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Loree Heller  
*Old Dominion University, lheller@odu.edu*

Peter Orlean

W. Lee Adair Jr.

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**Saccharomyces cerevisiae sec59** cells are deficient in dolichol kinase activity

Loree Heller*, Peter Orlean†, and W. Lee Adair, Jr.*‡

*Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, FL 33612; and †Department of Biochemistry, University of Illinois, Urbana, IL 61801

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**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 mannosyltransferase. Membranes prepared from sec59 cells that had been shifted to the restrictive temperature, however, made normal amounts of dolichyl phosphate mannosyltransferase. 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 in dolichol kinase activity. To identify this enzyme, the mutant sec59 was 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 dolichyl 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 [35S]SO4, remains normal for 2 hr while oligosaccharide synthesis is greatly decreased (2). The peptide forms accumulated in the endoplasmic reticulum (ER) have fewer oligosaccharide chains (2) and these are also shorter on average than those associated 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 mannosyltransferase (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 synthesis (6).

The gene complementing the sec59 mutation was cloned from a YEp13 yeast genomic library and sequenced (3). A highly hydrophilic 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).

**Materials and Methods**

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

**Enzyme Assays.** Cells were grown in minimal medium [0.17% yeast nitrogen base plus amino acids, 0.5% (NH4)2SO4, 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 CaCl2, 30 μM [32P]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 alkalizable lipids were hydrolyzed by incubation at 37°C for 25

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

*To whom reprint requests should be addressed: Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612.*
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 and the lower mass forms of M22-GlcNAc2 lessen (3). Since addition of the mannol residues is 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 [14C]mannose from GDP-[14C]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 mannos 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-[14C]Man (Fig. 1, lane 1), membranes prepared from cells that had been incubated at the restrictive temperature made no detectable Dol-P-[14C]Man (lane 2), suggesting that they had become depleted of the endogenous Dol-P that would normally have served as [14C]mannose acceptor. The same results were obtained when mannos 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 hamlet 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-[14C]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-[14C]Man was measured in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 20 μg of exogenous Dol-P. Radioalyzed lipids were extracted and separated by thin-layer chromatography on silica gel 60 with chloroform/methanol/water (65:25:4 by volume) as solvent (a, origin). Lane 5, Dol-P-[14C]Man standard made by incubation of membranes from wild-type yeast with GDP-[14C]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 polyenyl 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           | 24°C | 38°C |
| sec59 ura3-52 leu2-3,112 | 3.90 ± 1.36 | 0.79 ± 0.14 |
| S288C (wild type) | 8.09 ± 1.12 | 10.24 ± 1.65 |

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>cis-Prenyltransferase</th>
<th>Dol-PP phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C 38°C</td>
<td>24°C 38°C</td>
</tr>
<tr>
<td>S288C (WT)</td>
<td>100 ± 143</td>
<td>100 ± 123</td>
</tr>
<tr>
<td>sec59 ura3-52</td>
<td>105 ± 140</td>
<td>114 ± 90</td>
</tr>
</tbody>
</table>

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 G1 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 ovi-

![Fig. 2. Models for the metabolic role of dolichol kinase.](image-url)
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 polypropenyl 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 polypropenyl to yield dolichol. These cells underglycosylate protein yet maintain their ability to glycosylate any protein at all by making use of unsaturated polypropenyl phosphate derivatives in their lipid-dependent glycosylation reactions (26, 27). Polypropenols have been shown to be substrates for dolichol kinase (31–33) 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 α-gal3 mutant are viable but are blocked at the Dol-P-GlcNAC2Man1 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 α-gal3 and α-gal6, which are defective respectively in the synthesis of Dol-P-Glc and in the subsequent transfer of glucose to Dol-P-Man in N-linked 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 secretory pathway (41).