Evidence that the synthesis of glucosylphosphodolichol in yeast involves a 35-kDa membrane protein

(dolichol intermediates/N-linked glycoproteins/endoplasmic reticulum)

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ABSTRACT In an effort to identify the polypeptide chain of glucosylphosphodolichol synthase (EC 2.4.1.117), yeast microsomal membranes were allowed to react with 5-azido[β - 32 P]UDPGlc, a photoactive analogue of UDPGlc, which is a substrate for this enzyme. Upon photolysis the 32 P-labeled probe was shown to link covalently to a 35-kDa protein present in microsomal membranes prepared from several wild-type yeast strains. Binding was either reduced or absent in the microsomal membranes from two yeast mutants (*alg5* and *dpg1*) that are known to be defective in the synthesis of glucosylphosphodolichol. The microsomes isolated from a heterozygous diploid strain *alg5::dpg1* generated from these two mutants exhibited partial restoration of both the ability to photolabel the 35-kDa protein and the ability to catalyze the synthesis of glucosylphosphodolichol. Microsomal membranes from a mutant strain that synthesized glucosylphosphodolichol but lacked the ability to transfer the glucosyl residue to the growing lipid-linked oligosaccharide (*alg6*) exhibited labeling with 5-azido[β - 32 P]UDPGlc comparable to that found in microsomes from the wild-type strain. In all cases photoinsertion of the probe into the 35-kDa protein correlated with the level of synthase assayed in the microsomal membranes. These results strongly support the conclusion that the 35-kDa protein labeled in these experiments is a component of glucosylphosphodolichol synthase.

Direct biochemical characterization of the membrane-associated enzymes involved in the assembly of dolichol-linked oligosaccharides that function as intermediates in the N-glycosylation that occurs in the endoplasmic reticulum has been hampered by rapid loss of enzyme activity after membrane solubilization. To obviate this problem associated with conventional purification, active-site-directed photoaffinity labeling was used with a component of avian oligosaccharyltransferase (1), and more recently it has been used with rat liver glucosylphosphodolichol (Glc-*P*-Dol) synthase (EC 2.4.1.117) (2). The later studies, using 5-azido[β - 32 P]UDPGlc to label rat liver microsomal membrane proteins, led to the proposal that a 35-kDa membrane protein was involved in the synthesis of Glc-*P*-Dol (2).

To confirm and extend these observations in rat liver we have turned to yeast, because mutants have been described that are defective in synthesis of Glc-*P*-Dol. We have studied the binding of the photoprobe 5-azido[β - 32 P]UDPGlc to microsomal membrane proteins from two strains with mutations affecting asparagine-linked glycosylation, *alg5* and *alg6*. The *alg5* mutant is impaired in the synthesis of Glc-*P*-Dol, whereas the *alg6* mutant is capable of synthesizing Glc-*P*-Dol but lacks the enzyme that catalyzes transfer of the glucosyl unit to the growing lipid-linked oligosaccharide chain (3). In addition, labeling has been studied in another mutant, *dpg1*,

in which synthesis of Glc-*P*-Dol is markedly reduced (4). The results of the studies with these mutants and the wild-type strains from which they were derived revealed a direct correlation between the extent of photolabeling of a 35-kDa membrane protein with 5-azido[β - 32 P]UDPGlc and the level of Glc-*P*-Dol synthase activity measured in yeast microsomes. These findings provide strong evidence for the involvement of this protein in synthesis of Glc-*P*-Dol.

EXPERIMENTAL PROCEDURES

Materials. *Saccharomyces cerevisiae* haploid parental strain DBY 640 and *alg5* and *alg6* mutants were obtained from the laboratory of P. Robbins (Dept. of Biology, Massachusetts Institute of Technology, Boston). Parental strain X2180, mutant X2180-*dpg1*, and the heterozygous diploid *alg5::dpg1* were kindly supplied by C. Ballou (Dept. of Biochemistry, University of California, Berkeley). 5-Azido[β - 32 P]UDPGlc was synthesized according to Drake *et al.* (5). Dolichol phosphate (Dol-*P*) was obtained from the Institute of Biochemistry and Biophysics (Warsaw, Poland). UDP[3 H]glucose (10 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. All other chemicals were from commercial sources.

Methods. Yeast strains X2180 and *dpg1* were grown at 28°C on YPD medium (0.5% yeast extract/1% peptone/2% glucose). Strains DBY 640, *alg5*, and *alg6* were grown on YPAD medium (YPD medium containing in addition adenine sulfate at 40 μ g/ml). In either case cells were grown to mid-logarithmic phase (when they reached a density of 10^7 – 10^8 cells per ml), harvested by centrifugation, and washed with water. The washed cells were resuspended in 100 mM Tris-HCl, pH 7.6, containing 10 mM dithiothreitol, incubated at 30°C for 30 min, centrifuged, and resuspended at 30°C for 30 min in YPD medium containing 1.2 M sorbitol, 10 mM dithiothreitol, and yeast lytic enzyme (ICN; 35 mg/10 g of yeast). Formation of spheroplasts was monitored spectrophotometrically. Microsomes were prepared as described earlier (6).

Microsomal membranes (150–200 μ g of protein) were incubated at room temperature for 10–15 sec with 5 μ M 5-azido[β - 32 P]UDPGlc in the presence of 10 mM MgCl₂/10 mM CDPcholine/50 mM Tris-HCl, pH 7.6, containing 15 mM sucrose and 25 mM NaCl in a total vol of 100 μ l. The mixture was then irradiated with 254-nm light for 2 min, using a 14-W Rayonet photochemical reactor lamp 4 cm from the membrane suspension. Subsequently the protein was precipitated

Abbreviation: Glc-*P*-Dol, glucosylphosphodolichol.

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with 7% perchloric acid and collected by centrifugation. The pellet was neutralized with NaOH and dissolved in sample buffer containing 4 M urea and 0.65 M 2-mercaptoethanol and subjected to SDS/PAGE on a 10% gel. The ^{32}P -labeled proteins were detected by autoradiography.

RESULTS

Photoaffinity Labeling of Yeast Microsomes with 5-Azido- $[\beta\text{-}^{32}\text{P}]\text{UDPGlc}$. Membrane preparations from two yeast mutants either lacking or containing reduced Glc-*P*-Dol synthase activity and their respective parental wild-type strains were irradiated in the presence of the photoprobe. Subsequent SDS/PAGE followed by autoradiography revealed that the predominant labeled species was a protein of molecular mass of 35 kDa. As shown in Fig. 1, this labeled protein was present in roughly equivalent amounts in the membranes from two wild-type strains—i.e., X2180 and DBY 640. A much lower level of labeling of the 35-kDa protein was observed in membranes from a mutant derived from X2180, *dpg1* (4), that is known to be impaired in Glc-*P*-Dol synthesis. In the case of the other mutant, *alg5* (3), in which synthesis of Glc-*P*-Dol is not detectable, no labeling of the 35-kDa protein was observed. As shown in Fig. 2a, probe was in apparent excess because its photoinsertion into the 35-kDa protein in the membranes from wild-type X2180 shows relatively little dependence on probe concentration in the range from 1 to 10 μM . In fact, it was found that increasing the concentration of probe up to 50 μM did not increase the labeling of the 35-kDa protein, although it did increase nonspecific binding to many proteins (data not shown). Similar results were obtained for the membranes from the *dpg1* mutant (Fig. 2b); the low level of labeling of the 35-kDa protein that did occur was not increased by increasing the probe concentration. Moreover, as shown in Fig. 2c, labeling of the 35-kDa protein in membranes of the *alg5* mutant could not be detected even when the concentration of the probe was varied from 1 to 10 μM .

Because it seemed possible that the lack of labeling of 35-kDa protein in the microsomal membranes of the *alg5*

FIG. 1. Binding of 5-azido $[\beta\text{-}^{32}\text{P}]\text{UDPGlc}$ to the 35-kDa protein in microsomal membranes from the parental wild-type yeast strains DBY 640 and X2180 and the two mutant strains *alg5* and *dpg1* that are defective in Glc-*P*-Dol synthesis. Positions of molecular mass markers are indicated by arrows.

FIG. 2. Effect of concentration of 5-azido $[\beta\text{-}^{32}\text{P}]\text{UDPGlc}$ on labeling of 35-kDa protein in microsomal membranes from X2180 wild type (a), *dpg1* mutant (b), and *alg5* mutant (c).

mutant strain could be caused by the absence of a functional regulatory protein affecting the entire asparagine-linked glycosylation pathway we examined labeling in microsomes from the *alg6* mutant, which synthesizes Glc-*P*-Dol but cannot transfer its glucosyl moiety to the growing oligosaccharide chain (3). As shown in Fig. 3, labeling of the 35-kDa protein was readily detected in membranes from the *alg6* mutant. In fact, the level of labeling was higher than that observed with membranes from the wild-type parental strain DBY 640. We also examined photoprobe binding to microsomal membranes from the heterozygous diploid containing the *alg5* and *dpg1* genes. The results shown in Fig. 3 indicated that the 35-kDa protein was labeled in the diploid strain and that the level of labeling was higher than that in either the *dpg1* or *alg5* mutant membranes (see Fig. 1), but it did not reach that found in the X2180 wild-type membranes.

Although binding of the probe to the 35-kDa protein in yeast membranes was abolished by increasing the concentration of unlabeled UDPGlc, it was unaffected by addition of its hydrolysis product glucose 1 phosphate (data not shown). On the basis of this observation it seems unlikely that labeling occurs via a labeled breakdown product of the 5-azido $[\beta\text{-}^{32}\text{P}]\text{UDPGlc}$. These results agree with the earlier finding of labeling of a 35-kDa protein in rat liver microsomes (2) with this photoprobe.

Dol-*P*-Glc Synthase Activity in the Membranes from Parental and Mutant Yeast Strains. The ability to synthesize Glc-*P*-Dol was assayed *in vitro* by incubation of microsomes with UDP $[\text{H}]\text{Glc}$ followed by isolation of the total lipid fraction and analysis of the labeled products by thin-layer chromatography. In agreement with earlier findings (4, 7), two products were synthesized in membranes of wild-type yeast, $[\text{H}]\text{Glc-}P\text{-Dol}$ and sterol $[\text{H}]\text{glucoside}$.

FIG. 3. Binding of photoprobe to 35-kDa protein in microsomal membranes from the *alg6* mutant compared with that in the heterozygous diploid (*alg5::dpg1*) and the X2180 wild type. The broad band at the top of the gel in the case of the diploid was not routinely observed.

The effect of exogenous Dol-P on the formation of [^3H]Glc-*P*-Dol in wild-type and mutant strains was studied. Because the formation of Glc-*P*-Dol in yeast was shown to be inhibited by detergents (7), exogenous Dol-*P* was added by brief sonication of the membranes in the presence of this lipid. The amount of labeled glucose incorporated into Glc-*P*-Dol was only slightly increased by addition of exogenous Dol-*P* to membranes from wild-type strains X2180 and DBY 640, and Dol-*P* had no stimulatory effect on the formation of Glc-*P*-Dol by the membranes from the *dpg1* and *alg5* mutant strains (data not shown). When dependence of Glc-*P*-Dol

FIG. 4. Dependence of Glc-*P*-Dol synthase activity in microsomal membranes from wild type and *dpg1* and *alg5* mutant strains on UDP[^3H]Glc concentration.

synthesis on the concentration of labeled UDP-Glc was studied (Fig. 4), it was found that at 0.5 μM UDP[^3H]Glc the activity of Glc-*P*-Dol synthase measured in the membranes from the *dpg1* mutant reached approximately 25% of that measured in the wild-type parental strain X2180. In contrast, no activity could be detected in the membranes from the *alg5* mutant strain, even when the sugar nucleotide concentration was varied over the same range. As shown in Fig. 5, although synthesis of both Glc-*P*-Dol and sterol glucoside was inhibited by unlabeled 5-azido-UDPGlc, the synthesis of the sterol derivative was much less sensitive to inhibition. Thus, at a 5-azido-UDPGlc concentration of 0.5 μM (the concentration routinely used for the photolabeling), the formation of labeled Glc-*P*-Dol was inhibited almost completely (Fig. 5*b*), whereas that of labeled sterol glucoside was blocked by only 50% (Fig. 5*a*). Similar results were observed when unlabeled UDP-Glc was used instead of unlabeled photoprobe.

The synthesis of Glc-*P*-Dol and sterol glucoside was measured in membrane preparations from various yeast strains. The results in Table 1 suggest that mutations affecting synthesis of Glc-*P*-Dol—i.e., *dpg1* and *alg5*—had little effect on the formation of sterol glucoside. Interestingly, high activity of Glc-*P*-Dol synthase was observed in the microsomal membranes from the *alg6* mutant. It is also clear that the enzyme activity in the membranes from a heterozygous diploid containing the *alg5* and *dpg1* genes was higher than that obtained with the membranes from the *dpg1* mutant strain, but lower than in the wild-type strain.

Effect of Permeabilization of the Yeast Microsomes. In agreement with earlier observations (7) formation of both Glc-*P*-Dol and sterol glucoside in yeast membrane preparations was inhibited by the detergent Nonidet P-40, even at a concentration as low as 0.0025%. The inhibition by these low concentrations of detergents was reversed by addition of exogenous Dol-*P*. Interestingly, this inhibitory effect of the detergent on enzyme activity was not accompanied by reduced binding of the photoprobe to the 35-kDa membrane protein (data not shown). Treatment with nystatin, a polyene antibiotic that interacts specifically with the ergosterol in yeast membranes (8), would be expected to increase the permeability of the membranes and allow entry of UDPGlc. However, it was found that nystatin at a concentration up to 2 $\mu\text{g}/\text{mg}$ of protein neither stimulated synthesis of Glc-*P*-Dol nor enhanced binding of the probe to the 35-kDa protein (data not shown). The lack of a stimulatory effect of nystatin on photoprobe binding was also observed with the membranes from the mutant strains that showed either reduced (*dpg1*) or no (*alg5*) Glc-*P*-Dol synthase activity.

DISCUSSION

In this study we have used a ^{32}P -labeled photoreactive derivative of one of the substrates for Glc-*P*-Dol synthase, 5-azido-UDPGlc, to identify polypeptide(s) involved in the assembly of this dolichol-linked intermediate. Photolysis of 5-azido[β - ^{32}P]UDPGlc in the presence of yeast microsomes resulted in specific labeling of a 35-kDa membrane protein. Photolabeling of the 35-kDa protein was markedly reduced in microsomal membranes from the *dpg1* mutant strain, which is known to be impaired in synthesis of Glc-*P*-Dol (4), and was undetectable in the *alg5* mutant, which is devoid of this synthetic activity (3). These results might be explained either by the absence or reduction in amount of the 35-kDa protein in the membranes of the mutants, or by an alteration in the protein that reduced its affinity for the photoprobe. However, the latter explanation seems unlikely, since with neither mutant did an increase in the concentration of 5-azido[β - ^{32}P]UDPGlc increase the level of protein labeling. In the case of the *alg5* mutant functional synthase appears to be absent, since neither photolabeling of 35-kDa protein nor enzymatic

FIG. 5. Effect of unlabeled 5-azido-UDPGlc or unlabeled UDPGlc on synthesis of sterol glucoside (a) and Glc-P-Dol (b).

formation of Glc-P-Dol could be detected. With respect to the *dpg1* mutant the situation is more complex. In microsomes from this mutant *in vitro* formation of Glc-P-Dol was approximately 2% of synthesis in the membranes of wild-type strain X2180. However, upon an increase in the concentration of UDP[³H]Glc to optimal the amount of Glc-P-Dol synthesized reached as much as 25–30% of that synthesized in membranes of the wild-type strain. On the basis of this observation, coupled with the finding that covalent attachment of the sugar nucleotide photoprobe to the 35-kDa protein was not increased upon increasing the concentration of either the probe or Dol-P, it is possible that in this *dpg1* mutant more than just the initial binding step is impaired.

We also briefly considered the possibility that the 35-kDa protein might be a common regulatory protein affecting the entire N-linked glycosylation pathway in yeast. If this were the case decreased labeling might also be expected in strains containing other mutations in the pathway. However, photolabeling of the 35-kDa protein was not reduced in membranes from the *alg6* mutant, which is defective in transfer of glucosyl units from Glc-P-Dol to the oligosaccharide (3). In fact, it exceeded that in the wild type, a finding that corresponds well with the observation of an increased amount of Glc-P-Dol synthesized in the microsomal membranes from the *alg6* mutant.

The *dpg1* and *alg5* mutations have been reported to be nonallelic, and the *dpg1* gene has been shown to complement the *alg5* gene (4). In the present work, microsomal membranes from an heterozygous diploid containing *alg5* and *dpg1* genes were shown to contain a 35-kDa protein capable

of binding 5-azido[β -³²P]UDPGlc. However, the level of synthesis of Glc-P-Dol in this heterozygous diploid was not restored to that of the X2180 wild type (*dpg1* parental strain), although it did approach that of the DBY 640 wild type (*alg5* parental strain). This partial recovery of the enzyme activity in the membranes from the heterozygous diploid strain suggests that Glc-P-Dol synthase may be a multimeric protein made up of nonidentical subunits. Perhaps in the *alg5* mutant one of the subunits of the Glc-P-Dol synthase complex is missing or defective, and therefore only approximately 50% of active enzyme is recovered.

In any case, the fact that the level of labeling of 35-kDa protein in microsomal membranes corresponds to the level of Glc-P-Dol synthase activity in all strains tested supports our assumption first made on the basis of experiments in liver microsomes (2). These studies led us to conclude that a 35-kDa protein is involved in the synthesis of Glc-P-Dol. Because enhancement of the permeability of microsomal membranes by treatment with low concentrations of either Nonidet P-40 or nystatin had no stimulatory effect on the binding of the photoprobe to 35-kDa protein, it seems likely that in yeast the sugar nucleotide binding site of this 35-kDa protein faces the cytoplasmic side of the microsomal vesicles. This is consistent with earlier reports (9, 10) suggesting that the substrate binding site of Glc-P-Dol synthase is cytoplasmically oriented. Based on all of the observations in this study the simplest working hypothesis at the present time is that the 35-kDa protein is an integral component of Glc-P-Dol synthase, rather than a UDPGlc-translocating protein. We hope that preparation of an antibody to this protein will lead to a better understanding of its structural organization and function in assembly of oligosaccharide chains destined for attachment to nascent polypeptides synthesized in the endoplasmic reticulum.

Table 1. Synthesis of sterol glucoside and Glc-P-Dol in various yeast strains

Yeast strain	Glucolipid formed,* cpm	
	Sterol-Glc	Glc-P-Dol
X2180	65,300	91,770
<i>dpg1</i>	38,330	1,900
DBY 640	135,330	21,290
<i>alg5</i>	112,600	380
<i>alg6</i>	14,101	132,925
<i>alg5::dpg1</i>	37,644	17,300

*Amount of glucolipid formed is expressed as cpm of [³H]glucose incorporated into glucolipid product by 1 mg of membrane protein in 10 min in the presence of 10 μg of Dol-P.

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