**KEG1/YFR042w** Encodes a Novel Kre6-binding Endoplasmic Reticulum Membrane Protein Responsible for β-1,6-Glucan Synthesis in *Saccharomyces cerevisiae*

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KEG1/YFR042w of *Saccharomyces cerevisiae* is an essential gene that encodes a 200-amino acid polypeptide with four predicted transmembrane domains. The green fluorescent protein- or Myc₆-tagged Keg1 protein showed the typical characteristics of an integral membrane protein and was found in the endoplasmic reticulum by fluorescence imaging. Immunoprecipitation from the Triton X-100-solubilized cell lysate revealed that Keg1 binds to Kre6, which has been known to participate in β-1,6-glucan synthesis. To analyze the essential function of Keg1 in more detail, we constructed temperature-sensitive mutant alleles by error-prone polymerase chain reaction. The keg1-1 mutant cells showed a common phenotype with Δkre6 mutant including hypersensitivity to Calcofluor white, reduced sensitivity to the K1 killer toxin, and reduced content of β-1,6-glucan in the cell wall. These results suggest that Keg1 and Kre6 have a cooperative role in β-1,6-glucan synthesis in *S. cerevisiae*.

The fungal cell wall is an essential extracellular network composed of polysaccharides and proteins that contributes to protect the cell from internal turgor pressure and external harmful agents. It also contributes in the selective uptake and excretion of various materials and works in communication with the environment. The yeast *Saccharomyces cerevisiae* cell wall is composed of polysaccharides (~85%) and proteins (~15%) and represents ~30% of the cell dry weight (1). Polysaccharides form a basal fibril network with branches and covalent linkage among the components. The proteins are heavily glycosylated with N- and O-linked saccharides mostly composed of mannose. Many of these mannoproteins are covalently linked to polysaccharide networks through glycosylphosphatidylinositol-anchor remnant or alkali-labile linkage (1, 2). Some proteins are physically trapped in the mesh of networks or are chemically linked with other proteins by a disulfide bond. Polysaccharides are long fibrillar and tensile β-1,3-glucans of ~1500 glucose units/chain, amorphous and flexible β-1,6-glucans of ~350 glucose units/chain, and linear microfibril chitins of β-1,4-linked N-acetylglucosamine (1–2%). β-1,3-Glucan and chitin are synthesized by specific polymerases embedded in the plasma membrane using cytoplasmic UDP-sugars as the substrates. The localization and activity of polymerases are controlled by the actin cytoskeleton and secretory system according to the environmental stimuli and cell cycle regulatory system. Conversely, β-1,6-glucan synthesis is more complex, and the gene products whose mutations have defects in the β-1,6-glucan content are localized throughout the secretory pathway and at the cell surface (3). *KRE5* and *CNE1*, which encode UDP-glucose:glycoprotein glucosyltransferase- and calnexin-related proteins, *CWH41* and *ROT2*, which encode glucosidas I and II, respectively, fungus-specific genes *BIG1*, *ROTI*, *KRE9* and its homologue *KNH1*, *KRE6*, and *SKNI*, which encode type II membrane protein with sequence and structural similarity to family 16 glycoside hydrolases, and *KRE1*, which encodes a glycosylphosphatidylinositol-anchored cytoplasmic membrane protein, are involved. The relation among these gene products and their molecular roles in β-1,6-glucan synthesis remains obscure at present. The biochemical tools including *in vitro* biosynthetic assay are also still underdeveloped practically (4).

Recently, a number of large scale post-genome studies revealed the functional roles of many previously uncharacterized open reading frames in *S. cerevisiae* (5–8). However, a considerable number of genes still remain uncharacterized, because they are difficult to examine in a large scale analysis for various reasons. These include essential genes that are required for colony formation under the laboratory culture condition favorable for yeast. In many cases, the sequence homology or motif information did not suggest a possible candidate of their function, and the experiments available for such large-scale analyses as those that use promoter shutoff showed that the cells lost their colony-forming activity without showing a distinctive phenotype when the gene product was depleted. We have to study these remaining genes individually, employing approaches more specific for each gene to reach a more complete understanding of the *S. cerevisiae* genome.

*YFR042w* is one of the uncharacterized essential genes, and the predicted gene product is a 200-amino acid hydrophobic polypeptide with four possible transmembrane domains. Because we have been studying the transport, modification, and localization of secretory proteins, which occurs during the intracellular membrane traffic from the ER² via the Golgi to the

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2 The abbreviations used are: ER, endoplasmic reticulum; GFP, green fluorescent protein.
cell surface, we examined if the product of YFR042w works in the ER or Golgi. In this paper we show that it localizes in the ER, and that Keg1 works in the ER or Golgi. In this paper we show that it localizes in the ER, associates with Kre6 protein, and has a role in the biosynthesis of β-1,6-glucan in the cell wall. The gene was named KEG1 for a Kre6-binding ER membrane protein responsible for β-1,6-glucan synthesis.

**Experimental Procedures**

**Strains and Media**—S. cerevisiae used in this study are listed in Table 1. They were grown in YPD (1% Bacto yeast extract (BD Biosciences), 2% Bacto peptone (BD Biosciences), and 2% glucose), or SD (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, and appropriate supplements) medium at 30°C unless other temperatures were indicated. SC medium was SD containing 0.2% vitamin assay, the pH of YPD was acidified by adding 1/10 volume of 1N HCl to make a 5-fluoro-orotic acid plate for Ura3 selection of temperature-sensitive mutants. For K1 killer plate assay, we examined if the product of YFR042w works in the ER or Golgi. In this paper we show that it localizes in the ER, associates with Kre6 protein, and has a role in the biosynthesis of β-1,6-glucan in the cell wall. The gene was named KEG1 for a Kre6-binding ER membrane protein responsible for β-1,6-glucan synthesis.

**TABLE 1**

S. cerevisiae strains used in this study

| Strain | Genotype and plasmid | Origin |
|--------|---------------------|--------|
| KA31a  | MATα, his3Δ leu2Δ trp1Δ ura3Δ | Laboratory stock |
| KNY19  | KA31a, pCA106 (CEN, URA3 GFP-KEG1), pKN13 (CEN, TRP1 KRE6-Myc6) | This study |
| KNY20  | KA31a, pCA106 (CEN, URA3 GFP-KEG1), pH651 (CEN, TRP1 Myc6) | This study |
| KNY21  | KA31a, pCA120 (CEN, URA3 Myc6-KEG1), pKN27 (CEN, TRP1 KRE6-GFP) | This study |
| KNY22  | KA31a, pC25 (CEN, URA3 Myc6), pKN27 (CEN, TRP1 KRE6-GFP) | This study |
| BY4743 | MA Taα, his3Δ leu2Δ/leu2Δ met15Δ0/ met15Δ0 ura3Δ0 | This study |
| Y25856 | as BY4743, KEG1/KEG1Δ-kanMX4 | EUROSCARF |
| CAY207 | as Y25856, pCA106 (CEN, URA3 GFP-KEG1) | This study |
| CAY327 | as Y25856, pCA120 (CEN, URA3 Myc6-KEG1) | This study |
| BY4741 | MA Taα, his3Δ leu2Δ/leu2Δ met15Δ0/ met15Δ0 ura3Δ0 | EUROSCARF |
| KSY99  | as BY4741, MET15 | This study |
| KNY34  | pCA106 (CEN, URA3 GFP-KEG1) | This study |
| KNY60  | pK7 (CEN, URA3 KRE6-Myc6) | This study |
| CAY206 | as Y4741, Keg1Δ-kanMX4, pCA106 (CEN, URA3 GFP-KEG1) | This study |
| AKY17  | as KSY99, keg1-1 TRP1 | This study |
| KTY203 | CA47, pCA69 (CEN, URA3 KEG1) | This study |
| KTY205 | CA47, pK3316 (CEN, URA3) | This study |
| KNY14  | CA47, pCA106 (CEN, URA3 GFP-KEG1) | This study |
| KNY15  | CA48, pK56 (2μ, URA3 ROT1) | This study |
| Y05574 | as Y4741, kre6Δ-kanMX4 | EUROSCARF |
| KNY75  | Y05574, pK7 (CEN, URA3 KRE6-Myc6) | This study |
| KNY76  | Y05574, pKN29 (CEN, URA3 KRE6-GFP) | This study |
| NCY232 | K1 killer strain | ATCC60782 |

**TABLE 2**

Primers used in this study

| Primer | Gene | Restriction site | Direction | Sequence |
|--------|------|-----------------|-----------|----------|
| CA47   | KEG1 | BamHI           | Forward   | 5'-CGGGATCCCTGGGCCATATTTCGTAC-3' |
| CA48   | KEG1 | XhoI            | Reverse   | 5'-CCAGTCGTGGGTATCTATCTCGAGGG-3' |
| CA57   | KEG1 | BamHI           | Forward   | 5'-CGGGATCCCTGGGCCATATTTCGTAC-3' |
| YN228  | KEG1 | EcoRI           | Forward   | 5'-CGGGATCCCTGGGCCATATTTCGTAC-3' |
| NT38   | KRE6 | SalI            | Reverse   | 5'-CGGGATCCCTGGGCCATATTTCGTAC-3' |
| YN276  | KRE6 | XhoI            | Forward   | 5'-CGGGATCCCTGGGCCATATTTCGTAC-3' |

**Plasmid Construction**—The DNA fragments containing the KEG1 and KRE6 were prepared by PCR using the primers listed in Table 2. The nucleotide sequences were confirmed after cloning in the plasmid. Low copy KEG1 plasmid (pCA69) was made by ligation of the PCR amplification fragment from the genomic DNA using the primers CA47 and CA48 into the plasmid pRS316 (CEN, URA3). The T-nterminal tagging of green fluorescent protein (GFP) to Keg1 (pCA106) was made by ligation of the PCR amplification fragment using CA57 and CA48 into pCA22 (CEN, TRP1 YPT1 promoter-GFP). The N-terminal fusion of Myc6 to Keg1 (pCA120) was made by replacing the vector of pCA106 with pCA25 (CEN, URA3 YPT1 promoter-Myc6). The C-terminal fusion of GFP to Kre6 (pKN27 and pKN29) was made by ligation of the PCR amplification fragment using NT38 and YN276 into pCA114 (CEN, TRP1 GFP-TDH1 terminator), respectively.

**Construction of Temperature-sensitive Mutant Alleles of KEG1**—The 5'-355-bp fragment of KEG1 was obtained by cleaving pCA69 with HindIII, blunting the end by T4 DNA polymerase, and then cleaving with BamHI. This fragment was cloned in the BamHI-Smal site of pRS313 (CEN, HIS3). Then the 3'-259-bp fragment of pCA69 obtained by PCR amplification using primers YN228 and CA48 was inserted into the EcoRI-XhoI sites of the newly generated plasmid. This plasmid
was cleaved with EcoRI and mixed with DNA prepared by amplification under error-prone PCR conditions (10) using primers CA47 and CA48. CAY236 (∆keg1, pCA106) was transformed with this mixture to integrate the PCR fragments into the plasmid by gap repair. The His“ transformants were replicated to 5-fluoro-orotic acid plates to remove pCA106 and then replicated to Phloxine B plates and incubated at 37°C for 2 days. The cells that formed pink colonies were tested for single colony formation at 25 and 37°C. After confirming that the temperature sensitivity was due to the plasmid, the nucleotide sequences of the mutant keg1 were determined. The keg1-1 mutant gene with a single amino acid substitution H124L and good temperature-sensitive response was used to replace the mutant gene with a single amino acid substitution H124L and good temperature-sensitive response was used to replace the authentic KEG1 of KSY99 to obtain AKY17 with chromosomal keg1-1 gene.

Subcellular Fractionation and Solubility Test of Membrane Proteins—Cells were converted to spheroplasts and burst in B88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 200 mM sorbitol) containing protease inhibitors (1 μg/ml each of chymostatin, aprotinin, leupeptin, pepstatin A, antipain, and 1 mM phenylmethylsulfonyl fluoride). Unbroken cells were removed by centrifugation at 1000 × g for 5 min to obtain the total cell lysate. For subcellular fractionation, total cell lysate was centrifuged at 10,000 × g for 10 min to generate the pellet (P10) and supernatant (S10), and then S10 was centrifuged at 100,000 × g for 60 min to generate the pellet (P100) and supernatant (S100). Each fraction was adjusted to the original volume of the lysate, and the same amount was applied for SDS-PAGE and Western blotting.

Anti-Scs2 rabbit antiserum (11) was kindly provided by Dr. Kikai, Osaka). The cell wall fraction was collected by centrifugation (10,000 × g) for 1 h. The precipitate was removed by centrifugation at 10,000 × g for 60 min. The supernatant was mixed with anti-Myc monoclonal antibody and kept gently rotating at 4°C for 1 h. Protein A-Sepharose beads (Amersham Biosciences) washed with B88 containing 1% Triton X-100 were added, and incubation was continued at 4°C for an additional hour. The beads were washed 5 times with B88 containing 1% Triton X-100 and then suspended in the SDS sample buffer at 37°C for 5 min to obtain the SDS-PAGE samples.

Immunoprecipitation—Cells were converted to spheroplasts and burst in B88 containing protease inhibitors. Unbroken cells were removed by centrifugation at 1000 × g for 5 min. The cleared lysate was mixed with 1/10 volume of 10% Triton X-100 and kept on ice for 15 min. The mixture was centrifuged at 100,000 × g for 60 min. The supernatant was mixed with anti-Myc monoclonal antibody and kept gently rotating at 4°C for 1 h. Protein A-Sepharose beads (Amersham Biosciences) washed with B88 containing 1% Triton X-100 were added, and incubation was continued at 4°C for an additional hour. The beads were washed 5 times with B88 containing 1% Triton X-100 and then suspended in the SDS sample buffer at 37°C for 5 min to obtain the SDS-PAGE samples.

Immunofluorescence Microscopy—Fixation and permeabilization of the yeast cells for indirect immunofluorescence was performed as described by Wooding and Pelham (12), and subsequent steps were performed as described by Vashist et al. (13) with some modifications; a description of the procedure is as follows. Log-phase cells grown at 30°C were fixed by adding 2.5 ml of fresh 10% paraformaldehyde to 7.5 ml of yeast culture and pelleted by centrifugation. They were resuspended in 3.2 ml of 0.1 M potassium phosphate, pH 7.5, 1.8 ml of paraformaldehyde solution was added, and fixation was continued for an additional 15 min. Cells were then washed 4 times in 0.1 M potassium phosphate, pH 7.5 (PP) and resuspended in 1 ml of PP with 1.2 M sorbitol containing 100 mM dithiothreitol. 10 μl of lyticase was added, and incubation was continued at 30°C. The spheroplasts were harvested by centrifugation and resuspended in 50 mM NH4Cl in SPP (0.1 M potassium phosphate, pH 7.5, with 1.2 M sorbitol) and then in SPP before being transferred to poly-l-lysine-coated slides. The slides were immersed in methanol for 6 min and acetone for 30 s, both at −20°C, and then air-dried. The anti-Myc mouse monoclonal antibody was diluted to 1/40 in 1% skim milk, 0.1% bovine serum albumin, and 0.05% Tween 20 in Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl), and incubations were carried out overnight at 4°C. Slides were washed and incubated in the secondary antibodies (rhodamine-conjugated goat antibody to mouse immunoglobulin G) for 2 h at room temperature, washed with phosphate-buffered saline, and mounted as described by Kilmartin and Adams (14). Images were obtained using an FV500 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

RESULTS

Keg1/Yfr042w Is an ER-resident Integral Membrane Protein—KEG1/YFR042w is an essential gene because its disruptants could not form colonies under the standard laboratory culture conditions (17). According to the Saccharomyces Genome Database, it encodes a 200-amino acid polypeptide of \( M_r = 23,555 \) and pl = 10.5, the biochemical function of which remains unknown. Homologous sequences were found in several fungal genome databases by a FASTAP search (Fig. 1A), but no homologues were found in the data base of higher eukaryotes. The similar sequences of Candida glabrata, Ashbya gossypii, and
K. lactis have amino acid identities of 57, 50, and 45%, respectively. We routinely use two parental *S. cerevisiae* strains, KA31a in our laboratory stock and BY4741 obtained from the EUROSCARF Consortium. There are 10 base differences in the 2603-nucleotide fragment containing the whole *KEG1* gene. As a result, the KA31a *Keg1* protein has 3 amino acid substitutions, G3V, Y55C, and N84D, compared with the sequence of the BY4741 *Keg1* protein as registered in the sequence data base. The identical amino acids are highlighted with reverse letters. The asterisk indicates the H126L amino acid substitution position of the *keg1-1* allele. B, the hydrophobicity index was calculated according to Kyte and Doolittle (26), with a window size of 19 amino acids. Bars and asterisks indicate the hydrophobic segments and H126L position as in *A*.

**FIGURE 1.** Sequence alignment of Keg1 protein and its yeast homologues and the hydrophathy profile of Keg1 protein. A, amino acid sequence alignment of the Keg1 homologues. Sc, *S. cerevisiae* KA31a; Cg, *C. glabrata* CBS138 (XP_0452447.1); Ag, *A. gossypii* ATCC10895 (NP_985794.1); Kl, *Kluyveromyces* RRL Y-1140 (XP_451048.1). Four hydrophobic segments are indicated by bars over the sequences. The identical amino acids are highlighted with reverse letters. The asterisk indicates the H126L amino acid substitution position of the *keg1-1* allele. B, the hydrophobicity index was calculated according to Kyte and Doolittle (26), with a window size of 19 amino acids. Bars and asterisks indicate the hydrophobic segments and H126L position as in *A*.

Keg1 is an integral membrane protein as predicted by its amino acid sequence.

The intracellular localization of GFP fusion protein was observed by fluorescent microscopy. The fluorescence signal of GFP-Keg1 was found in a central ring and ribbons at the cell periphery close to the plasma membrane (Fig. 4). This is the typical pattern common to the ER-resident proteins that reside in the nuclear envelope and peripheral ER, distinct from the fluorescence images of the other large organelles such as the mitochondria and vacuole or the smaller organelles including the Golgi, endosomes, and peroxisome. The subcellular fractionation indicated that most of GFP-Keg1 was in the P10 fraction, and only a small portion was in the P100, although a small portion remained in the precipitate in comparison with the reference membrane proteins Ssc2 or tagged Kre6. We concluded that Keg1 is an integral membrane protein as predicted by its amino acid sequence.

Isolation of the Temperature-sensitive *keg1-1* Allele—To analyze the function of Keg1 protein in detail, we sought to construct temperature-sensitive *keg1* mutant alleles. Fifteen temperature-sensitive *keg1* mutant candidates were obtained by error-prone PCR mutagenesis as described under “Experimental Procedures.” The *keg1-1* allele was selected for further study because it has a single amino acid alteration H124L and good...
temperature-sensitive response. The 124th amino acid, His, is well conserved among the four Keg1-related sequences (Fig. 1A). Fig. 6A shows the temperature sensitivity of the keg1-1 mutation. The colony formation defect was complemented by introducing either of the Myc6-KEG1 or GFP-KEG1 plasmid, which also indicated that these tagged polypeptides were functional. The keg1-1 mutant had an increased sensitivity to chitin binding dye Calcofluor white, which was also reversed by introducing GFP-KEG1 to near the wild type (Fig. 6B). The remain-

**FIGURE 2. Activity of the tagged Keg1 derivatives.** A, activity of GFP-Keg1 protein. The heterozygous KEG1/keg1 Δ::kanMX4 diploid (Y25856) was transformed with plasmid pCA106 (CEN, URA3 GFP-KEG1), and the transformant CAY207 was sporulated. The tetrad progenies (colonies 1–4 and 5–8) were tested for growth on a YPD plate, requirement of pCA106 (growth on SC+5-FOA plate), and the presence of keg1 Δ::kanMX4 (growth on YPD+G418 plate). B, activity of Myc6-Keg1 protein. Y25856 was transformed with plasmid pCA120 (CEN, URA3 Myc6-KEG1), the transformant CAY327 was sporulated, and the tetrads were analyzed as in A.

**FIGURE 3. Solubilization test of tagged Keg1 and Kre6 proteins.** A, the cells having Myc6-Keg1 and Kre6-GFP (KNY21) were disrupted and subjected to the solubilization test in the buffer (B88) containing 1% Triton X-100 (Tx100), 0.1 M Na2CO3 (pH 11), or 2 M urea (Urea). After separating the supernatant (S) and precipitate (P) from the total cleared lysate (T), the tagged proteins and the ER membrane protein Scs2 were detected by Western blotting. B, cells having GFP-Keg1 and Kre6-Myc6 (KNY19) were tested as in A.

**FIGURE 4. Intracellular localization of Keg1 protein by fluorescent microscopic observation.** The cells having GFP-Keg1 and Kre6-Myc6 (KNY19) were fixed using paraformaldehyde and subjected to indirect immunofluorescent staining using anti-Myc monoclonal antibody and rhodamine-labeled secondary antibody. Fluorescent images of GFP-Keg1 and Kre6-Myc6 were shown with their merge and Nomarski images.

**FIGURE 5. Subcellular fractionation of Keg1 protein.** The cells having GFP-Keg1 and Kre6-Myc6 (KNY19) were converted to spheroplasts and disrupted, and the total cell lysate (TCL) was subjected to differential centrifugations to obtain the 10,000 × g supernatant (S10) and pellet (P10) and then 100,000 × g supernatant (S100) and pellet (P100). The sample volumes were adjusted to the concentration of total cell lysate, and the proteins shown on the left were detected by Western blotting.
Keg1 Participates in β-1,6-Glucan Synthesis

To get a clue to uncover the function of Keg1, we screened for the multicopy suppressor genes of the temperature sensitivity of the *keg1-1* mutant. The *keg1-1* mutant did not form colonies on the YPD plate at 37 °C. We introduced a 2-μm plasmid library into the *keg1-1* mutant cells, and obtained three transformants whose colony-forming activity at 37 °C was dependent on the introduced plasmids (data not shown). These plasmids had different DNA fragments, but the *ROT1* gene was common in all inserts, and the *ROT1* subclone had the multicopy suppressor activity for colony formation (Fig. 7A). Rot1 was previously reported as being a membrane protein required for maintaining normal levels of the cell wall β-1,6-glucan (18) and may function with BiP/Kar2 in protein folding (19). The Calcofluor white hypersensitivity of the *keg1-1* mutant was partially recovered by introducing the multicopy *ROT1* gene (Fig. 7B). The tagged version of *ROT1* with GFP or 3HA either at the N or C terminus did not have the suppressor activity, which made it difficult to examine the protein-protein interaction between Rot1 and Keg1 in further detail. Using a guinea pig anti-Rot1 antiserum generously provided by Dr. Kenji Kohno (Nara Institute of Science and Technology, Japan), the Rot1 signal was faintly detected in the negative control sample and not specifically enriched in the immunoprecipitate of Myc6·Keg1 (data not shown). Therefore, we could not conclude whether Rot1 and Keg1 have a physical interaction or not.

**Interaction and Colocalization of Keg1 Protein with Kre6**—A physical interaction between Keg1 and Kre6 proteins was reported in the yeast two-hybrid interaction screening (20). The *KRE6* gene was discovered by the killer toxin resistant mutant phenotype and was found to encode an 80-kDa type II integral membrane protein that is required for β-1,6-glucan biosynthesis (21, 22). To confirm the Keg1·Kre6 physical interaction biochemically by immunoprecipitation experiments, we constructed tagged *KRE6-Myc6* and *KRE6-GFP*. Introduction of *KRE6-Myc6* recovered the Calcofluor white sensitivity of Δkere6 partially, but *KRE6-GFP* did not have such activity, probably because a large GFP interferes with the Kre6 function (Fig. 6C). A Triton X-100-solubilized cleared lysate was prepared from the cells producing both GFP·Keg1 and Kre6-Myc6 and was subjected to immunoprecipitation using anti-Myc monoclonal antibody and rhodamine-labeled sec-
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FIGURE 8. Physical interaction between Keg1 and Kre6 proteins. A, co-immunoprecipitation of GFP-Keg1 with Kre6-Myc. Cells having GFP-Keg1 with or without KRE6-Myc were disrupted with glass beads and solubilized using 1% Triton X-100. Anti-myc monoclonal antibody and protein A-Sepharose beads were added to collect Kre6-Myc, and the associated materials. GFP-Keg1 in the bead-bound materials (B) comparing the 1/10 samples of starting sample (S) and unbound materials (U) were detected by Western blotting using an anti-GFP antibody. B, co-immunoprecipitation of Kre6-GFP with Myc-Keg1. Cells having KRE6-GFP with or without Myc-Keg1 were used to test the influence of tags, and Kre6-GFP was detected by Western blotting as in A.

FIGURE 9. Intracellular localization of Keg1 and Kre6 in the mutants of the interacting protein. A, fluorescence of GFP-Keg1 and Nomarski images of the cells of krei6Δ-kanMX4 and KRE6 strains were shown. B, indirect immunofluorescence of Kre6-Myc and Nomarski images of the cells of keg1-1 and KEG1 strains grown at 25 °C were shown.

ordary antibody and compared these results with the fluorescent images of GFP-Keg1 in the same cells. The signals of Kre6-Myc were clearly found around the nucleus and at the cell periphery, which suggested that the localization was in the ER rather than in the Golgi, which would give a punctate pattern (Fig. 4). The fluorescent images of GFP-Keg1 and Kre6-Myc closely resembled each other, and the merged images of the same cells showed that they are almost identical (Fig. 4). Subcellular fractionation indicated that Kre6-Myc had almost recovered in P10 and slightly recovered in P100 as GFP-Keg1 and the ER-marker Scs2, in contrast to the similar amounts of the early Golgi-marker Van1 being found in P10 and P100 (Fig. 5). Therefore, we conclude that GFP-Keg1 and Kre6-Myc co-localize in the same subcellular compartment, i.e. in the ER membrane, and are able to physically interact without solubilizing the membrane.

We examined whether the absence of Kre6 might have an influence on the localization or stability of the GFP-Keg1 protein using the Δkre6 null mutant. The typical fluorescent images of the ER-resident protein were similarly observed in either KRE6 or Δkre6 cells (Fig. 9A). Therefore, the presence of Kre6 was not required for localization or stability to occur in Keg1 in the ER. Similarly, the images of Kre6-Myc had no difference in both KEG1 and keg1-1 cells at the permissive temperature (Fig. 9B). At the nonpermissive temperature, we could not determine the localization of the immunofluorescent signal of Kre6-Myc even in the wild-type cells.

Characterization of KEG1 by the Phenotype of the Temperature-sensitive Mutant Allele keg1-1 at Permissive Temperatures—

We examined the intracellular traffic of the vacuolar carboxypeptidase Y, the plasma membrane glycosylphosphatidylinositol-anchored protein Gas1, and secretory invertase, but no significant defect was found in the keg1-1 mutant at its nonpermissive temperature (data not shown). In most temperature-sensitive mutants, the mutant protein is not as active and/or stable as the parent wild-type protein at any temperature. Therefore, the temperature-sensitive mutant often shows some defects even at the temperature that is permissive for colony formation of the mutant cells. The keg1-1 mutant showed increased sensitivity to the cell wall-digesting enzyme zymolyase. As shown in Fig. 10, the optical density of the suspension of the keg1-1 mutant cells grown at the permissive temperature decreased more rapidly than that of the wild-type cells. The suspension of the Δkre6 mutant cells was used as a control, which showed more rapid lysis by zymolyase. These results indicate a functional role of Keg1 in the maintenance of the cell wall integrity.

K1 killer toxin binds to a cell-wall receptor containing β-1,6-glucan and kills yeast (3). Because KRE6 was discovered by K1 killer resistance of the mutant, we next examined the sensitivity of the keg1-1 mutant to the K1 killer toxin. As shown in Fig. 11A, the size of the growth-inhibiting zone of the keg1-1 mutant
Keg1 Participates in β-1,6-Glucan Synthesis

The KEG1 gene encodes an ER-resident N-glycoprotein (19), and the Ror1 mutant showed a severe growth defect that was partially restored by the addition of sorbitol. The mutant also showed K1 killer toxin resistance and reduced β-1,6-glucan contents in the cell wall (18). Therefore, it is likely that there is some functional relationship between Keg1 and Ror1. Our attempts to detect a physical interaction between Keg1 and

![Figure 10. Zymolyase hypersensitivity of the keg1-1 cells. The wild-type (BY4741, circles), keg1-1 (AKY17, squares), and Δkre6 (Y05574, triangles) cells were grown in YPD at 25 °C until A{sub}600nm = 0.5. The cells were collected, washed, and suspended in 10 mM Tris-HCl, pH 7.4, at A{sub}600nm = 0.5. After the addition of the final 2 μg/ml of zymolyase at 25 °C, A{sub}600nm of the mixture was measured at the time indicated. Average values of the three experiments were plotted with the S.D.](Image 10)

![Figure 11. K1 killer toxin sensitivity and the glucan content of keg1-1 cells. A, the wild-type (WT) (BY4741), keg1-1 (AKY17), and Δkre6 (Y05574) were grown in YPD at 30 °C until A{sub}600nm = 1.0 and plated on a low-pH YPD plate. Five μl of a fresh overnight culture of the killer producer (NCYC232) grown at 20 °C was spotted, and the plates were incubated at 25 °C for 2 days to show the growth inhibitory zone around the killer producer spot. B, the cells were grown in YPD at 25 °C until A{sub}600nm = 1.0, and the amounts of the alkali-insoluble β-1,3-glucan (gray bars) and β-1,6-glucan (open bars) were determined as described under "Experimental Procedures." Average values of the three experiments were shown with the S.D.](Image 11)

size of halo became similar to that of wild type (data not shown), which indicated that this phenotype was temperature-dependent. This result suggests that Keg1 may have a functional role in the synthesis of β-1,6-glucan as Kre6.

**Cell Wall Glucan Contents of the keg1-1 Mutant Cells**—Next, we sought to examine the composition of the cell-wall glucan in the keg1-1 mutant. We examined the contents of the alkali-insoluble β-1,3-glucan and β-1,6-glucan by enzymatic methods as described under “Experimental Procedures.” As shown in Fig. 11B, the alkali-insoluble β-1,6-glucan of the keg1-1 mutant cell (8.4 ± 1.6 μg/ml culture) was approximately half that of the wild-type cell (17.5 ± 3.0 μg/ml culture) and was similar to that of the Δkre6 mutant cell (8.6 ± 0.3 μg/ml culture). These alterations are consistent with the K1 killer resistance of the mutant cells. Concomitantly with this decrease, the amounts of alkali-insoluble β-1,3-glucan significantly increased in the keg1-1 and the Δkre6 mutant cell (32.2 ± 5.6, 45.0 ± 5.8, and 43.0 ± 7.3 μg/ml culture in the wild-type, keg1-1, and Δkre6 cells, respectively). The alkali-soluble β-1,3-glucan content was determined by an aniline blue binding assay. It had also increased to 141 ± 28 and 137 ± 29% in the keg1-1 and the Δkre6 mutant cells, respectively, in the wild-type cells. These results suggest that the Keg1 protein, in combination with the Kre6 protein in the ER, participates in the synthesis of β-1,6-glucan in the cell wall.

**DISCUSSION**

The KEG1 studied in this report has been an essential but previously uncharacterized gene even after extensive large scale post-genomic studies. We selected this gene because our preliminary study showed that the GFP-tagged gene product could functionally rescue the colony-forming activity of the null mutant, and it was clearly localized in the ER. Because no significant phenotype was observed in the cells in which Keg1 protein was being depleted by promoter shut-off, we constructed a temperature-sensitive allele of KEG1 to characterize the gene product. The temperature-sensitive mutant protein would have many advantages over the loss of the gene product, as the temperature shift would be better to detect a rapid response in the phenotype, the mutant protein would have an altered activity that could appear in the phenotype even at the permissive temperatures, and its suppressor mutations or multicopy suppressor genes would give us valuable information about the uncharacterized gene of interest. As we had expected, the keg1-1 mutant showed a distinct phenotype including Calcofluor white hypersensitivity, K1 killer toxin resistance, and reduced content of the cell wall β-1,6-glucan.

Our multicopy suppressor screening found that the temperature-sensitive colony formation defect of keg1-1 could be suppressed by the introduction of multicopy ROT1. Calcofluor white sensitivity of the keg1-1 mutant also recovered partially. The ROT1 gene encodes an ER-resident N-glycoprotein (19), and the Ror1 mutant showed a severe growth defect that was partially restored by the addition of sorbitol. The mutant also showed K1 killer toxin resistance and reduced β-1,6-glucan contents in the cell wall (18). Therefore, it is likely that there is some functional relationship between Keg1 and Rot1.
Rot1 have been unsuccessful so far. Takeuchi et al. (19) reported that Rot1 may function as an ER chaperon in collaboration with Bip/Kar2. If so, there may be only a temporal interaction between Keg1 and Rot1, which would be difficult to detect by immunoprecipitation. Alternatively, Rot1 may help in the formation of a functional complex including Keg1 and Kre6, which we found in this study and assumed to be involved somehow in the β-1,6-glucan synthesis. We sought to examine if the interaction of Keg1 and Kre6 is altered in the absence of Rot1. However, cultivation of the Δrot1 cells was too difficult to obtain reliable immunoprecipitation data. Another membrane protein-encoding gene, BIG1, whose mutation also resulted in a reduction in β-1,6-glucan (24), did not show the multicopy suppressor activity of keg1-J.

The interaction between Keg1 and Kre6 suggested by yeast two-hybrid screening was confirmed by our immunoprecipitation experiments. This interaction seemed confusing at first because Kre6 was previously reported to be localized in the early Golgi compartment (23). This conclusion was based on the observation of Kre6-GFP expressed from a high copy plasmid because the attempts using a centromeric plasmid were reported to be unsuccessful. The Kre6-GFP signals were mostly co-localized with Och1, nearly half with Vrg4, and a few with Kex2. Our immunofluorescent data on Kre6 were all based on genes with the authentic promoter on the centromeric plasmids. Because tagging of Kre6 with GFP seemed to cause more damage to its activity than tagging with Myc6 (Fig. 6C), we believe that the Kre6-Myc6 data would be more reliable than the Kre6-GFP data. The Kre6-Myc6 signal gave the typical ER-resident protein profile (Figs. 4 and Fig. 9B) distinct from the Golgi compartment profiles (25). In addition, the result of subcellular fractionation was consistent with these microscopic observations (Fig. 5). The overexpression and possibly inadequate tagging of Kre6 might be the cause of the previous observation. We concluded that Kre6 is an ER membrane protein, although experiments using the Kre6-specific antibody and the wild-type KRE6 gene without tagging will be formally required to confirm this conclusion. The protein-protein interaction of Keg1 and Kre6 is the first example of a possible protein complex working in the process of β-1,6-glucan synthesis. We hope that this finding becomes a clue to uncover the molecular roles of ER-resident proteins in making the cell-wall β-1,6-glucan.

The molecular process of β-1,6-glucan synthesis is one of the remaining mysteries in the well studied field of yeast biology. The extensive genetic analyses indicated that a number of genes whose products work in various compartments of the secretory pathway are concerned in β-1,6-glucan synthesis as described in the Introduction. Therefore, the location of its synthesis is controversial. Although the bulk of β-1,6-glucan is only detected at the cell surface, some essential events seem to occur in the ER (1, 3). Limited development of the biochemical tools, including the in vitro β-1,6-glucan synthesis assay system is another obstacle preventing the elucidation of the exact roles of the gene products. Here, we have to add an essential gene KEG1 in the list of the participants. An essential gene in β-1,6-glucan synthesis is the first case, although BIG1 and ROT1 are regarded as essential in the absence of an osmotic stabilizer. Because the cell wall β-1,6-glucan is an essential component of many fungi and is absent in the higher eukaryote, the essential Keg1 protein and its homologues will be an ideal target of antifungal agents.

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