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

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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. *KRE6* and *SKN1* 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 *pkc1* lysis defect. When mutated, several *KRE* pathway genes and members of the *PKC1*-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 *pkc1* null wall, support a role for the *PKC1* pathway functioning 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). *pkc1* null cells possess a G_2-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 *pkc1* 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 though 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 *pkc1* cell lysis (Lee and Levin, 1992; Irie et al., 1991; Costigan et al.,

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1992). Multiple copies of either *MKK1* or *MKK2*, a pair of MAP kinase kinase homologs, are also able to suppress *pkc1* temperature-sensitive alleles from lysing (Irie et al., 1993). Furthermore, at elevated temperatures, null mutations of all members of the MAP kinase cascade also exhibit an osmotic-suppressible lysis phenotype (Errede and Levin, 1993). *MPK1(SLT2)*, the MAP kinase at the base of this pathway, has recently been isolated as a dosage-dependent suppressor 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 *pkc1* cell lysis when overproduced; these include *PPZ1* and *PPZ2*, 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 *SKN1* (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 *SKN1* has no effect on killer sensitivity, growth, or (1→6)-β-glucan levels. *SKN1* is, however, a functional homolog of *KRE6* and can suppress the *kre6* null phenotypes in a dosage-dependent manner. Deletion of both *KRE6* and *SKN1* results in a severe growth defect which can be lethal in some strain backgrounds. The slow growth defect of viable *kre6 skn1* null deletion strains can be partially alleviated by the accumulation of spontaneous extragenic suppressors. Such *kre6 skn1* null suppressed strains possess little, if any, (1→6)-β-glucan. Because *KRE6* and *SKN1* 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 *SKN1* have been proposed to function independently and to act early in the assembly of the polymer, possibly as glucan synthases.

The *KRE6* and *SKN1* gene products, Kre6p and Sknlp, have been characterized to further examine their role in (1→6)-β-glucan assembly. Here, *KRE6* and *SKN1* 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 *pkc1* 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 I. 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 Haguenauer-Tsapis 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 *pkc1*Δ cell wall proved difficult due to its cell lysis phenotype. Osmotic-stabilizing agents, although required for *pkc1*Δ cell growth, distort sample weights significantly and prevent polymer levels being normalized to dry weight of cells. *pkc1*Δ 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 Rinsun et al. (1991), and all cell wall polymer measurements were normalized per mg cell wall dry weight. The *pkc1*Δ 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 *kre6*Δ and *skn1*Δ haploid strains TR510 and TR511, respectively. TR512 is a congenic wild-type spore progeny from TR67. TR211 and TR213 are *kre6*Δ *skn1*Δ 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-SalI fragment in the multicopy plasmid, YEp24 (Roemer and Bussey, 1991). This same *KRE6* BamHI-SalI insert was subcloned into the centromeric plasmid, pRS315, and named pRS315-*KRE6* (Roemer and Bussey, 1991). YEp13-*KRE6* contains a 4.2-kb *KRE6* BamHI/Dra I fragment inserted into the BamHI-PvuII sites of the 2 μm-based plasmid, YEp13. *SKN1* was subcloned as a 5.5-kb SalI-HindIII fragment into the 2 μm plasmid, YEp352, and named YEp352-*SKN1* (Roemer et al., 1993). YEp13-*PKC1* is a 4.3-kb *PKC1* SphI fragment in YEp13.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
SEY6210	*MAT α leu2-3,112 ura3-52 his3-Δ200 lys2-80 trp1-Δ901 suc2-Δ9*	S. D. Emr
TR92	*MAT a kre6::HIS3 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	Roemer and Bussey, 1991
TR95	*MAT α kre6::HIS3 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	Roemer and Bussey, 1991
TR144	*MAT α kre6::Tn10 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	Roemer and Bussey, 1991
TR178	*MAT α skn1::LEU2 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	Roemer et al., 1993
HAB806	*MAT α kre11::URA3 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	Brown et al., 1993
TR520	*MAT α kre2::TRP1 leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*	M. Lussier
TR67	*MATa/MATα KRE6/kre6::HIS3 his3/his3 leu2/leu2/can1/can1*	Roemer and Bussey, 1991
TR510	*kre6::HIS3 his3 leu2 can1*	This work
TR512	*his3 leu2 can1*	This work
TR160	*MAT a/MATα SKN1/skn1::LEU2 his3/his3 leu2/leu2 can1/can1*	Roemer et al., 1993
TR511	*skn1::LEU2 his3 leu2 can1*	This work
TR211	*kre6::HIS3 skn1::LEU2 his3 leu2 can1*	Roemer et al., 1993
TR213	*kre6::HIS3 skn1:: LEU2 his3 leu2 can1*	Roemer et al., 1993
GPY1115	*MAT a pkc1::HIS3 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ9011 suc2-Δ9 ade2-101*	Paravicini et al., 1992
MHD93	*MATa slt2::URA3 ura3 leu2-3,112 his3-Δ1 trp1*	C. Nombela
3233-1B	*MATa mkk1::LEU2 mkk2::HIS3 ura3 leu2 his3 trp1*	K. Matsumoto
TR500	*MAT a/MATα KRE6/kre6::Tn10 PKC1/pkc1::HIS3 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101*	This work
TR501	*MAT a/MATα SKN1/skn1::LEU2 PKC1/pkc1::HIS3 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101*	This work
TR502	*MAT a/MATα KRE11/kre11::URA3 PKC1/pkc1::HIS3 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101*	This work
TR503	*MAT a/MATα KRE2/kre2::TRP1 PKC1/pkc1::HIS3 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101*	This work
TR504	*MAT a/MATα KRE2/kre2::TRP1 KRE6/kre6::HIS3 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801/lys2-801 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9*	This work
GPY100	*MAT a/MATαKRE6/kre6::HIS3 SLT2/slt2::URA3 leu2-3,12/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ1 LYS2/lys2-801 trp1-Δ901/trp1 SUC2/suc2-Δ9*	This work
GPY102	*MAT a/MATα KRE6/kre6::URA3 MKK1/mkk1::LEU2 MKK2/mkk2::HIS3 leu2-3,112/leu2 ura3-52/ura3 his3-Δ200/his3 LYS2/lys2-801 trp1-Δ901/trp1 SUC2/suc2-Δ9*	This work
GPY104	*MAT a/MATα KRE11/kre11::URA3 MKK1/mkk1::LEU2 MKK2/mkk2::HIS3 leu2-3,112/leu2 ura3-52/ura3-52 his3-Δ200/his3 LYS2/lys2-801 trp1-Δ901/trp1 SUC2/suc2-Δ9*	This work
LB3003-2Aa	*MATa ura3 his4 mnn9*	T. Stevens

Epitope Tagging

Standard molecular manipulations were as described by Sambrook et al. (1989). Epitope tagging of Kre6p 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 (TACCCATACGACGTCCCAGACTACGCTTCTGCA) and 430 (GAAGCGTAGTCTGGGACGTCGTATGGGTAT) and *KRE6* oligos 598 (GATCTCAACTACAACCCATACGACGTCCCAGACTACGCT) and 599 (GATCAGCGTAGTCTGGGACGTCGTATGGGTTGTAGTTGA). Oligos 429 and 430 were annealed and subcloned into the corresponding PstI site of YEp352-*SKN1*. Similarly, oligos 598 and 599 were annealed and ligated into the unique BglIII site of YEp24-*KRE6* and pRS315-*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-*SKN1*-HA, YEp24-*KRE6*-HA, and pRS315-*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 10^7 cells were grown up in appropriate minimal medium for ^{35}S- or ^{32}P-labeling, harvested, concentrated fourfold, and grown an additional 30 min in fresh minimal medium. Cells were labeled with either 100 μCi of Trans ^{35}S-label or carrier-free [^{32}P]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 $Na_2B_4O_7$ [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 molybdate, was also added in ^{32}P-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; PAS, 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 PMSF [including 5 mM sodium molybdate in ^{32}P-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) (CI-4B, Pharmacia LKB Biotechnology, Piscataway, NJ) was added and samples were 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 ^{32}P-labeling experiments, ~200 μg 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 ^{35}S-labeled samples was enhanced by bathing gels in a 1.0 M sodium salicylate solution before drying.

Labeling and immunoprecipitation of Kre6p-HA in the *pkc1* null strain GPY1115, as well as its isogenic wild-type parent strain, SEY6210, were carried out as described above, in low phosphate media supplemented with 0.5 M KCl. Kre6p-HA protein levels were determined by Western 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 ml sodium citrate buffer containing the above protease inhibitors, and digested with 10 mU Endo H (or mock digested) overnight at 37°C. Samples were then washed twice with WB, and processed as described for ^{35}S-labeled samples.

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 Na_2CO_3 (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 horseradish 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 ~ a 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*, *skn1Δ*, and *kre6Δ skn1Δ* 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 *kre1* and *kre9* null mutants (Fig. 1) (Boone et al., 1990; Brown and Bussey, 1993). The *kre6* 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 *kre6* 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. *kre6 skn1* null strains display a dramatic alteration in cell wall ultrastructure, lacking the darkly staining mannoprotein outer

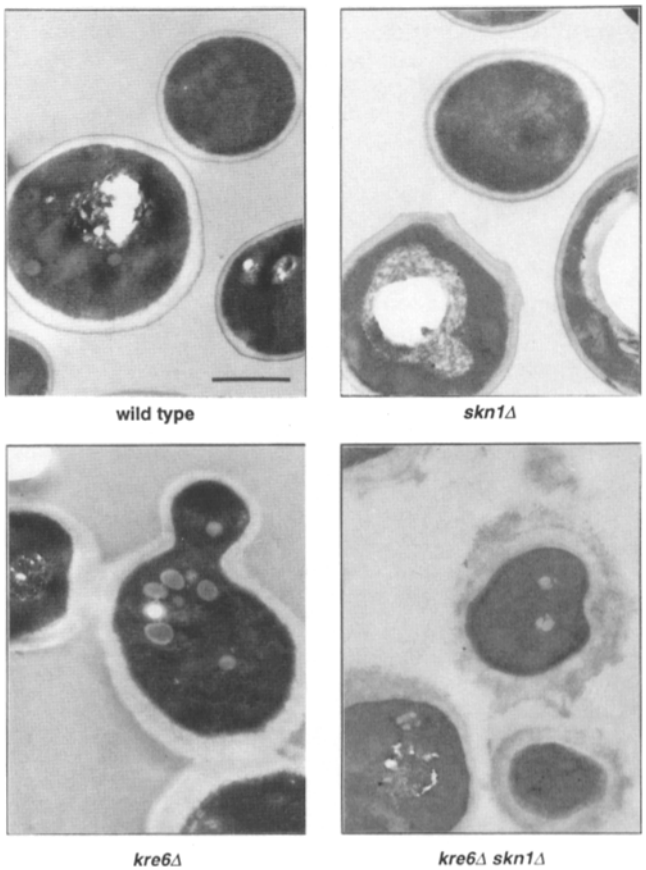

Figure 1. Cell wall electron micrographs of *kre6* and *skn1* mutants. (*top left*) Wild type (TR512); (*top right*) *skn1Δ* (TR511); (*bottom left*) *kre6Δ* (TR510); and (*bottom right*) *kre6Δ skn1Δ* (TR213). The *kre6 skn1* double null mutant shows these defects despite having acquired extragenic mutations capable of improving growth of the original double null strain. Bar, 1.0 μm.

layer and also appearing to be significantly thicker and often delaminated.

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, bglA (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-glucanases throughout its amino terminal domain and binds (1→3)-β-glucan to initiate an immune response against invading fungi. The bglA and FGA proteins share significant homology within two 100-amino acid stretches of both the Kre6p and Sknlp COOH-terminal domains. Kre6p and Sknlp also share similarity to a portion of the bacterial (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-

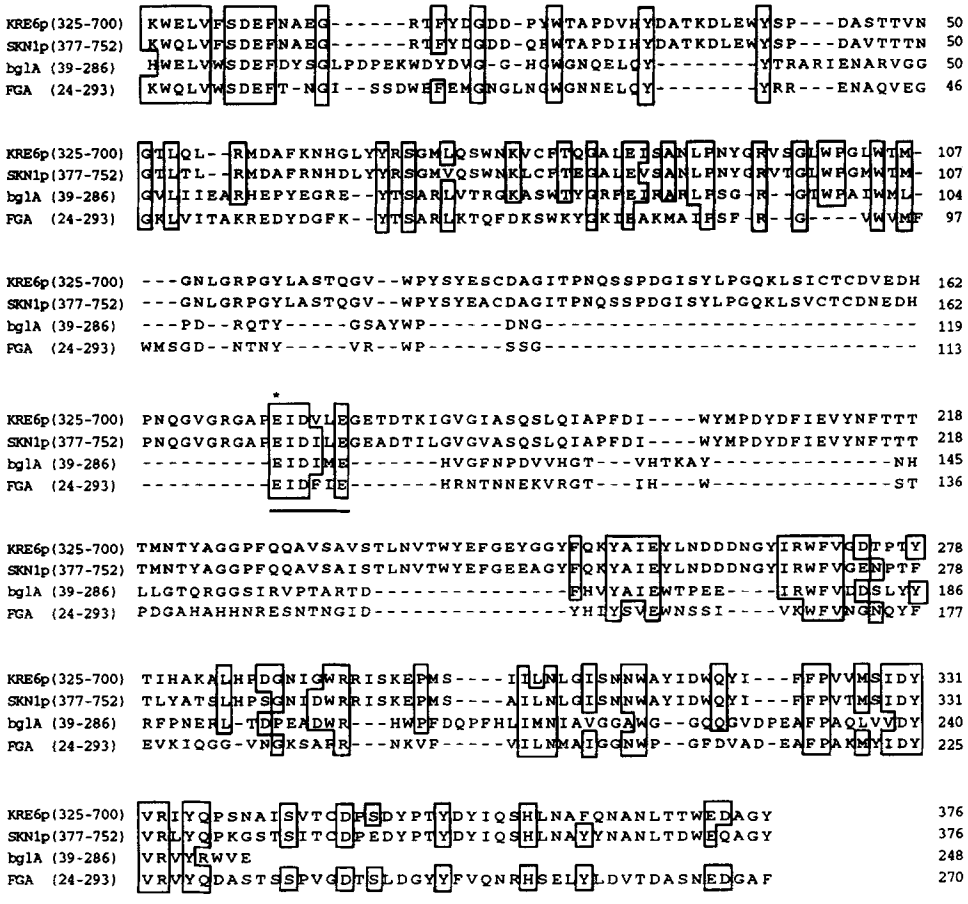

Figure 2. Similarity of Kre6p and Sknlp to FGA and bglA. Residues conserved by at least one member of each pair of protein sequences are boxed. Homology to the bacterial β-glucanase active site sequence, Glu-Ile-Asp-Ile-Glu, is underlined. An asterisk identifies the proposed catalytic nucleophile (Hoj et al., 1992; Planas et al., 1992). Gaps (---) have been introduced to improve alignments. Accession numbers for FGA and bglA are D16622 and U04836, respectively.

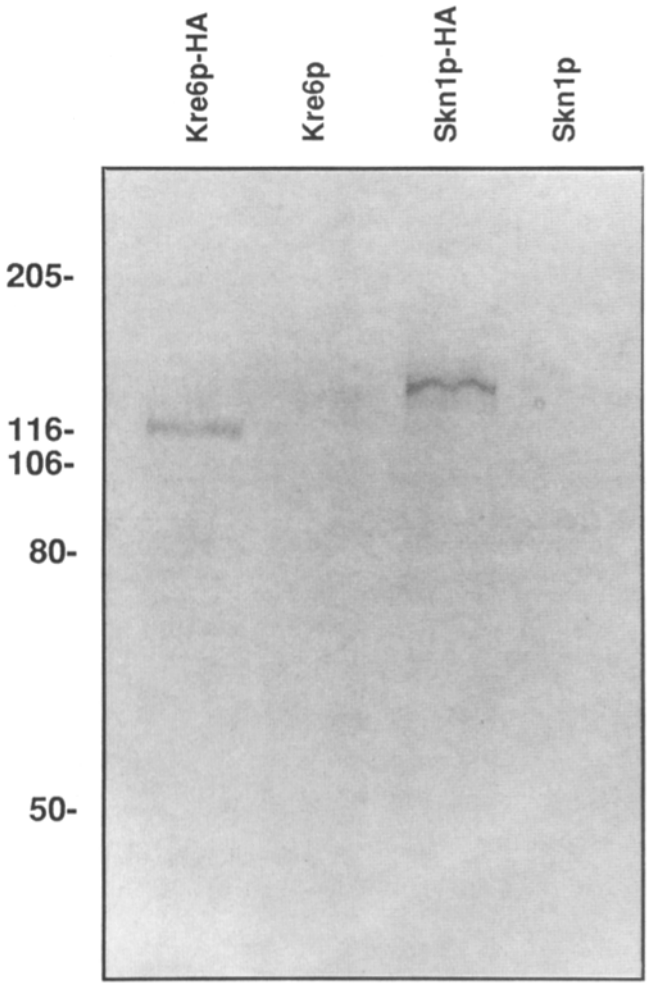

Figure 3. Immunodetection of Kre6p-HA and Sknlp-HA. Strain TR92, was transformed with epitope-tagged *KRE6* or *SKNI* plasmids, or their respective untagged control plasmids, and ^{35}S-labeled 10 min before immunoprecipitation with 12CA5 monoclonal antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Relative molecular masses are shown to the left in kD.

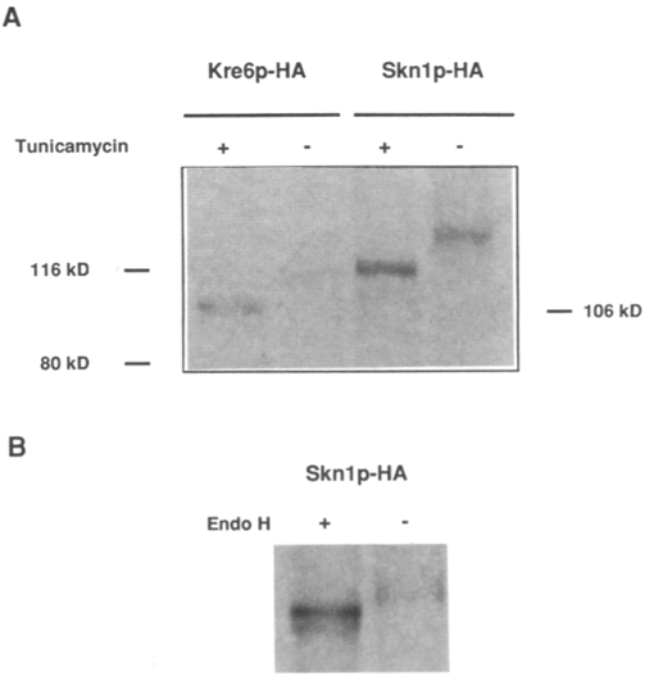

Figure 4. *N*-glycosylation of Kre6p-HA and Sknlp-HA. (*A*) Strain TR92, expressing either Kre6p-HA or Sknlp-HA, was grown in the presence or absence of tunicamycin 30 min before ^{35}S-labeling, immunoprecipitated with 12CA5, and analyzed by SDS-PAGE and fluorography. (*B*) Endo H treatment of Sknlp-HA 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 $GlcNAc_2Man_9Glc_3$ core moiety that is attached and modified in the ER (Abeijon and Hirschberg, 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 *kre2Δ* 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 alpha-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 Na_2CO_3 and separated by high speed centrifugation into supernatant and pellet fractions. Both Kre6p-HA and Sknlp-HA fractionated exclusively to the

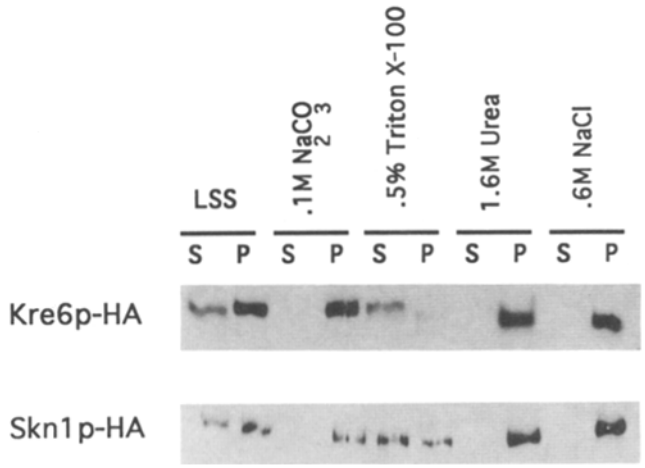

Figure 5. Membrane association of Kre6p-HA and Sknlp-HA. Cell lysates from strain TR92, expressing either Kre6p-HA or Sknlp-HA, were subjected to either a low speed spin (*LSS*), or incubated in 0.1 M Na_2CO_3, 0.5% Triton X-100, 1.6 M urea, or 0.6 M NaCl, as described in Materials and Methods. Cell lysates were then separated into pellet (*P*) and supernatant (*S*) fractions by high speed centrifugation, analyzed by Western blotting with 12CA5, and visualized by ECL fluorography.

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 *KRE5* (Meaden et al., 1990). *KRE5* 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 (Pelham 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 *KRE1* 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 [^{32}P]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. ^{32}P-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 ^{32}P-labeled Kre6p-HA and Sknlp-HA immunoprecipitates, and enhanced their electrophoretic mobility slightly when these proteins were ^{35}S-labeled (data not shown). As both proteins contain NH_2-terminal domains composed of 21% serine and threonine residues, several different phosphorylation site consensus sequences are evident; including those of *PKC1*, MAP kinase, cAMP-dependent protein kinase, and casein kinase (Kemp and Pearson, 1990).

KRE6 Suppresses the Lysis Phenotype of pkc1, 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 *KRE6*, *SKN1*, and *PKC1* were examined. Both centromeric and 2 μm-based *KRE6*-containing plasmids transformed into the *pkc1*Δ strain, GPY1115, are capable of alleviating the *pkc1* osmotic lysis lethality when grown on YPD (Fig. 8). No significant difference in the level of Kre6p-HA phosphorylation, however, was detected in the *pkc1*Δ background vs wild type

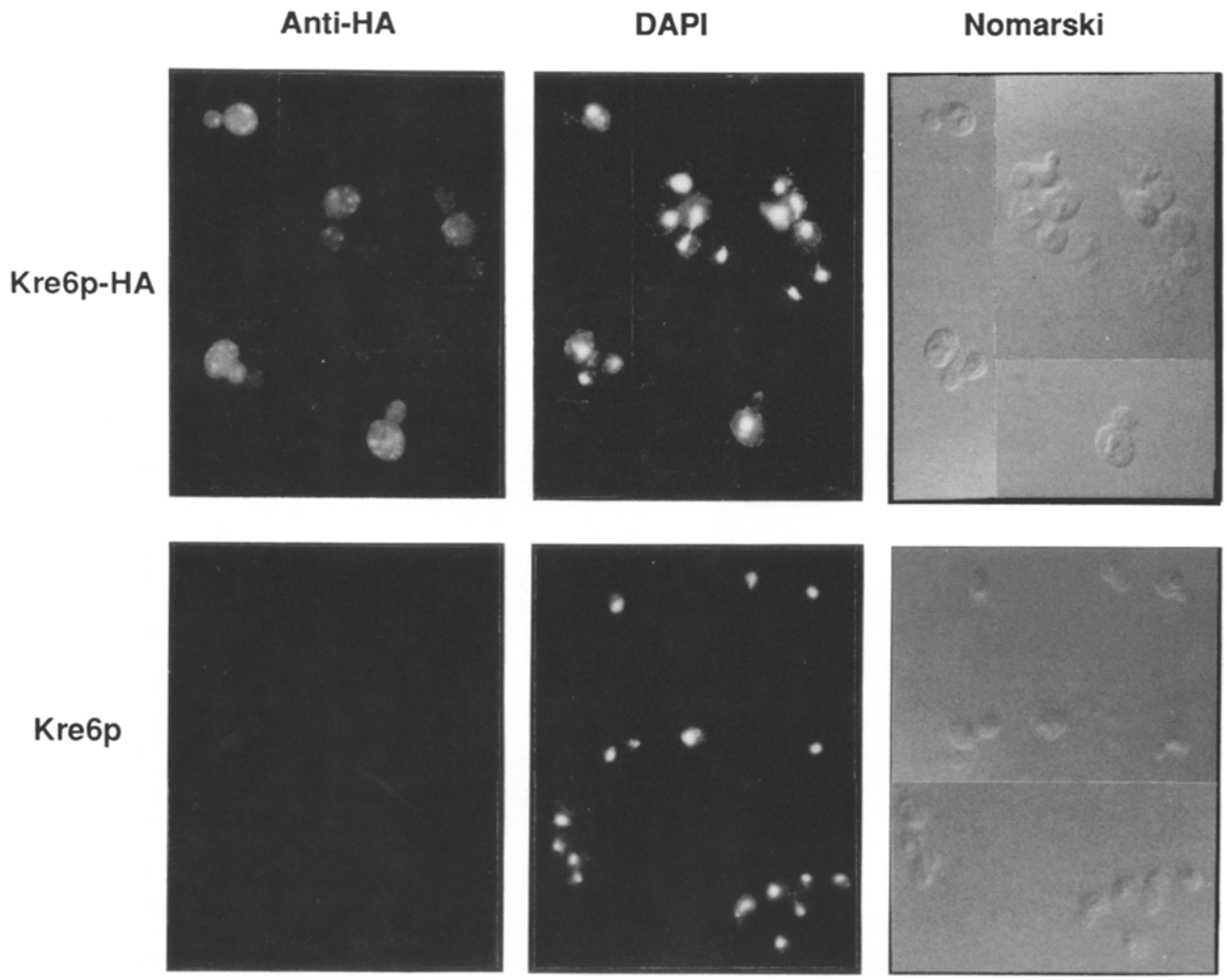

Figure 6. Indirect immunofluorescence of Kre6p-HA. Strain TR92, possessing either YEp24-*KRE6*-HA or untagged plasmid YEp24-*KRE6*, was prepared for immunofluorescence as described in Materials and Methods. (*left column*) Kre6p-HA staining observed with 12CA5 antibodies (*Anti-HA*); (*center column*)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

(data not shown). Overexpression of *SKN1*, failed to suppress the *pkc1* null lethality on non-osmotically supplemented media (data not shown). *PKC1*, 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 *pkc1* 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 *pkc1* lethality on YPD (data not shown).

The *PKC1* pathway is thought to be branched, and we asked whether *KRE6* could suppress defects in the MAP kinase branch of the *PKC1*-mediated pathway. Centromeric and 2 μm-based *KRE6* plasmids were transformed into *mpk1*, and *mkk1,2*, null strains, and examined for growth at 37°C in the absence of osmotic-stabilizing supplements. Neither *mpk1* nor *mkk1,2* 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 PKC1-mediated MAP Kinase Cascade

To investigate possible additional genetic interactions be-

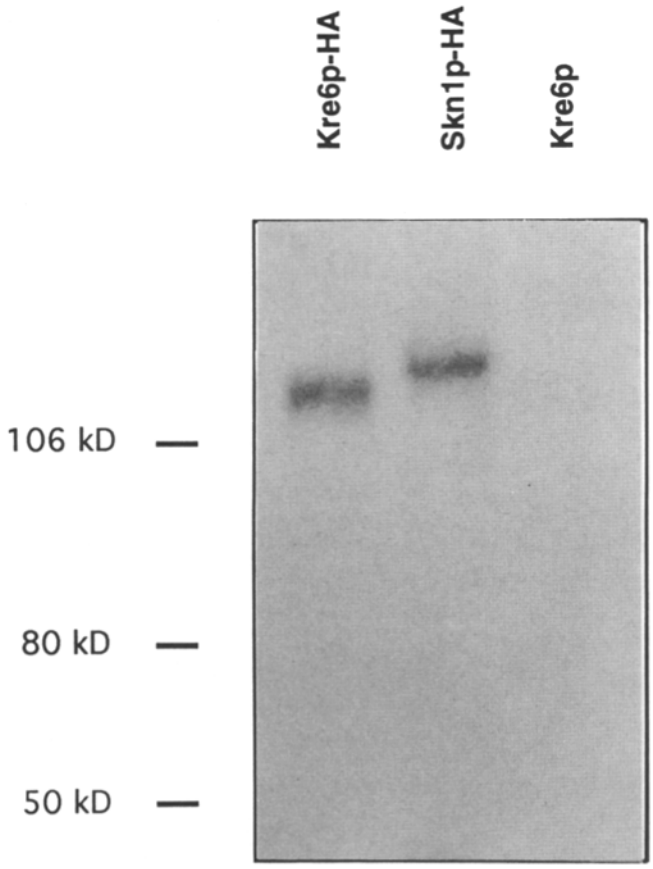

Figure 7. Kre6p-HA and Skn1p-HA are phosphorylated. Strain TR92, possessing either YEp24-*KRE6*-HA or YEp352-*SKN1*-HA, was grown in low phosphate medium and labeled with [^{32}P]orthophosphate before immunoprecipitation. TR92, maintaining the untagged plasmid, YEp24-*KRE6*, was similarly treated. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

tween *kre6*Δ and *pkc1*Δ, the *kre6*Δ *pkc1*Δ double heterozygote TR500, was sporulated and dissected onto osmotically supplemented YPD medium. Tetrad analysis of spore progeny indicated that the *pkc1 kre6* double mutant spores were inviable, either on sorbitol or KCl-supplemented YPD plates (Table II). The *pkc1 kre6* null spores germinated but invariably arrested with a small- to medium-sized bud. Additional

Table II. Synthetic Lethal Phenotype Summary

Genotype	Class*	Scored tetrads‡ PD	NPD	TT
*kre6*Δ *pkc1*Δ	SL	3	4	20
*skn1*Δ *pkc1*Δ	N	3	2	9
*kre11*Δ *pkc1*Δ	SL	4	4	7
*kre2*Δ *pkc1*Δ	SL	2	1	12
*kre6*Δ *mpk1(slt2)*Δ	SL	1	2	8
*kre6*Δ *mkk1*Δ *mkk2*Δ	SL	ND§	ND	ND
*kre11*Δ *mkk1*Δ *mkk2*Δ	SL	ND	ND	ND
*kre6*Δ *kre2*Δ	N	2	2	9

* Classification of the double mutants at 30°C on osmotically supplemented medium. *N*, normal growth; *SL*, synthetic lethal.
‡ *PD*, parental ditype; *NPD*, nonparental ditype; *TT*, tetratype. Synthetic lethal spore progeny were inferred by scoring markers from viable progeny.
§ *ND*, not determined.

synthetic lethal interactions were found between *kre6* and both *mpk1* and *mkk1,2* 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 *pkc1* and *kre6* is due specifically to an exaggerated (1→6)-β-glucan defect, or whether other cell wall perturbations in combination with *pkc1* can also cause lethality, genetic interactions between *PKC1* and other cell wall-related genes were examined. *KRE11*-deleted strains possess a (1→6)-β-glucan phenotype comparable to *kre6* null strains (Brown et al., 1993). Tetrad dissection of the *pkc1 kre11* double heterozygote diploid strain, TR502, revealed *pkc1 kre11* spores to be inviable on 1.2 M sorbitol-supplemented YPD (Table II). *pkc1 kre11* segregants invariably died after 2–5 cell divisions. A null mutation in *KRE2*, a mannosyltransferase required for correct O-linked glycosylation of mannoproteins (Hausler et al., 1992; Hill et al., 1992), also conferred a synthetic lethal phenotype in combination with *pkc1* on sorbitol-supplemented YPD. In contrast, *skn1* mutants which lack any obvious cell wall phenotype fail to show an exaggerated growth defect in combination with *pkc1* (Table II). In addition, combining *kre6* and *kre2* mutations lacked any synergistic growth defect (Table II).

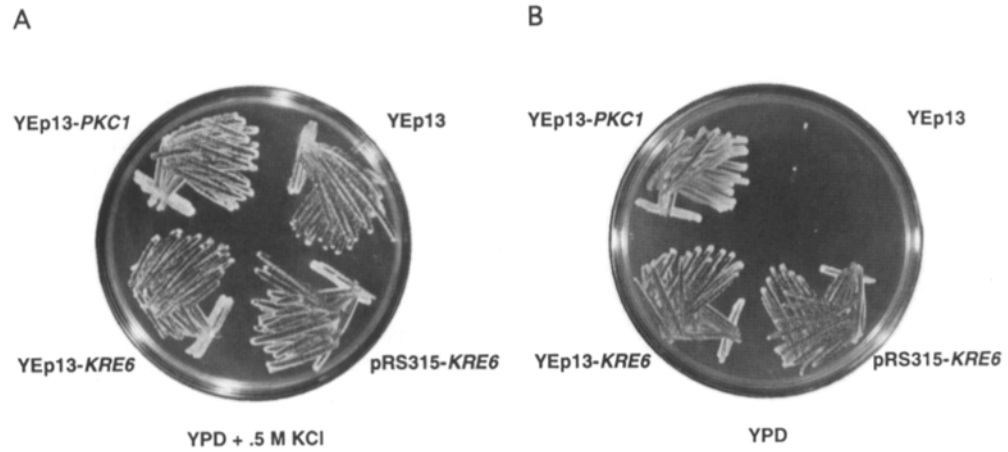

Figure 8. Suppression of the *pkc1*Δ defect by *KRE6*. The *pkc1*Δ strain GPY1115, was transformed with plasmids in the presence of 0.5 M KCl. Transformants were streaked onto (*A*) a YPD plate supplemented with 0.5 M KCl, and (*B*) a standard YPD plate. GPY1115 bears (*clockwise from the top*) 2 μm plasmid YEp13, CEN plasmid pRS315-*KRE6*, YEp13-*KRE6*, and YEp13-*PKC1*.

Table IIIA. β-Glucan Levels of pkc1Δ, KRE6-suppressed pkc1Δ, and Wild-Type Cell Walls

Strain‡	Plasmid	Alkali-insoluble* (1→6)-β-Glucan	Alkali-insoluble* (1→6) + (1→3)-β-Glucan	Alkali-soluble* (1→3)-β-Glucan
SEY6210	None	117.0 ± 4.7	657.2 ± 10.1	380
GPY1115	pRS315	67.2 ± 1.9	521.4 ± 28.8	225
GPY1115	pRS315-*KRE6* TF1§	67.1 ± 1.7	468.0 ± 3.0	208
GPY1115	pRS315-*KRE6* TF2	72.8 ± 3.5	566.0 ± 2.8	239
GPY1115	pRS315-*KRE6*-HA‖	74.9 ± 5.0	497.0 ± 23.0	199

* Concentration (μg/mg cell wall dry wt).
‡ GPY1115 is a *pkc1Δ* derived from the wild-type SEY6210.
§ TF1 and TF2; independent transformants.
‖ Centromeric-based epitope tagged *KRE6* construct.
Error represents 1 SD.

pkc1Δ 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 *pkc1Δ* 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-glucan 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 *kre6 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 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 *KRE6* and *KRE5* and *KRE6* and *KRE1* (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 *KRE1*-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 immunogold 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 xyloglucan 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; Gigeaut and Carpita, 1993).

A body of biochemical evidence suggests that at least some (1→6)-β-D-glucan occurs as a glucosyl moiety on glycoproteins (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 lumenal domains. Consistent with a type II membrane topology for Kre6p and Sknlp, where their COOH-terminal domains would be lumenal, 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 lumenal secretory processes to effect (1→6)-β-glucan synthesis.

We have examined the relationship between *KRE6* and *SKN1* and other *KRE* genes, with kinases implicated in the regulation of cell wall synthesis. Genetic interactions between *KRE2, KRE6, KRE11, SKN1*, and *PKC1* support the contention that *pkcl* cells lyse as a result of a fragile cell wall.

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 *krel1 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 *kre2* 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 *mpk1(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 Pkc1p, such that in the absence of Pkc1p, 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 (See Fig. 9 *A*) is that *KRE6* and the (1→6)-β-glucan pathway function in a manner that is independent of the *PKC1* path-

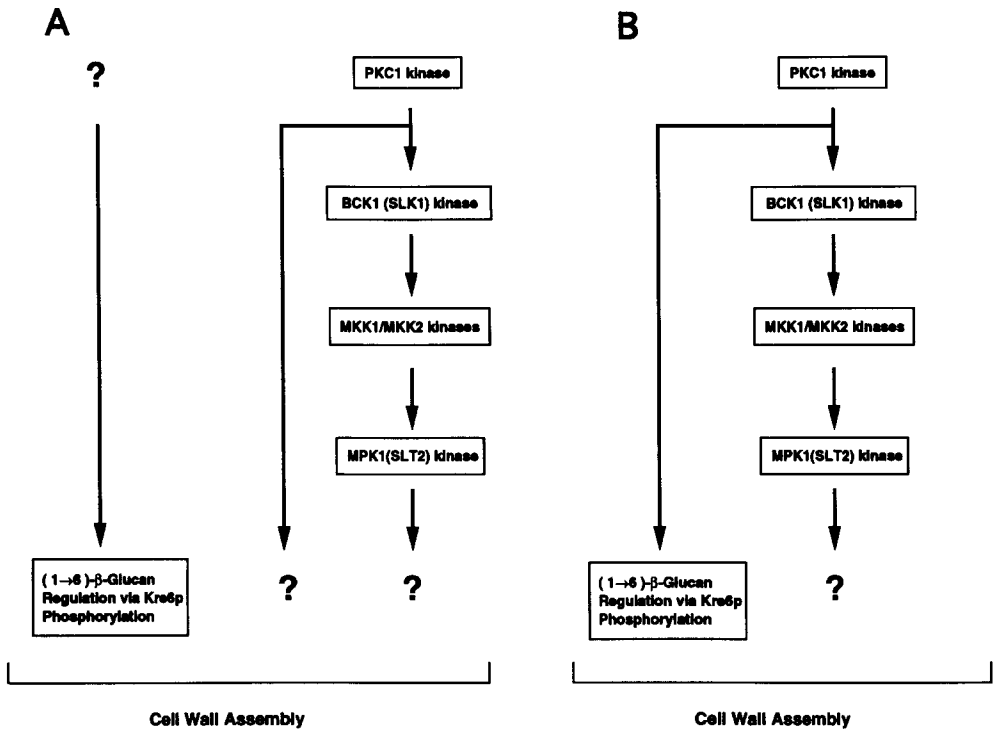

Figure 9. Models for the interactions between the *KRE6*/(1→6)-β-glucan pathway, and the *PKC1* pathway. See Discussion for details.

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 *pkc1* 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 *pkc1* 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 *pkc1* 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 (See 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 Pkc1p pseudosubstrate sequence (Antonsson et al., 1994; Watanabe et al., 1994). Kre6p could act downstream of Pkc1p, within or under the ill-defined second branch of the *PKC1* pathway, since Kre6p does not suppress *mpk1(slt2)* or *mkk1,2* null mutations of the MAP kinase branch. To explain the *kre6 pkc1* synthetic lethality, Kre6p must normally possess some residual activity in a *pkc1*Δ strain. Synthetic lethality between *kre6* and *mkk1,2* or *mpk1(slt2)* is consistent with creating lesions in both branches of the *PKC1* pathway. Suppression of *pkc1* 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 *pkc1* background. A prediction from this model is that Kre6p phosphorylation is dependent on *PKC1*. However, Kre6p remains phosphorylated in a *pkc1* 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 *pkc1* background vs wild type.

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