The *Pneumocystis* Ace2 Transcription Factor Regulates Cell Wall-remodeling Genes and Organism Virulence*

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Background: Ace2 transcription factors regulate fungal cell wall remodeling.

Results: *Pneumocystis carinii* Ace2 (PcAce2) is activated by lung matrix proteins and participates in fungal virulence.

Conclusion: PcAce2 represents a key component of life cycle regulation that is induced by *Pneumocystis* contact with host substrates.

Significance: This is the first example demonstrating a contact-induced activation of the Ace2 transcription factor.

*Pneumocystis carinii* (Pc) β-glucans are major components of the organism cell wall; yet, the regulation of Pc cell wall genesis and remodeling is not well understood. Ace2 transcription factors, which are present in many fungi, regulate glucanases and other enzymes needed for cell wall remodeling. The cloning and heterologous expression of PcAce2 in *ace2Δ* *Saccharomyces cerevisiae* demonstrated that PcAce2 can restore the defective glucanase and endochitinase gene expression of the mutant as well as regulate cell wall β-glucan biosynthetic genes. Furthermore, when a reconstructed yeast system was used, PcAce2 activated the transcription of the *Pneumocystis gsc1* β-glucan synthetase, confirming the activity of a Pc transcription factor on a native *Pc* promoter and gene for the first time. We further observed that *Pneumocystis* binding to host extracellular matrix proteins and lung epithelial cells induced the phosphorylation (activation) of the PcAce2 transcription factor. Finally, we present a novel method that confirms the role of PcAce2 in modulating organism virulence using *ace2Δ* *Candida glabrata* infection in neutropenic mice. Together, these results indicate that the adherence of Pc to lung matrix proteins and epithelial cells leads to the activation of the Ace2 transcription factor, which regulates cell wall degradation and biosynthesis genes that are required for cell wall remodeling.

*Pneumocystis* species are opportunistic fungal pathogens that inflict serious morbidity and mortality on immune-compromised hosts, with fatality rates ranging between 10 and 45% in patients with this infection (1). *Pneumocystis jirovecii* is the species that infects immune-compromised humans, whereas *Pneumocystis carinii* (Pc)² represents the parallel species utilized in the widely studied rodent models. Studies of Pc have proven extremely useful, as all of the therapies for the treatment of human disease have first been developed through studies of the rodent models (2). Of considerable concern is the development of potential drug resistance to sulfa compounds and other agents used widely to treat this devastating infection (3).

The development of new agents to treat *Pneumocystis* infections has been slowed by the lack of a reliable culture system, the absence of a means to directly genetically manipulate the organisms, and an incomplete understanding of the *Pneumocystis* life cycle. Classic ultrastructural studies have demonstrated a curious, tight interaction between *Pneumocystis* trophic forms and lung epithelial cells (4). Additional *in vitro* investigations by our group and others have implicated a strong role for host extracellular matrix proteins, such as fibronectin, and an integrin-like surface receptor on *Pneumocystis* termed Pclnt1 in mediating the binding of organisms to host substrates (5–8). Because of these observations, we performed a series of studies to determine the consequences of *Pneumocystis* binding on its life cycle regulatory mechanisms. These studies demonstrated that *Pneumocystis* recognition of lung epithelial cell surfaces or host matrix proteins such as fibronectin or vitronectin, a process termed thigmotropism, lead to enhanced expression and activity of unique signaling kinases including the PcSte20 MAP kinase and its downstream partner, the cell wall biosynthetic kinase termed PcCbk1 (8–10). These proteins have been shown to have roles in both organism proliferation and cell wall remodeling; however, the downstream mechanisms that are triggered by these kinases as they act on the *Pneumocystis* life cycle have remained obscure (9, 10).

To address this question, we next undertook a series of experiments to determine the role of an Ace2-like protein in mediating contact-induced signaling and cell wall remodeling in *Pneumocystis*. ACE2 genes encode fungal DNA-binding proteins that have been implicated in the control of cell cycle progression in *Saccharomyces cerevisiae* and in the regulation of cell wall-degrading enzymes in *S. cerevisiae* and other fungi, an essential process in life cycle progression and growth (11, 12). The process of cell wall remodeling requires the careful integration and regulation of enzymes that not only degrade the cell wall but also synthesize cell wall components. In addition, Ace2 proteins have been implicated in the pathogenesis of *Candida albicans* and *Candida glabrata*; strains with a deletion of ace2 exhibit enhanced virulence in animal models (13, 14). Data

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2 The abbreviations used are: Pc, *Pneumocystis carinii*; RACE, rapid amplification of cDNA ends; RAM, regulation of Ace2 and morphogenesis; OD, optical density.
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from *S. cerevisiae* studies indicate that the Cbk1 protein can phosphorylate and activate the Ace2 transcription factor (11). Analysis of the *Pneumocystis* Genome Project database indicated the potential presence of a partial *ace2*-like transcription factor gene, rendering it a potentially important target in the cell signaling that is induced by the organisms adhering to matrix proteins and lung epithelial cells.

In this report, we demonstrate that the binding of *Pneumocystis* to lung matrix proteins and epithelial cells activates PcAce2. This is the first demonstration of a contact-induced activation of Ace2 in any fungal species. In addition, we demonstrate not only that PcAce2 can mediate the expression of cell wall degradation enzymes, as previously observed in *S. cerevisiae* but also that PcAce2 activates the expression of target genes that participate in cell wall synthesis. Using a murine model of pathogenesis with *ace2Δ C. glabrata* as an additional vehicle for heterologous expression, we further provide evidence that PcAce2 has a role in organism virulence that is parallel to its *Candida* orthologue. This represents the first application of heterologous expression to test the potential virulence of a Pc gene. Based on these findings, our postulate is that PcAce2 represents a key component of contact-induced cell signaling and life cycle regulation that is induced by *Pneumocystis* contact with host cellular substrates.

**EXPERIMENTAL PROCEDURES**

**Reagents and Strains**—For these studies, Pc organisms were originally derived from American Type Culture Collection (ATCC, Manassas, VA) stocks and were propagated and purified from corticosteroid-treated rats as reported previously (15). Unless otherwise noted, all reagents were obtained from Sigma-Aldrich. Standard yeast genetics and molecular biology techniques were implemented to generate the yeast strains and plasmids used in this report.

**Cloning of the Complete Pc cDNA Sequence Encoding PcAce2**—Sequences encoding potential Ace2 fungal transcription factors were searched using the keyword “Ace2” in the Pc Genome Project database. Initially, 39 partial sequences were identified with homology to fungal *ACE2* genes in the genome database. Of these, we focused on an 871-bp sequence because it had the greatest similarity to other fungal Ace2 transcription factors. Sequence extension and full-length cloning were performed using the rapid amplification of cDNA ends (RACE) method according to the manufacturer’s instructions (GeneRacer® kit, Invitrogen). Total Pc RNA was prepared using TRIzol® reagent (Invitrogen) according to the manufacturer’s protocol. Aliquots of RACE products were resolved on 2% agarose gels. In this manner, we generated a complete 1146-bp *Pcace2* cDNA sequence. Sequence analysis was conducted with MacVector™ software (Accelrys Software, San Diego). Homology and zinc finger motifs were determined using the NCBI BLAST alignment program.

**Southern Hybridization of *Pc* Ace2 Sequences in *Pc* Genomic DNA**—Southern hybridization was used to confirm that the *Pcace2* sequence was truly represented within the Pc genome and was not related to the amplification of host nucleic acid contamination. A 336-bp *Pcace2* amplicon was used as a probe against freshly isolated Pc genomic DNA, which was prepared with the IsoQuick nucleic acid extraction kit (Orca Research Inc., Bothell, WA). The *Pcace2* probe was labeled with 32P using the random primer method (RadPrime, Invitrogen. Twenty micrometers of genomic DNA was digested with either EcoRI or HindIII and separated on a 1% agarose gel. The transfer and hybridization were performed as described (15).

**Pcace2 mRNA Expression over the Life Cycle of the Organism**—Separated Pc cystic and trophic forms were isolated by differential filtration through 3-μm filters as detailed previously (16). Total RNA was extracted from the separated life forms using the RNeasy Plus mini kit with gDNA eliminator spin columns to remove genomic DNA (Qiagen, Valencia, CA). Quantitative real-time PCR was performed on this RNA using the CFX96 real-time PCR detection system (Bio-Rad). Total RNA (5.0 μg) from the isolated Pc life forms was converted to cDNA for quantitative PCR analysis by SuperScript III reverse transcriptase (Invitrogen). The amplified nucleic acids were quantified using the SYBR® Green PCR Master Mix (Invitrogen). The conditions for the PCR reactions were as follows: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s; then 60 °C for 30 s and 72 °C for 30 s with SYBR® quantification. Primers (0.2 μM final concentration) for the amplification of *Pcace2* and Pc-β actin cDNA are listed in Table 1. To determine the relative expression levels of *Pcace2* in the different life forms, the mRNA levels of *Pcace2* were normalized to the level of Pc-β-actin mRNA using the comparative threshold cycle (Ct) method, in which the fold difference is 2 − (ΔCt of target gene − ΔCt of reference gene). Both PCR primer sets were tested using purified rat lung cDNA; they failed to amplify products, verifying their specificity for Pc mRNA.

**Complementation of ace2Δ *S. cerevisiae* Morphological and Sedimentation Defects by Heterologous Expression of *Pcace2***—The BY4741 *ace2Δ* (YLR131C, MATa his3Δ leu2Δ met15Δ ura3Δ ace2Δ) *S. cerevisiae* strain (17) was transformed with either a control vector (pYES2.1/V5-Has/lacZ) (8) or pYES2.1 containing the in-frame full-length *Pcace2* cDNA (pYES2.1/Pcace2/V5/6×His). Cells were grown overnight in synthetic complete medium containing 2% glucose and supplemented with appropriate amino acids but lacking uracil to select and maintain the plasmids. The measurement of sedimentation rates was conducted as described previously (11). The sedimentation half-time was calculated as the time for the optical density (OD) at a wavelength of 600 nm to fall to one-half of the difference between the initial and final OD values. All of the sedimentation assays were performed in triplicate. The colony morphology studies were conducted by plating the appropriate *S. cerevisiae* mutant or PcAce2-complemented strains on synthetic complete medium without uracil, and colonies were observed after growing the strains for 3 days at 30 °C.

**Cell Wall Degradative and Synthetic Gene Expression Confirmed by *Pcace2* and *Sace2* in ace2Δ *S. cerevisiae* Cells**—Overnight cultures of *S. cerevisiae* strains grown at 30 °C in synthetic medium without uracil and in the presence of 2% galactose to drive the transgene expression were recovered by centrifugation at 2500 × g for 5 min. The growth medium was removed, RLT Plus buffer (Qiagen) was added with acid-washed glass beads (~0.50 g, 425–600 μm size), and the culture was placed in a TissueLyser LT (Qiagen) for 5 min at 50 oscillations/s to
PCR primers were designed using MacVector™ software. The primers used in this study are as follows:

| Primer name           | Primer sequence (5’→3’) | Purpose                                      |
|-----------------------|-------------------------|----------------------------------------------|
| PcAce2 Start          | ATGCCCTTAA7CAACAAAGTTTATAT | Forward and reverse primers for amplifying PcAce2 |
| PcAce2 Stop           | TTATACCTCCTGCTCTGTCACCTCG | Reverse primer for amplifying Southern blot probe used with PcAce2 start primer |
| PcAce2−356Rt          | GCCTCTGAAAGCCTAGCTCTTGG | Primers for quantitative PCR analysis of PcAce2 mRNA levels in P. coccoides forms |
| PcAce2F1              | TCAGGCTCCAGAAAGGTCGGA   | Forward and reverse primers for amplifying S. cerevisiae CTS1 product for quantitative PCR |
| PcAce2R1              | CAAGAGCGCAACACTAGAGG   | Forward and reverse primers for amplifying S. cerevisiae DSE2 product for quantitative PCR |
| PcBactinF1            | ATAGAGACAGACATTTCTCTC  | Forward and reverse primers for amplifying S. cerevisiae KRE6 product for quantitative PCR |
| PcBactinR1            | ATGTCACAACAACAGGATTATAC | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| ScCTS1F1              | TGGCCGACCAAAATGTCATTAC  | Forward and reverse primers for amplifying S. cerevisiae KRE1 product for quantitative PCR |
| ScCTS1R1              | GCAGGAGGACACACTGACGGAC  | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| ScDS2F1               | AAAGATTCAGAGCCCCAGAAGC  | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| ScDS2R1               | GGCTCTCCTGCTCGACTTGGG   | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| ScKRE6F1              | GGGGAGTGACGAAGAATCTAATGC | Endogenous control for quantitative PCR analysis |
| ScKRE6R1              | GCTGCTTGGTAAGAATGCTGAC  | Endogenous control for quantitative PCR analysis |
| ScActinF1             | ATGTACGAAGGATAGCAGTCTTC | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| ScActinR1             | GACGACGAGGATAGCAGTCTTC  | Forward and reverse primers for amplifying S. cerevisiae GAS1 product for quantitative PCR |
| PcAce2/Gsc1F1         | CAGACTF1                 | Forward and reverse primers for amplifying P. coccoides FKS1 product for quantitative PCR |
| PcAce2/Gsc1R1         | CGACTR1                  | Endogenous control for quantitative PCR analysis |
| PcAce2–336R           | GCTTCAAAACGAACTACTGG    | Forward and reverse primers for amplifying C. glabrata actin product for quantitative PCR |
| PcAce2 Stop           | ATGGCCTTAATACAAAAGATTTATAT | Forward and reverse primers for amplifying C. glabrata CTS1 product for quantitative PCR |
| trp1–63 his3–200 leu2–1 | (18) containing the pYES2.1/PcAce2/V5/6×His plasmid by incubation in the presence of 2% galactose for 18 h at 30 °C. The cells were lyzed by a combination of treatment with Y-PER lysis reagent (Pierce Chemical Co.) and a French press. The expressed fusion protein was purified using the Y-PER His tag fusion protein purification system (Pierce Chemical Co.). |

**Phosphorylation of PcAce2 by the Upstream Cell Wall-remodeling Pckb1 Kinase**—Total Pck organisms (1 × 10^7) were suspended in Ham’s F-12 medium containing 10% fetal bovine serum and applied to lung epithelial cells (A549, ATCC), extracellular matrix-coated plastic, or control tissue culture insert substrates (30 mm; BD Biosciences) for 3 h at 37 °C with 5% CO₂. The organisms were lysed in Y-PER reagent (Pierce) in the presence of protease inhibitors. Protein lysates (1.0 mg) were precleared with protein A-Sepharose for 30 min at 4 °C. The synthetic peptide antibody recognizing Pckb1 was then incubated with the protein lysate overnight (10), and protein A-Sepharose was used to capture Pckb1 antibody-PcCbk1 protein complexes. These complexes were washed four times with 500 μl of KLB (40 mM Tris, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na₃VO₄, 1 mM DTT, and protease inhibitors) followed by a wash with the Pckb1 kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM MnCl₂). The subsequent kinase reactions were performed in Pckb1 kinase buffer containing 10 μCi of [γ-³²P]ATP (6000 Ci/mmol) and 20 μM ATP in the presence of 3.0 μg of PcAce2-His substrate (heat-inactivated protein to abrogate endogenous autophosphorylation) at 30 °C for 1 h. After 1 h, 2× Laemmli buffer was added, and the samples were boiled and loaded onto a 15% SDS-polyacryl-
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amide gel to determine the phosphorylation status of the substrate proteins.

Analysis of *C. glabrata* CTS1 Expression with PcAce2 in *C. glabrata* ace2Δ Cells—*C. glabrata* wild type strain BG2008 (CBS138 his3Δ) and Ace2Δ strain BG2180 (CBS138 his3Δ ace2Δ) were the kind gift of Dr. Brendan Cormack at Johns Hopkins University. *C. glabrata* was transformed with the pGRB2.1-PDC1 vector, which contains the hygromycin resistance gene (*hgy*) driven by the constitutive *C. glabrata* PDC1 promoter containing a *Pace2* insert that includes the start and stop regions of *Pace2* and XbaI and XhoI restriction enzyme sites for directional cloning (pGRB2.1-PDC1/PcAce2). Transformed cells were plated on YPD (yeast extract-peptone-dextrose) plates containing 200 μg/ml hygromycin B for selection. Clones containing the desired construct were grown overnight in YPD broth with 200 μg/ml hygromycin B at 30 °C, and total RNA was harvested. Quantitative PCR was conducted as described above for *CgCTS1*. For comparison, PCR was also performed under identical conditions as for *C. glabrata* actin1 (*CgACT1*).

Virulence Studies of PcAce2 Utilizing PcAce2-complemented *C. glabrata* ace2Δ Strains—All animal experimentation was approved and conducted in accordance with the Mayo Clinic Institutional Animal Care and Use Committee. Female BALB/c mice (Charles River Laboratories, Wilmington, MA), 20–25 g, were maintained in groups of up to 5 animals/cage with food and water provided *ad libitum*. Transient neutropenia was induced in the mice by injecting cyclophosphamide (150 mg/kg) intraperitoneally 3 days prior to and the day of lateral tail vein injection of *C. glabrata* (∼1 × 10⁵ cells in 100 μl). Because *C. glabrata* ace2Δ cells clump when cultured, 2 units/ml chitinase was included in the culture medium for the duration of growth as described previously (19). *C. glabrata* cells were counted using a hemocytometer and by plating them onto agar at the time of the mouse inoculation to confirm the number of viable organisms and inoculum dose. The mice were weighed daily and terminated humanely when the body weight fell by >20% or the animals showed evidence of significant distress or serious illness. The dates of demise or termination of animals were used to determine survival after challenge.

Statistical Analysis—All data are expressed as the mean ± S.E. Differences between data groups were first determined using analysis of variance, and Student’s t tests were subsequently analyzed on paired data groups. The corresponding nonparametric statistics were applied for non-Gaussian distribution data. For survival curve comparisons, log-rank Mantel-Cox testing was applied. Statistical testing was performed using GraphPad Prism version 5.0b software, and differences were considered to be statistically significant at *p* < 0.05.

**RESULTS**

*P. carinii* Has a Differentially Expressed *Pace2* Gene with Homology to Fungal ace2 DNA-binding Transcription Factors—Our previous studies indicated that specific signaling kinases, notably PcSte20 and downstream PcCbk1, are activated following the binding of Pc to lung epithelial cells and extracellular matrix in a process termed thigmotropism (8). To further explore the mechanisms by which Pc contact with the lung microenvironment may regulate the life cycle of *Pneumocystis*, we sought to identify downstream transcription factors that regulate gene expression following the binding of the organisms to lung cells or matrix proteins. We specifically focused our attention on Ace2 proteins because they are known to be downstream phosphorylation targets of Cbk1 and to bind DNA. Interrogation of the Pc genome database revealed a partial sequence for an *ace2*-like gene. To further characterize this protein, we isolated the remaining portions of the *Pace2* gene using the RACE method. Analysis of the full-length *Pace2* cDNA sequence revealed an open reading frame encoding a predicted 380-amino acid protein with a theoretical mass of 42,020 Da (GenBank accession number JF292576). Protein sequence alignments of PcAce2 and other fungal Ace2 orthologues are shown in Fig. 1. The multiple sequence alignment of fungal Ace2 transcription factors demonstrates that the amino acid sequence of the predicted PcAce2 has the greatest homology to the Ace protein from *Schizosaccharomyces pombe* (61% homology by BLASTX). The predicted amino acid sequence of PcAce2 also contained two zinc finger motifs, which are typical of DNA-binding proteins, and are also found in the Ace2 proteins of *S. pombe* and *S. cerevisiae*.

Following identification of the complete *Pace2* coding sequence, a 336-bp radiolabeled probe was generated and hybridized to restriction endonuclease-digested Pc genomic DNA to verify the presence of these sequences within Pc organisms (Fig. 2). The *Pace2* probe demonstrated strong localization to EcoRI- and HindIII-digested Pc genomic DNA fragments, but it failed to hybridize to digested DNA from uninfected rat lung, indicating that the amplification product was specifically represented within the Pc genome and was not the product of carryover host nucleic acid amplification.

We next sought to determine the relative expression of *Pace2* over the life cycle of the organism by assessing the expression levels in the two major morphological forms of Pc: the cystic and trophic forms (Fig. 3). *Pace2* mRNA expression was significantly higher in trophic forms than in cystic forms (*p* = 0.0059). Trophic forms are found at the life cycle stage that is most closely attached to and associated with lung epithelial cells (2, 20). A negligible PCR signal was detected using rat lung RNA as template. Taken together, these data indicate that the *Pace2* gene has homology to other Ace2 DNA-binding proteins and is differentially expressed over the organism’s life cycle, with the greatest expression in the trophic form.

PcAce2 Phosphorylation by PcCbk1 Is Induced following Interactions of Pc with Lung Extracellular Matrix Proteins and Epithelial Cells—Ace2 proteins are activated to function as transcription factors via phosphorylation. Therefore, we assessed whether the activation of the PcCbk1 kinase that occurs in response to contact with lung cells and extracellular matrix results in the downstream phosphorylative activation of PcAce2. To accomplish this, *Pneumocystis* organisms were incubated for 3 h either on uncoated plastic surfaces or on surfaces coated with matrix proteins or A549 lung epithelial cells as described previously (8, 9). The organisms were lysed, the total PcCbk1 protein was immunoprecipitated from the organisms, and the precipitated proteins were tested for their ability to phosphorylate recombinant expressed PcAce2 (Fig. 4A).
Compared with uncoated plastic surfaces, the Pc organisms cultured on surfaces coated with either the mammalian matrix proteins (fibronectin or collagen) or A549 lung epithelial cells exhibited significantly increased levels of PcAce2 phosphorylation \( (p < 0.05). \) Thus, the interactions between Pc organisms and lung environmental surfaces induce the activation of PcCbk1, which can subsequently phosphorylate PcAce2. To exclude the possibility that these increases in phospho-PcAce2 were related simply to increased amounts of total PcCbk1, we performed Western blots of the immunoprecipitates (Fig. 4).

Over the time frame of the interactions of the Pneumocystis organisms with the various coated surfaces (3 h), the protein levels of PcAce2 did not change appreciably. Thus, the significant increases of PcCbk1 phosphorylation induced by Pneumocystis binding to extracellular matrix-coated surfaces or lung cells were not due simply to increased expression of total PcCbk1 protein but were instead due to activation of available PcCbk1 in the organisms.

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**FIGURE 1.** Alignment of the predicted PcAce2 amino acid sequences with sequences from related fungal species. Amino acid alignments are shown as follows: PcAce2, P. carinii Ace2; SpAce2, S. pombe Ace2; ScAce2, S. cerevisiae Ace2. Multiple sequence alignments of the fungal Ace2 transcription factors performed with ClustalW (MacVector 8.1.2) demonstrate significant amino acid homology. The underlined sequence regions represent the predicted PcAce2 zinc finger motifs. The sequence data for PcAce2 are available from GenBank™ (accession number JF292576).

**FIGURE 2.** PcAce2 is represented in the Pc genomic DNA. Pc genomic DNA was freshly isolated and digested with the indicated restriction endonucleases. The digestion products were separated by electrophoresis and transferred to nitrocellulose. The 336-bp PcAce2 probe hybridized to specific locations of EcoRI- and HindIII-digested Pc DNA. In contrast, hybridization was not detected in the digested or undigested rat lung genomic DNA samples.

**FIGURE 3.** Pcace2 steady state mRNA is differentially expressed in separated Pc life cycle forms. To analyze the Pcace2 mRNA transcription profile of separated cystic and trophic life forms of the organisms, the various populations were separated by differential filtration, and quantitative real-time PCR for Pcace2 was performed. A significant alteration of Pcace2 expression was evident between cystic and trophic forms. Both trophic forms and cysts expressed significantly higher levels of Pcace2 than control uninfected rat lung \( (p = 0.0059 \text{ compared with control}) \). Moreover, the steady state mRNA expression of Pcace2 was significantly higher in the Pc trophic form than in the cystic form, indicating differential expression over the life cycle of the fungus \( (p = 0.0059 \text{ compared with cystic form mRNA levels}) \).
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FIGURE 4. The interaction between Pneumocystis organisms and lung extracellular proteins or lung epithelial cells induces phosphorylation of PcAce2 by PcCbk1. A, freshly isolated Pc organisms were allowed to interact with surfaces coated with A549 lung cells, fibronectin (Fn), or collagen for 3 h, and total Pc protein lysates were obtained and immunoprecipitated with a PcCbk1 antibody. The precipitates were assayed for phosphorylation of purified PcAce2/6×His by PcCbk1. (*, p < 0.05 compared with lysates from control uncoated plastic surfaces.) B, to determine whether increases in phosphorylated PcAce2 were related simply to increased amounts of total PcCbk1, we performed Western blots of the PcAce2 organism immunoprecipitates using anti-PcCbk1 antibody following incubation of the organisms on the indicated surfaces for 3 h.

FIGURE 5. Pcace2 cDNA can restore the sedimentation defect in S. cerevisiae ace2Δ cells. Wild-type cells (ACE2), ace2Δ cells, and ace2Δ cells complemented with PcAce2 (ace2Δ/PCace2) were grown in selective synthetic complete liquid medium. The sedimentation half-time (T1/2) was calculated as the time for the OD at a wavelength of 600 nm to fall to one-half of the difference between the initial and final OD values. The data shown are from a representative sedimentation assay of three independent experiments. The expression of PcAce2 in ace2Δ S. cerevisiae restored the sedimentation half-time to that of wild type S. cerevisiae, which has normal Ace2 function. (See “Results” for statistical comparisons.)

PcAce2 Can Modulate the Expression of Fungal Cell Wall Degradative and Synthetic Enzymes and Can Induce the Expression of the Pneumocystis Glucan Synthetase Gene, Pcgsc1, Directly—Prior studies have revealed that Ace2 can regulate the expression of cell wall degradative enzymes such as chitinases and glucanases and other cell wall-remodeling enzymes (12). To evaluate this activity of PcAce2, we analyzed the overexpression of PcAce2 and ScAce2 in S. cerevisiae ace2Δ cells and found that it altered the expression of cell wall degradative gene transcripts and cell wall biosynthetic gene transcripts (Fig. 7). Indeed, S. cerevisiae ace2Δ cells expressing PcAce2 demonstrated significantly increased expression of the cell wall degradative enzyme transcripts CTS1 (chitinase), DSE2, and SCW11 (glucanases) compared with S. cerevisiae ace2Δ cells without PcAce2 under similar conditions (p < 0.05 comparing ace2Δ S. cerevisiae cells with empty vector to ace2Δ S. cerevisiae cells expressing PcAce2, t test). In addition, we observed significantly enhanced expression of the cell wall biosynthetic enzyme transcripts FKS1 (β-1,3-glucan synthesis), GAS1 (glucosyltransferase), and KRE6 (β-1,6-glucan synthesis) (p < 0.05 comparing ace2Δ S. cerevisiae cells with empty vector to ace2Δ S. cerevisiae cells expressing PcAce2, t test). In these studies, the expression of FKS2 and KRE1 transcripts was not significantly augmented with PcAce2 expression. In addition, wild type ScAce2 was also expressed in the ace2Δ cells, using a parallel vector construct as an overall confirmation of the relative activity of PcAce2 under these conditions. Taken together, these data indicate that PcAce2 can alter the expression of genes that drive cell wall remodeling when it is expressed in S. cerevisiae.

With accumulating evidence that PcAce2 functions as a transcription factor, our next step was to investigate whether PcAce2 could alter the activity of the Pcgsc1 gene, a gene that we had documented previously as being engaged in Pneumocystis β-glucan cell wall synthesis (16). To accomplish this objective, PcAce2 was overexpressed in ace2Δ S. cerevisiae cells that also harbored a construct containing the Pcgsc1 promoter region and a partial Pcgsc1 cDNA (Fig. 8). With induction by the GAL1 promoter, abundant PcAce2 was expressed. Enhanced expression of PcAce2 resulted in significantly increased steady state mRNA level of the major Pc β-1,3-glucan synthesis gene, Pcgsc1 (p = 0.03, Wilcoxon test). This strongly indicates a potential role for PcAce2 in glucan cell wall remodeling in Pc. To our understanding, this represents the first illustration of the role of

ace2Δ S. cerevisiae expressing PcAce2 had a sedimentation half-time of 46.8 ± 2.8 min (p = 0.0002 by analysis of variance). The sedimentation rates of the wild type S. cerevisiae and ace2Δ S. cerevisiae expressing PcAce2 were not significantly different (p = 0.54, t test).

In addition, ace2Δ S. cerevisiae colonies have a “rough” morphology when cultured on selective synthetic complete solid medium (11). S. cerevisiae ace2Δ cells transformed with an empty plasmid exhibited the typical rough colony appearance (Fig. 6). However, S. cerevisiae ace2Δ cells complemented with the PcAce2 display a “smooth” colony appearance (Fig. 6, right panel) similar to that of the wild type S. cerevisiae strain. Taken together, PcAce2 can function fully when heterologously expressed to restore cell wall defects in ace2Δ S. cerevisiae.

cells and measuring the sedimentation of the cells. We observed that the expression of PcAce2 in ace2Δ S. cerevisiae restored the sedimentation rates to levels that were statistically equivalent to those seen in wild type S. cerevisiae with normal Ace2 function. Over the course of three sedimentation assays, the sedimentation half-time of S. cerevisiae expressing wild type Ace2 was 49.2 ± 2.0 min; S. cerevisiae with mutated Ace2 (ace2Δ) had a sedimentation half-time of 19.2 ± 2.5 min, and
a putative Pc transcription factor in regulating the expression of another Pneumocystis gene.

**PcAce2 Modulates Organism Virulence in Immune-deficient Mice Infected with ace2Δ C. glabrata—**Our inability to directly manipulate Pc genes within *Pneumocystis* has limited our ability to test gene function as it relates to organism virulence in whole animal models of infection. It is noteworthy, however, that Ace2-deficient strains of *C. glabrata* exhibit altered levels of virulence in immunosuppressed mice. The neutropenic mouse has been used extensively to study mutations in the *C. glabrata* genome and their potential role in virulence (13, 14). As a first step, we expressed PcAce2 in *C. glabrata ace2Δ* cells (Fig. 9). We observed that the expression of the major chitinase *CTS1*, as assessed by quantitative PCR, was significantly higher in *C. glabrata ace2Δ* with PcAce2 than in *C. glabrata ace2Δ* alone (*p* = 0.0045 comparing *Cgace2Δ* and *Cgace2Δ + PcAce2*, *t* test). However, PcAce2 did not quite fully restore the *CTS1* expression level to that seen in wild type *C. glabrata*. In comparison, *C. glabrata* actin1 (*CgACT1*) was not altered in expression in the various strains studied (Fig. 9, bottom panels). We next tested the effects of *C. glabrata ace2Δ* cells expressing PcAce2 on virulence in mice (Fig. 10). Transient neutropenia was induced in mice with cyclophosphamide prior to the mice being challenged with the various *C. glabrata* strains. Consistent with earlier reports (12, 13), we observed that the *C. glabrata ace2Δ* strain exhibited enhanced mortality over the course of 7 days following infection compared with animals infected with wild type *C. glabrata* (*p* < 0.0001, log-rank test). In contrast, *C. glabrata ace2Δ* cells expressing PcAce2 were less virulent than *C. glabrata ace2Δ* cells without PcAce2, and they caused a level of mouse mortality that was similar to that from wild type *C. glabrata* (*p* = 0.36, log-rank test). Hence, we posited that PcAce2 could alter the state of organism virulence when it is heterologously expressed in *C. glabrata ace2Δ* cells.

**DISCUSSION**

The continued importance of *Pneumocystis* pneumonia in immune-compromised patients, such as those with AIDS, malignancies, and organ transplantation, makes the development of effective antifungal agents to combat this lethal infection of critical importance. The currently available treatment regimens are not completely effective, as the mortality for *Pneumocystis* pneumonia remains as high as 40% in immune-compromised patients without AIDS (1, 2). Furthermore, emerging drug resistance is becoming an increasing concern.
for this organism, and potential molecular resistance mechanisms have been identified for each of the currently employed therapeutic agents (3, 24, 25). The incomplete understanding of the Pneumocystis life cycle regulation has limited the development of new agents to treat this infection.

Components of the life cycle regulation in Pneumocystis are slowly being revealed. Studies from our group and others have supported the concept that the adhesion of Pc to lung epithelial cells and matrix proteins is a central component of the life cycle of the organism that promotes its proliferation (9, 20, 26). Indeed, short-term (but non-sustainable) proliferation of Pc has been documented on lung epithelial cell substrates (26, 27). In that light, our group initiated studies to identify cell-signaling mechanisms that are activated by the binding of Pc organisms to lung substrates, leading to the identification of the PcSte20 and PcCbk1 cell-signaling kinases that are activated by organism contact in a process that we likened to thigmotropism in plant species. These kinases exhibit activity in the further regulation of organism proliferation and morphological changes in fungi (9, 10).

In this study, we gained insights into the control of the Pneumocystis life cycle and the response of the organism to surface contact. Specifically, we have demonstrated that Pneumocystis expresses an Ace2 family DNA-binding protein that is capable of regulating downstream enzymes involved in cell wall remodeling. We further observed that the PcAce2 transcription factor is phosphorylated by the upstream PcCbk1 cell wall biosynthesis kinase, the expression and activity of which is further upregulated following the binding of Pc to lung cells and matrix proteins (8, 10). These investigations, together with our previous observations, support an emerging model of this unique thigmotropic response in Pneumocystis (8). Our previous studies support the synthesis gene, 23900 JOURNAL OF BIOLOGICAL CHEMISTRY

FIGURE 8. Overexpression of PcAce2 in ace2Δ S. cerevisiae that also contain the Pcgsc1 promoter and Pcgsc1 cDNA successfully induces transcription of Pcgsc1 cDNA. PcAce2 expression driven by the inducible GAL1 promoter of pYES2.1 resulted in increased mRNA of the major Pc β-1,3-glucan synthesis gene, Pcgsc1, further supporting a potential role for PcAce2 in regulating the expression glucan cell wall-remodeling genes. The top panel illustrates the quantitative PCR results of Pcgsc1 transcription equalized to endogenous transcription levels of the housekeeping gene actin (*, p < 0.05). The middle panel verifies by Western blotting with anti-V5 antibody that PcAce2/V5 was strongly expressed under the control of the GAL1 promoter. The bottom panel shows the partial Pcgsc1 cDNA PCR amplicon that was generated during the quantitative PCR procedure.

FIGURE 9. PcAce2 expression in ace2Δ C. glabrata cells restores expression of the major chitinase CTS1. Top panel, expression of the major chitinase CTS1 in C. glabrata strains was determined by real-time PCR analysis. The expression of CTS1 in ace2Δ C. glabrata was significantly lower than that in wild type C. glabrata (CgAce2) (*, p = 0.0004). In addition, Cgace2Δ cells expressing PcAce2 (CgAce2Δ/PcAce2) demonstrated significant recovery of CgCTS1 expression compared with the Cgace2 mutant strain, indicating the functional activity of the PcAce2 transcription factor in C. glabrata (**, p = 0.0045). Bottom panels, PCR was also conducted in parallel for CgACT1 (C. glabrata actin1), which showed no differences in expression in the ace2Δ C. glabrata or in the Cgace2Δ cells expressing PcAce2. In contrast, PCR confirmed the reduced expression of CgCTS1 in ace2Δ C. glabrata and restoration of CgCTS1 expression in Cgace2Δ cells expressing PcAce2.

FIGURE 10. Survival curves for neutropenic mice challenged with the Ace2-altered strains of C. glabrata. The survival of mice infected with C. glabrata wild type (circles), C. glabrata ace2Δ (squares), and C. glabrata ace2Δ expressing PcAce2 (triangles) strains. Mice infected with ace2Δ C. glabrata exhibit enhanced mortality (p < 0.0001). However, C. glabrata cells expressing PcAce2 induce mortality in mice similar to that induced by wild type C. glabrata cells (p = 0.36, not significantly different).
idea that at least a portion of the initial binding of Pc to lung epithelial surfaces occurs through PcInt1, a surface integrin-like molecule that facilitates the binding of the fungus to fibronectin (5). Following binding, PcSte20 expression and kinase activity are enhanced in a process requiring the collaborative assistance of a small GTPase termed PcCdc42, allowing the phosphorylatory activation of the cell wall-remodeling kinase PcCbk1 (8, 15). Additionally, this work reveals that the binding of Pc to lung epithelial cells and matrix further stimulates the activation of PcAce2, a putative transcription factor that enhances the expression of both cell wall synthetic and cell wall degradative genes. These data together suggest that one major impact of Pc-sensing contact with lung cell surfaces is the downstream regulation of genes involved in cell wall remodeling. We do not propose that this is the only response. Other systems involved in organism proliferation are likely also to be activated by Pc binding, although the molecular mechanisms of the responses remain unknown.

Ace2 proteins represent a central component of the regulation of Ace2 and morphogenesis (RAM) network, a kinase-signaling pathway that is strongly conserved throughout eukaryotes from fungi to mammals. The RAM network has been widely investigated in S. cerevisiae and has been implicated in a variety of processes controlling cell cycle progression, cell separation, cell growth, cell wall integrity, and fungal mating (12). In S. cerevisiae, other members of the RAM network include two protein kinases, ScCbk1 and ScCIC1, and at least four other associated proteins, ScMob2, ScTao3, ScHym1, and ScSog2. At present, the only other component of the RAM pathway reported in Pneumocystis has been our description of PcCbk1, the kinase that acts as a downstream target of PcSte20 (a contact-regulated kinase in Pc) and functions as the immediate upstream kinase for Ace2 proteins. It has been proposed that the phosphorylation of the zinc finger regions of Ace2 proteins permits them to act as DNA-binding proteins, promoting the transcription of various genes including genes involved in cell wall remodeling (28). Our studies further demonstrate the functions of PcAce2 in regulating the expression of both cell wall degradative enzymes and cell wall synthetic enzymes. Moreover, we demonstrate (albeit heterologously) that PcAce2 can increase the expression of Pegsc1, a significant Pc cell wall β-glucan synthetic gene. The relative balance of synthetic and degradative enzymes in cell wall remodeling clearly requires a fine level of global and regional regulation. It is clear that other factors beyond PcAce2 will participate in the cell wall remodeling of this fungus. However, this study demonstrates the feasibility of PcAce2 acting as a cognate participant in Pneumocystis cell wall remodeling that is activated by organism contact with the lung environment.

This study also demonstrates a new strategy to assess the potential roles of Pneumocystis genes on pathogenesis in animal models of infection. In C. glabrata, ace2A strains exhibit enhanced virulence during systemic infections in neutropenic mice (13). The reasons for enhanced mortality in these animals are presumably related to greater amounts of fungal cell wall carbohydrate being exposed or sloughed off in the knock-out strains, resulting in a greater systemic proinflammatory state rather than being related to the clumping or aggregation of the organisms during infection (13). Interestingly, we demonstrated that PcAce2 complemented CgAce2 function with respect to regulating the expression of the downstream CTS1 chitinase gene (Fig. 9). We demonstrated additionally that when PcAce2 was expressed in ace2Δ C. glabrata, it suppressed the enhanced virulence that was observed in a systemic infection model of ace2Δ C. glabrata (Fig. 10). This provided the first virulence assessment of any heterologously expressed Pneumocystis protein.

It is not possible to study the effects on gene knockouts directly in Pneumocystis because of the lack of both a reliable culture system and transformation system. Although it would be interesting to determine the role of PcAce2 in Pneumocystis itself or in animal models of Pc infection, these possibilities, unfortunately, remain technically unfeasible. Nevertheless, the multiple lines of investigation that we present strongly support a role for the activation of the PcAce2 transcription factor following the contact of the organism with host cell or matrix substrates. To our knowledge, this contact-dependent activation of a RAM pathway component is the first such description in any fungus. Furthermore, we provide evidence that PcAce2 functions in the control of cell wall remodeling, an essential life cycle activity during the proliferation and growth of fungi. Taken together, we believe that PcAce2 represents a significant component of the Pneumocystis mechanism that recognizes and responds to the mammalian lung, which is a central microenvironment for the life cycle of this elusive fungus.

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