Glycobiology vol. 11 no. 1 pp. 89–98, 2001

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

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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 polyprenyl 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 (Δ*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 Δ*rer2* cells overexpressing *SRT1* was 7- to 17-fold higher than in microsomes from Δ*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/polyprenol 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 polyprenyl 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 mannosylphosphoryldolichol (Man-P-Dol), glucosylphosphoryldolichol (Glc-P-Dol) and $Man_5GlcNAc_2$-P-P-Dol. The assembly of $Glc_3Man_9GlcNAc_2$-P-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).

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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 hypo-glycosylated CPYs. Furthermore, some Δ*rer2* 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 Δ*rer2* cells overexpressing *SRT1*. The phenotypes of Δ*rer2* revertant cells and Δ*rer2* 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* promoter. 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 CPYs (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 various CPY glycoforms to a single species migrating at ~51 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

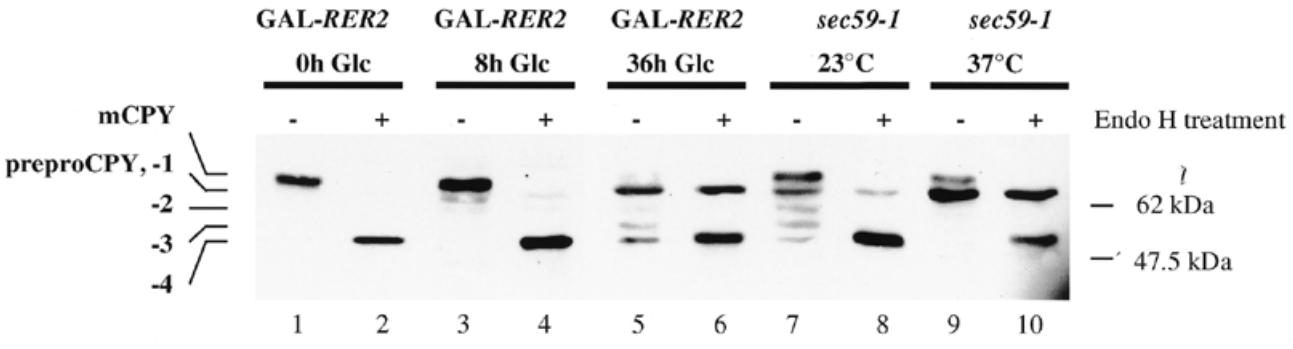

Fig. 1. Analysis of CPY processing in a strain depleted for Rer2p and in a *sec59-1* mutant strain grown at permissive and nonpermissive temperature. Strain YG991 (GAL-*RER2*, lanes 1–6) that expresses the *RER2* protein under the control of the glucose-repressible *GAL1-10* promoter was grown in galactose containing medium and shifted to glucose containing medium at the time 0 h. Protein extracts were prepared from cells grown on glucose medium for different amounts of time (indicated above each lane) and incubated in absence (-) or presence (+) of Endo H. Extracts from strain YG736 (*sec59-1*) cells grown either at permissive (23°C, lanes 7, 8) or restrictive (37°C for 3 h, lanes 9, 10) temperature were analyzed as a control. Proteins were separated by SDS–PAGE and CPY molecules were visualized by Western blot analysis using CPY-specific antiserum. 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.

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 Δ*rer2* cells were grown for 5–7 days on solid medium, the appearance of fast growing colonies was observed. Some of these stable revertants of Δ*rer2* were analyzed, and their growth rates were comparable to those of wild type cells or of Δ*rer2* cells overexpressing the *SRT1* locus (Figure 2A). When the expression of CPY was analyzed in both revertant and *SRT1*-suppressed Δ*rer2* 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 Δ*rer2* revertant cells and *SRT1*-suppressed Δ*rer2* cells was due to increased levels of dolichol and Dol-P, these lipids were analyzed in wild type, Δ*rer2*, Δ*rer2* revertant cells and Δ*rer2* cells overexpressing *SRT1*. In our analytical system, polyprenols could be separated from dolichols and comigration experiments showed that dolichol and not polyprenol 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 Δ*rer2* 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 Δ*rer2* cells; however, we do not know the chemical nature of these components. The analysis of Dol-P in Δ*rer2* 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 Δ*rer2* revertant cells and in Δ*rer2* 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-Ps were altered in Δ*rer2* revertants and *SRT1* overexpressing cells compared to Δ*rer2* 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 polyisoprenyl chains longer than the size typically found in wild type cells. Overexpression of *SRT1* in Δ*rer2* 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, Δ*rer2*, Δ*rer2* revertant

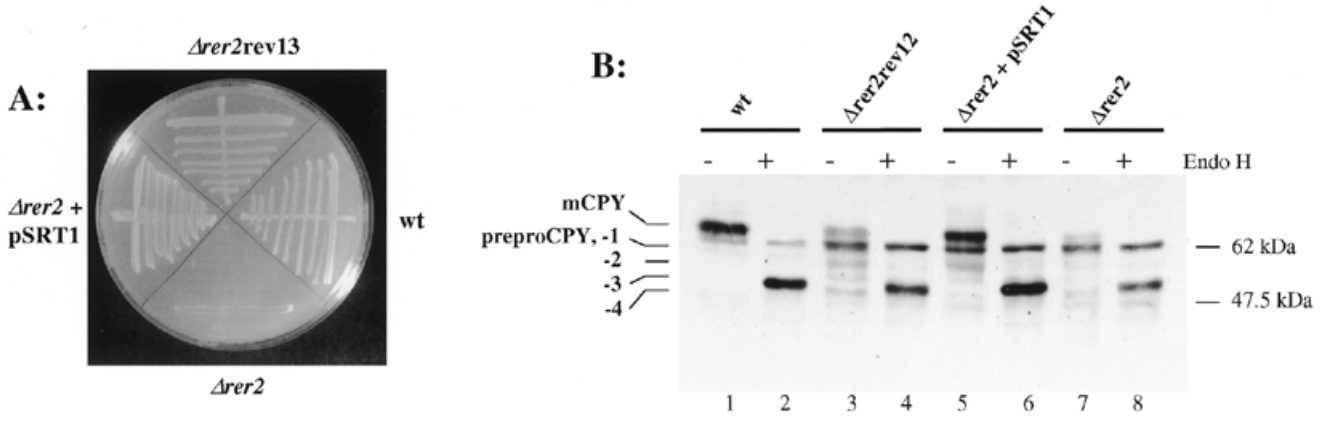

Fig. 2. Analysis of growth and CPY processing in Δ*rer2* revertant cells and Δ*rer2* cells overexpressing *SRT1*. (**A**) Cells of the strains SS328 (wt, wild type), YG932 (Δ*rer2*), YG1118 (Δ*rer2*+pSRT1, the Δ*rer2* strain overexpressing *SRT1)* and YG1114 (Δ*rer2*rev13, a revertant of Δ*rer2* deleted cells) were grown for 3 days on a YPD plate at 30°C. A picture of the agar plate is shown. (**B**) Strains SS328 (wt, wild type), YG1113 (Δ*rer2*rev12, a revertant of Δ*rer2* deleted cells), YG1118 (Δ*rer2*+pSRT1, the Δ*rer2* strain overexpressing *SRT1)* and YG932 (Δ*rer2*) 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.

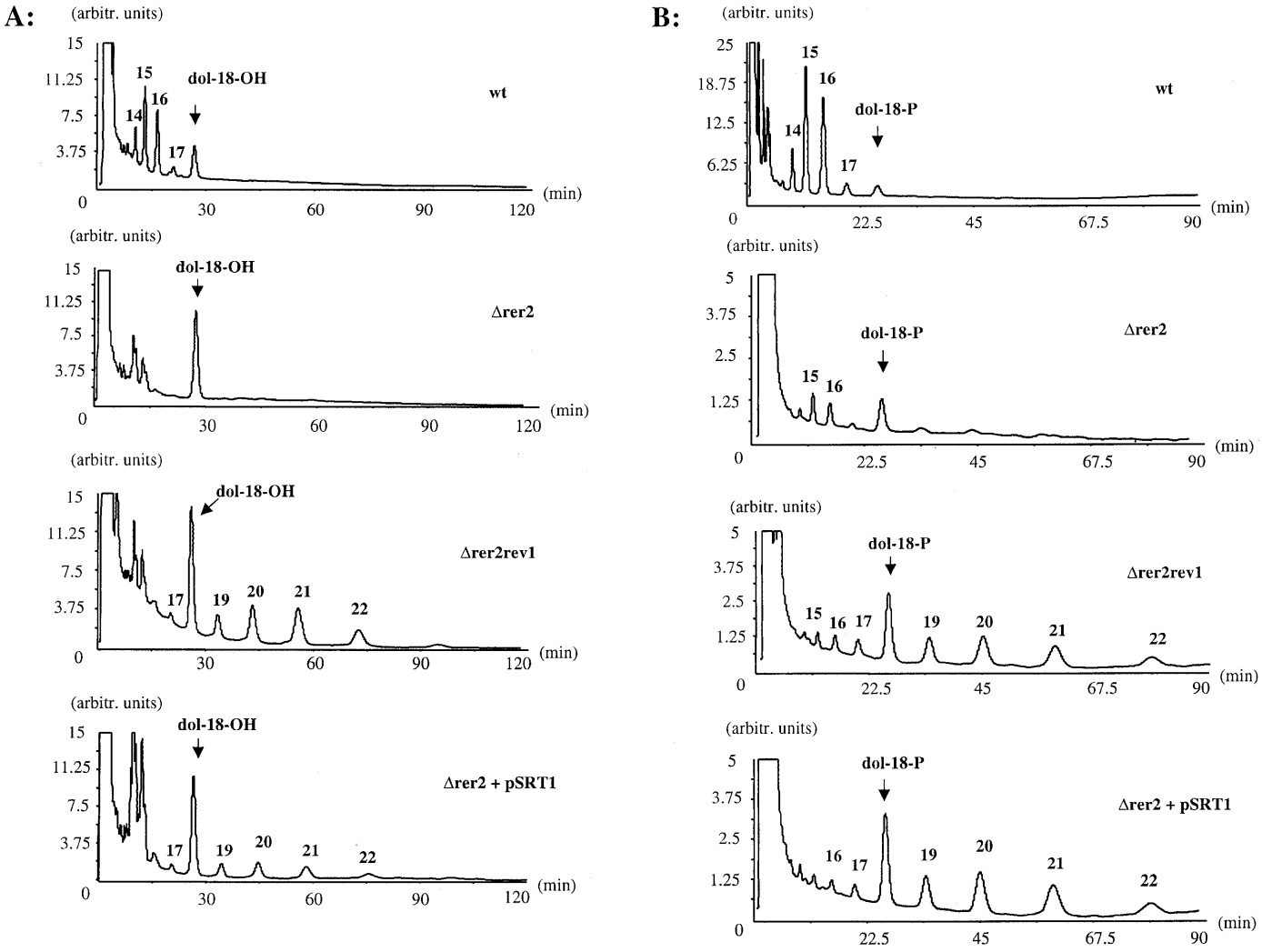

Fig. 3. Analysis of dolichol and Dol-P levels in different strains. Dolichol (**A**) and Dol-P (**B**) were extracted from 500 OD-equivalents of cells from strains SS328 (wt, wild type), YG932 (Δ*rer2*), YG1111 (Δ*rer2*rev1, a revertant of Δ*rer2* deleted cells) and YG1118 (Δ*rer2*+pSRT1, the Δ*rer2* 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.

cells, and Δ*rer2* cells overexpressing *SRT1* were analyzed. Lipid-linked oligosaccharides were metabolically labeled *in vivo* with [^{3}H]mannose. The [^{3}H]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, $Glc_3Man_9GlcNAc_2$-P-P-Dol, whereas in Δ*rer2* cells very low levels of lipid-linked oligosaccharides were detected (Figure 4A). This finding is compatible with the marked hypoglycosylation observed in Δ*rer2* mutant cells. Interestingly, both the Δ*rer2* revertant cells, as well as Δ*rer2* cells overexpressing *SRT1*, accumulated $Glc_3Man_9GlcNAc_2$-P-P-Dol. However, the levels of $Glc_3Man_9GlcNAc_2$-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 Δ*rer2* cells is due to a deficiency in lipid-linked oligosaccharide production and provide indirect evidence that the longer chain dolichols found in Δ*rer2* revertant cells and Δ*rer2* cells overexpressing *SRT1* are used for the biosynthesis of $Glc_3Man_9GlcNAc_2$-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 Δ*rer2* revertant cells and Δ*rer2* 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.

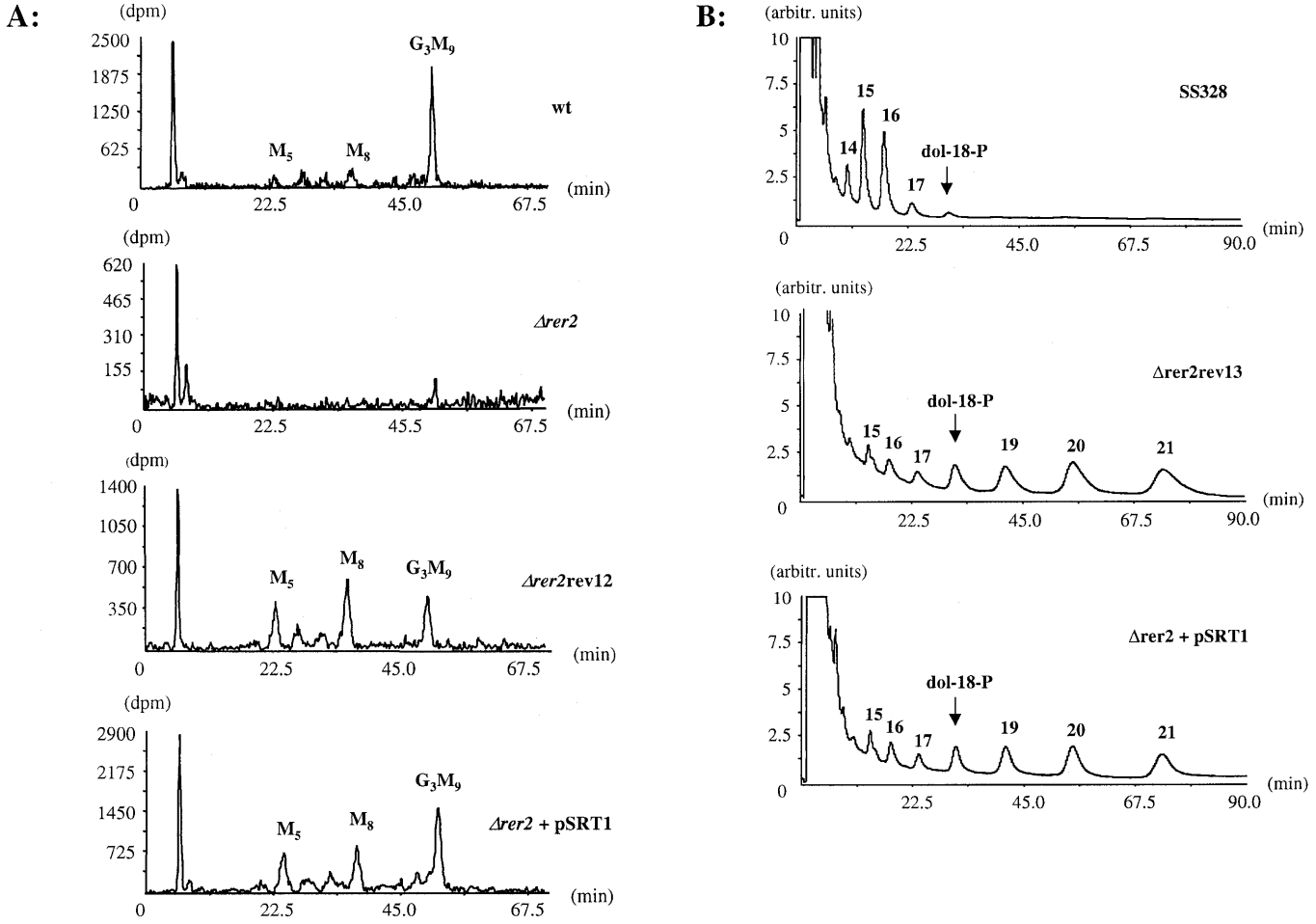

Fig. 4. Analysis of lipid-linked oligosaccharides (LLOs) and their Dol-P moieties in different strains. (**A**) Labeled LLOs were isolated from strains SS328 (wt, wild type), YG932 (Δ*rer2*), YG1113 (Δ*rer2*rev12, a revertant of Δ*rer2* deleted cells) and YG1118 (Δ*rer2* + pSRT1, the Δ*rer2* strain overexpressing *SRT1*). [^{3}H]oligosaccharides were released by mild acid hydrolysis and analyzed by HPLC. Elution was monitored by a radiodetector. The elution positions of $Glc_3Man_9GlcNAc_2$ (G_3M_9), $Man_8GlcNAc_2$ (M_8) and $Man_5GlcNAc_2$ (M_5) are indicated. (**B**) LLOs were isolated from the strains SS328 (wt, wild type), YG1114 (Δ*rer2*rev13, a revertant of Δ*rer2* deleted cells) and YG1118 (Δ*rer2*+pSRT1, the Δ*rer2* strain overexpressing *SRT1*). The Dol-P moiety was cleaved from the oligosaccharide by strong base hydrolysis and analyzed by HPLC. A UV detector at 214 nm was used. The chain length of the isoprenologues is indicated above the peaks. Dol-18-P served as a standard.

Overexpression of SRT1 in Δrer2 cells restored cis-IPTase activity

The occurrence of long chain dolichols in Δ*rer2* 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 Δ*rer2* cells produced a 7- to 17-fold increase in *cis*-IPTase activity *in vitro* as compared to Δ*rer2* 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 Δ*rer2* 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 Δ*rer2* 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

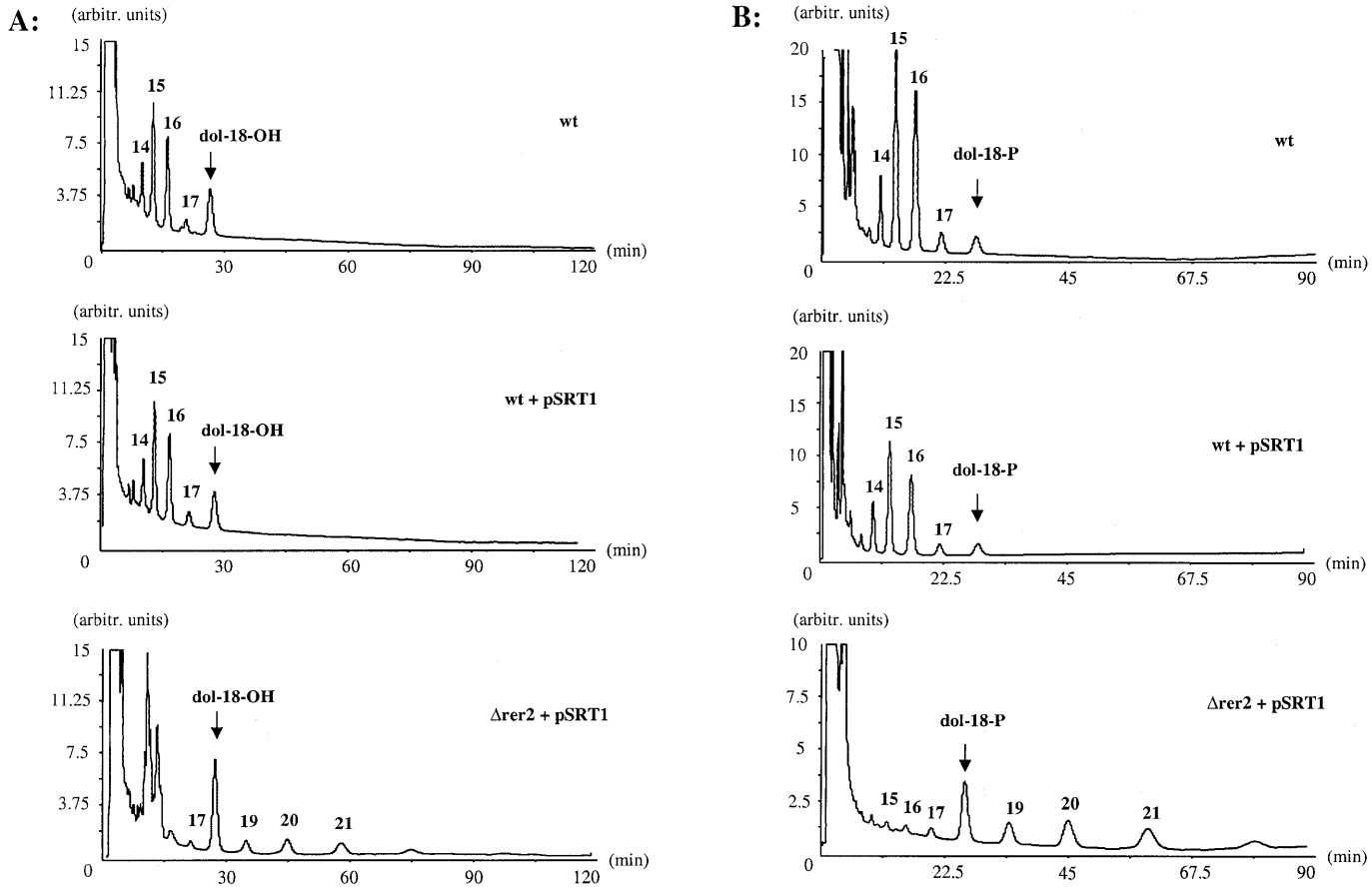

Fig. 5. Analysis of dolichol and Dol-P levels in strains overexpressing *SRT1*. Dolichol (**A**) and Dol-P (**B**) were extracted from 500 OD-equivalents of cells from strains SS328 (wt, wild type), YG1120 (wt + pSRT1, the wild type strain overexpressing *SRT1*) and YG1118 (Δ*rer2* + pSRT1, the Δ*rer2* 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.

Table I. Comparison of *cis*-IPTase activity in microsomal fractions of different strains

	cis-IPTase activity initiated with:	
	t,t-F-P-P	*t,t,c,*-GG-P-P
Strain	(% of wt activity)	
wt	100	100
wt + *RER2*	153	n.d.
Δ*rer2*	18	7
Δ*rer2* + *SRT1*	150	121
Δ*rer2*rev13	77	89

Assays were performed as described in *Materials and methods* using microsomal fractions from strains YG964 (wt, wild type strain containing the vector plasmid), YG965 (wt + pRER2, wild type overexpressing *RER2*), YG991 (Δ*rer2*), YG1120 (wt + pSRT1, wild type overexpressing *SRT1*) and YG1114 (Δ*rer2*rev13, Δ*rer2* revertant cells). *cis*-IPTase activity was initiated with *t,t*-farnesyl pyrophosphate (*t,t*-F-P-P) or *t,t,c,*-geranylgeranyl pyrophosphate (*t,t,c,*-GG-P-P) added at 50 μM. The rates for wild type are 4350 c.p.m./min/mg protein with *t,t*-F-P-P and 4300 c.p.m./min/mg protein with *t,t,c,*-GG-P-P, respectively, after subtraction of a zero time control. Values are given as % of wild type activity. The rates were determined by duplicate analyses and represent the mean value of two independent experiments. n.d., Not determined.

Δ*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 Δ*rer2* strains (Sato *et al.*, 1999). Overexpression of *SRT1* in Δ*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 Δ*rer2* cells having a single copy of *SRT1*. The ability of *SRT1* to act as a high copy number suppressor of Δ*rer2* also explains the high reversion rates observed in Δ*rer2* 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 Δ*rer2* revertant cells contained unusually long chain dolichols and Dol-Ps strongly supported this conclusion.

These long chain dolichols were exclusively found in Δ*rer2* revertant cells and Δ*rer2* 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 polyprenols 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 Δ*rer2* cells overexpressing *SRT1*, but protein translocation was impaired to a similar extent in Δ*rer2* cells and in Δ*rer2* 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 polyprenol 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 KKXX 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 biological 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 polyprenols of the Polish Academy of Sciences, Warzawa, Poland. Sep-Pak columns were from Waters, Switzerland and [1-^{14}C]isopentenyl pyrophosphate (55 mCi/mmol), *t,t*-farnesyl pyrophosphate and *t,t,c*-geranylgeranyl pyrophosphate were from ICN.

Strains

SS328 (*MATα ade2-101 ura3-52 his3Δ200 lys2-801*) (Vijayraghavan *et al.*, 1989), YG964 (*MATα ade2-101 ura3-52 his3Δ200 lys2-801* pYEp352), YG965 (*MATα ade2-101*

ura3-52 his3Δ200 lys2-801 p*RER2*), YG991 (*MAT*a Δ*rer2::GAL1-10-RER2-URA3 ade2-101 ura3-52 his3Δ200 lys2-801*), YG932 (*MAT*α Δ*rer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801*), YG938 (*MAT*α Δ*srt1::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801*), SS328×SS330 (*MAT*a/α *ade2-101/ade2-101 ura3-52/ura3-52 his3Δ200/his3Δ200 lys2-801/+ tyr1/+*), YG1111, YG1113, YG1114: three randomly chosen revertants of YG932, YG1117 (*MAT*α Δ*rer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801* YEp352), YG1118 (*MAT*α Δ*rer2::kanMX4 ade2-101 ura3-52 his3Δ200 lys2-801* p*SRT1*), YG1120 (*MAT*α *ade2-101 ura3-52 his3Δ200 lys2-801* p*SRT1*), YG736 (*MAT*a *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 CAG TAA AAA TAG GGT AAA CAC AGG TAA AAG ACG GT**T CGA TGA ATT CGA GCT C**-3′, primer 2: 5′-TAC TCT ATA AAT ATC TAT GCC ATG TGG TAG GAA AAA AAT GCA GAC CTT TC**C 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 Δ*rer2* 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 GGC TGC CTT TCA AAC ATA GGA CGT TTC TGT TGA CCA TA**C TGA TGA ATT GAG CTC**-3′, primer 2: 5′-TTT ACA TTT TAT TAG CTA TAC ACT TGG CGC AAG GAT ATT TTA TAT ACC GG**C 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 OD_{546} 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 OD_{546} between 1 and 1.5. The metabolic labeling with [^{3}H]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 (OD_{546} = 1–2) were pelleted and resuspended in 12 ml H_2O. 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 N_2, and the lipids were dissolved in 10 ml methanol/water (98:2) containing 20 mM H_3PO_4. A C-18 Sep-Pak (2 ml) was equilibrated with 10 ml methanol/water (98:2) containing 20 mM H_3PO_4, and the lipid extract was applied to the column. The column was washed with 10 ml methanol/water (98:2) containing 20 mM H_3PO_4 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% NH_4OH (v/v). A silica Sep-Pak (2 ml) was equilibrated with 40 ml chloroform/methanol (2:1) containing 0.5% NH_4OH and the dolichol 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% NH_4OH, and Dol-P was eluted with 30 ml chloroform/methanol/water (10:10:3). Samples were dried under N_2 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

20 mM H_3PO_4 for Dol-P and isopropanol/methanol/water (75:20:5) containing 20 mM H_3PO_4 for dolichol, were run at a flow rate of 1 ml/min. Samples were injected using an autosampling device (Merck/Hitachi AS-2000A), and standard dolichols with 18 and 21 isoprene units were used for comigration experiments (not shown). Dolichol and Dol-P were detected by absorption at 214 nm with a Merck/Hitachi L-4250 UV-VIS detector.

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 ml chloroform/methanol (2:1) to remove the bulk membrane lipids. Lipid-linked oligosaccharides were then extracted three times with 60 ml 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 as 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-IPTase activity was assayed as described previously (Crick *et al.*, 1991). Reaction mixtures contained 25 mM Tris–HCl (pH 8.5), 5 mM $MgCl_2$, 1.25 mM DTT, 2.5 mM sodium orthovanadate, 50 µM farnesyl pyrophosphate, 90 µM [1-^{14}C]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 N_2. The amount of [^{14}C]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-IPTase, *cis*-isoprenyltransferase; CPY, carboxypeptidase Y; Dol-P, dolichyl monophosphate; Poly-P-P, fully unsaturated, long chain polyprenyl 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.

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