Characterization of the Yeast (1→6)-β-Glucan Biosynthetic Components, Kre6p and Skn1p, and Genetic Interactions between the PKC1 Pathway and Extracellular Matrix Assembly

Terry Roemer, Gerhard Paravicini,* Mark A. Payton,* and Howard Bussey

Biology Department, McGill University, Montreal, Quebec, Canada, H3A 1B1; and *GLAXO Institute for Molecular Biology, Chemin des Aulx, Geneva, Switzerland

Abstract. A characterization of the S. cerevisiae KRE6 and SKN1 gene products extends previous genetic studies on their role in (1→6)-β-glucan biosynthesis (Roemer, T., and H. Bussey. 1991. 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. Proc. Natl. Acad. Sci. USA. 88:11295-11299; Roemer, T., S. Delaney, and H. Bussey. 1993. SKN1 and KRE6 define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. Mol. Cell. Biol. 13:4039-4048). KRE6 and SKN1 are predicted to encode homologous proteins that participate in assembly of the cell wall polymer (1→6)-β-glucan. Kre6p and Sknlp are predicted to encode phosphorylated integral-membrane glycoproteins, with Kre6p likely localized within a Golgi subcompartment. Deletion of both these genes is shown to result in a dramatic disorganization of cell wall ultrastructure. Consistent with their direct role in the assembly of this polymer, both Kre6p and Sknlp possess COOH-terminal domains with significant sequence similarity to two recently identified glucan-binding proteins.

Deletion of the yeast protein kinase C homolog, PKC1, leads to a lysis defect (Levin, D. E., and E. Bartlett-Heubusch. 1992. Mutants in the S. cerevisiae PKC1 gene display a cell cycle-specific osmotic stability defect. J. Cell Biol. 116:1221-1229). Kre6p when even mildly overproduced, can suppress this pkcl lysis defect. When mutated, several KRE pathway genes and members of the PKCl-mediated MAP kinase pathway have synthetic lethal interactions as double mutants. These suppression and synthetic lethal interactions, as well as reduced β-glucan and mannan levels in the pkcl null wall, support a role for the PKC1 pathway in cell wall assembly. PKC1 potentially participates in cell wall assembly by regulating the synthesis of cell wall components, including (1→6)-β-glucan.

Bud growth in S. cerevisiae requires regulated cell wall synthesis (Cabib et al., 1982). Regulation of cell cycle events subsequent to START includes a coordinated regulation of cell wall biosynthetic genes responsible for new cell wall synthesis (Shaw et al., 1991) and polarization of the cytoskeleton towards the site of cell wall growth (Adams and Pringle, 1984; Madden et al., 1992; Lew and Reed, 1993). Phosphorylation of biosynthetic components involved in cell wall synthesis represents a potential level of regulation that integrates the cell cycle with changes within the wall and ensuing morphological events.

PKC1 encodes a yeast homolog of the mammalian protein kinase C family (Levin et al., 1990). pkcl null cells possess a G2-specific terminal lysis phenotype believed to be a consequence of a fragile cell wall (Levin and Bartlett-Heubusch, 1992). PKC1-deleted cells rapidly release their contents into the medium, and as judged by electron microscopy, possess thin cell walls which burst at the bud tip (Errede and Levin, 1993). The lysis phenotype of pkcl can be partially prevented by the presence of osmotic-stabilizing agents in the medium (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). PKC1 has been proposed to regulate cell wall synthesis through a bifurcated pathway comprising a MAP kinase cascade on one branch, and a second less well understood branch (Lee and Levin, 1992; Errede and Levin, 1993; Lee et al., 1993). PKC1 is thought to act as an upstream regulator of this MAP kinase cascade, based on the isolation of dominant gain of function alleles of the MAP kinase kinase kinase, BCK1 (SLK1/SSP31), which suppress pkcl cell lysis (Lee and Levin, 1992; Irie et al., 1991; Costigan et al.,...
pressor of a bck1 null mutant (Lee et al., 1993) and independently isolated according to its cell lysis phenotype (Torres et al., 1991). MPK1 is suspected to phosphorylate a variety of substrates, including transcription factors, in a manner analogous to the FUS3/KSS1-dependent activation of STE12 (Errede and Levin, 1993). Additional components of the PKC1-mediated MAP kinase cascade suppress pckl cell lysis when overproduced; these include PP21 and PP2Z, a pair of serine/threonine phosphatases, and BCK2, a gene of unknown function (Lee et al., 1993). Despite the requirement for PKC1 in maintaining correct cell wall ultrastructure (Paravicini et al., 1992), its role in regulating cell wall synthesis remains unknown.

Many genes involved in the assembly of cell wall components such as chitin, mannan, and β-glucan have been identified (for reviews see Bulawa, 1993; Ballou, 1990; Klis, 1994). However, how genes involved in cell wall synthesis are regulated, is poorly understood. Among known cell wall–related genes are a number of K1 killer toxin resistant, or KRE genes, involved in the synthesis of the cell wall polymer (1→6)-β-glucan (Boone et al., 1990; Meaden et al., 1990; Bussey, 1991; Hill et al., 1992; Brown et al., 1993; Brown and Bussey, 1993). The cloned and characterized KRE genes, include two highly homologous genes, KRE6 and SKNI (Roemer and Bussey, 1991; Roemer et al., 1993). Disruption of KRE6 results in slow growth and killer resistance, presumably due to a 50% reduction in the wild-type level of cell wall (1→6)-β-glucan. Loss of SKNI has no effect on killer sensitivity, growth, or (1→6)-β-glucan levels. SKNI is, however, a functional homolog of KRE6 and can suppress the kre6 null phenotypes in a dosage-dependent manner. Deletion of both KRE6 and SKNI results in a severe growth defect which can be lethal in some strain backgrounds. The slow growth defect of viable kre6 skni null deletion strains can be partially alleviated by the accumulation of spontaneous extragenic suppressors. Such kre6 skni null suppressed strains possess little, if any, (1→6)-β-glucan. Because KRE6 and SKNI are required for the majority of this polymer, and since single disruptions of either gene lead to structurally wild-type (1→6)-β-glucan, KRE6 and SKNI have been proposed to function independently and to act early in the assembly of the polymer, possibly as glucan synthases.

The KRE6 and SKNI gene products, Kre6p and Sknlp, have been characterized to further examine their role in (1→6)-β-glucan assembly. Here, KRE6 and SKNI are shown to encode phosphorylated integral-membrane glycoproteins that are likely localized to the Golgi apparatus. The topology implied by the posttranslational modifications of Kre6p and Sknlp, offers the potential for both proteins to link cytoplasmic regulation with the secretory pathway-based assembly of the (1→6)-β-glucan polymer. The observed phosphorylation of both Kre6p and Sknlp prompted an examination for genetic interactions with suspected cell wall regulating kinases. KRE6-dependent suppression of the pckl lysis defect, as well as synthetic lethal interactions between several KRE genes and members of the PKC1-mediated MAP kinase pathway, support the contention that a role of the PKC1 pathway is to act on cell wall assembly.

### Materials and Methods

#### Yeast Strains, Media, and Methods

Yeast strains used in this study are listed in Table 1. Media for yeast growth and sporulation were as described in Guthrie and Fink (1991).YPD is yeast complex medium; YNB is a synthetic medium that was supplemented with appropriate nutrients. Low phosphate minimal medium was prepared according to Hagenauer-Flaps and Hinnen (1984). Yeast mating, sporulation, and tetrad analysis were performed as described in Guthrie and Fink (1991). Resistance and sensitivity to K1 killer toxin was scored by the seeded plate assay of Bussey et al. (1982) and Hutchins et al. (1983). Yeast transformations were by the lithium acetate method of Ito et al. (1983) using 100 μg of sheared, denatured carrier DNA (Schiestl and Gietz, 1989).

#### Cell Wall Analysis

Analysis of the composition of the pcklΔ cell wall proved difficult due to its cell lysis phenotype. Osmotic-stabilizing agents, although required for pcklΔ cell growth, distorted sample weights significantly and prevent polymer levels being normalized to dry weight of cells. pcklΔ cell lysis also precluded the option of washing away the osmotic supplement before sample weighing. Normalizing cell wall polymer levels to the wet weight of cells is also likely to be inaccurate with such lytic strains. To attempt to overcome these problems, cell walls from the various strains were isolated, as described by Van Rinsven et al. (1991), and all cell wall polymer measurements were normalized per mg cell wall dry weight. The pcklΔ strain, GPY1115, was extremely sensitive to glass bead agitation; lysing completely after a few brief vortex pulses. 500-ml cultures of GPY1115 harboring either pRS315 or pRS315-KRE6 plasmids were grown to mid log in YNB supplemented with 0.5 M KCl. Two 100-ml aliquots of each culture were processed to provide an accurate cell wall dry weight determination. Three 100-ml aliquots of each culture were processed to determine β-glucan levels. Alkali-insoluble (1→6)-β-glucan and (1→3)-β-glucan were isolated after NaOH extractions according to Boone et al. (1990). Alkali-soluble (1→3)-β-glucan levels were estimated from NaOH extraction supernatants by precipitating carbohydrates with two volumes of ethanol at −20°C. Carbohydrates were measured as hexose as described by Badin et al. (1953). Mannoprotein was isolated from 250-ml cultures of GPY1115 transformed with KRE6, PKC1, or control plasmids grown to mid log in 0.5 M KCl-supplemented YNB, according to Ballou (1990). Mannan levels were determined by measuring hexose, and normalized to the cell wall dry weight determined from an additional 100-ml of each culture.

#### Electron Microscopy

Tetrads from strains TR67 and TR160 were dissected to isolate fresh kre6Δ and sknlΔ haploid strains TR510 and TR511, respectively. TR512 is a congenic wild-type spore progeny from TR67. TR211 and TR213 are kre6Δ sknlΔ strains possessing independently derived extragenic mutations which partially suppress their slow growth phenotype. 10-ml cultures of TR211, TR213, TR510, TR511, and TR512, were grown in YPD to a cell density of 10^7/ml and processed as described by Boone et al. (1990) with the single modification that samples were embedded in Epon. Sections were viewed on a Philips EM410 electron microscope at an opening voltage of 80 kV. TR211 and TR213 cell wall ultrastructure phenotypes were indistinguishable.

#### Plasmids

Previously, YEp24-KRE6 was constructed as a 4.6-kb KRE6 BamHI–Sall fragment in the multicopy plasmid, YEp24 (Roemer and Bussey, 1991). This same KRE6 BamHI–Sall insert was subcloned into the centromeric plasmid, YPl351, and named pRS315-KRE6 (Roemer and Bussey, 1991). YEp3-3-KRE6 contains a 4.2-kb KRE6 BamHI/Dra I fragment inserted into the BamHI–PvuII sites of the 2 μm-based plasmid, YEp3. SKNI was subcloned as a 5.5-kb Sail–HindIII fragment into the 2 μm plasmid, YEp352, and named YEp352-SKN1 (Roemer et al., 1993). YEp3-3-PKC1 is a 4.3-kb PKC1 Sphi fragment in YEp3.
Table I. Yeast Strains Used in This Study

| Strain        | Genotype                        | Source               |
|---------------|---------------------------------|----------------------|
| SEY6210       | MAT α leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | S. D. Emr           |
| TR92          | MAT α kre6::HIS3 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Roemer and Bussey, 1991 |
| TR95          | MAT α kre6::HIS3 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Roemer and Bussey, 1991 |
| TR144         | MAT α kre6::Tr10 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Roemer and Bussey, 1991 |
| TR178         | MAT α skn1::LEU2 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Roemer et al., 1993 |
| HAB806        | MAT α kre1::URA3 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Brown et al., 1993  |
| TR520         | MAT α kre2::TRP1 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | M. Lussier          |
| TR67          | MATαMATα KRE6/kre6::HIS3 his3Δ32 sus2-Δ3 ade2-101 | Roemer and Bussey, 1991 |
| TR510         | kre6::HIS3 his3Δ32 leu2 can1   | This work            |
| TR512         | his3 leu2 can1                  | This work            |
| TR150         | MAT αMATα SKN1/skn1::LEU2 his3Δ32 leu2 can1 | This work            |
| TR511         | skn1::LEU2 his3Δ32 leu2 can1    | This work            |
| TR211         | kre6::SKN1/skn1::LEU2 his3Δ32 leu2 can1 | Roemer et al., 1993 |
| TR213         | kre6::SKN1/skn1::LEU2 his3Δ32 leu2 can1 | Roemer et al., 1993 |
| GPY1115       | MAT α pkc1::HIS3 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 succ2-Δ9 | Paravincini et al., 1992 |
| MHD93         | MATαslt2::URA3 ura3-3,112 his3Δ31 trpl | This work |
| 3233-1B       | MATα mkk1::LEU2 mkk2::HIS3 ura3Δ31 trpl | This work |
| TR500         | MAT αMATα KRE6/kre6::Tr10 PKC1/pkc1::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 ade2-101 | This work |
| TR501         | MAT αMATα SKN1/skn1::LEU2 PKC1/pkc1::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 ade2-101 | This work |
| TR502         | MAT αMATα KRE11/skn1::URA3 PKC1/pkc1::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 ade2-101 | This work |
| TR503         | MAT αMATα KRE2/kre2::TRP1 PKC1/pkc1::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 ade2-101 | This work |
| TR504         | MAT αMATα KRE2/kre2::TRP1 KRE6/kre6::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 ade2-101 | This work |
| GPY100        | MAT αMATα KRE6/kre6::HIS3 SLT2/slt2::URA3 ura3-3,12 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 | This work |
| GPY102        | MAT αMATα KRE6/kre6::URA3 MKK1/mkk2::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 | This work |
| GPY104        | MAT αMATα KRE11/skn1::URA3 MKK1/mkk2::HIS3 ura3-3,112 leu2-3,112 ura3-52 his3Δ200 lys2-801 trpl Δ901 trpl Δ901 succ2-Δ9 | This work |
| LB3003-2Aa    | MATα ura3Δ33 can1 mevs9         | T. Stevens          |

**Epitope Tagging**

Standard molecular manipulations were as described by Sambrook et al. (1989). Epitope tagging of Kρ6p and Sknlp was performed as described by Kolodziej and Young (1991). The following complementary oligonucleotides encoding the influenza virus hemagglutinin (HA)1 sequence in frame with SKN1 and KRE6 open reading frames were designed: SKN1 oligos 429 (TACCCATACGCTCAGACTTCTTCGC) and 430 (GAGCGGCTAGTCTCGAGGCTGTAGTGTTGTTAGTAT) and KRE6 oligos 598 (GATGTCGTCACATTAAACACATAGCCTAGGTCAGTCGATCC) and 599 (GATGTCGTCAGCTGAGGCTGTAGTGTTGTTAGTAT). Oligos 429 and 430 were annealed and subcloned into the corresponding PstI site of YEp24-SKI. Similarly, oligos 598 and 599 were annealed and ligated into the unique BglII site of YEp24-KRE6 and pRS115-KRE6. Subclones possessing the epitope insertion could be identified by restriction mapping a unique AatII site (shown in bold) present in the oligonucleotide sequence. A selection for positive subclones that possessed the oligonucleotide sequence in the correct orientation, was facilitated by introducing an in-frame stop codon (shown underlined) to truncate the protein when the oligonucleotides were ligated in the incorrect orientation. Thus, transforming AatII+ subclones into the kre6Δ strain, TR92, yielded two populations of transformants: one of fast growing killer toxin sensitive transformants that harbor the correctly tagged construct, and a second population of slow growing killer toxin resistant transformants possessing AatII+ subclones whose oligonucleotides were ligated in the incorrect orientation. Functional epitope tagged versions of these plasmids were named YEp352-SKI-HA, YEp24-KRE6-HA, and pRS115-KRE6-HA, respectively.

**Cell Labeling and Immunoprecipitations**

Cell labeling and immunoprecipitations were performed essentially as described by Cooper and Bussey (1989). 10-ml cultures of 107 cells were grown up in appropriate minimal medium for 35S- or 32P-labeling, harvested, concentrated fourfold, and grown an additional 30 min in fresh minimal medium. Cells were labeled with either 100 μCi of Trans 35S-label or carrier-free 32P-orthophosphate (ICN Biochemicals, Irvine CA) for 15 min with shaking. Tunicamycin-treated cultures (10 μg/ml) were preincubated with the drug 30 min before labeling. After labeling, cultures were pelleted by centrifugation, and washed with ice-cold breakage buffer (BB) (150 mM NaCl, 10 mM Na2 B407 (pH 8.0)). Cells were resuspended in 65 μl BB, supplemented with 1.5 μg/ml leupeptin, 3 μg/ml pepstatin A, 1 mM PMSF, and a 50-100-μl vol of acid-washed glass beads (0.45-0.5-mm pore diameter) was added. (A phosphatase inhibitor, 5 mM sodium molydate, was also added in 32P-labeling experiments.) Samples were left on ice for 10 min, and then vortexed strongly 15-20 times for 20 s intervals, with alternating short incubations on ice. Membrane proteins were solubilized with the addition of 10 μl 10% SDS, and incubated for 5 min at 95°C. Samples

1. Abbreviations used in this paper: BB, breakage buffer; HA, influenza virus hemagglutinin; Kre6p-HA, epitope-tagged version of Kre6p; P56, protein A Sepharose; Sknlp-HA, epitope-tagged version of Sknlp; WB, wash buffer.
were cooled to room temperature, centrifuged 1 min, and supernatants collected and diluted 10-fold in 1.0 ml reaction buffer (RB) (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mg/ml BSA, 1.5 µg/ml leupeptin, 3 µg/ml pepstatin A, 1 mM PMSE [including 5 mM sodium molybdate in 32P-labeling experiments]). Samples were centrifuged again for 1 min and supernatants transferred to fresh tubes. To diminish the nonspecific binding of radiolabeled proteins, 100 µl protein A-Sepharose (PAS) (Cl-4B, Pharmacia LKB Biotechnology, Piscataway, NJ) was added and samples incubated at 4°C for 1 h with rotation. Supernatants were collected and transferred to a fresh tube, to which a 1:150 dilution of the 12CA5 monoclonal antibody (Berkeley Ab Company, Berkeley, CA) was added. Samples were incubated with 12CA5 at 4°C with rotation for 2 h, and then immunoprecipitated after a 1-h incubation at 4°C with rotation, with the addition of 50 µl PAS. To remove radiolabeled RNA from 32P-labeling experiments, ~200 µl RNase A was added and incubated for an additional 30 min at 4°C. After immunoprecipitations, samples were subjected to a brief centrifugation to pellet PAS beads. Immunoprecipitates were washed four times in wash buffer (WB) (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton-X-100), and finally suspended in 50 µl Laemmli loading buffer, boiled 5 min, and analyzed by SDS-PAGE. Fluorography of 32P-labeled samples was enhanced by baling gels in a 1.0 M sodium carbonate solution before drying.

Labeling and immunoprecipitation of Kre6p-HA in the pckl null strain was carried out as described above, in low phosphate media supplemented with 0.5 M KCl. Kre6p-HA protein levels were determined by Western blot analysis.

Endo H Digestion

Immune complexes bound to PAS were washed three times with WB, followed by two additional washes in 1.0 ml of 100 mM sodium citrate (pH 5.5). Samples were then resuspended in 200 µl sodium citrate buffer containing the above protease inhibitors, and digested with 10 µl Endo H (or mock digested) overnight at 37°C. Samples were then washed twice with WB, proteins were transferred to fresh tubes, added to 1:150 dilution of the 12CA5 monoclonal antibody (Berkeley Ab Company, Berkeley, CA), added. Samples were incubated with 12CA5 at 4°C with rotation for 2 h, and then immunoprecipitated after a 1-h incubation at 4°C with rotation, with the addition of 50 µl PAS. (To remove radiolabeled RNA from 32P-labeling experiments, ~200 µl RNase A was added and incubated for an additional 30 min at 4°C). After immunoprecipitations, samples were subjected to a brief centrifugation to pellet PAS beads. Immunoprecipitates were washed four times in wash buffer (WB) (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton-X-100), and finally suspended in 50 µl Laemmli loading buffer, boiled 5 min, and analyzed by SDS-PAGE. Fluorography of 32P-labeled samples was enhanced by baling gels in a 1.0 M sodium carbonate solution before drying.

Extraction of Membrane Proteins

Membrane association of both Kre6p and Sknlp was determined as described by Ljungdahl et al. (1992). Protein extracts from 50-ml cultures expressing Kre6p-HA or Sknlp-HA were prepared by glass bead lysis, and centrifuged at 1,000 g to remove nonlysed cells. Supernatants were removed and split into five separate 80-µl fractions. Four fractions were adjusted to a final concentration of either 0.1 M Na2CO3 (pH 11.0), 0.5% Triton X-100, 1.6 M urea, or 0.6 M NaCl, in a final volume of 200 µl. These samples were incubated 15 min on ice, and fractionated by high speed centrifugation (150,000 g for 15 min at 4°C). A fifth sample was diluted to 200 µl with BSB, and subjected to a low speed spin of 14,000 g for 10 min. The pellet and supernatant fractions for all samples were collected and solubilized in Laemmli buffer at 95°C before analysis by SDS-PAGE. Western blots were performed using a 1:2,000 dilution of 12CA5 antibody, and 1:1,000 of horse-radish peroxidase-conjugated goat anti-mouse secondary antibody, and developed using the ECL chemiluminescence detection kit (Amersham Canada, Oakville, Ontario).

Indirect Immunofluorescence Microscopy

Exponentially growing wild-type yeast strains harboring plasmids encoding either epitope-tagged Kre6p-HA constructs, or native Kre6p were fixed with 3.7% formaldehyde, and treated for immunofluorescence microscopy using standard techniques (Pringle et al., 1991). Antibody dilutions were 1:3,000 and 1:1,000 for 12CA5 and Texas red-conjugated goat anti-mouse secondary antibody, respectively. Cells expressing epitope-tagged Kre6p (Kre6p-HA) were viewed under Texas red excitation wavelengths to indicate the subcellular localization of Kre6p, and under 4',6-diamidino-2-phenylindole (DAPI) excitation irradiation to visualize DNA. Images were obtained using a Zeiss Axioplan Microscope, and represent ~2,000-fold magnification.

Homology Search

Amino acid sequences of Kre6p and Sknlp were compared with all entries in GenBank non-redundant protein sequence database release 81 (February 15, 1994) (Altschul et al., 1990). Computer alignment was created using GENE WORKS (Intelligenetics, Inc., Mountain View, CA).

Results

**KRE6 and SKN1 Are Required for Normal Cell Wall Ultrastructure**

To further explore phenotypes associated with the loss of the KRE6 and SKN1 genes, the cell wall ultrastructure of kre6Δ, sknlΔ, and kre6Δ sknlΔ strains was examined by electron microscopy. A correlation was found between the loss of cell wall integrity and the severity of mutant phenotypes. The kre6 null strain has an altered cell wall ultrastructure reminiscent of that previously seen in both krel and krel9 null mutants (Fig. 1) (Boone et al., 1990; Brown and Bussey, 1993). The krel6 null cell wall lacks a darkly staining outer layer through to be composed primarily of mannoproteins (Zlotnik et al., 1984). The thick central layer of the cell wall, composed largely of β-glucan (Zlotnik et al., 1984; Horisberger and Clerk, 1987), was also noticeably different in krel6 null cells, appearing more amorphous than wild-type cell walls. In contrast, the strain deleted for SKN1, which alone shows no pronounced cell wall phenotype, possessed a cell wall ultrastructure similar to that of the wild-type. krel6 sknlΔ null strains display a dramatic alteration in cell wall ultrastructure, lacking the darkly staining mannoprotein outer...
Kre6p and Sknlp Share Homology with Glucan-binding Proteins

Kre6p and Sknlp share similarity to portions of the recently identified Rhodothermus marinus (1→3)-β-glucanase, bgI A (Spilliaert, R. G. O. Hreggvidsson, J. K. Kristjansson, G. Eggertsson, and A. Palsdottir, unpublished results) and the (1→3)-β-glucan clotting factor, FGA, from horseshoe crab (Seki et al., 1994) (Fig. 2). FGA shares similarity to (1→3)-β-glucanase active site amino acid sequence, Glu*-Ile-Asp-Ile-Glu; including its proposed active site catalytic nucleophile Glu* (Hoj et al., 1992; Planas et al., 1992).

Immunodetection of Kre6p and Sknlp

We sought to identify the KRE6 and SKN1 gene products, as an initial step in their characterization. Epitope-tagging of Kre6p and Sknlp was carried out by inserting a 9-amino acid segment of the HA protein (Kolodziej and Young, 1991) into the amino-terminal domain of each protein. Epitope-tagged versions of both Kre6p (Kre6p-HA) and Sknlp (Sknlp-HA) functioned as the wild-type gene products, as judged by both growth restoration and the ability to confer toxin sensitivity in the kre6Δ strain, TR92 (data not shown).

Immunoprecipitations from whole cell extracts using anti-HA monoclonal antibody, 12CA5, specifically detected Kre6p-HA and Sknlp-HA proteins (Fig. 3). Kre6p and Sknlp are predicted to encode 80- and 85-kD proteins, respectively. Their actual mobilities are substantially slower than predicted; in the apparent molecular mass range of 120 kD for Kre6p-HA and 125-130 kD for Sknlp-HA. Interestingly, Sknlp-HA migrates as a doublet, with the major form migrating slightly slower than the minor species. Whether these two forms of Sknlp-HA are the result of proteolysis, or represent some other protein modifications has not been determined.

Kre6p and Sknlp are quite acidic, with predicted pI's of 4.2 and 4.4, respectively. As often reported for acidic proteins (Cooper et al., 1989), their anomalous apparent molecular mass could partly be a reflection of reduced SDS binding and distortion of their charge/mass ratio. However, Kre6p and Sknlp are also predicted to possess substantial cytoplasmic and lumenal domains accessible to a variety of potential posttranslational modifications which may also contribute to their mobility on SDS-PAGE.

Kre6p and Sknlp Are N-Glycoproteins

Glycosylation could contribute to the anomalous mobility of Kre6p and Sknlp. To examine this possibility, the mobilities of both Kre6p and Sknlp from both tunicamycin-treated and non-treated cell cultures were compared. Tunicamycin...
N-glycosylation of Kre6p-HA and Sknlp-HA. (A) Strain TR92, expressing either Kre6p-HA or Sknlp-HA, was grown in the presence or absence of tunicamycin 30 min before 35S-labeling, immunoprecipitated with 12CA5 monoclonal antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Relative molecular masses are shown to the left in kD.

Figure 4. N-glycosylation of Kre6p-HA and Sknlp-HA. (A) Strain TR92, expressing either Kre6p-HA or Sknlp-HA, was grown in the presence or absence of tunicamycin 30 min before 35S-labeling, immunoprecipitated with 12CA5, and analyzed by SDS-PAGE and fluorography. (B) Endo H treatment of Sknlp-HA immunoprecipitate.

treated cell cultures expressing either Sknlp-HA or Kre6p-HA show a 10-kD increase in mobility relative to untreated control cultures (Fig. 4 A). Similar mobility shifts were detected when treating immunoprecipitated Sknlp-HA or Kre6p-HA (data not shown) with the endoglycosidase, Endo H (Fig. 4 B).

Asparagine-linked glycoproteins contain a GlcNAc2Man9Glc3 core moiety that is attached and modified in the ER (Abeijon and Hirshberg, 1992), and often extended with outer chains later in the secretory pathway (Herscovics and Orlean, 1993). Strains possessing a mnn9 mutation are unable to elaborate N-linked core oligosaccharides (Ballou, 1990), and allow one to discern whether such outer chain modifications occur in a given N-glycoprotein. Kre6p-HA and Sknlp-HA were expressed in the mnn9 strain LB3003-2Aa, and their electrophoretic mobilities compared to those in wild-type strain SEY6210 (data not shown). The absence of any detectable mobility shift in the mnn9 strain, compared with wild type, suggests that both Kre6p and Sknlp are exclusively core-glycosylated. As each asparagine-linked core oligosaccharide is composed of ~2.5-kD of carbohydrate and as no mnn9-dependent core elaboration was detected, the 10-kD N-linked glycosylation mobility shift suggested that 4–5 core oligosaccharides are attached to both Kre6p and Sknlp. This number is consistent with the 5 and 6 potential N-linked glycosylation sites within the respective COOH-termini of Kre6p and Sknlp.

N-linked glycosylation does not appear to be solely responsible for the slow mobility of Kre6p and Sknlp, since both tunicamycin-treated species continue to migrate significantly more slowly than their protein sequences predict. Possible O-linked glycosylation was examined using a null mutation of KRE2 which encodes a mannosyltransferase required for complete O-linked glycosylation (Hausler et al., 1992; Hill et al., 1992). Both Kre6p-HA and Sknlp-HA were produced into the kre2A strain, TR520, and their mobilities compared with those in a wild-type strain, SEY6210. No obvious KRE2-dependent mobility shift was detected (data not shown).

Kre6p and Sknlp Are Integral Membrane Proteins

Kre6p and Sknlp both possess a conserved stretch of 30 hydrophobic amino acid residues predicted to adopt an helical secondary structure capable of spanning a membrane. To examine possible membrane association, TR92 whole cell extracts containing Kre6p-HA or Sknlp-HA were treated with 0.1 M Na2CO3 and separated by high speed centrifugation into supernatant and pellet fractions. Both Kre6p-HA and Sknlp-HA fractionated exclusively to the
Kre6p Appears Localized to the Golgi Apparatus

urea). Conversely, detergent treatment using 0.5% Triton membrane pellet after high speed centrifugation (Fig. 5). Similar fractionation profiles for both proteins were seen using high salt (0.6 M NaCl) or denaturing conditions (1.6 M protein possessing the COOH-terminal amino acid sequence, HDEL; a retention signal for soluble ER proteins section of KRE5 (Meaden et al., 1990). KRE5 lacks any detectable early in the secretory pathway based on the characteriza-

membrane pellet after high speed centrifugation (Fig. 5). Similar fractionation profiles for both proteins were seen using high salt (0.6 M NaCl) or denaturing conditions (1.6 M urea). Conversely, detergent treatment using 0.5% Triton X-100 solubilized the majority of Kre6p-HA and Sknlp-HA.

Kre6p Appears Localized to the Golgi Apparatus

Initial synthesis of (1→6)-β-glucan has been proposed to occur early in the secretory pathway based on the characterization of KRES (Meaden et al., 1990). KRES lacks any detectable (1→6)-β-glucan when deleted, and encodes a secretory protein possessing the COOH-terminal amino acid sequence, HDEL; a retention signal for soluble ER proteins (Petham et al., 1988). As Kre6p and Sknlp also define an early event in the polymer's synthesis, the localization of these secretory proteins was sought. Indirect immunofluorescence microscopy of TR92 maintaining a 2 μm-based Kre6p-HA construct revealed a punctate-staining pattern suggestive of Golgi localization in yeast (Fig. 6) (Preuss et al., 1992; Redding et al., 1991; Nothwehr et al., 1993; Cooper and Bussey, 1992). A variability in both the number of stained punctate spots per cell and the number of cells stained per field was found. Such variability in staining likely reflects variation in protein abundance; a phenomenon commonly seen using 2 μm-based plasmids (Redding et al., 1991). The specificity of the immunofluorescence signal to Kre6p-HA was demonstrated by the observation that identical strains transformed with the same 2 μm-based Kre6p construct lacking the HA epitope and identically processed, failed to provide any immunofluorescence signal.

Attempts to localize Kre6p-HA expressed from a centromeric-based plasmid proved unsuccessful. Although overexpression can lead to mislocalization, overexpression of secretory membrane proteins is not believed to result in a mislocalization to the Golgi; instead mislocalization to the vacuole, and some accumulation in the ER are documented (Roberts et al., 1992; Nothwehr et al., 1993; Cooper and Bussey, 1992). Of over 1,100 Kre6p-HA cells giving a clear staining pattern, more than 1,000 showed strong punctate staining, while only ~150 showed strong punctate plus weaker perinuclear (ER) staining. Less than 1% of Kre6p-HA cells showed faint perinuclear staining only, and no cells had vacuolar or cell surface staining. These observations suggest that Kre6p resides within a Golgi compartment.

Preliminary experiments using Sknlp-HA rule out a cell surface localization, where under identical conditions we detect an equivalently abundant HA-tagged KREI protein (to be published elsewhere). We are, however, unable to distinguish whether Sknlp-HA resides in an ER or Golgi intracellular location.

Kre6p and Sknlp Are Phosphorylated Proteins

Both Kre6p and Sknlp are predicted to possess substantial cytoplasmic domains that are amino-terminal to their transmembrane domain (Harthann et al., 1989; Parks and Lamb, 1991) and that are potential substrates for protein kinases. To examine possible phosphorylation of these proteins, TR92 transformed with either Kre6p-HA or Sknlp-HA expressing plasmids, or an untagged control construct was grown in the presence of [32P]orthophosphate and immunoprecipitated (Fig. 7). Kre6p and Sknlp are both phosphorylated as shown by appropriately sized signals on SDS-PAGE that are absent from control samples. [32P]-labeled Kre6p-HA and Sknlp-HA were similarly detected in a mnn9 background and in tunicamycin-treated cell cultures, indicating that the phosphorylation of Kre6p and Sknlp is due neither to phosphodiester linkages in N-linked outer chains nor to phosphorylation of N-linked core oligosaccharides (data not shown). Instead, Kre6p and Sknlp are phosphorylated on either serine/threonine or tyrosine residue(s). Consistent with this, phosphatase digestions removed radiolabel from [32P]-labeled Kre6p-HA and Sknlp-HA immunoprecipitates, and enhanced their electrophoretic mobility slightly when these proteins were [35S]-labeled (data not shown). As both proteins contain NH2-terminal domains composed of 21% serine and threonine residues, several different phosphorylation site consensus sequences are evident; including those of PKC, MAP kinase, cAMP-dependent protein kinase, and casein kinase (Kemp and Pearson, 1990).

KRE6 Suppresses the Lysis Phenotype of pkclA

but Not Defects in the MAP Kinase Branch of the PKC1 Pathway

Recent work has implicated the yeast PKC1 pathway in regulating cell wall growth (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). Disruption of PKC1 results in a lysis phenotype, which can be prevented by the addition of osmotic support to the medium (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). As both Kre6p and Sknlp are phosphoproteins required for cell wall synthesis, possible genetic interactions between the KRES, SKNI, and PKC1 were examined. Both centromeric and 2 μm-based KRES-containing plasmids transformed into the pkclΔ strain, GPY1115, are capable of alleviating the pkcl Δ osmotic lysis lethality when grown on YPD (Fig. 8). No significant difference in the level of Kre6p-HA phosphorylation, however, was detected in the pkclΔ background vs wild type.
Indirect immunofluorescence of Kre6p-HA. Strain TR92, possessing either YEp24-KRE6-HA or untagged plasmid YEp24-KRF_, was prepared for immunofluorescence as described in Materials and Methods. (left column) Kre6p-HA staining observed with 12CA5 antibodies (Anti-HA); (center column) DAPI staining of nuclei and mitochondria; and (right column) cells viewed by Nomarski optics.

Figure 6. Indirect immunofluorescence of Kre6p-HA. Strain TR92, possessing either YEp24-KRE6-HA or untagged plasmid YEp24-KRF_, was prepared for immunofluorescence as described in Materials and Methods. (left column) Kre6p-HA staining observed with 12CA5 antibodies (Anti-HA); (center column) DAPI staining of nuclei and mitochondria; and (right column) cells viewed by Nomarski optics.

(data not shown). Overexpression of SKNI, failed to suppress the pkcl null lethality on non-osmotically supplemented media (data not shown). PKCI, when overexpressed from a multicopy construct, did not suppress the slow growth phenotype or killer resistance of a kre6 null mutation (data not shown).

As KRE6 is one of several KRE genes participating in (1→6)-β-glucan synthesis, additional KRE genes were tested for their ability to suppress the pkcl lysis phenotype. KRE11, encoding a candidate regulatory component localized to the cytoplasm (Brown et al., 1993; Brown, J., personal communication), as well as KRE1 and KRE9, encoding secretory proteins involved in (1→6)-β-glucan assembly (Boone et al., 1991; Brown and Bussey, 1993), were all incapable of rescuing pkcl lethality on YPD (data not shown).

The PKCI pathway is thought to be branched, and we asked whether KRE6 could suppress defects in the MAP kinase branch of the PKCI-mediated pathway. Centromeric and 2 μm-based KRE6 plasmids were transformed into mpkl, and mkl.2, null strains, and examined for growth at 37°C in the absence of osmotic-stabilizing supplements. Neither mpkl nor mkl.2 null strains were rescued from their temperature-sensitive lysis phenotype on YPD by additional copies of KRE6 (data not shown).

Synthetic Lethal Interactions between KRE6 and Components of the PKCI-mediated MAP Kinase Cascade

To investigate possible additional genetic interactions be-
Table II. Synthetic Lethal Phenotype Summary

| Genotype          | Scored tetrad | Class*  | PD | NPD | TT |
|-------------------|---------------|---------|----|-----|----|
| kre6A pkclΔ       |               | SL      | 3  | 4   | 20 |
| skn1Δ pkclΔ       |               | N       | 3  | 2   | 9  |
| kre2ΔΔ pkclΔ      |               | SL      | 2  | 1   | 12 |
| kre6A mpklΔ       |               | SL      | 1  | 2   | 8  |
| kre6A mkk1Δ mkk2Δ |               | SL      | ND | ND  | ND |
| krellΔ mkk1Δ mkk2Δ|               | SL      | ND | ND  | ND |
| kre6A kre2Δ       |               | N       | 2  | 2   | 9  |

* Classification of the double mutants at 30°C on osmotically supplemented medium. N, normal growth; SL, synthetic lethal.

Figure 7. Kre6p-HA and Sknlp-HA are phosphorylated. Strain TR92, possessing either YEp24-KRE6-HA or YEp352-SKN1-HA, was grown in low phosphate medium and labeled with [32P]orthophosphate before immunoprecipitation. TR92, maintaining the untagged plasmid, YEp24-KRE6, was similarly treated. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Figure 8. Suppression of the pkclΔ defect by KRE6. The pkclΔ strain GPY115, was transformed with plasmids in the presence of 0.5 M KC1. Transformants were streaked onto (A) a YPD plate supplemented with 0.5 M KC1, and (B) a standard YPD plate. GPY115 bears (clockwise from the top) 2 μm plasmid YEp13, CEN plasmid pRS315-KRE6, YEp13-KRE6, and YEp13-PKC1.

tween kre6Δ and pkclΔ, the kre6Δ pkclΔ double heterozygote TR500, was sporulated and dissected onto osmotically supplemented YPD medium. Tetrad analysis of spore progeny indicated that the pkclΔ kre6Δ double mutant spores were inviable, either on sorbitol or KCl-supplemented YPD plates (Table II). The pkclΔ kre6Δ null spores germinated but invariably arrested with a small- to medium-sized bud. Additional synthetic lethal interactions were found between kre6 and both mpklΔ and mkk1Δ,2 double nulls at 30°C, which were also not rescued by osmotic supplements (Table II).

Synthetic Lethal Interactions between PKC1 and Other Cell Wall-related Genes

To address if synthetic lethality between pkclΔ and kre6Δ is due specifically to an exaggerated (1→6)-β-glucan defect, or whether other cell wall perturbations in combination with pkclΔ can also cause lethality, genetic interactions between PKC1 and other cell wall-related genes were examined. KRE2-deleted strains possess a (1→6)-β-glucan phenotype comparable to kre6Δ null strains (Brown et al., 1993). Tetrad dissection of the pkclΔ krellΔ double heterozygote diploid strain, TR502, revealed pkclΔ krellΔ spores to be inviable on 1.2 M sorbitol-supplemented YPD (Table II). pkclΔ krellΔ segregants invariably died after 2-5 cell divisions. A null mutation in KRE2, a mannosyltransferase required for correct O-linked glycosylation of mannosproteins (Hausler et al., 1992; Hill et al., 1992), also conferred a synthetic lethal phenotype in combination with pkclΔ on sorbitol-supplemented YPD. In contrast, skn1Δ mutants which lack any obvious cell wall phenotype fail to show an exaggerated growth defect in combination with pkclΔ (Table II). In addition, combining kre6Δ and kre2Δ mutations lacked any synergistic growth defect (Table II).
pkclΔ Cells Have an Altered Cell Wall

Growth in osmotic-supplemented media does not result in substantial changes to the cell wall of pkcl null cells since transfer to a low osmoticum after growth in osmotic-supplemented conditions leads to immediate pkcl cell lysis (Paravicini et al., 1992). To examine the composition of the pkcl null cell walls, GPy1115 and the isogenic wild-type strain SEY6210 were grown in the presence of osmotic support, and their cell walls purified. Fractionation and quantitation of the various cell wall components revealed that pkclΔ cells lack a significant amount of all β-glucan polymers when compared with wild type (Table III A). These results, showing an overall reduction of 30% in total β-glcucan in a pkcl mutant, are very similar to those reported recently by Shimizu et al. (1994). Mannan, the other major wall component, was modestly reduced by 20% in this pkcl-deleted background (Table III B).

To address whether KRE6 suppression occurs through possible remodeling of the pkcl cell wall, GPy1115 strains harboring a variety of KRE6 or control plasmids were grown in 0.5 M KCl-supplemented media and their cell wall polymer levels compared. No significant increase in (1→6)-β-glucan, (1→3)-β-glucan, or mannan were seen in GPy1115 transformed with KRE6 (Table III, A and B). Thus, although the cell wall of GPy1115 is very different from wild type, possessing reduced levels in all β-glucan polymers and mannan, the partial KRE6 suppression of the pkcl osmotic lysis phenotype is not accompanied by an obvious restructuring of the wall.

Discussion

Our study addresses a number of new issues in the synthesis and regulation of an extracellular matrix polymer in S. cerevisiae. Previous work indicates that Kre6p and Sknlp are candidates for β-glucan biosynthetic enzymes (Roemer and Bussey, 1991; Roemer et al., 1993). The β-glucan and cell wall ultrastructural effects seen in k6 sknl mutants, and the sequence similarity of regions of Kre6p/Sknlp to β-glucan-binding domains of other proteins, support this contention. The Golgi localization of Kre6p/Sknlp to β-glucan-binding domains of other proteins, support this contention. The Golgi localization of Kre6p is consistent with, and extends, previous work suggesting a sequential secretory pathway-based synthesis of (1→6)-β-glucan beginning in the ER, continuing within the Golgi, and being completed at the cell surface (Boone et al., 1990; Meaden et al., 1990). The relative frequency of Kre6p staining patterns in secretory organelles is inconsistent with an alternative interpretation that Kre6p is normally localized to the ER, but is mislocalized to the Golgi once the ER is saturated. Epistatic relationships between KRE5 and KRE6 and KREI (Boone et al., 1990; Meaden et al., 1990) are consistent with a Golgi localization of Kre6p; with Kre6p acting downstream of a KRE5-dependent ER event, and being epistatic to the KREI-dependent cell surface event. A secretory pathway location for (1→6)-β-glucan synthesis is also supported by work of Horisberger and Clerc (1987) who have shown by immunofluorescence labeling of (1→6)-β-glucan, that both intracellular and cell wall staining are observed. (1→6)-β-glucan assembly in yeast likely parallels extracellular matrix polysaccharide synthesis in higher plants, where for example xyloglu-
can and pectin are synthesized within the secretory pathway and deposited within the cell wall (Moore and Staehelin, 1988; Zhang and Staehelin, 1992; Levy and Staehelin, 1992; Driouich et al., 1993; Gigaut and Carpi, 1993).

A body of biochemical evidence suggests that at least some (1→6)-β-glucan occurs as a glucosyl moiety on glycopolypeptides (Van Rinsum et al., 1991; Klis, 1994) and the β-glucosylasparagine linkage has recently been found on laminin (Schreiner et al., 1994). Our work identifying genes whose products act to assemble this glucan polymer, could well be explained by a set of components involved in such protein glucosylation. This view of (1→6)-β-glucan biosynthesis as a newly described form of protein glycosylation would place the polymer in a conventional biochemical context. In yeast, both N- and O-mannosylation of proteins are paradigm examples of glycans synthesized sequentially in the secretory pathway, and subsequently secreted and assembled into the cell wall (Kukuruzinska et al., 1987).

Demonstration of Kre6p and Sknlp as membrane proteins with both phosphorylation and N-glycosyl modifications, indicates that these proteins possess both cytoplasmic and luminal domains. Consistent with a type II membrane topology for Kre6p and Sknlp, where their COOH-terminal domains would be luminal, the COOH-termini of both proteins possesses substantial homology with a pair of secreted/ extracellular glucan-binding proteins. Such a type II topology has the potential to allow Kre6p and Sknlp to couple cytoplasmic and luminal secretory processes to effect (1→6)-β-glucan synthesis.

We have examined the relationship between KRE6 and SKNL and other KRE genes, with kinases implicated in the regulation of cell wall synthesis. Genetic interactions between KRE2, KRE6, KRE7, SKNL, and PKC1 support the contention that pkcl cells lyse as a result of a fragile cell wall.

Table III. β-Glucan Levels of pkclΔ, KRE6-suppressed pkclΔ, and Wild-Type Cell Walls

| Strain* | Plasmid | (1→6)-β-Glucan | (1→3)-β-Glucan |
|---------|---------|----------------|----------------|
| SEY6210 | None    | 117.0 ± 4.7    | 380            |
| GPy1115 | prS315  | 67.2 ± 1.9     | 225            |
| GPy1115 | prS315-KRE6 TF1¶ | 67.1 ± 1.7 | 208            |
| GPy1115 | prS315-KRE6 TF2 | 72.8 ± 3.5 | 239            |
| GPy1115 | prS315-KRE6-HA3 | 74.9 ± 5.0 | 199            |

* Concentration (μg/mg cell wall dry wt).
¶ GPY1115 is a pkclΔ derived from the wild-type SEY6210.
§ TF1 and TF2, independent transformants.
‖ Centromeric-based epitope tagged KRE6 construct.

Error represents 1 SD.
Table IIIB. Mannan Levels in pkc1Δ vs KRE6 and PKC1 Transformants

| Strain     | Plasmid          | Mannan (µg/mg cell wall dry wt) |
|------------|------------------|---------------------------------|
| GPY1115    | pRS315           | 386.6 ± 29.8*                   |
| GPY1115    | YEp13            | 377.7 ± 23.9                    |
| GPY1115    | pRS315-KRE6      | 444.2 ± 55.2                    |
| GPY1115    | YEp13-KRE6       | 421.7 ± 36.0                    |
| GPY1115    | YEp13-PKC1       | 518.6 ± 11.0                    |

* Error represents 1 SD.

The basis of synthetic lethality for the kre6 pkc1 and kre11 pkc1 double mutants is not known, but is consistent with a further weakening of the pkc1 cell wall, presumably by exaggerating the (1-→6)-β-glucan defect. Colethality between krec2 and pkc1 suggests that the reduced synthesis of O-mannosyl moieties of glycoproteins exacerbates the pkc1 cell wall defect. Moreover, as mutants with defects in either the KRE2 or KRE11 genes alone grow quite well, their synthetic lethality with a defective PKC1 underscores the fragile nature of the pkc1 cell wall. Osmotic stabilizers are insufficient to rescue these synthetically lethal combinations, probably because the biosynthesis of wall components has been too severely affected.

Expression of KRE6 from a centromeric plasmid is sufficient to partially suppress the osmotic lysis phenotype of a pkc1 null mutant. Of the cell wall–related genes tested, only KRE6 has this ability; other participants in the (1-→6)-β-glucan synthetic pathway, including Sknlp, cannot suppress the pkc1 lysis phenotype when overexpressed. Despite this evidence of a special role for KRE6, we have no direct evidence that Kre6p acts as a glucan synthase component in the suppression. KRE6 transformants in the pkc1 strain showed no obvious increase in (1-→6)-β-glucan, or other cell wall polymer. The possibility remains, however, that a modest (1-→6)-β-glucan or other cell wall alteration does occur with additional copies of KRE6, and that this does improve the condition of the cell wall, but that this alteration is below our relatively crude levels of detection.

A number of lines of evidence strongly indicate that PKC1 affects multiple cell wall synthetic events, and does not act on (1-→6)-β-glucan synthesis alone. We have shown the pkc1 null mutant lacks a significant amount of all cell wall β-glucan polymers, as well as mannan. This reduction in wall mass likely reflects the thinner, more fragile nature of the pkc1 wall. pkc1Δ cells also lack the characteristic cell wall ultrastructure evident in krel, kre6 and kre9-deleted strains, and are osmotically fragile, unlike kre mutants. In addition, suppression of pkc1 defects by KRE6 is only partial.

PKC1 is thought to act through a MAP kinase cascade suspected of regulating multiple functions, and a second unknown regulatory branch. KRE6 suppression of pkc1 does not act primarily downstream of this MAP kinase branch of the PKC1 pathway, since KRE6 does not suppress the conditional lysis phenotype of mpkl(slt2) or mkk1,2 null strains. We have not, however, ruled out the possibility that the MAP kinase pathway partially regulates KRE6 expression.

Is there a relationship between the KRE6 homolog, SKN1, and PKC1? The lack of either colethality or suppression of pkc1 by SKN1 could indicate a direct activation of Sknlp by Pkclp, such that in the absence of Pkclp, Sknlp is completely inactivated. Alternatively, there may be no relationship between SKN1 and PKC1, and the absence of any genetic interaction simply reflects the minor role SKN1 plays relative to KRE6 during vegetative growth.

Our results suggest two plausible relationships between PKC1 and KRE6 in (1-→6)-β-glucan assembly. One model (Fig. 9A) is that KRE6 and the (1-→6)-β-glucan pathway function in a manner that is independent of the PKC1 pathway.
way, but with both pathways affecting cell wall synthesis as a common process. (1→6)-β-glucan synthesis would be viewed as a parallel cell wall assembly pathway to those regulated by PKC1. The in vivo level of all cell wall polymer classes, including (1→6)-β-glucan, appear reduced in pckl strains, and further diminution of (1→6)-β-glucan levels by loss of Kre6p would be lethal. In this model, another, as yet unknown, protein kinase(s) would regulate (1→6)-β-glucan synthesis through phosphorylation of Kre6p and/or Sknlp. PKC1 could function in such "global" cell wall regulation directly or could indirectly perturb these events in a pleiotropic manner, for example by disrupting cytoskeletal organization (Mazzoni et al., 1993). Very recently pckl mutants have been shown to overproduce an extracellular β-glucanase (Shimizu et al., 1994) which may contribute to their loss of osmotic integrity. Overproduction of Kre6p a putative glucan synthase may have the capacity to make more glucan and to partially ameliorate the deleterious effects of the overproduced glucanase, resulting in partial in vivo suppression of the pckl mutant phenotype.

A second possibility, for which we have no direct experimental support, is that (1→6)-β-glucan synthesis is regulated directly, or indirectly, by PKC1-dependent phosphorylation of Kre6p through the less defined second branch of the PKC1 bifurcated pathway (Fig. 9 B). Protein kinase C phosphorylation of membrane proteins occurs in mammalian cells (Hunter et al., 1984; Li et al., 1993). Although the yeast PKC1 phosphorylation consensus sequence is not known, Kre6p does possess several RXXS/T sequences (where X symbolizes any amino acid and S/T is phosphorylated) found in the in vivo Pcklp pseudosubstrate sequence (Antonsson et al., 1994; Watanabe et al., 1994). Kre6p could act downstream of Pcklp, within or under the ill-defined second branch of the PKC1 pathway, since Kre6p does not suppress mpkl(st2) or mkkl,2 null mutations of the MAP kinase branch. To explain the kre6 pckl synthetic lethality, Kre6p must normally possess some residual activity in a pcklΔ strain. Synthetic lethality between kre6 and mkkl,2 or mpkl(st2) is consistent with creating lesions in both branches of the PKC1 pathway. Suppression of pckl by additional copies of KRE6 may occur by elevating the residual activity of Kre6p enough to compensate for the non-stimulated form of Kre6p in a pckl background. A prediction from this model is that Kre6p phosphorylation is dependent on PKC1. However, Kre6p remains phosphorylated in a pckl null background, indicating that a non-PKC1-dependent phosphorylation of Kre6p must also exist. Confirmation of this model would require identifying the Kre6p phosphorylated residue(s) and demonstrating distinct in vivo phosphorylation patterns of Kre6p in a pckl background vs wild type.

We thank Jeff Brown, Bo Jiang, and Marc Lussier for critical reading of the manuscript, Carol Smith and Guy L'Heureux for photographic work, and Kathy Hewitt for electron microscopy expertise. Thanks also to Stella DeTiani for strain constructions, Diane Oki for assistance in manuscript preparation, and Matthew Spottswood for assistance with protein alignment. Strain MHD93 was kindly provided by Cesar Nobela, and strain 3233-1B by Kunihiro Matsumoto.

The work was supported by Operating and Strategic grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), T. Roemer is a NSERC postgraduate fellow.

Received for publication 12 May 1994 and in revised form 14 July 1994.

References

Abejon, C., and C. B. Hirschberg. 1992. Topology of glycosylation reactions in the endoplasmic reticulum. Trends Biochem. Sci. 17:32-36.

Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in Saccharomyces cerevisiae. J. Cell Biol. 98:934-945.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Antonsson, B., S. Montessuit, Friedle, M. A. Payton, and G. Paravincini. 1994. Protein kinase C in yeast. Characterization of the Saccharomyces cerevisiae PKC1 gene product. J. Biol. Chem. 269:16821-16828.

Badin, J., C. Jackson, and M. Schubert. 1953. Improved method for determination of plasma poly saccharides with triphenyl. Proc. Soc. Exp. Biol. Med. 84:228-291.

Ballou, C. E. 1990. Isolation, characterization, and properties of Saccharomyces cerevisiae mann mutants with nonconditional protein glycosylation defects. Methods Enzymol. 191:41-472.

Boone, C., S. S. Sommer, A. Hensel, and H. Bussey. 1990. Yeast KRE genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. 110:1833-1843.

Brown, J. L., and H. Bussey. 1993. The yeast KRE5 gene encodes an α-glycoprotein involved in cell surface β-glucan assembly. Mol. Cell. Biol. 13:6346-6356.

Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey. 1993. A mutational analysis of killer toxin resistance in S. cerevisiae identifies new genes involved in cell wall (1→6)-β-glucan synthesis. Genetics. 133:837-849.

Bulawa, C. E. 1993. Genetics and molecular biology of chitin synthesis in fungi. Annu. Rev. Microbiol. 47:505-532.

Bussey, H. 1991. K1 killer toxin, a pore-forming protein from yeast. Mol. Microbiol. 5:2339-2343.

Bussey, H., W. Sacks, D. Galley, and D. Saville. 1982. Yeast killer mutations affecting toxin secretion and activity and toxin immunity function. Mol. Cell. Biol. 2:346-354.

Cahib, E., R. Roberts, and B. Bowers. 1982. Synthesis of the yeast cell wall and its regulation. Annu. Rev. Biochem. 51:763-793.

Cooper, A., and H. Bussey. 1989. Characterization of the yeast KEX1 gene product: a carboxypeptidase involved in processing secreted precursor proteins. Mol. Cell. Biol. 9:2706-2714.

Cooper, A., and H. Bussey. 1992. Yeast Kex1p is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. J. Cell Biol. 119:1459-1468.

Costigan, C., S. Gehrung, and M. Snyder. 1992. A synthetic lethal screen identifies SLD1, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. Mol. Cell. Biol. 12:1162-1178.

Driouch, A., L. Faye, and L. A. Stachelin. 1993. The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biotechnol. 18:210-215.

Errede, B., and D. E. Levin. 1993. A conserved kinase cascade for MAP kinase activation in yeast. Curr. Opin. Cell Biol. 5:254-260.

Gigaut, D. M., and N. C. Carpita. 1993. Synthesis of (1→3)-(1→4)-β-galactan in the Golgi apparatus of maize coleoptiles. Proc. Natl. Acad. Sci. USA. 90:3849-3854.

Guthrie, C., and G. R. Fink. 1991. Guide to yeast genetics and molecular biology. Methods Enzymol. 194:3-93.

Haugenauer-Tuapis, R., and A. Hinnen. 1984. A deletion that includes the signal peptide cleavage site and N-glycosylation sites of the 65K subunit of yeast cell surface yeast asparaginase. Mol. Cell. Biol. 4:2668-2675.

Harthanne, E., T. A. Rapoport, and H. F. Lodish. 1989. Predicting the orientation of eukaryotic membrane-spanning domains. Proc. Natl. Acad. Sci. USA. 86:5786-5790.

Häusler, A., L. Ballou, C. E. Ballou, and P. W. Robbins. 1992. Yeast glycoprotein biosynthesis: MNT1 encodes an α-1,2-mannosyltransferase involved in O-glycosylation. Proc. Natl. Acad. Sci. USA. 89:6846-6850.

Hercovics, A., and P. Orlean. 1993. Yeast glycoprotein synthesis. FASEB (Fed. Am. Soc. Exp. Biol.) J. 7:540-550.

Hill, K., C. Boone, M. Goebel, R. Paccia, A.-M. Sedu, and H. Bussey. 1992. Yeast KRE2 defines a new gene family encoding probable secretory proteins, and is required for the correct N-glycosylation of proteins. Genetics. 130:273-283.

Hoj, P. B., R. Condron, J. C. Traeger, J. C. McAuliffe, and B. A. Stone. 1992. Identification of glutamic acid 105 at the active site of Bacillus amyloliquefaciens 1,3-1,4-β-glucan 4-glucanohydrolase using epoxide-based inhibitors. J. Biol. Chem. 267:25039-25066.

Horisberger, M., and M.-F. Clerk. 1987. Cell wall architecture of a Saccharomyces cerevisiae mutant with a truncated carbohydrate outer chain in the mannoprotein. Eur. J. Cell Biol. 45:62-71.

Hunter, T., N. Ling, and A. Cooper. 1984. Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature ( Lond.). 311:480-483.

Hutchings, K., and H. Bussey. 1983. Cell wall receptor for yeast killer toxin: involvement of (1→6)-β-glucan. J. Bacteriol. 154:161-169.

Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto, and Y. Ohashi. 1993. MKK1 and MKK2, which encode Saccharomyces cerevisiae migas-activated protein kinase-migas homologs, function in the pathway mediated by protein kinase C. Mol. Cell. Biol. 13:3076-3083.
Irie, K., H. Araki, and Y. Oshima. 1991. A new protein kinase, SSP31, modulating the SMP3 gene-product involved in plasmid maintenance in Saccharomyces cerevisiae. Gene (Amst.) 108:139--144.

Ito, H., M. Fukada, M. Murata, and A. Kimura. 1983. Transformation of intact yeast cells with alkali cations. J. Bacteriol. 153:63--68.

Kemp, B. E., and R. B. Pearson. 1990. Protein kinase recognition sequence motifs. Trends Biochem. Sci. 15:342--346.

Klis, F. M. 1994. Cell wall assembly in yeast. Yeast. 10:851--869.

Kolodziej, P. A., and R. A. Young. 1991. Epitope tagging and protein surveillance. Methods Enzymol. 194:508--519.

Kukurnzinska, M. A., M. L. E. Bergh, and B. J. Jackson. 1987. Protein glycosylation in yeast. Annu. Rev. Biochem. 56:915--944.

Lee, K., and D. Levin. 1992. Dominant mutations in a gene encoding a putative protein kinase (BCK1) bypass the requirement for a Saccharomyces cerevisiae protein kinase C homolog. Mol. Cell. Biol. 13:3067--3075.

Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D. E. Levin. 1993. A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. 13:3067--3075.

Lee, K. S., L. Hines, and D. Levin. 1993. A pair of functionally redundant yeast genes (PPZ1 and PPZ2) encoding type 1-related protein phosphatases function within the PKC1-mediated pathway. Mol. Cell. Biol. 13:5843--5853.

Levin, D. E., and E. Bartlett-Heubusch. 1992. Mutants in the S. cerevisiae PKCI gene display a cell cycle-specific osmotic stability defect. J. Cell Biol. 116:1221--1229.

Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thurmier. 1990. A candidate protein kinase C gene, PKCI, is required for the S. cerevisiae cell cycle. Cell. 62:213--224.

Levy, S., and L. A. Stachelin. 1992. Synthesis, assembly and function of plant cell wall macromolecules. Curr. Opin. Cell Biol. 4:856--862.

Lew, D. J., and S. R. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation of Cdc28 and cyclins. J. Cell Biol. 120:1305--1320.

Li, M., J. W. West, R. Numann, B. J. Murphy, T. Scheuer, and W. A. Catterall. 1993. Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. Science (Wash. DC). 261:1439--1442.

Ljungdahl, P. O., C. J. Gimeno, C. A. Styles, and G. R. Fink. 1993. SHR3: a novel component of the secretory pathway specifically required for localization of amino acid permeases in yeast. Cell. 71:463--478.

Madden, K. C., Costigan, and M. Snyder. 1992. Cell polarity and morphogenesis in Saccharomyces cerevisiae. Trends Cell Biol. 2:21--29.

Mazzoni, C., P. Zarzov, A. Rambourg, and C. Mann. 1993. The SLT2 (MPK1) MAP kinase is involved in polarized cell wall growth in Saccharomyces cerevisiae. J. Cell Biol. 123:1821--1833.

Meaden, P., K. Hill, J. Wagner, D. Slippetz, S. S. Sommer, and H. Bussey. 1990. The yeast KRE5 gene encodes a probable endoplasmic reticulum protein required for (1-6)-β-glucan synthesis and normal cell growth. Mol. Cell. Biol. 10:3013--3019.

Moore, P. J., and L. A. Stachelin. 1988. Immunogold localization of the cell-wall matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in Trifolium pratense L.; implication for secretory pathways. Planta. 174:433--445.

Nathwehr, S. F., C. J. Roberts, and T. H. Stevens. 1993. Membrane protein retention in the golgi apparatus: diphtetidox mnanopeptidase A is retained by a cytoplasmic receptor recognizing aromatic residues. J. Cell Biol. 121:1197--1209.

Paravicini, G., M. Cooper, L. Frieled, D. J. Smith, J.-L. Carpenterier, L. S. Klig, and M. A. Payton. 1992. The osmotic integrity of the yeast cell requires a functional PKC1 product. Mol. Cell. Biol. 12:4896--4905.

Pelham, H. R. B., K. G. Hardwick, and M. J. Lewis. 1988. Sorting of soluble enzymes in yeast. EMBO (Eur. Mol. Biol. Organ.) J. 7:1757--1762.

Planas, A., M. Juncoa, J. Lloberas, and E. Querol. 1992. Essential catalytic role of Glu134 in endo-β,1,3,1,4-D-glucan 4-glucanohydrolase from B. licheniformis as determined by site-directed mutagenesis. FEBS (Fed. Eur. Biochem. Soc.) Lett. 308:141--145.

Preuss, D., J. Mulholland, A. Franzussof, N. Segev, and D. Botstein. 1992. Characterization of the Saccharomyces cerevisiae Golgi complex through the cell cycle by immunoelectron microscopy. Mol. Biol. Cell. 3:789--803.