycin was concomitant with a parallel decrease in protein mannosylation, indicating that most of the sugar is transferred to protein via the carrier lipid. Results are discussed in terms of the role of Dol-P-Man in protein glycosylation in C. albicans.

Keywords: Candida albicans, dolichol phosphate mannose, mannoproteins, protein glycosylation

INTRODUCTION

Dolichol phosphate mannose synthase (EC 2.4.1.83) transfers mannose from GDP-Man to the polyisoprenoid dolichol phosphate forming Dol-P-Man, a key intermediate in protein glycosylation in both multicellular and unicellular eukaryotes. Most of our knowledge of Dol-P-Man synthase comes from studies in Saccharomyces cerevisiae, where the enzyme participates in vivo in all three glycosylation pathways, namely N-glycosylation, O-glycosylation and glycosyl phosphatidylinositol mannosilation.

Abbreviations: MMF, mixed membrane fraction; LLO, lipid-linked oligosaccharides; Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate mannose.
lationship of glycoprotein metabolism to the yeast–mycelium morphological transition and the long-term goal of detecting a glycoprotein-related pathogenicity determinant that might be used as a potential target to control candidiasis.

We describe here the presence and some properties of Dol-P-Man synthase activity in cell-free extracts of C. albicans and the role of the mannosylated lipid in the glycosylation of membrane proteins.

**METHODS**

**Strain and culture conditions.** *Candida albicans* ATCC 26555 was used in this study. It was maintained by periodic transfer on slants of yeast extract-peptone-dextrose (YPD) medium (Barlinski-García & Nickerson, 1962) containing 2% (w/v) agar. For propagation, inocula were obtained by resuspending the cells from a 24 h slant culture in 5 ml of the above medium. Four 2 litre Erlenmeyer flasks containing 600 ml YPD medium were inoculated with the yeast suspension to give a density of about 6–7 x 10^8 cells ml^-1 and the flasks were shaken aerobically at 120 r.p.m. for 10 h at 28 °C.

**Preparation of the mixed membrane fraction (MMF).** Cells were collected by centrifugation at 7000 g (r_w) for 10 min, washed twice with 50 mM Tris/HCl buffer, pH 7.5 (Tris buffer), and the pellet (routinely 15–17 g wet weight; 3–4 x 10^11 cells) was resuspended in 20 ml of the same buffer. The yeast suspension was mixed with an equal volume of glass beads (0.45–0.90 mm diameter) and broken in an MSK cell homogenizer (Braun) for 2 min while cooling with a stream of CO_2. Breakage was assessed by phase-contrast microscopy. The homogenate was centrifuged at 1300 g for 5 min to remove cell walls and unbroken cells. The resulting cell-wall-free supernatant was centrifuged at 118000 g (r_w) for 60 min. The supernatant was discarded and the membrane pellet was resuspended in Tris buffer and used as the source of mannosyltransferase activity.

**Solvents.** The following solvent systems were used (all ratios by vol.): A, chloroform/methanol (1:1); B, chloroform/methanol/ammonium hydroxide (75:25:4); C, chloroform/methanol/water (65:25:4); D, chloroform/methanol/water (3:48:47); E, chloroform/methanol (3:2); F, chloroform/methanol/water (10:10:3); G, ethyl acetate/pyridine/water (12:5:4); H, chloroform/methanol/acetic acid/water (25:15:4:2).

**Dol-P-Man synthase assay.** Unless otherwise indicated, standard reaction mixtures contained 5 mM MgCl_2, MMF (1–4–1.9 mg protein), 45000–55000 c.p.m. GDP-[14C]Man (Amersham; 303 Ci mol^-1, 11.2 TBq mol^-1 ) and 50 mM Tris/HCl buffer, pH 7.5, in a total volume of 300 μl. After 30 min incubation at 28 °C, the mixture was made 1:1:1 (chloroform:methanol:water) by the addition of 0.7 ml water and 2 ml solvent A. The mixture was vortexed vigorously and the phases were separated by low-speed centrifugation. The lower, chloroform phase was removed and saved, and more chloroform (1 ml) was added to the mixture. After vortexing, the chloroform phase was separated by centrifugation from the upper phase and the interphase. The chloroform phase was combined with the previous one. The pooled chloroform extract was washed with 1 ml solvent D to remove any water-soluble contaminants; the organic phase, which contained the radio-labelled mannolipid, was evaporated to dryness under a current of hot air, mixed with 10 ml scintillation fluid (0.1 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 20 g 2,5-diphenyl-oxazole dissolved in 1000 ml toluene) and its radioactivity measured in a liquid scintillation spectrometer. The upper phase and interphase were saved and used to determine mannoproteins and lipid-linked oligosaccharides (LLO) as described below.

**Determination of mannoproteins and LLO.** In experiments where LLO were not determined, mannoproteins were measured as follows. The upper layer and interphase obtained above after chloroform extraction of the mannolipid were mixed and filtered through Millipore type 1 glass fibre filters (diameter, 2.5 cm; retention, 1–6 μm). The filters were successively washed twice with 5% (w/v) TCA, 10% TCA and 66% (v/v) ethanol, dried for 30 min at 80 °C and their radioactivity measured (Flores-Carreón & Sentandreu, 1990). In those experiments where LLO were measured, they were extracted as described by D’Souza et al. (1992). Briefly, the upper layer and the interphase were mixed with enough methanol to give a single phase, and the pellet was isolated by centrifugation. The pellet was washed successively twice with 2 ml methanol and 2 ml solvent E. The pellet was then extracted three times with 2 ml solvent F, then the extracts were pooled, air-dried and their radioactivity measured as above to determine the amount of LLO. The remaining pellet was homogenized in water and filtered as above to measure mannoproteins.

**Analysis of the mannolipid by TLC.** In order to have enough material for analysis, radiolabelled mannolipid was synthesized in reaction mixtures that were scaled up fivefold. It was extracted as described above, concentrated by evaporation under vacuum and dissolved in a small amount of solvent A. Aliquots were then analysed by TLC on 20 x 20 cm Silica Gel 60 plates (Merck) using three different solvents: B (basic), C (neutral) and H (acid). Air-dried plates were cut into 1 cm segments and their radioactivity measured.

**Ion-exchange chromatography of mannolipid in DE52 cellulose.** A crude preparation of the mannolipid was layered on a column (1 x 7 cm) of the anion-exchanger DE52 (Whatman) equilibrated with solvent F. The column was washed with the same solvent and the sample was eluted with 10 mM ammonium formate in solvent F. Fractions of 2 ml were collected and radioactivity was measured in 200 μl aliquots. Alternatively, binding to the anion-exchanger was carried out by batch chromatography. In this case, a known amount (about 3–4 x 10^6 c.p.m.) of the mannolipid was gently shaken for 5–10 min with DE52 that had been washed with solvent F. Following centrifugation at low speed, all radioactivity was retained in the resin pellet. This was washed with solvent F and the mannolipid was then eluted with 10 mM ammonium formate in the same solvent.

**β-Elimination of mannoproteins and analysis of released products.** Filters that had been scintillation-counted to measure radiolabelled mannolipid as described above were taken from the vials and washed several times by dipping into toluene. They were dried at 80 °C for 30 min and placed in 2 ml 0.1 M NaOH. After 24 h at room temperature, the alkali was removed and the filters were washed several times with water, dried, and the remaining radioactivity measured. For analysis of β-eliminable products, the alkaline hydrolysate (2 ml) was saved and neutralized with Dowex 50W, H⁺ form (Bio-Rad). After centrifugation, the supernatant was freeze-dried and resuspended in a small amount of water. A 20 μl aliquot of this sample was spotted on a 4 x 57 cm strip of Whatman no. 1 paper and the chromatogram was developed for 20 h in descending fashion with solvent G. A control chromatogram spotted with a standard mixture containing 100 μg each of glucose, mannose, galactose, fucose and maltose was run in parallel. After drying, the sample chromatogram was cut into 1 cm segments that were
scintillation-counted to locate radioactivity. The position of the standards in the control chromatogram was revealed with silver (Trevelyan et al., 1950).

**Mild acid hydrolysis of the mannolipid and identification of the released sugar.** A sample of the mannolipid was dried under nitrogen and hydrolysed in 0.05 M HCl in 50% (v/v) 2-propanol at 100 °C for 15 min. The hydrolysate was washed with chloroform to remove lipids and the water phase was concentrated to dryness and dissolved in a small amount of water. The sugar was identified by paper chromatography in the solvent and using the conditions described above for the β-eliminatable products.

### RESULTS AND DISCUSSION

**Dol-P-Man synthase activity and protein mannosylation in the MMF**

When incubated with GDP-[14C]Man, MMF from *Candida* yeast cells catalysed the incorporation of mannose into mannolipid and mannoproteins in a manner dependent on the concentration of protein (Fig. 1). While synthesis of the mannolipid was not affected by temperature of incubation in the range of 20–37 °C (Fig. 1a), transfer of the sugar to mannoproteins was more efficient at the physiological temperatures (Fig. 1b). This finding may merely reflect differences in catalytic properties between Dol-P-Man synthase and the protein mannosyltransferases. It should be pointed out that results from standard 30 min assays as those shown in Fig. 1(a) (and later in Fig. 5) do not represent initial-rate measurements since at this time point mannolipid accumulation levels off (see Fig. 6). The role of Dol-P-Man as donor of the last four mannose residues during the assembly of the N-linked oligosaccharide precursor and of the first O-linked manno moiety is well documented (Orlean, 1990, 1992; Herscovics & Orlean, 1993). In our assays, most of the sugar (87–93%) in the mannoproteins was O-linked as judged by its susceptibility to β-elimination (Fig. 1c). The remaining low percentage of non-β-eliminatable mannose is likely to correspond to mannosaccharides resulting from the transfer of mannose to pre-existing pools of N-linked acceptors. No further attempt was made to identify these species. Paper chromatography of β-eliminated sugars of mannoproteins from different experiments in solvent G revealed the presence of mannose as the sole product (Fig. 2). As mentioned above, it is known that only the first mannose residue in O-glycosylation is donated by Dol-P-Man; the second and subsequent mannose residues are transferred directly from GDP-Man (Tanner & Lehle, 1987). Failure to detect mannobiose and/or mannooligosaccharides reflects the inability of the MMF to elongate O-linked mannoproteins, a result probably due to the requirement of some particular conditions not met by our assay method.

**Characterization of the mannolipid as Dol-P-Man**

The mannolipid synthesized from GDP-Man by the membranes was identified as Dol-P-Man by the following criteria. (a) The product, which migrated as a single species on TLC, exhibited a mobility which was typical for mannosyl-phosphoryl-polyprenols (Forsee & Elbein, 1973). Thus, as shown in Fig. 3, the lipid migrated rapidly in an acidic solvent (Fig. 3c, Rf 0.94) and slowly in a basic solvent (Fig. 3a, Rf 0.28) but had an intermediate mobility in a neutral solvent (Fig. 3b, Rf 0.72). (b) Over 90% of the radioactivity was rendered water-soluble following mild acid hydrolysis of the mannolipid. Paper chromatography of the water phase in solvent G revealed that the sugar was mannose (not shown). (c) As expected for a negatively charged molecule, the mannolipid quantitatively bound to the anion-exchanger DE52 cellulose and could be eluted with 10 mM ammonium formate in the form of a single, symmetrical peak of radioactivity. A typical elution profile is shown in Fig. 4. Essentially similar results were observed when binding to the anion-exchanger was measured by batch chromatography as
Fig. 2. Paper chromatography of products released after β-elimination of mannoproteins. A sample of radiolabelled mannoproteins synthesized during a 30 min incubation period in an experiment similar to that shown in Fig. 6 was subjected to β-elimination. The hydrolysate was neutralized and analysed by descending paper chromatography in solvent G as described in Methods. The position of mannose (Man) and maltose (Mal) standards is indicated. Under these conditions, a trisaccharide such as raffinose should migrate to a distance around 8–9 cm. Mannose was also identified as the sole radioactive product in hydrolysates obtained after β-elimination of mannoproteins from experiments similar to that shown in Fig. 1(c).

Fig. 3. TLC analysis of the mannolipid in three different solvents. A sample of the radiolabelled mannolipid dissolved in solvent A was streaked along a 1 cm line 2 cm from the edge in Silica Gel 60 plates which were developed in solvent B (a), C (b) or H (c) as described in Methods. Mean Rf values from two separate analyses were 0.28, 0.72 and 0.94 in solvents B, C and H, respectively.

Fig. 4. Anion-exchange chromatography of mannolipid. A sample of the radiolabelled lipid carrier was layered on the top of a column (1 × 7 cm) of DE52 cellulose equilibrated with solvent F. The column was washed with the same solvent and the charged lipid was eluted with 10 mM ammonium formate in solvent F as indicated. Elution of radioactivity was monitored by scintillation counting of 200 µl aliquots of each fraction. The elution profile shown is representative of at least three experiments. Binding of the mannolipid to the exchanger was also assessed by batch chromatography as indicated in the text.

indicated in Methods. (d) Amphotomycin is a lipopeptide antibiotic that inhibits Dol-P specific glycosyltransferases by forming an inactive complex with the phosphorylated lipid in a reaction enhanced by calcium (Kang et al., 1978; Banerjee, 1989). In this study, the drug blocked Dol-P-Man formation as well as the transfer of mannose to the protein in a manner proportional to its concentration (see below).

Effect of exogenous Dol-P and GDP-Man concentration on Dol-P-Man synthase

It has been described that exogenous Dol-P activates Dol-P-Man synthesis in yeast (Schützbach et al., 1993) and mammalian (Heifetz & Elbein, 1977; Jensen & Schützbach, 1985) enzyme preparations. In agreement with these observations, addition of increasing amounts of a commercial sample of the polyisoprenoid phosphate to our assay mixtures brought about a proportional stimulation of Dol-P-Man formation. In the experiment depicted in Fig. 5, maximum stimulation (four- to fivefold) was observed with 80 µg of the acceptor lipid in the assay mixture. It is worth noting that the optimum concentration of Dol-P may vary with different MMF batches depending on the endogenous levels of the acceptor lipid, which remained undetermined in this study. A Kₘ of 0.3 µM for Dol-P has been reported for a purified mammalian Dol-P-Man synthase (Jensen & Schützbach, 1985). Because of the crude nature of the extracts used in this study, no attempt was made to calculate this constant.

The question then arose whether the MMF had the ability to use exogenous Dol-P-Man as a sugar donor. To investigate this, a known amount (5–10 x 10⁶ c.p.m.) of a
Mannosyltransferases in *Candida albicans*

Dol-P-Man preparation partially purified by DE52 chromatography was dispersed in 0.025% Nonidet P-40 and incubated with the MMF under standard conditions except for the absence of GDP-Man. In several experiments, incorporation of the label into mannoproteins never exceeded 6–7% of the supplied radioactivity after 15–30 min incubation (not shown). These findings suggest that the topology and mechanism of operation of protein mannosyltransferases in the membrane domain may impose restrictions upon the recognition and utilization of exogenously added mannolipid as sugar donor. This implies further that a close functional relationship between Dol-P-Man synthase and protein mannosyltransferases must exist in the membrane for efficient protein mannosylation to occur.

When Dol-P-Man synthase activity was measured as a function of increasing concentrations of GDP-Man, a typical Michaelian kinetic plot was obtained (not shown). A *K*ₘ value of 0.36 μM for the sugar donor was estimated from the double reciprocal plots of data. Some reported *K*ₘ values are 0.4 μM, 0.69 μM and 2.7 μM for, respectively, the Dol-P-Man synthases from porcine aorta (Heifetz & Elbein, 1977) and rat liver microsomes (Jensen & Schutzbach, 1985), and the purified yeast enzyme reconstituted in phospholipids (Schutzbach et al., 1993).

**Time course of Dol-P-Man synthesis, protein mannosylation and LLO formation**

We next determined the kinetics of both lipid and protein mannosylation as a function of time of incubation. Following incubation of membranes with GDP-[¹⁴C]Man, synthesis of Dol-P-Man was paralleled by protein mannosylation up to 30 min. Thereafter, and coinciding with the time at which Dol-P-Man levels reached a steady value, labelled mannoproteins rapidly accumulated, reaching values 1.8- and 2.6-fold higher than those of the mannolipid, after 45 and 60 min incubation, respectively (Fig. 6). In this experiment, mannoproteins obtained after the different periods of incubation were also subjected to β-elimination as described in the legend to Fig. 1. Results were essentially the same in terms of their susceptibility to this treatment (not shown). LLO were detected in low but significant amounts but they remained at fairly constant levels throughout the experimental period. These species may represent endogenous N-linked intermediates that grew further following incubation of membranes with the mannose donor.

**Is all mannose detected in mannoproteins transferred through Dol-P-Man?**

To determine whether some of the sugar in mannoproteins may result from a direct transfer from GDP-Man, synthesis of Dol-P-Man by MMF was specifically blocked by increasing amounts of amphomycin, which inhibits glycosylation reactions involving Dol-P. Levels of labelled mannoproteins and LLO were determined in parallel. As illustrated in Fig. 7, the drug inhibited Dol-P-Man synthesis in a concentration-dependent manner. Values of inhibition were 51%, 63% and 89% with 25, 50 and 100 μg of amphomycin, respectively. This effect was concomitant with a parallel decrease in the levels of labelled mannoproteins. It should be noted, however, that a small amount of labelled proteins was detected even in the presence of 200 μg of the antibiotic, a condition fully inhibitory of Dol-P-Man synthesis. LLO remained at trace, constant levels throughout the experimental period. These results indicate that in our assay conditions most, if not all, mannose residues are transferred to proteins via Dol-P-Man.

---

Fig. 5. Effect of exogenous Dol-P on Dol-P-Man synthase activity. Synthesis of Dol-P-Man was measured as described in Methods as a function of increasing concentration of Dol-P. All other components of the assay mixture remained unchanged. After 30 min at 28°C, radioactivity incorporated into mannolipid was measured.

Fig. 6. Time course of incorporation of mannose from GDP-Man into mannolipid (○), mannoproteins (□) and LLO (△). MMF was incubated at 28°C with the sugar donor in standard reaction mixtures. After the indicated times, radioactivity incorporated into the different products was determined as described in Methods. Results shown are representative of two independent experiments.

---

2293
Although no experiments were done to associate the enzyme with a particular membrane system, it is likely to be located in the endoplasmic reticulum as is the case in other systems and in particular in S. cerevisiae. Attempts to solubilize, purify and characterize Dol-P-Man synthase from C. albicans membranes are currently in progress in this laboratory. Characterization of purified enzyme preparations will lead to a better understanding of enzyme properties and regulation.

ACKNOWLEDGEMENTS

This work was partially supported by grants from SEP and CONACyT, Mexico. Thanks are given to Oswaldo Harris for the preparation of the figures and to QBP Ana Gómez for technical assistance.

REFERENCES


Received 31 January 1995; revised 24 April 1995; accepted 11 May 1995.