Topology of nucleotide-sugar:dolichyl phosphate glycosyltransferases involved in the dolichol pathway for protein glycosylation in native rat liver microsomes

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Activities of nucleotide-sugar:dolichyl phosphate glycosyltransferases (UDP-*N*-acetylglucosamine:dolichyl phosphate *N*-acetylglucosaminyl 1-phosphotransferase, UDP-glucose:dolichyl phosphate glucosyltransferase and GDP-mannose:dolichyl phosphate mannosyltransferase) are not fully expressed in native microsomes and can be enhanced by pretreatment of the microsomes with detergent. To examine whether the latency of dolichyl phosphate glycosyltransferases in native microsomes reflects a luminal orientation of the catalytic centre, we examined the effect of proteinase treatment of native microsomes on enzymic activity and investigated the relationship between enzymic activity and alteration of the permeability of the microsomal membrane barrier. The enzymic activities catalysing transfer of *N*-acetylglucosamine and glucose to lipid acceptors

were proteinase-sensitive in native sealed microsomes. When various detergents were used to disrupt the membrane barrier, we found no relationship between activity of dolichyl phosphate glycosyltransferases and the latency of mannose-6-phosphatase, which is a marker of the permeability properties of the microsomal membrane. Permeabilization of the endoplasmic reticulum membrane by the pore-forming *Staphylococcus aureus* α -toxin did not affect glycosyltransferase activities. These results do not support the hypothesis that latency of the transferase activities is dependent on the permeability properties of the endoplasmic-reticulum membrane. Collectively our findings can best be explained by postulating that the active centres of the transferases are cytoplasmically oriented, while activation by detergent may be conformation-dependent.

INTRODUCTION

Asparagine-linked glycosylation involves *en bloc* transfer of the preformed branched tetradecasaccharide (GlcNAc₂Man₉Glc₃), the major glycosylation precursor, linked to a polyisoprenoid carrier (dolichyl pyrophosphate, Dol-PP), to a nascent polypeptide chain. *N*-Acetylglucosamine and the first five mannose residues are added directly from UDP-*N*-acetylglucosamine (UDP-GlcNAc) and GDP-mannose (GDP-Man) to the lipid-linked carrier. These nucleotide-sugar donor substrates are synthesized in the cytosol. Dolichyl phosphate mannose (Dol-P-Man) and dolichyl phosphate glucose (Dol-P-Glc) serve as donor substrates for the addition of all glucose and the additional four mannose moieties to the growing oligosaccharide chain. The reactions of this dolichol cycle occur in the endoplasmic reticulum (ER) [for reviews, see Presper and Heath (1983), Kornfeld and Kornfeld (1985) and Roth (1987)]. Dol-P-Glc also acts as natural glucosyl donor for bile acid glucosidation (Matern and Matern, 1987). Dol-P-Man is involved in O-mannosylation (Tanner and Lehle, 1987) and in formation of a glycosylphosphoinositol anchor (DeGasperi et al., 1990).

Although it is now widely accepted that the oligosaccharide pyrophosphate dolichol is present on the luminal face of the rough-ER membranes (Katz et al., 1977; Lingappa et al., 1978; Hanover and Lennarz, 1980; Snider and Robbins, 1982; Snider and Rogers, 1984), there continues to be controversy regarding the topological organization of the initiating steps of the dolichol

pathway and of the reactions leading to formation of Dol-P-Glc and Dol-P-Man. It has been speculated that the active centres of Dol-P *N*-acetylglucosaminyl 1-phosphotransferase, *N*-acetylglucosaminyltransferase, glucosyltransferase and mannosyltransferase, which catalyse the synthesis of, respectively, dolichyl pyrophosphate *N*-acetylglucosamine (Dol-PP-GlcNAc) and dolichyl pyrophosphate *NN'*-diacetylchitobiose [Dol-PP-(GlcNAc)₂], Dol-P-Glc and Dol-P-Man reside on the cytoplasmic side of the ER membrane (Snider et al., 1980; Haselbeck and Tanner, 1982; Spiro and Spiro, 1985; Clarke et al., 1989; Abeijon and Hirschberg, 1990; Kean, 1991; Drake et al., 1992). The opposite view holds a luminal orientation of Dol-PP-(GlcNAc)₂ (Hanover and Lennarz, 1978, 1982; Godelaine et al., 1983; Perez and Hirschberg, 1986), glucosyltransferase (Perez and Hirschberg, 1986; Clarke et al., 1989) and mannosyltransferase (Deglon et al., 1991). It should be pointed out that demonstration of carrier-mediated transport for UDP-GlcNAc (Perez and Hirschberg, 1985) and UDP-Glc (Perez and Hirschberg, 1986; Vanstapel and Blanckaert, 1988a) across the ER membrane does not validate a deductive inference concerning the sidedness of the active sites of glycosyltransferases. The cDNA sequences encoding hamster *N*-acetylglucosamine 1-phosphotransferase (Zhu and Lehrman, 1990) and mannosyltransferase (Orlean et al., 1988) have recently become available. Such primary-structure information suggests that these enzymes most likely correspond to integral membrane proteins with membrane-spanning regions. However, there is as yet no con-

Abbreviations used: CHAPSO, 3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; Dol-P, dolichyl phosphate; Dol-P-Glc, dolichyl phosphate glucose; Dol-P-Man, dolichyl phosphate mannose; Dol-PP-GlcNAc, dolichyl pyrophosphate *N*-acetylglucosamine; Dol-PP-(GlcNAc)₂, dolichyl pyrophosphate *NN'*-diacetylchitobiose; glucosyltransferase, UDP-glucose dolichyl phosphate glucosyltransferase (EC 2.4.1.117); *N*-acetylglucosaminyltransferase, UDP-*N*-acetylglucosamine:dolichyl pyrophosphate *N*-acetylglucosamine *N*-acetylglucosaminyltransferase (EC 2.4.1.141); *N*-acetylglucosamine 1-phosphotransferase, UDP-*N*-acetylglucosamine:dolichyl phosphate *N*-acetylglucosamine 1-phosphotransferase (EC 2.7.8.15); ER, endoplasmic reticulum; mannosyltransferase, GDP-mannose:dolichyl phosphate mannosyltransferase (EC 2.4.1.83); PtdCho, L- α -phosphatidylcholine.

clusive evidence available on whether the catalytic site for these enzymes is exposed on the luminal or on the cytoplasmic side of the ER membrane.

To examine the topology of the active sites of the glycosyltransferases, we disrupted the structural integrity of the ER membranes by treatment of microsomes with either detergent or the pore-forming *Staphylococcus aureus* α -toxin. The aim of this approach was to examine a possible relationship between latency of Dol-*P* glycosyltransferases and mannose-6-phosphatase, for which a luminal orientation in the microsomal membrane is well established. We found that latency was preserved in permeabilized microsomes, and that there is no relationship between latency of the Dol-*P* glycosyltransferases and latency of mannose-6-phosphatase. These findings fail to support a luminal orientation of the Dol-*P* glycosyltransferases. On the basis of the results of additional proteinase-sensitivity studies, we postulate that the active centres of the various Dol-*P* glycosyltransferases are exposed at the cytoplasmic side of the ER.

MATERIALS AND METHODS

Chemicals

UDP-[³H]GlcNAc (699.3 GBq/mmol) was purchased from New England Nuclear Co. (Boston, MA, U.S.A.). UDP-[³H]Glc (540 GBq/mmol) and GDP-[¹⁴C]Man (11.1 GBq/mmol) were from Amersham (Amersham, Bucks., U.K.). UDP-GlcNAc sodium salt, GDP-Man sodium salt, UDP-Glc sodium salt, L- α -phosphatidylcholine (PtdCho) (from egg yolk), ATP, phosphocreatine, BSA (fraction V), Hepes and Dol-*P* (grade III; C₈₀-C₁₀₅) came from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Creatine kinase was obtained from Boehringer Mannheim Biochemicals. GSH was purchased from Jansen Chimica (Beerse, Belgium). 3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of reagent grade quality, and aqueous solutions were made with deionized water. *Staph. aureus* α -toxin (from strain Wood 46) was purified by a pore-glass-adsorption procedure (Harshman et al., 1988).

Preparation and treatment of microsomes

The microsomal fraction of male Wistar rat liver was prepared as described by Vanstapel et al. (1986). The final pellet was resuspended and washed with 0.25 M sucrose containing 5 mM Hepes and 1 mM EDTA (pH 7.4).

The native microsomes were exposed to various types of pretreatment. Disruption of microsomes by Triton X-100 or CHAPSO was done by preincubating the microsomes (1.05 mg of protein/ml) for 60 min at 0 °C in incubation medium (see below) containing the detergent. Permeabilization of the microsomal membrane using *Staph. aureus* α -toxin (125 μ g of α -toxin/mg of protein) was done as outlined elsewhere (Vanstapel et al., 1987). For treatment with proteinase, microsomes (1.05 mg of protein/ml) were incubated at 37 °C for 30 min with subtilisin (0.25 mg of proteinase/mg of microsomal protein).

Preparation of liposomes

To avoid disruption of the microsomes by detergent, the Dol-*P* acceptor substrate was solubilized with PtdCho liposomes. A mixture of Dol-*P*/PtdCho (1:25, w/w) was exposed at ambient temperature to a stream of nitrogen to evaporate organic solvent. The dried lipid was hydrated in deionized water and sonicated by

using an exponential probe sonicator (20 kHz, amplitude 12 μ m), twice for 20 s, to obtain a dispersion of liposomes.

Dol-*P* glycosyltransferase assays

Incorporation of *N*-acetylglucosamine into lipid acceptors

Microsomes (1 mg of protein/ml) were incubated at 37 °C with UDP-[³H]GlcNAc and Dol-*P* in 400 μ l of medium containing the following components: 20 mM Hepes, 140 mM potassium glutamate, 5 mM GSH, 0.5 mM EGTA, 9 mM MgCl₂, 5% dextran (w/v), 2.5 mM MnCl₂, radiolabelled nucleotide sugar, Dol-*P* and an ATP-regenerating system consisting of 3 mM ATP, 7 mM phosphocreatine and 0.05 mg/ml creatine kinase (added after adjustment of pH to 7.6 with KOH). The Dol-*P* concentration was 40 μ g/ml unless otherwise specified in the legends to Figures and Tables. Reactions were started by the addition of 20 μ l of UDP-[³H]GlcNAc (14 μ M, unless stated otherwise). Incubation was continued at 37 °C for up to 6 min, with withdrawal of two 150 μ l portions at 3 min intervals. Each portion was transferred without delay to 1.5 ml of ice-cold chloroform/methanol (3:2, v/v), with subsequent addition of 240 μ l of 4 mM MgCl₂, as described by Godelaine et al. (1977). After mixing thoroughly, the phases were separated by centrifugation. The aqueous upper layer was removed, and the lower layer was washed twice with 750 μ l of chloroform/methanol/4 mM MgCl₂ (1:16:16, by vol.).

Thereafter, 600 μ l of the lower organic phase was pipetted into a counting vial. The solvent was evaporated to dryness, and the radioactivity was measured in a Packard 1900CA liquid-scintillation counter (Packard Instruments, Downers Grove, IL, U.S.A.).

Glucosyltransferase assay

The assay conditions were as described for incorporation of *N*-acetylglucosamine into lipid acceptors, except that Mn²⁺ was omitted from the reaction mixture. The Dol-*P* concentration was 80 μ g/ml unless stated otherwise. Reactions were started by addition of UDP-[³H]Glc (5 μ M, unless specified otherwise).

Mannosyltransferase assay

We used the same incubation conditions as for the glucosyltransferase assay, except that the reaction was initiated with GDP-[³H]Man (5 μ M, unless specified otherwise). The Dol-*P* concentration was 50 μ g/ml, unless stated otherwise. Reactions were terminated after 30 s and 60 s.

In all three glycosyltransferase assays, product formation curves were non-linear. Initial rates were estimated by calculation of the second-degree polynomial equation which fitted the product formation curve as a function of incubation time. Enzymic activities, which represent formation of labelled sugar incorporated in lipid species, are expressed as pmol of monosaccharides transferred from corresponding nucleotide diphosphate sugars to Dol-*P* by 1 mg of protein and after 1 min of incubation.

Uptake assay

Uptake of radiolabelled substrate into microsomal vesicles was assayed by an ultrafiltration technique, as described by Vanstapel and Blanckaert (1988a). The uptake medium and stop buffer contained 150 mM KCl, 6 mM MgCl₂, 250 mM sucrose and 10 mM Hepes.

Kinetic and statistical analysis

Experimental data were fitted and kinetic parameters determined by the non-linear regression data-analysis program ENZFITTER (Elsevier Biosoft, Cambridge, U.K.).

All results are expressed as means $\pm$ S.E.M. for the number of experiments indicated. Differences between means were analysed by Student's *t* test.

Other assays and analyses

When labile phosphate esters (e.g. ATP) were present in the incubation medium, we used a radioactive assay for measurement of mannose-6-phosphatase activity (Kitcher et al., 1978). [3 H]Mannose 6-phosphate was prepared by ATP-dependent phosphorylation of [3 H]mannose catalysed by hexokinase, as described by Koerner et al. (1974). Total protein concentration was determined by the protein-Coomassie Blue dye-binding method using BSA for assay calibration (Bradford, 1976).

RESULTS AND DISCUSSION

Intactness of the microsomal preparations was verified by measurement of mannose-6-phosphatase latency

Nucleotide sugars cannot freely permeate across hydrophobic membranes. The ER membrane can, on that ground, constitute a major determinant of the regulation of membrane-bound enzymes. Therefore it was imperative to verify systematically the intactness of the membrane permeability barrier and to confirm the correct topological orientation of the microsomal preparations. Structural integrity of the ER vesicles was routinely assessed by assaying the latency of mannose-6-phosphatase activity (Vanstapel et al., 1986). In our native untreated microsomal preparations, the mannose-6-phosphatase latency was consistently $> 95\%$ ($97.72 \pm 0.05\%$, $n = 9$), indicating that over 95% of the vesicles were sealed.

Dol-P supplemented via PtdCho liposomes enhances activity of Dol-P glycosyltransferases in native microsomes

Endogenous Dol-P is present at low concentration in the ER (0.3 $\mu\text{g/ml}$) (Chojnacki and Dallner, 1988) and limits the rate of mannosyl transfer (Richards and Hemming, 1972; Tkacz et al., 1974), of glucosyl transfer (Behrens and Leloir, 1970; Herscovics et al., 1977) and of Dol-PP-GlcNAc formation (Ghalambor et al., 1974). With few exceptions, previous studies of the Dol-P glycosyltransferases have used detergent for dispersion of exogenous Dol-P, added to reach a near-saturating substrate concentration. Such experimental approach obviously prohibits examination of the enzymic properties of the glycosyltransferases in native sealed microsomes. To avoid this problem, we supplemented the lipid acceptor to the reaction medium by dissolution of Dol-P in phospholipid vesicles. It has been argued that such approach is the method of choice for introduction of amphipaths into incubation mixtures (Heirwegh, 1992).

It has been reported that addition of PtdCho does not change mannosyl- (Jensen and Schutzbach, 1985) and *N*-acetylglucosaminyl 1-phosphotransferase activity (Chandra et al., 1991). We found that PtdCho liposomes exerted no statistically significant ($P > 0.3$) stimulatory effect on *N*-acetylglucosamine, glucose and mannose incorporation in microsomes. In assay mixtures without added liposomes and in incubation mixtures that were supplemented with PtdCho liposomes (1 mg/ml), *N*-acetylglucosamine incorporation was, respectively, 4.3 ± 4 pmol/min per mg of protein ($n = 8$) and 4.3 ± 0.5 pmol/min per mg of protein ($n = 8$); glucose incorporation was, respectively,

Figure 1 Kinetic analysis of initial rates of glucosyl- and mannosyl-transferase activities as a function of exogenous Dol-P concentration and of nucleotide-sugar concentration

Initial rates of Dol-P-Glc and Dol-P-Man formation were measured in native microsomes incubated with various concentrations of exogenous Dol-P at a fixed 5 μM concentration of UDP-Glc (a) or GDP-Man (b). UDP-Glc (c) and GDP-Man (d) was varied at a fixed concentration of Dol-P (91 μg of Dol-P/ml for glucosyltransferase and 80 μg of Dol-P/ml for mannosyltransferase). Results are means $\pm$ S.E.M. for four to six determinations in three to four separate microsomal membrane preparations. The initial reaction rate, v_i , is expressed in pmol/min per mg of protein.

6.7 ± 0.4 pmol/min per mg of protein ($n = 13$) and 7.6 ± 0.7 pmol/min per mg of protein ($n = 20$); and mannose incorporation was, respectively, 28 ± 5 pmol/min per mg of protein ($n = 8$) and 33 ± 5 pmol/min per mg of protein ($n = 8$).

Figures 1(a) and 1(b) show how the initial rates of glucose and mannose incorporation in lipid acceptors depended on the concentration of exogenously added Dol-P. The estimated apparent $K_m^{\text{Dol-P}}$ values for glucosyltransferase and mannosyltransferase were, respectively, 33 ± 4 $\mu\text{g/ml}$ ($n = 6$) and 38 ± 8 $\mu\text{g/ml}$ ($n = 4$). Kinetic analysis of microsomal glucosyl- and mannosyl-transferase was also performed by varying the nucleotide sugar concentration in the presence of a fixed Dol-P concentration, added via liposomes (Figures 1c and 1d). For glucosyl- and mannosyl-transferase, the apparent V_{max} values were, respectively, 88 ± 12 pmol/min per mg of protein ($n = 5$) and 2.19 ± 0.22 nmol/min per mg protein ($n = 4$). The corresponding apparent $K_m^{\text{nucleotide-sugar}}$ values were, respectively, 1.9 ± 0.3 μM ($n = 5$) and 3.5 ± 0.5 μM ($n = 4$). Kinetic experiments dealing with *N*-acetylglucosamine incorporation in native microsomes are presented in the accompanying paper (Bossuyt and Blanckaert, 1993).

Effect of proteinase treatment on Dol-P glycosyltransferase activities

In a first approach to investigate the topology of Dol-P glycosyltransferases, we assessed the effect of subtilisin on transferase activities in intact microsomes. If native sealed

Table 1 Effect of proteinase treatment on glycosyltransferases in native microsomes

Native microsomes were preincubated in the presence or absence of subtilisin (see the Materials and methods section), and initial rates of incorporation of *N*-acetylglucosamine, glucose and mannose into lipid acceptors were assessed. Data from four different microsomal preparations are presented as means $\pm$ S.E.M. for the number of determinations shown in parentheses. Where indicated by an asterisk the mean is statistically significant different ($P < 0.0002$) from mean of control microsomes.

Preincubation of the microsomes	Dol- <i>P</i> glycosyltransferase activities (pmol/min per mg of protein)		
	GlcNAc transfer	Glc transfer	Man transfer
Without subtilisin	37 $\pm$ 4 (8)	35 $\pm$ 2 (7)	1188 $\pm$ 35 (7)
With subtilisin	4 $\pm$ 1 (8)*	4 $\pm$ 1 (7)*	1313 $\pm$ 109 (7)

microsomes are sensitive to the proteinase, which is an impermeant probe, then this will suggest that the enzyme's active centre is exposed at the cytosolic side of the ER membrane. Table 1 shows that the transfer to Dol-*P* of glucose and of *N*-acetylglucosamine, but not of mannose, was sensitive to subtilisin

treatment. Subtilisin equally failed to affect mannosyltransferase when the microsomes were disrupted by treatment with Triton X-100 (0.025 % by vol.) (results not shown; $n = 4$). These findings support a cytoplasmic localization of the catalytic sites involved in incorporation of *N*-acetylglucosamine and glucose into microsomes. Because the mannosyltransferase was insensitive to subtilisin in both sealed and disrupted microsomes, no topological information could be obtained for this enzyme by such approach. It should be noted that the results of proteinase experiments cannot lead to definitive conclusions with regard to enzyme topology. Proteinases can affect (i) enzymes with cytoplasmic catalytic sites, (ii) enzymes with transmembrane catalytic sites or (iii) transmembrane enzymes with luminal active sites and cytoplasmic proteinase-sensitive domains. Therefore complementary evidence was sought by investigating the effects of various detergents and of the pore-forming *Staph. aureus* α -toxin and on the Dol-*P* glycosyltransferases.

Lack of correlation between latency of Dol-*P* glycosyltransferases and latency of a luminal marker enzyme

Detergent activation profiles were investigated to determine whether transferase latency was a direct function of membrane integrity. We tested two detergents: Triton X-100, a non-ionic detergent which is commonly used to study Dol-*P* glycosyl-

Figure 2 Effect of detergents on activities of microsomal Dol-*P* glycosyltransferases and of mannose-6-phosphatase

Incorporation of *N*-acetylglucosamine (○), glucose (□) and mannose (△) was determined after treatment of the microsomes with either Triton X-100 or CHAPS. The final detergent concentration was as specified along the abscissa. In all parts of the Figure corresponding mannose-6-phosphatase activities are shown by ■. Activities of mannose-6-phosphatase and of Dol-*P* glycosyltransferases are indicated on the right and left ordinates respectively. Values are means $\pm$ S.E.M. of four determinations (mannose-6-phosphatase activity, *N*-acetylglucosamine and glucose incorporation), seven determinations (CHAPS profile of mannose incorporation) and ten determinations (Triton X-100 profile of mannose incorporation) in four different microsomal membrane preparations.

transferases, and the zwitterionic detergent CHAPSO. Membrane permeability properties were assessed by measuring mannose-6-phosphatase activity.

Our results in Figure 2 show that the latency of mannose-6-phosphatase vanished at CHAPSO concentrations above 2 mM, where a plateau was reached. By contrast, CHAPSO concentrations that were optimal for mannose-6-phosphatase activity exerted an inhibitory effect on the Dol-*P* glycosyltransferases. Secondly, Triton X-100 markedly augmented the transferase activities. The Triton X-100 concentration at which transfer of *N*-acetylglucosamine, glucose and mannose to lipid acceptors was maximally stimulated were, respectively, 0.25%, 0.016% and 0.025% (all v/v). For comparison, the highest mannose-6-phosphatase activity was reached at 0.05%.

These results show that, for each of the transferases, no direct relationship could be established between 'activation' of transferase activity and activity of the luminal marker enzyme mannose-6-phosphatase. Elimination of the membrane barrier does not seem to be a determinant of enzymic activity for any of the glycosyltransferases. It has been shown by others that the Triton X-100 concentration at which Dol-*PP*-(GlcNAc)₂ is formed at maximal rate is twice the concentration required for the release of esterase (Godelaine et al., 1983). Such detergent concentration causes extensive changes in the biochemical properties of microsomal vesicles and in their morphology (Kreibich et al., 1982). We speculate that the activation by detergent may be conformation-dependent. In addition to enhancing the microsomal membrane permeability, detergent treatment can stimulate enzymic activity of a microsomal enzyme by improving substrate availability, as has been described for diacylglycerol cholinephosphotransferase (Vance et al., 1977). In this manner, even enzymes with cytoplasmic disposition can exhibit latency.

***Staph. aureus* α -toxin permeabilizes the microsomal membrane for sucrose and nucleotide sugars**

In a third experimental approach we eliminated the membrane permeability barrier for nucleotide sugars by treating the microsomes with *Staph. aureus* α -toxin. This toxin forms hexameric aggregates delineating a pore in the membrane (Füssle et al., 1981) that allows free diffusion of small water-soluble compounds. Such permeabilization occurs without disruption of the membrane bilayer, as can be seen by electron microscopy (Lange and Arion, 1986). By contrast, elimination of the membrane permeability barrier by detergent leads to profound alteration of membrane structure, which may affect catalytic properties of membrane-imbedded enzymes.

The latency of mannose-6-phosphatase decreased with increasing *Staph. aureus* α -toxin concentration and was completely eliminated at 100 μ g of toxin/mg of microsomal protein (Figure 3), indicating that the toxin-treated microsomes had become freely permeable to mannose-6-phosphate. This conclusion can be extended to sugar-nucleotides, since mannose-6-phosphatase and UDP-glucuronyltransferase activities increased in parallel when the membrane barrier is permeabilized by the α -toxin (Vanstapel and Blanckaert, 1988b). Furthermore, pretreatment of native microsomes with α -toxin abolished endogenous conjugation of bilirubin by dissipation of an intravesicular pool of UDPGlc (Vanstapel and Blanckaert, 1987). These observations provide indirect evidence that the α -toxin permeabilizes the microsomal membrane for both UDP-GlcA and UDP-Glc.

Direct evidence that *Staph. aureus* α -toxin makes microsomal membranes pervious to small molecules was obtained as follows.

Figure 3 Effect of *Staph. aureus* α -toxin on mannose-6-phosphatase latency

Mannose-6-phosphatase activity was determined in microsomes pretreated with various concentrations of *Staph. aureus* α -toxin. Results are means $\pm$ S.E.M. for three different microsomal preparations.

Figure 4 Effect of *Staph. aureus* α -toxin on microsomal uptake of sucrose

Uptake of sucrose was determined in α -toxin-pretreated microsomes ($\bullet$) and in controls ($\blacktriangle$) in which no toxin had been added to the vesicles. Uptake was started by addition of [¹⁴C]sucrose (857 Bq), and the amount of vesicle-associated label was monitored as a function of time. Results are means $\pm$ S.E.M. of six determinations in three different microsomal preparations.

Permeabilization of the membrane should preclude microsomal retention of small substances for which the ER membrane constitutes a permeability barrier. Figure 4 illustrates that, upon incubation of native microsomes with radiolabelled sucrose, the disaccharide became associated with the vesicles in a time-dependent manner. By contrast, microsomes treated with *Staph. aureus* α -toxin were unable to retain sucrose. Similarly, we found that α -toxin-treated microsomes could retain neither UDP-Glc nor UDP-GlcNAc, whereas native microsomes took up and retained these nucleotide-sugars (results not shown). These findings unequivocally demonstrate that *Staph. aureus* α -toxin effectively permeabilized the ER membrane, thereby permitting free passage of nucleotide-sugar through pores created in the membrane.

Dol-*P* glycosyltransferases remain latent despite membrane permeabilization in *Staph. aureus* α -toxin-treated microsomes

If the catalytic centre of Dol-*P* glycosyltransferases were located at the luminal face of the microsomes and confined behind a

Table 2 Effect of permeabilization of microsomes by *Staph. aureus* α -toxin on Dol-P glycosyltransferases and mannose-6-phosphatase

Dol-P glycosyltransferase activities with UDP-GlcNAc, UDP-Glc, and GDP-Man were determined in native microsomes and in microsomes that had been subjected to α -toxin treatment (see the Materials and methods section). Mannose-6-phosphatase was assayed in parallel, using [3 H]mannose 6-phosphatase as substrate. Data from at least four different microsomal preparations are presented as means $\pm$ S.E.M. for the number of determinations in parentheses. When indicated by an asterisk ($P < 0.01$) or a double asterisk ($P < 0.0002$), the mean is statistically significant different from mean for control microsomes.

Enzyme	Treatment...	Activity (pmol/min per mg of protein)	
		None	α -Toxin
Dol-P glycosyltransferase			
GlcNAc transfer		39 $\pm$ 3.5 (8)	36 $\pm$ 2.5 (8)
Glc transfer		52 $\pm$ 4 (9)	49 $\pm$ 3 (9)
Man transfer		1259 $\pm$ 36 (9)	1069 $\pm$ 49 (9)*
[3 H]Mannose-6-phosphatase		0.66 $\pm$ 0.05 (9)	9.84 $\pm$ 0.5 (9)**

membrane barrier, α -toxin-pretreated microsomes should lose latency, since the co-substrate then would have free access to the enzyme's catalytic centre. The results shown in Table 2 demonstrate that permeabilization of the ER membrane by α -toxin had little or no influence on the glycosyltransferase activities. By contrast, mannose-6-phosphatase was $\pm$ 15-fold enhanced by membrane permeabilization.

These observations militate against a luminal disposition of the catalytic sites of glycosyltransferases. On the other and, they are consistent with the hypothesis that biosynthesis of Dol-P occurs at the cytoplasmic side of the ER (Adair and Cafmeyer, 1983).

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