

Yeast *KRE* Genes Provide Evidence for a Pathway of Cell Wall β -Glucan Assembly

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Abstract. The *Saccharomyces cerevisiae KRE1* gene encodes a Ser/Thr-rich protein, that is directed into the yeast secretory pathway, where it is highly modified, probably through addition of O-linked mannose residues. Gene disruption of the *KRE1* locus leads to a 40% reduced level of cell wall (1 \rightarrow 6)- β -glucan. Structural analysis of the (1 \rightarrow 6)- β -glucan frac-

tion, isolated from a strain with a *krel* disruption mutation, showed that it had an altered structure with a smaller average polymer size. Mutations in two other loci, *KRE5* and *KRE6* also lead to a defect in cell wall (1 \rightarrow 6)- β -glucan production and appear to be epistatic to *KRE1*. These findings outline a possible pathway of assembly of yeast cell wall (1 \rightarrow 6)- β -glucan.

β -GLUCANS, homopolymers of glucose, are an abundant class of polysaccharides that includes cellulose, and appears to serve structural, functional, and morphological roles at the cell surface of fungi, bacteria, and plants (Fleet and Phaff, 1981; Sharp et al., 1984; Inon de Iannino and Ugalde, 1989; Kato, 1981). Despite their widespread occurrence, there has been surprisingly little work to address the basis of cell wall glucan biosynthesis at the genetic and molecular level in eukaryotes. In vitro enzymatic reactions resulting in glucan synthesis have been defined and partially characterized for several systems (Kang and Cabib, 1986; Aloni et al., 1982), although components of the synthetic machinery have eluded purification. The isolation of mutants defective in the production of cell wall glucan should define genes that encode biosynthetic enzymes as well as other products, for example those that regulate glucan synthesis or generate glucan precursors. A mutant approach has been valuable in understanding the synthesis of such other cell wall polysaccharides, as mannan (Ballou, 1982) and chitin (Silverman et al., 1988; Bulawa et al., 1986).

Mixed linked β -D-glucans consisting of glucopyranosyl residues joined through (1 \rightarrow 3) and (1 \rightarrow 6)-linkages are common to fungi belonging to the *Ascomycetes*, *Basidiomycetes*, and *Oomycetes* (Wessels and Sietsma, 1981). Fractionation studies of the *Saccharomyces cerevisiae* cell wall demonstrated the presence of several glucan subclasses, which could be structurally distinguished by polymer length and the ratio of (1 \rightarrow 3) to (1 \rightarrow 6)- β -D-linkages (Fleet and Manners, 1976). Much of the yeast cell wall glucan is isolated from whole cells as an alkali insoluble fraction that was found to contain two distinct types of polymers. The most abundant alkali insoluble glucan consists predominantly of repeating units of linear (1 \rightarrow 3)- β -linked residues, 3% of which are branched through a (1 \rightarrow 6)- β -linkage (Manners et al., 1973a). This glucan has a degree of polymerization esti-

mated to be 1,500 and has been proposed to determine the shape and stability of the yeast cell wall (Zlotnik et al., 1984). The other alkali-insoluble glucan has a degree of polymerization estimated to be 140 and contains residues that are predominantly connected through linear (1 \rightarrow 6)- β -linkages (Manners et al., 1973b). This glucan will be referred to as (1 \rightarrow 6)- β -glucan, although in addition to linear (1 \rightarrow 6)-linked units it is composed of some linear (1 \rightarrow 3)-linked residues and a relatively high proportion of (1 \rightarrow 3, 1 \rightarrow 6)-linked branched residues (14%). Yeast (1 \rightarrow 6)- β -glucan accounts for \sim 20% of the alkali insoluble glucan or 3% of the total cellular dry weight.

The K1 killer toxin of *S. cerevisiae* provides a selection scheme for the isolation of mutants defective in (1 \rightarrow 6)- β -D-glucan production. This toxin is a protein secreted by killer yeast strains which kills sensitive (nonkiller) strains. K1 toxin displays a lectin-like affinity for linear (1 \rightarrow 6)- β -D-glucan and must bind to the wall of sensitive yeast in order to initiate the killing process (Bussey et al., 1979). Mutations in the *KRE1* gene result in killer toxin resistance and are associated with an abnormal production of the cell wall (1 \rightarrow 6)- β -glucan (Hutchins and Bussey, 1983).

We describe here that the *KRE1* gene encodes a protein directed into the yeast secretory pathway. The (1 \rightarrow 6)- β -glucan fraction which remained in a *krel* mutant yeast strain had an altered structure with a smaller average polymer size and suggests that (1 \rightarrow 6)- β -glucan is synthesized in a stepwise manner. We address this possibility through the isolation of additional killer resistant mutants, some of which are required for (1 \rightarrow 6)- β -glucan biosynthesis and appear to be epistatic to *KRE1*. Gene products required for fungal cell wall biosynthesis have been recognized as potential targets for specific antifungal antibiotics and the *KRE* genes are discussed in this context.

Materials and Methods

Yeast Strains and Procedures

S484, S486 and S442 are isogenic strains of *S. cerevisiae* derived from S331 as previously described (Ridley et al., 1984). S484 has a genotype *MAT α ural met13 can1 cyh2 mkl1* [HOK] [NEX], whereas S486 is similar but lacks [HOK] [NEX]. The genotype of S442 is *MAT α lys2 cyh2 can1 mkl1* [HOK] [NEX]. The killer-resistant strains were isolated by selecting for mutants of S484 or S486. Once obtained, the resistant mutants were characterized through crosses with S442 followed by tetrad analysis. The strains presented in Table III result from spore progeny obtained from crosses of mutants with S442. Each strain in Table III is *MAT α lys2 cyh2 can1 mkl1* [HOK] [NEX]; in addition, S706 and S731 are *met 13*.

TA405, *MAT α /MAT α his3/his3 leu2/leu2 can1/can1*, is an isogenic diploid strain (Whiteway and Szostak, 1985). The strains 463-1A, 463-1B, 463-1C, and 463-1D presented in Table I were obtained as the spore progeny from a TA405 diploid made heterozygous for a *krel* disruption mutation (*KREI/krel::HIS3*). The genotype of strains 463-1A and 463-1B is *MAT α leu2 his3 krel::HIS3*, whereas the genotype of strains 463-1C and 463-1D is *MAT α leu2 his3*. Some of the other strains used throughout this work and their corresponding genotypes are as follows: 11A *MAT α krel-1 ura3*; T158C/S14a *MAT α /MAT α his4c-864/HIS4 ade2-5/ADE2* [KIL-KI] (Bussey et al., 1979); HABI50-1 *MAT α /MAT α krel-3/krel::HIS3 his3/HIS3 leu2/LEU2 lys2/LYS2*; 7B *MAT α gcl1 his3 ura3*.

Growth conditions and media (YEPD, complete and Halvorson's) were as described previously by Bussey et al. (1982) and Wickner (1978). Standard techniques were used for diploid construction and sporulation (Sherman et al., 1982). Transformation was performed using the lithium acetate technique of Ito et al. (1983).

(1 \rightarrow 6)- β -Glucan Quantification

Yeast cells were grown as 5–10-ml cultures in YEPD or minimal media (if plasmid selection was required) until stationary phase. Cells were harvested, washed once with distilled water, and then extracted three times with 0.5 ml of 3% NaOH at 75°C (1 h per extraction). After alkali extraction, the cells were washed once with 1 ml of 100 mM Tris-HCl, pH 7.5, and once with 1 ml of 10 mM Tris-HCl, pH 7.5. The washed cells were then digested for 16 h at 37°C, with 1 mg of Zymolyase 100,000 (ICN Biomedicals, Inc., Costa Mesa, CA), in 1 ml of 10 mM Tris-HCl, pH 7.5. Approximately 90% of the glucose-containing carbohydrate was released into the supernatant by this digestion. Zymolyase does not contain a (1 \rightarrow 6)- β -glucanase activity (Hutchins and Bussey, 1983). The insoluble pellet that remains after Zymolyase digestion was removed by centrifugation, and the supernatant was dialyzed against distilled water, using Spectra/por tubing with a 6,000–8,000-D pore size (Spectrum Medical Industries, Inc., Los Angeles, CA), for 16 h. The total yield of glucan was determined by the sum of the carbohydrate content of both the Zymolyase-insoluble pellet and the solubilized supernatant after dialysis. Analysis of the carbohydrate content of the retained fraction after dialysis determined the proportion of (1 \rightarrow 6)- β -glucan. Total carbohydrate, of each fraction, was measured as hexose by the borosulfuric acid method (Badin et al., 1953).

Plasmids

Vector YCp50 and the yeast genomic library constructed by M. Rose were provided by B. Futcher (Cold Spring Harbor Laboratory). Plasmid, pFL44, was obtained from F. Lacroute (Centre Nationale de la Recherche Scientifique, Gif sur Yvette, France) and is a yeast 2- μ m based, multicopy, shuttle vector with *URA3* and *Ap'* markers, which contains the pUC19 polylinker. The plasmid pFL44 was used for subcloning DNA fragments of YCp50:KREI. Bluescript+ and Bluescript- vectors (Stratagene Corp., La Jolla, CA) were used for various recombinant DNA constructions and for production of single stranded DNA. The yeast expression vector, PVT100U, contains the *f1* origin of replication, also allowing the production of single-stranded DNA, and was provided by T. Vernet et al. (1987). Plasmid PBSK:HIS3 was created by ligating a 1.7-kb Bam HI fragment containing the *HIS3* gene (Struhl, 1985) into Bluescript+. Another Bluescript+ based plasmid, p486, contains the 0.5-kb Eco RV-Hinc II fragment of *KREI* (Fig. 1) ligated into these same restriction sites of the Bluescript polylinker. Plasmid p492 contains the 0.4 kb Spe I-Nsi I fragment which spans the end of the *KREI* open reading frame, ligated into the Spe I and Pst I sites of the Bluescript+ polylinker. Plasmid p143 was constructed from Bluescript- through ligation of the 2-kb Nhe I-Pst I fragment of the *KREI* locus into the Spe I-Pst I digested vector. Plasmid p339 was derived from PUC19, and contains a modified

Bam HI-Sal I fragment (containing the prepro- α factor structural gene) from pJK6 (Kurjan and Herskowitz, 1982) ligated into the polylinker. The modification concerns the insertion of a Bgl II restriction site (5'-AGATCT-3') six nucleotides before the initiation codon of prepro- α factor (Kurjan and Herskowitz, 1982).

DNA Purification and Recombinant DNA Techniques

Plasmid DNA was purified from *Escherichia coli* as described by Maniatis et al. (1982). Yeast DNA was isolated according to Davis et al. (1980). Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase and Klenow fragment were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, MD) or New England Laboratories, Inc. (Beverly, MA) and were used as recommended by the suppliers. Southern blot hybridization and nick translations were carried out as described by Dmochowska et al. (1987). Oligonucleotide-directed mutagenesis was carried out according to Kunkel (1985).

Cloning the Yeast *KREI* Gene

Strain 11A was transformed with a YCp50-based yeast genomic library (Rose et al., 1987) and uracil prototrophs were selected. Transformants were replica-plated to minimal media, Halvorson's 1 \times pH 4.7 agar, which had been seeded with 75 μ l/liter of a stationary culture of the diploid killer strain T158C/S14a and contained 0.002% of the vital stain methylene blue. After replica plating, the methylene blue plates were incubated at 18°C for 3–4 d; at the end of this period the *Kre*+ transformants had stained a dark blue color, whereas *krel*- colonies remained white. Individual cells were isolated from the blue staining colonies and these were later grown for plasmid recovery.

DNA Sequencing

Subclones of the *KREI* yeast genomic DNA were made in Bluescript vectors or in PVT100U. Plasmids containing subclones were transformed into the bacterial strain, UT580, and single-stranded DNA was made using M13K07 helper phage (Vernet et al., 1987). Sequencing was by the dideoxy method (Sanger et al., 1977) and was determined for both strands, using the Sequenase Kit (US Biochemicals, Cleveland, OH) with [α -³⁵S]dATP (Amersham Canada Limited, Oakville, Ontario, Canada) as a substrate. DNA primers were either Bluescript-specific primers or synthesized to be complementary to parts of the *KREI* DNA sequence.

KREI Disruption

To create a *krel::HIS3* disruption construct, a *HIS3* containing fragment was ligated into the Spe I and Kpn I sites, situated within the *KREI* coding sequence (Fig. 1) as described below. The *KREI* Bam HI-Pst I fragment was introduced into an altered PUC19 plasmid, in which the Kpn I site of the polylinker had been removed, to create p411. Plasmid p411 was digested with Asp718 (an isoschizomer of Kpn I), made blunt ended with Klenow fragment, and then ligated with a nonphosphorylated Xho I linker (5'-CCCCTCAGGGG-3'), to generate p458. The *HIS3* gene could be isolated from PBSK:HIS3 as a Spe I-Xho I fragment and ligated into p458 also digested with Spe I and Xho I. The ligation product of this last reaction was called p463, digestion of p463 with Nco I and Sph I, which cut within the *KREI* portion of the insert but not the *HIS3* portion, allowed disruption of the *KREI* locus upon transformation.

Mapping *KREI*

A Southern blot of chromosomes separated by pulse-field electrophoresis (Carle and Olson, 1985) was probed with *KREI* DNA. The *KREI* sequence hybridized to chromosome XIV (data not shown). Tetrad analysis provided the following linkage for *KREI*: the *krel-pha2* map distance is 8 cM (41 parental ditypes [PD], 0 nonparental ditypes [NPD], and 8 tetratypes [TT]), the *krel-met2* map distance is 34 cM (34 PD, 1 NPD, and 56 TT), the *krel-pet2* map distance is 48 cM (12 PD, 2 NPD, and 27 TT). Of seven tetrads examined where *krel* was recombinant with *pha2*, five tetrads were also recombinant for *krel* with *met2* and *pet2*, suggesting the order *krel pha2 met2 pet2*. The map distances were calculated according to Mortimer and Schild (1985).

Electron Microscopy

The conditions presented below represent a modified version of the proce-

published by Zlotnik et al. (1984). Cells were grown in minimal media 1× Halvorson's salts to stationary phase, harvested and washed with distilled water. Cell pellets were fixed in a solution containing 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 70 min. After fixation, cell pellets were rinsed in buffer, then postfixed for 1 h in 1% OsO₄ in 0.1 M sodium phosphate buffer (pH 7.2) and then rinsed again. Cell pellets were subsequently dehydrated through a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin (Spurr, 1969). Gold- and silver-colored sections were mounted on formvar-coated grids and sections were stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate (Reynold, 1963). Sections were viewed on a Philips EM410 electron microscope at an operating voltage of 80 kV.

pVT:KRE1

To create the pVT:KRE1 insert, blunt-end restriction sites were introduced into subclones of the *KRE1* locus and the resultant constructs reassembled to form an uninterrupted open reading frame. Single-stranded DNA was prepared from p486 and in combination with oligo IB (5'-CAATCAA-AAACCCGGGAAAATGATC-3'), an Sma I restriction site was introduced three nucleotides before the ATG of the *KRE1* open reading frame, resulting in plasmid p567. Plasmid p567 was then digested with Sma I and religated so that most of the 5'-untranslated region of *KRE1* was removed and the introduced Sma I site was situated next to a Bam HI site of the Bluescript poly-linker (p585). Single-stranded DNA was also prepared from p492 and used in combination with oligo 3B (5'-GTCTTATAAAGGCCATTATTTATTC-3') to insert a Stu I restriction site just after the *KRE1* open reading frame resulting in p563. The 0.4-kb Spe I-Hind III fragment of p563 was isolated and ligated into Spe I, Hind III digested p143 to create p580. Plasmid p580 was digested with Sal I and followed by Hinc II and the resultant 0.8-kb fragment was purified. This fragment was ligated into p585 after digestion with Xho I and Hinc II to give p596. Plasmid p607 was made when p596 was digested with Stu I and Eco RV, and the vector fragment (containing *KRE1*) religated. This procedure situated the Hind III site of the Bluescript poly-linker just after the *KRE1* open reading frame. The *KRE1* containing 1-kb (Bam HI-Hind III) fragment of p607 was purified and ligated into Bam HI and Hind III digested pVT100U, to generate pVT:KRE1.

pVT:Δ24/KRE1

Single-stranded DNA from p486 was used for oligonucleotide specific mutagenesis with oligo 6B (5'-GCTGCGGTCGTAAACATGGCAGCT-3') to form p647. Oligo 6B directs the introduction of a Hpa I site just before Met 25 of Kre1p. The plasmid p647 was digested with Hpa I and Sma I, and religated, so as to remove the *KRE1* leader encoding DNA (p676). This process also situates the 5' end of the leader deleted *KRE1* fragment next to a Bam HI restriction site in the Bluescript polylinker. The Sst I-Hinc II (0.25 kb) fragment of p676 was ligated into the plasmid p607, which had also been digested with Sst I and Hinc II, creating p688. The leader deleted *KRE1* construct of p688 was isolated via Bam HI and Hind III digestion, then ligated into similarly digested pVT100U to generate pVT:Δ24/KRE1.

pVT:KRE1/SP and pVT:Δ24/KRE1/SP

Plasmids p607 and p688 were digested with Sna BI and Hind III, and the vector containing fragment isolated for each digestion. These fragments were ligated with the complementary oligonucleotides 15B (5'-GACTCG-CAGTCTTCGGCCATGTA-3') and 16B (5'-AGCTTACATGAGG-CCGAAGAACTGCGAGTC-3'), to create *KRE1*-substance P epitope fusion constructs p715 and p718. The oligos (15B and 16B) basepair to form a small segment of DNA encoding the peptide DSQFFGLM followed by a stop codon, the last six amino acids are part of the neuropeptide, substance P. Plasmids p715 and p718 were digested with Bam HI and Hind III and the *KRE1* substance P fusion fragments were introduced into pVT100U to create pVT:KRE1/SP and pVT:Δ24/KRE1/SP, respectively.

pVT:α20/KRE1

Single-stranded DNA was prepared from pVT:KRE1 and oligonucleotide 6B (see above) was used for directed mutagenesis to insert a Hpa I site just before the codon encoding Met 25 of Kre1p. The newly created plasmid was designated p758. The 0.9-kb Hpa I-Hind III (*KRE1* fragment without the leader) was isolated and ligated into Hinc II-Hind III digested p339 vector fragment to give p771. A segment of DNA with the prepro-α factor leader spliced in frame with a deleted *KRE1* gene fragment can be removed from

p771 as a 1.0-kb Bgl II-Hind III fragment. Ligation of this Bgl II-Hind III fragment into Bam HI-Hind III digested pVT100U generated pVT:α20/KRE1.

Seeded Plate Assay for Killer Resistance

Yeast strains were grown to stationary phase in liquid media (under plasmid-selective conditions if necessary) and 30 μl of this culture was used to inoculate 10 ml of minimal media, 1% agar, 1× Halvorson's, pH 4.7. Concentrated toxin (7 μl of 1,000× concentrated media from S14a/T158C; Bussey et al., 1983) was introduced onto the solidified agar and the plates incubated at 18°C overnight, followed by a 30-°C incubation for 24 h.

Western Analysis of Substance P Hybrid Proteins

Approximately 1 × 10⁷ transformed yeast cells (grown in minimal media under plasmid selection) were harvested in log phase and the cellular contents prepared for electrophoresis as described by Segev et al. (1988). Electrophoretic transfer blots were analyzed with anti-substance P antibody (NC1/34 HL; Accurate Chemical & Scientific, Westbury, NY) as described by Munro and Pelham (1984), in combination with an alkaline phosphatase immunoblot detection kit (Bio-Rad Laboratories, Richmond, CA).

Large-Scale (1→6)-β-Glucan Preparation

Yeast (1→6)-β-glucan was isolated from a 2-liter culture of wildtype cells (strain 7B) or a 5-liter culture of *krel* mutant cells (strain 3), each grown to stationary phase in YEPD, 1× Halvorson's salts. The cells were harvested, (strain 3 cells were split into two samples each treated as given below) washed with distilled water, and stored at -70°C. Mannoprotein and alkali soluble glucan was removed via five 100-ml extractions with 3% NaOH, each for 1 h at 70°C. After alkali extraction the cell walls were neutralized (with phosphate buffer, pH 6.8), and digested with 33 mg of Zymolyase 100,000 in 10 mM sodium phosphate buffer pH 6.8 (with a 40-ml final volume containing 0.01% sodium azide) for 16 h at 37°C. After this digestion, insoluble material was removed by centrifugation (12,000 rpm) and the supernatant treated with 20 μl amylase (10 mg/ml, Boehringer Mannheim Canada Ltd., Dorval, Quebec) for 2 h at room temperature. After amylase treatment the glucan containing solution was extracted twice with 5-ml portions of phenol. Several 10-ml ether extractions removed residual phenol. The aqueous phase was collected and dialyzed against distilled water in Spectra/por tubing with a pore size of 6,000-8,000 D (Spectrum Medical Industries, Inc.) for 5 h, then freeze-dried. The freeze-dried material was solubilized in 5 ml of distilled water and further dialyzed in Spectra/por tubing with a 2000 D pore size for 30 h before a second freeze drying. The water-soluble material, which remains after this procedure was used for structural analysis. 2 liter of a culture of wild-type cells yielded 40-50 mg of (1→6)-β-glucan and 5 liter of a culture of *krel* mutant cells produced an equivalent amount.

[¹³C]Nuclear Magnetic Resonance (NMR) Spectroscopy

[¹³C]NMR spectra were obtained using 10-mm-diam tubes, with 40 mg of glucan dissolved in 3 ml D₂O. Data were collected under conditions of proton decoupling, using a Bruker spectrometer (model WH 400; Bruker Instruments, Billerica, MA) operated in the Fourier-transform mode, at 100.62 MHz, with a sweep width of 6493.5 Hz and an acquisition time of 0.631 s. The pulse angle was 73° and the pulse interval was 4.0 s, during which the decoupler was gated off. The probe temperature was maintained at 19°C. Each spectrum was recorded several times, from independent glucan samples, with ~10,000 scans. The reference for the chemical shift values was external Dioxane at 67.4 ppm.

Gel Filtration Chromatography

A Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) column of dimensions 110.0 × 1.0 cm was used at a flow rate of 16 ml/h. The eluent was 0.1 M NaOH and 0.4-ml fractions were collected. Calibration of the column was carried out using dextran blue (Pharmacia Fine Chemicals) to indicate the void volume and several dextrans of known molecular weights (Sigma Chemical Co., St. Louis, MO; Fig. 6). Determination of the carbohydrate content of each fraction was carried out by the phenol-sulfuric acid method (Dubois et al., 1956).

1. *Abbreviations used in this paper:* NMR, nuclear magnetic resonance.

NheI

GCTAGCAGTTATTTCACTTTTCATTACAGCATCCCTCATGTTTATTATCTCTTTATCTAATATAAATAGGAACATAAATAAT -360

CCCCTCACCGTATAAAGCGACAGTTCCGTGACGGTTACTATTATGAATATCTCAACGGAAGAGGGCATTAAAGATCATAATAGTTGGTACTCTCGTATTTTATATATATATCACT -240

EcoRV

ATATTTTAACTTTTACTGCTCAATTGTGCCATATACTTCGCCTTATTGCGTACATTCTTCACCTTGATCCCCCTACCTCAGCGTGTATGGTGATATCGCGTTTTTCATAAACTGA -120

GAATGGGGCTTTTTCTATAACGTGATTATGAAAAAAGAAAAATAAAAAATCAAGAATTAAGCACTTGATATGCTACAGTAAAGACCTCTTCAACTTCTGCAAGACAATCAAAAAA -1

ATG ATG CGT CGC ACG CTA TTA CAT TCA TTC GCT ACG CTG CTA CTT TCT TTG TCG TTG TGG TCA GCT GCG GTC ATG GCA GCT GTG ACA ACT 90
Met Met Arg Arg Thr Leu Leu His Ser Phe Ala Thr Leu Leu Ser Leu Ser Leu Trp Ser Ala Ala Val Met Ala Ala Val Thr Thr 30

CAG GTT ACA GTG GTA ACA AAT GTC GCA GGG GCC CTG GTT ACG GAG ACC ACA ATA TGG GAC CCT GCC ACC GCT GCT GCT GCT ACA ACT 180
Gln Val Thr Val Val Thr Asn Val Ala Gly Ala Leu Val Thr Glu Thr Thr Ile Trp Asp Pro Ala Thr Ala Ala Ala Ala Thr Thr 60

HincII

ACC GCT CAA ACA GGT TTC TTC ACT ACG GTA TTC ACT ACC ACT AAC GAT GTC GGA ACC ACC GTC ACT CTT ACT CAG ACA GTC AAC AGA GCC 270
Thr Ala Gln Thr Gly Phe Phe Thr Thr Val Phe Thr Thr Thr Asn Asp Val Gly Thr Thr Val Thr Leu Thr Gln Thr Val Asn Arg Ala 90

ACT ATG CTA CCA ACC ACG ACG ACT TCT ACC TCA TCT ACT GGT AAG ACA ACC ACC ACT GTT CCT ACC GCA ACT TCA TCG TTG TCT TCG GGA 360
Thr Met Leu Pro Thr Thr Thr Ser Thr Ser Ser Thr Gly Lys Thr Thr Thr Thr Val Pro Thr Ala Thr Ser Ser Leu Ser Ser Gly 120

KpnI

CTG TAT TTA TCT ACA GTT ACC ACG ACA AAC GAT TTG GGT ACC ACA GTT ACA TTG ACT CAA ACG TTC ACA CAT TCT AGC ACC AGT GCT ACT 450
Leu Tyr Leu Ser Thr Val Thr Thr Thr Asn Asp Leu Gly Thr Thr Val Thr Leu Thr Gln Thr Phe Thr His Ser Ser Thr Ser Ala Thr 150

TCA TCC GCC TCC TCG TCT GTG TCC TCG TCT GTA TCT TCG TCT GGT TCA TCC TCC AGT GTA AAG ACG ACC ACA TCG ACA GGG AGC GCA GTA 540
Ser Ser Ala Ser Ser Ser Val Ser Ser Ser Val Ser Ser Ser Gly Ser Ser Ser Ser Val Lys Thr Thr Thr Ser Thr Gly Ser Ala Val 180

SpeI

GCT GAA ACA GGC ACC AGG CCA GAC CCC TCC ACA GAC TTC ACA GAA CCT CCT GTG TCT GCT GTC ACT AGT CTA TCT ATT GAC TCA TAC ATT 630
Ala Glu Thr Gly Thr Arg Pro Asp Pro Ser Thr Asp Phe Thr Glu Pro Pro Val Ser Ala Val Thr Ser Leu Ser Ile Asp Ser Tyr Ile 210

ACC ATC ACT GAA GGT ACA ACC TCC ACT TAC ACA ACC ACA CGT GCG CCA ACG TCC ATG TGG GTC ACT GTT GTT AGA CAG GGC AAC ACT ATC 720
Thr Ile Thr Glu Gly Thr Thr Ser Thr Tyr Thr Thr Thr Arg Ala Pro Thr Ser Met Trp Val Thr Val Val Arg Gln Gly Asn Thr Ile 240

SnaBI

ACT GTG CAA ACT ACT TTT GTC CAG CGT TTC TCC TCC CAG TAC GTA ACA GTC GCT TCT CCC TCC GTG GGG TCT ATT GGG ATG GGT ACT TTA 810
Thr Val Gln Thr Thr Phe Val Gln Arg Phe Ser Ser Gln Tyr Val Thr Val Ala Ser Pro Ser Val Gly Ser Ile Gly Met Gly Thr Leu 270

ACC GGT ACT GTA GGC GTT ATT AAA TCT GCA ATA AAG AAA ACA GTT TCG CAT AAT GAG GCC CAG CAT CTA GGT ATG AGT TCG TTT ACT TCA 900
Thr Gly Thr Val Gly Val Ile Lys Ser Ala Ile Lys Lys Thr Val Ser His Asn Glu Ala Gln His Leu Gly Met Ser Ser Phe Thr Ser 300

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ATT TTG GGT GGG CTA TTA ACG GTT TTA ATT TGG TTC TTA TAA ATTTTATTTCAGAAATAAACACAAACATATACATATATAAGAGTAAAAATAAAAAATAAAAA 1005
Ile Leu Gly Gly Leu Leu Thr Val Leu Ile Trp Phe Leu 313

NsiI

AATTTTACAGGGTTAAAAATAAAGAAACCATCACTCCTTTTCTATTTCATAATCCATGACAAACTTGATGCAT 1079

Figure 1. The nucleotide sequence of an Nhe I-Nsi I restriction fragment, isolated from YCp50::KRE1, is shown, with the predicted amino acid sequence of KRE1 below. Both strands of the DNA sequence were obtained as described in Materials and Methods. Arrows show the position of predicted signal cleavage sites determined using the rules of von Heijne (1984). A 15-amino acid direct repeat is underlined by a dashed line. The carboxy-terminal hydrophobic sequence of the KRE1 gene product is underlined by a solid line. Asterisks show the positions of restriction sites inserted using site specific mutagenesis (see Materials and Methods). Various restriction sites used for recombinant DNA constructs are designated above the DNA sequence. These sequence data are available from EMBL/Genbank/DBJ under accession number X51729.

Isolation of Killer-resistant Mutants

To isolate mutants resistant to K1 killer toxin, 1 x 10^7 cells of S486 or S484 were mixed with 2 x 10^8 of a nonreverting, homozygous leu2 K1+ diploid strain and plated on complete media lacking leucine, pH 4.7. After 8 d, colonies of resistant S486 and S484 could be seen above a lawn of initially plated cells. The colonies were purified and tested for resistance by replica plating onto methylene blue medium (0.003% methylene blue), which had just been inoculated with diploid K1 killer cells (~1 x 10^7 cells spread onto the agar surface and allowed to dry). After incubation for 1-2 d at 25°C, resistant colonies were white or light blue (depending on the particular mutant allele), whereas sensitive colonies were dark blue. Except for the initial experiment, the following was done to ensure isolation of inde-

pendent mutants: (a) only one resistant colony was taken per killer selection plate and (b) each 10^7 cells of input S484 and S486 were derived from single colonies.

Results

Isolation of the KRE1 Gene

To identify the KRE1 gene product and initiate a study of its function, we isolated the wild-type KRE1 locus. The krel-1 ura3 yeast strain 11A was transformed with a yeast genomic

library in the *URA3*-containing centromeric vector YCp50, and uracil prototrophs were selected (Rose et al., 1987). Transformants were screened for a killer-sensitive phenotype (*Kre*⁺) as described in Materials and Methods. Two independent *Kre*⁺ transformants were obtained and found to be unstable for both the *Kre*⁺ and *Ura*⁺ phenotypes when grown under nonselective conditions in YEPD. A unique plasmid was isolated from each of these transformants that could complement the *krel-1* mutation. One plasmid, YCp50:KRE1, contained a 6.5-kb insert of yeast genomic DNA and restriction endonuclease mapping revealed that this DNA fragment was contained within a larger (11 kb) insert, of the other complementing plasmid. Genetic analysis showed that the complementing fragment contained the *KRE1* locus (see below).

Nucleotide Sequence of *KRE1*

Subcloning of the insert of plasmid YCp50:KRE1 determined that a 3.9-kb Bam HI-Pst I restriction fragment could complement the *kre-*, phenotype of strain 11A. However, subclones on either side of an internal Kpn I site failed to complement, suggesting that the Kpn I site is located within the *KRE1* functional region. Further subcloning experiments localized the complementing activity to a 1.5-kb Nhe I-Nsi I fragment, the DNA sequence of this fragment (Fig. 1) was determined using the dideoxy nucleotide method of Sanger et al. (1977). This sequence contains a single extended open reading frame that spans the Kpn I site. This open reading frame would encode a protein of 313 amino acids with a molecular weight of 32,000 (Fig. 1).

The protein, Kre1p, displays a striking abundance of threonine (25%) and serine (15%) residues. The amino terminus of Kre1p is hydrophobic and resembles the signal sequences of secreted proteins. There are two potential signal cleavage sites (von Heijne, 1984) found after amino acid residues 23 and 27 (Fig. 1). The last 21 amino acid residues of Kre1p also form a hydrophobic sequence. No sites for N-linked glycosyl attachment were observed, however, the abundance of serine and threonine residues may provide sites for O-linked glycosylation (Tanner and Lehle, 1987). Kre1p contains an internal repeat of 15 amino acids. Comparison of both the *KRE1* nucleotide sequence, and the deduced primary amino acid sequence with those from available data bases, has not revealed any sequences with significant similarities to *KRE1*.

Disruption of *KRE1*

A null mutation of the *KRE1* locus was generated by the one step gene disruption procedure using *HIS3* as a selective marker (Rothstein, 1983). The *krel::HIS3* disruption construct is described in Materials and Methods. The diploid TA405, homozygous for a *his3* mutation, was transformed with a restriction fragment of the cloned DNA containing a disruption of the *KRE1* coding region. *His*⁺ transformants were sporulated and subjected to tetrad analysis. Several independent transformants gave rise to two *His*⁺ *kre*⁻ segregants and two *His*⁻ *Kre*⁺ segregants (18 out of 18 tetrads analyzed). The killer-resistant segregants consistently formed slightly smaller colonies upon spore germination when compared with the killer sensitive segregants, but individual cells were of normal size and morphology as judged by light microscopy. The structure of the integrated *krel::HIS3* deletion

replacement was confirmed by Southern analysis of the chromosomal DNA from disrupted haploids (data not shown). The diploid HABI50-1 (*krel-3/krel::HIS3*) was sporulated for tetrad analysis, 22 of 23 tetrads were parental ditype for killer resistance and 1 was a tetratype. These results show that the cloned sequence is tightly linked to the *KRE1* locus and that *KRE1* is nonessential for both mitotic growth and meiotic spore formation. In further experiments, we have determined the location of *KRE1* on the yeast genetic map (see Materials and Methods). Closest linkage was with the *PHA2* locus (required for phenylalanine biosynthesis), analysis of recombinants between *krel* and *pha2* suggests that *krel* is the distal most known marker on the left arm of chromosome XIV.

Cell wall (1→6)-β-glucan can be isolated from the alkali insoluble glucan fraction following acid extraction or treatment with an endo-(1→3)-β-glucanase (Manners et al., 1973b). Yeast strains with a mutant *krel-1* allele were found to display an ~40% reduced level of the (1→6)-β-glucan fraction when isolated by either protocol (Hutchins and Bussey, 1983), however, the yield was greater with the glucanase method. To avoid any subfractionation that may occur upon incomplete acid extraction, (1→6)-β-glucan was isolated using the endoglucanase technique (see Materials and Methods). Analysis of the (1→6)-β-glucan levels of the spore progeny from a tetrad heterozygous for the *krel::HIS3* disruption mutation, demonstrated that the level of this glucan was reduced in progeny with a disrupted allele and the reduction was ~40% of wild-type levels (Table I). This finding suggests that the mutation that defines the *krel-1* allele leads to a null phenotype. Consistent with this idea, *krel-1* mutant yeast strains display a complete killer resistant phenotype, which appears similar to the phenotype of *krel::HIS3* mutant strains. However, small in-frame insertion mutations or deletions of the *KRE1* coding sequence can lead to a partial resistant phenotype (see Fig. 3, below).

Electron Microscopy of *krel* Mutant Cell Walls

The *krel::HIS3* mutant yeast cells were examined by EM and compared with wild-type cells. Under the conditions used, wild-type cells were found to have a finely delineated dark-staining outer layer. This layer was missing from *krel* mutant cells and the outer surface appeared rough in texture (Fig. 2). The mutant cell wall material also stained more intensely, especially in the outer half of the wall. These struc-

Table I. (1→6)-β-Glucan Levels in *krel* Mutant Strains

Yeast strain	Allele at <i>KRE1</i> locus	(1→6)-β-Glucan μg/mg dry wt
463-1A	<i>krel::HIS3</i>	24.9 ± 3.5
463-1B	<i>krel::HIS3</i>	19.3 ± 2.0
463-1C	<i>KRE1</i> +	34.2 ± 3.2
463-1D	<i>KRE1</i> +	34.5 ± 0.6
7B	<i>KRE1</i> +	27.0 ± 0.8
3	<i>krel::HIS3</i>	17.2 ± 2.2

The levels of (1→6)-β-glucan were analyzed for the spore progeny of a tetrad, from the isogenic diploid TA405 made heterozygous for a *krel::HIS3* disruption mutation, (*KRE1/krel::HIS3*). Strains 463-1A and 463-1B display a reduced level of (1→6)-β-glucan and carry the *krel::HIS3* mutation. Disruption of the *KRE1* locus of the haploid strain, 7B (*glc1, ura3, his3*), resulted in strain 3 (*glc1, ura3, his3, krel::HIS3*). Error represents 1 SD.

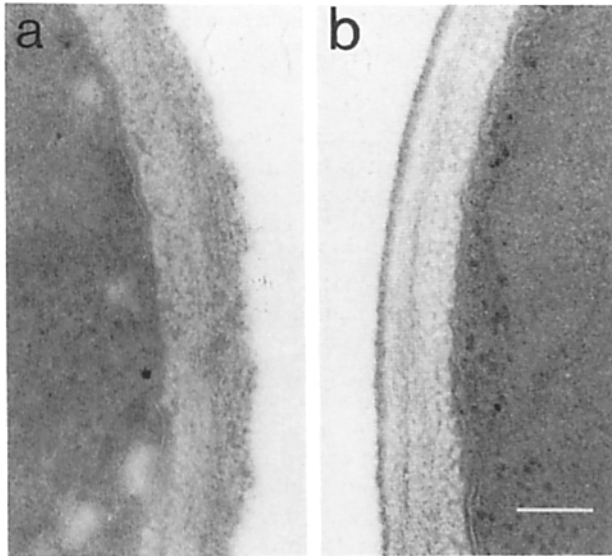


Figure 2. Cell wall electron micrographs of a *krel::HIS3* mutant strain (463-1B) (a), and the *KRE1* strain (463-1C) (b). Cells were treated exactly as described in Materials and Methods. Bar, 0.15 μ m.

tural alterations were found to segregate 2:2 in a tetrad obtained from a TA405 diploid made heterozygous for a *krel* disruption mutation (*KRE1/krel::HIS3*).

The *KRE1* Gene Encodes a Product with a Functional Signal Peptide

Restriction endonuclease sites were introduced three nucleotides before, and immediately after, the *KRE1* open reading frame using site-specific mutagenesis (Fig. 1). Introduction of these new sites facilitated the ligation of the open reading frame, into a 2- μ m based expression vector, pVT100U, which contains the *ADHI* promoter and terminator (Vernet et al., 1987). Upon transformation of a *krel-1* mutant, the resultant plasmid, pVT:KRE1, fully complemented the *kre-* phenotype and led to (1 \rightarrow 6)- β -glucan levels equivalent to those induced by YCp50:KRE1 (Table II). Transformation of a wild-type (*Krel+*) strain with pVT:KRE1 did not lead to an increased amount of (1 \rightarrow 6)- β -glucan.

To determine whether the *KRE1* sequence encoded a functional signal peptide, a deletion was made of the first 72 nucleotides of the open reading frame (predicted to encode 24 NH₂-terminal amino acids of Krelp, Fig. 1). The resultant construct was introduced into pVT100U, positioning Met 25 of Krelp next to the *ADHI* promoter. When transformed into yeast cells mutant at the *KRE1* locus, the leader-deleted construct (pVT: Δ 24/KRE1) did not complement the *kre-* phenotype. However, if the leader deleted portion of the *KRE1* sequence was replaced with a segment of DNA which encodes the first 20 amino acids of the alpha factor precursor (Kurjan and Hershowitz, 1982) (pVT: α 20/KRE1), a *Kre+* phenotype was observed (Fig. 3).

Another hybrid gene was constructed that replaced the DNA segment of *KRE1* encoding the last 59 amino acids of Krelp, with a sequence that codes for a six-amino acid portion of the neuropeptide substance P. This construct was introduced into pVT100U (pVT:KRE1/SP) and allowed partial complementation of the *krel* mutant strain (Fig. 3). The sub-

stance P portion provides an epitope that can be detected by an mAb (Munro and Pelham, 1984). Yeast strain 11A was transformed with the *ADHI* expression vector carrying hybrid constructs both with and without (pVT: Δ 24/KRE1/SP), the Krelp signal peptide. Electrophoretic transfer blot analysis of total protein isolated from transformed yeast cells showed that the leader allowed a 50-kD modification of the Krel-substance P hybrid protein (Fig. 4). This sizing is approximate because extended electrophoresis of the modified polypeptide resulted in smearing of the immunoreactive band. Similar analysis of concentrated yeast culture media revealed that only the modified hybrid protein was exported (data not shown). The leader-deleted Krel-substance P hybrid protein has a predicted molecular weight of \sim 25,000, while the apparent size as determined by SDS-PAGE was found to be 30 kD (Fig. 4). A similar discrepancy has been observed for other serine- and threonine-rich proteins, suggesting that it is associated with a high content of hydroxy-amino acids (Early et al., 1988). The observed modification of the Krel-substance P hybrid protein is probably the result of O-linked mannose addition. Evidence to support this conjecture comes from immunoprecipitation experiments, using other fusion constructs, where the modification was found to be endoglucosaminidase H resistant (data not shown).

Structural Analysis of (1 \rightarrow 6)- β -Glucan from a *krel* Mutant

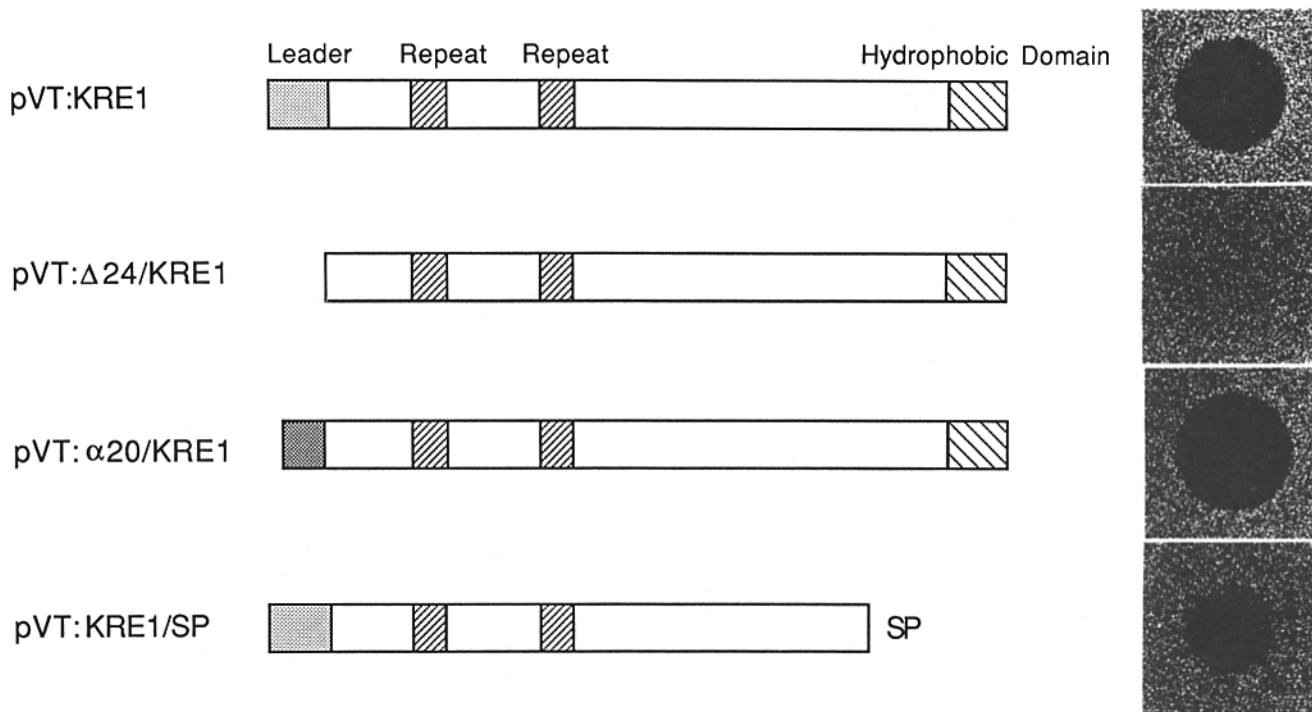
To facilitate [¹³C]NMR analysis of the (1 \rightarrow 6)- β -glucan fraction isolated from a *krel::HIS3* disruption strain (mutant glucan), a large-scale procedure for the purification of \sim 50 mg of Zymolyase-resistant glucan was designed (see Materials and Methods). The yeast strain 7B (*his3 ura3 glc1*) used for wild-type glucan purification carried the *glc1* mutation to minimize glycogen contamination (Tkacz, 1984); disruption of the *KRE1* locus in this strain created a *krel* null mutant, (strain 3), with a reduced amount of (1 \rightarrow 6)- β -glucan (Table I).

The proton decoupled [¹³C]NMR spectrum of glucan purified from the wild-type strain (7B), is presented in Fig. 5 A. The data for this spectrum were obtained under conditions where the signal area reflects relative amounts of the constituent carbon atom(s) (Shimamura, 1989). The wild-

Table II. Plasmid-dependent Maturation of Cell Wall (1 \rightarrow 6)- β -Glucan

Yeast strain (allele at <i>KRE1</i> locus)	Transformation plasmid	(1 \rightarrow 6)- β -Glucan μ g/mg dry wt
11A (<i>krel-1</i>)	YCp50	15.7 \pm 1.4
11A (<i>krel-1</i>)	YCp50:KRE1	41.4 \pm 4.7
11A (<i>krel-1</i>)	pVT100U	17.5 \pm 1.1
11A (<i>krel-1</i>)	pVT:KRE1	42.6 \pm 4.1
7B (<i>KRE1</i>)	pVT100U	38.9 \pm 3.9
7B (<i>KRE1</i>)	pVT:KRE1	45.2 \pm 6.4

Yeast (1 \rightarrow 6)- β -glucan levels were analyzed for various 11A (*krel-1*, *ura3*) transformants. Plasmid YCp50:KRE1 contains a yeast genomic insert that complements the *krel-1* mutation ligated into the centromeric (single copy) vector YCp50. Plasmid pVT:KRE1 contains the *KRE1* open reading frame ligated into the 2- μ m derived (multicopy) expression vector pVT100U. Transcription of the *KRE1* from pVT:KRE1 occurs via the *ADHI* promoter. Yeast (1 \rightarrow 6)- β -glucan levels were also analyzed for transformants of strain 7B (*ura3*, *his3*, *glc1*). Error represents 1 SD.



KRE1p Leader: *MMRRTLLHSFATLLLSLSLWSAA*[∇]

Prepro- α Factor Leader: *MRFPSIFTAVLFAASSALAA*[∇]

Substance P epitope (SP): *DSQFFGLM*

Figure 3. Leader-dependent function of the *KRE1* gene product. Inserts of various pVT:100U-derived vectors are designated by the plasmid name and drawn schematically, indicating structural features as described in the text. The amino acid sequence of the Kre1p leader is compared with the sequence of the prepro- α factor leader. The signal cleavage site of the prepro- α factor leader and a site predicted for Kre1p are indicated. Examples of the seeded plate assay used to assess complementation of the killer-resistant phenotype of transformants, of strain 11A (*krel-1*, *ura3*) are also shown (see Materials and Methods). Plasmids, pVT:KRE1 and pVT: α 20/KRE1, completely complement the *kre-* phenotype. Plasmid pVT:KRE1/SP can only partially complement and pVT: Δ 24/KRE1 does not complement the *kre-* phenotype.

type glucan showed predominant signals at 103.8, 76.4, 75.7, 73.8, 70.3, and 69.6 ppm (Fig. 5 A; C-1, C-3, C-5, C-2, C-4, and C-6 linked, respectively). These chemical shifts are characteristic of linear (1 \rightarrow 6)- β -glucan (Gopal et al., 1984; Bassieux et al., 1977; Saito et al., 1977). Several minor signals can be ascribed to the presence of linear (1 \rightarrow 3)-linked, branched (1 \rightarrow 3, 1 \rightarrow 6)-linked and terminal β -glucopyranosyl residues in the polymer. For example, the signal with a chemical shift of 61.5 ppm is the result of residues unsubstituted at C-6 (Fig. 5 A; C-6), as found for terminal β -glucopyranosyl residues or those which have a linear (1 \rightarrow 3)-linked structure, other assignments are presented in Fig. 5. Integration analysis suggests that 82% of the residues are O-substituted at the C-6 position.

The proton decoupled [¹³C]NMR spectrum of glucan purified from the *krel* mutant strain (3), is presented in Fig. 5 B. Each of the signals of this spectrum was found to have a signal of equivalent chemical shift present in the spectrum

of wild type glucan (cf. Fig. 5, A with B). Therefore, each glucan contains a similar set of linked residues. A noticeable difference between the two spectra is the relative ratio of signals within a given spectrum. The spectrum of the mutant glucan contains a higher proportion of signals corresponding to linear (1 \rightarrow 3)-linked, branched and terminal β -glucopyranosyl residues than the wildtype. Integration analysis predicts that 64% of the residues are O-substituted at C-6.

Hence both the mutant and wild-type glucans give rise to [¹³C]NMR spectra consistent with a branched (1 \rightarrow 6)- β -glucan structure. The mutant glucan differs from the wild type in having fewer residues O-substituted at C-6. These results were confirmed by methylation analysis, which also indicated that the reduction of C-6, O-substituted residues was due to fewer linear (1 \rightarrow 6)-linked glucopyranosyl units (data not shown).

Gel filtration chromatography of mutant and wild-type glucans over a Sepharose CL-6B column demonstrated that

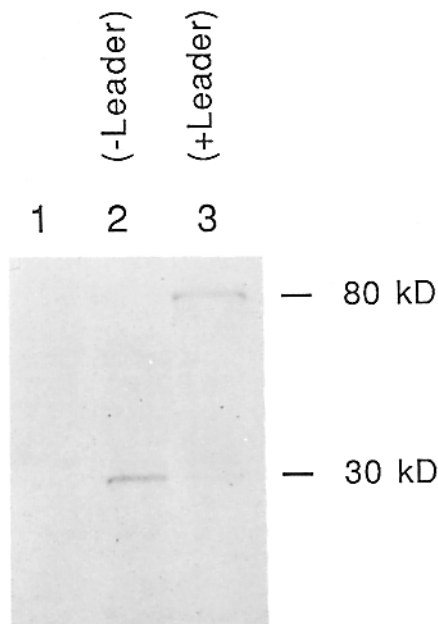


Figure 4. Western blot analysis of the products of Krel-substance P fusion constructs was carried out as described in Materials and Methods. Plasmids pVT:KRE1/SP and pVT: Δ 24/KRE1/SP contain similar DNA inserts, encoding Krel-substance P fusion proteins (with the substance P epitope replacing the last 59 amino acids of Krelp), except that the insert of pVT: Δ 24/KRE1/SP is deleted for DNA encoding the predicted leader of Krelp. Strain 11A transformed with pVT:KRE1/SP (see Fig. 3) produces an immunoreactive band that migrates with a molecular mass of 80 kD (lane 3). Strain 11A transformed with pVT: Δ 24/KRE1/SP leads to an immunoreactive band which migrates with a predicted molecular mass of 30 kD (lane 2). Strain 11A transformed with the expression vector pVT100U provided a control that did not give rise to an immunoreactive band (lane 1).

the mutant glucan had a smaller average degree of polymerization than the wild type (Fig. 6). The wild-type glucan displayed a range of different-sized material, with an average predicted molecular mass of 40 kD. The mutant glucan displayed a range of material with smaller predicted molecular masses and an average of 20 kD. These results suggest that the average degree of polymerization of the wild-type glucan was \sim 200, whereas that of the mutant glucan was 100.

Killer-resistant Mutants Identify a Group of Genes Required for Cell Wall (1 \rightarrow 6)- β -Glucan Production

The observation that *krel::HIS3* mutant yeast strains produced an altered form of cell wall (1 \rightarrow 6)- β -glucan with a smaller average polymer size, suggests that *KRE1* could be required for the stepwise synthesis of the mature polymer. Additional genes required for the production of cell wall (1 \rightarrow 6)- β -glucan were identified through further selection of mutants resistant to killer toxin (see Materials and Methods). 44 resistant mutants were characterized by performing genetic analysis in an isogenic background. Six complementation groups were defined by recessive mutations, each of which segregated as a defect in a single gene. Two of the complementation groups were found to be equivalent to *KRE1* and *KRE2* described by Al-Aidroos and Bussey (1978). The other complementation groups designated *KRE4*, *KRE5*,

KRE6, and *KRE8* are novel. Segregation analysis indicated that the *krel*, 2, and 5 mutations identify three separate loci, and that *KRE5* and *KRE6* are not allelic. The mutants that define the *KRE4* and *KRE8* complementation groups proved to be only weakly resistant, and will not be considered further here.

Mutants in three complementation groups were found to have reduced levels of cell wall (1 \rightarrow 6)- β -glucan (Table III). The level in strain S708 that harbors the *krel-3* allele was reduced 40% in agreement with the previous observations for mutants containing *krel-1* or a *krel:HIS3* disruption mutation. Strains S726 and S731 carrying mutations at the *KRE5* and *KRE6* loci respectively showed a significant reduction in (1 \rightarrow 6)- β -glucan and demonstrated a slow growth phenotype which cosegregated with the killer resistance when compared with wild-type strains or the *krel* mutant, S708. The level of (1 \rightarrow 6)- β -glucan was not altered in the *krel-2* strain S706 (Table III).

Double mutants were constructed for the strains that showed a reduced level of cell wall (1 \rightarrow 6)- β -glucan. The level of (1 \rightarrow 6)- β -glucan in double mutants of *krel* with *kre5* or *kre6* was not significantly lower than that found in *kre5* or *kre6* single mutants. This result suggests that mutations at both the *kre5* and *kre6* loci lead to killer toxin resistance because they are epistatic to *KRE1*. In contrast, *kre5* and *kre6*

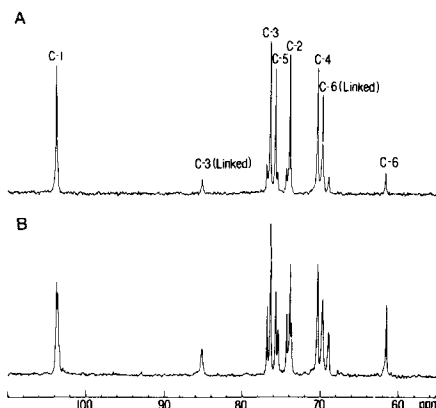


Figure 5. (A) [^{13}C]NMR spectrum of (1 \rightarrow 6)- β -glucan purified from a wild type (*Kre+*) yeast strain. The predominant signals (A; C-1, C-3, C-5, C-2, C-4, and C-6 linked) have chemical shifts that are characteristic of linear (1 \rightarrow 6)- β -glucan as described in the text. Presently, there are insufficient reference data to assign identities to each of the minor signals; however, some can be assigned as presented below. The signal at 85 ppm (A; C-3 linked) corresponds to O-substituted at C-3 found in branched and linear (1 \rightarrow 3)-linked residues (Yoshioka et al., 1985). The signal at 68.8 ppm can be assigned to C-4 of residues O-substituted at C-3 and the signal at 61.5 ppm results from residues unsubstituted at C-6, the latter are found in linear (1 \rightarrow 3)-linked and terminal β -glucopyranosyl residues (Bruneteau et al., 1988). Some of the expected minor signals coincide with a major signal. For example, the signal with a chemical shift of 69.6 ppm is the result of residues O-substituted at C-6 (Fig. 5A; C-6 linked), as found for those which have a linear (1 \rightarrow 6)-linked or branched structure. Assignment of the signal at 69.6 and 61.5 ppm as the result of a CH_2 group was confirmed by a DEPT NMR pulse sequence (Doddrell et al., 1982). The area of the assigned minor signals was similar predicting that the relative proportion of branched and terminal β -glucopyranosyl residues was approximately equal, as expected. (B) [^{13}C]NMR spectrum of the (1 \rightarrow 6)- β -glucan fraction purified from a *krel* mutant.

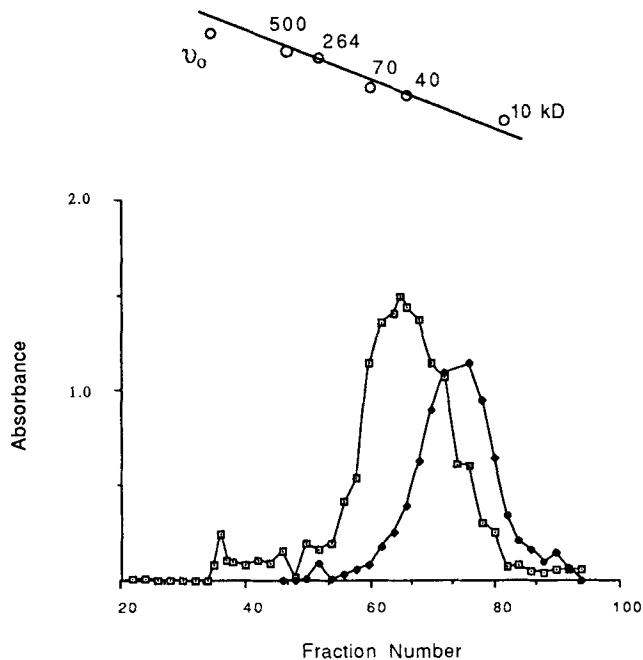


Figure 6. Gel filtration chromatography of purified (1→6)- β -glucan isolated from the *krel* mutant strain 3, (◆); or the *KRE1* strain 7B (□) on Sepharose CL-6B. The dextran standards and chromatography conditions used are described in Materials and Methods.

double mutants displayed a further reduction in the cell wall (1→6)- β -glucan level (reduced by ~80% over wild type), and are associated with a severe growth impairment.

Discussion

We have cloned the *KRE1* gene from *S. cerevisiae* and shown that a disruption of the *KRE1* locus results in an ~40% reduction of cell wall (1→6)- β -glucan. Haploid yeast strains with a disrupted *krel* allele grow somewhat more slowly than wild type and were found to have an unusual cell wall ultrastructure. Yeast cell wall (1→6)- β -glucan is a highly branched glucose polymer composed mostly of linear (1→6)-linked residues as well as some linear (1→3)-linked residues. Branching occurs through triply linked (1→3, 1→6)- β -glucopyranosyl residues. Structural analysis of the (1→6)- β -glucan, which remains in a *krel* mutant (mutant glucan) when compared with the glucan purified from isogenic wild type cells, showed that each glucan was composed of a similar set of linked residues. However, the mutant glucan contained fewer (1→6)-linked residues, which were incorporated into a polymer of smaller average size. It is possible that the *KRE1* gene product is required for the addition of extended chains, composed predominantly of linear (1→6)- β -glucan, onto a highly branched acceptor glucan.

We favor this interpretation because *krel* mutants are completely resistant to the K1 killer toxin of *S. cerevisiae*. The killer toxin displays a lectin-like affinity for linear (1→6)- β -glucan chains and unlike the cell walls of wild type yeast strains, *krel* mutant cell walls lack a component with similar toxin affinity (Bussey et al., 1979).

A potential pathway of gene products necessary for yeast (1→6)- β -glucan biosynthesis is implicated by the finding that

other mutants are resistant to killer toxin. Mutations at either the *KRE5* or *KRE6* loci result in killer resistance and a reduced amount of cell wall (1→6)- β -glucan. This reduction is not affected by a *krel* mutant allele, suggesting that mutations at the *KRE5* or *KRE6* loci are epistatic to *KRE1*. Mechanistically it seems reasonable that the *KRE5* and *KRE6* gene products could be required for the production of an acceptor glucan, which is defined by the (1→6)- β -glucan fraction that remains in a *krel* mutant (Fig. 7). This interpretation implies that the mutant *kre5-1* or *kre6-1* alleles lead to the production of an altered acceptor glucan, which cannot be extended in a *KRE1*-dependent fashion and therefore result in killer toxin resistance. Recent experiments have shown that disruption of the *KRE5* locus leads to a yeast strain which is not impaired for (1→3)- β -glucan biosynthesis, but has an extremely slow growth rate, and appears to lack cell wall (1→6)- β -glucan (Meaden, P., unpublished results). The lack of (1→6)- β -glucan in yeast strains carrying a *kre5* null mutation further indicates that mutations at the *KRE5* locus are epistatic to *KRE1*.

The *KRE1* gene product (Krelp) has a functional amino-terminal signal sequence that directs the protein into the yeast secretory pathway, where it is extensively modified probably through the addition of O-linked mannose residues. Yeast mating-type agglutinin proteins (Lasky and Ballou, 1988; Watzel et al., 1988) and a large proportion of the bulk cell wall protein (Frevort and Ballou, 1985) are serine/threonine-rich and O-glycosylated. Therefore by analogy, Krelp may also be localized at the yeast cell surface. In support of this idea, fusion constructs which place a leader-deleted *KRE1* fragment next to the carboxy terminus of the *PHO5* open reading frame (Meyhack et al., 1982), lead to a fusion protein that partially complements a *krel* mutant and directs acid phosphatase activity to the cell surface (data not shown). The 21 carboxy-terminal amino acid residues of Krelp form a hydrophobic sequence, which may serve as a membrane spanning domain or provide a signal for attachment of a glycosyl-phosphatidylinositol membrane anchor (Conzelmann et al., 1988).

The appearance of *krel* mutant cells, as examined using EM, revealed that the outer portion of the wall was abnormal. Particularly noticeable was the lack of a finely delineated dark staining region, thought to be a surface layer of mannoprotein (Zlotnik et al., 1984). This alteration, although possibly enhanced by the fixation procedure, may have functional significance, as *krel* mutants are more sensitive to

Table III. (1→6)- β -Glucan Levels of *kre*-Strains

Yeast strain	<i>KRE</i> allele	(1→6)- β -Glucan μ g/mg dry wt
S442	<i>KRE+</i>	29.4 \pm 2.0
S484	<i>KRE+</i>	30.7 \pm 1.3
S708	<i>krel-3</i>	16.1 \pm 3.3
S706	<i>kre2-2</i>	26.3 \pm 2.1
S726	<i>kre5-1</i>	11.6 \pm 1.5
S731	<i>kre6-1</i>	11.9 \pm 0.5

Cell wall (1→6)- β -glucan levels were determined for killer-resistant mutants isolated in the S442 and S484 genetic background. Total alkali-insoluble glucan was not significantly different for any of these strains (with an average of 134 \pm 28 μ g/mg dry wt), except for S726, which showed a modest increase (175.2 \pm 6.8 μ g/mg dry wt). Error represents 1 SD.

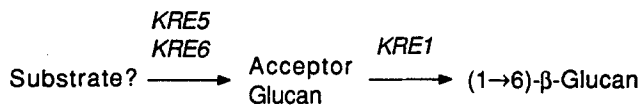


Figure 7. A model for the functional role of the *KRE1* gene product is shown. It is postulated that extended chains, composed predominantly of linear (1→6)-linked β-glucopyranosyl residues, are attached to a highly branched acceptor glucan, in a *KRE1* dependent fashion. The acceptor glucan is defined by the (1→6)-β-glucan fraction that remains in a *kre1* mutant and cannot interact with the killer toxin. The acceptor glucan appears to be made in a *KRE5*-, and *KRE6*-, dependent fashion. The acceptor glucan and the chains containing linear (1→6)-linked residues together make up what is referred to in the text as yeast (1→6)-β-glucan, which acts as a killer toxin receptor.

zymolyase treatment than wild-type cells (data not shown), and over secrete proteins normally found in the growth medium (Bussey et al., 1983). It is likely that wild-type cells release a certain portion of wall-localized proteins into the growth medium and this process is exaggerated in *kre1* mutant cells. However, *kre1* mutants do not show significant reduction in total wall mannoprotein (Hutchins, 1982), indicating that the bulk of the mannoprotein is efficiently targeted within the wall (Valentin et al., 1987) or periplasmic space. In addition, the *kre1* mutant cell walls were found to stain more intensely, especially in the outer half of the wall, leading to a bipartite appearance. This may suggest that a *kre1* mutant is particularly defective in the assembly of the outer wall, which could be the region of (1→6)-β-glucan localization (Cabib et al., 1982).

Efficient *in vitro* synthesis of chitin and linear (1→3)-β-glucan has been observed with membrane preparations and UDP-charged substrates (Kang and Cabib, 1986; Cabib et al., 1982), but an analogous system for yeast (1→6)-β-glucan or branched (1→3)-β-glucan synthesis has not yet been achieved. Although it is not known if *Kre1p* functions directly in (1→6)-β-glucan biosynthesis, that *Kre1p* is targeted to the yeast secretory pathway and potentially localized on the cell surface supports this possibility. In accord with this idea, kinetic experiments after hyphal cell wall biosynthesis of *Schizophyllum commune* suggested that cell wall deposited (1→3)-β-glucan could be subsequently modified by attachment of (1→6)-β-linked branches (Sietsma et al., 1985). Multiple copies of the *KRE1* gene under the control of the *ADH1* promoter did not lead to the overproduction of (1→6)-β-glucan, but this does not rule out the possibility of a glucan synthase or transferase function. For instance, the gene products required for the synthesis of an acceptor glucan could be rate limiting for (1→6)-β-glucan biosynthesis (Glazebrook and Walker, 1989). Indirect mechanisms may lead to the observed phenotypes of *kre* mutants. For example, each of the *kre* mutants could be required for preservation rather than synthesis of yeast (1→6)-β-glucan. *Kre1p* could then function as an inhibitor of a putative cell wall glucanase, with an activity towards linear (1→6)-β-glucan, resulting in partial degradation of the polymer. Several glucanase activities have been reported to occur in *S. cerevisiae*, but their functions are unknown (Kuranda and Robbins, 1987).

Cell wall (1→6)-β-glucan has been reported to occur among species from taxonomically diverse genera of yeasts including *Candida albicans* (Manners et al., 1974). *C. albi-*

cans is of particular interest because of its dimorphic nature and pathogenicity. Glucan accounts for 50–70% of the *C. albicans* cell wall and appears to function as the main structural component of both the yeast and mycelial forms (Fleet, 1985). As was observed for *S. cerevisiae* most of the cell wall glucan was isolated from whole cells as an alkali insoluble fraction which was found to contain two glucan subclasses. One glucan subclass closely resembled the *S. cerevisiae* (1→6)-β-glucan and while the other was found to contain relatively more (1→3)-linked glucopyranosyl residues, both types of glucan appear to be highly branched and composed predominantly of (1→6)-linked residues (Gopal et al., 1984). We have recently isolated a DNA fragment from the *C. albicans* genome capable of complementing the *kre-* phenotype of an *S. cerevisiae kre1* mutant. It is likely that *C. albicans* homologues of the *S. cerevisiae KRE* genes described here, have a similar function in the production or assembly of the *C. albicans* cell wall. However, the greater abundance of (1→6)-linked residues in the total cell wall glucan of *C. albicans* may imply that *KRE* homologues are associated with additional structural or morphological roles in this fungus. Partly because of the functional similarity of gene products required for most eukaryotic cellular processes, it has been difficult to devise specific antifungal antibiotics. Identification of the synthetic machinery for components, like fungal cell wall β-glucans, that are absent in mammalian cells, should reveal proteins that are excellent potential targets for specific antifungal inhibitors.

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