Yeast KRE2 Defines a New Gene Family Encoding Probable Secretory Proteins, and Is Required for the Correct N-Glycosylation of Proteins

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ABSTRACT

We have cloned, sequenced and disrupted the KRE2 gene of Saccharomyces cerevisiae, identified by killer-resistant mutants with a defective cell wall receptor for the toxin. The KRE2 gene is close to PHO8 on chromosome 4, and encodes a predicted 49-kD protein, Kre2p, that probably enters the secretory pathway. Haploid cells carrying a disruption of the KRE2 locus grow more slowly than wild-type cells at 30°, and fail to grow at 37°. At 30°, kre2 mutants showed altered N-linked glycosylation of proteins, as the average size of N-linked outer chains was reduced. We identified two other genes, YUR1 on chromosome 10, and KTR1 on chromosome 15, whose predicted products share 36% identity with Kre2p over more than 300 amino acid residues. Yur1p has an N-terminal signal sequence like Kre2p, while Ktr1p has a predicted topology consistent with a type 2 membrane protein. In all cases the conserved regions of these proteins appear to be on the luminal side of secretory compartments, suggesting related function. KRE2, KTR1 and YUR1 define a new yeast gene family.

KILLER strains of Saccharomyces cerevisiae contain a double-stranded RNA virus encoding a secreted protein toxin capable of killing sensitive strains (Wickner 1986). Toxin action is thought to occur in at least two stages; cell wall binding, followed by action at the cell membrane (Zhu and Bussey 1991). K1 killer toxin displays an affinity for (1→6)-β-D-glucan, and binding to this cell wall polysaccharide appears to be the initial step in the action of the toxin (Hutchins and Bussey 1983). The toxin molecule forms ion channels in sensitive yeast spheroplasts and these lethal channels are thought to be the basis of toxin action at the plasma membrane (Martinac et al. 1990).

K1 killer toxin binds to linear chains of (1→6)-β-glucan in vitro, and such polymers act as competitive inhibitors of toxin action in vivo. In addition mutants resistant to the toxin show defects in (1→6)-β-glucan synthesis (Boone et al. 1990). Although (1→6)-β-glucan is a component of the toxin receptor the precise structure of the toxin receptor in the cell wall remains unknown and may contain further components. Mutants defective in the cell wall receptor of killer toxin permit an analysis of genes involved in assembling the receptor. We hope that information gained from such an analysis can extend what is known of the structure, biosynthesis and functional interactions of yeast cell wall components. These include glucan, mannoprotein and chitin; and mutants defective in their biosynthesis have been valuable in establishing the molecular biology of the fungal cell wall (Balou 1982; Silverman 1988; Bulawa et al. 1986).

The kre2-1 mutation (Al-Aidroos and Bussey 1978) affects the cell wall binding site for the toxin; kre2 cells are resistant and were found to bind less toxin than wild-type cells, but were sensitive as spheroplasts. kre2 mutants differed phenotypically from other wall mutants such as krel, 5 and 6 in having apparently normal levels of (1→6)-β-glucan and in retaining sensitivity to a second killer toxin, K2 (Rogers and Bevan 1978; Boone et al. 1990). Through an analysis of the KRE2 gene we attempt to determine the basis of the killer-resistant phenotype in kre2 cells. Evidence is presented which shows that the kre2 cell surface is altered with reduced N-linked glycosylation of proteins. We also describe two unlinked yeast genes, KTR1 and YUR1, whose products have extensive sequence identity with KRE2. These genes define a new family encoding putative secretory proteins.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains used in this work are listed in Table 1. Killer-resistant strains were isolated from S442 as described by Boone et al. (1990). Sensitivity or resistance to K1 killer toxin was scored by the seeded assay test as described by Bussey et al. (1982). Growth conditions and media for yeast and bacterial propagation were as described by Bussey et al. (1982) and Cooper and Bussey (1989). Standard techniques were employed for construction and sporulation of diploid strains (Sherman, Fink and Hicks 1982). Yeast transformations were per-
formed using the lithium acetate method of Ito et al. (1983).

**Plasmids:** Plasmid YCP50 and the yeast genomic library of Rose et al. (1987) were used for cloning. The BlueScript vectors of M13mp19 (Stratagene, San Diego, California) were used as vectors for bacterial transformation and production of single-stranded DNA.

**DNA purification and recombinant DNA techniques:** Plasmid DNA was prepared from Escherichia coli as described by Maniatis et al. (1989). Yeast DNA was isolated by the method of Davis et al. (1980). Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase and Klenow fragment were purchased from either Bethesda Research Laboratories, Inc. (Gaithesburg, Maryland), or New England Biolabs, Inc. (Beverly, Massachusetts), and were used as recommended by the suppliers.

**DNA sequencing:** Subclones of pKre2 for sequencing were made in the BlueScript vectors of M13mp19. Bacterial strain UT580, with helper phage M13K07 (Vernet, Ngard and Thomas 1987), was used for transformation of plasmids containing subclones and production of single-stranded DNA.

Sequencing was by the dideoxy method of Sanger, Nicklen and Coulson (1977), with the Sequenase kit (U.S. Biochemical, Cleveland, Ohio) using [(U-32P]ATP (Amershalm Canada Limited, Oakville, Ontario) as a substrate. DNA primers used were either the Bluescript universal or reverse primer or those synthesized to be complementary to parts of the KRE2 sequence.

**Gene disruption:** The structure of the KRE2 disruptions used in this work are shown in Figure 3. Strain A41 (kreb2Δ) contains a disruption at the KRE2 locus in which a 1.8-kb EcoRI fragment of pKre2 in PBSK* was replaced by a 1.8-kb fragment carrying the HIS3 gene. The plasmid construct was digested with HpaI and XbaI before transformation into TA405 by the one-step disruption technique of Rothstein (1985). Strain A71 (kreb2::LEU2) contains a disruption at the KRE2 locus in which a 2.1-kb fragment carrying the LEU2 gene was inserted into a Pep11 site (*). This work

**Preparation and analysis of purified (1→6)-β-glucan:** (1→6)-β-Glucan was purified from strain 7B/6, a derivative of strain 7B that had been disrupted at the KRE2 locus using the kreb2Δ1 plasmid construct. Glucan was extracted

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**Table 1** Yeast strains used

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<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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<td>A41</td>
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<td>This work</td>
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<tr>
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<td>This work</td>
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<td>leu2 his3 can1</td>
<td>This work</td>
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</tr>
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</tr>
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<td>TA405</td>
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<td>Whiteway and Szostak (1985)</td>
</tr>
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</table>
from this strain as described by BOONE et al. (1990). Purified (1→6)-β-glucan (70 mg) was purified from 5 liters of stationary phase cells and was resuspended in 3 ml D2O before examination by 13C and 2H NMR. Spectra were obtained under the conditions described by BOONE et al. (1990). For quantification, glucan was measured as hexose by the borsulfuric method of BADIN, JACKSON and SCHUBERT (1953).

Preparation and analysis of purified cell wall manno-protein: Bulk mannan, (mannoprotein), was isolated from spore clones from two tetrads of A41 as described by NAKAJIMA and BALLOU (1974). Briefly, yeast cells were grown in 1 liter YEPD until stationary phase. Mannoprotein was isolated by extraction with hot citrate buffer followed by precipitation of the mannoprotein first with methanol and then as a borate complex with Cetavlon (hexadecyltrimethylammonium bromide, Sigma Chemical Co., St. Louis, Missouri). Alcian blue binding to cells was by the method of FRIIS and OTTOLENGHI (1970). Measurement of phosphate on isolated mannoprotein was by the method of BARLETT (1959). Antibodies raised against (1→3)-α-mannosyl- and (1→6)-α-mannosyl-linked polysaccharides were gift from A. FRANZUSOFF.

Identification of GcNACMan8 and outer chaia oligo-saccharides: S. cerevisiae A41-13D and A41-13A were grown at 26° to early logarithmic phase in YEPD containing 1% glucose, concentrated fourfold and labeled for 10 min with 400 μCi of [2-'H]mannose (ICN, 28Ci/mmol). Cell pellets obtained by centrifugation were sequentially extracted with CHCl3/MeOH (2:1), H2O and CH3Cl/MeOH/H2O (1:1:0.3) and protein pellets were dissolved in sodium dodecyl sulfate (SDS) and Endo-H-treated as previously described (BYRD et al. 1982). Endo-H-released oligosaccharides were fractionated on a Bio-Gel P-6 column and the fractions corresponding to the GcNAcMan8 peak were pooled and chromatographed with 13C-labeled standards on a column (29 cm × 0.46 cm) of 5-μ particle size Aminopernerisorb (Phase Separations, packed by Chromatography Sciences Co., Canada) with a Varian model 5000 liquid chromatograph, as described by ROMERO, SAUNIER and HERSCOVICS (1985). The fractions corresponding to the void volume of the Bio-Gel P-6 column were pooled and rechromatographed on Bio-Gel P-60 (100–200 mesh).

Mapping of KRE2: Membranes containing separated chromosomes of S. cerevisiae were purchased from Clontech (Palo Alto, California). A 2.5-kb Hpal-XbaI fragment of pKre2 was labeled by nick translation using α-32P-dCTP (Amersham) as a substrate and then used to probe the membrane by the method of SOUTHERN (1975). A location on chromosome IV was indicated by the blot. In genetic mapping, kre2 was scored by toxin resistance in the seeded plate assay test and the pho8-1 allele scored by alkaline phosphatase assay as described by TOH-E, NAKAMURA and OSHIMA (1976).

RNA isolation and Northern analysis: Total RNA was isolated from a Kre+ strain (A41-1A) and an isogenic derivative (A41-1B) as described by ELDER, LOH and DAVIS (1983). The RNA blots were hybridized with 500 ng of a 1.8-kb HindIII-XbaI fragment of pKre2 that had been labeled with [α-32P]dCTP (Amersham). Subclones of pKre2 were cloned into vector YCp50 and tested for their ability to complement the killer-resistant phenotype of the kre2-1 mutation. Complementing activity of subclones is also shown in Figure 1. The smallest subclone capable of complementing kre2-1 was the 2.5-kb Hpal-XbaI fragment.

Nucleotide and predicted amino acid sequence of KRE2: The sequence of the entire 2.5-kb Hpal-XbaI fragment was determined by the dideoxy method of SANGER, NICKLEN and COULSON (1977) and one open reading frame of 1299 bp was found (Figure 2). It was of interest that this open reading frame was entirely encompassed in the Hpal-PvuII subclone, some 1.9 kb (Figure 1), which did not complement kre2-1.

The predicted protein from the KRE2 open reading frame, Kre2p, comprises 433 amino acids with a molecular mass of 49,888. One hydrophobic region was found at the N terminus of the protein with characteristics of a signal sequence (VON HEIJNE 1984). Cleavage by signal peptidase was predicted between the
The yeast DNA found in pKre2 is schematically represented. The KRE2 gene is shown as a black box, the truncated PHO8 gene by a striped box, and the spotted box represents ORF1 from KANEKO et al. (1987). Arrows indicate the direction of transcription for each of the genes. The wavy line represents the approximate position of the KRE2 transcript. The complementing activity of various fragments of pKre2 is also shown. Restriction endonuclease abbreviations are as follows: BamHI (B), BglII (Bg), ClaI (C), DraI (D), EcoRI (E), HindIII (H), HpaI (Hp), NcoI (N), PvuII (P), SalI (S), XbaI (X), XhoI (X). P* denotes the PvuII site referred to in the text.

30th and 31st amino acids (Figure 2). It is likely, therefore, that Kre2p enters the secretory pathway. Three sites for N-linked glycosylation were found within the sequence.

The protein sequence of KRE2 was compared to known sequences in the GenBank, EMBL, and NBRF protein databases, but no homology was found. Considerable identity was, however, detected between Kre2p and two predicted yeast proteins. One is from an open reading frame (KTRI), found in the 5' region upstream from the NUP1 gene (DAVIES and FINK 1990). The second is from an open reading frame.
HpaI–XbaI fragment construct in which a 1.2-kb EcoRI fragment subclone was deleted and replaced by the
(a) Southern analysis with a labelled 2.5-kb HpaI–XbaI fragment from pKre2. (b) Spore progeny from a diploid heterozygous for the
KRE2 locus, are resistant to killer toxin, and are Leu+.
Spores B1, B4, C1 and C4 contain the kre2Δ1 disruptant with the kre2::LEU2 construct, are partially resistant to killer toxin, and are Leu+.
Spores B1, B4, C1 and C4 contain the kre2::LEU2 construct, are partially resistant to killer toxin, and are Leu+.
Spores B1, B4, C1 and C4 contain the kre2::LEU2 construct, are partially resistant to killer toxin, and are Leu+.
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bp 5' to KRE2. Southern analysis of genomic DNA from strain TA405 indicated that a restriction fragment encompassing both PHO8 and KRE2 hybridized to probes containing the KRE2 or PHO8 genes. In a genetic test of the physical association, linkage between the pho8-l and kre2-1 alleles was examined. These alleles segregated in meiotic tetrads as follows: [PD = 34, TT = 14, NPD = 1]. This corresponds to a genetic distance of approximately 20 cm, which is higher than that expected from the physical distance (<1 cm). Possibilities for this discrepancy include polymorphisms between the two strains used in the cross, the fact that both genes are members of gene families, or the location of the fragment near the right telomere of chromosome 4, but have not been explored. The physical mapping and sequencing data leave little doubt that in strain TA405, the KRE2 and PHO8 genes are situated as shown in Figure 1.

Northern analysis of pKre2: The insertion of the reporter LEU2 gene 3' to the KRE2 open reading frame resulted in a partial phenotype and suggested the possibility that the KRE2 transcript was larger than the approximately 1.3 kb expected based on the size of the open reading frame. A longer transcript would also explain the noncomplementation of the kre2-1 allele by a subclone that encompassed the entire open reading frame of KRE2. To examine the length and number of transcripts encoded on the 2.5-kb HpaI-XbaI complementing fragment, a Northern blot was performed. Total RNA was isolated from a Kre+ strain (A41-1A). RNA was probed with a 1.8-kb HindIII-XbaI fragment of pKre2 as described in MATERIALS AND METHODS. Only one transcript of approximately 2 kb was detected, and was correspondingly missing in a disrupted strain (data not shown). This indicated that the transcript for the KRE2 gene is some 630 bp larger than the open reading frame and likely extends beyond the stop codon, although its exact position remains to be determined.

Structural analysis of (1→6)-β-D-glucan from kre2Δ1 cells: To determine if an altered (1→6)-β-D-glucan was the basis of toxin resistance in kre2Δ1 cells, a structural analysis of purified (1→6)-β-D-glucan was performed by 13C NMR as described by Boone et al. (1990). (1→6)-β-D-Glucan was isolated from strain 7B/6, which had been disrupted at the KRE2 locus with the kre2Δ1 construct, and was compared to glucan isolated from the wild-type parent strain, 7B (Boone et al. 1990). The proton-decoupled spectrum obtained from 7B/6 was consistent with a branched (1→6)-β-D-glucan structure and was indistinguishable from the spectrum of the wild-type 7B (1→6)-β-glucan. The amount of glucan purified from each strain was also similar and these results indicated that alteration of the (1→6)-β-D-glucan was not the cause of toxin resistance in kre2Δ1 cells. (1→6)-β-D-Glucan purified from strain 7B/6 was examined for its ability to competitively inhibit toxin-mediated cell death as described by Hutchins and Bussey (1988). 7B/6 glucan was found to be as effective as wild-type glucan to competitively bind killer toxin (F. McCaw, unpublished results). Earlier work showed that kre2-1 cells bind less K1 killer toxin than wild-type cells, due to a reduced affinity for the toxin (Al-Aidroos and Bussey 1978). Whole cells that had been disrupted at the KRE2 locus with the kre2Δ1 construct were examined for their ability to bind killer toxin. A reduced affinity for toxin binding was found, with results similar to those obtained for the kre2-1 allele. Taken with our finding that kre2Δ1 cells were sensitive to killer toxin as spheroplasts, the resistance seen in whole kre2Δ1 cells is likely due to an altered cell surface that was a less efficient receptor for the toxin molecule. However, the structure of the (1→6)-β-glucan did not seem to be the basis of this receptor defect.

Cell wall mannoprotein analysis: The other major cell surface component, mannoprotein, was examined for alteration in kre2Δ1 cells. Cells from spore clones from six tetrads derived from the disruption heterozygote were examined for their ability to bind the dye Alcian blue, a dye thought to bind to a phosphate moiety of mannoprotein (Friis and Ottolenghi 1970). The kre2Δ1 cells bound less Alcian blue than wild type, and appeared pale blue as compared to cells of the darkly blue staining wild-type spore clones (data not shown). However, comparable ratios of phosphate to mannoprotein were found in both wild-type and disruptant segregants (20 mannose:1 phosphate). Wild-type proportions and configuration of phosphate in the kre2Δ1 mannoprotein raised the possibility that accessibility of Alcian blue to the phosphate may be altered in kre2Δ1 cells leading to reduced binding of the dye, or that the absolute amount of phosphate was reduced in kre2Δ1 cells (see below).

To examine the mannoprotein component of the cell wall, bulk mannoprotein was purified from kre2Δ1 disruptant spore clones by the method of Nakajima and Ballou (1974). Approximately a third less mannoprotein was recovered from the disruptants as compared to the wild type: average, 6.97 mg (+0.2 sd) mannoprotein/g wet weight for the wild type vs. 4.69 mg (+0.5) for disruptants in two tetrads examined. The structure of mannoprotein from progeny of a disruptant spore was examined by 1H NMR and compared to that from wild type. Both mannoprotein profiles appeared similar, and indicated that the structure of the outer chain was not grossly altered in kre2Δ1 cells.

Altered N-linked glycosylation in kre2Δ1 cells: To look more closely at a possible glycosylation defect in kre2Δ1 cells, we examined [35S]methionine-labeled acid phosphatase and invertase that had been immu-
noprecipitated from segregants from a disruption heterozygote. SDS-PAGE analysis indicated that these proteins, when produced in kre2ΔI cells, displayed an apparent molecular mass smaller than the wild-type proteins. Acid phosphatase had an apparent molecular mass of 150 kD in the disruptant compared to 180 kD in the wild type (Figure 4A). Invertase was reduced to 120 kD in the mutant as compared to 130 kD in wild-type cells (data not shown). The alteration was specific to N-linked glycosylation, as treatment with Endo-H reduced the proteins from both wild-type and disruptant strains to the same apparent molecular mass (Figure 4B).

To directly explore the altered pattern of N-glycosylation in kre2 mutants we analyzed the N-glycan chains. Following labeling with [3H]mannose, and cleavage from protein using Endo-H, the N-glycan chains were fractionated by gel filtration (see MATERIALS AND METHODS). The glycoprotein core structures retained by a Bio-Gel P-6 column were analyzed by HPLC. Both the mutant and wild-type cores contained 8 mannose residues, indicating that core synthesis per se was not altered in the kre2 null. The larger N-linked chains eluting in the void volume of the Bio-Gel P-6 column were fractionated by gel filtration on a Bio-Gel P-60 column, see Figure 5. The average size of the glycan chains is considerably reduced in the kre2 mutant. The bulk of these chains is assembled by a series of Golgi reactions (FRANZUSOFF and SHERMAN 1989), and thus it seems that the glycosylation defect affects the size of these Golgi-assembled outerchains.

To further examine the effect of kre2 on N-glycans, we made use of mnn mutants that have an altered outer chain mannoprotein content. These were tested for sensitivity to killer toxin and for their ability to complement the kre2-1 mutation. All of those examined, mnn1, mnn2, mnn6 and mnn9, were completely sensitive to toxin and complemented kre2-1. Cells carrying the kre2ΔI disruption were also tested for their ability to agglutinate with anti α(1→3)-mannose antibodies. Both wild-type and disruptant cells were agglutinated with the antibody, indicating the presence of outer chain α(1→3)-mannose linkages in mannoproteins from kre2ΔI cells. In addition, kre2ΔI and wild-type cells were tested for their sensitivity to KT28 toxin, which employs cell wall outer chain mannoprotein as a receptor (SCHMITT and RADLER 1988). mnn2 and mnn5 mutants have been found to be resistant to KT28 toxin, indicating that the outer α(1→3)-linked mannose residues of mannoprotein are required for toxin binding. Both wild-type and kre2ΔI cells were fully sensitive to KT28 toxin (M. SCHMITT and F. RADLER, unpublished observation).

To determine if the KRE2 product interacted with other genes known to perturb the mannoprotein bio-

**FIGURE 4.**—Immunoprecipitation of acid phosphatase from kre2 mutant and wild type cells. A. Acid phosphatase was immunoprecipitated from cells as described in MATERIALS AND METHODS. Lane 1 is an immunoprecipitate from wild type, and lane 2 from a kre2ΔI mutant. B. Immunoprecipitated acid phosphatase as for (A) above was digested with Endo-H prior to SDS-PAGE. Lane 1, wild type; lane 2, kre2ΔI mutant.

**FIGURE 5.**—Bio-Gel P-60 (100–200 mesh) chromatography of Endo-H-sensitive, [3H]mannose-labeled oligosaccharides excluded from Bio-Gel P-6. The Bio-Gel P-60 column (1.0 X 114 cm) was equilibrated and eluted (flow rate 7 ml/hr) with 0.1 M pyridine acetate buffer (pH 5.0). Fractions of 0.7 ml were collected and an aliquot was assayed for radioactivity. Oligosaccharides extracted from the wild type A41–13D (upper panel), and kre2ΔI strain A41–13A (lower panel), were analyzed. The arrows indicate the positions of the void volume (V0) determined with bovine serum albumen, and mannose (Man).
altering glycosylation could affect function of a glucan resistant to toxin, indicating that tants. In each case, the haploid double mutant was receptor that requires the KREl product for its syn-

edge; both diploids gave killer resistance comparable to that seen prior to or independent of mnn9, the most severe outer chain mutant examined. Interaction between kre2 and kre1: A possible interaction between these two genes was investigated to extend the earlier observation that kre2Δ1 and kre1–1 mutants did not fully complement (K. AL-AIDROOS 1975). Such work might explain how kre2 mutations altering glycosylation could affect function of a glucan receptor that requires the KRE1 product for its synthesis (BOONE et al. 1990). Isogenic, doubly heterozygous strains were made harboring the kre2Δ1 and kre1Δ1 null mutations, and examined for killer toxin sensitivity (see Table 2). The double heterozygote was found to be more resistant than either of the single heterozygotes alone, with a smaller zone size and a less sharp zone edge. Based on this failure to fully complement, there is a genetic interaction between the KRE1 and KRE2 genes, possibly at the level of the polysaccharides made when the gene products are wild type.

KTR1: We found a fragment of an open reading frame with extensive homology to Kre2p in the published sequence 5′ to the yeast NUP1 gene on chromosome 15 (DAVIS and FINK 1990). A clone was obtained (gift from L. DAVIS) that contained the NUP1 gene and a region of 5′ DNA that contained the homologous open reading frame, called KTR1 (for Kre Two Related). The complete sequence of KTR1 was determined and identified an open reading frame of 398 amino acid residues with a predicted molecular mass of 46,070 (see Figure 2). The predicted protein has no N-terminal signal sequence, but from residues 17–34 there is a hydrophilic potential membrane-spanning domain, preceded by a pair of charged Lys residues, suggesting that this domain spans a membrane and defines a type 2 orientation, with the C-terminal portion of the protein beyond residue 34 in the lumen of a secretory compartment (PARKS and LAMB 1991). The homology extends through the central region of both proteins; from residue 79–337 of Ktrlp (see Figure 2) the proteins share 54% identity. Disruption of KTR1: To examine the possible function of KTR1, a disruption of the gene was made by the one-step method of ROThSTEIN (1983) (see MATERIALS AND METHODS). Diploids of TA405 containing a heterozygous disruption were sporulated and dissected; in 8 tetrads, spores containing a disruption at the KTR1 locus grew normally, and had no obvious phenotype. Disruptants were tested for a range of phenotypes associated with the kre2 null mutant, including killer resistance, Alcian blue staining, temperature sensitivity, and agglutination with antisera to (1→3)-α and (1→6)-α mannosyl linked polymers. In all of these tests the His+ ktr1 disruptants behaved as their isogenic wild types. Strains with a null mutation of KRE2 grow slowly and are temperature sensitive. If KTR1 provides a partially redundant function for Kre2p, then a double kre2 ktr1 disruptant strain should show a more extreme phenotype than the kre2 null alone. Haploid spore progeny harbouring kre2 ktr1 double disruption mutations were made. These mutants were identical in phenotype on YEPD plates to the kre2 null mutant strains.

YUR1: The YUR1 gene was identified as an open reading frame between TIF2 and RPB4 on yeast chromosome 10 (FOREMAN, DAVIS and SACHS 1991). In overall structure its predicted product strongly resembles Kre2p. Yur1p is a predicted protein of 420 amino acid residues with a molecular mass of 49,852 (see Figure 2). The protein has only one hydrophobic domain, which forms an amino terminal signal sequence, and thus Yur1p probably enters the yeast secretory pathway; it contains five possible sites for N-glycosylation. From residue 98–362 Yur1p has 42% identity with Kre2p. Yur1p has sequence similarity with Ktr1p also, with 47% identity from residue 89–396. The three proteins are compared in Figure 2; all share 96% identity over more than 300 amino acid residues.

### TABLE 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Average zone size (mm)</th>
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<tbody>
<tr>
<td>TA405</td>
<td>a/α leu2/leu2 his3/his3 can1/can1</td>
<td>17.7 ± 0.2</td>
</tr>
<tr>
<td>A41</td>
<td>a/α KRE2/kre2Δ1::HIS3 leu2/leu2 his3/his3 can1/can1</td>
<td>17.8 ± 0.3</td>
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<tr>
<td>HAB 505</td>
<td>a/α KRE1/kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1</td>
<td>18.3 ± 0.4</td>
</tr>
<tr>
<td>HAB502A</td>
<td>a/α KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1</td>
<td>16.3 ± 0.3</td>
</tr>
<tr>
<td>HAB 502B</td>
<td>a/α KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1</td>
<td>16.3 ± 0.3</td>
</tr>
<tr>
<td>HAB502C</td>
<td>a/α KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1</td>
<td>16.3 ± 0.3</td>
</tr>
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</table>

The sensitivity to K1 killer toxin of strain which are disrupted at the KRE2 and KRE1 loci are compared with strains which are disrupted for each of the single loci alone. Error represents one standard deviation. An isogenic homozygous kre1Δ1::HIS3 diploid HAB680-3 gave no zone in this test, and an isogenic homozygous kre2Δ1::HIS3 diploid HAB660-2 gave a zone of 7 mm with a fuzzy edge; both diploids gave killer resistance comparable to that seen in isogenic haploid strains.

synthetic pathway, a possible genetic relationship between mnnn mutants and kre2Δ1 was examined. Double mutants were made between krecΔ1 and each of mnn1, mnn2, mnn6 and mnn9 (see METHODS AND MATERIALS), and tested for K1 killer toxin sensitivity to indicate any epistatic relationship between the mutants. In each case, the haploid double mutant was resistant to toxin, indicating that krec2Δ1 was acting prior to or independent of mnn9, the most severe outer chain mutant examined.

### Notes

1. Although the KTR1 gene product was identified as an open reading frame between TIF2 and RPB4 on yeast chromosome 10, it was later determined that YUR1 is the homologous yeast gene (FOREMAN, DAVIS and SACHS 1991). The predicted protein of 420 amino acids has a molecular mass of 49,852. It contains five potential N-glycosylation sites and has sequence similarity with Kre2p, particularly in the N-terminal region. The YUR1 gene is located on chromosome 10 and shares 96% identity with Kre2p over most of its sequence.

2. The KTR1 gene was initially identified as a disrupted gene in a yeast strain, and its predicted protein was found to have significant homology with the Kre2p protein. The KTR1 gene product has a molecular mass of 49,852 and contains five potential N-glycosylation sites. It has sequence similarity with Yur1p, with 47% identity from residue 89–396.

3. The sensitivities of various strains to the K1 killer toxin are summarized in Table 2. The sensitivity of a strain with a disruption in both KRE2 and KRE1 genes is compared with the sensitivity of single mutant strains. The error represents one standard deviation.

4. Interaction between KRE2 and KRE1: A possible interaction between these two genes was investigated to extend the earlier observation that kre2Δ1 and kre1–1 mutants did not fully complement (K. AL-AIDROOS 1975). The work explained how kre2 mutations altering glycosylation could affect function of a glucan receptor that requires the KRE1 product for its synthesis. Isogenic, doubly heterozygous strains were made harboring the kre2Δ1 and kre1Δ1 null mutations, and examined for killer toxin sensitivity. The double heterozygote was found to be more resistant than either of the single heterozygotes alone, with a smaller zone size and a less sharp zone edge. There was a genetic interaction between the KRE1 and KRE2 genes, possibly at the level of the polysaccharides made when the gene products are wild type.

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6. YUR1: The YUR1 gene was identified as an open reading frame between TIF2 and RPB4 on yeast chromosome 10 (FOREMAN, DAVIS and SACHS 1991). In overall structure its predicted product strongly resembles Kre2p. The predicted protein of 420 amino acids has a molecular mass of 49,852 and contains five potential N-glycosylation sites. It has sequence similarity with Kre2p, with 47% identity from residue 89–396. The three proteins are compared in Figure 2; all share 96% identity over more than 300 amino acid residues.
DISCUSSION

Taking a genetic approach to yeast cell surface biology, we have isolated the KRE2 gene through functional complementation of a killer-resistant allele. The sequence of KRE2 has led to the discovery of a new gene family encoding putative secretory proteins. While our genetic, physiological and biochemical work has demonstrated that defects in Kre2p perturb the synthesis of the N-glycan moiety of glycoproteins, we remain ignorant of the primary function of this gene and of its homologs, KTR1 and YUR1. A brief discussion of our findings on KRE2 function and the gene family follow.

Mutants with a kre2 mutation are killer toxin resistant, with reduced toxin binding to the cell wall receptor. A component of this receptor is a β-glucan, but the structure and amount of this polymer appear normal in a kre2 null mutant. Because β-glucan is known to cross-link with mannoprotein in the yeast cell wall, we examined mannoprotein in kre2 mutants. We found the mutants stained poorly with the mannoprotein-phosphate dye, Alcian blue. Although the phosphate/mannose ratio was normal, the absolute amount of the polymer was reduced in a kre2 null mutant. Examination of the Golgi-assembled, N-glycan-linked chains from glycoproteins indicated that these were of reduced size in the kre2 mutant. However, structural studies on the N-linked glycan chains and epistatic analysis with mnn mutants suggested that kre2 was not acting directly at the level of N-glycan synthesis and that the observed defect was probably an indirect consequence of the kre2 mutation. There are several precedents for genes with indirect effects on protein glycosylation; mutants in both the PMR1 gene coding for a calcium-dependent ATPase and the ERD1 gene involved in endoplasmic reticulum, (ER) sorting show a glycosylation defect in outer N-glycan chain extension (Rudolph et al. 1989; Hardwick et al. 1990).

Mutations in the PMR1 or ERD1 genes cause a glycosylation phenotype like mnn9 mutants; with N-linked chains of only 10–14 mannose residues. Recently it has been shown that mnn9 mutations themselves probably lead indirectly to glycosylation defects, as mutants are vanadate resistant and may be defective in protein sorting (Balou et al. 1991). A series of other mnn or vanadate resistant mutants, vrg, in four complementation groups, also perturb N-linked outer chain glycosylation (Balou et al. 1991). All but the mnn10, vrg2 group show more severe outerchain glycosylation defects than kre2A1, but it remains a possibility that KRE2 may be one of these genes. The sequence of KRE2 indicates that Kre2p is predicted to be a glycoprotein that enters the secretory pathway. Apart from a signal sequence, the protein contains no known targeting signals and its cellular location is unknown. The Kre2p protein does have a high propensity for β-turns, and may associate with a membrane through β-coils in a way analogous to outer membrane proteins in Gram-negative bacteria (Jahnig 1990).

The slow growth of the kre2 null mutant at 30° and the temperature-sensitive growth phenotype of this mutant at 37° point to an important function for this gene. The phenotype could be explained on the basis that one or many glycoproteins are required to be correctly glycosylated at all temperatures, but that correct glycosylation is essential for growth at 37°. Alternatively some essential glycoprotein is made at 37° that requires a normal pattern of glycosylation. However, as discussed above, protein glycosylation can be perturbed by mutation without leading to lethality at high temperature. Thus the growth and temperature sensitive phenotypes could be caused by the defect in the unknown primary function of Kre2p, and be independent of glycosylation. The cell wall receptor for killer toxin is defective in kre2 mutants, and this leads to resistance. The genetic interaction of kre2 mutants with krel mutants involved in assembling the (1→6)-β-D-glucan component of the receptor suggests an interaction between glucan and N-linked glycoproteins at the cell surface as has been suggested by others (Pastor et al. 1984). Possibly the reduced level of glycosylation seen in kre2 mutant cells leads to a reduced number of mannoprotein attachments to glucan, and this alters the structure or assembly of the toxin receptor so that it is defective.

The KRE2 gene family: The family of the unlinked KRE2 homologs, KTR1 and YUR1 provides an intriguing aspect of this study. Both Ktr1p and Yur1p appear, like Kre2p, to be associated with the yeast secretory pathway. Yur1p and Kre2p have classical signal sequences for ER entry, whereas Ktr1p appears to be a type 2 membrane protein. Consequently, the conserved domain of striking identity among the three proteins is predicted in all cases to be within the lumen of a secretory compartment. This localization, plus the conserved nature of the luminal domains, hints at a similar function for these proteins. However, despite considerable biochemical and genetic knowledge of prokaryotic and eukaryotic secretory systems, they represent a wholly new group. In terms of function, the fact that kre2 mutants have a phenotype implies that there is not complete functional redundancy among the group. Consistent with this, the ktr1 null mutant is not killer resistant, has no obvious growth defect, and does not appear to interact with Kre2p, as the krel/ktr1 double null mutant is no more extreme than the kre2 null mutant alone. We have no information on the role of the YUR1 gene. Ultimately, further studies on the products of these genes may
extend this genetic work to determine their functions in the secretory system.

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