Pneumocystis carinii BCK1 functions in a mitogen-activated protein kinase cascade regulating fungal cell-wall assembly

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Abstract ***Pneumocystis*** **pneumonia remains the most common AIDS-defining opportunistic infection in people with HIV. The process by which** ***Pneumocystis carinii*** **constructs its cell wall is not well known, although recent studies reveal that molecules such as β-1-3-glucan synthetase (*GSC1*) and environmental pH-responsive genes such as *PHR1* are important for cell-wall integrity. In closely related fungi, a specific mitogen-activated protein kinase (MAPK) cascade regulates cell-wall assembly in response to elevated temperature. The upstream mitogen-activated protein kinase kinase kinase (MAPKKK, or MEKK), BCK1, is an essential component in this pathway for maintaining cell-wall integrity and preventing fungal cell lysis. We have identified a *P. carinii* MEKK gene and have expressed it in *Saccharomyces cerevisiae* to gain insights into its function. The *P. carinii* MEKK, *PCBCK1*, corrects the temperature-sensitive cell lysis defect of *bck1Δ* yeast. Further, at elevated temperature *PCBCK1* restored the signaling defect in *bck1Δ* yeast to maintain expression of the temperature-inducible β-1-3-glucan synthetase gene, *FKS2*. *PCBCK1*, as a functional kinase, is capable of autophosphorylation and substrate phosphorylation. Since glucan machinery is not present in mammals, a better understanding of this pathway in *P. carinii* might aid in the development of novel medications which interfere with the integrity of the *Pneumocystis* cell wall.**

Key words: Mitogen-activated protein kinase; Glucan; AIDS

1. Introduction

Pneumocystis pneumonia remains the most common AIDS-defining opportunistic infection in people infected with the human immunodeficiency virus (HIV) [1–4]. Once inhaled from the environment into the lungs of an immunosuppressed host, *Pneumocystis carinii* binds to the alveolar epithelium and causes damage through an elaborate induction of host inflammatory responses [5–9]. Although the natural reservoir for *P. carinii* is not known, its nucleic acids have been isolated in the air in hospitals and unexpected environments such as apple orchards and ponds [10–16]. It is unknown whether *P. carinii* has the ability to adapt to different environmental stresses such as temperature, however a phylogenetically related ascomycete, *Saccharomyces cerevisiae*, activates a specific mitogen-activated protein kinase (MAPK) cascade upon a temperature increase from 25°C to 37°C (similar to the transition from room temperature to body temperature). The *S. cerevisiae* mitogen-activated protein kinase kinase kinase (MAPKKK, or MEKK) BCK1 is critical for maintaining cell-wall integrity at elevated temperature [17–22]. Disruption of the cell-wall integrity MAPK pathway results in fungal death at the elevated temperature due to loss of inducible β-1-3-glucan synthetase function within 2 h, however addition of an osmotic stabilizing agent, such as sorbitol, will ameliorate this effect and allow the fungi to grow at the elevated temperature [18,20,22].

The principal component of the fungal cell wall is glucan, which is comprised of homopolymers of glucose molecules with a β-1,3-linked carbohydrate core and side chains of β-1,6- and β-1,4-linked glucose [23–27]. Indeed, the glucan present in the *P. carinii* cell wall is responsible for the significant inflammatory response in the lungs of the infected host [6]. In *S. cerevisiae*, *FKS1* and *FKS2* encode subunits of the β-1-3-glucan synthetase, which mediate the polymerization of uridine 5′-diphosphoglucose into β-1,3-glucan. We have previously isolated a *P. carinii* β-1-3-glucan synthetase gene, *GSC1*, which has homology to both *FKS1* and *FKS2* [23]. *FKS1* is predominantly expressed under optimal growth conditions at temperatures ranging from 25°C to 30°C [28–30]. *FKS2* is induced within 20 min in response to elevated temperature (37°C or greater) to increase the stability of the yeast cell wall. Dual pathways participate in the induction of *FKS2* expression, a calcineurin-mediated pathway and the cell-wall integrity MAPK pathway, however maintenance of *FKS2* expression after 60 min of continued heat stress requires a functioning cell-wall integrity MAPK pathway [22,31]. Perturbation of fungal cell-wall assembly represents an attractive target for the treatment of fungal infections, since the biosynthetic machinery for generating glucan is not present in mammals [32–34].

Our laboratory has demonstrated that *P. carinii* molecules such as the β-1-3-glucan synthetase *GSC-1* and environmental pH-responsive genes such as *PHR1* participate in cell-wall maintenance [23,24]. Unfortunately, the inability to culture *P. carinii* outside of the infected host and the inability to overexpress or disrupt genes in *P. carinii* make direct analysis of these pathways impossible. Hence, we report the identifica-

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tion of a *P. carinii* gene encoding a MEKK, *PCBCK1*, and we investigate *PCBCK1* effects on cell-wall integrity signaling in *S. cerevisiae bck1Δ* mutants.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Restriction endonucleases, *Taq* DNA polymerase and Platinum *Pfx* DNA polymerase were purchased from Invitrogen. PHAS-I substrate was purchased from Stratagene. [γ^{-32}P]ATP was obtained from ICN Pharmaceuticals.

2.2. Preparation of P. carinii organisms

All studies described in this report were approved by the institutional animal care and use committee. *P. carinii* pneumonia was induced in Harlan Sprague–Dawley rats by immunosuppression with dexamethasone as previously reported [23,24,35–38]. Lungs from moribund rats were minced and homogenized in HBSS and *P. carinii* were purified from host lung cells by filtration through a 10-μm filter (Millipore). *P. carinii* organisms were confirmed by Wright–Giemsa staining and samples containing contaminating bacterial or fungal organisms were discarded.

2.3. Cloning and chromosomal location of the PCBCK1 gene

A degenerate polymerase chain reaction (PCR)-based approach was used to obtain a partial clone of the *P. carinii BCK1*, followed by library screening to obtain the full-length cDNA sequence. Degenerate PCR was performed in a 50-μl reaction that included 1× PCR buffer, 50 μM dNTPs, 2 μM each of the primers ELMAVKQV (5′-GARYTNATGGCWGTWAARCARGT-3′) and NAGKIDRH (5′-TTWGCWCCYTTWATRTCNCGRTG-3′), 250 ng of *P. carinii* genomic DNA, and 1.25 units of *Taq* DNA polymerase. Samples were amplified for 30 cycles at 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s, and the PCR products were subjected to electrophoresis on 2% agarose. A single 373-bp amplicon was visualized by ethidium bromide staining and was ligated into a pGEM T-Easy plasmid (Promega), and completely sequenced. The PCR amplicon was used as a probe to screen a rat-derived *P. carinii* cDNA library in the Uni-ZAP XR bacteriophage as previously described (NIH AIDS Research Reagent Program, Bethesda, MD, USA) [35,36]. The phagemid was excised using the M13 helper phage and the *P. carinii BCK1* insert was sequenced. The entire *P. carinii BCK1* cDNA was amplified by PCR using the proofreading Platinum *Pfx* DNA polymerase and the following primers: 5′-ATGGATTATTTAAAGAAAGT-3′ (sense) and 5′-TTTTTTTGCATCAGTCAAAG-3′ (antisense). Following amplification, 1 unit of Taq DNA polymerase was added at 72°C for 30 min and the gene was ligated into the yeast expression plasmid pYES2.1 TOPO TA (Invitrogen), then sequenced entirely to confirm the absence of PCR errors and to confirm correct orientation. PYES2.1 TOPO TA has the galactose-regulated promoter *GAL1*, which induces transcription when the yeast are grown in galactose as a carbon source, and a carboxyl V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr), allowing identification of fusion proteins with an anti-V5 antibody (Invitrogen). The stop codon for *PCBCK1* was not included to allow in-frame fusion to the C-terminal V5 epitope tag. The chromosomal location of *PCBCK1* was determined by first labeling the *PCBCK1* 373-bp PCR amplicon with [^{32}P]α-dATP by the random primer method, followed by hybridization to *P. carinii* chromosomes separated by contour-clamped homogenous field electrophoreses (CHEF).

2.4. Site-directed mutagenesis

The lysine in PCBCK1 at position 575 corresponds to the conserved lysine which is essential for catalytic activity in the kinase domain of protein kinases. We used site-directed mutagenesis to change the lysine to an arginine, which has been previously shown to abolish kinase activity but not lead to instability of the protein [39–41]. To create PCBCK1Δ^{K575R}, we generated a sense mutagenesis primer 5′-GGAGAATTGATGGCAGTACGACAAGTTGAAATACCGTCT-3′ and an antisense mutagenesis primer 5′-AGACGGTATTTCAACTTGTCGTACTGCCATCAATTCTCC-3′ to create the desired mutation. These oligonucleotides were annealed to *PCBCK1* in the pYES2.1 plasmid and the mutations were generated by PCR following the instructions by the manufacturer (Stratagene QuikChange Multi Site-Directed Mutagenesis). Following mutagenesis the plasmid was sequenced to verify the correct mutation was generated.

2.5. Yeast strains, plasmids, and transformation

S. cerevisiae used in this study was obtained from ATCC: *bck1Δ* (*MATa his3-1*, *leu2-0*, *ura3-0*, *met15-0*, *bck1::kanR*). For the purposes of positive controls, the *S. cerevisiae BCK1* gene was amplified by PCR with the proofreading Platinum *Pfx* DNA polymerase using the following primers: 5′-ATGCCCTTTTTGAGGAAAATAGCGGGGAC-3′ (sense) and 5′-TTCAGTTTTATTCTCCTGAGAGGTTATCCTT-3′ (antisense) and ligated into the yeast expression plasmid pYES2.1 TOPO TA (Invitrogen), then sequenced to confirm the absence of PCR errors. The stop codon for *SCBCK1* was not included to allow in-frame fusion to the C-terminal V5 epitope tag. Yeast were grown overnight in YEPD media containing 200 mg l^{-1} G418 at 30°C to an OD_{600} of 1.0, then transformed with the plasmids pYES2.1/*PCBCK1*, pYES2.1/*SCBCK1*, pYES2.1/*PCBCK1*Δ^{K575R}, or pYES2.1/*LACZ* by electroporation as previously described [24,36,37]. Transformants were selected on glucose minimal media lacking uracil at 30°C.

2.6. Northern analysis

Expression of *PCBCK1* was determined by northern analysis. *P. carinii* total RNA was isolated with Trizol Reagent (Invitrogen), and 10 μg were separated on 1.0% formaldehyde-agarose gels, transferred to Nytran Plus membranes (Schleicher and Schuell; Keene, NH), and probed with radiolabeled *PCBCK1*. Following exposure to autoradiography film, the membranes were re-probed using a radiolabeled *P. carinii* actin probe. Temperature induction of *FKS2* mRNA expression was performed following the procedure described by Zhao et al. [22]. *S. cerevisiae bck1Δ* yeast and *bck1Δ* yeast transformed with pYES2.1/*PCBCK1* were grown to mid-log phase (OD_{600} = 0.8) at 25°C in YEPD containing 200 mg l^{-1} G418 or galactose minimal media, respectively. An immediate temperature shift to 37°C was achieved by adding an equal volume of media prewarmed at 55°C to the cultures and placing them in a 37°C shaking water bath. Cultures were removed at various time points, pelleted, and total RNA was isolated with YeastarRNA (Zymo Research) following the instructions of the manufacturer. Five micrograms of total RNA was separated on 1.0% formaldehyde-agarose gels and transferred to Nytran Plus membranes. *S. cerevisiae FKS1* and *FKS2* probes were generated by PCR using the primers, respectively: 5′-ATGAACACTGATCAAC-3′ (sense) and 5′-AATTACCGTAAATTGG-3′ (antisense) and 5′-ATGTCCTACAACGATCC-3′ (sense) and 5′-GAACCATCTTGATCAGG-3′ (antisense) [22]. *SCFKS1*, *SCFKS2* and *ACTIN* probes were labeled by the random primer method using [^{32}P]α-dATP, hybridized at 68°C using Clontech Express Hybridization Solution (Clontech, Inc.; Palo Alto, CA, USA), and exposed to autoradiography film.

2.7. Temperature-induced cell lysis assays

S. cerevisiae bck1Δ yeast transformed with pYES2.1/*PCBCK1*, pYES2.1/*SCBCK1*, pYES2.1/*PCBCK1*Δ^{K575R}, or pYES2.1/*LACZ* were grown on minimal media plates at 30°C and 37°C containing galactose or galactose supplemented with 1 M sorbitol (used as an osmotic stabilizing agent). The plates were photographed after 7 days.

2.8. Immunoblotting

Protein lysates were extracted from *S. cerevisiae* using the YPER reagent (Pierce) containing a protease/phosphatase inhibitor cocktail (1 μg ml^{-1} each of leupeptin, aprotinin, and pepstatin; 1 mM each of phenylmethylsulfonyl fluoride and sodium orthovanadate; and 50 mM sodium fluoride). Soluble proteins were boiled in Laemmli buffer, separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with TBS containing 5% milk prior to incubation with the anti-V5 antibody for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) following the procedures recommended by the manufacturer.

2.9. Autophosphorylation and substrate kinase assays

S. cerevisiae bck1Δ yeast transformed with pYES2.1/*PCBCK1* or

A.

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   1 ATGGATTATT TAAAGAAAGT GAGATTATGG AGTGAGGAAG AAGTGGGAGA ATGGCTTGAG
  61 TCGAATAATT TTGGAGATTA TATGGATATT TTTAAAGAAA ACAATATTAA TGGAGATATT
 121 CTATTAGAAT GTAATGCGGC GGTGCTGAAG GAGCTTGGTG TTAAGAAACT AGGGGATCGT
 181 ATTAGGCTTT CTGTGTGTAT TAAGGGATTA AGGGAGAAAT GTATTGAATC GGCAAGAAAA
 241 TCGAAAATGA GCTTTTTAAT GATAGATAAT CAGATACAAG GGTTTTCATT TACATTACAG
 301 AGTCCTACGG ATCCTTTAAT TCCGGTTAAA TCGAGTGAAT TAGCGTCGCC GACATCAAAT
 361 TTCCAACTTG ATAATTCATA TAGCGATGCG TGTTGTTTTG ATTCATTAAA CAATTTCCAT
 421 TCAGTTCTTT CTAATATATC ATGTTCTACT AAAAGTATAA GTAGAAATTC ATCTATTAGT
 481 ACACAAAAAG TTTTTGAAAA ATCACCTATA TTTCTTGAGA CAAATGTTAT GCAATCAGAT
 541 TCCATTAAAA AGAATTGTCT TAAATTTATT GGAGAAAAAG GGCAAACTCG AATTGTAAAT
 601 ATTAGTCTTT GTTATACGGC AGATGCTATT TTATCTAAGG CGCTTAAAAA ATTCAATATA
 661 ACAGAAAATC CTAGTGAATG GAGAGTTTTT ATTACGAATG AAGATGGATC ATTTGAATGT
 721 ATTTCTAATG ATACGCTTTT GACCATTTCT CGTCAATTAT CAAGGCCAGA AAGAGAGCGA
 781 CTTATTTTAA AAAGAAAAAG TGATCCATTA ACACAAGAAG AATTTAAAAA ATCACAAACG
 841 ATTGCGAGAG AACAACGAGA TGCTATATAT CATACAGCTG TAATGGCAAA AACGTCAGGT
 901 AACTTGAGAA AATTGGAAAC ATTTTTTGGA GAAAAACTTG CTCCGACACT TACATCATCT
 961 TTTACTACAC CATTACCTTC ACCATTACCG AAAGATAATA AATATGGGAA TATAACACAT
1021 ATAAGAAATT TTTTTGGTCA GCGTCCTCCT AGCGAACTTA TTAATTCAAA TTTAGCAGAG
1081 TATTTTCCTG GTCATGAGAA AAAAGTTTTA GAACAAACAA TTAAAAATTC TATTAAATCT
1141 AATCATTTAA ATTCTTTTAA AGGATCAAAT TTCGACACAC CTAGTTCTAC TTTAACATAT
1201 GATCTTTCAT CTATTCCTGC AGTAGGTGAA GAACGGATTC AATATGAAGG CCGAAGATTA
1261 TCAGGAGTTA GTCATACACT TTCTTTATCA CGTTTTATTT CTACTCGATT TCCTACATTA
1321 CTTGAATCAT CTATTTTAAG GGAAAATAGT TCTCATCATA CATTATCTTC TTCATCAAAA
1381 TTGAATTCAG AAGGTAACAC ACAAGATAGC AAACCTAGTT TTCGAAAAAA GTTAGAAGAA
1441 AAGGCTTCTT CAGATGAAAC GTTTAAATCA GAATCACCTA ATATGTTAAT GGAGCCTTCA
1501 ATACTTTCAG ATTTTTATGA TGCAAGTTTG GAAACAGATA GAGATCTCTT GGATGATAAT
1561 ATTTTTGAAA ATAATGAAGC TAAAGACTTT AATACTGAAA AAGAAATAGA AAATAATAAT
1621 AGCGGCCCTA CAAGATGGAT TAAGGGTGCT TTAATAGGAA GCGGATCTTT TGGAAGTGTA
1681 TTTTTAGGAA TGAACGCTTT AAGTGGAGAA TTGATGGCAG TAAAACAAGT TGAAATACCG
1741 TCTATTGATA TTCAAGGATG TAAAAGAAAA AGGGCCATGT TGGATGCCTT ACAAAGAGAG
1801 ATTTCACTAC TTAAAGAATT GCATCATGAA AATATAGTTC AATATCTTGG ATCAAGCATG
1861 GACGAAACAC ATTTAACCTT CTTTCTTGAA TATGTTCCTG GGGGATCCGT TACTGCATTA
1921 TTAAATAATT ATGGGGCTTT TGAAGAGCCT TTAATTAGAA ATTTTGTGCG ACAAATTCTT
1981 AAAGGATTAA ACTATTTACA CAACAAAAAA ATCATTCATA GGGACATTAA GGGTGCCAAT
2041 ATTTTAGTTG ATAACAAAGG AGGAATTAAA ATATCAGATT TTGGTATATC TAAAAAAGTT
2101 GAAGCTAACC TATTATCTAT GACAAGAAAT CAACGCCCAT CTCTACAAGG ATCTGTTTAT
2161 TGGATGGCAC CTGAAGTTGT AAAACAAACT TTATATACCA GAAAAGCCGA TATATGGTCA
2221 CTCGGCTGTT TAATAGTAGA AATGTTTACA GGAAAACATC CTTTTCCGAA AATGAATCAA
2281 TTACAAGCCA TATTTAAGAT TGGACAATAC GTTTCCCCTG ATATACCAGA ACATTGTACT
2341 TCAGAAGCTA GACATTTTTT AGAAAAAATA TTTGAGCCAG ATTATCACGC ACGACCTACT
2401 GCAGCAGATT TACTTAAATA TAGTTTTTTA GGACCTATGG TTTCTAGTCC TTTGACTGAT
2461 GCAAAAAAAT AA
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B.

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   1 MDYLKKVRLW SEEEVGEWLE SNNFGDYMDI FKENNINGDI LLECNAAVLK ELGVKKLGDR  SAM
  61 IRLSVCIKGL REKCIESARK SKMSFLMIDN QIQGFSFTLQ SPTDPLIPVK SSELASPTSN
 121 FQLDNSYSDA CCFDSLNNFH SVLSNISCST KSISRNSSIS TQKVFEKSPI FLETNVMQSD
 181 SIKKNCLKFI GEKGQTRIVN ISLCYTADAI LSKALKKFNI TENPSEWRVF ITNEDGSFEC  RAS
 241 ISNDTLLTIS RQLSRPERER LILKRKSDPL TQEEFKKSQT IAREQRDAIY HTAVMAKTSG
 301 NLRKLETFFG EKLAPTLTSS FTTPLPSPLP KDNKYGNITH IRNFFGQRPP SELINSNLAE
 361 YFPGHEKKVL EQTIKNSIKS NHLNSFKGSN FDTPSSTLTY DLSSIPAVGE ERIQYEGRRL
 421 SGVSHTLSLS RFISTRFPTL LESSILRENS SHHTLSSSSK LNSEGNTQDS KPSFRKKLEE
 481 KASSDETFKS ESPNMLMEPS ILSDFYDASL ETDRDLLDDN IFENNEAKDF NTEKEIENNN
 541 SGPTRWIKGA LIGSGSFGSV FLGMNALSGE LMAVKQVEIP SIDIQGCKRK RAMLDALQRE  KINASE
 601 ISLLKELHHE NIVQYLGSSM DETHLTFFLE YVPGGSVTAL LNNYGAFEEP LIRNFVRQIL
 661 KGLNYLHNKK IIHRDIKGAN ILVDNKGGIK ISDFGISKKV EANLLSMTRN QRPSLQGSVY
 721 WMAPEVVKQT LYTRKADIWS LGCLIVEMFT GKHPFPKMNQ LQAIFKIGQY VSPDIPEHCT
 781 SEARHFLEKI FEPDYHARPT AADLLKYSFL GPMVSSPLTD AKK
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Fig. 1. Nucleotide and predicted amino acid sequence of *PCBCK1*. NCBI GenBank accession number AF312696. The open reading frame of *PCBCK1* is 2472-bp (A), which encodes a protein of 823 amino acids with a predicted molecular weight of 92.9 kDa. Conserved amino acids motifs are shaded gray, and include SAM from amino acids 21–79, the Ras association domain from amino acids 192–268, and the serine/threonine protein kinase domain from amino acids 546–810 (B). Multiple sequence alignment demonstrates homology in the carboxy terminal of these proteins (C).

pYES2.1/*PCBCK1Δ*^{K575R} were grown to mid-log phase (OD_{600} = 0.8) at 25°C in galactose minimal media and rapidly shifted to 37°C as described above. The yeast were lysed in YPER reagent (Pierce) containing 1 μg ml^{-1} each of leupeptin, aprotinin, and pepstatin; 1 mM each of phenylmethylsulfonyl fluoride and sodium orthovanadate; and 50 mM sodium fluoride for 20 min at room temperature. The suspension was clarified by centrifugation at 13 000 × *g* for 10 min and the protein concentration of the lysates was determined spectrophotometrically using the BCA method (Pierce). Protein lysate (500 μg) was precleared with a 50% slurry of protein A–Sepharose at 4°C for 30 min, and the protein was immunoprecipitated at 4°C for 2 h using the anti-V5 antibody (dilution 1:5000). The immunocomplexes were captured with protein A–Sepharose and were washed twice in lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} each of leupeptin, aprotinin, and pepstatin) and twice in kinase buffer [50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol (DTT)]. The immunocomplexes were added to a 40 μl reaction containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 μg of PHAS-I, 20 μM ATP, and 10 μCi [^{32}P]γ-ATP. In vitro kinase reactions to test substrate phosphorylation were incubated at 30°C for 30 min., while autophosphorylation was performed without the addition of PHAS-I to the kinase reaction and incubation was at 30°C for 2 h. Kinase reactions were stopped with the addition of Laemmli buffer, boiled for 5 min, resolved by electrophoresis on 12% SDS–polyacrylamide gels, and submitted to autoradiography.

3. Results

3.1. Cloning, chromosomal location, and mRNA expression of PCBCK1

Molecular cloning of *PCBCK1* was performed using a degenerate PCR strategy and traditional library screening (Fig. 1A). We designed degenerate oligonucleotide primers derived from conserved amino acid sequences of fungal MEKK proteins. An initial 373-bp PCR amplicon was obtained and was found to be unique on NCBI GenBank analysis but homologous to *S. cerevisiae BCK1*. To further confirm that the PCR product was specifically represented within the *P. carinii* genome, the 373-bp PCR amplicon was hybridized to *P. carinii* chromosomes separated by CHEF. The probe hybridized to a single chromosomal band (Fig. 2), demonstrating that *PCBCK1* is represented within the *P. carinii* genome. Using of the 373-bp PCR amplicon as a probe, a 2472-bp full-length cDNA was obtained by screening a *P. carinii* cDNA library in the bacteriophage Uni-Zap XR (GenBank accession number AF312696). Using this same probe, we identified two mRNA transcripts for *PCBCK1* (Fig. 3). The observed transcript sizes are 2500 and 2600 bp, which might indicate alternative splicing of the *PCBCK1* gene.

C.

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PCBCK1 : mdylkkvrlwseeevgewlesnnfgdymdifkenningdillecnaavlkelgvkklgdrirlsvcikglrekciesark
SCSTE11: ------------------------------------------------------------------------meqtqtaegt
SPBYR2 : ----------------------------------------------------------------------------------
SCBCK1 : ----------------------------------------------------------------------------------
HSMEKK1: ----------------------------------------------------------------------------------

PCBCK1 : skmsflmidnqiqgfsftlqsptdplipvksselasptsnfqldnsysdaccfdslnnfhsvlsniscstksisrnssis
SCSTE11: dlligdektndlpfvqlfleeigctqyldsfiqcnlvteeeikyldkdilialgvnkigdrlkilrksksfqrdkrieqv
SPBYR2 : ------------------------------------------------------------meyytskevaewlksiglek
SCBCK1 : ----------------------------------------------------------------------------mpflrkia
HSMEKK1: ----------------------------------------------------------------------------------

PCBCK1 : tqkvfekspifletnvmqsdsikknclkfigekgqtrivnislcytadailskalkkfnitenpsewrvfitnedgsfec
SCSTE11: nrlknlmekvsslstatlsmnselipekhcvifilndgsakkvnvngcfnadsikkrlirrlphellatnsngevtkmvq
SPBYR2 : yieqfsqnniegrhlnhltlpllkdlgientakgkqflkqrdylrefprpcilrfiacngqtravqsrgdyqktlaialk
SCBCK1 : gtahthsrsdsnssvkfghqptssvastkssskspratsrksiyddirsqipnltpnstssqfyestpvieqsfnwttdd
HSMEKK1: ----------------------------------------------------------------------------------

PCBCK1 : isndtlltisrqlsrpererlilkrksdpltqeefkksqtiareqrdaiyhtavmaktsgnlrkletffgeklaptltss
SCSTE11: dydvfvldytknvlhllydvelvtichandrveknrlifvskdqtpsdkaistskklylrtlsalsqvgpsssnllaqnk
SPBYR2 : kfsledaskfivcvsqssrikliteeefkqicfnssperdrliivpkekpcpsfedlrrsweielaqpaalssqsslsp
SCBCK1 : hisagtlenptsftnssykndngpsslsdsrkssggnsvnslsfdklilswdptdpdewtmhrvtswfkfhdfpeswilf
HSMEKK1: ----------------------------------------------------------------------------------

       :                              ********
PCBCK1 : fttplpsplpkdnkygnithirnffg**QRPPSE**l**I**nsnlaeyfpghekkvleqtiknsiksnhlnsfkgsnfdtpsstlty
SCSTE11: gishnnaegklridntekdrirqifn**QRPPSE**f**I**stnlagyfphtdmkrlqktmresfrhsarlsiaqrrplsaesnnig
SPBYR2 : klssvlptstqkrsvrsnnakpfesy**QRPPSE**l**I**nsrisdffpdhqpkllektisnslrrnlsirtsqghnlgnfgqeil
SCBCK1 : fkkhqlfghrfikllaydnfavyekylpqtktasytrfqqllkktmtknvtnshirqksasklkssrsssesiksklkns
HSMEKK1: ----------------------------------------------------------------------------------

PCBCK1 : dlssipavgeeriqyegrrlsgvshtlslsrfistrfptllessilrenssh--------------------------
SCSTE11: dillkhsnavdmallqgldqtrlsskldttkipklahkrpedndaisnqlellsvesgeeedhdffgedsdivslptkia
SPBYR2 : prssrrarpselvcplsslrisvaedvnrlpridrgfdppltvsstqrisrppslqksitmvgveplyqsngneksskyn
SCBCK1 : ksqedisnsrstsesplsptksgpsktdekiflhststhqktksasslyrrsfislrgssssnassakspsniklsipar
HSMEKK1: -------menketlkglhkmddrpeermireklkatcmpawkhewlerrnrrgpvvvkpipvkgdgsemnhlaaespgev

       :               *********
PCBCK1 : ----------------------------------------------------------------------------------
SCSTE11: tpkn------------------------------------------------------------------------------
SPBYR2 : vfsesahgnhqvl**S**f**SP**gs**SPS**fieqpspisptsttsedtntleedtddqsik-----------------------
SCBCK1 : phsiiesnstltk**S**a**SP**pa**SPS**ypsifrr-----------------------------------------------
HSMEKK1: qasaaspaskgrr**S**p**SP**gn**SPS**grtvksespgvrrkrvspvpfqsgritpprrapspdgfspyspeetnrrvnkvmrarl

PCBCK1 : ----------------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------------
SCBCK1 : ----------------------------------------------------------------------------------
HSMEKK1: yllqqigpnsfliggdspdnkyrvfigpqncscargtfcihllfvmlrvfqlepsdpmlwrktlknfeveslfqkyhsrr


PCBCK1 : ----------------------**H**tl**SSSS**klnsegntqdskpsfrkkleekassdetfksespnmlmepsilsdfydasl
SCSTE11: ----------------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------------
SCBCK1 : ----------------------**H**hk**SSSS**essllnslfgsgigeeaptkpnpqghslssenlakgkskhyetnvssplkq
HSMEKK1: ssrikapsrntiqkfvsrmsns**H**tl**SSSS**tstsssensikdeeeqmcpicllgmldeesltvcedgcrnklhhhcmsiwa

PCBCK1 : etdrdllddnifenneakdfntekeiennnsgptr-------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------------
SCBCK1 : sslptsddkgnlwnkfkrksqigvpspntvayvtsqetpslksnsstatltvqtadvnipspsssppppipktanrslevi
HSMEKK1: eecrrnreplicplcrskwrshdfyshelsspvdspsslraaqqqtvqqqplagsrrnqesnfnlthygtqqippaykdl

PCBCK1 : ----------------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------------
SCBCK1 : stedtpkissttasfketypdcinpdktvpvpvnnqkysvknflldqkfyplkktglndsenkyilvtkdnvsfvplnlk
HSMEKK1: aepwiqvfgmelvgclfsrnwnvremalrrlshdvsgalllangestgnsggssgsspsggatsgssqtsisgdvveacc
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Fig. 1 (*Continued*).

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PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : svaklssfkesaltklginhknvtfhmtdfdcdigaaipddtleflkkslflntsgkiyikdqmklqqkpkpapltsenn
HSMEKK1: svlsmvcadpvykvyvaalktlramlvytpchslaeriklqrllqpvvdtilvkcadansrtsqlsistllelckgqage

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : vplksvkskssmrsgtssliastddvsivtsssditsfdehasgsgrstpkprvitmtevsntnpteelnywnikevlsh
HSMEKK1: lavgreilkagsigiggvdyvlncilgnqtesnnwqellgrlclidrlllefpaefyphivstdvsqaepveirykklls

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : eenapkmvfktspklelnlpdkgsklniptpiteneskssfqvlrkdegteidfnhrreapytkpevapkrespkppant
HSMEKK1: lltfalqsidnshsmvgklsrriylssarmvttvphvfskllemlsvsssthftrmrrrlmaiadeveiaeaiqlgvedt

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : spqrtlstskqnkpirlvrastkisrskrskplppqllsspieasssspdsltssytpasthvlipqpykgandvmrrlk
HSMEKK1: ldgqqdsflqasvpnnylettensspectvhlektgkglcatklsassediserlasisvgpsssttttttttteqpkpmv

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : tdqdstsnspslkmkqkvnrsnsnvstsnsifyspspllkrgnskrvvsstsaadifeenditfadappmfdsddsddds
HSMEKK1: qtkgrphsqclnssplshhsqlmfpalstpssstpsvpagtatdvskhrlqgfipcripsaspqtqrkfslqfhrncpen

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : sssddiiwskkktapetnnenkkdeksdnssthsdeifydsqtqdkmerkmtfrpspevvyqnlekffpranldkpiteg
HSMEKK1: kdsdklspvftqsrplpssnihrpkpsrptpgntskqgdpsknsmtldlnssskcddsfgcssnssnavipsdetvftpv

PCBCK1 : ----------------------------------------------------------------------------
SCSTE11: ----------------------------------------------------------------------------
SPBYR2 : ----------------------------------------------------------------------------
SCBCK1 : iasptspksldsllspknvassrtepstpsrpvppdssyefiqdglngknkplnqaktpkrtktirtiaheaslarknsv
HSMEKK1: eekcrldvntelnssiedlleasmpssdttvtfksevavlspekaenddtykddvnhnqkckekmeaeeeealaiamams

       :                                                 *********************************
PCBCK1 : ------------------------------------------------WiKGAlIGSGSFGsVfLGMNAlsGELMAVKQVei
SCSTE11: ------------------------------------------------WlKGAcIGSGSFGsVYLGMNAhtGELMAVKQVei
SPBYR2 : ------------------------------------------------WirGAlIGSGSFGqVYLGMNAssGELMAVKQVil
SCBCK1 : klkrqntkmwgtrmvevtenhmvsinkaknskgeykefa-------WmKGemIGkGSFGaVYLclNvttGEmMAVKQVev
HSMEKK1: asqdalpivpqlqvengediiiiqqdtpetlpghtkakqpyredteWlKGqqIGlGafsscyqaqdvgtGtlMAVKQVty

       :                                                                          *****
PCBCK1 : psidiqgckrkramldalqr-------------------------------------------------------EisLL
SCSTE11: knnnigvptdnnkqansdenneqeeqqekiedvgavshpktnqnihrkmvdalqh--------------------EmnLL
SPBYR2 : dsvseskdrhaklldalag---------------------------------------------------------EiaLL
SCBCK1 : pkyssqneailstvealrsevst-----------------------------------------------------L
HSMEKK1: vrntsseqeevvealreeirmmshlnhpniirmlgatceksnynlfiewmaggsvahllskygafkesvvinyte-----

       : *************          *****************  *********       **********************
PCBCK1 : KELhHENIVqYlGssmdeth---LtfFLEYVPGGSVtaLLnnYGaFeEpLIrnfvr--QiLKGLnYLHnKkIiHRDIKGA
SCSTE11: KELhHENIVtYyGasqeggn---LniFLEYVPGGSVssmLnnYGpFeEsLItnftrqi--LiGvaYLHkKnIiHRDIKGA
SPBYR2 : qELsHEhIVqYlGsnlnsdh---LniFLEYVPGGSVagLLtmYGsFeEtLvknfik--QtLKGLeYLHsrgIvHRDIKGA
SCBCK1 : KdLdHlNIVqYlGfenknniysl---FLEYVaGGSVgsLirmYGrFdEpLIkhltt--QvLKGLaYLHsKgIlHRDmKad
HSMEKK1: -------------------------------------------------------QlLrGLsYLHenqIiHRDvKGA

       : ********************                                    **********************
PCBCK1 : NILvDnKGgiKIsDFGISKKveanllsmtrnq------------------------RpSlQGSVyWMAPEVVKQTlyTr
SCSTE11: NILiDiKGcvKItDFGISKKlsplnkkqnk--------------------------RaSlQGSVFWMsPEVVKQTatTa
SPBYR2 : NILvDnKGkiKIsDFGISKKlelnststktgga------------------------RpSfQGSsFWMAPEVVKQTmhTe
SCBCK1 : Nllldqdgickisdfgisrkskdiysnsdmtmr----------------------------GtVFWMAPEmVdtkqgys
HSMEKK1: NlLiDstgqrlriadfgaaarlaskgtgagefqgqllgtiafmapevlrgqqygrsc-----------------------
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Fig. 1 (*Continued*).

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       :  *  ********************                                          *********
PCBCK1  :  Ka-DiWSlGClivEMFTGKHPfPkmn-----------------------------------------QlQAIFKIGqyv
SCSTE11:  Ka-DiWStGCvvIEMFTGKHPfPdfs-----------------------------------------QmQAIFKIGtnt
SPBYR2  :  Kt-DiWSlGClvIEMlTsKHPyPncd-----------------------------------------QmQAIFrIGeni
SCBCK1  :  akvDiWSlGCivlEMFaGKrPwsnlevva---------------------------------------AmFKIGksk
HSMEKK1:  ---DvWSvGCaiIEMacakppwnaekhsnhlalifkiasattapsipshlspglrdvalrclelqpqd------------

       :                                  ***********
PCBCK1  :  spdipehctsearhflekifepdyha-----RPTAadLLkysflgpmvsspltdakk-----------------------
SCSTE11:  tpeipswatsegknflrkafeldyqy-----RPsAlELLqHPwldahii-------------------------------
SPBYR2  :  lpefpsnisssaidflektfaidcnl-----RPTAsELLsHPfvs-----------------------------------
SCBCK1  :  sappipedtlplisqigrnfldacfeinpekRPTAnELLsHPfsevnetfnfkstrlakfiksndklnssklritsqenk
HSMEKK1:  -------------------------------RPpsrELLkHPvfrttw---------------------------------

PCBCK1  :  --
SCSTE11:  --
SPBYR2  :  --
SCBCK1  :  te
HSMEKK1:  --
```

Fig. 1 (*Continued*).

3.2. PCBCK1 *contains conserved protein domains*

Analysis of the translated cDNA of *PCBCK1* demonstrates a molecule of 823 amino acids and predicted molecular mass of 92.9 kDa (Fig. 1B). PCBCK1 has 47% identity to *S. cerevisiae* BCK1 using the NCBI database BLASTX (translated nucleotide query to protein database). Additional homology includes 39% identity to *S. pombe* BYR2 (M74293), and 35% identity to *S. cerevisiae* STE11 (U19103). The putative protein sequence has three highly conserved protein domains. In the N-terminal region there is a sterile alpha motif (SAM) present from amino acids 21–79. SAM is a protein domain found in signaling molecules which can act as a binding site for SH2-containing proteins such as scaffolding proteins and facilitate native protein homodimerization [20,42–44]. The PCBCK1 SAM domain is homologous to the fungal SAM domains found in *S. cerevisiae* BCK1, STE11, BOB1, BOI2, *S. pombe* Byr2, and *Kluyveromyces lactis* BCK1 (NCBI Conserved Domain Database). The amino acid sequence from 192–268 of PCBCK1 contains a Ras association domain (RalGDS/AF-6) which has been shown in some cases to bind RasGTP. This domain is homologous to the RAS domains found in fungal adenylyl cyclase proteins from *S. cerevisiae*, *Magnaporthe grisea*, *S. pombe*, *Podospora anserina*, and *Neurospora crassa*, and to the *S. cerevisiae* proteins STE4 and STE50 [45,46].

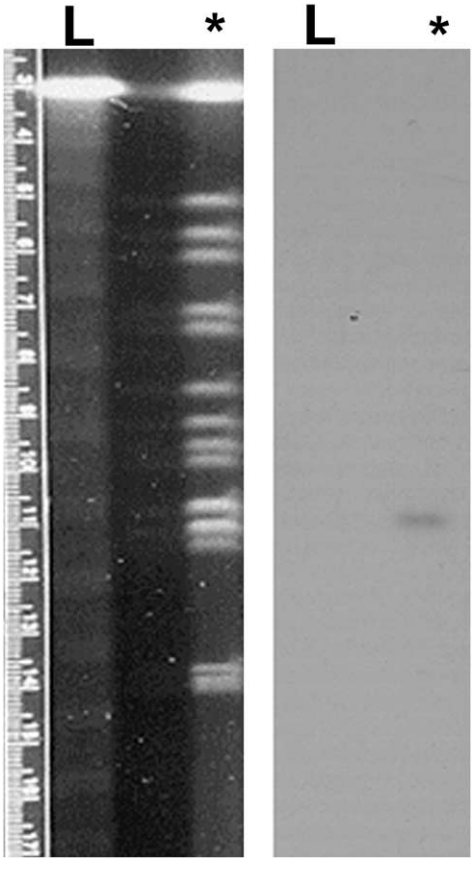

Fig. 2. Chromosomal location of *PCBCK1*. *P. carinii* chromosomes were separated by CHEF. *PCBCK1* hybridized to a single chromosome. L indicates the molecular weight ladder and the *PCBCK1* hybridization is indicated by *.

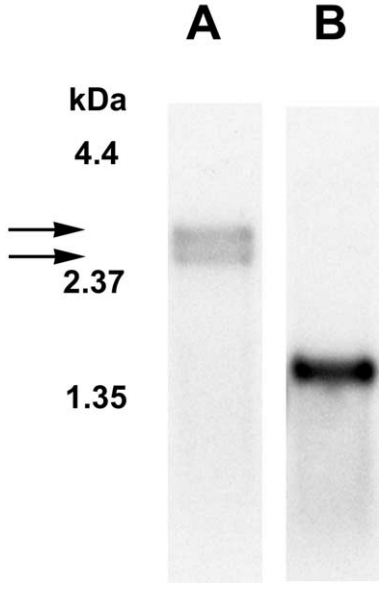

Fig. 3. mRNA expression of *PCBCK1*. Two transcripts are detected from RNA isolated from *P. carinii* organisms hybridized with a radioactively labeled *PCBCK1* (lane A). The approximate sizes are 2500 and 2600 bp (arrows). Lane B demonstrates the same blot reprobed with *P. carinii* actin.

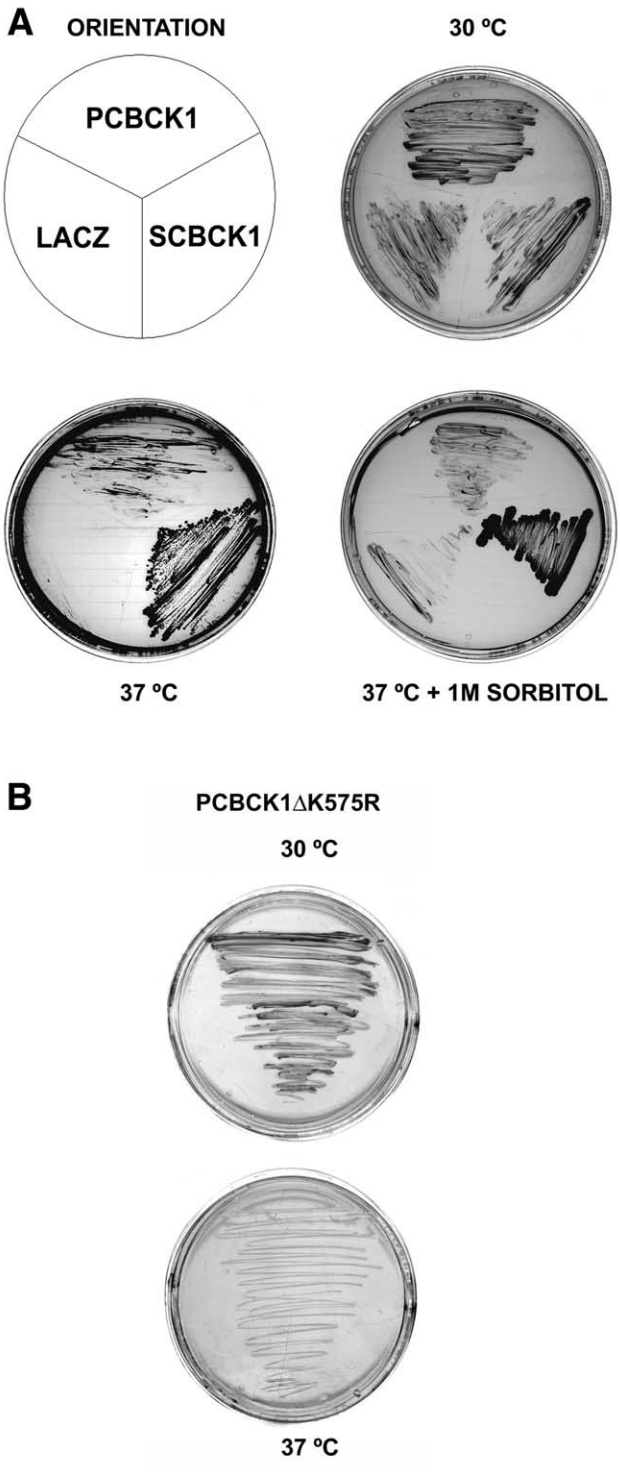

Fig. 4. PCBCK1 complements cell-wall integrity signaling to allow fungal growth at elevated temperature. *S. cerevisiae bck1Δ* yeast transformed with *PCBCK1*, *SCBCK1*, or *LACZ* grow normally at 30°C (A). *PCBCK1* expression supports growth at 37°C as does the wild-type *SCBCK1* (as a positive control), however the mutant yeast expressing LACZ (as a negative control) do not grow at the elevated temperature. Addition of 1 M sorbitol to the plates ameliorates this effect by osmotic stabilization of the cell wall. *S. cerevisiae bck1Δ* yeast transformed with PCBCK1Δ^{K575R}, the kinase dead mutant, grow normally at 30 °C but do not grow at the elevated temperature (B).

Toward the carboxyl terminal there is a highly conserved serine/threonine protein kinase catalytic domain (amino acids 546–810). Protein alignment of PCBCK1 with *S. cerevisiae* STE11 and BCK1, *S. pombe* Byr2, and human MEKK1 reveals significant homology occurring toward the carboxy terminal of the proteins (Fig. 1C).

3.3. PCBCK1 *complements cell-wall integrity signaling in* S. cerevisiae bck1Δ *yeast*

We expressed *PCBCK1* in *S. cerevisiae bck1Δ* mutant yeast in order to gain insight in the function of this gene. These mutant yeast, with their *BCK1* deleted, will lyse at the non-permissive temperature of 37°C due to disruption of MAPK signaling at the level of the absent *BCK1* gene. Growth can occur at the non-permissive temperature if an osmotic stabilizing agent such as sorbitol is added to the yeast [18,20]. *PCBCK1* allows growth of the *bck1Δ* yeast at 37°C, as does the positive control, *S. cerevisiae BCK1* (Fig. 4A). As expected, the *LACZ* gene expressed in these yeast fails to complement the signaling defect and growth cannot occur at the elevated temperature. To access whether the observed complementation was specific for PCBCK1, we created a kinase dead mutant, PCBCK1Δ^{K575R}, and tested growth at both temperatures. As shown in Fig. 4B, PCBCK1Δ^{K575R} grows normally at 30 °C but not at 37 °C. This single site mutation in the kinase domain, changing the conserved lysine to an arginine, has been shown to abolish kinase activity in analogous kinases [39–41]. For further confirmation, we verified expression of PCBCK1 at the elevated temperature by immunoblotting protein lysates from these yeast grown at 25°C and 37°C (Fig. 5).

3.4. PCBCK1 *is a functional kinase*

PCBCK1 was immunoprecipitated and assayed for kinase activity with and without PHAS-I as a substrate. PCBCK1 is

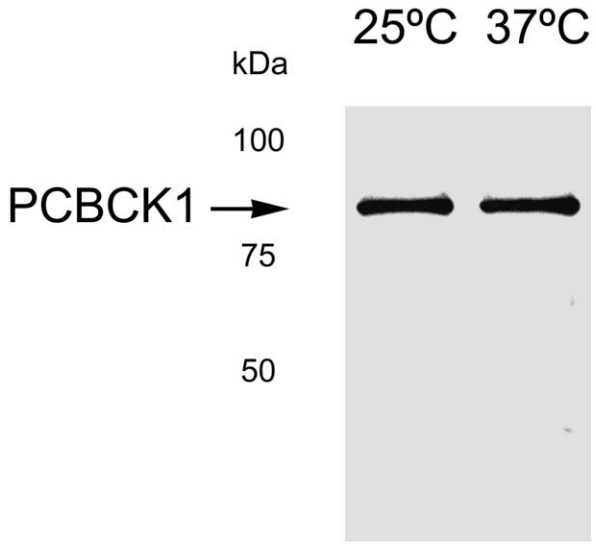

Fig. 5. Protein expression of PCBCK1 at elevated temperature. To further verify that *PCBCK1* functionally complements cell-wall integrity in yeast at elevated temperature, we immunoblotted protein lysates from *S. cerevisiae bck1Δ* yeast expressing *PCBCK1* which were grown at 25°C or 37°C in galactose minimal media for 72 h. An appropriately sized 92-kDa protein is observed under both growth conditions.

an active kinase as judged by its observed autophosphorylation (Fig. 6A), and its ability to phosphorylate PHAS-I (Fig. 6B). No difference in phosphorylation of PHAS-I was observed in yeast grown at 25°C or temperature-shifted to 37°C. This is likely due to autophosphorylation of PCBCK1 and is consistent with the similar observation that *S. cerevisiae* STE11 (the yeast MEKK that functions in pheromone-induced mating) does not have increased kinase activity nor enhanced mRNA expression after exposure to mating pheromone [41]. Testing of the kinase dead mutant, PCBCK1Δ^{K575R}, under identical conditions reveals no observed autophosphorylation (Fig. 6A) or PHAS-I substrate phosphorylation (Fig. 6B).

3.5. PCBCK1 *restores cell-wall integrity signaling to maintain expression of the temperature-inducible glucan synthetase,* FKS2*, at elevated temperature*

FKS2 mRNA expression during elevated temperature requires an intact cell-wall integrity MAPK pathway if the heat stress persists beyond 60–120 min, which functions to prevent fungal lysis due to cell-wall instability [22]. To determine if *PCBCK1* complements this signaling defect in *bck1Δ* yeast to maintain expression of *FKS2* at elevated temperature, we analyzed *FKS2* mRNA expression in *S. cerevisiae bck1Δ* yeast expressing *PCBCK1* grown at 25°C or temperature-shifted to 37°C at various time points. *PCBCK1* restored the MAPK signaling defect to maintain *FKS2* mRNA expression after 60 min of elevated temperature (Fig. 7A). This is in contrast to *S. cerevisiae bck1Δ* yeast which demonstrate reduced *FKS2* expression at 60 min and absence of *FKS2* expression at 120 min at the elevated temperature (Fig. 7B). These data support the function of *PCBCK1* in the cell-wall integrity MAPK pathway within *S. cerevisiae*.

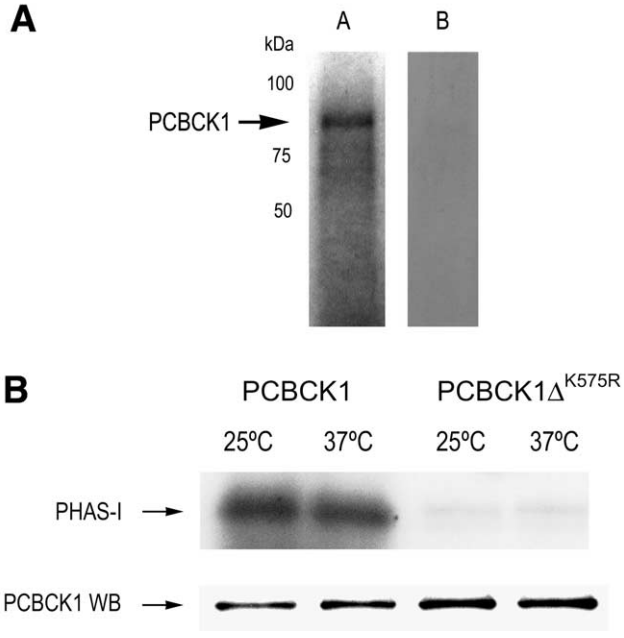

Fig. 6. PCBCK1 exhibits functional kinase activity. PCBCK1 was immunoprecipitated from *S. cerevisiae bck1Δ* yeast and tested in a kinase reaction without a substrate to demonstrate autophosphorylation (A, lane A). The kinase dead mutant, PCBCK1Δ^{K575R}, does not have an observed autophosphorylation (A, lane B). Next, PCBCK1 was immunoprecipitated from *S. cerevisiae bck1Δ* yeast grown at 25°C or temperature-shifted to 37°C and tested in a kinase reaction using PHAS-I as a phosphorylation substrate. As shown in B, there was no difference in observed phosphorylation at the two temperatures. Likewise, the kinase dead mutant, PCBCK1Δ^{K575R}, cannot phosphorylate the substrate. The 37°C time point shown is at 2 h, and is no different from time points tested at 30 or 60 min.

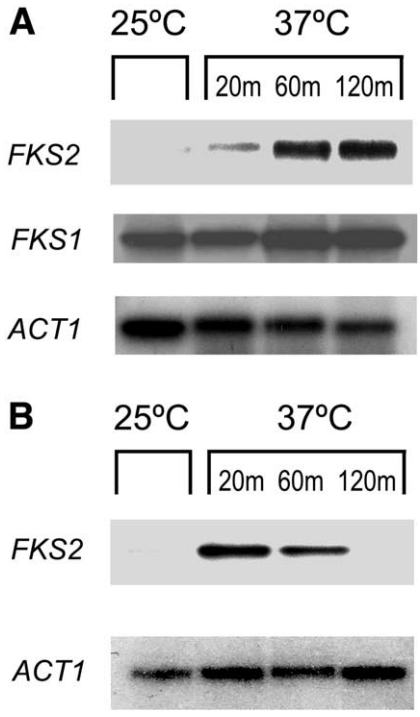

Fig. 7. PCBCK1 complements cell-wall integrity signaling to allow maintenance of *FKS2* mRNA expression at elevated temperature. *FKS2* expression is not detected in *S. cerevisiae bck1Δ* yeast expressing *PCBCK1* grown at 25°C, however *FKS1* expression is present. When these yeast are shifted to 37°C, we identify *FKS2* expression starting in 20 min, increasing at 60 min, and stabilizing at 120 min (A). This is in contrast to the mutant yeast which have a disruption in cell-wall integrity MAPK signaling due to deletion of their *BCK1* gene (*S. cerevisiae bck1Δ*). These yeast demonstrate *FKS2* induction at 37°C in 20 min, a reduction at 60 min, and loss of *FKS2* expression at 120 min (B). *ACT1* indicates *S. cerevisiae* actin.

4. Discussion

The opportunistic fungus *P. carinii* remains an important cause of pneumonia in patients infected with HIV, and in patients with altered cellular immunity [4,47,48]. Our laboratory has been investigating components of the *P. carinii* cell wall, and we and others have found that the *P. carinii* cyst has a cell wall enriched in β-glucans, chitins, and a family of glycoproteins designated as gpA or MSG [6,23,49–53]. We have previously identified and characterized a β-1-3-glucan synthetase from *P. carinii*, *GSC1*, which is capable of incorporating 5′-diphosphoglucose into glucan [23]. In this investigation we describe the identification of a *MEKK* gene from *P. carinii*, *PCBCK1*, which functions in the cell-wall integrity MAPK cascade in *S. cerevisiae*. Yeast with the *BCK1* gene deleted are defective in cell-wall assembly and lyse at the non-permissive temperature of 37°C. Our results show that expression of *PCBCK1* complements the *bck1Δ* defect in *S. cerevisiae*, restoring growth at the elevated temperature. Further, restoration of cell-wall integrity MAPK signaling with *PCBCK1* specifically allows maintenance of the yeast down-

stream temperature-inducible β-1-3-glucan synthetase *FKS2* with elevated temperature.

Activation of the cell-wall integrity MAPK pathway is a survival mechanism for fungi which experience environmental stress, such as elevated temperature. The fungal cell wall, comprised primarily of glucan, is in constant flux during normal cellular growth [54]. In *S. cerevisiae*, subunits of the β-1-3-glucan synthetase enzymes are encoded by the genes *FKS1* and *FKS2* [28,29]. These enzymes are the principal means that glucose gets incorporated in the fungal cell wall as β-1,3-glucan. Under normal growth conditions, *FKS1* is the predominantly expressed subunit and its level fluctuates slightly with the cell cycle. When the yeast are exposed to thermal stress, *FKS2* is induced through dual mechanisms involving calcineurin and the cell-wall integrity MAPK pathway. Induction of *FKS2* mRNA occurs within 20 min of heat shock, and continued expression of *FKS2* mRNA requires a functional cell-wall integrity MAPK pathway [22].

P. carinii exists in the alveolar spaces of the infected host. Human-to-human transmission is the suspected route of acquisition of this infection in immunocompromised individuals, however this is controversial [15,16,55–57]. It is uncertain whether *P. carinii* has a natural environment similar to many other fungi. It is intriguing that *P. carinii* nucleic acids have been identified in natural settings outside of the lung, but a definite ecological niche has not yet been identified [10–12,58]. If *P. carinii* experiences temperature shifts, ranging from 25°C (room temperature) to 37°C (body temperature), we can envision that *P. carinii* utilizes a MAPK cell-wall integrity pathway to stabilize its cell wall similar to other fungi. Interference of the cell-wall integrity MAPK pathway in *P. carinii* is an attractive target for novel drug development because the machinery for glucan biosynthesis is not present within mammals. Since it is impossible to culture *P. carinii*, and the methodology for gene disruption and isolation of clonal populations of mutant *P. carinii* organisms is not currently possible, we had to analyze the function of the *P. carinii* MEKK gene in a closely related yeast, *S. cerevisiae*. Our results suggest that *P. carinii* uses signaling molecules to maintain cell-wall integrity similar to those found in closely related fungi. Within the infected lung, *P. carinii* would be exposed to temperatures of 37°C or higher during fever. The function of *PCBCK1* is likely necessary to maintain the integrity of the *P. carinii* cell wall under these conditions, similar to the function of the *S. cerevisiae* BCK1 protein.

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