Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*

(*Saccharomyces cerevisiae* gene/gene disruption/membrane protein/β-glucan synthase)

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ABSTRACT   The *KRE6* gene product is required for synthesis of the major β-glucans of the yeast cell wall, as mutations in this gene confer reduced levels of both the (1→6)- and (1→3)-β-D-glucan polymers. Cloning and sequencing of *KRE6* reveals a gene encoding a predicted 80-kDa protein with a central transmembrane domain and the topology of a type II membrane protein. Null mutants of *KRE6* grow slowly, have larger cells, and show a reduction in alkali-insoluble wall glucans. The mutants show good viability and are not osmotically sensitive, but they are more susceptible to β-glucanase digestion and mechanical stress than wild-type cells. The specific activity of the GTP-dependent, membrane-associated, *in vitro* (1→3)-β-glucan synthase is reduced 50% in *kre6* null mutants, and this reduction correlates with the mutation in meiotic tetrads. Transformants of *kre6* null mutants with a *KRE6* gene expressed from a centomere-based vector show a 4- to 5-fold increase in *in vitro* (1→3)-β-glucan synthase activity over transformants with the vector alone. The phenotype and structure of the *KRE6* product, Kre6p, suggest that Kre6p may be a β-glucan synthase, and if so, it implies that β-glucan synthases are functionally redundant in yeast. Alternatively, Kre6p may be part of a single multiprotein glucan synthase or modulate its activity. Use of *KRE6* should permit a genetic analysis of eukaryotic (1→3)-β-glucan synthesis.

β-Glucan polysaccharides are widely found as cell surface polymers in bacteria, fungi, and plants. The biological role of these substances remains unclear, though they have been implicated in a range of processes, including providing osmotic and mechanical protection, selective permeation of macromolecules, cell extension, growth and division, and cell–cell interactions (1). In *Saccharomyces cerevisiae*, two classes of alkali-insoluble β-glucans exist. (1→3)-β-Glucan, making up approximately 25% of the cell wall dry weight, has a degree of polymerization estimated as 1500 residues and is attached predominantly as 1→3 linkages and branched with occasional 1→6 linkages (2). (1→6)-β-Glucan is a more complex, smaller, alkali-insoluble polymer with an average degree of polymerization of 140 residues and makes up approximately 7% of the cell wall dry weight (3). It is composed of a 1→6-linked core, branched by occasional 1→3 linkages, with 1→6 side chains providing many terminal glucopyranosyl residues (3). Despite the natural abundance of these polymers and the apparent simplicity of their structure as glucose homopolymers, little is known about the mechanism of β-glucan biosynthesis. Recently the operon for cellulose synthesis in the bacterium *Acetobacter xylinum* has been analyzed, and four gene products, including two membrane proteins, have been shown to be required for the synthesis of this (1→4)-β-glucan (4, 5). In plants, the com-

ponents of cellulose synthesis also appear to be membrane associated but have proved difficult to analyze biochemically. In synthesis of the plant (1→3)-β-glucan callose, a 57-kDa polypeptide has been implicated as a component of callose synthase by partial purification and photoaffinity labeling of this protein with an analog of the substrate UDP-glucose (6). In fungi (1→3)-β-glucans are common cell wall components, and a considerable body of biochemical and physiological information exists on the (1→3)-β-glucan synthase (7). The synthase assayed *in vitro* is membrane associated, requires UDP-glucose as a substrate, and is stimulated by a soluble GTP-activated protein. Purification of these components has not been achieved. Unlike (1→3)-β-glucan synthase, (1→6)-β-glucan synthase activity has not been detected *in vitro*. As with bacteria, genetics has been useful in identifying components in polysaccharide biosynthesis in fungi; examples include the N-linked glycosylation of proteins (8) and synthesis of chitin (9–11) and glycogen (12). However, the approach has been less informative for (1→3)-β-glucan synthesis, where searches for conditional lethals with a lysis phenotype or for resistance to drugs thought to inhibit (1→3)-β-glucan synthesis have not identified synthase mutants. In yeast both chitin and glycogen synthesis use more than one synthase gene, and the resulting functional redundancy has complicated a genetic analysis. Here we describe an *S. cerevisiae* gene, *KRE6*, that encodes a predicted type II membrane protein involved in β-glucan synthesis *in vivo*[†] and is required for full activity of β-glucan synthase *in vitro*.

METHODS

Yeast Strains and Methods. The killer-resistant mutants HAB224-2B and 2C (*kre6-1 ura3 his3*) were as described by Meaden *et al.* (13). The isogenic diploid strain TA405 (*MAT*a/α *his3/his3 leu2/leu2*) (13) was used for a *kre6::HIS3* disruption and SEY6211 (*MAT*a *leu2 ura3 his3 trp1 ade2 suc2*) for allelism tests. An additional *KRE6* null was constructed in a SEY6210 strain (*MAT*α *leu2 ura3 his3 lys2 trp1 suc2*) autodiploidized by following the HO plasmid method described by Murray and Szostak (14). SEY6210 and SEY6211 were generously provided by Scott Emr (California Institute of Technology). ¹³C NMR analysis of (1→6)-β-glucan was performed from strain 7B-3 (*MAT*a *kre6::HIS3 ura3 glc1*) and 7B (*MAT*a *his3 ura3 glc1*). Mannoproteins were estimated from an isogenic SEY6210 *kre6* null mutant and *KRE6* strain as described by Nakajima and Ballou (15). Chitin levels of *KRE6* and *kre6* null strains were assessed *in vitro* after incubating late-logarithmic-phase cells for 3 hr in

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Abbreviation: GTP[γS], guanosine 5'-[γ-thio]triphosphate.
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[†]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80657).

the fluorescent brightener calcofluor white (Sigma) (ref. 16, p. 159). Estimation of β-glucans secreted into the medium was performed by centrifuging 10-ml cultures of the above strains grown on YEPD medium (ref. 16, p. 163), dialyzing (6000–8000 molecular weight cutoff) equivalent amounts of supernatant, and measuring hexose by the borosulfuric acid assay (17). Growth conditions and media for yeast and bacterial propagation were as previously described (18). Yeast transformations were by the lithium acetate method of Ito *et al.* (19). Resistance and sensitivity to K1 killer toxin was scored by the seeded plate assay of Bussey *et al.* (18). An allelism test was performed by ligating a 6.0-kilobase (kb) *Hind*III fragment, isolated from a larger *KRE6* complementing fragment, into the dephosphorylated *Hind*III site of the integrating plasmid, pRS306 (20). This construct was linearized within the insert by digesting with *Bgl* II and used to transform HAB224-2B. Three killer-toxin-sensitive integrants were crossed with SEY6211 and induced to sporulate, and tetrads were analyzed.

Plasmids. The YCp50-based yeast genomic library of Rose *et al.* (21) was used. The position of an introduced *Bam*HI site in two larger complementing *KRE6* fragments was conveniently used for subclonings. pRS315, a centromere-based plasmid (20), containing a 5.4-kb *Bam*HI/*Sal* I *KRE6* fragment inserted into the corresponding polylinker sites was used for *in vitro* assays of (1→3)-β-glucan synthase. This same *KRE6* fragment was subcloned in PBSK+ and used to probe a chromosome blot. Overexpression of *KRE6* was performed by using the *LEU2* 2μ-based plasmid pVTL100 described by Vernet *et al.* (22); pVTL100 possesses a defective *LEU2* promoter, allowing leucine prototrophy only at high copy number. *KRE6* was inserted into the *Bam*HI/*Pvu* II sites of the pVTL100 polylinker as a 5.0-kb *Bam*HI/*Dra* I fragment. *KRE6* overexpression was further examined with this same fragment ligated into the *Bam*HI/*Pvu* II sites of the 2μ-based YEp13 plasmid. Both of the above described *KRE6* fragments complement *kre6* mutants fully when expressed from centromeric plasmids. Additional *KRE6* subclonings were performed in YCp50, YEp24, and YEp13 vectors. Single-stranded DNA was prepared from Bluescript vectors +/− (Stratagene).

DNA Purification and Recombinant DNA Techniques. Plasmid DNA was prepared from *Escherichia coli* as described by Sambrook *et al.* (23). Yeast DNA was isolated by the method of Hoffman and Winston (24). Restriction endonucleases, T4 DNA ligase, DNA polymerase Klenow fragment, and calf intestinal alkaline phosphatase were purchased from either Bethesda Research Laboratories or New England BioLabs and were used according to the instructions of the supplier. Tn*10*-LUK transposon mutagenesis mapping of *KRE6* was performed as described by Huisman *et al.* (25), using the target plasmid p504 (see Fig. 1*A*). *KRE6* was mapped to chromosome XVI by using a *KRE6* nick-translated probe and a gel wafer of separated *S. cerevisiae* chromosomes purchased from Clontech.

DNA Sequencing. Subclones of *KRE6* were made in Bluescript vectors of M13mp19 and used to transform the bacterial strain UT580, and single-stranded DNA was prepared by using M13K07 helper phage (22). Sequencing was by the dideoxy method (26), using the Sequenase kit and Bluescript-specific primers (United States Biochemical) and deoxyadenosine 5′-[α-^{35}S]thio]triphosphate as a substrate. Nested deletions were made using the Erase-A-Base Kit (Promega) and the complete sequence was obtained on both strands.

KRE6 Disruption. To create a *kre6::HIS3* disruption construct, a *KRE6 Bam*HI/*Sal* I fragment was subcloned in a previously modified PBSK+ polylinker, where the *Eco*RI site had been removed by filling in the 5′ *Eco*RI overhang with Klenow fragment and religated using T4 DNA ligase. This construct was then digested with *Eco*RI to remove a

1.3-kb *Eco*RI fragment within the *KRE6* open reading frame, gel purified by using GeneClean (Bio 101, La Jolla, CA), and treated with alkaline phosphatase, and a *HIS3*-containing *Eco*RI fragment was inserted. The ligation product from this reaction was digested with *Hpa* I and *Sal* I to yield a 5-kb *kre6::HIS3* fragment that, when used to transform TA405, SEY6210-15B, or 7B, allowed disruption of the *KRE6* locus by one-step gene replacement (27). After growth on YEPD at 30°C for 3 days, wild-type spore colonies were 3 mm in diameter compared to 1 mm for *kre6* null colonies. Gene disruptions were confirmed by Southern blot analysis (23). Genomic DNA was isolated from TA405 and SEY6210 tetrads, 7B and 7B-3, digested with *Eco*RV, and probed with an [α-^{32}P]dCTP-labeled (Oligolabelling Kit, Pharmacia) *Hind*III/*Nde* I *KRE6* fragment.

(1→6)-β-Glucan Analysis. Alkali-insoluble glucan was isolated from stationary-phase cultures grown in minimal media with appropriate supplements. (1→6)-β-Glucan was prepared after digesting alkali-insoluble glucan with (1→3)-β-glucanase (Zymolyase; ICN) by the method of Boone *et al.* (28). Total alkali-insoluble (1→3)- and (1→6)-β-glucan was determined as the sum of the carbohydrate content of both the Zymolyase-insoluble pellet and the solubilized supernatant before dialysis. Analysis of the carbohydrate retained after dialysis gave the amount of alkali-insoluble (1→6)-β-glucan. Carbohydrate was measured as hexose (17). (1→6)-β-Glucan was purified on a large scale for ^{13}C NMR analysis as described by Boone *et al.* (28). Gel filtration chromatography to estimate the average size of *kre6* null (1→6)-β-glucan was performed on a Sepharose CL-6B column as described by Boone *et al.* (28), and the hexose content of eluted fractions was determined (17).

(1→3)-β-Glucan Synthase Assay. Cell-free extracts were prepared from early logarithmic-phase cells after homogenization with glass beads and were stored in buffer A [50 mM Tris·HCl, pH 7.5/0.5 mM EDTA/33% (vol/vol) glycerol] (7). The *kre6* deletion mutants were found to break more easily by this procedure than wild-type cells. Protein concentrations were estimated by using the Bio-Rad protein assay (Bio-Rad). (1→3)-β-Glucan synthase activities were determined as outlined by Cabib and Kang (29), with the following modifications: (*i*) activity was not enriched by a 60-min centrifugation at 165,000 × *g* and (*ii*) bovine serum albumin and EDTA were omitted. Assays were carried out at 30°C for 60 min in 40 μl of 5 mM UDP-glucose/0.5 mM guanosine 5′-[γ-thio]triphosphate (GTP[γS])/25 mM KF/1 mM 2-mercaptoethanol and 3.0 μl of 7 μM UDP-[^{14}C]glucose (355 mCi/mmol, Amersham; 1 Ci = 37 GBq), plus protein extract in buffer A. Specific activities are expressed as nmol of glucose incorporated per mg of protein per hr. Fractionation of the (1→3)-β-glucan synthase activity into membrane and soluble fractions was performed by centrifugation (165,000 × *g* for 1 hr) in the absence of Tergitol but otherwise as described by Kang and Cabib (7). All glucan synthase activity was pelleted with the membrane fraction, and no β-glucan synthase activity was detected in the soluble fractions of wild-type or mutant extracts. UDP-[^{14}C]glucose incorporated into trichloroacetic acid-insoluble material was measured as described by Szaniszlo *et al.* (30). The glucan product of this β-glucan synthase reaction was similar to that reported by Shematek *et al.* (31) and was solubilized by (1→3)-β-glucanase but not by α-amylase.

Papulacandin and Zymolyase Sensitivity. The glucan synthase inhibitor papulacandin B (obtained from G. Maclachlan, McGill University, Montreal; 4 μl of a 1 mg/ml solution in ethanol) was spotted onto 5 ml of YEPD with 2% agar and 0.003% methylene blue that had been seeded with the yeast to be tested at 1 × 10^6 cells per ml and incubated at 30°C overnight, and the zone of inhibition was measured. For Zymolyase sensitivity, yeast cells at approximately 1 × 10^6
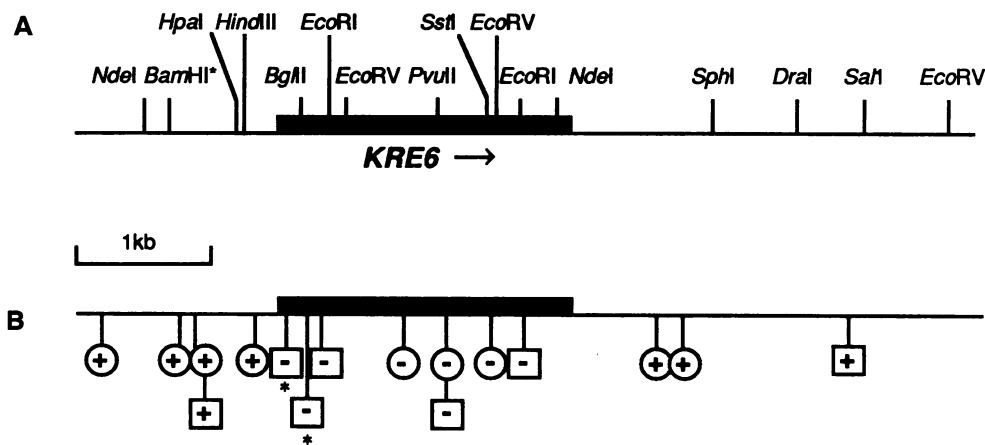
FIG. 1. (A) Restriction map of p504. The position of KRE6 is indicated by the black box, and the direction of its transcription by an arrow. The site of an introduced BamHI site, not present in p504, is indicated by an asterisk; this site was frequently used for KRE6 subclonings. (B) Map of transposon insertions into p504. Squares represent the position of Tn10:LUK integrated in the forward orientation (i.e., IS10$_L$ LUK IS10$_R$) with respect to KRE6, and circles represent integration in the reverse orientation (IS10$_R$ KUL IS10$_L$). A plus or minus identifies an insertion which does, or does not, allow kre6 complementation by p504 when the p504-Tn10:LUK plasmid is transformed into the kre6-1 mutant strain HAB224-2B. Asterisks below squares indicate the two forward insertions that displayed β-galactosidase activity, indicating KRE6:lacZ chimeras.

cells per ml were incubated in water or in water containing Zymolyase 60,000 at 60 μg/ml and incubated at room temperature for 90 min; 5 μl of treated cells was spotted to YEPD plates and incubated at 30°C for 24 hr, and growth was scored.

RESULTS

The KRE6 gene was identified by a recessive mutant allele partially resistant to killer toxin. Killer toxin uses a β-glucan as a cell wall receptor, and the kre6-1 mutant showed a reduced level of (1→6)-β-glucan and a receptor defective in binding the toxin (28). To explore genes involved in the synthesis of β-glucans, we cloned KRE6. Cloning was effected by functional complementation of the resistance phenotype with a yeast genomic library in YCp50, (21, 28). After screening of 45,000 transformants, 8 independent Kre⁺ transformants were found to be unstable for both the Kre⁺ and Ura⁺ phenotypes when grown under nonselective conditions. A plasmid was isolated from each Kre⁺ transformant; these contained a common 5.3-kb sequence amongst three different-sized genomic inserts. To identify the complementing open reading frame within the smallest complementing insert, called p504, a mini-Tn10:LUK transposon mutagenesis map of KRE6 was constructed (25) (Fig. 1). A cluster of noncomplementing integration events delineated a 2-kb region of p504, and complementing insertions bordering this region spanned 3 kb, indicating the open reading frame spanned 2–3 kb. Two KRE6:lacZ fusions, generated by the chance integration of Tn10:LUK in the correct orientation and reading frame within KRE6, yielded detectable β-galactosidase activity, and revealed the transcription direction of this open reading frame (Fig. 1).

The fragment was established to be KRE6 by an allelism test. The cloned wild-type fragment containing a URA3 marker (see Methods) was inserted by homologous integration in tandem with the kre6-1 allele. Crossing this integrant with a KRE6 ura3-1 strain and subsequent tetrad analysis indicated cosegregation of the Ura⁺ and Kre⁺ (toxin sensitive) phenotypes; of 21 tetrads scored all segregated 4:0 for sensitivity and 2:0 for Ura⁺, showing that the inserted fragment was closely linked to the kre6-1 allele. By using hybridization to a yeast chromosomal blot, KRE6 was shown to map to chromosome XVI.

The gene was sequenced, and it was found to encode a 720-amino acid residue, 80-kDa protein with the predicted characteristics of a type II membrane protein (Fig. 2). Such proteins have a cytoplasmic N terminus, a transmembrane domain, and a C terminus within the lumen of a secretory compartment or within the periplasm for a yeast plasma membrane protein (32, 33). Features of the protein include an N-terminal 248 residues which are presumably cytoplasmic and contain a pair of basic residues that may assist in determining a cytoplasmic location (33), followed by a 31-amino acid residue hydrophobic region that is capable of forming a transmembrane domain. C-terminal to the transmembrane domain is a putative lumenal tract, residues 280–720, containing five potential sites for N-glycosylation and eight for tyrosine sulfation. Interestingly, all 6 cysteine and 14 tryptophan residues localize to the predicted lumenal side of the protein. No homology was found between KRE6 or its product with sequences in the GenBank data base as of October 1991. If Kre6p is a glucan synthase, it should contain a substrate (UDP-glucose) binding site; there is a reasonable

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  1   MPLRNLTETHNFSSTNLDTDGTGDDHDGAPLSSSPSFGQQNDNSTNDNAGLTNPFMGSDEESNARDGESLSSSVHYQPQG   80

 81   SDSSLLHDNSRLDLSQNKGVSDYKGYYSRNNSRAVSTANDNSFLQPPHRAIASSPSLNSNLSKNDILSPPEFDRYPLVGS  160

161   RVTSMTQLNHHGRSPTSSPGNESSASFSSNPFLGEQDFSPFGGYPASSFPLMIDEKEEDDYLHNPDPEEEARLDRRRFID  240

241   DFKYMDKR[SASGLAGVLLLFLAAIFIFIVLPALTFTGAI]DHESNTEEVTYLTQYQYPQLSAIRTSLVDPETPDTAKTREA  320
                                                   *
321   MDGSKWELVFSDEFNAEGRTFYDGDDPYWTAPDVHYDATKDLEWYSPDASTTVNGTLQLRMDAFKNHGLYYRSGMLQSWN  400
                                          *
401   KVCFTQGALEISANLPNYGRVSGLWPGLWTMGNLGRPGYLASTQGVWPYSYESCDAGITPNQSSPDGISYLPGQKLSICT  480
                                                       *
481   CDVEDHPNQGVGRGAPEIDVLEGETDTKIGVGIASQSLQIAPFDIWYMPDYDFIEVYNFTTTTMNTYAGGPFQQAVSAVS  560
                                                        *
561   TLNVTWYEFGEYGGYFQKYAIEYLNDDDNGYIRWFVGDTPTYTIHAKALHPDGNIGWRRISKEPMSIILNLGISNNWAYI  640
        *
641   DWQYIFFPVVMSIDYVRIYQPSNAISVTCDPSDYPTYDYIQSHLNAFQNANLTTWEDAGYTFPKNILTGKCTSSKFKLSS  720
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FIG. 2. Predicted protein sequence of the KRE6 product, Kre6p. The boxed residues represent a putative transmembrane domain, and two basic residues implicated in cytoplasmic localization are shown in bold letters immediately N-terminal to the boxed hydrophobic domain. A UDP-glucose-binding consensus site is underlined. Potential sites for asparagine-linked glycosylation are indicated with asterisks.

consensus site on the lumenal domain of Kre6p: -Arg-Val-Ser-Gly- at 420, which resembles -Arg-Ser-Gly-Gly- in yeast glycogen synthase 1 (12).

Disruption of the gene by one-step gene replacement into an isogenic diploid and tetrad analysis of the progeny indicated that haploid cells with a deletion of *KRE6* gave slow-growing colonies reduced in alkali-insoluble levels of both (1→3)- and (1→6)-β-glucan (see Table 1), suggesting a role of this gene in the synthesis of both polymers. Preliminary data suggest the level of alkali-soluble glucan was also reduced in the *kre6* disruptant. A similar reduction in alkali-insoluble β-glucans was found with *kre6* null mutants from strains 7B and SEY6210. To test if glucan was produced and secreted, rather than incorporated into the wall, we measured polysaccharide in the growth medium. The amount detected in the media of the *kre6* null mutant was comparable to that of the wild type. Analysis by ^{13}C NMR and by gel filtration of the 1→6 polymer produced in a *kre6::HIS3* mutant indicated it was wild type in structure and size, consistent with production of a reduced amount of a normal polymer. Cells from *kre6::HIS3* mutants had wild-type levels of the mannoprotein wall component. In addition, *kre6* nulls demonstrated wild-type staining for chitin, as judged by calcofluor fluorescence. These results argue against a primary general role for Kre6p in affecting membrane proteins or altering membrane structure or composition. Light microscopy of disruptants indicated that the cells were larger than wild type and oddly shaped and tended to clump. Yeast cells harboring the disruption also grew more slowly than the wild type in media containing 1.2 M sorbitol, and so were not apparently osmotically fragile. Cells grown in aqueous media were examined for viability with the vital stain methylene blue and showed >99% viability, not significantly different from wild-type cells. Disruptants were more zymolyase sensitive and more readily broken with glass beads than wild-type cells (see *Methods*). Mutants with a *kre6::HIS3* disruption grew on glycerol and galactose and were thus not dependent on glucose as a carbon source. In addition, *kre6* null mutants were sensitive to the glucan synthase inhibitor papulacandin B and also grew on YEPD at 37°C.

The phenotype of the *kre6* mutants and the topology of the protein suggested the possibility that Kre6p was a glucan synthase component, and we explored this by using the *in vitro* assay of Cabib and Kang (29). The specific activity of β-glucan synthase was reduced in *kre6* disrupted mutants from four isogenic tetrads tested (Table 2). We found that the level of the synthase was considerably lower from cells in stationary phase but that the level in the mutant remained proportionately low (data not shown). To explore the possibility that the residual activity in *kre6* null mutants was qualitatively different from that in the wild type, we tested the properties of their glucan synthases. In both cases the glucan synthase activity was membrane associated and GTP activated (Table 3).

We attempted to overproduce the *in vitro* (1→3)-β-glucan synthase activity by expressing *KRE6* from plasmids of

Table 2. (1→3)-β-Glucan synthase activity in wild type and *kre6* deletion mutants

Yeast strain	Allele at *KRE6* locus	Specific activity of (1→3)-β-glucan synthase, nmol/mg per hr
	Tetrad 1	
98	*kre6::HIS3*	356.8 ± 6.0
99	*KRE6*	531.0 ± 23.7
100	*kre6::HIS3*	297.7 ± 2.4
101	*KRE6*	515.9 ± 40.3
	Tetrad 2	
103	*kre6::HIS3*	386.7 ± 6.6
104	*KRE6*	518.2 ± 8.1
105	*KRE6*	515.8 ± 110.5
106	*kre6::HIS3*	281.7 ± 4.6
	Tetrad 3	
108	*kre6::HIS3*	253.6 ± 6.0
109	*KRE6*	337.2 ± 22.5
110	*KRE6*	325.6 ± 12.3
111	*kre6::HIS3*	211.8 ± 1.6
	Tetrad 4	
113	*KRE6*	473.2 ± 2.8
114	*kre6::HIS3*	248.6 ± 1.3
115	*KRE6*	458.0 ± 12.4
116	*kre6::HIS3*	189.4 ± 2.5

Four independent isogenic TA405 diploids were made heterozygous for the *kre6::HIS3* disruption mutation and spore progeny from four meiotic tetrads were measured for (1→3)-β-glucan synthase specific activity. Error represents 1 SD.

differing copy number used to transform a *kre6* null mutant. Glucan synthase activity of *kre6* mutant 99, transformed with *KRE6* on the centromeric vector pRS315, was elevated 4.5-fold over the null mutant, which represents a 2-fold increase in *KRE6*-dependent (1→3)-β-glucan synthase activity (Table 4). Expression of *KRE6* from higher copy number plasmids failed to lead to greater glucan synthase activity when transformed into *kre6* mutant cells. For YEp13 *KRE6* transformants, the increase was 2.5-fold over the null mutant, or an 80% increase in *KRE6*-dependent activity. The nominally very high copy number construct pVTL100-KRE6 did not elevate the synthase activity in the *kre6* null mutant to the wild-type level (Table 4). We examined the ability of the above transformants to complement the killer resistance phenotype of a *kre6::HIS3* mutant; full complementation was seen with the centromeric plasmid expressing *KRE6*, but this was reduced with the *KRE6* gene on YEp13, and abolished on pVTL100 plasmids. In an extension of these findings, expression of *KRE6* from the pVTL100 plasmid in a wild-type cell conferred a dominant killer-resistant phenotype, while expression of the gene from a centromeric plasmid had little effect on the killer sensitivity of the wild type. This suggests that overexpression of Kre6p leads to a decrease in glucan synthase activity *in vivo*.

Table 3. GTP-activated (1→3)-β-glucan synthase in *KRE6* and *kre6* mutants

Yeast strain	Allele at *KRE6* locus	Specific activity of β-glucan synthase, nmol/mg per hr	
		With GTP[γS]	Without GTP[γS]
113	*KRE6*	356.3 ± 2.9	72.0 ± 6.2
114	*kre6::HIS3*	160.4 ± 5.6	33.1 ± 1.8
115	*KRE6*	328.3 ± 12.1	56.6 ± 1.5
116	*kre6::HIS3*	104.4 ± 3.7	16.8 ± 1.7

Strains 113–116 are from meiotic tetrad 4 described in Table 2. The GTP[γS] concentration in the assay is 0.5 mM. Error represents 1 SD.

Table 1. Levels of alkali-insoluble β-glucans in wild type and a *kre6* mutant

Yeast strain	Allele at *KRE6* locus	β-Glucan, μg/mg dry wt	
		(1→6)	(1→3) + (1→6)
103	*kre6::HIS3*	16.0 ± 0.1	63.4 ± 2.2
104	*KRE6*	35.0 ± 2.5	92.6 ± 1.3
105	*KRE6*	36.0 ± 0.1	101.1 ± 0.1
106	*kre6::HIS3*	17.1 ± 1.3	71.6 ± 0.6

β-Glucan levels were measured from the spore progeny of a tetrad from an isogenic diploid TA405 strain made heterozygous for a *kre6::HIS3* disruption mutation (*KRE6/kre6::HIS3*). Error represents 1 SD.

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Table 4. Plasmid-based *KRE6* expression

Yeast strain	Allele at *KRE6* locus	Plasmid	Specific activity of $(1\rightarrow3)$-β-glucan synthase, nmol/mg per hr
99	*KRE6*	—	465.7 ± 12.8
100	*kre6::HIS3*	pRS315	161.6 ± 6.2
100	*kre6::HIS3*	pRS315 *KRE6*	792.7 ± 36.0
100	*kre6::HIS3*	pVTL100	183.0 ± 18.6
100	*kre6::HIS3*	pVTL100 *KRE6*	283.3 ± 7.1
99	*KRE6*	pVTL100	375.2 ± 12.7
99	*KRE6*	pVTL100 *KRE6*	279.2 ± 13.3

Plasmid pRS315 *KRE6* is a centromere-based plasmid containing *KRE6*. pVTL100 *KRE6* is a 2μ-based plasmid designed for high copy number maintenance and containing *KRE6*. Strains 99 and 100 are two spore progeny from an isogenic diploid TA405. Error represents 1 SD.

DISCUSSION

We have identified a yeast gene encoding a putative type II membrane protein involved in β-glucan synthesis. The protein has a topology similar to that seen with glucosyltransferases in mammalian cells (34) but no homology with such transferases. Disrupted mutants in the *KRE6* gene show reduced levels of both β-glucans in the cell wall, and extracts from such mutants show a reduced specific activity of glucan synthase *in vitro*. Efforts to overproduce Kre6p led to modest elevation of the *in vitro* $(1\rightarrow3)$-β-glucan synthase levels, but use of high copy plasmids to express *KRE6* not only did not raise the activity further but actually reduced both the *in vitro* level of the synthase and the *in vivo* complementation of killer resistance. Such experiments, and the dominant killer resistance phenotype seen with overexpression of *KRE6* in a wild-type strain, suggest that Kre6p overproduction is deleterious to its activity in yeast. Despite lacking a significant amount of wall β-glucan, *kre6* disrupted mutants are viable, but they grow slowly and have abnormally large cells. An explanation of our results is that Kre6p is one of several glucan synthases in *S. cerevisiae*. There is functional redundancy among both chitin and glycogen synthases in yeast (9–11). Such a redundancy in glucan synthases would explain the inability to obtain yeast mutants in glucan synthesis with a conditional lysis phenotype, as these would require mutations in more than one gene.

A key assumption in our work is that we have disrupted the *KRE6* gene and generated a null mutation, and that the remaining glucan synthase activity and cellular glucan synthesis are not dependent on residual Kre6p activity. We have deleted over 60% of the gene, including the membrane-spanning domain, and the remaining *in vitro* glucan synthase activity is membrane associated and GTP activated. Thus it is unlikely to be encoded by the deleted *KRE6* gene, which would be expected to produce only a small cytoplasmic product. It is also difficult to see how such a truncated product could function to allow β-glucan synthesis *in vivo*.

An alternative explanation for our results is that Kre6p is a membrane protein modulating a single glucan synthase, perhaps as a component of a synthase complex, and its absence reduces the activity of the enzyme *in vitro* and the amount of glucan produced *in vivo*. Although we have overproduced the *in vitro* $(1\rightarrow3)$-glucan synthase activity by overexpressing *KRE6*, the 4- to 5-fold activity increase found does not definitively eliminate either of these models. Distinguishing between them will require considerably more knowledge about both the genetics and the biochemistry of the yeast glucan synthetic components. Kre6p appears to be involved in both $(1\rightarrow3)$-, and $(1\rightarrow6)$-β-glucan synthesis. As $(1\rightarrow6)$-β-glucan is actually a mixed-linked polymer containing some

$1\rightarrow3$-linked glucopyranosyl residues, Kre6p may simply be required to synthesize such $1\rightarrow3$ chains, which are essential to allow synthesis of the $1\rightarrow6$ moiety of the polymer. Examination of the $1\rightarrow6$ polymer in the *kre6* null mutant shows that it is wild type in structure, indicating a reduced level of a completed polymer. This finding is consistent with the reduced synthesis expected if one of two or more glucan synthases was deleted. *KRE6* should now permit genetic analysis and identification of further components involved in β-glucan synthesis.

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