An alternative cis-isoprenyltransferase activity in yeast that produces polyisoprenols with chain lengths similar to mammalian dolichols

Barbara Schenk, Jeffrey S. Rush, Charles J. Waechter, and Markus Aebi

Institute for Microbiology, ETH Zurich, CH-8092 Zurich, Switzerland, and 1Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536, USA

Received on August 1, 2000; revised on August 22, 2000; accepted on August 22, 2000

Dolichyl monophosphate (Dol-P) is a polyisoprenoid glycosyl carrier lipid essential for the assembly of a variety of glycoconjugates in the endoplasmic reticulum of eukaryotic cells. In yeast, dolichols with chain lengths of 14–17 isoprene units are predominant, whereas in mammalian cells they contain 19–22 isoprene units. In this biosynthetic pathway, t,t-farnesyl pyrophosphate is elongated to the appropriate long chain polypropenyl pyrophosphate by the sequential addition of cis-isoprene units donated by isopentenyl pyrophosphate with t,t,c-geranylgeranyl pyrophosphate being the initial intermediate formed. The condensation steps are catalyzed by cis-isoprenyltransferase (cis-IPTase). Genes encoding cis-IPTase activity have been identified in Micrococcus luteus, Escherichia coli, Arabidopsis thaliana, and Saccharomyces cerevisiae (RER2). Yeast cells deleted for the RER2 locus display a severe growth defect, but are still viable, possibly due to the activity of an homologous locus, SRT1. The dolichol and Dol-P content of exponentially growing revertants of RER2 deleted cells (∆rer2) and of cells overexpressing SRT1 have been determined by HPLC analysis. Dolichols and Dol-Ps with 19–22 isoprene units, unusually long for yeast, were found, and shown to be utilized for the biosynthesis of lipid intermediates involved in protein N-glycosylation. In addition, cis-IPTase activity in microsomes from ∆rer2 cells overexpressing SRT1 was 7- to 17-fold higher than in microsomes from ∆rer2 cells. These results establish that yeast contains at least two cis-IPTases, and indicate that the chain length of dolichols is determined primarily by the enzyme catalyzing the chain elongation stage of the biosynthetic process.

Key words: endoplasmic reticulum/cis-isoprenyltransferase/polyisoprenol biosynthesis/protein N-glycosylation

Introduction

Long chain polyisoprenoids are ubiquitous membrane components known to play an important role as glycosyl carrier lipids in the biosynthesis of cell wall components in bacteria (Lennarz and Scher, 1972; Hemming, 1985) and of several glycoconjugates in eukaryotic cells (Kornfeld and Kornfeld, 1985; Rip et al., 1985; Hirschberg and Snider, 1987; Waechter, 1989; Krag, 1998; Burda and Aebi, 1999). Protein N-glycosylation of membrane and secretory glycoproteins is an essential process that takes place in the endoplasmic reticulum (ER) in eukaryotes, following a highly conserved pathway utilizing dolichyl monophosphate (Dol-P) as a glycosyl carrier lipid.

Dolichols are a family of polyisoprenoid lipids of different chain lengths. The chain length is species-specific and can vary from 14 to 17 isoprene residues in Saccharomyces cerevisiae and Schizosaccharomyces pombe (Quellhorst et al., 1998) to 19 to 22 isoprene residues in mammalian cells (Rip et al., 1985). In animal and fungal cells, dolichol synthesis is initiated with t,t-farnesyl pyrophosphate (F-P-P), a common intermediate in protein isoprenylation and the biosynthesis of sterols, ubiquinone and dolichol (Grunler et al., 1994). Cis-isoprenyltransferase (cis-IPTase) catalyzes the sequential addition of multiple cis-isoprene units with isopentenyl pyrophosphate (I-P-P) serving as the isoprene donor (Daleo et al., 1977; Grange and Adair, 1977; Wong and Lennarz, 1982; Adair and Cafmeyer, 1987; Crick et al., 1991; Ericsson et al., 1992; Szkopinska et al., 1996). The end product of the chain elongation stage is a long chain, fully unsaturated polyisoprenyl pyrophosphate (Poly-P-P), which subsequently undergoes dephosphorylation, reduction of the α-isoprene unit and re-phosphorylation by dolichol kinase. In yeast, dolichol kinase is encoded by the SEC59 locus (Ferro-Novick et al., 1984a,b; Bernstein et al., 1989; Heller et al., 1992).

The biosynthesis of the Dol-P-P-linked precursor for N-linked oligosaccharides is initiated on the cytoplasmic face of the ER where nucleotide-activated sugars serve as direct glycosyl donors for the synthesis of mannolipophosphoryldolichol (Man-P-Dol), glucosylphosphoryldolichol (Glc-P-Dol) and Man₇GlcNAc₂-P-Dol. The assembly of Glc₃Man₇GlcNAc₂-P-Dol, the oligosaccharyl donor, is then completed after these three intermediates are translocated to the lumenal face of the ER (Hirschberg and Snider, 1987). The precursor oligosaccharyl unit is subsequently transferred to specified asparagine residues of nascent polypeptide chains by the enzyme complex oligosaccharyltransferase (Silberstein and Gilmore, 1996; Knauer and Lehle, 1999; Yan and Lennarz, 1999). The mannolipid intermediate, Man-P-Dol, is also required for O-mannosylation of yeast glycoproteins, GPI-anchor synthesis and C-mannosylation of some proteins (Takeda and Kinoshita, 1995; Doucey et al., 1998; Strahl-Bolsinger et al., 1999).

© 2001 Oxford University Press

89

1To whom correspondence should be addressed
There is convincing evidence that the level of Dol-P in the ER is one rate-controlling factor in lipid intermediate synthesis and protein N-glycosylation (Harford et al., 1977; Lucas and Levin, 1977; Harford and Waechter, 1980; Hubbard and Robbins, 1980; Carson et al., 1981; Spiro and Spiro, 1986; Rosenwald et al., 1990). Moreover, developmental studies in embryonic rat brain (Crick and Waechter, 1994), proliferating murine B lymphocytes (Crick et al., 1994) and 8-bromo-cAMP-treated JEG-2 choriocarcinoma cells (Konrad and Merz, 1996) have documented large increases in cis-IPTase activity and Dol-P synthesis that preceded the induction of lipid intermediate biosynthesis. Thus, elucidating the structure and regulation of this class of cis-IPTases is important to understand thoroughly all of the factors regulating the protein N-glycosylation process.

The cis-IPTases are a family of proteins conserved throughout the kingdoms. Three eubacterial and plant members of this family have been described, the undecaprenyl diphosphate synthases from Micrococcus luteus and Escherichia coli (Apfel et al., 1999; Kato et al., 1999; Shimizu et al., 1998) and the cis-IPTase from Arabidopsis thaliana (Oh et al., 2000). In yeast, the locus encoding cis-IPTase activity has been identified as RER2 (Sato et al., 1999). Yeast cells deleted for the RER2 locus are viable, but grow slowly, and they are defective in protein N-glycosylation. SRT1 was shown to be an homologous locus in yeast sharing 30% overall identity with RER2. Although SRT1 was isolated as a high copy suppressor of rer2-1 mutant cells, its precise function was not established.

In this paper, we show that cells deleted for SRT1 exhibit no growth and no CPY-glycosylation phenotype, although cells deleted for RER2 accumulate the unglycosylated prepro glycoform of carboxypeptidase Y (CPY) together with hypoglycosylated CPYs. Furthermore, some Δrer2 deleted cells undergo a reversion event leading to a much faster growth rate. Interestingly, these revertant cells contain dolichols of longer chain length than is usual for yeast cells, but common for mammalian cells. The same long chain dolichols were found in Δrer2 cells overexpressing SRT1. The phenotypes of Δrer2 revertant cells and Δrer2 cells overexpressing SRT1 are described, and the atypically long chain dolichols produced in these cells are shown to be utilized for lipid intermediate synthesis and protein N-glycosylation. Evidence that SRT1 encodes an alternative cis-IPTase is presented. The possible functional significance and regulation of this novel enzyme in yeast are discussed.

**Results**

**Accumulation of preproCPY upon Rer2p depletion**

Yeast strains deleted for the RER2 locus were viable, but grew very slowly (Sato et al., 1999). To monitor the effect of Rer2p-depletion, a strain was constructed expressing the RER2 locus under the control of the GAL1-10 promotor. Upon a shift from induced to repressed conditions, the expression of CPY, a vacuolar protease, was monitored. This protein can serve as an indicator for deficiencies in the secretory pathway as well as in the process of N-linked glycosylation (Hasilik and Tanner, 1978; Stevens et al., 1982; te Heesen et al., 1992).

Depletion of Rer2p resulted in the appearance of hypoglycosylated CPY's (Figure 1, lanes 3 and 5). Discrete CPY bands represent proteins lacking one or more complete N-linked oligosaccharide chains (-1 to -4). Treatment of the protein extract with endoglycosidase H (Endo H) converted all the CPY glycoforms (-1 to -4). Treatment of the protein extract with endoglycosidase H (Endo H) converted all the CPY glycoforms to a single species migrating at 47.5 kDa (Figure 1, lanes 2, 4, 6, 8, 10). Additionally, we detected a band corresponding to totally unglycosylated CPY whose mobility was not affected by Endo H treatment (Figure 1, lanes 5 and 6). The band believed to be unglycosylated CPY had the same mobility as preproCPY, which did not translocate into the ER and therefore lacked N-linked oligosaccharides (Stevens et al., 1982). Mutant cells carrying the sec59-1 allele, altered in dolichol kinase activity, were reported to reveal a similar phenotype (Ferro-Novick et al., 1984a,b). Indeed, sec59-1 mutant cells grown at permissive temperature accumulated...
hypoglycosylated CPY (Figure 1, lane 7) and preproCPY when shifted to non-permissive temperature (Figure 1, lane 9). We conclude that the depletion of cis-IPTase and the inactivation of dolichol kinase resulted in two distinct phenotypes: a hypoglycosylation of secretory proteins and an inhibition of protein translocation into the ER.

**Glycosylation is partially restored in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1**

In contrast to the SEC59 locus, RER2 was not essential, but a deletion resulted in a very slow growth (Sato et al., 1999; Figure 2). Cells deleted for both RER2 and the RER2 homologue SRT1 were not viable, suggesting that SRT1 at least partially replaced RER2. Indeed, SRT1 was isolated as a high copy number suppressor of an rer2 mutation (Sato et al., 1999).

When Δrer2 cells were grown for 5–7 days on solid medium, the appearance of fast growing colonies was observed. Some of these stable revertants of Δrer2 were analyzed, and their growth rates were comparable to those of wild type cells or of Δrer2 cells overexpressing the SRT1 locus (Figure 2A). When the expression of CPY was analyzed in both revertant and SRT1-suppressed Δrer2 cells, increased N-glycosylation was observed (Figure 2B, lanes 3–6). Significant levels of fully glycosylated CPY were resolved, although preproCPY was still present.

**Analysis of dolichol and dolichyl monophosphate in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1**

To assess whether the partially restored level of N-glycosylation in Δrer2 revertant cells and SRT1-suppressed Δrer2 cells was due to increased levels of dolichol and Dol-P, these lipids were analyzed in wild type, Δrer2, Δrer2 revertant cells and Δrer2 cells overexpressing SRT1. In our analytical system, polyisoprenols could be separated from dolichols and comigration experiments showed that dolichol and not polyisoprenol accumulated in the cells analyzed (data not shown).

Wild type cells accumulated dolichol and Dol-P with chain lengths of 14–17 isoprene units (Figure 3). In Δrer2 cells, UV absorbing material eluted in the range of dolichol-14 and dolichol-15; however, no comigration was observed with either of these two dolichol species. These two peaks were specific for the elution profiles obtained from Δrer2 cells; however, we do not know the chemical nature of these components. The analysis of Dol-P in Δrer2 cells revealed strongly reduced levels of Dol-P within the normal size range. However, very low levels of Dol-P with 19 and 20 isoprene units were also resolved. These long chain Dol-Ps became very prominent in Δrer2 revertant cells and in Δrer2 cells overexpressing SRT1 (Figure 3B). In addition, these long chain dolichols were also present in the dolichol fraction of these cells (Figure 3A).

These data showed that both the content and chain length of dolichols and Dol-P were altered in Δrer2 revertants and SRT1 overexpressing cells compared to Δrer2 or wild type cells. Though the total levels of dolichol and Dol-P were still reduced compared to wild type cells, it was sufficient for growth and normal rates of N-glycosylation. Importantly, these results showed that S. cerevisiae contains an activity capable of synthesizing polysoprenyl chains longer than the size typically found in wild type cells. Overexpression of SRT1 in Δrer2 cells was sufficient to induce the synthesis of the dolichols with chains containing 3–5 isoprene units more than the dolichols usually found in yeast, suggesting that this locus encodes an alternative cis-IPTase.

**Lipid-linked oligosaccharide (LLO) analysis in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1**

To verify that the long chain dolichols observed in SRT1-overexpressing cells were utilized for lipid intermediate biosynthesis, lipid-linked oligosaccharides in wild type, Δrer2, Δrer2 revertant

---

**Fig. 2. Analysis of growth and CPY processing in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1.** (A) Cells of the strains SS328 (wt, wild type), YG932 (Δrer2), YG1118 (Δrer2+pSRT1, the Δrer2 strain overexpressing SRT1) and YG1114 (Δrer2 rev13, a revertant of Δrer2 deleted cells) were grown for 3 days on a YPD plate at 30°C. A picture of theagar plate is shown. (B) Strains SS328 (wt, wild type), YG1113 (Δrer2 rev12, a revertant of Δrer2 deleted cells), YG1118 (Δrer2+pSRT1, the Δrer2 strain overexpressing SRT1) and YG932 (Δrer2) were grown in supplemented minimal medium. Total protein extracts were prepared and incubated in absence (-) or presence (+) of Endo H. SDS–PAGE and Western blot analysis of CPY molecules was as described for Figure 1. The positions of the mature CPY (mCPY), non-translocated CPY (preproCPY) and mature CPY protein lacking 1 to 4 N-glycan chains (-1, -2, -3, -4) are given on the left, the positions of marker proteins with the indicated molecular weight are shown on the right.
cells, and Δrer2 cells overexpressing SRT1 were analyzed. Lipid-linked oligosaccharides were metabolically labeled in vivo with [3H]mannose. The [3H]oligosaccharides were liberated by mild acid hydrolysis, isolated, and analyzed by HPLC. Wild type cells are known to accumulate the full length lipid-bound oligosaccharide, Glc3Man9GlcNAc2-P-P-Dol, whereas in Δrer2 cells very low levels of lipid-linked oligosaccharides were detected (Figure 4A). This finding is compatible with the marked hypoglycosylation observed in Δrer2 mutant cells. Interestingly, both the Δrer2 revertant cells, as well as Δrer2 cells overexpressing SRT1, accumulated Glc3Man9GlcNAc2-P-P-Dol. However, the levels of Glc3Man9GlcNAc2-P-P-Dol were lower in revertant and SRT1 overexpressing cells, and precursor lipid intermediates accumulated (Figure 4A).

These results indicate that hypoglycosylation of proteins observed in Δrer2 cells is due to a deficiency in lipid-linked oligosaccharide production and provide indirect evidence that the longer chain dolichols found in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1 are used for the biosynthesis of Glc3Man9GlcNAc2-P-P-Dol.

To address this hypothesis directly, the Dol-P moiety of lipid-linked oligosaccharides isolated from these strains was analyzed after the carrier lipid was liberated by strong alkaline hydrolysis. In wild-type cells, LLO had Dol-P moieties with the same chain length (primarily 15–16 isoprene units) as total cellular dolichol or Dol-P (Figure 4B). In contrast to the wild type strain, LLOs from Δrer2 revertant cells and Δrer2 cells overexpressing SRT1 contained Dol-Ps with unusually long chain lengths. Very low amounts of Dol-P were detected without alkaline hydrolysis (data not shown), demonstrating that the Dol-P detected originated from LLOs. This observation is consistent with the conclusion that the relatively long Dol-Ps, atypical for S.cerevisiae, were used as substrates for LLO biosynthesis.

---

Fig. 3. Analysis of dolichol and Dol-P levels in different strains. Dolichol (A) and Dol-P (B) were extracted from 500 OD-equivalents of cells from strains SS328 (wt, wild type), YG932 (Δrer2), YG1111 (Δrer2rev1, a revertant of Δrer2 deleted cells) and YG1118 (Δrer2-pSRT1, the Δrer2 strain overexpressing SRT1) and analyzed by HPLC. Dolichol (A, dol-18-OH) or Dol-P (B, dol-18-P) composed of 18 isoprene units was added to the cells prior to extraction and served as qualitative and quantitative markers. Isoprenologues were separated by HPLC and detected using an UV detector at 214 nm. The chain length of the isoprenologues is indicated above the peaks.
Alternative cis-isoprenyltransferase activity in yeast

Overexpression of SRT1 in Δrer2 cells restored cis-IPTase activity

The occurrence of long chain dolichols in Δrer2 revertants suggested the presence of a cis-IPTase activity. To address this possibility, cis-IPTase activity was assayed in vitro with crude microsomes using either t,t-F-P-P or t,t,c-GG-P-P to initiate the reaction with I-P-P (Table I). As reported previously (Sato et al., 1999), overexpression of RER2 in wild-type cells increased the cis-IPTase activity about 1.5-fold, whereas a deletion of this gene virtually abolished the activity. Overexpression of SRT1 in Δrer2 cells produced a 7- to 17-fold increase in cis-IPTase activity in vitro as compared to Δrer2 cells. These results indicate that Srt1p, the Rer2p homologue, is an alternative cis-IPTase in vivo. Since increased synthesis of Poly-P-P in microsomes overexpressing Srt1p could be initiated with either t,t-F-P-P or t,t,c-GG-P-P, it is very likely that a single enzyme catalyzes the conversion of t,t-F-P-P to Poly-P-P (Table I).

A preliminary investigation of the nature of the membrane-association of the yeast cis-IPTases was also conducted with microsomes from the appropriate strains. Neither the cis-IPTase encoded by SRT1 or RER2 could be released from the microsomal fraction by washing with 1 M NaCl, indicating that these enzymes were firmly membrane-bound, but apparently not by electrostatic interactions.

Deletion of the RER2 locus is a prerequisite to reveal the expression of the cis-IPTase activity encoded by SRT1 in vivo

Although long chain dolichols are not observed in wild-type cells, small amounts are present in Δrer2 cells (Figure 3). This observation suggested that a deletion of the RER2 locus was essential for expression of SRT1 activity in vivo. To test this hypothesis directly, SRT1 was overexpressed in wild-type (RER2) and Δrer2 cells. When dolichol and Dol-P were isolated from these strains and analyzed (Figure 5), dolichols and Dol-Ps with 19–22 isoprene units were only present in...
∆rer2 cells overexpressing SRT1. This result establishes that overexpression of SRT1 and the absence of Rer2p are required for the production of significant levels of the dolichols and Dol-Ps with atypically long polyisoprenol chains in vivo.

**Discussion**

This paper presents evidence that SRT1 encodes an alternative cis-IPTase activity capable of synthesizing dolichols and Dol-Ps with polyisoprenol chains significantly longer than those normally found in wild type strains of S.cerevisiae. SRT1 is a high copy number suppressor of a deletion of the RER2 locus and is essential to support the growth of ∆rer2 strains (Sato et al., 1999). Overexpression of SRT1 in ∆rer2 cells resulted in a restoration of cis-IPTase activity and the synthesis of atypically long chain dolichols and Dol-Ps. Low levels of relatively long chain Dol-Ps were also detected in ∆rer2 revertant cells. Amplification of the chromosomal SRT1 locus or increased expression of SRT1 would suppress the phenotype of the RER2 deletion. However, this amplification...
was not tested directly, but the observation that Δrer2 revertant cells contained unusually long chain dolichols and Dol-Ps strongly supported this conclusion.

These long chain dolichols were exclusively found in Δrer2 revertant cells and Δrer2 cells overexpressing SRT1, but not in wild type cells overexpressing SRT1. Therefore, the production of long chain dolichols depended not only on the presence of the SRT1 locus, but also on the absence of the RER2 locus. We hypothesized that there was an additional factor necessary for maximal cis-IPTase activity utilized by both enzymes. This hypothesis is supported by the fact that overexpression of the RER2 locus on a high copy plasmid in wild type cells resulted only in a 1.5-fold increase in cis-IPTase activity (Table I), whereas normally an up to 20-fold increase of activity can be achieved by expression from a 2μ-derived high copy number plasmid. This observation suggested that the putative factor became limiting in wild type cells and cells overexpressing either RER2 or SRT1 and that its affinity towards RER2 was much higher as compared to SRT1. Alternatively, the fact that only dolichols containing 14–17 isoprene units were detected in wild type cells overexpressing the SRT1 locus could be explained by a high affinity of the RER2 protein towards either F-P-P or I-P-P in vivo. Interestingly, the production of unusually long chain polyisoprenyls was observed previously in a yeast strain defective in squalene synthase and overexpressing a mutant form of F-P-P synthase, and it was proposed that cis-IPTase required direct interaction with F-P-P synthase for activity (Szkopinska et al., 1997).

It remains to be determined why there are two distinct cis-IPTases in yeast. Wild type yeast cells grown to exponential or stationary phase did not contain detectable levels of dolichols with lengths longer than 15–16 isoprene units. The presence of two genes encoding cis-IPTase activities in the genome of S.cerevisiae suggests that the relatively longer dolichols might have a specific biochemical role. Although the precise role is still speculative, dolichol and Dol-P may fulfill additional functions aside from serving as glycosyl carrier lipids in the various glycosylation processes. In this regard, depletion of dolichol kinase and cis-IPTase resulted in a deficiency of protein translocation into the ER lumen (Ferro-Novick et al., 1984a,b) and rer2 mutant cells were found to be impaired in protein sorting (Sato et al., 1999). A specific chain length might be optimal for these cellular processes. Indeed, we observed a partial restoration of N-linked protein glycosylation in Δrer2 cells overexpressing SRT1, but protein translocation was impaired to a similar extent in Δrer2 cells and in Δrer2 cells overexpressing SRT1 (Figure 2B).

Analytical studies clearly show that longer chain Dol-Ps are used as glycosyl carrier lipids for the assembly of the lipid-linked oligosaccharide (Figure 4). The chain length distribution of the Dol-P isolated from the pool of lipid-linked oligosaccharides was basically the same as the dolichols and Dol-Ps obtained after hydrolysis of total cell lipid extracts. Thus, the abnormally long dolichols (Dol-Ps) were utilized by the enzymes catalyzing the formation of the various intermediates, providing in vivo evidence that these enzymes have a fairly broad specificity with respect to the chain length of the lipid substrates. Instead, in vitro studies showed that yeast GPT has a preference towards long chain Dol-P (Palamarczyk et al., 1980). Although it is possible that dolichol chain length affects O-linked glycosylation (Strahl-Bolsinger et al., 1999) and GPI-anchor biosynthesis (Takeda and Kinoshita, 1995), the effect of the atypically long dolichols on these processes was not examined.

The observations that both RER2 and SRT1 encoded separate enzymes and that the in vivo products differed with respect to the chain length, suggest that chain length of the polyisoprenol products is determined primarily by the cis-IPTases. Reports about the mechanism of chain length determination are available for trans-prenyltransferases (Ohnuma et al., 1993), but these enzymes do not share any sequence homology with the yeast cis-prenyltransferase reported here and RER2 (Sato et al., 1999). Furthermore, chain lengths of the trans-prenyltransferase products vary to a much lesser degree than is the case for cis-IPTase activity.

It is intriguing that the chain lengths of the polyisoprenols formed by SRT1 are longer than the chains produced by RER2 and very similar to mammalian dolichols. Although the structure of the mammalian enzyme has not yet been reported, it will be very interesting to see if these cis-IPTases are structurally more closely related to SRT1 and the Arabidopsis enzyme (Oh et al., 2000) than to RER2 and the bacterial cis-IPTases (Shimizu et al., 1998; Apfel et al., 1999; Kato et al., 1999). As the information on the structures of the cis-IPTases in prokaryotic and eukaryotic cells increases, it may be possible to determine how the chain elongation stage is regulated by each enzyme system to produce polyisoprenols with the appropriate chain length. It will be particularly interesting to learn how the bacterial cis-IPTases, which are apparently soluble enzymes, terminate the chain elongation process after adding the correct number of isoprene units with high fidelity.

Although Rer2p and Srt1p do not contain cleavable signal sequences or putative transmembrane domains, they appear to be firmly associated with the microsomal fraction and are not released by washing with 1 M NaCl. The RER2 protein is proposed to be cytoplasmically located, but associated with the ER membrane. Although Rer2p contains a C-terminal KXXX motif, known as the ER retrieval sequence of ER membrane proteins (Townsley and Pelham, 1994), the role of these residues in Rer2p function or its association with the ER has not been experimentally addressed.

Hopefully, future studies will provide a better understanding of the nature of the membrane-association and regulation of expression of the alternative, “latent” cis-IPTase, SRT1, as well as the biochemical function of the atypically long dolichols and Dol-Ps formed by this enzyme in S.cerevisiae.

Materials and methods

Materials

Dolichol and dolichyl monophosphate (dol-18-OH and dol-18-P) with 18 isoprene units were purchased from the collection of polyisoprenols of the Polish Academy of Sciences, Warsaw, Poland. Sep-Pak columns were from Waters, Switzerland and long dolichols (Dol-Ps) were utilized by the enzymes catalyzing the formation of the various intermediates, providing in vivo evidence that these enzymes have a fairly broad specificity with respect to the chain length of the lipid substrates. Instead, in vitro studies showed that yeast GPT has a preference towards long chain Dol-P (Palamarczyk et al., 1980). Although it is possible that dolichol chain length affects O-linked glycosylation (Strahl-Bolsinger et al., 1999) and GPI-anchor biosynthesis (Takeda and Kinoshita, 1995), the effect of the atypically long dolichols on these processes was not examined.

The observations that both RER2 and SRT1 encoded separate enzymes and that the in vivo products differed with respect to the chain length, suggest that chain length of the polyisoprenol products is determined primarily by the cis-IPTases. Reports about the mechanism of chain length determination are available for trans-prenyltransferases (Ohnuma et al., 1993), but these enzymes do not share any sequence homology with the yeast cis-prenyltransferase reported here and RER2 (Sato et al., 1999). Furthermore, chain lengths of the trans-prenyltransferase products vary to a much lesser degree than is the case for cis-IPTase activity.

It is intriguing that the chain lengths of the polyisoprenols formed by SRT1 are longer than the chains produced by RER2 and very similar to mammalian dolichols. Although the structure of the mammalian enzyme has not yet been reported, it will be very interesting to see if these cis-IPTases are structurally more closely related to SRT1 and the Arabidopsis enzyme (Oh et al., 2000) than to RER2 and the bacterial cis-IPTases (Shimizu et al., 1998; Apfel et al., 1999; Kato et al., 1999). As the information on the structures of the cis-IPTases in prokaryotic and eukaryotic cells increases, it may be possible to determine how the chain elongation stage is regulated by each enzyme system to produce polyisoprenols with the appropriate chain length. It will be particularly interesting to learn how the bacterial cis-IPTases, which are apparently soluble enzymes, terminate the chain elongation process after adding the correct number of isoprene units with high fidelity.

Although Rer2p and Srt1p do not contain cleavable signal sequences or putative transmembrane domains, they appear to be firmly associated with the microsomal fraction and are not released by washing with 1 M NaCl. The RER2 protein is proposed to be cytoplasmically located, but associated with the ER membrane. Although Rer2p contains a C-terminal KXXX motif, known as the ER retrieval sequence of ER membrane proteins (Townsley and Pelham, 1994), the role of these residues in Rer2p function or its association with the ER has not been experimentally addressed.

Hopefully, future studies will provide a better understanding of the nature of the membrane-association and regulation of expression of the alternative, “latent” cis-IPTase, SRT1, as well as the biochemical function of the atypically long dolichols and Dol-Ps formed by this enzyme in S.cerevisiae.
ura3-52 his3Δ200 lys2-801 pRER2), YG991 (MATα Δrer2::GAL1-10-RER2-URA3 ade2-101 ura3-52 his3Δ200 lys2-801), YG932 (MATα Δrer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801), YG938 (MATα Δαt1::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801), SS328×SS330 (MATα αtde2-101 ade2-101 ura3-52 his3Δ200 lys2-801/ + tyr1+), YG1111, YG1113, YG1114: three randomly chosen revertants of YG932, YG1117 (MATα Δrer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801 YEp352), YG1118 (MATα Δrer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801 pSRT1), YG1120 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 pSRT1), YG736 (MATα ade2-101 ura3-52 his3Δ200 sec59-1).

Yeast manipulations

Standard yeast media and genetic techniques (Guthrie and Fink, 1991) were used for growth of yeast cultures.

Isolation of the ORF RER2

The screen for high copy number suppressors was performed as described previously (Fleischmann et al., 1996). YG736 was transformed with a YEp352-based yeast genomic library (Fleischmann et al., 1996) and colonies growing at 37°C on minimal medium lacking uracil were isolated. Plasmids conferring growth at elevated temperature were recovered in E.coli and the DNA inserts were identified by determining the terminal sequences. The open reading frames found on the DNA inserts were subcloned and retransformed to identify the ORF responsible for suppression.

Construction of strains

Disruption of the RER2 and SRT1 loci: To construct YG932, the RER2 locus was replaced by homologous recombination using a PCR product containing the complete kanamycin resistance gene (kanMX4) flanked by RER2 specific regions (Wach et al., 1994). The kanMX4 sequence was amplified using the pFA6a-kanMX4 plasmid and two primers (primer 1: 5’-TCA ATA GAA CTT AAG CAA AAA TAG GGT AAA CAC AGG TAA AAG ACG GTT CGA GTA ATT CGA GCT C-3′; primer 2: 5’-TAC TCT ATA AAT ATC TAT GCC ATG TGG TAG GAA AAA AAT GCA GAC CTT TCC GTA CGC TGC AGG TCG AC-3′, bold sequences indicate homology to the kanMX gene). The resulting PCR fragment was transformed into the diploid wild type strain SS328×SS330 selecting for resistance towards G418 (200 µg/ml). Transformants were screened for correct replacement of the RER2 ORF by whole cell PCR (Sathe et al., 1991). Heterozygous diploids were induced to sporulate, asci were dissected and resulting haploid Δrer2 strains were analyzed by whole cell PCR.

The same procedure was used to disrupt the SRT1 locus (YG938), the primers being as follows: primer 1: 5’-TTA TAA AGA ACA GCC TGG TCT TTA ACA AAT AGA GGA GTT TTC TGT TGA CCA TAC TGA TGA ATT GAG CTC-3′; primer 2: 5’-TTT ACA TTT TAG CTG TCA ACT TGG CGC AAG GAT ATT TTA TAT ACC GCC GTA CGC TGC AGG TCG AC-3′; bold sequences indicate homology to the kanMX gene. To obtain a strain that expresses the RER2 protein under control of the GAL1-10 promoter, the following plasmid was constructed: a truncated version of RER2 lacking 300 bp at the 3′ end of the ORF was placed directly downstream of the glucose-repressible and galactose-inducible GAL1-10 promoter of S.cerevisiae (te Heesen et al., 1992), followed by the URA3-marker gene. The plasmid was cut within the truncated RER2 region to direct insertion into the RER2 locus and transformed into the diploid wild type strain SS328×SS330. Transformants were selected for growth on plates lacking uracil and analyzed by whole cell PCR (Sathe et al., 1991). Heterozygous diploids with correct insertion were induced to sporulate, asci were dissected, and the resulting haploid GAL1-RER2 strain (YG917) was analyzed by whole cell PCR to confirm the configuration of the modified RER2 locus.

Detection of CPY and Endoglycosidase H (Endo H) treatment

Cells were grown in minimal medium supplemented with the appropriate amino acids at 30°C to an OD660 between 1 and 1.5. Total protein extracts were prepared and Endo H treatment was performed as described previously (Burda et al., 1996). Western blotting techniques and detection of CPY have been described previously (te Heesen et al., 1992).

In vivo labeling and extraction of lipid-linked oligosaccharides

Cells were grown in minimal medium supplemented with the appropriate amino acids at 30°C to an OD660 between 1 and 1.5. The metabolic labeling with [3H]mannose, extraction, and analysis of lipid-linked oligosaccharides (LLO) by HPLC were performed as described previously (Zufferey et al., 1995).

Analysis of cellular dolichol and dolichyl monophosphate

Dolichol and Dol-P were extracted as described (Elmberger et al., 1989). Two liters of logarithmically growing yeast cells (OD660 = 1–2) were pelleted and resuspended in 12 ml H2O. Dolichol-18-OH (12 µg) in a small volume of hexane was added as an internal standard. The cell suspension was then subjected to strong alkaline hydrolysis (3 M KOH in 40% methanol, 100°C, 60 min). Dolichol and Dol-P were extracted by the addition of 12 ml methanol and 48 ml dichloromethane to the hydrolysate, and the mixture was incubated for 1 h at 40°C. The organic (lower) phase was removed and washed four times with equal volumes of dichloromethane/methanol/water (3:48:47). The washed organic phase was dried under N2, and the lipids were dissolved in 10 ml methanol/water (98:2) containing 20 mM H3PO4. A C-18 Sep-Pak (2 ml) was equilibrated with 10 ml methanol/water (98:2) containing 20 mM H3PO4 and the lipid extract was applied to the column. The column was washed with 10 ml methanol/water (98:2) containing 20 mM H3PO4 and then with 10 ml methanol/water (98:2). Dolichol and Dol-P were eluted with chloroform/methanol (98:2) containing 20 mM H3PO4 and the eluate was applied to the column. The column was washed with 10 ml methanol/water (98:2) containing 20 mM H3PO4 and then with 10 ml methanol/water (98:2). Dolichol and Dol-P were eluted with chloroform/methanol (2:1) and the eluate was adjusted to 0.5% NH4OH (v/v). A silica Sep-Pak (2 ml) was eluted with 40 ml chloroform/methanol (2:1) containing 0.5% NH4OH and the eluates and Dol-P containing sample was applied to the column. Dolichol was recovered from the silica Sep-Pak in 20 ml chloroform/methanol (2:1) containing 0.5% NH4OH, and Dol-P was eluted with 30 ml chloroform/methanol/water (10:1:0.3). Samples were dried under N2 and resuspended in the mobile phase solvent mixture.

Dolichol and Dol-P fractions were separated into single isoprenologues on a Merck LiChrospher 100 reversed phase C-18 column (5 µm, 4 × 12.5 mm) equilibrated with the mobile phase for 1 h using a Merck/Hitachi L-6200A Intelligent pump. The mobile phases, isopropanol/methanol/water (65:30:5) containing...
Analysis of the Dol-P moiety of lipid-linked oligosaccharides

Logarithmically growing cells were harvested, broken with glass beads and extracted three times with 60 mM chloroform/methanol (2:1) to remove the bulk membrane lipids. Lipid-linked oligosaccharides were then extracted three times with 60 mM chloroform/methanol/water (10:10:3). The pooled extracts were dried and Dol-P was released by strong base hydrolysis (3 M KOH in 40% methanol, 100°C, 60 min). Dol-P was then recovered and analyzed as described above.

In vitro assay for cis-isoprenyltransferase

Logarithmically growing yeast cells were disrupted in buffer A (0.1 M Tris pH 7.5, 0.25 M sucrose, 10 mM β-mercaptoethanol, 1 mM EDTA) with a cell homogenizer (B.Braun Biotech, Melsungen, Germany). The homogenate was centrifuged at 5000 × g (10 min, 4°C) to remove cellular debris, and then at 100,000 × g (1 h, 4°C) to sediment crude microsomes. The microsomal pellet was resuspended in buffer A using a 2 ml Dounce homogenizer.

cis-ITPase activity was assayed as described previously (Crick et al., 1991). Reaction mixtures contained 25 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 1.25 mM DTT, 2.5 mM sodium orthovanadate, 50 μM farnesyl pyrophosphate, 90 μM [1-¹⁴C]isopentenyl pyrophosphate (55 mCi/mmol) and 200 μg microsomal protein in a total volume of 100 μl. After incubation at 30°C for the indicated period of time, the reaction was stopped by the addition of 2 ml chloroform/methanol (2:1). Lipid products were separated from water soluble substrates by partitioning after addition of 0.9 ml 0.9% NaCl. The organic (lower) phase was washed three times with chloroform/methanol/water (3:48:47) and dried under N₂. The amount of [¹⁴C]Poly-P-P formed was determined by scintillation spectrometry.

Acknowledgments

We thank Balasz Magyar for technical assistance setting up the HPLC analyses and Dr. F. Fernandez for helpful suggestions during the course of this study. This work was partially supported by NIH Grant GM30365 awarded to C. J. W., and by Grant 31–57082.99 from the Swiss National Science Foundation to M. A.

Abbreviations

cis-ITPase, cis-isoprenyltransferase; CPY, carboxypeptidase Y; Dol-P, dolichyl monophosphate; Poly-P-P, fully unsaturated, long chain polyenyl pyrophosphate; Endo H, endoglycosidase H; ER, endoplasmic reticulum; Glc-P-Dol, glucosylphosphoryldolichol; Man-P-Dol, mannosylphosphoryldolichol; t,t-F-P-P, trans,trans-farnesyl pyrophosphate; t,t,c-GG-P-P, trans,trans,cis-geranylgeranyl pyrophosphate.

References


