8-1992

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Original Publication Citation

Heller, L., Orlean, P., & Adair, W. L., Jr. (1992). Saccharomyces cerevisiae sec59 cells are deficient in dolichol kinase activity. *Proceedings of the National Academy of Sciences of the United States of America, 89*(15), 7013-7016. doi:10.1073/pnas.89.15.7013

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7013–7016, August 1992
Biochemistry

Saccharomyces cerevisiae sec59 cells are deficient in dolichol kinase activity

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Communicated by Phillips W. Robbins, April 3, 1992

ABSTRACT **The temperature-sensitive *Saccharomyces cerevisiae* mutant *sec59* accumulates inactive and incompletely glycosylated protein precursors in its endoplasmic reticulum at the restrictive temperature. O-mannosylation and glycosyl phosphatidylinositol membrane anchoring of protein are also abolished, consistent with a deficiency in dolichyl phosphate mannose. Membranes prepared from *sec59* cells that had been shifted to the restrictive temperature, however, made normal amounts of dolichyl phosphate mannose when exogenous dolichyl phosphate was supplied, but dolichyl phosphate mannose synthesis was severely depressed in the absence of exogenous dolichyl phosphate. Quantitative measurements of dolichyl phosphate in *sec59* cells showed that the levels were decreased to 48% of wild type at the permissive temperature and to <10% at the restrictive temperature. Assays of enzymes from the dolichyl phosphate synthetic pathway, *cis*-prenyltransferase and dolichyl pyrophosphate phosphatase, gave wild-type levels. However, dolichol kinase activity was greatly decreased. When *sec59* cells were transformed with a plasmid that overexpresses the wild-type gene, dolichol kinase activity increased 10-fold over wild-type levels. These results strongly suggest that the *sec59* gene encodes dolichol kinase.**

Saccharomyces cerevisiae secretory (*sec*) mutants stop dividing and become enlarged and dense at the restrictive temperature, 37°C (1), a property that allowed the selection of secretory mutants by density gradient centrifugation. The mutant *sec59* was isolated in this manner and characterized as a class B secretory mutant, one which accumulates inactive and incompletely glycosylated secretory proteins at the restrictive temperature (2). Protein synthesis, as measured by radiolabeling with $^{35}SO_4^{2-}$, remains normal for 2 hr while oligosaccharide synthesis as measured by [^{3}H]mannose incorporation is decreased (2). The peptide forms accumulated in the endoplasmic reticulum (ER) bear fewer oligosaccharide chains (2) and these are also shorter on average than those attached at the permissive temperature (3).

In addition to this effect on N-glycosylation, *sec59* cells are also completely blocked in O-mannosylation and in the synthesis of glycosyl phosphatidylinositol (GPI) membrane anchors (4, 5), processes that require dolichyl phosphate mannose (Dol-*P*-Man) as donor. In these respects, *sec59* cells have a very similar biochemical phenotype to that of the yeast class B mutant, *sec53*, which is defective in phosphomannomutase, and hence in GDP-mannose supply (6).

The gene complementing the *sec59* mutation was cloned from a YEp13 yeast genomic library and sequenced (3). A highly hydrophobic 59-kDa protein was predicted, containing a sequence resembling the putative dolichol binding region of three glycosyltransferases, Leu-Phe-Val-Xaa-Phe-Xaa-Xaa-Ile-Pro-Phe-Xaa-Phe-Tyr (7).

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The fact that the predicted *SEC59* gene product contains a putative dolichol binding region, as well as the apparent decrease in the levels of Dol-*P*-Man, suggested that the deficiency in *sec59* cells could be in an enzyme involved either in the synthesis of Dol-*P*-Man or in the turnover of Dol-*P*. To identify the defect in *sec59* cells, quantitation of Dol-*P* was performed, as well as assays of the enzymes involved in the synthesis of Dol-*P* and dolichol.

MATERIALS AND METHODS

Materials. *S. cerevisiae* haploid strain S288C (α *mal gal2*) was used as wild type. Mutant strains PRY132 (α *sec59*) and PRY134 (a *sec59 ura3-52*) were gifts from P. Robbins (Massachusetts Institute of Technology) and originally constructed in the laboratory of R. Schekman (2). Mutant strain α *sec59 ura3-52 leu2-3,112* was derived from PRY134. The plasmid pSEC5920 was a gift from R. Schekman (University of California, Berkeley).

Enzyme Assays. Cells were grown in minimal medium [0.17% yeast nitrogen base plus amino acids, 0.5% $(NH_4)_2SO_4$, and 2% dextrose with uracil (20 μg/ml) and/or leucine (30 μg/ml) as needed] or YPD medium (1% Bacto-yeast extract plus 2% Bacto-Peptone and 2% dextrose) to mid-logarithmic phase, washed, suspended in 25 mM Tris·HCl, pH 7.5/20 mM 2-mercaptoethanol (Tris/ME), and then broken by two passes through a French pressure cell (15,000 psi; 1 psi = 6.89 kPa) or by vortex mixing with glass beads. Cell walls and any unbroken cells were removed from the broken cell slurry by centrifugation at 2000 × *g*, and membranes were sedimented from the resulting supernatant by centrifugation at 100,000 × *g*. The pelleted membranes were resuspended in Tris/ME and the centrifugation step was repeated. Washed membranes were resuspended in Tris/ME and protein was determined by the method of Lowry *et al.* (8).

Dol-*P*-Man synthase activity was measured in washed yeast membranes (9). Incubation mixtures contained 0.3% (vol/vol) Triton X-100. When included, 20 μg of pig liver Dol-*P* (Sigma) was added to assay mixtures. Incubation was for 8 min at 37°C.

cis-Prenyltransferase (10) and dolichyl pyrophosphate (Dol-*PP*) phosphatase (11) were assayed as described. Dolichol kinase assays were modified from the procedure of Keller *et al.* (12). Membrane fractions were incubated in a total volume of 100 μl containing 0.05 M Tris·HCl (pH 7.5), 0.01 M UTP, 0.1 M $CaCl_2$, 30 μM [γ-^{32}P]CTP (5 Ci/mmol, ICN; 1 Ci = 37 GBq), 0.1% Triton X-100, and 2 μg of pig liver dolichol for 20 min at 24°C. The reaction was terminated by the addition of 750 μl of 1 M KOH in methanol and alkali-labile lipids were hydrolyzed by incubation at 37°C for 25

Abbreviations: Dol-*P*, dolichyl phosphate; Dol-*P*-Man, dolichyl phosphate mannose; Dol-*P*-Glc, dolichyl phosphate glucose; GPI, glycosyl phosphatidylinositol; ER, endoplasmic reticulum.

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min. This step is required to hydrolyze phosphatidic acid produced in a significant competing reaction (13). The lipids were extracted by the Folch procedure (14) and the organic phase was applied to a DEAE-cellulose column. Acidic lipids were eluted with 0.5 M ammonium acetate in chloroform/methanol (1:1, vol/vol) and ^{32}P incorporation into Dol-*P* was determined by scintillation counting in CytoScint (ICN).

Dol-*P* Quantitation. A modification of the protocol of Adair and Cafmeyer (10) was used. Cells were grown in YPD to an OD_{600} of ≈1, washed, and pelleted at 2000 × *g*. A standard of C_{60} polyprenyl phosphate (Sigma) was used. One gram (wet weight) of cells were boiled for 1 hr in 2 M KOH in 50% methanol and cooled to room temperature. The pH was adjusted to neutrality by the addition of glacial acetic acid. The cell debris was pelleted and extracted once with methanol and then with chloroform/methanol (1:1, vol/vol). The liquid phases were pooled with the original liquid phase and extracted by the method of Folch. One half volume methanol was added to the lower phase and this phase was applied to a DEAE-cellulose column. The DEAE-cellulose was washed with chloroform/methanol (2:1, vol/vol) and Dol-*P* was eluted with 0.3 M ammonium acetate in chloroform/methanol (2:1, vol/vol). Quantitation was by reverse-phase HPLC using a Brownlee RP18 Spheri-5 column. The solvent was 2-propanol/methanol (1:2.5) containing 0.1% H_3PO_4.

RESULTS

At nonpermissive temperature, *sec59* cells are blocked in three glycosylation pathways in which Dol-*P*-Man serves as mannosyl donor. O-mannosylation and GPI membrane anchoring are completely abolished in consequence, while the pattern of the Dol-*PP*-linked precursor oligosaccharides in N-glycosylation is abnormal and consists of $Man_{5-6}GlcNAc_2$ (3). Since addition of the mannosyl residues needed to form the full-length Dol-*PP*-linked precursor oligosaccharide is dependent on Dol-*P*-Man, a decrease in Dol-*P*-Man synthase activity could explain the truncated N-linked chains seen in *sec59* cells. We therefore determined first whether membranes prepared from *sec59* cells after a shift to the restrictive temperature had Dol-*P*-Man synthase activity. In the same experiment, we also used transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to Dol-*P* as a probe to assess levels of endogenous Dol-*P* in *sec59* membranes.

Membranes prepared from *sec59* cells that had been incubated at the restrictive temperature for 1 hr were fully capable of Dol-*P*-Man synthesis when exogenous Dol-*P* was provided as acceptor (Fig. 1, lane 4). The defect in *sec59* cells is therefore not in Dol-*P*-Man synthase, consistent with the failure of the gene encoding Dol-*P*-Man synthase, *DPM1*, to complement the temperature sensitivity of the *sec59* mutant when introduced into *sec59* cells on a high-copy plasmid (C. Albright and P.O., unpublished work). The activity seen in the presence of exogenous Dol-*P* confirmed the observations of Lehle, reported by Bernstein *et al.* (3). However, when no Dol-*P* was added to incubations, and transfer of mannose to *endogenous* Dol-*P* alone was assessed, membranes prepared from *sec59* cells with or without a shift to the restrictive temperature showed a striking difference. Whereas membranes from cells kept at permissive temperature made Dol-*P*-[^{14}C]Man (Fig. 1, lane 1), membranes prepared from cells that had been incubated at the restrictive temperature made no detectable Dol-*P*-[^{14}C]Man (lane 2), suggesting that they had become depleted of the endogenous Dol-*P* that would normally have served as [^{14}C]mannose acceptor. The same results were obtained when mannosyl transfer to endogenous Dol-*P* was measured in the absence of any detergent, so it is unlikely that the endogenous Dol-*P* had merely become inaccessible in the lumen of *sec59* microsomes. Control experiments with wild-type cells showed that the temperature shift itself did not result in this apparent depletion in Dol-*P*.

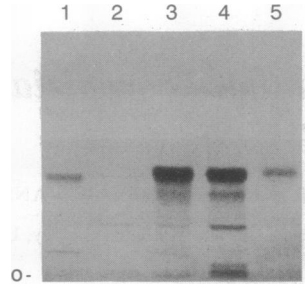

FIG. 1. Synthesis of Dol-*P*-[^{14}C]Man by *sec59* membranes. Washed membranes were prepared from *sec59* cells that had been incubated 24°C (lanes 1 and 3) or shifted to 38°C (lanes 2 and 4) for 1 hr. Synthesis of Dol-*P*-[^{14}C]Man was measured in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 20 μg of exogenous Dol-*P*. Radiolabeled lipids were extracted and separated by thin-layer chromatography on silica gel 60 with chloroform/methanol/water (65:25:4 by volume) as solvent (o, origin). Lane 5, Dol-*P*-[^{14}C]Man standard made by incubation of membranes from wild-type yeast with GDP-[^{14}C]mannose and mammalian Dol-*P*.

To show that levels of endogenous Dol-*P* are indeed much lower in membranes from *sec59* cells, Dol-*P* was extracted from wild-type and *sec59* cells grown at 24°C and from cells incubated for 2 hr at 38°C. At the permissive temperature of 24°C, Dol-*P* levels in *sec59* cells were 48% of those in wild-type cells (Table 1), but when *sec59* cells were shifted to their restrictive temperature of 38°C, their Dol-*P* levels decreased to <10% of wild-type levels. These results show unequivocally that *sec59* membranes are depleted of Dol-*P*. A concomitant increase in the level of free dolichol is not seen, however, because the level of free dolichol in *S. cerevisiae* is already 3- to 4-fold higher than that of the corresponding Dol-*P*. Current methods of dolichol analysis do not permit the predicted 25% increase in dolichol levels in *sec59* cells to be detected.

To pinpoint the defect in *sec59* membranes that leads to a decrease in Dol-*P* pools, the activities of enzymes involved in Dol-*P* synthesis and turnover were measured. In the *de novo* pathway for Dol-*P* synthesis, *cis*-prenyltransferase condenses farnesyl pyrophosphate and 11–15 isopentenyl pyrophosphate units in yeast to form polyprenyl pyrophosphate (10), while Dol-*PP* phosphatase dephosphorylates Dol-*PP* to Dol-*P*. Both of these enzymes would be predicted to contain an isoprenoid recognition sequence, as does the SEC59 protein. Neither *cis*-prenyltransferase nor Dol-*PP* phosphatase showed any decrease in activity in membranes prepared from *sec59* cells (Table 2), whether from cells shifted to the restrictive temperature or from cells kept at the permissive temperature. We therefore conclude that *sec59* cells are not defective in either of these two enzymes.

A third enzyme involved in dolichol metabolism that must recognize dolichol is dolichol kinase, which catalyzes the CTP-dependent phosphorylation of free dolichol to Dol-*P* (13), but whose exact role in dolichol metabolism is not clear. Dolichol kinase activity in membranes from the *sec59* mutant was 5% of wild-type levels in membranes from cells kept at permissive temperature and only 3% of wild-type levels in membranes from shifted cells (Table 3). While *in vitro* dolichol kinase activities are very low in membranes from

Table 1. Dolichyl phosphate levels in wild-type and *sec59* cells

Strain	Dol-*P*, μg per 10^{10} cells	
	24°C	38°C
S288C (wild type)	8.09 ± 1.12	10.24 ± 1.65
sec59 ura3-52 leu2-3,112	3.90 ± 1.36	0.79 ± 0.14

Logarithmic-phase cells were maintained at 24°C or shifted to 38°C for 2 hr and Dol-*P* was extracted and quantitated.

Table 2. *cis*-Prenyltransferase and Dol-*PP* phosphatase activity in wild-type (WT) and *sec59* cells

Strain	*cis*-Prenyltransferase		Dol-*PP* phosphatase	
	24°C	38°C	24°C	38°C
S288C (WT)	100	143	100	123
sec59 ura3-52	105	140	114	90

Logarithmic-phase cells were kept at 24°C or shifted to 38°C for 1 hr. Membrane fractions from these cells were assayed for *cis*-prenyltransferase and Dol-*PP* phosphatase activity. Values are expressed as percentages of WT activity at 24°C. Average WT activity (38°C): *cis*-prenyltransferase, 844 pmol/hr per mg of protein; Dol-*PP* phosphatase, 249 pmol/hr per mg of protein.

sec59 cells, levels of dolichol kinase activity *in vivo* in cells growing at permissive temperature are high enough to maintain growth. Since we have not directly measured *in vivo* dolichol kinase activity, this low *in vitro* activity may be an artifact incurred during the preparation of the enzyme.

These results show that *sec59* cells are defective in dolichol kinase, and lead to the prediction that the *SEC59* gene encodes dolichol kinase. Indeed, when the *sec59* strain was transformed with the multicopy plasmid pSEC5920, which bears the *SEC59* gene, the resulting transformants had dolichol kinase activity 10- to 14-fold higher than that of wild-type cells (Table 3). This overproduction of dolichol kinase activity, together with the presence of a dolichol recognition sequence in the SEC59 protein, is consistent with the notion that the *SEC59* gene encodes dolichol kinase.

DISCUSSION

We have shown that cells of the temperature-sensitive yeast secretion mutant *sec59* are defective in CTP-dependent dolichol kinase. Dolichol kinase activity was first detected by Allen *et al.* (15) in bovine liver, mouse plasmacytoma, and Chinese hamster ovary cells and was found to be CTP-dependent. The counterpart of this enzyme in *S. cerevisiae* was first described by Palamarczyk and coworkers (13, 16). The role of dolichol kinase in cellular metabolism, however, has been obscure. Specific dolichol kinase activities have been found to vary with stage of the cell cycle, during differentiation, and between different subcellular fractions. Thus, in mouse L1210 cells, dolichol kinase activity rises during the G_1 phase of the cell cycle to a maximum in S phase (17). The rate of Dol-*P* synthesis in developing sea urchin embryos increases 7-fold prior to gastrulation (18, 19), and dolichol kinase activity increases during estrogen-induced differentiation in chick oviduct cells (20), during maturation of erythrocytes (21), and during development of central nervous tissue (22, 23).

Table 3. Dolichol kinase activity

Strain	pmol/hr per mg of protein (%)	
	24°C	38°C
S288C (wild type)	58.9 ± 9.8 (100)	63.9 ± 19.5 (108)
sec59 ura3-52 leu2-3,112	3.1 ± 0.3 (5)	1.6 ± 0.1 (3)
sec59 ura3-52 leu2-3,112 containing pSEC5920	843.0 ± 57.2 (1431)	619.2 ± 29.8 (1051)

Cells were prepared as in Table 2 and assayed for dolichol kinase. Values in parentheses are percentages relative to wild-type activity at 24°C.

Studies of the subcellular distribution of dolichol kinase activity performed with calf brain showed that heavy microsomes had the highest specific activity (24). The membrane topography of dolichol kinase has been probed by using rat liver microsomes, and it was shown that the active site of the enzyme was located at the cytoplasmic surface of the microsomal membranes (25). These results, and the facts that Dol-*P* participates in glycosylation pathways in the ER, that a dolichol kinase deficiency blocks three ER glycosylation pathways, and that the secretory pathway is blocked at the level of the ER, lead to a working model in which dolichol kinase is localized predominantly in the ER membrane, where it catalyzes the phosphorylation of dolichol at the cytoplasmic face of the membrane.

Our finding that membranes from *sec59* cells become depleted in Dol-*P* raises questions as to how this depletion comes about, and how dolichol kinase actually participates in cellular metabolism. Thus, Dol-*P* functions as a carrier and lipid intermediate in ER glycosylation pathways but is regenerated in all the glycosyl-transfer reactions it is known to participate in as donor, and therefore formally serves only a catalytic role. This being so, one would not expect Dol-*P* to become depleted in membranes as rapidly as it does. In *sec59* cells a Dol-*P* deficiency should arise only gradually by dilution as cells continue to grow and divide.

There are three models to explain how a dolichol kinase defect could give rise to a Dol-*P* deficiency at the cytoplasmic face of the ER. Dolichol kinase could be required as a component of a Dol-*P* translocation system (Fig. 2*A*), for *de novo* synthesis of Dol-*P* (Fig. 2*B*), or for mobilizing preexisting dolichol pools (Fig. 2*C*). In the first model, Dol-*P* released in luminal glycosyl-transfer reactions becomes dephosphorylated in the lumen of the ER by Dol-*P* phosphatase, a step that then permits ready translocation of the polyisoprenoid back across the membrane as the nonpolar,

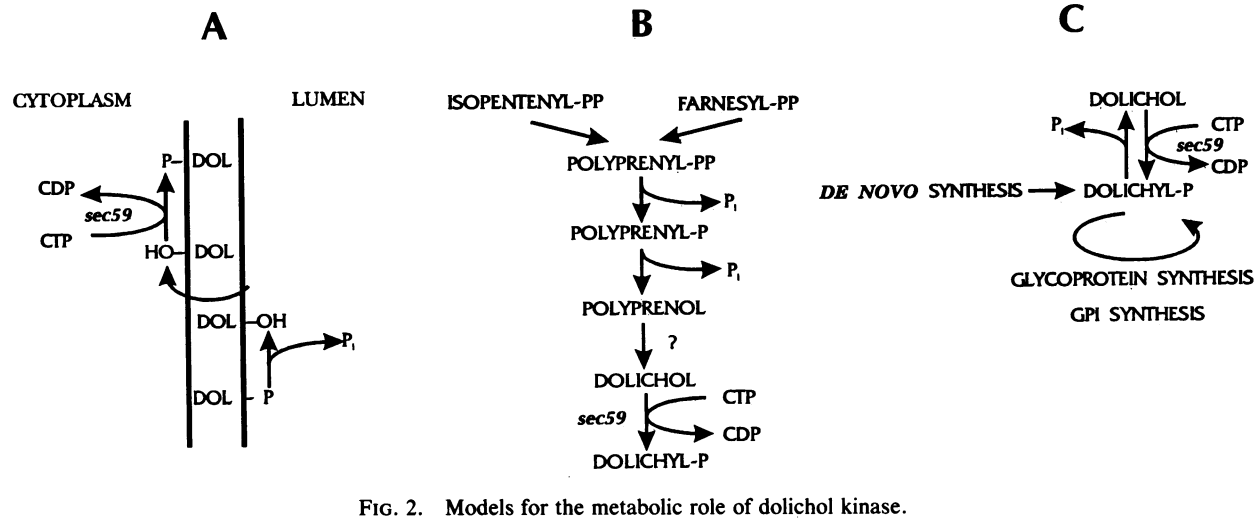

FIG. 2. Models for the metabolic role of dolichol kinase.

free alcohol. Dolichol kinase is then required as a component of this translocation mechanism in order to rephosphorylate dolichol at the cytoplasmic surface of the ER membrane so that it can serve there as a substrate for glycosyltransferases. In the second model, phosphorylation of dolichol is an obligatory step in the *de novo* synthesis of Dol-*P*. This assumes that Dol-*P* synthesis proceeds via the intermediate polyprenyl pyrophosphate, which is dephosphorylated, reduced at its α-isoprene unit, then rephosphorylated to yield Dol-*P*. In the third model, dolichol kinase is not required for translocation *per se* but for recruitment of dolichol to serve in glycosylation pathways.

While the ability to phosphorylate the polyisoprenoid alcohol is an essential function, it is possible that the requirement for the α-saturated dolichol is not absolute. A mutant Chinese hamster ovary cell line has been described that fails to reduce the terminal isoprene unit in polyprenol to yield dolichol. These cells underglycosylate protein yet maintain their ability to glycosylate any protein at all by making use of unsaturated polyprenyl phosphate derivatives in their lipid-dependent glycosylation reactions (26, 27). Polyprenols have been shown to be substrates for dolichol kinase (12) and therefore could substitute for dolichol in this mutant, yet viable, mammalian cell line.

Regardless of how Dol-*P* becomes depleted in the *sec59* mutant, there are a number of reasons to expect this depletion of Dol-*P* to be lethal. Prevention of Dol-*P*-Man synthesis will abolish O-mannosylation and GPI anchoring, either of which may prove to be essential processes in yeast. Consistent with this is the fact that Dol-*P*-Man synthase itself is an essential protein in yeast (4). It seems, though, that the Dol-*P*-Man-dependent steps in N-glycosylation alone are not required for viability of eukaryotes. Thus, a number of mutant mammalian cell lines, certain species of protozoa, and the yeast *alg3* mutant are viable but are blocked at the Dol-*PP*-$GlcNAc_2Man_5$ stage in N-glycosylation (28–32).

A decrease in Dol-*P* would also explain the reduced number of N-linked oligosaccharide chains transferred to protein in the *sec59* mutant, for the cells would be deficient in the production of both Dol-*P*-Man and dolichyl phosphate glucose (Dol-*P*-Glc). Studies on the efficiency of oligosaccharide transfer to protein as a function of composition and size indicate that the presence of the three Dol-*P*-Glc-derived glucose residues greatly enhances transfer rates (33–39). Further, the yeast mutants *alg5* and *alg6*, which are defective respectively in the synthesis of Dol-*P*-Glc and in the subsequent transfer of glucose to Dol-*PP*-$GlcNAc_2Man_9$, transfer fewer oligosaccharides to protein (30, 40). Thus, when *sec59* cells are shifted to the restrictive temperature, there will be a progressive depletion of their Dol-*P*-Glc and Dol-*P*-Man pools, resulting in the synthesis of truncated oligosaccharide chains that are transferred to protein with lowered efficiency, before N-glycosylation ceases altogether.

How does the block in all three glycosylation pathways that characterizes class B *sec* mutants *sec53* and *sec59* give rise to the defect in secretion that allowed these mutants to be isolated in the first place? These mutants, which are defective respectively in phosphomannomutase (6) and in dolichol kinase, are thus blocked in the synthesis of the immediate precursors of Dol-*P*-Man. However, temperature-sensitive Dol-*P*-Man synthase (*dpm1*) mutants do not behave as secretion mutants (4, 41). The only apparent difference between the biochemical phenotypes of the *sec53* and *sec59* mutants, on the one hand, and that of the *dpm1* mutant, on the other, is that *dpm1* cells still make an oligosaccharide (albeit a truncated one) that can be transferred to protein, but *sec53* and *sec59* cells cannot. This has led to the notion that attachment of an N-linked chain of a minimum size to protein suffices to allow cells to secrete such proteins and to maintain a functional secretary pathway (41).

In summary, we have shown that cells of the yeast *sec59* mutant are depleted in Dol-*P* and defective in dolichol kinase. These defects are lethal and show that the role of dolichol kinase in cellular metabolism is an essential one. The phenotype of *sec59* can now be attributed to the cells' inability to phosphorylate dolichol, which in turn results in a block in N-glycosylation.

The advice and support for this project provided by Phillips W. Robbins, Massachusetts Institute of Technology, are gratefully acknowledged. We thank Dr. Randy Schekman for the gift of strains and plasmids and Dr. Charles Albright for useful discussions. This work was supported by a National Institutes of Health grant (GM41003) to W.L.A.

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