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

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 glycosylation of protein are also abolished, consistent with a deficiency in dolichyl phosphate mannosyltransferase. Assays of enzymes from the sec9 mutant, which is increased in the absence of exogenous dolichyl phosphate, showed that the levels were decreased to <10% of wild type at the permissive temperature and to <10% of wild type at the restrictive temperature. Assays of enzymes from the dolichol phosphate synthetase pathway, cis-prenyltransferase and dolichyl phosphate 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 See mutants stop dividing and become enlarged and dense at the restrictive temperature, 37°C (1), a property that allowed the selection of See mutants by density gradient centrifugation. The mutant sec59 was isolated in this manner and characterized as a class B See mutant, one which accumulates inactive and incompletely glycosylated See protein at the restrictive temperature (2). Protein synthesis, as measured by radiolabeling with 35S042-, remains normal for 2 hr while oligosaccharide synthesis as measured by [3H]mannose incorporation is decreased (2). The peptide forms accumulated in the endoplasmic reticulum (ER) are expressed in 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.

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 supply (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-Xaa-Phe-Tyr (7).

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 (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. Schekman (2). Mutant strain a sec59 ura3-52 leu2-3,112 was derived from PRY134. The plasmid pSEC920 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% (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 pellet 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 pyrophosphatase (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 alkali-labile lipids were hydrolyzed by incubation at 37°C for 25...

Abbreviations: Dol-P, dolichyl phosphate; Dol-P-Man, dolichyl phosphate mannosyl; 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.
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 ³²P incorporation into Dol-P was determined by scintillation counting in CytoScint (ICN).

**Dol-P Quantitation.** A modification of the protocol of Adair and Casmeyer (10) was used. Cells were grown in YPD to an OD₆₀₀ of ~1, washed, and pelleted at 2000 × g. A standard of C₆₀ polypropenyl 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% H₃PO₄.

**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 normal in sec59 cells (5). ³²P incorporation into Dol-P-Man is greatly increased in sec59 cells in the presence of isoprenyl pyrophosphate (10), whereas Dol-P-Man synthesis is not detectable in sec59 cells in the absence of isoprenyl 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>
<thead>
<tr>
<th>Strain</th>
<th>Dol-P, µg per 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>S288C (wild type)</td>
<td>8.09 ± 1.12</td>
</tr>
<tr>
<td>sec59 ura3-52 leu2-3,112</td>
<td>3.90 ± 1.36</td>
</tr>
</tbody>
</table>

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

**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. 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. 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 polypropenyl 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.
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 placmyctoma, 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 ovis-

**Table 3.** Dolichol kinase activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>pmol/hr per mg of protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C</td>
</tr>
<tr>
<td>S288C (wild type)</td>
<td>58.9 ± 9.8 (100)</td>
</tr>
<tr>
<td>sec59 ura3-52 leu2-3,112</td>
<td>3.1 ± 0.3 (5)</td>
</tr>
<tr>
<td>sec59 ura3-52 leu2-3,112 containing pSEC5920</td>
<td>843.0 ± 57.2 (1431)</td>
</tr>
</tbody>
</table>

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. 2A), for de novo synthesis of Dol-P (Fig. 2B), or for mobilizing preexisting dolichol pools (Fig. 2C). In the first model, Dol-P released in luminal glycosyl-transfer reactions becomes phosphorylated 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,
free alcohol. Dolichol kinase is then required as a component of this translocation mechanism in order to phosphorylate 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 \textit{de novo} synthesis of Dol-P. This assumes that Dol-P synthesis proceeds via the intermediate polyenyl pyrophosphate, which is dephosphorylated, reduced at its \(\alpha\)-isoprene unit, then rephosphorylated to yield Dol-P. In the third model, dolichol kinase is not required for translocation \textit{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 \(\alpha\)-saturated dolichol is not absolute. A mutant Chinese hamster ovary cell line has been described that fails to reduce the terminal isoprene unit in polyenol to yield dolichol. These cells undergoglycosylate protein yet maintain their ability to glycosylate any protein at all by making use of unsaturated polyenol phosphate derivatives in their lipid-dependent glycosylation reactions (26, 27). Polyenols have been shown to serve as 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 syntheses 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 \(a_{3}\) mutants that are viable but are blocked at the Dol-P-GlcNAC\(2\)Man\(2\) 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 \(a_{3}\) and \(a_{6}\), which are defective respectively in the synthesis of Dol-P-Glc and in the subsequent transfer of glucose to Dol-P-Man, are less capable of transferring 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 characterize 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).