Disordered Cell Integrity Signaling Caused by Disruption of the kexB Gene in Aspergillus oryzae†

Osamu Mizutani, Akira Nojima, Morimasa Yamamoto, Kentaro Furukawa, Tomonori Fujioka, Youhei Yamagata, Keietsu Abe,* and Tasuku Nakajima

Laboratory of Enzymology, Department of Molecular and Cell Biology, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

Received 8 January 2004/Accepted 12 April 2004

We isolated the kexB gene, which encodes a subtilisin-like processing enzyme, from a filamentous fungus, Aspergillus oryzae. To examine the physiological role of kexB in A. oryzae, we constructed a kexB disruptant (ΔkexB), which formed shrunken colonies with poor generation of conidia on Czapek-Dox (CD) agar plates and hyperbranched mycelia in CD liquid medium. The phenotypes of the ΔkexB strain were restored under high osmolarity in both solid and liquid culture conditions. We found that transcription of the mpkA gene, which encodes a putative mitogen-activated protein kinase involved in cell integrity signaling, was significantly higher in ΔkexB cells than in wild-type cells. The ΔkexB cells also contained higher levels of transcripts for cell wall-related genes encoding β-1,3-glucan transferase and chitin synthases, which is presumably attributable to cell integrity signaling through the increased gene expression of mpkA. As expected, constitutively increased levels of phosphorylated MpkA were observed in ΔkexB cells on the CD plate culture. High osmotic stress greatly downregulated the increased levels of both transcripts of mpkA and the phosphorylated form of MpkA in ΔkexB cells, concomitantly suppressing the morphological defects. These results suggest that the upregulation of transcription levels of mpkA and cell wall biogenesis genes in the ΔkexB strain is autoregulated by phosphorylated MpkA as the active form through cell integrity signaling. We think that KexB is required for precise proteolytic processing of sensor proteins in the cell integrity pathway or of cell wall-related enzymes under transcriptional control by the pathway and that the KexB defect thus induces disordered cell integrity signaling.

Some secretory proteins of eukaryotic cells are converted from the precursor proteins to the mature proteins after modification through processes such as various glycosylation reactions and limited proteolysis within the Golgi apparatus. The modification process is well conserved from yeast to mammals, and the target proteins of the process are expanded to peptide hormones, neuropeptides, serum proteins, cell growth factors, and cell growth factor receptors. Therefore, elucidation of the protein modification process serves a clue to understand the physiological meaning of the posttranslational process. Kexin protein modification process is well conserved from yeast to mammals, and the target proteins of the process are expanded to peptide and cell growth factor receptors. Therefore, elucidation of the protein modification process serves a clue to understand the physiological meaning of the posttranslational process. Kexin is a Ca2+-dependent transmembrane protease prokine that cleaves the secretory proproteins on the carboxyl side of Lys-Arg and Arg-Arg in a late Golgi compartment (15, 46). Kexin-like enzymes have been found from yeast to mammals (16, 19, 54, 65). Because filamentous fungi, including Aspergillus species, secrete large amounts of proteins that are predicted to be modified through proteolysis in a Golgi compartment, kexins also are thought to be key enzymes of the proteolytic process in the Golgi compartment of the fungi. Aspergillus kexins are found in Aspergillus nidulans (39) and A. niger (29).

Disruption of the A. niger kexB gene causes various morphological alterations such as shorter and multibranched hyphae (29, 60). Recent studies have shown that Kex2p in the dimorphic yeast Candida glabrata is required for cell surface integrity (3). A bioinformatics approach, using the C. albicans genome database, assigned 147 open reading frames (ORFs) as encoding potential substrates for Kex2p in C. albicans (56). Among these ORF products, some predicted Kex2p targets were cell wall-related proteins and sensor proteins including GAS1 and WSC2 homologs. In light of the prediction from the bioinformatics approach using C. albicans and the morphological defects of the A. niger kexB disruptant, kexin activity in Aspergillus species also seems to be concerned primarily with formation of the cell wall and morphogenesis. Therefore, maintenance of morphogenesis in fungi is likely to be one of the biologically important functions in which kexin plays some roles. However, the mechanism underlying the involvement of kexB in morphogenesis in fungi is still unclear.

Although the Saccharomyces cerevisiae kex2-null mutant (kex2Δ) does not exhibit morphological defects compared with the phenotypes of other filamentous fungi, additional genetic defects in MPK1 (SLT2), together with the kex2Δ mutation, cause lethality (62). Since the yeast MPK1 gene encodes a mitogen-activated protein (MAP) kinase in the cell integrity pathway that plays an essential role in maintaining the biogenesis and integrity of the cell wall (41), kexin seems to be involved in cell wall integrity in S. cerevisiae. The cell integrity pathway is induced in response to several environmental stimuli, resulting in the increased expression of numerous genes, many of which encode integral cell wall proteins (glycosylphosphatidylinositol proteins, PIR [proteins with internal repeats] family proteins, and others) or enzymes, including β-1,3-glucan synthases (Fks1p and Fks2p) and chitin synthases, required for...

* Corresponding author. Mailing address: Laboratory of Enzymology, Department of Molecular and Cell Biology, Graduate School of Agricultural Science, Tohoku University, I-1 Amamiya, Tsutsumi-dori, Aobaku, Sendai 981-8555, Japan. Phone: 81-22-717-8776. Fax: 81-22-717-8778. E-mail: kabe@biochem.tohoku.ac.jp.
† Supplemental material for this article may be found at http://ec.asm.org.
cell wall biogenesis in *S. cerevisiae* (13, 31, 67, 68). The *A. nidulans* *mpkA* gene that is a counterpart of the yeast *MPK1* (*SLT2*) was cloned and characterized (7). An *mpkA* deletion mutant (*ΔmpkA*) was constructed, and its morphological defects suggested that the kinase is involved in germination of conidial spores and polarized growth. As described above, the apparent morphological changes observed in the *A. niger* *kexB* disruptant suggest the possibility that involvement of kexin in cell integrity is more significant in *Aspergillus* fungi than *S. cerevisiae*. Consequently, our studies focus on gaining an insight into the roles of kexin in morphogenesis and cell wall integrity in *Aspergillus* fungi.

*A. oryzae*, which is an economically important filamentous fungus, as well as *A. niger*, is used in the manufacture of fermented foods and in the production of enzymes for medical and food-grade use (10, 27, 71). Because its industrial importance has fortunately accelerated the establishment of platforms of fungal genomics, including an expressed sequence tag (EST) database and genome sequence information, *A. oryzae* genomics is now one of the most advanced platforms of fungal genomics and thus is also valuable for cell biology research on filamentous fungi (43; http://www.aist.go.jp/RIODB/flip/index.html).

As a part of the *A. oryzae* genome projects, we manufactured *A. oryzae* cDNA microarrays consisting of approximately 2,000 cDNA clones by using information in the *A. oryzae* EST database (44). In the present study, we isolated an *A. oryzae* *kexB* knockout (*ΔkexB*) strain that had remarkable morphological defects, and the mutant phenotypes, surprisingly, were suppressed under highly osmotic conditions. We investigated the function of *kexB* in morphogenesis by using the *ΔkexB* knockout strain and our cDNA microarrays. Here we report (i) cloning of the *A. oryzae* kexin gene (*kexB*) and enzymatic characterization of KexB in the membrane fraction isolated from a KexB-overexpressing *A. oryzae* strain, (ii) construction of the *ΔkexB* strain and observation of its phenotype, (iii) comparison of gene expression profiles between the *ΔkexB* strain and the wild type under solid-culture conditions by using our cDNA microarrays and Northern blot analysis, and (iv) demonstration of constitutive upregulation of both transcription levels of the *mpkA* gene and phosphorylation levels of MpkA in the *A. oryzae* *ΔkexB* strain on the solid culture. Our results suggest that disruption of *kexB* in *A. oryzae* leads to morphological changes attributable to disordered cell integrity signaling. We discuss the contribution of *A. oryzae* *kexB* to the cell integrity pathway and morphogenesis.

### MATERIALS AND METHODS

**Strains, media, and growth conditions.** We used *A. oryzae* RIB40 (ATCC 42149) as the wild type and for constructing the *kexB* knockout mutant. This strain was also used for the *A. oryzae* EST and genome sequencing projects (43). *A. oryzae* niaD300 (niaD), a niaD mutant derived from RIB40, was used as a recipient strain for transformation and protein expression. These strains were grown in YPD complete medium (1% yeast extract, 2% polypeptone, 2% dextrose) or Czapek-Dox minimal medium (CD) (52). Instead of glucose, maltose was added to CD medium as an inducer for overexpression by recombinant *A. oryzae*. CD medium supplemented with 0.1 μg of porythiamine (*TaKaRa Bio Inc., Tokyo, Japan*) per ml was used as the selection medium for the *kexB* knockout derivatives of *A. oryzae*. To isolate niaD mutants from the *kexB* knockout strain of *A. oryzae*, 500 mM chlorate and either nitrite (NO$_3$), hypoxanthine, glutamate, or ammonium chloride as a nitrogen source were added to CD medium.

**Molecular cloning and sequencing of *kexB***. For subcloning, *Escherichia coli* XL1-blue (hsdR17 supE44 recA1 endA1 gyrA thi relA1 lacF proAB + lacIq

lacZAM15::Tn10, Ter1) cells and pBlueScript II SK (+) plasmid (TOYOBO Inc., Tokyo, Japan) were used as host and vector, respectively, for DNA manipulation. The vector pGEM-T Easy (Promega Co., Tokyo, Japan) was used for TA cloning of PCR products. All basic molecular biology procedures were carried out as described by Sambrook et al. (52). To clone the kexB gene, we searched the *A. nidulans* EST database of the University of Oklahoma (http://www.genome.ou.edu/fungal.html) with the yeast (*S. cerevisiae*) *KEX2* gene and found an approximately 600-bp homologous sequence. The nucleotide identity between the yeast *KEX2* gene and the obtained *A. nidulans* DNA fragment was about 40%. PCR primers (5’-CGCTTCTGGGAAGATCAACAAATC-3’ and 5’-CTGACTTCTCGCGAGCTC-3’) were designed on the basis of the sequence of the fragment, and PCR was performed using genomic DNA from *A. nidulans* as the template. We obtained a 581-bp fragment, which we used as a probe for screening an *A. nidulans* cDNA library. Three positive clones were obtained from 2,000 plaques, and the positive clone with the longest insert was sequenced. The insert contains a 2,460-bp ORF encoding a single polypeptide comprising 819 amino acid residues. A FASTA search against the *A. oryzae* EST database (http://www.aist.go.jp/RIODB/flip/index.html) was performed with the full-length sequence of the *A. nidulans* *kexB* gene (DDBJ/EMBL/GenBank accession no. AB056726). Clone 6-58 in the *A. oryzae* EST database was homologous, and the DNA sequence of this clone was completed using the ABI PRISM BigDye Terminator cycle-sequencing ready reaction kit version 2.0 (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 377 sequencer (Applied Biosystems Japan). The initiation codon of the *A. oryzae* *kexB* gene was predicted by comparison with the initiation codon of the *A. niger* *kexB* gene (24) and the position of the discovery of a stop codon 33 bp upstream of the initiation codon in the *A. oryzae* *kexB* cDNA.

**Creation of the *kexB*-overexpressing strain.** The overexpression plasmid pNXK1 was constructed as follows. The *kexB* gene was PCR amplified using *Z*. *Taq* DNA polymerase (*TaKaRa*) and a pair of primers, 5’-AAGCTTATA TGCGGCTTTCCGAAAG-3’ and 5’-AAGCTTGGATACGGGAAATCGAAGCC-3’. Each primer was designed to introduce a HindIII site (underlined). *Eco* RI clone 6-58 of *A. oryzae* was used as the template. The amplified fragment was inserted into the pGEM-T Easy vector (Promega) and sequenced. The plasmid was digested with HindIII, and the digested fragment was ligated into the HindIII sites of the pNGA142 vector that has the gla412 promoter (24). The constructed expression plasmid was named pNXK1. Transformation of *A. oryzae* niaD300 (niaD) was performed using the modified protoplast-polyethylene glycol method (20) and pNXK1 digested with BamHI. The BamHI-digested pNGA142 also was introduced into the niaD300 strain as a control (pNGA strain). For protoplast formation, 5 mg of lysing enzyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 10 mg of cellulase Onozuka R-10 (Yakult Co., Tokyo, Japan) per ml, and 10 mg of Yatalase (*TaKaRa*) per ml were used. Transformants were subcultured at least three times on CD agar plates to obtain homokaryotic strains.

**Enzyme assay.** The cells were grown in 50 ml of CD liquid medium with shaking for 2 days at 30°C. They were then transferred to CD liquid medium containing 2% maltose and cultured for 1 h at 30°C. They were collected using glass filters (Asahi Techno Glass Co., Funabashi, Japan) and ground in a mortar and pestle. The ground cells were resuspended in 0.2 M HEPEs (pH 7.6) containing 1 mM EDTA and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was further centrifuged at 100,000 × g for 90 min at 4°C. The precipitate was resuspended in 50 mM HEPEs (pH 7.6) containing 1 mM EDTA, 50 mM NaCl, 2% sodium deoxycholate, and 20% glycerol, and the mixture was centrifuged for 90 min at 4°C and 100,000 × g. The supernatants were pooled as the membrane protein fraction. Enzyme assays were performed as described previously (45), but 20 mM Tris-HCl (pH 7.0) was used as the assay buffer.

**Creation of the *kexB* disruption mutant.** The plasmid for *kexB* gene disruption (pPΔkexB-B) was constructed as follows. The *kexB* fragment was obtained by PCR with primers 1 (5’-TAATGGCGGCTTTCCGGAAGTCCCGGTTAG-3’) and 2 (5’-TAGGGTGAAAGCATAAGATGCAGAGTCCG-3’) by using EST clone 6-58 of *A. oryzae* as a template. These primers were designed to introduce PstI and HindIII sites (underlined) that site-specifically cleave the target region, respectively. The fragment was digested with PstI and HindIII and ligated into the Pst-HindIII fragment of the pPTRI vector (*TaKaRa*), which contains the porythiamine resistance gene (*ptra*). The constructed plasmid was named pPXkexB. In addition, a stop codon linker (5’-CTAGTGTGTTAGATAAGGAGTCCATA-3’) was inserted into the NheI site of pPXkexB. The linker had termination codons in every frame, and the expression of the wild-type plasmid was named pPXkexB-st (Fig. 1A). After transduction of *A. oryzae* RIB40 (wild type) was performed as described above by using pPXΔkexB-st digested with Xhol (Fig. 1B). *A. oryzae* transformants were screened for
resistance to pyridhamine (0.1 μg/ml) and were subcultured at least three times on CD agar plates containing pyridhamine.

Candidates for the knockout strain were selected by colony PCR by using primer sets 1 (Δsense 1 [5'-GTGGTGTAACGCAGGTTCATCC-3'] and Δantisense 2 [5'-CGCGGAAGTCATCTTGACAGG-3']) and primers (7) (DDBJ/EMBL/GenBank accession no. AAD24428), which expression is under the control of the galactose-inducible GAL1 promoter (30). A fragment containing the complete ORF of the A. oryzae mpkA cDNA was ligated with HindIII and XbaI and ligated into the HindIII-XbaI fragment of plasmid YEp24, which was used as the template. The amplified fragment was digested with HindIII and SpeI, and the digested fragment was ligated into the plasmid YEp24. The resulting plasmid was used as the template.

Northern blot hybridization. mRNAs were prepared as described above from the kexB disruptant and wild-type strains cultured on CD agar plates at 30°C for 50, 70, and 105 h or a CD agar plate plus 0.8 M NaCl at 30°C for 105 h. mRNAs (50 ng each) were denatured through an aqueous-spray method (64) and transferred to Hybond-N nylon membranes (Amersham Biosciences) by using 7.5 mM NaOH. Blotted membranes were hybridized with the probes for chs, chb, gelA, or gelB of A. oryzae. The probe for the histone H2B gene was used as a quantitative control. Probes for chsC, chbB, gelA, or gelB were prepared by PCR using the primers 5'-GAGCTCTATCGGAGGACCACT TACTC-3' (forward) and 5'-CTTGGATATCCGGTGTAAGGAGCG-3' (reverse), 5'-ATGGGCTTACCAAGGCCCCTGAAAAGG-3' (forward) and 5'-AT GTTTCACTCGGAGGAGGAAGAAG-3' (reverse), 5'-ATATATATATA TICTCAGAGGATCTCGC-3' (forward) and 5'-TCAAGAAGAAAAATCAT CAAAACAAATAGCCC-3' (reverse) and 5'-CCOATAGTAGCCACAGT ATC CCGGGAAG-3' (forward) and 5'-GAAAGACATATAGGTT AAAAAAGAAC-3' (reverse). mRNAs were removed, and the transcript was produced through a point drying method. Dried colonies were coated with platinum-vanadium and observed under an S-700 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 15.0 kV.

mRNA isolation from mycelia in solid and liquid culture. Spores (10⁴) were inoculated on a sterile nylon mesh filter (Nippon Rikagaku Kikan Co., Ltd., Tokyo, Japan) on a CD plate and grown at 30°C for 4 days. The nylon mesh filter with mycelia of A. oryzae was then removed from the plate and frozen in liquid N₂. The mycelia were collected with a spatula and ground to a fine powder in a mortar. Total RNA was prepared from powdered cells by using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) as specified by the manufacturer. mRNA was isolated from the total RNA by using Message Maker (Invitrogen Co., Tokyo, Japan) as specified by the manufacturer. mRNA was also prepared from liquid cultures. Conidia (5 x 10⁷) were inoculated into 100 ml of CD medium and cultured at 30°C for 22 h, and mRNA was isolated as described above.

Microarray analyses. Details of A. oryzae cDNA microarrays were described by Maeda et al. (44), and 2,000 cDNA clones on the cDNA microarray were chosen as highly expressed genes from approximately 5,000 nonredundant EST clones of the A. oryzae EST libraries (http://www.aist.go.jp/RIODB/ffdb/index.html) (43). Comparative microarray analyses between the ΔkexB and wild-type strains cultured on CD agar plates and on CD agar plates containing 0.8 M NaCl were performed. First, we examined the transcription levels of the histone H2B and H4 genes by using Northern blot analysis. Under both conditions, the transcription levels of the histone genes were the same in both the ΔkexB and wild-type strains. The data did not suggest that values are appropriate controls for microarray analyses. Fluorescently labeled cDNA was prepared from each mRNA by using Cy3-dUTP or Cy5-dUTP (Amersham Biosciences Inc., Tokyo, Japan) and the CyScribe first-strand cDNA labeling kit (Amersham Biosciences) as specified by the manufacturer. Cy3- and Cy5-labeled cDNAs (20 μl each) were mixed and purified using the Microcon-30 sample reservoir (Millipore Co., Tokyo, Japan). Human Cot-1 DNA (60 μg; Invitrogen) was added to the cDNA, which was then concentrated to 28 μl. Blocking reagents, including 4 μl of yeast tRNA (10 μg/μl; Invitrogen), 16 μl of poly(dA) (1 μg/μl; Invitrogen), 10.2 μl of 20X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 1.8 μl of 10% sodium dodecyl sulfate (SDS), were added to the mixture and boiled for 1 min. The mixture was cooled to room temperature. The labeled DNA was applied to a microarray slide (Asahi Techno Glass Co.) in a cassette chamber, and the chamber was incubated at 55°C for 9 h. The slide was washed sequentially with 2X SSC to 0.1% SDS at room temperature, 0.2X SSC-0.1% SDS at 40°C, and 0.2X SSC at room temperature for 5 min per wash. The slide was dried by centrifugation for 1 min at 300 x g.

Fluorescent DNA bound to the microarray was detected and quantified with a Genecipel 4000B microarray scanner (Axon Instruments, Foster City, Calif.) using the Genecipe Pro 3.0 software package to locate spots in the microarray. Data are means of triplicate determinations from three different experiments. Spots were quantified as the median of all pixel values in the spot region. The background was quantified for each spot separately as the median of all pixel values in the background region. Background values were subtracted from the respective pixel values to obtain net signal values. Net signal values greater than 1 were set to 1. Probes with net signal values below 1.000 for both channels were eliminated from further calculation. Spots that showed net signal values less than 5% of that of histone H2 were also omitted. The merged signal values were used for subsequent comparisons and assessed with the Genomic Profiler software package (Mitsui Knowledge Industry, Tokyo, Japan) and a Student’s t test by the method of Arfin et al. (2). We performed both global normalization and internal normalization with each chip as the control. We performed the analysis only for data that showed a net difference greater than 1.00 in each spot. The analysis showed that this approach had a smaller difference between the results from the two normalization methods (44).

Download from http://ec.asm.org/
ORF for mychis-tagged MpkA was digested with HindIII and Nhel from the pNmpkA-4mhm and ligated into the HindIII and XbaI sites of pYES2, resulting in the vector pNmpkA-4mhm expressing mychis-tagged MpkA.

To add a niaD mutation to the A. oryzae \( \Delta kexB \) strain, the nitrile auxotrophs derived from the \( \Delta kexB \) strain were screened for resistance to chloride by using a CD agar plate containing nitrite (NO\(_2\)) instead of nitrate (NO\(_3\)) as a nitrogen source in the presence of 500 mM chloride. We further screened the niaD-mutating \( \Delta kexB \) strains that were unable to utilize nitrate but could use nitrite, hypoxanthine, glutamate, or ammonium chloride as nitrogen sources. Strains \( \Delta kexB \) niaD (obtained from the selection agar plates) were performed as described above by using pNmpkA-4mhm digested with BamHI. A. oryzae transformants \( \Delta kexB \) mpkA and wt-mpkA-4mhm strains were screened as prototrophs.

Transformants, \( \Delta kexB \) mpkA-4mhm and wt-mpkA-4mhm strains of A. oryzae, were obtained by colony PCR by using primers 5'-GCAAAGGAGTCGAAAAG-3' and 5'-GTAGAATCACGAAATGAGACCTTTGACGACC-3' to confirm that the BamHI-digested pNmpkA-4mhm fragment was inserted into the niaD locus of the genome. Genomic DNAs isolated from the transformants as described above were used as templates for colony PCR. When the BamHI-digested pNmpkA-4mhm fragment was inserted into the niaD locus, a 2,186-bp fragment was amplified by these primers.

Preparation of cell extracts and immunoblot analysis. Cell extracts were prepared by the same method as described for the mRNA isolation from solid cultures of the \( \Delta kexB \), \( \Delta kexB \) mpkA-4mhm, wild-type, and wt-mpkA-4mhm strains grown on CD agar plates at 30°C for 50, 70, and 105 h or a CD agar plate plus 0.8 M NaCl at 30°C for 105 h. The mycelia were ground to a fine powder in a mortar and immediately suspended in prewarmed SDS sample buffer (120 mM Tris HCl [pH 8.8], 5% SDS, 5% mercaptoethanol, 10% glycerol, 1 mM sodium vanadate) without dye. The samples were vortexed at 10 s quickly and boiled at 100°C for 10 min, and the cell debris was removed by centrifugation for 10 min at 15,000 × g. Each sample (50 μg of protein) was subject to SDS-polyacrylamide gel electrophoresis analysis. Protein concentrations were determined by the method of Schaffner and Weissman (64) with bovine serum albumin as a standard. After the transfer of proteins to a Pall Fluoro Trans membrane (NIPPON Genetics Co. Ltd., Tokyo, Japan) using a semidry blotting apparatus (Bio-Rad), the membrane was used for immunoblotting with anti-phospho-p44/42 MAP kinase (Cell Signalling Technology, Inc., Beverly, Calif.) antibodies followed by immunoconjugation with the alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (NACALAI TESQUE, Inc., Kyoto, Japan) and visualization of the immune complexes with the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. To detect the MpkA MAP kinase containing the mychis tag, immunoblots were probed with the anti-myc monoclonal antibody or anti-His monoclonal antibody (Vector Laboratories Inc., Burlingame, Calif.). The underlined amino acid residues are identical to the late-Golgi retention signal (consensus, YXX/XXX) in the cytoplasmic tail of the S. cerevisiae Kex2p (69). To confirm whether the A. oryzae kexB gene product has protein-processing activities like those of known kexins, kexB was integrated at the niaD locus in the A. oryzae niaD300 strain and expressed using the A. oryzae glaA142 promoter. Southern blot analysis confirmed, in addition to the authentic kexB locus, the integration of an extra copy of kexB at the niaD locus in the transformant (data not shown). The transformed strain in which kexB was overexpressed did not show a detectable or noteworthy phenotype for hyphal development or morphology under induced conditions in either liquid or agar plate culture. We fractionated a cell lysate of the kexB-overexpressing strain and found kexin-like enzymatic activities in the membrane fraction. The solubilized membrane fraction from the kexB-overexpressing strain showed higher hydrolysis of fluorogenic peptides than did that from the control strain harboring the expression vector lacking the kexB insert (Table 1). In particular, A. oryzae KexB has a preference for peptides containing dibasic residues, as follows: Boc-Gln-Arg-Arg-MCA (4-methylcoumaryl-7-amide). Basic amino acids in bold.

<table>
<thead>
<tr>
<th>Peptide substrate</th>
<th>Cleavage activity (nkat/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4 P3 P2 P1 P1’</td>
<td>pNPGAl42</td>
</tr>
<tr>
<td>Boc-Gln-Arg-Arg-MCA</td>
<td>0.07</td>
</tr>
<tr>
<td>Boc-Leu-Lys-Arg-MCA</td>
<td>0.06</td>
</tr>
<tr>
<td>Boc-Leu-Arg-Arg-MCA</td>
<td>0.07</td>
</tr>
<tr>
<td>Boc-Arg-Val-Arg-Arg-MCA</td>
<td>0.05</td>
</tr>
<tr>
<td>Boc-Val-Pro-Arg-MCA</td>
<td>0.04</td>
</tr>
<tr>
<td>Boc-Gly-Lys-Lys-MCA</td>
<td>0.03</td>
</tr>
<tr>
<td>Boc-Gly-Arg-Arg-MCA</td>
<td>0.01</td>
</tr>
<tr>
<td>Boc-Leu-Ser-Thr-Arg-MCA</td>
<td>0.02</td>
</tr>
<tr>
<td>Boc-Leu-Gly-Arg-MCA</td>
<td>0.00</td>
</tr>
<tr>
<td>Boc-Gly-Arg-MCA</td>
<td>0.01</td>
</tr>
<tr>
<td>Boc-Glu-Lys-Lys-MCA</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The arrow indicates the cleavage site by A. oryzae KexB. Boc, b-tryguxinocarbonyl-; MCA, 4-methylcoumaryl-7-amide. Basic amino acids are in bold.

which is necessary for enzymatic activities in other proteases (42, 55). Hydropathy analysis suggested the presence of two highly hydrophobic regions that are a signal peptide and transmembrane domain required for anchoring to Golgi membranes, similar to those in the amino acid sequence of yeast kexin. A. oryzae has a Kex2p-like gene by using the Sequence database under accession no. AB056727 and AB056726, respectively. The se-
linker downstream of the XhoI site (Fig. 1B). The stop codon linker had multiple stop codons to cover all reading frames. The ΔkexB strain was isolated from about 400 colonies of the transformants by the colony PCR method as described by van Zeijl et al. (66) with primers 1 and 2, as described in Materials and Methods. The ΔkexB candidate was further confirmed by PCR and Southern analysis (Fig. 1C). The probes for both kexB and ptrA indicated the expected two hybridization signals to digested genomic DNA isolated from the ΔkexB candidate and the single hybridization signal to that from the wild-type strain, suggesting that the homologous recombination successfully took place at the authentic kexB locus. Reverse transcription-PCR failed to reveal transcripts derived from kexB in the ΔkexB strain (data not shown).

The ΔkexB strain formed shrunken colonies and scarcely differentiated conidia on CD agar plates (Fig. 2A). The detailed morphology of the ΔkexB strain was further compared with that of the wild type by using scanning electron microscopy (Fig. 2C to G). ΔkexB cells grown for 4 days on CD agar plates formed neither conidiophores nor conidia. The hyphae of the ΔkexB strain became finer, denser and hyperbranched in comparison with those of the wild type. Hyphal tips of the ΔkexB strain were thicker and multibranched (Fig. 2F). The disruptant grown in CD liquid culture medium also showed highly branched mycelia (data not shown). We carried out a rescue experiment in which the mutant phenotypes of the A. oryzae ΔkexB (ΔkexB niaD) strain were almost fully restored by transformation with the pNAX1 plasmid containing the wild-type kexB gene (see Fig. S1 in the supplemental material). However, the A. oryzae ΔkexB (ΔkexB niaD) strain transformed with the pNGA142 vector without the kexB insert maintained the mutant phenotypes (see Fig. S1 in the supplemental material). These results suggested that the phenotypes of the ΔkexB strain are attributable to the defect of the kexB gene. Surprisingly, although the ΔkexB strain exhibited the various described morphological defects on CD agar plates, the defects were suppressed on high-osmolarity CD agar plates containing 0.8 M sodium chloride (Fig. 3), 0.8 M potassium chloride, or 1.2 M sorbitol (data not shown). The morphological defects of the ΔkexB strain also were restored in a high-osmolarity CD liquid culture medium (data not shown).

cDNA microarray analysis of the ΔkexB disruptant. Because the ΔkexB strain showed the various described phenotypes, we wondered how transcription profiles reflected the phenotypes and therefore compared the gene expression profiles of the ΔkexB and wild-type strains by using A. oryzae cDNA microarrays. We analyzed transcripts from the two strains cultured on CD agar plates for 105 h at 30°C as well as on CD agar plates containing 0.8 M NaCl (Table 2). On CD agar plates, a large number of genes were more upregulated in the ΔkexB strain than in wild-type cells whereas gene expression levels of the ΔkexB strain were similar to those of the wild type in the presence of high osmotic pressure (~0.8 M NaCl). The ΔkexB type (lanes 3, 4, 7, and 8). The enzymes used were PstI and SphI (lanes 1 and 3), ApaLI and NspV (lanes 2 and 4), SpeI (lanes 5 and 7), and PstI (lanes 6 and 8). Hybridization was performed with the kexB probe (see Materials and Methods) (left) and the ptrA probe (right).
strain exhibited 4.9- and 55-fold-lower levels of brlA (70) and rodA (57) transcripts, respectively, than did the wild type. Because brlA encodes a transactivator to promote the formation of conidia and rodA encodes a hydrophobic protein necessary to form conidiophores, the downregulation of brlA expression in the ΔkexB strain might be one of the reasons for its poor generation of conidia. We were unable to assign identities to other genes that showed markedly reduced transcript levels in the ΔkexB strain, because the annotations of some genes in the cDNA microarray remain unknown.

Because of the morphological defects of the ΔkexB strain, we paid further attention to the following eight genes which are involved in cell wall biogenesis: chsC (50), chsA (unpublished data; DDBJ/ENBL/GenBank accession no. BAB85683), chsB (51), chsY, chsZ (9), gelA, gelB (48), and fksA (34) (Fig. 4). chsC, chsA, and -B, chsY, and chsZ encode type I, III, V, and VI chitin synthases, respectively (9). According to tBlastx analysis, gelA and gelB are predicted to be the A. oryzae counterparts of the A. fumigatus gel1 and gel2 genes, which encode putative glucanosyltransferases that are thought to be involved in cell wall biosynthesis (49). fksA is the putative A. oryzae 1,3-glucan synthase gene (34). On CD agar plates, transcription of chsC, chsB, and gelB of the ΔkexB strain was markedly upregulated and their transcription levels were 3.2, 4.6, and 4.3 times higher, respectively, than those of the wild type. In contrast, high osmotic pressure (0.8 M NaCl) in the culture plate suppressed the upregulation of these three genes in the ΔkexB strain.

**Northern blot analysis of the ΔkexB strain.** Cell wall biogenesis in S. cerevisiae is thought to be under the control of the ΔkexB strain, because the annotations of some genes in the cDNA microarray remain unknown.

Because of the morphological defects of the ΔkexB strain, we paid further attention to the following eight genes which are involved in cell wall biogenesis: chsC (50), chsA (unpublished data; DDBJ/ENBL/GenBank accession no. BAB85683), chsB (51), chsY, chsZ (9), gelA, gelB (48), and fksA (34) (Fig. 4). chsC, chsA, and -B, chsY, and chsZ encode type I, III, V, and VI chitin synthases, respectively (9). According to tBlastx analysis, gelA and gelB are predicted to be the A. oryzae counterparts of the A. fumigatus gel1 and gel2 genes, which encode putative glucanosyltransferases that are thought to be involved in cell wall biosynthesis (49). fksA is the putative A. oryzae 1,3-glucan synthase gene (34). On CD agar plates, transcription of chsC, chsB, and gelB of the ΔkexB strain was markedly upregulated and their transcription levels were 3.2, 4.6, and 4.3 times higher, respectively, than those of the wild type. In contrast, high osmotic pressure (0.8 M NaCl) in the culture plate suppressed the upregulation of these three genes in the ΔkexB strain.

**TABLE 2. Number of genes whose expression levels were altered by deletion of kexB in A. oryzae**

<table>
<thead>
<tr>
<th>Relative expression ratios (ΔkexB/wild type) (fold)</th>
<th>CD</th>
<th>CD + 0.8 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;4.0</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>2.0–4.0</td>
<td>291</td>
<td>25</td>
</tr>
<tr>
<td>0.5–2.0</td>
<td>1,087</td>
<td>1,736</td>
</tr>
<tr>
<td>0.25–0.5</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>&lt;0.25</td>
<td>27</td>
<td>0</td>
</tr>
</tbody>
</table>

* After three independent analyses, 1,521 spots on the cDNA microarrays for CD plates and 1,762 spots on the cDNA microarrays for CD plates with 0.8 M NaCl were statistically verified by using the Genomic profiler program.
mpkA functionally complements yeast MPK1 (T. Fujioka, K. Furukawa, O. Mizutani, K. Abe, and T. Nakajima, unpublished data). We also found an A. oryzae mpkA gene homolog in the A. oryzae EST database and A. oryzae genome information through homology searches with the nucleotide sequence of A. nidulans mpkA. Because our cDNA microarray analyses showed upregulation of the transcription levels of genes involved in cell wall biogenesis in A. oryzae ΔkexB, we further examined whether transcription of mpkA is also upregulated in this strain. We performed transcriptional analyses of mpkA and cell wall-related genes such as chsC, chsB, gelA, and gelB by Northern blotting at various times after inoculation (Fig. 5A and B). The wild-type strain began to form conidiophores and conidia 70 h after inoculation and had formed fully mature conidiophores with plenty of conidia at 105 h on CD plates. Transcription of the histone H2B gene seemed to be constitutive and was used as a control at all time points. Transcription levels of chsC, chsB, and gelB were higher in the ΔkexB strain than in the wild type at the three time points assayed. As expected, the transcription of mpkA in the ΔkexB strain also was increased at all time points. However, the transcription levels of gelA were almost same in both strains at all time points. Because cell integrity signaling is inactivated by high osmotic stress in S. cerevisiae (11, 25, 26), we examined whether high osmotic stress downregulates the high level of transcription of mpkA in the ΔkexB strain to the transcription level of mpkA in the wild-type strain. We performed transcriptional analyses of mpkA by Northern blotting under conditions of high osmotic stress (Fig. 5C). As expected, high osmotic stress apparently downregulated the transcription level of mpkA in the ΔkexB strain to a level similar to that in the wild-type strain. Therefore, the morphological defects concomitant with the marked upregulation of mpkA and the osmoresponse suppression of the morphological defects with the simultaneous downregulation of transcription of mpkA and genes for cell wall biogenesis may indicate that kexB is involved in a signal transduction pathway, particularly cell integrity signaling.

Cloning of the mpkA gene from A. oryzae and time course of MpkA phosphorylation in the ΔkexB strain. Since transcription levels of the mpkA gene in the ΔkexB strain are constitutively upregulated under normal culture conditions and downregulated in the presence of osmotic stress, we further examined phosphorylation levels of MpkA protein in the ΔkexB strain under normal and high-osmosis conditions. From the A. oryzae cDNA library, we isolated a positive clone by PCR. We determined the complete nucleotide sequence of this clone, which contained a 1,272-bp ORF encoding an MpkA protein of 423 amino acid residues. The catalytic domain of MpkA possesses all the subdomains found in protein kinases (23). In addition, MpkA has a TEF tripeptide dual phosphorylation motif characteristic of MAP kinases, which is known to be required for activation of MAP kinases. The putative amino acid sequence showed about 90% identity to that of the putative MpkA identified in A. nidulans (7), and thus the A. oryzae gene was designated mpkA.

Because MpkA of A. oryzae is also related to the yeast Mpk1p, we investigated the in vivo functionality of MpkA and its derivative mychis-tagged MpkA by using an S. cerevisiae mpk1 mutant. We confirmed that expression of both A. oryzae mpkA and mpkAmh genes in the S. cerevisiae mpk1 disruptant

FIG. 4. Expression levels of genes involved in cell wall biogenesis. The bar graphs indicate the expression levels of the genes encoding cell wall synthesis-related proteins from wild-type and ΔkexB strains grown on CD agar plates (A) and CD agar plates containing 0.8 M NaCl (B). The gray and white bars indicate the relative intensities of transcription of the genes in the wild-type and ΔkexB strains, respectively, on the basis of the cDNA microarray analyses. The relative intensities of the examined genes were calculated using the intensity of histone H2B as an internal standard (1.0). The genes were as follows: 1, chsC (chitin synthase C) (50); 2, chsA (chitin synthase A) (our unpublished data; DDBJ/ENBL/GenBank accession no. BAB85683); 3, chsB (chitin synthase B) (51); 4, chsY (chitin synthase Y) (9); 5, chsZ (chitin synthase Z) (9); 6, gelA (glycosylphosphatidylinositol-anchored glucanosyltransferase) (48); 7, gelB (glycosylphosphatidylinositol-anchored glycanosyltransferases) (48); and 8, kexA (β[1-3] glucan synthesis) (34).

signal transduction pathways including the cell integrity pathway. The S. cerevisiae MPK1 (SLT2) gene encodes a MAP kinase in the cell integrity pathway (26). In S. cerevisiae, when cell wall biosynthesis is inhibited or the cell wall is damaged, transcription levels of MPK1 and genes required for cell wall biogenesis are simultaneously upregulated by the transcription factor Rlm1p, which is a phosphorylation target of Mpk1p (13, 31, 67, 68). Bussink et al. (7) isolated the A. nidulans mpkA gene which is the counterpart of yeast MPK1. Recently we confirmed that expression of A. nidulans mpkA cDNA in a temperature-sensitive S. cerevisiae mpk1 disruptant suppressed the mpk1 disruption mutation, suggesting that A. nidulans
suppressed the temperature sensitivity attributed to the mpk1 mutation, suggesting that both mpkA and mpkA mh functionally complement the yeast MPK1 (data not shown). To demonstrate whether MpkA is phosphorylated in the ΔkexB strain, we examined the phosphorylation levels of MpkA and MpkAmh expressed in ΔkexB cells grown on the CD agar plates. We detected a significant increase in the phosphorylation levels of both MpkA and MpkAmh in the A. oryzae ΔkexB cells grown for 105 h on the CD agar plates by using anti-phospho-p44/42 MAP kinase antibodies (Fig. 6A, lanes 1 and 2), although MpkA in the wild-type strain was scarcely phosphorylated, regardless of osmotic stress (lanes 3, 4, 7, and 8). The phosphorylation levels of MpkA and MpkAmh in the ΔkexB strain were apparently downregulated by high osmotic stress (lanes 5 and 6), with concomitant downregulation of the transcription levels of mpkA (Fig. 5C). Phosphorylation of MpkA was observed when the wild-type cells were subjected to hypotonic stress (Fig. 6A, lanes 9 and 10), indicating that MpkA is capable of transducing the same types of stress signals through the putative cell integrity pathway as well as that of S. cerevisiae. The ΔkexB-mpkA mh and wt-mpkA mh strains used as controls of expression quantity of MpkA showed the same phenotypes as the ΔkexB and the wild-type strains on the CD agar plate culture, respectively. Furthermore, the phosphorylation levels of MpkAmh and the authentic MpkA in the ΔkexB-mpkA mh cells significantly increased at the three culture time points examined whereas MpkA and its derivative in the wt-mpkA mh strain were poorly phosphorylated (Fig. 6B). The phosphorylation levels of MpkAmh and the authentic MpkA in the ΔkexB-mpkA mh strains at 70 and 105 h of cultivation were higher than those at 50 h. Although the phosphorylation levels of MpkAs at each growth stage

FIG. 5. Gene expression analysis of chsC, chsB, gelA, gelB, and mpkA by Northern blotting. (A) Time course of the phenotypic change of the ΔkexB and wild-type strains on CD agar plates. The left, middle, and right panels show colonies cultivated for 50, 70, and 105 h, respectively. (B) Gene expression analysis of chsC, chsB, gelA, gelB, and mpkA over time (50, 70, and 105 h) by Northern blotting. A histone gene was used as a control. wt, wild type. (C) Gene expression analysis of mpkA on CD agar plates plus 0.8 M NaCl for 105 h by Northern blotting. A histone gene was used as a control.
were different in the ΔkexB-mpkAmh cells, the MpkAs in the ΔkexB-mpkAmh were remarkably phosphorylated at all time points after inoculation, suggesting that the KexB defect in A. oryzae causes constitutive activation of the cell integrity pathway.

**DISCUSSION**

In the present study, we cloned the A. oryzae kexB gene, which encodes 836 amino acid residues and confirmed that the encoded protein has processing activity (Table 1). To study kexB function, we constructed a kexB gene disruptant strain (ΔkexB; Fig. 1). The ΔkexB strain showed shrunken colonies with poor generation of conidia on CD agar plates (Fig. 2), and hyperbranched mycelia occurred in liquid culture. Interestingly, the morphological defects derived from the ΔkexB geno-

type were restored under conditions of high osmosis (Fig. 3). In A. oryzae ΔkexB, the transcription levels of genes for cell wall biogenesis and an mpkA homolog that presumably encodes a MAP kinase involved in cell integrity signaling were higher than those in the wild type (Table 2; Fig. 4 and 5), and high osmotic pressure downregulated the transcription levels of A. oryzae mpkA and the cell wall-related genes in the ΔkexB strain to levels similar to those in the wild type (Fig. 4 and 5). Then we cloned the A. oryzae mpkA gene and confirmed that its expression suppressed the temperature sensitivity of the S. cerevisiae mpk1 disruptant (data not shown), suggesting the in vivo functionality of A. oryzae MpkA. As expected, constitutively elevated phosphorylation levels of MpkA in ΔkexB cells on the CD agar plate culture were demonstrated by using anti-phospho-p44/42 MAP kinase antibodies, and high osmotic stress downregulated the increased phosphorylation levels of MpkA in the ΔkexB strain to the same as those observed in the wild type (Fig. 6). These results suggest that the upregulation of transcription levels of mpkA and cell wall-related genes in the ΔkexB strain is mediated by phosphorylated MpkA as an active form through cell integrity signaling.

The phenotypes of A. oryzae ΔkexB are different from those of kexB disruptants of A. niger (29, 60) and A. nidulans (K. Furukawa, O. Mizutani, T. Fujioka, Y. Yamagata, K. Abe, K. Gomi, and T. Nakajima, unpublished). The A. niger kexB disruptant formed conidiophores and conidia on agar plates (29). The A. nidulans ΔkexB strain showed shrunken colonies on agar plates but had differentiated conidiophores and conidia. According to the phenotypes of the ΔkexB strains of these three Aspergillus species, the function of the kexB gene in A. oryzae is probably more essential for cell growth and especially for cell wall biogenesis. The differences of the ΔkexB phenotypes among these three Aspergillus species may be attributable to the processing targets of KexB in each species but not to the substrate specificity of each KexB protein, because the substrate specificity of the A. oryzae product was similar to those of A. niger (29) and A. nidulans (39). Moreover, it is noteworthy that the various morphological phenotypes derived from the ΔkexB genotype in A. oryzae were restored under high osmotic stress in both solid and liquid culture. However, the number of conidia in the ΔkexB strain was 70% of that in the wild type even under high osmotic pressure, suggesting that osmotic suppression of ΔkexB phenotypes is incomplete. Although high osmotic pressure did not suppress the phenotypes of kexin gene disruptants of S. cerevisiae or C. albicans (36, 56), the osmotic restoration of ΔkexB phenotypes occurs in A. oryzae and A. nidulans (Furukawa et al., unpublished). It remains unknown whether the ΔkexB phenotypes of A. niger are suppressed under high osmotic conditions.

In our comprehensive and comparative analysis of gene expression between the A. oryzae ΔkexB and wild-type strains by using cDNA microarrays, the transcription levels of a large number of genes were higher in the ΔkexB strain than in the wild type on CD agar plates (Table 2). These results imply that disruption of the kexB gene affects the transcription levels of a broad range of genes and that KexB of A. oryzae probably processes key proteins required for maintenance of normal morphogenesis and cell growth. In addition, we found that the expression levels of chsC, chsB, and gelB were markedly higher in the A. oryzae ΔkexB strain than in the wild type (Fig. 4).
Cell wall biogenesis in *S. cerevisiae* is thought to be under the control of various signal transduction pathways including the cell integrity pathway (26). Cell integrity signaling is activated, with different timing and kinetics, by hypoosmotic shock (11, 32), by heat shock (32), during bud emergence (72), on exposure to mating pheromone (6, 72), and on various treatments leading to perturbation of the cell wall (4, 35, 67). The pathway organizes changes in cellular morphology by controlling the expression of genes encoding enzymes involved in cell wall metabolism. The central pathway concerned with cell integrity is the Mpk1p MAP kinase cascade, and Mpk1p phosphorylates and consequently activates the transcription factor Rlm1p, whose transcriptional targets are genes encoding cell wall biogenesis proteins, cell wall proteins, and Mpk1p itself (13, 31, 68). Northern blot analyses of cell wall-related genes and *mpkA* revealed that transcription levels of the examined genes were simultaneously upregulated in *A. oryzae* genome sequences (O. Mizutani, K. Abe, and T. Nakajima, unpublished data). The cell integrity pathway in the Δ*kexB* strain is constitutively activated (A), although the pathway is not activated (resting) unless the wild-type strain senses some stress such as hypoosmolarity (B). *A. oryzae* KexB is predicted to be required for precise proteolytic processing of sensor proteins in the cell integrity pathway and/or of cell wall-related enzymes whose genes are under transcriptional control by the pathway.

We propose the following two scenarios to explain why and how cell integrity signaling is activated in the *A. oryzae* Δ*kexB* strain. In the first scenario, if KexB processes enzymes required for cell wall biogenesis, then the Δ*kexB* strain fails to process these enzymes, resulting in perturbation of cell wall assembly. Various sensor proteins might detect perturbation of the cell wall as a stressor in *A. oryzae* Δ*kexB* and consequently activate the cell integrity pathway that upregulates the transcription of some cell wall-related genes required for restoration of the damaged cell wall. Sensor proteins such as Mid2p and Wsc1p in *S. cerevisiae* are thought to directly sense alterations of certain cell wall properties to mediate the activation of the cell integrity pathway via G-protein reactions (26). Putative ORFs homologous to the yeast sensors occur among *A. oryzae* genome sequences (data not shown). In the second scenario, if KexB processes putative cell surface sensors of the cell integrity pathway, incorrect processing of the sensor proteins by the Δ*kexB* mutation might be responsible for the constitutive activation of cell integrity signaling and for the consequent upregulation of the transcription levels of cell wall-related genes in *A. oryzae*. The yeast BCK1 and MKK1 orthologs have not yet been isolated from *Aspergillus* species; however, putative ORFs (*bckA, mkkA*) homologous to the two genes are found in *A. oryzae* genome sequences (O. Mizutani, K. Abe, and T. Nakajima, unpublished data). The cell integrity pathway in the Δ*kexB* strain is constitutively activated (A), although the pathway is not activated (resting) unless the wild-type strain senses some stress such as hypoosmolarity (B). *A. oryzae* KexB is predicted to be required for precise proteolytic processing of sensor proteins in the cell integrity pathway and/or of cell wall-related enzymes whose genes are under transcriptional control by the pathway.

![Schematic model for implication of the cell integrity pathway in the Δ*kexB* (A) and wild-type (B) strains. The cell integrity MAP kinase pathway is shown by the dotted box. *pkcA* (47), *mpkA* (7), and *rlmA* (Fujioka et al., unpublished; accession no. BAD01583) were isolated from *Aspergillus* fungi. The yeast BCK1 and MKK1 orthologs have not yet been isolated from *Aspergillus* species; however, putative ORFs (*bckA, mkkA*) homologous to the two genes are found in *A. oryzae* genome sequences (O. Mizutani, K. Abe, and T. Nakajima, unpublished data). The cell integrity pathway in the Δ*kexB* strain is constitutively activated (A), although the pathway is not activated (resting) unless the wild-type strain senses some stress such as hypoosmolarity (B). *A. oryzae* KexB is predicted to be required for precise proteolytic processing of sensor proteins in the cell integrity pathway and/or of cell wall-related enzymes whose genes are under transcriptional control by the pathway.](http://ec.asm.org/ on February 7, 2021 by guest)
related genes. The morphological defects of the ΔkexB strain might be caused by upregulation of cell wall-related genes through activation of cell integrity signaling. A bioinformatics approach using the C. albicans genome database assigned 147 ORFs as putative Kex2p substrates, and these ORFs included a gene encoding a Wsc2p homologue that seems to be a sensor protein in the cell integrity pathway (56). Applying this analogy to A. oryzae, the sensor/signal-like proteins of the pathway in A. oryzae might be processed by KexB.

Although KexB may process cell wall-related enzymes and/or cell surface sensors (Fig. 7), we currently prefer the first scenario in light of the following predictions. Although the yeast KEX2 disruptant (kex2Δ) has no noteworthy morphological phenotype, the Pck1-Mpk1 pathway is known to be activated in the kex2Δ strain, resulting in perturbation of cell wall structure (62). Consequently, additional defects of MPK1 yeast scenario in light of the following predictions. Although the yeast KEX2 disruptant (kex2Δ) has no noteworthy morphological phenotype, the Pck1-Mpk1 pathway is known to be activated in the kex2Δ strain, resulting in perturbation of cell wall structure (62). Consequently, additional defects of MPK1 together with the kex2Δ mutation, cause cell death (62). This synthetic lethality suggests that the perturbation of cell wall assembly in the kex2Δ strain is probably suppressed by activation of the cell integrity pathway by impaired sensors is unavailable even for S. cerevisiae (21, 28, 35, 67). However, we cannot exclude the possibility that the transcription level of mpkA might be upregulated by the influence of another signal transduction pathway.

The kex2Δ mpk1Δ phenotype of S. cerevisiae is rescued by growth on high-osmolality medium (62). The phenotype of the A. oryzae ΔkexB strain also was restored under high osmotic pressure (Fig. 3). We propose the following two explanations for why the phenotypes of the ΔkexB strain were suppressed under high osmotic pressure. First, instead of KexB, perhaps other processing proteases inducible under high osmotic pressure suppress the ΔkexB mutation. YPS1 and YPS2, which encode aspartyl proteases, are multicopy suppressors of the kex2Δ mutation in S. cerevisiae (14, 36). The expression level of YPS1 in S. cerevisiae cells treated for 10 min with 0.4 M NaCl is 15.7 times higher than that in cells without the NaCl treatment (58). We found two genes homologous to YPS1 and YPS2 in A. oryzae (38), and NaCl may induce these homologs and suppress the ΔkexB mutation. Second, the restoration of the ΔkexB phenotypes might depend on mechanisms mediated by osmotic stress, such as a cascade similar to the yeast Hog1 MAP kinase pathway (22, 26, 33, 59). The cell integrity pathway in S. cerevisiae is thought to be negatively regulated by osmotic pressure (11, 25, 26), and activation of the Hog1p pathway mediates adaptive rearrangements in cell wall composition and architecture in S. cerevisiae and C. albicans (1, 18). A similar scenario might explain the osmotic restoration of the ΔkexB phenotypes in A. oryzae. To verify restoration of the altered phenotypes by increased osmotic pressure in ΔkexB, in vivo functional studies, such as exploration of suppressors of the ΔkexB mutation or construction of a mutant in which the HOG pathway is constitutively activated in the ΔkexB genetic background, are necessary and in progress.

In conclusion, we predict that A. oryzae KexB is required for precise proteolytic processing of sensor proteins in the cell integrity pathway or of cell wall-related enzymes whose genes are under transcriptional control by the pathway. Thus, the KexB defect leads to disordered cell integrity signaling.

ACKNOWLEDGMENTS

We thank Katsuya Gomi and Hiroyuki Horiiuchi for helpful suggestions. We also thank Osamu Hatamoto, Hiroshi Maeda, Junichi Maruyama, Taruji Satou, Takamitsu Maruyama, Yoshihiko Matsuura, Kanako Suzuki, Motoaki Sano, and Masayuki Machida for helpful discussions and/or technical assistance.

This work was supported in part by a Grant-in-Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan (BDP-03-VI-1-7).

REFERENCES


