

Research Article

Functional, comparative and cell biological analysis of *Saccharomyces cerevisiae* Kre5p

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Abstract

Saccharomyces cerevisiae *kre5*Δ mutants lack β-1,6-glucan, a polymer required for proper cell wall assembly and architecture. A functional and cell biological analysis of Kre5p was conducted to further elucidate the role of this diverged protein glucosyltransferase-like protein in β-1,6-glucan synthesis. Kre5p was found to be a primarily soluble N-glycoprotein of ~200 kDa, that localizes to the endoplasmic reticulum. The terminal phenotype of Kre5p-deficient cells was observed, and revealed a severe cell wall morphological defect. *KRE6*, encoding a glucanase-like protein, was identified as a multicopy suppressor of a temperature-sensitive *kre5* allele, suggesting that these proteins may participate in a common β-1,6-biosynthetic pathway. An analysis of truncated versions of Kre5p indicated that all major regions of the protein are required for viability. Finally, *Candida albicans* *KRE5* was shown to partially restore growth to *S. cerevisiae* *kre5*Δ cells, suggesting that these proteins are functionally related. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: *Saccharomyces cerevisiae*; cell wall; glucan synthesis; ER protein

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Introduction

The cell wall of *Saccharomyces cerevisiae* is an essential organelle, accounting for up to 30% of the dry weight of the cell (Orlean, 1997). It is involved in many cellular processes, ranging from protection from external osmotic stress to mating and sporulation (reviewed in Klis, 1994; Orlean, 1997). The cell wall is a highly complex, ordered structure, composed of mannoprotein, β-1,3-glucan, β-1,6-glucan and low levels of chitin. β-1,3-glucan is the major β-glucan and is a predominantly linear polymer composed of about 1500 glucose residues, while β-1,6-glucan is a shorter, highly branched molecule, consisting of about 450 glucose residues (Manners *et al.*, 1973a, 1973b; Kollár *et al.*, 1997). Although β-1,6-glucan is only a minor cell wall component (Dijkgraaf *et al.*, 2002), it has been shown to be a highly important polymer for cell wall biosynthesis and architecture. For example, Kollár *et al.* (1997) have shown that β-1,6-glucan is covalently cross-linked to mannoprotein, β-1,3-glucan and chitin, and have therefore proposed that

β-1,6-glucan is the central molecule of a cell wall building block, interconnecting all the various cell wall components.

The importance of cell wall β-1,6-glucan was initially realized through screens that were used to identify mutant strains deficient to varying extents in β-1,6-glucan synthesis. These screens involved the use of the K1 killer toxin, a lethal pore-forming protein that binds to a β-1,6-glucan-containing cell wall receptor, thus allowing for selection of strains resistant to this toxin, based on their reduced levels of cell wall β-1,6-glucan (reviewed in Bussey, 1991). The use of this screen has resulted in the identification of several *KRE* (killer resistant) genes in which mutation leads to lowered levels of β-1,6-glucan, as well as slow growth phenotypes of varying severity (Al-Aidroos and Bussey, 1978; Boone *et al.*, 1990; Brown *et al.*, 1993). The products of these genes localize along the secretory pathway, suggesting that β-1,6-glucan synthesis may be initiated in the endoplasmic reticulum (ER), continue

along the secretory pathway, and be completed at the cell surface (reviewed in Shahinian and Bussey, 2000). Of the *KRE* genes so far identified, *KRE5* is of particular interest, as its disruption leads to a virtually complete lack of cell wall β -1,6-glucan, as well as a highly severe growth phenotype or lethality, depending on the strain background (Meaden *et al.*, 1990; Shahinian *et al.*, 1998; Azuma *et al.*, 2002). In addition, the product of the *KRE5* gene is predicted to localize to the ER, and deletion of *KRE5* is epistatic to all other *KRE* genes so far described, suggesting an early and important role in β -1,6-glucan synthesis (Meaden *et al.*, 1990; Shahinian *et al.*, 1998). Little is known about the Kre5 protein, despite its apparent importance for β -1,6-glucan synthesis. Based on *KRE5* sequence analysis, it is predicted to be a relatively large protein of 156 kDa in its mature form, with 13 potential *N*-glycosylation sites (Meaden *et al.*, 1990). Furthermore, the presence of an N-terminal signal sequence, a C-terminal HDEL ER-retention signal and a lack of any predicted transmembrane domains suggest that Kre5p is a soluble ER-luminal protein (Meaden *et al.*, 1990).

The only clue to the biochemical activity of Kre5p comes from its limited but significant sequence similarity to UDP-glucose:glycoprotein glucosyltransferase (UGGT; Parker *et al.*, 1995; Fernández *et al.*, 1996; Tessier *et al.*, 2000). UGGT has been shown in some other organisms to be intimately involved in the process of quality control of protein folding in the ER, whereby it monoglucosylates misfolded or unfolded proteins, allowing them to bind the chaperones calnexin or calreticulin, facilitating another round of protein folding, and preventing exit of the protein from the ER until it has been properly folded (reviewed in Parodi, 1999). Despite the sequence similarity between Kre5p and UGGT, some lines of evidence suggest that Kre5p does not function as a classic UGGT. Of the UGGT proteins so far identified, Kre5p is the least similar at the amino acid level (Parodi, 1999). In fact, sequence analysis shows that *KRE5* has diverged at unexpectedly high rates when compared to its counterparts in *Schizosaccharomyces pombe* and other organisms (Aravind *et al.*, 2000). Experimental evidence also supports a functional dissimilarity between Kre5p and the UGGTs. It has been demonstrated

by Shahinian *et al.* (1998) that the essential activity of Kre5p is not that of a classic UGGT, and no UGGT activity has been found in *S. cerevisiae* (Fernández *et al.*, 1994; Jakob *et al.*, 1998). Thus, while Kre5p may have diverged from the UGGTs to the point where it is no longer involved in quality control of protein folding, it remains possible that Kre5p functions as a glycosyltransferase with different target protein and/or sugar donor specificity than the UGGT proteins, and that this modified activity is involved in β -1,6-glucan synthesis.

To examine the role of Kre5p in β -1,6-glucan biosynthesis, we have conducted a functional and cell biological analysis of Kre5p, and show it to be a primarily soluble, ER-localized, *N*-glycoprotein of approximately 200 kDa. Kre5p-deficiency results in a severe cell wall morphological defect, and the lethality of a *kre5* Δ strain can be partially complemented by the *Candida albicans* *KRE5* (*CaKRE5*) gene.

Materials and methods

Strains and culture conditions

Yeast strains used in this study are listed in Table 1. Standard yeast media and growth conditions were used as described in Bussey *et al.* (1982). Genetic crosses, sporulation of diploids, and dissection of tetrads were conducted using standard methods (Sherman *et al.*, 1982). Plasmid transformations in yeast were carried out using the one-step method of Chen *et al.* (1992), unless otherwise specified. Transformation of gap vector and PCR products was done using the high-efficiency method of Gietz *et al.* (1995). Transformants were selected on synthetic minimal media with auxotrophic supplements, with the exception of selection using the *kanMX2* module, where YPD containing 200 μ g/ml geneticin (GIBCO, Burlington, ON) was used. Plasmids were propagated using the *Escherichia coli* strain DH10B, and to prepare uracil-containing DNA used for site-directed mutagenesis (Kunkel *et al.*, 1987), the *E. coli* strain CJ236 (*dut*⁻, *ung*⁻, F') was used. Bacterial cells were cultured and transformed using standard media and methods (Sambrook *et al.*, 1989).

Table 1. Strains used in this study

| Strain | Genotype | Source |
|---------------------|---|----------------------------|
| TA405 | <i>MATa/α leu2/leu2 his3/his3 can1/can1</i> | Whiteway and Szostak, 1985 |
| HAB927 ^a | <i>MATa</i> | This study |
| HAB928 ^a | <i>MATa ura3::kanMX2</i> | This study |
| HAB929 ^a | <i>MATa/α ura3::kanMX2/ura3::kanMX2</i> | This study |
| HAB930 ^a | <i>MATa/α ura3::kanMX2/ura3::kanMX2 kre5::HIS3/KRE5</i> | This study |
| HAB931 ^a | <i>ura3::kanMX2 kre5::HIS3</i> | This study |
| SEY6210 | <i>MATa/α leu2-3 112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9</i> | S. D. Emr |
| HAB932 ^b | Haploid | This study |
| HAB933 ^b | <i>MATa/α kre5::HIS3/KRE5</i> | This study |
| HAB934 ^b | <i>kre5::HIS3 + pRS314 KRE5</i> | This study |
| HAB935 ^b | <i>kre5::HIS3 + pRS314 kre5-ts1</i> | This study |
| HAB936 ^b | <i>kre5::HIS3 + pRS314 kre5-ts2</i> | This study |
| HAB937 ^b | <i>kre5::HIS3 + YCp50 KRE5</i> | This study |
| Pmt3/4 ^b | <i>MATα pmt3::HIS3 pmt4::TRP1</i> | Gentzsch and Tanner, 1996 |
| Pmt2/4 ^b | <i>MATα pmt2::LEU2 pmt4::TRP1</i> | Gentzsch and Tanner, 1996 |
| Pmt6 ^b | <i>pmt6::URA3</i> | W. Tanner |
| Pmt1/2 ^b | <i>pmt1::HIS3 pmt2::LEU2</i> | Lussier et al., 1995 |

^a Derived from TA405.

^b Derived from SEY6210.

DNA purification and recombinant DNA techniques

Yeast DNA was isolated by the method of Hoffman and Winston (1987). Plasmid minipreps were prepared from *E. coli* using either the boiling or alkaline lysis method (Sambrook *et al.*, 1989). Restriction endonucleases, calf intestinal alkaline phosphatase, shrimp alkaline phosphatase, T4 DNA ligase, sequenase, T4 polynucleotide kinase, Klenow fragment, and the Gene 32 protein were purchased from Bethesda Research Laboratories (Gaithersburg, MD), Pharmacia LKB Biotechnology (Piscataway, NJ), New England Biolabs (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN), or US Biochemicals (Cleveland, OH), and were used according to the manufacturers' directions.

Oligonucleotide primers and plasmid constructs

Oligonucleotide primers used in this study are described in Table 2. The plasmid constructs used are summarized in Table 3. Single-stranded, uracil-containing DNA for use in site-directed mutagenesis was prepared using the method of Sambrook *et al.* (1989). Single-stranded pRS316 *KRE5* was used as template for all site-directed mutagenesis reactions, except for the generation of RUGT HDEL. Regions surrounding sites of mutagenesis

and junctions created between blunted ends in the open reading frames (ORFs) of plasmid constructs were verified by sequencing.

Site-directed mutagenesis was carried out using oligonucleotide primers 1, 2, 3 and 4 to generate pRS316 *kre5Δ1*, pRS316 *kre5Δ2*, pRS316 *kre5Δ3* and pRS316 *kre5Δ4*, respectively. The C-terminal HEEL sequence of pRS314 *KRE5*-RUGT was converted to HDEL via site-directed mutagenesis, using oligonucleotide primer 5 and single-stranded pRS314 *KRE5*-RUGT as template. This resulted in the generation of pRS314 *KRE5*-RUGT HDEL. A clone from a *C. albicans* YEp352 genomic bank (Boone *et al.*, 1991) containing a 6.2 kb insert with the *CaKRE5* ORF was obtained. For construction of pRS314 *KRE5*-*CaKRE5*, a novel *SmaI* site was generated in the *KRE5* ORF of pRS314 *KRE5 HindIII*-*SalI* using oligonucleotide primer 6. This construct was digested with *SmaI* and *KpnI* to remove a ~2.4 kb *KRE5* C-terminal fragment, which was replaced by a ~1.5 kb *DraI*-*KpnI* C-terminal *CaKRE5* fragment from YEp352 *CaKRE5*. A cassette encoding three tandem copies of the influenza virus haemagglutinin epitope (HA₃) was used to construct a functional tagged version of Kre5p. The polylinker *NotI* site was removed from pRS316 *KRE5* by digestion with *NotI*, creating blunt ends with Klenow fragment, and religating. A novel

Table 2. Oligonucleotide primers used

| Primer number | Description | Sequence |
|-------------------|---|---|
| 1 | <i>kre5Δ1</i> deletion | GCTTACGATATGGCATAAATGGACAGAATTGG |
| 2 | <i>kre5Δ2</i> deletion | GCTAGGTCTCTATATTGCTTTGAAAAGATTG |
| 3 | <i>kre5Δ3</i> deletion | TTTGGCTCTATTCATCAATTTTCATACCGAC |
| 4 | <i>kre5Δ4</i> deletion | ACCTTTGGCTATGGACATGACGAATTATAAC |
| 5 ^a | HEEL to HDEL | GGATCTCAGAAGCATGACGAATTATGATCTCTG |
| 6 ^b | <i>SmaI</i> insertion in <i>KRE5</i> | ACCGTTCAGTCATTTTTCC CCCGGG GGATCTACTGACGGGCAT |
| 7 ^b | <i>NotI</i> insertion in <i>KRE5</i> | GGCTACTATACTAAA GGCGGCCGCA ATGAAGAATACAATTTA |
| 8 | 5' <i>URA3</i> disruption | AGTATTCTTAACCCAACTGCACAGAACAAAAACCTGCAGGAAACGAAGAT AAATCCAGCTGAAGCTTCGTACGC |
| 9 | 3' <i>URA3</i> disruption | TTGAAGCTCTAATTTGTGAGTTTAGTATACATGCATTTACTTATAATACA GTTTTGCATAGGCCACTAGTGGATCTG |
| 10 | Insertion of <i>KRE5</i> into pFastBact1 (5') | GCTCGACGGTCCGTTACAACATGAGACTACTTGCGTTGG |
| 11 ^{b,c} | Insertion of <i>KRE5</i> into pFastBact1 (3') | TCCGACAATGCCGCTCCTCTGCATCACCATCACCATCACTAATGA GGTACC CGGTGC |
| 12 | Generation of <i>kre5-ts1</i> (5'-1) | ATGAGACTACTTGCGTTG |
| 13 | Generation of <i>kre5-ts1</i> (3'-1) | TCAGGAAGCCATTTATGAAGGC |
| 14 | Generation of <i>kre5-ts1</i> (5'-2) | CGTATGCTCAGAAAGAATAGGC |
| 15 | Generation of <i>kre5-ts1</i> (3'-2) | TTCTTTCACGCTCTTGGAACCGTGC |
| 16 | Generation of <i>kre5-ts2</i> (5') | ATGAGACTACTTGCGTTG |
| 17 | Generation of <i>kre5-ts2</i> (3') | TCAGGAAGCCATTTATGAAGGC |

^a Underlined nucleotide indicates the point mutation responsible for an E to D substitution.

^b Inserted restriction sites are indicated in bold.

^c Italicized nucleotides indicate the insertion of a 6XHIS tag.

Table 3. Plasmid constructs used in this study

| Plasmid | 5' Junction ^a | 3' Junction ^b | Vector | Insert | Source |
|------------------------|---|---|---------------------------|---|---------------------------|
| pRS315 <i>KRE5</i> | | | | | Meaden et al., 1990 |
| pSM491 | | | | | Kolodziej and Young, 1991 |
| pRS316 | | | | | Sikorski and Hieter, 1989 |
| pRS316 <i>KRE5</i> | <i>HindIII</i> – <i>HindIII</i> | <i>Sall</i> – <i>Sall</i> | pRS316 | 6.8 kb <i>KRE5</i> fragment from pRS315 <i>KRE5</i> | This study |
| pRS316 <i>KRE5-HA</i> | <i>NotI</i> – <i>NotI</i> | <i>NotI</i> – <i>NotI</i> | See Methods and materials | HA ₃ from pSM491 | This study |
| pRS316 <i>kre5Δ1</i> | | | | | This study |
| pRS316 <i>kre5Δ2</i> | | | | | This study |
| pRS316 <i>kre5Δ3</i> | | | | | This study |
| pRS316 <i>kre5Δ4</i> | | | | | This study |
| pVT102u | | | | | Vemet et al., 1987 |
| pVT102u <i>KRE5</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 6 kb <i>KRE5</i> fragment from pRS315 <i>KRE5</i> | This study |
| pVT102u <i>KRE5-HA</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 6.1 kb <i>KRE5-HA</i> fragment from pRS316 <i>KRE5-HA</i> | This study |
| pVT102u <i>kre5Δ1</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 5.1 kb <i>kre5Δ1</i> fragment from pRS316 <i>kre5Δ1</i> | This study |
| pVT102u <i>kre5Δ2</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 5 kb <i>kre5Δ2</i> fragment from pRS316 <i>kre5Δ2</i> | This study |
| pVT102u <i>kre5Δ3</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 5 kb <i>kre5Δ3</i> fragment from pRS316 <i>kre5Δ3</i> | This study |
| pVT102u <i>kre5Δ4</i> | <i>PvuII</i> – <i>AccI</i> ^c | <i>AccI</i> ^c – <i>PvuII</i> | pVT102u | 4.9 kb <i>kre5Δ4</i> fragment from pRS316 <i>kre5Δ4</i> | This study |

Table 3. Continued

| Plasmid | 5' Junction ^a | 3' Junction ^b | Vector | Insert | Source |
|--|---|---|--|---|---------------------------|
| YCp50 <i>KRE5</i> | | | | | Meaden et al., 1990 |
| pcDNA3 RUGT | | | | | D. Dignard |
| pRS314 | | | | | Sikorski and Hieter, 1989 |
| pRS314 <i>KRE5</i> <i>Bst</i> XI | <i>Sst</i> I– <i>Bst</i> XI | <i>Bst</i> XI– <i>Kpn</i> I | pRS314 | 5.3 kb <i>KRE5</i> fragment from YCp50 <i>KRE5</i> | This study |
| pRS314 <i>KRE5</i> <i>Hind</i> III– <i>Sal</i> I | <i>Sma</i> I– <i>Hind</i> III | <i>Sal</i> I– <i>Sal</i> I | pRS314 | 7 kb <i>KRE5</i> fragment from YCp50 <i>KRE5</i> | This study |
| pRS314 <i>kre5-ts1</i> | | | | | This study |
| pRS314 <i>kre5-ts2</i> | | | | | This study |
| pRS314 <i>KRE5</i> –RUGT | <i>Pst</i> I ^d – <i>Xho</i> I ^d | <i>Apa</i> I– <i>Apa</i> I | pRS314 <i>KRE5</i> <i>Hind</i> III– <i>Sal</i> I | 1.3 kb RUGT fragment from pcDNA3 RUGT | This study |
| pRS314 <i>KRE5</i> –RUGT HDEL | | | | | This study |
| pRS424 | | | | | Christianson et al., 1992 |
| pRS424 <i>KRE5</i> –RUGT | <i>Spe</i> I– <i>Spe</i> I | <i>Apa</i> I– <i>Apa</i> I | pRS424 | 5.3 kb <i>KRE5</i> –RUGT fragment from pRS314 <i>KRE5</i> –RUGT | This study |
| pRS424 <i>KRE5</i> –RUGT HDEL | <i>Pst</i> I– <i>Pst</i> I | <i>Apa</i> I– <i>Apa</i> I | pRS424 <i>KRE5</i> –RUGT | 1.3 kb RUGT HDEL fragment from pRS314 <i>KRE5</i> –RUGT HDEL | This study |
| p424 ADH | | | | | Rönicke et al., 1997 |
| p424 ADH RUGT | <i>Sma</i> I– <i>Kpn</i> I ^d | <i>Apa</i> I ^d – <i>Sma</i> I | p424 ADH | 4.6 kb RUGT fragment from pcDNA3 RUGT | This study |
| p424 ADH RUGT HDEL | <i>Xho</i> I– <i>Xho</i> I | <i>Xba</i> I– <i>Xba</i> I | p424 ADH RUGT | 1.2 kb RUGT HDEL fragment from pRS314 <i>KRE5</i> –RUGT HDEL | This study |
| p424 ADH RUGT– <i>KRE5</i> | <i>Xho</i> I ^d – <i>Pst</i> I ^d | <i>Pst</i> I ^d – <i>Xho</i> I ^d | p424 ADH RUGT | 1.7 kb <i>KRE5</i> fragment from pRS314 <i>KRE5</i> <i>Hind</i> III– <i>Sal</i> I | This study |
| YEp352 <i>CaKRE5</i> | | | | | Boone et al., 1991 |
| pRS314 <i>KRE5</i> – <i>CaKRE5</i> | <i>Sma</i> I– <i>Dra</i> I | <i>Kpn</i> I– <i>Kpn</i> I | See Materials and Methods | 1.5 kb <i>CaKRE5</i> fragment from YEp352 <i>CaKRE5</i> | This study |
| pRS424 <i>KRE5</i> – <i>CaKRE5</i> | <i>Bam</i> HI– <i>Bam</i> HI | <i>Kpn</i> I– <i>Kpn</i> I | pRS424 | 5.7 kb <i>KRE5</i> – <i>CaKRE5</i> fragment from pRS314 <i>KRE5</i> – <i>CaKRE5</i> | This study |
| YEp24 <i>KRE6</i> | | | | | Roemer and Bussey, 1991 |
| pRS426 | | | | | Christianson et al., 1992 |
| pRS426 <i>KRE6</i> | <i>Bam</i> HI– <i>Bam</i> HI | <i>Sal</i> I– <i>Sal</i> I | pRS426 | 5.4 kb <i>KRE6</i> fragment from YEp24 <i>KRE6</i> | This study |
| pBSK <i>kre5</i> ΔHDEL | | | | | Meaden et al., 1990 |
| pFastBact I <i>kre5</i> ΔHDEL | | | | | This study |

^a This represents the 5' junction between vector and insert.

^b This represents the 3' junction between vector and insert.

^c Blunt ends were created with Klenow fragment DNA polymerase I.

^d Blunt ends were created with T4 DNA polymerase.

*Not*I site was introduced into the *KRE5* ORF of the resulting construct by site-directed mutagenesis, using oligonucleotide primer 7. A *Not*I–*Not*I HA₃ fragment from pSM491 (Kolodziej and Young, 1991) was inserted into the novel *Not*I site, generating pRS316 *KRE5*–HA. Functionality of this construct was confirmed by its ability to complement the lethality of a *KRE5* disruption in haploid

SEY6210, and to restore K1 killer toxin sensitivity of HAB931 to wild-type levels.

Gene disruption

The entire *URA3* gene of HAB927 was disrupted with *kanMX2* by PCR, using a construct made

with oligonucleotide primers 8 and 9, generating strain HAB928. From this, a homozygous diploid strain was generated (HAB929). One copy of *KRE5* in HAB929 was disrupted with *HIS3*, using the *kre5Δ1::HIS3* construct from Meaden *et al.* (1990), creating strain HAB930. Heterozygous disruption of *KRE5* with *HIS3* in diploid SEY6210 was carried out using the *kre5Δ1::HIS3* construct from Meaden *et al.* (1990), generating the strain HAB933. All gene disruptions produced in this study were verified by Southern blot analysis.

Production of anti-Kre5p serum

A ~4.1 kb *kre5ΔHDEL* insert was amplified from pBSK *kre5ΔHDEL* with Expand HF (Boehringer Mannheim Canada), using oligonucleotide primers 10 and 11, replacing the C-terminal HDEL with a 6XHIS tag. A *CpoI*–*KpnI* fragment from this insert was subcloned into pFastBact1, creating pFastBact1 *kre5ΔHDEL*. This construct was verified by PCR, and used as described by the manufacturer of the Bac-to-Bac system (Gibco/Life Technologies, Inc.) to produce recombinant *kre5ΔHDEL* baculovirus.

Sf9 insect cells were used to produce 6XHIS-tagged Kre5pΔHDEL, according to Tessier *et al.* (2000). Protein was obtained from both medium and cell extracts. The cells were removed by centrifugation and the medium was adjusted to 40 mM Tris–HCl, pH 7.5, 0.5 M NaCl. The medium was concentrated ~10-fold at 4 °C on YM30 membranes (Amicon) in the presence of protease inhibitors [2 μg/ml aprotinin, leupeptin, pepstatin A, and E-64 (Boehringer Mannheim Canada)]. Two buffer exchanges were performed using Buffer ATC/PI (40 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl₂, protease inhibitors). The concentrated solution was centrifuged at 3000 × *g* for 5 min to remove some precipitated proteins, and imidazole was added to a final concentration of 5 mM. Pelleted cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 1% Triton X-100, protease inhibitors) and were lysed on ice for 20 min. Nuclei and other debris were removed by centrifugation at 3000 × *g* for 15 min, and the supernatant was treated on ice with DNase I in the presence of 10 mM MgCl₂ for 30 min. This was then centrifuged at 10 000 × *g* for 15 min and the medium was adjusted to 40 mM Tris–HCl,

pH 7.5, 0.5 M NaCl, 5 mM CaCl₂ and 5 mM imidazole. Separately, the concentrated medium and the cell extract were loaded by gravity flow onto 1 ml Ni²⁺-NTA Superflow columns (Qiagen, Inc.) which had been equilibrated in buffer ATC/PI and 10 mM imidazole. The columns were washed with 20 column volumes of the same buffer. The column was further washed with 10 column volumes of the same buffer at 2 ml/min and protein eluted with 20 column volumes of buffer ATC/PI and 200–400 mM imidazole at the same flow rate. Fractions were collected and analysed by SDS–PAGE and Western blot analysis. Kre5pΔHDEL was further purified by polyacrylamide gel electrophoresis.

Antibodies were raised against Kre5pΔHDEL in rabbits. Initially, rabbits were injected with 500 μg purified protein in Freund's complete adjuvant, followed by four boost injections with equivalent amounts of protein in Freund's incomplete adjuvant. Crude serum was used for all experiments in this study.

Preparation of total cell extracts, analysis of glycosylation and membrane association, and Western blot analysis

Total cell extracts were prepared from log-phase cultures grown in selective media. The cells were harvested and broken with glass beads in ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Germany)]. Cell wall material and unbroken cells were removed by centrifugation at 800 × *g* at 4 °C for 5 min.

For analysis of *N*-glycosylation, treatment of whole cell extracts with an endo-β-*N*-acetylglucosaminidase H/maltose-binding-protein fusion protein (endo H_f; New England Biolabs) or a mixture of endoglycosidase F and peptide-*N*-glycosidase F [endo F/PNGase F; Oxford Glycosciences (Bedford, MA)] was carried out according to manufacturers' instructions.

For membrane association testing, whole cell extracts were treated at 4 °C for 30 min with either 0.1 M Na₂CO₃, 0.6 M NaCl, 1.6 M urea, 1% Triton X-100 or 0.5% SDS. Treated extracts were then ultracentrifuged at 90 000 × *g* at 4 °C for 60 min, with the resulting pellet and supernatant separated. The pellet fraction was resuspended in a volume of lysis buffer equal to the supernatant.

Protein samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis

(SDS-PAGE; Laemmli, 1970) using 6% polyacrylamide gels, and then transferred to Hybond-C nitrocellulose membranes (Amersham, Oakville, ON, Canada). Western blot analysis was carried out using either a 1:1000 dilution of mouse anti-HA monoclonal antibody HA•11 (Berkeley Antibody Company) or a 1:3000 dilution of rabbit anti-Kre5p polyclonal serum (this study). 1:2000 dilutions of horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were used. Detection was conducted using the Amersham ECL chemiluminescence detection kit.

Immunofluorescence microscopy

Cells from exponentially growing cultures grown in selective media were prepared for indirect immunofluorescence microscopy, as described in Pringle *et al.* (1991). Mouse anti-HA monoclonal antibody HA•11 (Berkeley Antibody Company) and rabbit anti-Kar2p polyclonal antibody (kindly provided by Dr R. Schekman, Berkeley CA) were used at 1:50 dilutions. Oregon green-conjugated goat anti-mouse and Texas red-conjugated goat anti-rabbit secondary antibodies (Molecular Probes) were used at 1:100 dilutions. DAPI was used to visualize the cell nuclei. The cells were visualized with a Zeiss fluorescence microscope (Germany) equipped with a Spot Digital Camera (Diagnostic Instruments, Michigan, USA).

Isolation of temperature-sensitive *kre5* alleles

Error-prone PCR mutagenesis was carried out according to Muhlrad *et al.* (1992), with the following adaptations: (a) the concentrations of C/T and G/A dNTP stock solutions were 20 mM and 4 mM, respectively; and (b) an altered PCR program was used [94 °C for 2 min; 94 °C for 1.5 min, 50 °C for 2 min, 72 °C for 3 min (cycled 34 times); 94 °C for 1.5 min; 50 °C for 2 min; 72 °C for 10 min]. pRS314 *KRE5 BstXI* was used as a template for PCR mutagenesis using oligonucleotide primers 12, 13, 14 and 15, whereas pRS314 *KRE5 HindIII-SalI* was used as template for PCR mutagenesis using oligonucleotide primers 16 and 17. Gap vector was obtained by digestion with *EcoRI* and *BglII* in the case of pRS314 *KRE5 BstXI*, and *EcoRI* and *NsiI* in the case of pRS314 *KRE5 HindIII-SalI*.

Mutagenized PCR products, along with gap vector, were then transformed into HAB937, and transformants were allowed to grow at 30 °C for 4 days. YCp50 *KRE5* was shuffled out by replica plating onto 5-fluoroorotic acid (5-FOA)-containing media (1 g/l) at room temperature for 3–4 days. Colonies were then replica plated in duplicate onto selective media and allowed to grow at either 22 °C or 37 °C. Transformants that grew at 22 °C but not at 37 °C were chosen for further analysis. DNA was extracted from these transformants and retransformed into HAB933, which was then sporulated and dissected to verify plasmid-dependency. The temperature-sensitive allele generated using the pRS314 *KRE5 BstXI* template was called *kre5-ts1*, whereas the pRS314 *KRE5 HindIII-SalI* template gave rise to *kre5-ts2*.

Growth and viability analysis

Growth analysis was carried out for HAB934, HAB935 and HAB936 by pre-growing cells in selective liquid media at 22 °C and diluting them into two cultures, one grown at 22 °C and one at 37 °C. Samples were taken from these cultures at 3, 6, 12, 24 and 48 h, and their optical density measured. Viability was determined by plating aliquots of cells from the growth experiments onto rich media, incubating at 22 °C, and counting the number of resulting colonies. The number of colonies for each strain was standardized to the optical density of the plated aliquots, and the logarithmic transformation of these values was plotted against incubation time.

Electron microscopy

Yeast cells were grown to log-phase in liquid media at 22 °C, and diluted into two cultures that were subsequently grown at 22 °C or 37 °C for up to 24 h. Samples from these cultures were fixed in an equal volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (0.1 M cacodylate in 0.1% CaCl₂, pH 7.4) at room temperature for 1 h. The cells were then washed three times with 0.1 M sodium cacodylate buffer and fixed in 1% OsO₄ for 1.5 h. This was followed by several washes in water, dehydration in an acetone–water series, and finally the cells were embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a JEOL 2000 electron microscope.

Multicopy suppressor screen for the *kre5-ts2* growth phenotype

HAB936 was transformed via the high-efficiency method with a YEpl3-based *S. cerevisiae* genomic bank, and transformants were allowed to form colonies for 3 days at 22 °C. These transformants were replica-plated in duplicate onto selective minimal media, incubating one set at 22 °C and the other at 37 °C. Plasmid DNA was extracted from clones that grew at both 22 °C and 37 °C, and retransformed into HAB936, and the process repeated to determine plasmid dependency.

Results

Characterization and subcellular localization of Kre5p

To examine Kre5p, which is predicted to have a molecular weight of 156 kDa (Meaden *et al.*, 1990), whole cell extracts obtained from cells overexpressing Kre5p and Kre5p-HA were analysed via SDS-PAGE and Western blot analysis. Kre5p or HA epitope-tagged Kre5p (Kre5p-HA) were shown to have an apparent molecular weight of ~200 kDa (Figure 1A). Given the discrepancy between the apparent and predicted molecular weights of Kre5p, the presence of multiple potential *N*-glycosylation sites, and the prediction that Kre5p is a secretory pathway protein (Meaden *et al.*, 1990), we examined glycosylation of Kre5p-HA. The presence of *N*-linked glycosylation was examined by digesting whole cell extracts obtained from cells overexpressing Kre5p-HA with either endo-H_f or an endo-F/PNGase F mixture, and subsequently looking for increased mobility on SDS-PAGE. Kre5p-HA treated with either endo-H_f or endo-F/PNGase F migrated with an apparent molecular weight of ~185 kDa, less than that of mock-treated Kre5p-HA (Figure 1B), indicating the presence of *N*-linked glycosylation on the protein. As endoglycosidase-treated Kre5p-HA still has an apparent molecular weight greater than that predicted from the DNA sequence, the possibility of *O*-glycosylation was examined. The protein was expressed in *S. cerevisiae* strains deficient in various stages of *O*-glycosylation, namely the *kre2Δktr1Δktr3Δ* and *pmtΔ* mutant strains. The former strain is deficient for steps of *O*-glycosylation occurring in the Golgi (Lussier *et al.*,

1997), while the *pmtΔ* strains comprise a group of mutants deficient for steps of *O*-glycosylation carried out in the ER (Gentzsch and Tanner, 1996, 1997). Kre5p-HA expressed in *kre2Δktr1Δktr3Δ*, as well as in cells disrupted for various combinations of *PMT* genes, was analysed by SDS-PAGE and Western blot analysis, and its migration was compared to that of Kre5p-HA expressed in a wild-type background. No shift in mobility was detected in any of the mutant strains examined (data not shown), consistent with a lack of *O*-glycosylation.

As it lacks potential transmembrane domains, Kre5p is thought to be a soluble protein (Meaden *et al.*, 1990). To confirm this experimentally, whole cell extracts expressing Kre5p-HA from a centromere-based plasmid were fractionated by ultracentrifugation, and analysed by SDS-PAGE and Western blot analysis (Figure 1C). Much of the Kre5p signal was present in the soluble fraction (supernatant); however, some was present in the membrane fraction (pellet). To determine the nature of the pellet-associated fraction, extract was treated with Na₂CO₃, NaCl, urea, Triton X-100 or SDS prior to ultracentrifugation. Only treatment with SDS and, to a slightly lesser extent, Na₂CO₃, were able to solubilize the protein present in the membrane fraction (Figure 1C). This latter result suggests that Kre5p is primarily a soluble protein.

Kre5p is thought to localize to the ER, as it has a predicted N-terminal signal peptide and C-terminal HDEL ER-retention signal (Meaden *et al.*, 1990). To test this prediction, immunofluorescence microscopy was carried out on wild-type cells overexpressing Kre5p-HA. The cells were stained with both an anti-HA antibody and an antibody against the known soluble ER protein, Kar2p (Figure 2; Rose *et al.*, 1989). Staining with the anti-HA antibody revealed a perinuclear staining pattern, consistent with ER localization in yeast (Rose *et al.*, 1989; Deshaies and Schekman, 1990; Feldheim *et al.*, 1992). Furthermore, anti-HA fluorescence overlapped with anti-Kar2p fluorescence, indicating co-localization of the two proteins. No anti-HA signal was detected when cells overexpressing untagged *KRE5* were examined, and Kre5p-HA was undetectable when expressed from a centromeric plasmid (data not shown).

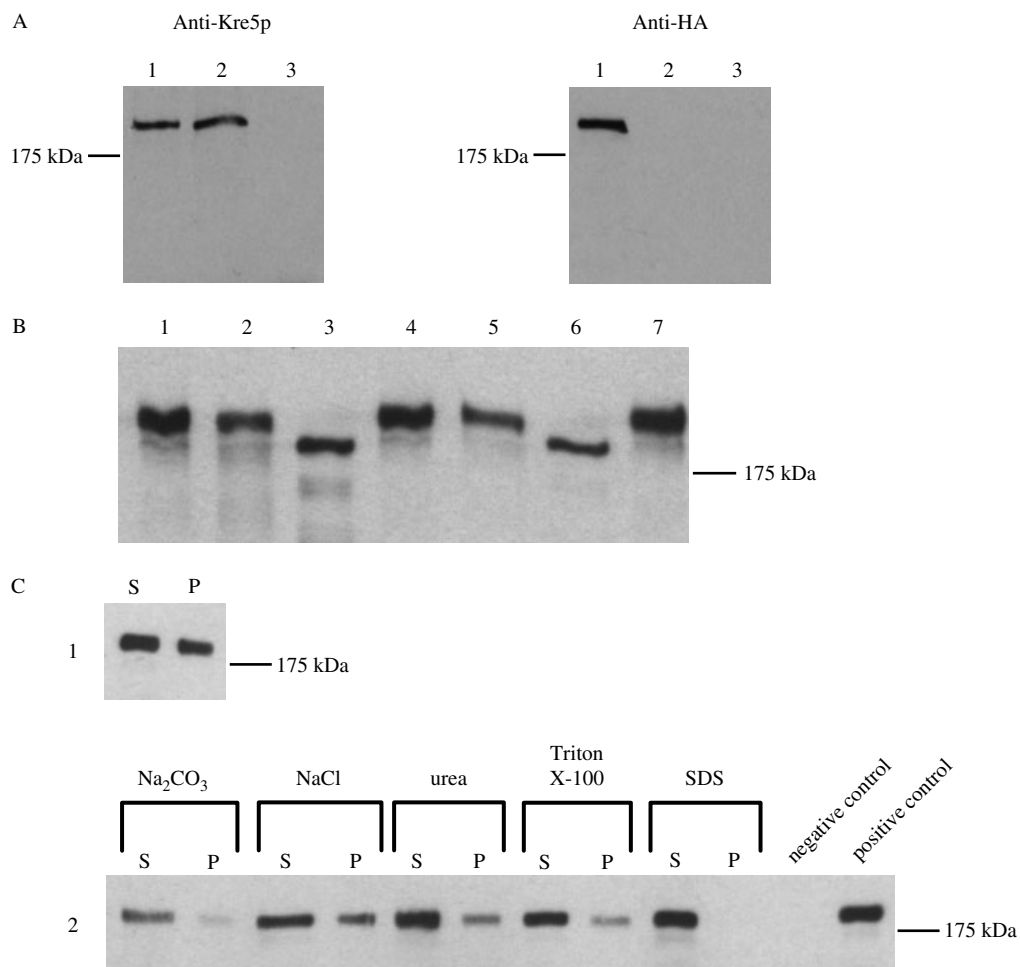


Figure 1. Characterization of Kre5p. (A) Whole cell extracts were obtained from wild-type cells (HAB932) carrying pVT102u *KRE5*-HA (lane 1), pVT102u *KRE5* (lane 2), or pVT102u (lane 3), and are shown following SDS-PAGE and Western blot analysis. Anti-Kre5p serum and anti-HA antibodies were used for blotting. (B) *N*-glycosylation analysis. Whole cell extracts from wild-type cells (HAB932) expressing Kre5p-HA in pVT102u were treated with either endo H_f (lane 3) or an endo F/PNGase F mixture (lane 6), and were subsequently analysed as for (A) above. Enzyme was omitted from mock endo H_f (lane 2) and mock endo F/PNGase F (lane 5) samples. Untreated extract was used as a control in lanes 1, 4 and 7. Blotting was carried out with anti-HA antibody. (C) Solubility analysis. Whole cell extracts from wild-type cells (HAB932) expressing *KRE5*-HA in pRS316 were mock-treated (1) or treated with the indicated chemical (2), and subsequently ultracentrifuged at $90\,000 \times g$. Supernatant (S) and pellet (P) fractions were separated and are shown following SDS-PAGE and Western blot analysis. Untreated, uncentrifuged extract obtained from cells expressing *KRE5* or *KRE5*-HA in pRS316 were used as negative and positive controls, respectively. Blotting was carried out using anti-HA antibody

Isolation of temperature-sensitive *kre5* alleles

Using error-prone PCR mutagenesis, two temperature-sensitive alleles of *KRE5* were isolated, and are diagrammed in Figure 3A. Haploid SEY6210 *kre5*Δ cells carrying plasmid-borne *kre5-ts1* or *kre5-ts2* (referred to hereafter as the *kre5-ts1* or *kre5-ts2* strains, respectively) grow similarly to *kre5*Δ cells carrying wild-type *KRE5* at the

permissive temperature (22 °C), but are non-viable at the restrictive temperature (37 °C; Figure 3B, C). Furthermore, both *kre5-ts* strains were more resistant to K1 killer toxin than *kre5*Δ cells carrying wild-type *KRE5* at the permissive and restrictive temperatures, indicating that the proteins generated from the temperature-sensitive alleles are likely only partially functional at the permissive temperature, and that the *kre5-ts* alleles may affect

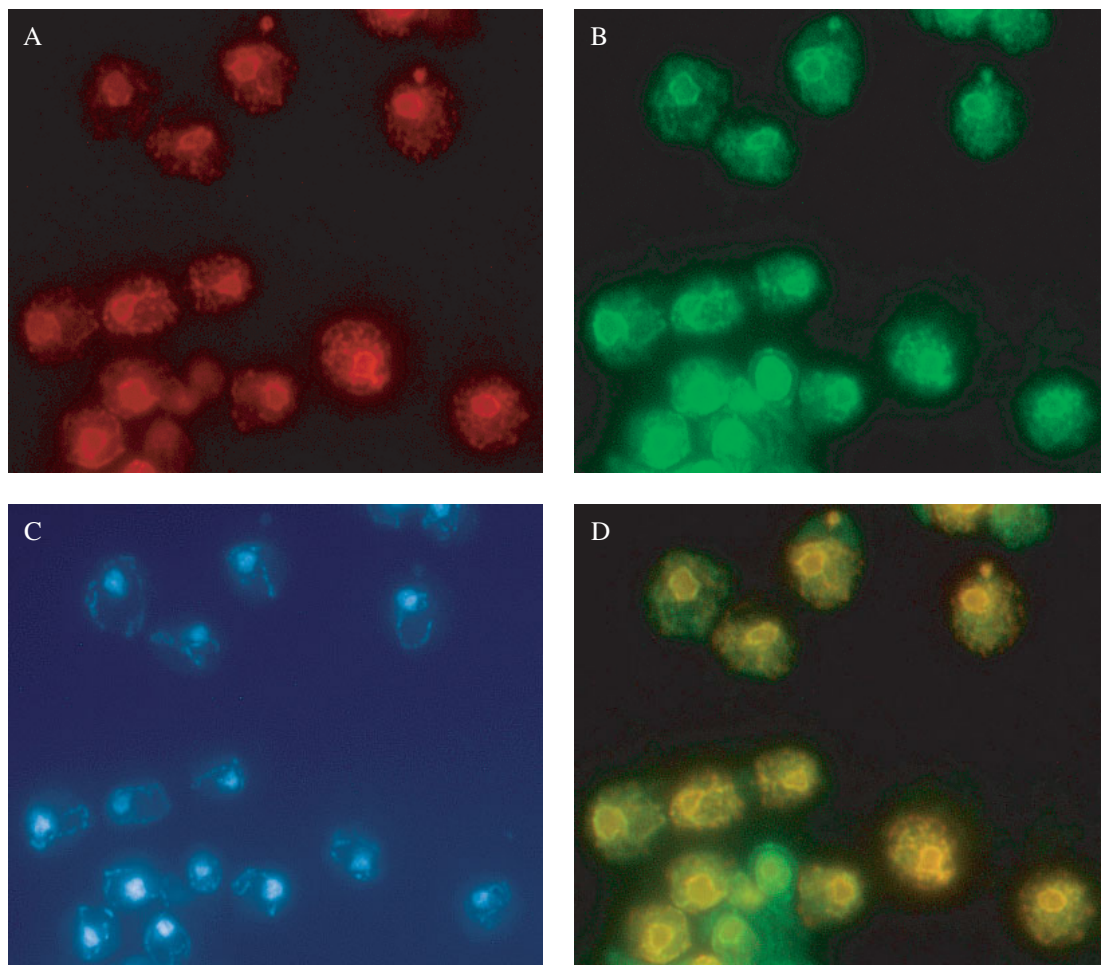


Figure 2. Subcellular localization of Kre5p-HA. Wild-type cells (HAB932) expressing *KRE5-HA* in pVT102u were analysed by indirect immunofluorescence microscopy, staining with either anti-HA (A) or anti-Kar2p (B) antibodies, or DAPI (C). Panel D is an overlap of panels A and B. Images are magnified 2000 \times

cell wall β -1,6-glucan levels in addition to growth. To determine the point mutations present in each *kre5-ts* allele, both alleles were fully sequenced. The location and nature of individual point mutations are indicated in Figure 3A. Point mutations in *kre5-ts1* are clustered in the C-terminal portion of the protein, whereas those in *kre5-ts2* are limited to the N-terminal half.

Morphological analysis of Kre5p-deficient cells

Electron microscopic analysis has previously been carried out on a viable *kre5* Δ strain carrying second site suppressor mutations (Simons *et al.*, 1998). To determine which morphological defects occur specifically due to a loss of functional Kre5p, the

kre5-ts strains were examined by transmission electron microscopy at the permissive and restrictive temperatures. The cells were examined after having been shifted to the respective temperature for 24 h. *kre5* Δ cells harbouring either *kre5-ts* allele show severe morphological defects at the restrictive temperature, with the cell walls having an apparently greater volume relative to cytoplasm, as compared to wild-type cells and to the *kre5-ts* cells at the permissive temperature (Figure 4). This phenotype was observed in 177 out of 199 counted *kre5-ts1* cells (89%), and 208 out of 228 counted *kre5-ts2* cells (91%) at the restrictive temperature, while it was virtually absent in wild-type cells and *kre5-ts* cells at the permissive temperature [11 out of 853 control cells counted (1%) showed

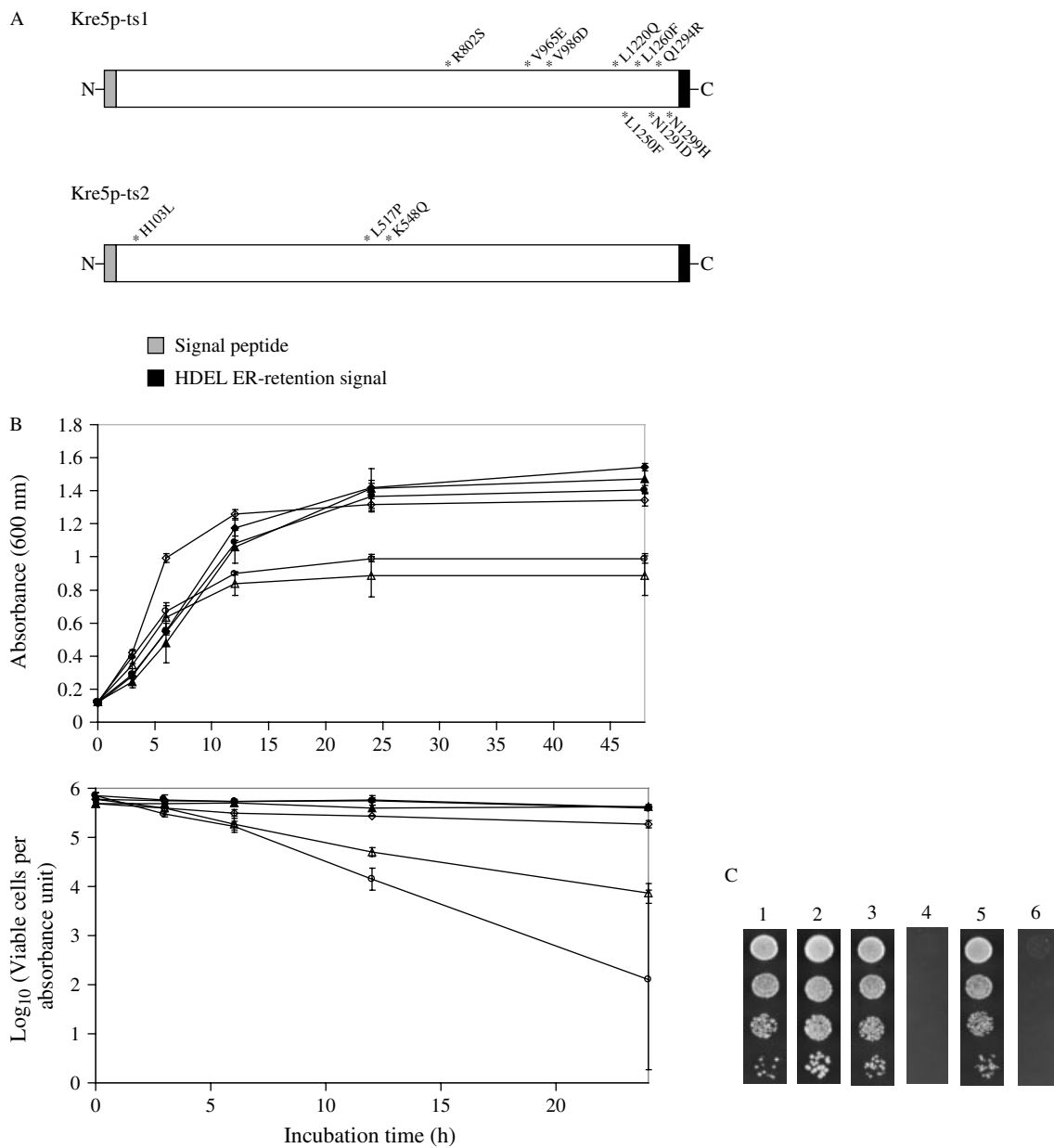


Figure 3. Isolation of temperature-sensitive *kre5* alleles. (A) Schematic diagram of the temperature-sensitive Kre5 proteins generated in this study. Mutagenesis was carried out over the entire protein in the case of Kre5p-ts1, while it was carried out only on the N-terminus (amino acids 7–753) in the case of Kre5p-ts2. Asterisks mark sites of point mutated amino acid residues. (B) Growth (top) and viability (bottom) curves for the temperature-sensitive alleles. At 37 °C, *kre5Δ* strains carrying *kre5-ts1* or *kre5-ts2* (HAB935 and HAB936, respectively) arrested growth between 12 and 24 h, whereas *kre5Δ* with wild-type *KRE5* (HAB934) grew similarly to all three strains incubated at 22 °C. A greater proportion of cells die at 37 °C in the case of the temperature-sensitive alleles than in the case of wild-type *KRE5* or the three alleles at 22 °C. Error bars indicate standard deviations. Diamond: *KRE5* strain; triangle: *kre5-ts1* strain; circle: *kre5-ts2* strain. Closed shape: 22 °C; open shape: 37 °C. (C) Spotting assay of the same strains. *KRE5* (1 and 2), *kre5-ts1* (3 and 4), and *kre5-ts2* (5 and 6) strains were pregrown at 22 °C to log-phase, diluted down to an OD₆₀₀ of 0.5, serial diluted 10-fold three times, and 5 μl was spotted onto selective media and incubated at the permissive (1, 3 and 5) and restrictive (2, 4 and 6) temperatures

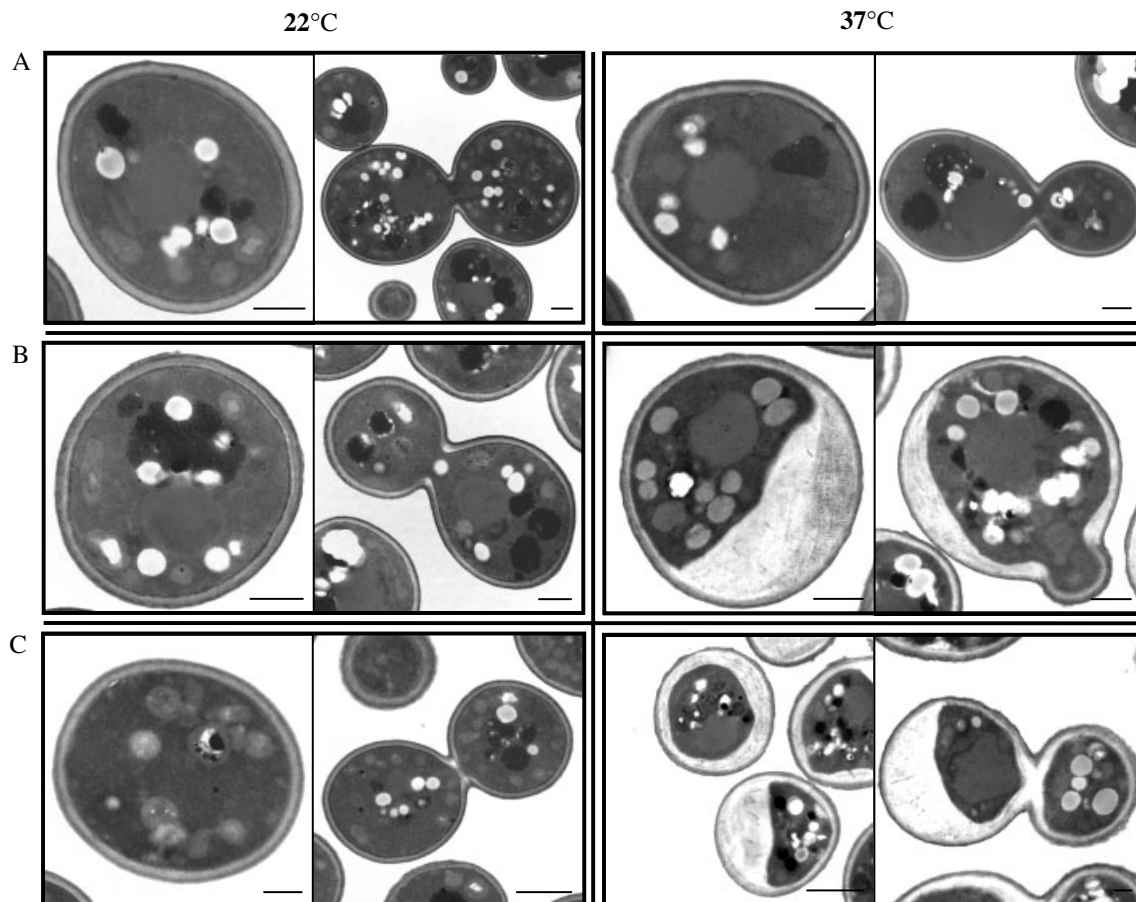


Figure 4. Electron microscopic analysis of Kre5p-deficient cells. *kre5* Δ cells harbouring *KRE5* (HAB934; A), *kre5-ts1* (HAB935; B), or *kre5-ts2* (HAB936; C) in pRS314 were grown for 24 h at the indicated temperature, and examined by transmission electron microscopy. Horizontal bars represent 2 μ m

traits resembling the mutant phenotype]. Furthermore, the observed thick wall phenotype at the restrictive temperature decreased in both frequency and severity at earlier incubation times, suggesting a progression of the defect in the population of cells (data not shown). This wall phenotype is not an artefact of cells being improperly embedded in the resin, as this phenomenon was easily distinguishable when it occurred (data not shown).

Multicopy suppression of Kre5p-deficiency by *KRE6*

In an attempt to identify genetic interactors of *KRE5*, a screen for multicopy suppressors of the lethality of the *kre5-ts2* strain at the restrictive

temperature was carried out. This screen identified *KRE6*, whose ability to suppress the Kre5p-deficient phenotype was further examined. *KRE6* overexpression is capable of partially restoring growth to the *kre5-ts2* strain at the restrictive temperature (Figure 5); however, it rescues neither the slow growth nor the K1 killer toxin sensitivity of a TA405 *kre5* Δ strain (data not shown). Overexpression of *SKN1*, the functional homologue of *KRE6* (Roemer *et al.*, 1993), was unable to rescue either the lethality of the *kre5-ts2* strain or the slow growth and K1 killer toxin-resistant phenotypes of TA405 *kre5* Δ (data not shown).

Deletional analysis of Kre5p

Kre5p is a large protein of 1348 amino acid residues, and we attempted to identify distinct

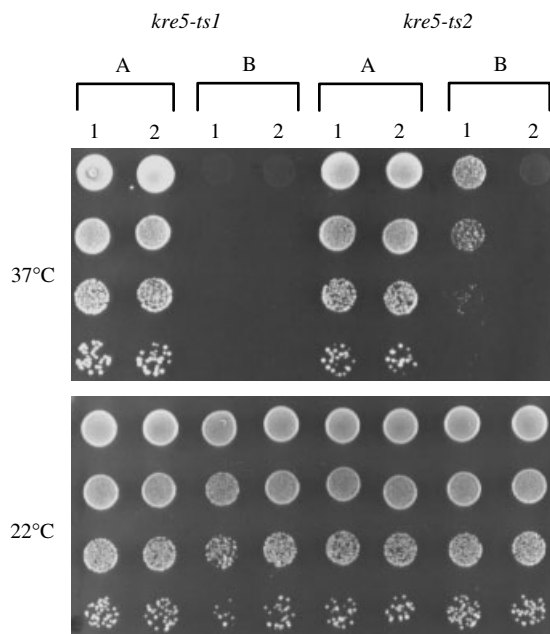


Figure 5. Multicopy suppression of the *kre5-ts2* growth defect by *KRE6*. Wild-type (A) and *kre5* Δ (B) haploid SEY6210 cells carrying pRS314 *kre5-ts1* or pRS314 *kre5-ts2* were transformed with pRS426 *KRE6* (1) or pRS426 (2). These cells were then pregrown at 22 °C to log-phase, diluted to OD₆₀₀ 0.5, serial-diluted 10-fold three times, and 5 μ l was spotted onto selective media and incubated at the restrictive and permissive temperatures

functional domains within it by conducting a deletion analysis of the protein. Deletions of \sim 340 amino acid residues (\sim 25% of the protein) were made throughout Kre5p, which are diagrammed in Figure 6A. Expression of these constructs was confirmed via SDS-PAGE and Western blot analysis (Figure 6B). Their ability to complement the lethality of haploid SEY6210 *kre5* Δ cells was used to test functionality. Three independently obtained constructs were tested for each deletion. None of the deletion constructs restored viability to the mutant cells when expressed behind the constitutive ADH promoter on a multicopy plasmid (data not shown). Thus, all four deleted regions spanning the protein are required for Kre5p function.

Kre5p is similar in size to UGGTs from other organisms and has a domain of weak but significant sequence similarity with the C-terminal-portion of the UGGT proteins (Parodi, 1999). As the active site of rat UGGT (RUGT) resides in the C-terminal-most 37 kDa (Tessier *et al.*, 2000), Kre5p may carry out an activity similar to that of

the UGGT proteins. To explore the functional interchangeability of domains within Kre5p and RUGT, RUGT and *KRE5*-RUGT hybrid constructs were made and their expression verified (see Materials and methods). None of the constructs were able to complement the lethality of SEY6210 *kre5* Δ cells (data not shown).

Comparison of Kre5p with *C. albicans* Kre5p

C. albicans Kre5p (CaKre5p) shows significant sequence similarity with UGGTs (45% identity with RUGT over the C-terminal 400 amino acid residues) and with Kre5p (18% identity over the C-terminal 400 amino acid residues) as well. We therefore wished to examine the functional relationship between Kre5p and CaKre5p. To do so, we generated CaKre5p and Kre5p-CaKre5p hybrid constructs (Figure 7A), which were then tested for their ability to rescue the lethal *S. cerevisiae* *kre5* Δ phenotype. CaKre5p was found to partially restore viability to *kre5* Δ cells, resulting in microcolony growth (Figure 7B), while Kre5p-CaKre5p failed to do so (data not shown). However, rescued *kre5* Δ cells expressing *CaKRE5* displayed resistance to K1 killer toxin, comparable to that seen in a viable *kre5* Δ strain not expressing *CaKRE5* (data not shown).

Discussion

Kre5p is predicted to be a large ER-luminal glycoprotein involved in cell wall β -1,6-glucan biosynthesis (Boone *et al.*, 1990; Meaden *et al.*, 1990). Disruption of the *KRE5* gene leads to severe slow growth or lethality, a complete reduction in alkali-insoluble β -1,6-glucan and other peripheral phenotypes, at least some of which are likely a consequence of the β -1,6-glucan defect (Meaden *et al.*, 1990; Shahinian *et al.*, 1998; reviewed in Shahinian and Bussey, 2000). Here, a further characterization of Kre5p and phenotypes associated with its absence has been carried out to attempt to clarify its role in β -1,6-glucan synthesis.

We have shown Kre5p to have an apparent molecular weight of \sim 200 kDa on SDS-PAGE. As this is substantially greater than the predicted molecular weight (Meaden *et al.*, 1990), the presence of *N*- and *O*-linked glycosylation was examined. A moderate degree of *N*-glycosylation was

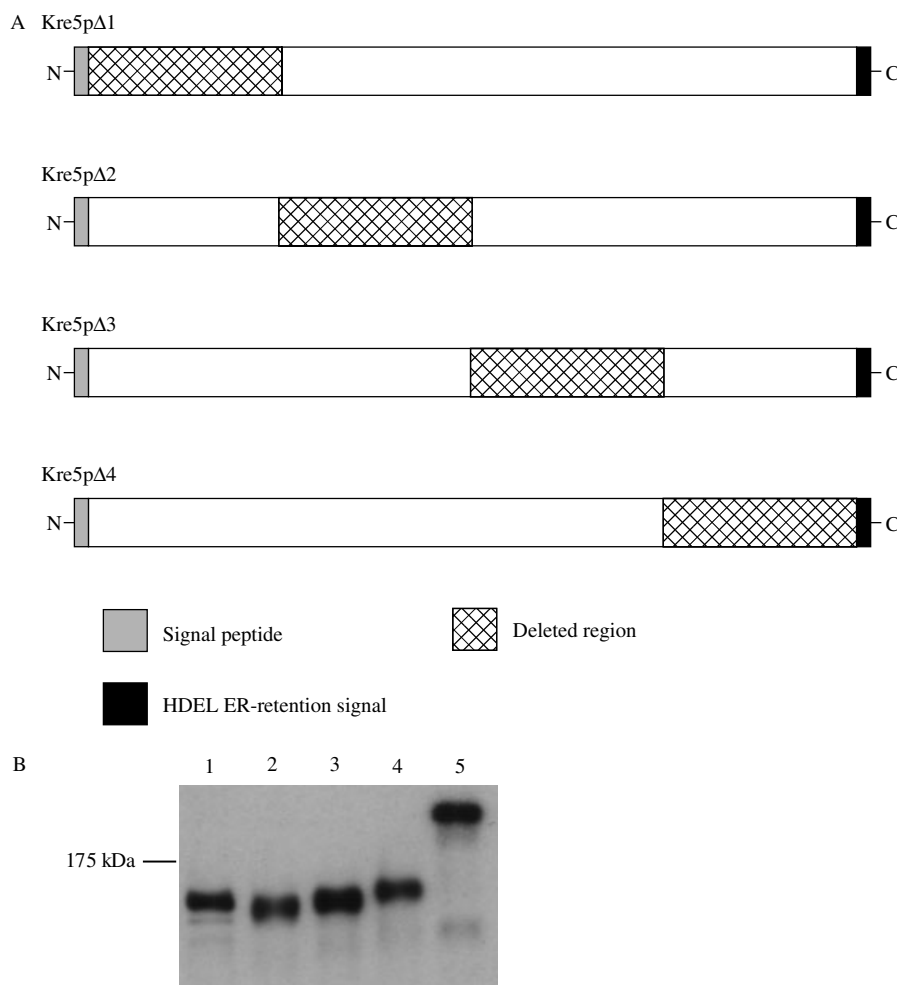


Figure 6. Construction of the Kre5p deletion set. (A) Schematic diagram of the Kre5p deletion constructs. (B) Expression of the Kre5p deletion constructs. Whole cell extracts were obtained from wild-type SEY6210 cells (HAB932) expressing *kre5Δ1* (1), *kre5Δ2* (2), *kre5Δ3* (3), *kre5Δ4* (4), or *KRE5* (5) on pVT102u, and were analysed by SDS-PAGE and Western blot analysis. Blotting was carried out using anti-Kre5p serum

found, whereas *O*-glycosyl chains appeared to be absent. It is possible, however, that *O*-glycosyl chains present on Kre5p are not affected in the *O*-glycosylation mutants examined. Kre5p-HA was shown to be associated in large part with the soluble fraction upon ultracentrifugation, consistent with the absence of any predicted transmembrane domains. However, a subset of it appeared in the membrane fraction. When Kre5p-HA was treated prior to centrifugation, only Na_2CO_3 and SDS effectively released it from the membrane fraction. This suggests that much of the membrane-association of Kre5p-HA was due to it being trapped in membrane-bound structures, perhaps

due to incomplete lysis during the making of the cell extracts, or because the protein forms insoluble inclusion bodies. The subcellular localization of Kre5p-HA was investigated by indirect immunofluorescence microscopy. A perinuclear staining pattern and co-localization with the ER protein Kar2p (Rose *et al.*, 1989) were observed, suggesting that Kre5p-HA is localized to the ER. This data must be interpreted cautiously, as Kre5p-HA was overexpressed in these experiments, and overexpression of secretory pathway proteins can result in their accumulation in the ER, as is the case for Chs3p (Trilla *et al.*, 1999). However, as Kre5p possesses a signal sequence and

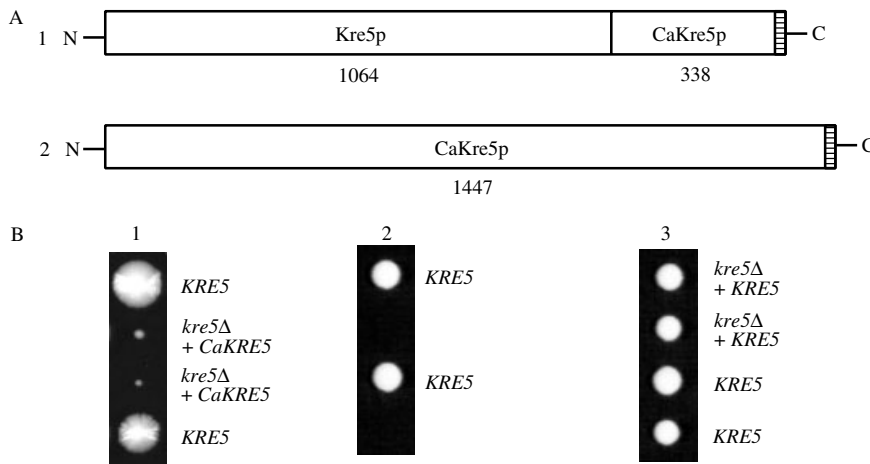


Figure 7. *CaKRE5* is able to restore viability to *kre5Δ* cells. (A) Schematic diagram of the *CaKre5p* and *Kre5p*–*CaKre5p* hybrid constructs used in this study. 1, *Kre5p*–*CaKre5p*, N-terminal portion of *Kre5p* fused to C-terminal portion of *CaKre5p*; 2, *CaKre5p*, full-length *CaKre5p*. Numbers indicate fragment lengths in amino acids. pRS424 was used to express 1, while YEp352 was used to express 2. (B) Representative tetrads of SEY6210 *kre5Δ/KRE5* cells transformed with YEp352 *CaKRE5* (1), pRS316 (2), or pRS316 *KRE5* (3). Plasmid and disruption markers were verified

C-terminal HDEL sequence, and as the *KRE5*–*HA* construct is functional, *Kre5p* is likely a soluble, ER-resident protein.

Electron microscopic analysis of *kre5Δ* cells has shown them to have rough cell walls lacking the outer mannoprotein layer (Simons *et al.*, 1998). However, viable *KRE5*-disrupted cells have second-site suppressor mutations, and it is therefore unclear whether the observed phenotypes are due specifically to the absence of functional *Kre5p*. A different approach was taken in this study, whereby a SEY6210 *kre5Δ* strain carrying either of two temperature-sensitive *kre5* alleles was used, allowing the terminal phenotype of *Kre5p*-deficient cells to be examined. It was determined that these non-viable cells have severely abnormal, enlarged cell walls. This terminal phenotype is more severe than seen previously in β -1,6-glucan mutants with a slow growth phenotype, where compensatory changes in wall structure have occurred (Roemer *et al.*, 1993; Dijkgraaf *et al.*, 1996; Shahinian *et al.*, 1998; Kapteyn *et al.*, 1999). The enlarged walls seen in these arrested *kre5-ts* mutants may represent the consequence of a failure to cross-link cell wall polymers via β -1,6-glucan.

A screen for multicopy suppressors of the lethality of *kre5-ts2* was conducted, identifying the *KRE6* gene. Based on the subcellular locations of the proteins involved in β -1,6-glucan synthesis, this process is thought to occur along the

secretory pathway (Shahinian and Bussey, 2000). As *Kre5p* and *Kre6p* are localized to the ER and Golgi, and encode a putative glycosyltransferase and glucanase, respectively (this study; Montijn *et al.*, 1999; Roemer *et al.*, 1994), and as *kre5Δ* is epistatic to *kre6Δ* (Shahinian *et al.*, 1998), these proteins may act sequentially in the presumed β -1,6-glucan biosynthetic pathway. The multicopy suppression effect of *KRE6* observed in this study therefore lends further support to the idea that sequential glucose processing events early in the secretory pathway are required for β -1,6-glucan synthesis.

Although *Kre5p* shows significant sequence similarity with UGGT proteins, evidence has accumulated suggesting that *Kre5p* is not a classic UGGT, in the sense that it does not monoglucosylate protein *N*-chains and does not appear to be involved in quality control of protein folding (reviewed in Parodi, 1999). When we tested various RUGT and *KRE5*–RUGT hybrid constructs for their ability to replace *S. cerevisiae KRE5*, none were able to do so, supporting the idea that *Kre5p* has functionally diverged from the UGGTs. In addition, although the C-terminal portion of RUGT is sufficient to obtain UGGT activity *in vitro* (Tessier *et al.*, 2000), a *Kre5p* deletion analysis revealed that overexpression of *Kre5p* constructs lacking various parts of the protein but containing an intact C-terminal

region is not sufficient to allow viability in *S. cerevisiae* cells. This indicates that there are no major regions (~340 or more amino acids) within Kre5p that are dispensable for viability but, more specifically, it may be an indication that Kre5p has a different functional structure than its rat counterpart. It may also be the case, however, that any UGGT-like activity carried out by the C-terminal region of Kre5p alone is insufficient to complement the lethality of the loss of the wild-type protein in *S. cerevisiae* cells. Despite the apparent differences between Kre5p and RUGT, full-length CaKRE5 was able to replace the *S. cerevisiae* gene, restoring viability to the *kre5* Δ mutant. This effect was only partial; however, this may simply be due to differing codon usage in *C. albicans* and *S. cerevisiae* (Santos et al., 1997). Although CaKre5p has not been tested for UGGT activity, it shows a much higher degree of sequence similarity to the UGGTs than does *S. cerevisiae* Kre5p. Thus, while Kre5p and RUGT functions are not sufficiently similar to allow interchangeability between the proteins in a viability assay, the apparent functional similarity between Kre5p and CaKre5p suggests that the *S. cerevisiae* protein may carry out an activity that is related to that of the UGGTs. For example, Kre5p may have diverged from the UGGTs such that it is now involved specifically in β -1,6-glucan synthesis. As proposed by Shahinian and Bussey (2000), Kre5p may glucosylate the GPI-moiety of proteins destined for the cell wall, signalling them for later attachment of β -1,6-glucan. Investigation of CaKre5p activity or UGGT activity in general in *C. albicans* may elucidate the relationship between CaKre5p and the UGGTs, which may in turn provide insight into the activity of *S. cerevisiae* Kre5p.

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