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 lumenal 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 (BALLOU 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 *kre1*, *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-

TABLE 1

Yeast strains used

Strains	Genotype	Source
A41	**a**/α *KRE2/kre2Δ1::HIS3 leu2/leu2 his3/his3 can1/can1*	This work
A41-13A	*Matα kre2Δ1::HIS3 leu2 can1*	This work
A41-13B	*Mata kre2Δ1::HIS3 leu2 can1*	This work
A41-13C	*Mata leu2 his3 can1*	This work
A41-13D	*Matα leu2 his3 can1*	This work
A41-1A	*leu2 his3 can1*	This work
A41-1B	*kre2Δ1::HIS3 leu2 can1*	This work
A41-1C	*leu2 his3 can1*	This work
A41-1D	*kre2Δ1::HIS3 leu2 can1*	This work
A71	**a**/α *KRE2/kre2::LEU2 leu2/leu2 his3/his3 can1/can1*	This work
AL32-3D	*MATa pho3-1 pho8-2 leu1 ELF-52*	Y. OSHIMA
AH216	*MATa pho3 pho5 leu2 his3*	
LB1-3B	*MATa mnn2-1*	C. BALLOU
LB1425-1B	*MATa mnn6-1*	C. BALLOU
LB2134-3B	*MATa mnn9*	C. BALLOU
KP35-5A	*Mata pho3 pho5 kre2-1 his3 ade2*	This work
KP35-10B	*MATa pho3 pho5 kre2-1 leu2*	This work
KP35-12C	*MATa pho3 pho5 kre2-1 leu2 his3*	This work
KP35-2D	*MATa pho3 pho5 kre2-1 leu2 ade2*	This work
KP35-7B	*MATa pho3 pho5 kre2-1 ade2*	This work
HAB660-2	Diploid from A41-13A × A41-13B	This work
HAB680-3	**a**/α *kre1Δ1::HIS3/kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1*	This work
H3A	*ade2 ura3 kre2-1*	H. BUSSEY
7B	*MATα ura3 glc1 his3*	BOONE *et al.* (1990)
7B/6	*MATα ura3 glc1 kre2Δ1::HIS3*	This work
S442	*MATa lys2 can1 cyh2 mkt1* [HOK] [NEX]	S. SOMMER
S14a	*MATa ade2*	H. BUSSEY
S14.14	*MATa ade2 kre2-1*	H. BUSSEY
SEY6210	*MATα leu2-3, 112 ura3-52 his3Δ200 trp1Δ901 lys2-801 suc2Δ9*	S. EMR
T158C/S14a	**a**/α *his4c-864/HIS4 ade2-5/ADE2* [*KIL-K1*]	BUSSEY *et al.* (1979)
TA405	**a**/α *his3/his3 leu2/leu2 can1/can1*	WHITEWAY and SZOSTAK (1985)

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, DIGNARD 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 [α-^{35}S]dATP (Amersham 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 (*kre2Δ1*) contains a disruption at the *KRE2* locus in which a 1.8-kb *Eco*RI fragment of pKre2 in PBSK$^+$ was replaced by a 1.8-kb fragment carrying the *HIS3* gene. The plasmid construct was digested with *Hpa*I and *Xba*I before transformation into TA405 by the one-step disruption technique of ROTHSTEIN (1983). Strain A71 (*kre2::LEU2*) contains a disruption at the *KRE2* locus in which a 2.1-kb fragment carrying the *LEU2* gene was inserted into a *Pvu*II site (*) in Figure 1 of pKre2 in PBSK$^+$. The plasmid construct was digested with *Xba*I and *Hin*dIII before transformation as described above. Disruption of *KTR1* was effected by insertion of a 1.8-kb *HIS3* containing fragment into the *Eco*RI site 719 bp into the *KTR1* open reading frame. Strain TA405 was disrupted in this way, and the insertion checked by genomic Southern hybridization.

Southern analysis: The structure of each disruption was confirmed by Southern analysis (SOUTHERN 1975). Total DNA was extracted from strains A41, and A71, digested with either *Hin*dIII (A41) or *Eco*RI (A71), separated on agarose gels and transferred to nitrocellulose. The probe in all cases was a 2.5-kb *Hpa*I-*Xba*I fragment of pKre2 that had been labeled using the nonradioactive labeling and detection kit from Boehringer Mannheim Biochemicals (Indianapolis, Indiana).

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 *kre2Δ1* plasmid construct. Glucan was extracted

from this strain as described by BOONE *et al.* (1990). Purified (1→6)-β-glucan (70 mg) was purified from 3 liters of stationary phase cells and was resuspended in 3 ml D_2O before examination by ^{13}C and ^{31}P NMR. Spectra were obtained under the conditions described by BOONE *et al.* (1990). For quantitation, glucan was measured as hexose by the borosulfuric method of BADIN, JACKSON and SCHUBERT (1953).

Preparation and analysis of purified cell wall mannoprotein: 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 (hexadecyltrimethyl-ammonium 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 a gift from A. FRANZUSOFF.

Identification of GlcNAcMan8 and outer chain oligosaccharides: *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-^{3}H]mannose (ICN, 28Ci/mmol). Cell pellets obtained by centrifugation were sequentially extracted with CH_3Cl/MeOH (2:1), H_2O and CH_3Cl/MeOH/H_2O (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 GlcNAcMan8 peak were pooled and chromatographed with ^{14}C-labeled standards on a column (25 cm × 0.46 cm) of 5-μ particle size Aminospherisorb (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 *Hpa*I-*Xba*I fragment of pKre2 was labeled by nick translation using [α-^{32}P]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 disruptant (A41–1B) as described by ELDER, LOH and DAVIS (1983). The RNA blots were hybridized with 500 ng of a 1.8-kb *Hin*dIII-*Xba*I fragment of pKre2 that had been labeled with [α-^{32}P-]dCTP (Amersham).

Cell labeling and immunoprecipitation: Labeling of yeast cells with ^{35}S-*trans*-label (70% [^{35}S]methionine, ICN), immunoprecipitation of proteins, digestion with Endo-H, SDS-polyacrylamide gel electrophoresic (PAGE) analysis, and protein visualization, were as described by COOPER and BUSSEY (1989). Immunoprecipitation was either with acid phosphatase antibodies or invertase antibodies (gift of Dr. David Williams). For immunoprecipitation of invertase, cells were grown to mid-log phase in minimal medium containing amino acid requirements and 2% sucrose. The cells were concentrated fourfold and grown for 30 min in 0.1% glucose prior to a 20-min pulse and 30-min chase. Cells were lysed and the SDS-solubilized extracts were "precleared" with 150 μl 20% protein-A Sepharose (Pharmacia). Affinity purified invertase antibodies were "preadsorbed" to solubilized extracts from SEY6210 (Δ*SUC2*) prior to immunoprecipitation. Extracts were then resolved on a 7% SDS-PAGE gel. For immunoprecipitation of acid phosphatase, cells were grown in low phosphate medium and then treated as above except without preclearing or preadsorbing treatments.

***mnn kre2Δ1::HIS3* strain construction:** Strain A41–1B (*kre2Δ1*) was crossed with either LB1–3B (*mnn2–1*), or LB1425–1B (*mnn6–1*), or LB2134–3B (*mnn9*) by single cell mating. Diploids were taken and sporulated and tetrads dissected. Spores were scored for the *kre2Δ1* disruption and mnn phenotypes; agglutination with (1→3)-mannose or (1→6)-mannose antibodies, or binding of Alcian blue.

RESULTS

Isolation of the *KRE2* gene: The wild-type *KRE2* gene locus was isolated by complementation of the *kre2–1* allele. Strain H3A (*kre2–1 ura3 ade2*) was transformed with a yeast DNA library prepared in the centromeric vector YCp50, which carries the *URA3* gene as a selectable marker (ROSE *et al.* 1987). Approximately 14,000 uracil prototrophs were screened for a killer-sensitive phenotype (Kre$^+$) as described by BOONE *et al.* (1990). Three transformants were found to have a plasmid-dependent, toxin-sensitive phenotype. One plasmid, designated pKre2, contained a yeast DNA insert of 5.1 kb and was a smaller subclone of the other two complementing plasmids, pMCG121 (9.6 kb), and pMCG128 (8.6 kb). pKre2 yeast DNA is shown in Figure 1. Insert DNA was found to also contain a truncated portion of the *PHO8* gene (KANEKO *et al.* 1987) and a small open reading frame (ORF1), of unknown function (KANEKO *et al.* 1987). 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 *Hpa*I-*Xba*I fragment.

Nucleotide and predicted amino acid sequence of *KRE2*: The sequence of the entire 2.5-kb *Hpa*I-*Xba*I complementing 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 *Hpa*I-*Pvu*II 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

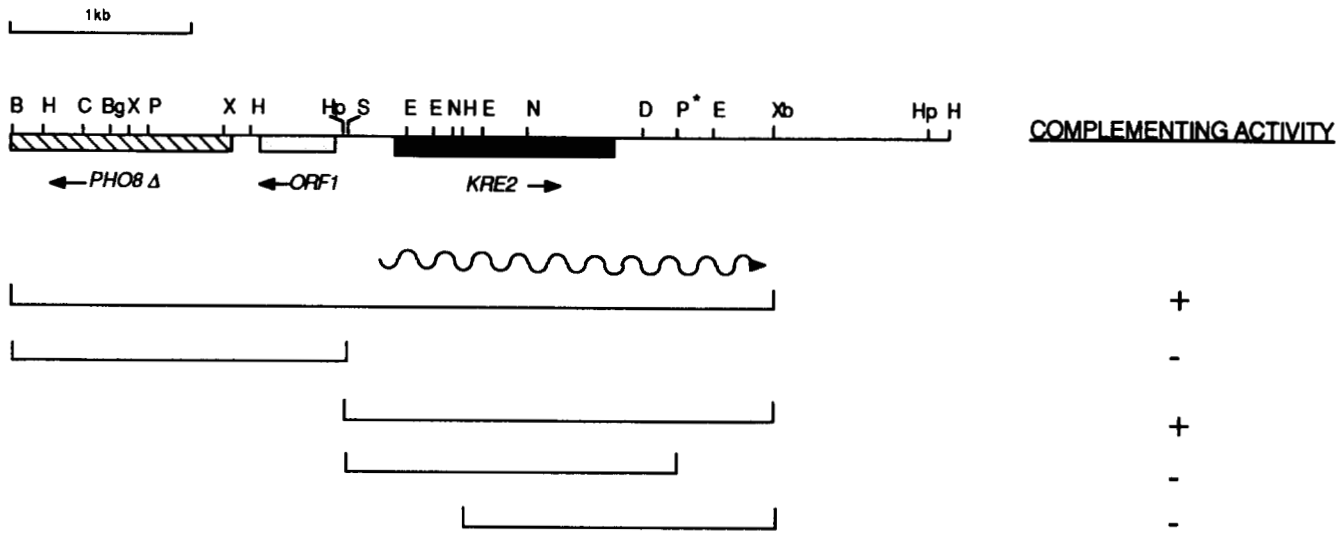

FIGURE 1.—Restriction map and subcloning of pKRE2. The yeast DNA found in pKre2 is schematically represented. The *KRE2* gene is shown as a black box, the truncated *PHO8* gene by a striped box, and the spotted box represents *ORF1* from KANEKO *et al.* (1987). Arrows indicate the direction of transcription 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: *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Dra*I (D), *Eco*RI (E), *Hin*dIII (H), *Hpa*I (Hp), *Nco*I (N), *Pvu*II (P), *Sac*I (S), *Xba*I (Xb), *Xho*I (X). P* denotes the *Pvu*II 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 (*KTR1*), found in the 5′ region upstream from the *NUP1* gene (DAVIES and FINK 1990). The second is from an open reading frame

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Kre2p   1 MALFLSKRLLRFTVIAGAVIVLLLTLNSNSRNSAIYSEFRSPAAFDFTSGSISPGQQVISEE  62
Ktr1p   1                                                   MAKIMIPAS   9
Yur1p   1                                          MAKGGSLYIVGIFLPIWTFMIYIFGKELF  29

Kre2p  63 NDAKKLEQSALNSEASEDSEAMDEESKALKAAAEKADAPDQTLKQPWITSLHLLLTKLVS 122
Ktr1p  10 KQPVYKKLGLLLVAVFTVYVFFHGARYARGSAPSPKYSTVLSSGSGYKYSKVELPKYTGP  69
Yur1p  30 LIRKYQKIDSTYTALSQRVKEQYDTSRRRNYFPKVKLSRNSYDDYTLNYTRQNDSDSFHL  89

Kre2p 123 QKLVTSLC-VRNKELKGLLSSIKYVENKINKKFPYPWVFLNDEPFTEEFKEAVTKAVSSE 181
Ktr1p  70 REKATFVTLVRNRDLYSLAESIKSVEDRFNSKFNYDWVFLNDEEFTDEFKNVTSALVSGT 129
Yur1p  90 RENATILMLVRNSELEGALDSMRSLEDRFNNKYHYDWTFLNDVPFDQQDFIEATTSMASGK 149

Kre2p 182 VKFGILPKEHWSYPEWINQTKAAEIRADAATK-YIYGGSESYRHMCRYQSGFFWRHELLE 250
Ktr1p 130 TKYGVIPKEHWSFPEWIDEEKAAQVRKEMGEKRIIYGDSISYRHMCRFESGFFYRHPLMD 189
Yur1p 150 TQYALIPPEDWNRPQWINDTLFEERLRVMEDEGVLYGGSKSYRNMCRFNSGFFFRQSILD 209

Kre2p 251 EYDGTGVWNQTSSYTVILITTF--FKWMQENEKVYGFTVSIHEYEVTIPTLWQTSMDFIK 308
Ktr1p 190 DYDW--YWRVEPDIKLHCDIDYDVFKFMKDNKKKYAFAISIKEYEATIPTLWETTRKFME 247
Yur1p 210 NYDYYFRVEPNVKYYCDFPYDP--FRVMRLKGKKYGFVISLYEYEETIPTLWDAVEEYLV 267

Kre2p 309 KNPEYLDRKQPDEFSFERYGKT----------YNLCHFWSNFEIANLNLWRSPAYRE 355
Ktr1p 248 AHPELIHENNMLDFVSDDQGLS----------YNLCHFWSNFEIAALDLWRSPAYSA 294
Yur1p 268 ASEETILRKEDSAYAFLTDSGLVGKHYPVVEANSDYNLCHFWSNFEIGDLNFFRSDEYKH 327

Kre2p 356 YFDTLDHQGGFFYEDRGDAPVHSIAAALFLPKDKIHYSSDIGYIIHLMITAHWTRRSITV 415
Ktr1p 295 YFDYLDREGGFFYERWGDAPVHSIGAALFLDRSEIHHFGDIGYYHVPFHSCPIDTSIRLA 354
Yur1p 328 FFETLDAKGGFYYERWGDAPVHSIGVSLLLRPDEIIHFDELGYFHSPFGTCPASYAVRLD 367

Kre2p 416 TTVNVTKVMISLSKVTLVVRNIMMLKGW STOP                              448
Ktr1p 355 NKCDCDPSKDFTWHSYSCTTKFYNINKLPKPAGWQNHMG STOP                   398
Yur1p 368 QRCRCKSDDESVIDITPHSCLMRWWKNGSGKYFLKEEQDEI STOP                 413
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FIGURE 2.—Sequence and comparison of the *KRE2, KTR1* and *YURI* encoded proteins. The proteins are shown in the single letter amino acid code, and are aligned to show identities, which are boxed. The protein names are shown to the left, adjacent to the amino acid residue numbers. Gaps introduced to improve alignment are shown by dashes. The EMBL accession numbers of *KRE2* and *KTR1* are, respectively, X62647 and X62941.

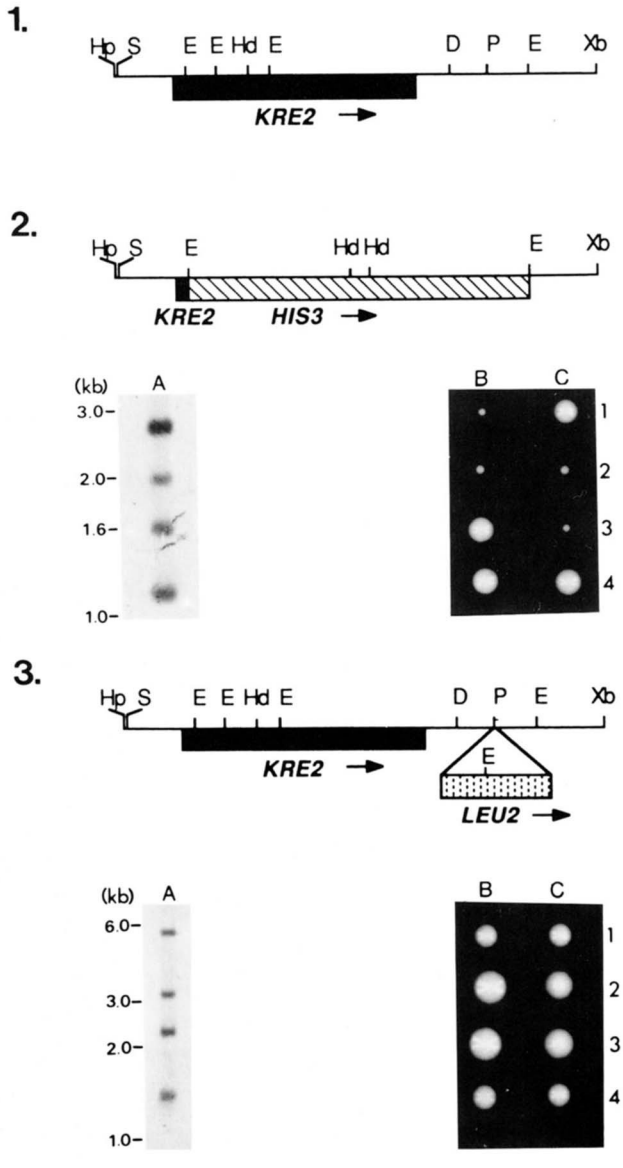

FIGURE 3.—Disruptions of the *KRE2* locus. 1, Structure of the *Hpa*I-*Xba*I fragment of pKre2 with the *KRE2* open reading frame indicated by a black box (see Figure 1). The direction of transcription of *KRE2* is indicated by an arrow. 2, Structure of the *kre2Δ1* construct in which a 1.2-kb *Eco*RI fragment of the *Hpa*I-*Xba*I subclone was deleted and replaced by the *HIS3* gene (striped box). (a) Southern analysis of total DNA isolated from a diploid heterozygous for the *kre2Δ1* disruption, digested with *Hin*dIII, and probed with a labelled 2.5-kb *Hpa*I-*Xba*I fragment from pKre2. (b) Spore progeny from a diploid heterozygous for the *kre2Δ1* construct. Tetrads were dissected and the spores allowed to germinate on YEPD at 30°. Spores B1 through 4 are the meiotic progeny from a single tetrad as are spores C1 through 4. Spores B3, B4, C1 and C4 are wild type for the *KRE2* locus, are sensitive to killer toxin and are His$^-$. Spores B1, B2, C2 and C3 contain the *kre2Δ1* construct at the *KRE2* locus, are resistant to killer toxin and are His$^+$. 3, Structure of the *kre2::LEU2* construct in which a *Hpa*I fragment containing the *LEU2* gene (dashed box) was inserted into the *Pvu*II site 3′ to the *KRE2* open reading frame. (a) Southern analysis of DNA isolated from a diploid made heterozygous at the *KRE2* locus with the *kre2::LEU2* construct, digested with *Eco*RI, and probed as above. (b) Spore progeny from a diploid made heterozygous at the *KRE2* locus with the above construct. Spores B1 through 4 are the meiotic progeny from a single tetrad as are spores C1 through 4. Spores B2, B3, C2 and C3 are wild type at the *KRE2* locus, are sensitive 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$^+$.

(*YUR1*) (FOREMAN, DAVIS and SACHS 1991). These similarities are shown in Figure 2, and discussed further in the relevant sections below.

Disruptions of *KRE2*: The constructs used to disrupt the *KRE2* locus are depicted in Figure 3. Histidine or leucine prototroph transformants of TA405 were sporulated and the tetrad progeny examined for growth at 23°, 30° and 37° as well as for resistance to K1 killer toxin. Analysis of several independent kre2Δ1 disruptions of TA405 showed a 2:2 segregation for growth and resistance to killer toxin at 23°, and 30° (25 out of 25 tetrads examined) (Figure 3, 2B and 2C). *kre2Δ1* null alleles (histidine prototrophs) showed a slow growth phenotype compared to wild-type spores from the same tetrad, and were resistant to killer toxin. These slow growing *kre2Δ1* spore progeny took on a pronounced yellowish color within a few days, compared to the creamy white of the isogenic wild type. At 37°, *kre2Δ1* spores germinated but failed to divide beyond an approximately 20-cell stage. The structure of the integrated *kre2Δ1* construct was confirmed by Southern analysis of genomic DNA isolated from heterozygous diploids (A41 and A51). Additional bands of 1.6 kb and 1.9 kb were observed due to two *Hin*dIII sites within the *HIS3* gene, as well as wild-type bands of 2.6 kb and 1.2 kb (Figure 3, 2A).

An insertional disruption was also constructed in TA405 in which the *LEU2* gene was inserted into the *Pvu*II site (* in Figure 1) 3′ to the *KRE2* gene. Upon sporulation and dissection, *kre2::LEU2* colonies showed a partial phenotype with respect to both resistance to killer toxin and growth (20 out of 20 tetrads examined) (Fig. 3, 3B and 3C). The structure of the integrated *kre2::LEU2* construct was also confirmed by Southern analysis of genomic DNA isolated from a disrupted diploid (A71). Additional bands of 2.3 kb and 1.4 kb were detected due to the disruption as well as those of the wild type locus (5.3, 3.3 and 1.4 kb) (Figure 3, 3A).

To test allelism of the *kre2Δ1* disruptant with the *kre2-1* mutation, *kre2-1 kre2Δ1* diploids were made by crossing S14.14 with either A41-1B or A41-1D. Diploids were sporulated, dissected and the tetrad progeny examined for resistance or sensitivity to killer toxin. All tetrad progeny examined (40 tetrads) were resistant to killer toxin, indicating close linkage and that the cloned gene was *KRE2*. The location of *KRE2* on the genomic map was determined. A 2.5-kb *Hpa*I-*Xba*I *KRE2* carrying fragment from pKre2 was found to hybridize to chromosome *4*. pKre2 was found by sequence analysis to contain a portion of *PHO8*, see Figure 1, with the open reading frame starting 910

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 *pho8–1* 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 *Hpa*I-*Xba*I 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 *Hin*dIII-*Xba*I 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-glucan was the basis of toxin resistance in *kre2Δ1* cells, a structural analysis of purified (1→6)-β-glucan was performed by ^{13}C NMR as described by BOONE *et al.* (1990). (1→6)-β-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-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-glucan was not the cause of toxin resistance in *kre2Δ1* cells. (1→6)-β-Glucan purified from strain 7B/6 was examined for its ability to competitively inhibit toxin-mediated cell death as described by HUTCHINS and BUSSEY (1983). 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 ^{1}H 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 *kr2Δ1* cells: To look more closely at a possible glycosylation defect in *kre2Δ1* cells, we examined [^{35}S]methionine-labeled acid phosphatase and invertase that had been immu-

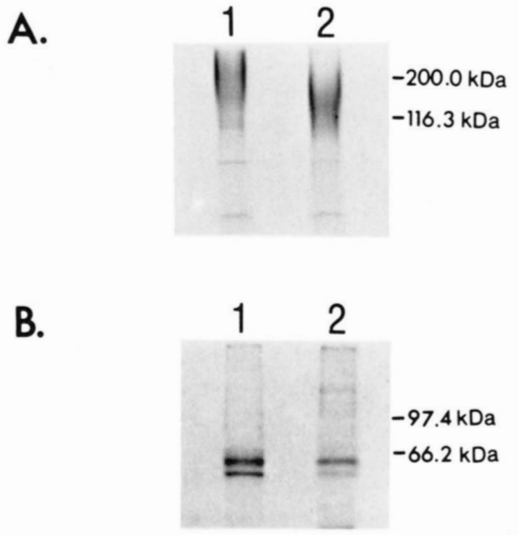

FIGURE 4.—Immunoprecipitation of acid phosphatase from *kre2* mutant and wild type cells. A, Acid phosphatase was immunoprecipitated from cells as described in MATERIALS AND METHODS. Lane 1 is an immunoprecipitate from wild type, and lane 2 from a *kre2Δ1* mutant. B, Immunoprecipitated acid phosphatase as for (A) above was digested with Endo-H prior to SDS-PAGE. Lane 1, wild type; lane 2, *kre2Δ1* mutant.

noprecipitated from segregants from a disruption heterozygote. SDS-PAGE analysis indicated that these proteins, when produced in *kre2Δ1* 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 [^{3}H]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 SCHEKMAN 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,

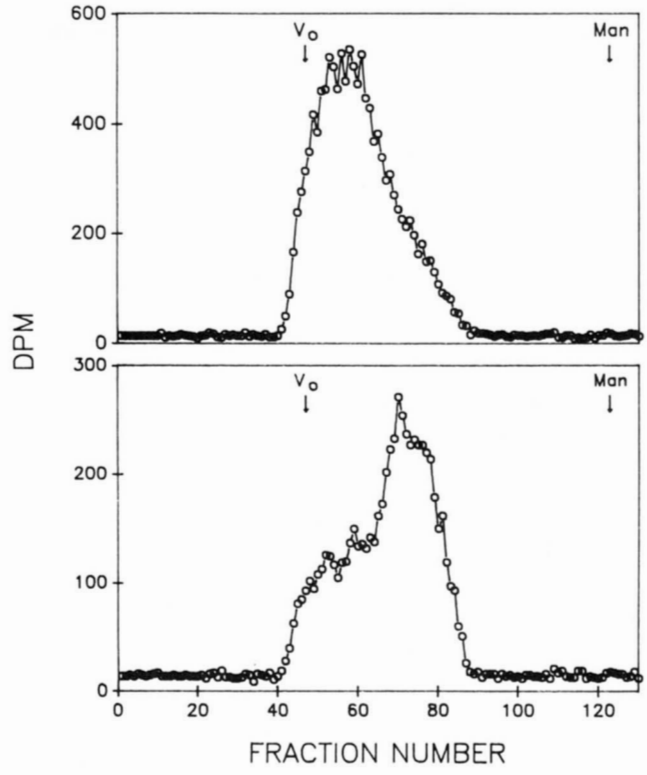

FIGURE 5.—Bio-Gel P-60 (100–200 mesh) chromatography of Endo-H-sensitive, [^{3}H]mannose-labeled oligosaccharides excluded from Bio-Gel P-6. The Bio-Gel P-60 column (1.0 × 114 cm) was equilibrated and eluted (flow rate 7 ml/hr) with 0.1 M pyridine acetate buffer (pH 5.0). Fractions of 0.7 ml were collected and an aliquot was assayed for radioactivity. Oligosaccharides extracted from the wild type A41–13D (upper panel), and *kre2Δ1* strain A41–13A (lower panel), were analyzed. The arrows indicate the positions of the void volume (V_0) determined with bovine serum albumen, and mannose (Man).

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 *kre2Δ1* 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 *kre2Δ1* cells. In addition, *kre2Δ1* 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 *kre2Δ1* 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-

TABLE 2

Interaction between *kre2* and *kre1* mutants on killer toxin sensitivity

Strain	Genotype	Average zone size (mm)
TA405	a/α *leu2/leu2 his3/his3 can1/can1*	17.7 ± 0.2
A41	a/α *KRE2/kre2Δ1::HIS3 leu2/ leu2 his3/his3 can1/can1*	17.8 ± 0.3
HAB 503	a/α *KRE1/kre1Δ1::HIS3 leu2/ leu2 his3/his3 can1/can1*	18.3 ± 0.4
HAB502A	a/α *KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1*	16.3 ± 0.3
HAB 502B	a/α *KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1*	16.3 ± 0.3
HAB502C	a/α *KRE2/kre2Δ1::HIS3 KRE1/ kre1Δ1::HIS3 leu2/leu2 his3/his3 can1/can1*	16.3 ± 0.3

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 *kre2Δ::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 *mnn* mutants and *kre2Δ1* was examined. Double mutants were made between *kre2Δ1* 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 *kre2Δ1* was acting 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 *kre2–1* 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 *K*re *T*wo *R*elated). 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 hydrophobic 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. Yurlp 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 Yurlp probably enters the yeast secretory pathway; it contains five possible sites for *N*-glycosylation. From residue 98–362 Yurlp has 42% identity with Kre2p. Yurlp has sequence similarity with Ktrlp also, with 47% identity from residue 89–396. The three proteins are compared in Figure 2; all share 36% 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 moeity 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 (BALLOU *et al.* 1991). A series of other *mnn* or vanadate resistant mutations, *vrg*, in four complementation groups, also perturb *N*-linked outer chain glycosylation (BALLOU *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 targetting 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 analagous 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 *kre1* 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 lumenal 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 *kre2/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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