Characterization of PCEng2, a β -1,3-Endoglucanase Homolog in *Pneumocystis carinii* with Activity in Cell Wall Regulation

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Pneumocystis jirovecii pneumonia is an opportunistic fungal infection that causes severe respiratory impairment in immunocompromised patients. The viability of *Pneumocystis* organisms is dependent on the cyst cell wall, a structural feature that is regulated by essential cell wall-associated enzymes. The formation of the glucan-rich cystic wall has been previously characterized, but glucan degradation in the organism—specifically, degradation during trophic excystment—is not yet fully understood. Most studies of basic *Pneumocystis* biology have been conducted in *Pneumocystis carinii* or *Pneumocystis murina*, the varieties of this genus that infect rats and mice, respectively. Furthermore, all known treatments for *P. jirovecii* were initially discovered through studies of *P. carinii*. Accordingly, in this study, we have identified a *P. carinii* β -1,3-endoglucanase gene (PCEng2) that is demonstrated to play a significant role in cell wall regulation. The cDNA sequence contained a 2.2-kb open reading frame with conserved amino acid domains homologous to similar fungal glycosyl hydrolases (GH family 81). The gene transcript showed up-regulation in cystic isolates, and the expressed protein was detected within both cyst and trophic forms. Complementation assays in Eng2-deleted *Saccharomyces cerevisiae* strains showed restoration of the cell wall separation defect during proliferation, demonstrating the importance of PCEng2 protein during fungal growth. These findings suggest that regulation of cyst cell wall β -glucans is a fundamental process during completion of the *Pneumocystis* life cycle.

Keywords: *Pneumocystis carinii*; cell wall; β -glucan; glucanase; β -1,3-endoglucanase

Immunocompromised patients with malignancies, organ transplantation, immunosuppressive therapy for inflammatory conditions, congenital immunodeficiency disorders, and acquired immune deficiency syndrome (AIDS) remain at risk for developing *Pneumocystis* pneumonia (PcP), a serious lung infection caused by the opportunistic fungal pathogen, *Pneumocystis jirovecii*. In human immunodeficiency virus (HIV) disease, PcP remains among the most prevalent AIDS-defining illness, even after the advent of highly active antiretroviral therapy, with an incidence of approximately 4.7 cases per 100 person-years (1, 2). Although PcP is less common in immunocompromised patients who are HIV negative, studies have reported yearly PcP incidences of up to 6.5% per 1,000 intensive care unit admissions in 2006 (3). In individuals who do not display symptoms of PcP, but have evidence of subclinical carriage, as detected by PCR, rates of colonization have been

CLINICAL RELEVANCE

Pneumocystis remains an important cause of pneumonia in patients with impaired immunity. In this study, we define the first known β -glucanase in *Pneumocystis*, which is responsible for cell wall regulation.

reported to be as high as 69% in HIV-positive patients (4) and 16% in HIV-negative patients (5). Thus, PcP continues to be of considerable clinical importance, and studies continue to define the life cycle and new methods to prevent and treat this important opportunistic fungal pathogen.

Many components of the *Pneumocystis* life cycle, including transmission, colonization, progression, and clearance of infection, remain poorly understood due to the fact that *Pneumocystis* cannot be cultured outside of the host lung (6). Although it is not clear that there is a single, well defined life cycle, morphological studies have revealed that *Pneumocystis* organisms primarily exist as morphologically distinct trophic and cystic life forms (7–9). Recent molecular analyses of the *Pneumocystis* putative life cycle have begun to elucidate mechanisms of attachment of the trophic form to host alveolar epithelial cells (10, 11), mating and pseudohyphal growth and pheromone signaling (10, 12, 13), meiotic and mitotic signaling during trophic cell division and replication (14, 15), cell division cycle control molecules (15–17), and cystic cell wall biosynthesis (18–22).

Central to the regulation of the life cycle is the formation of the thick cyst wall of *Pneumocystis* rich in β -glucan that is believed to be important in maintaining organism viability when outside the host lung (22–24). We have previously characterized, in part, the β -glucan synthetic machinery mediated by the *Pneumocystis carinii* glucan synthetase catalytic protein PCGsc1 (25). However, the process of β -glucan degradation is essential to the process of excystment and organism proliferation, but remains fully unknown at the present time. Thus, in our evolving understanding of the life cycle of this enigmatic organism, excystment of the trophic forms from mature cysts represents a critical process for regulation of growth and in the establishment of the infection.

The cell wall and associated enzymes of fungal species are integral structural components that influence the organism's ability to survive harsh environments and to proliferate (26). Fungal cell walls are composed of chitin, glucan, mannan, and proteins that encase the organism's plasma membrane or serve as the outer casing of a spore case or cyst. Glucans are major components of the cell wall, and typically consist of varying lengths of β -1,3-glucan polysaccharides as a core backbone, with β -1,6-glucan and β -1,4-glucan side chains (27–29). In addition, α -glucans are also present as relatively minor components (30). Cell wall-associated enzymes, such as glucanases, are therefore important regulators of this dynamic structure. Glucanases

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expressed by fungi function as glycosyl hydrolases that cleave glycosidic bonds by acid catalysis, and have activity either in the cytoplasm or in the immediate extracellular environment. They can be grouped into two major classes—the exohydrolases and the endohydrolases—and are further classified based on glucan substrate specificity (31–34).

Therefore, to better understand cell wall and life cycle regulation in *Pneumocystis carinii*, these studies were undertaken to identify and characterize potential β -1,3-endoglucanase molecules expressed by these intractable organisms. The closest phylogenetic relatives of *Pneumocystis* species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been demonstrated to be useful models in studying glucanase activity, and, accordingly, *S. cerevisiae* has been used in this study to characterize *P. carinii* glucanase function.

MATERIALS AND METHODS

Pneumocystis isolation

P. carinii (Pc) organisms were isolated from female Long Evans rats (Harlan, Inc., Indianapolis, IN) lungs. Rats were immunosuppressed with dexamethasone (1.2 mg/L) addition to their drinking water for 2 weeks before intratracheal inoculation of Pc. Infection was allowed to progress for 8–12 weeks. Pc was extracted for subsequent assays by resection of the lung, followed by mincing and homogenization in normal saline. The homogenate was filtered through gauze to separate large cellular debris. The filtrate was then centrifuged at $6,600 \times g$ for 10 minutes at 4°C . The pellets were resuspended in sterile water and incubated at room temperature for 5 minutes to allow lysis of remaining host cells, and then centrifuged again. Subsequently, the pellets were resuspended in 20 ml normal saline, passed through a $10\text{-}\mu\text{m}$ nitrocellulose filter, and centrifuged to collect a purified population of Pc organisms. For experiments requiring separation of the cysts and trophic forms, differential filtration through a $3\text{-}\mu\text{m}$ filter was performed, as we have previously reported (18). Such $3\text{-}\mu\text{m}$ filtration resulted in 99.5% pure trophic forms and greater than 40-fold-enriched cysts (18). The Pc forms were then either processed immediately for further analysis, or flash frozen in a dry ice/methanol bath and stored at -80°C .

Identifying the *P. carinii* β -1,3-Endoglucanase Complete Coding Sequence and Genomic Region

Partial sequences were initially identified from the *Pneumocystis* Genome Project database (<http://pgp.cchmc.org/>) with sequence close homology to other fungal β -glucanases. These partial sequences were used to derive primers for rapid amplification of cDNA ends (RACE) (GeneRacer; Invitrogen, Carlsbad, CA). From the initially identified fragment sequence, the full-length coding region of *P. carinii* β -1,3-endoglucanase (*PCEng2*) was generated with the RACE strategy in a manner parallel to our previously studies (18). This sequence was cloned into pCR-BluntII-TOPO (Invitrogen) and fully sequenced (GenBank accession no. EU814521). From PCR amplification of purified genomic DNA, the genomic region, including the 5' and 3' untranslated regions, was also identified and completely sequenced (GenBank accession nos. EU938375, GQ249166, and GQ249167).

Southern and Chromosomal Hybridization of the *PCEng2* Coding Sequences

To further verify the presence of the *PCEng2* coding sequence within the *Pneumocystis* genome, a specific *PCEng2* sequence was radiolabeled and hybridized to digested Pc genomic DNA and to Pc chromosomes separated by contour-clamped homogeneous electrophoresis field (CHEF) blot, as previously described (16, 35). A specific nucleic acid probe for *PCEng2* was generated by PCR amplification of a 600-bp sequence of *PCEng2*. The probe was radiolabeled with the RadPrime DNA labeling system (Invitrogen) and α - ^{32}P -ATP, followed by filtration through sepharose mini QuickSpin columns (Roche, Inc., South San Francisco, CA). The primer sequences employed for probe generations were: forward, 5'-ACGGAACGACGTTAAAGGCAAC-3', and reverse, 5'-CCTCGTCTGTACAATTCCACGCCATG-3'.

For Southern blot analysis, *P. carinii* was obtained from American Type Culture Collection (PRA-159; Manassas, VA). Genomic DNA was extracted with the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Inc., Madison, WI) according to the manufacturer's protocol. Pc genomic DNA (10 μg) was digested with 50 U restriction enzyme (*HindIII* or *EcoRI*; Promega, Inc., Madison, WI) for 4 hours at 37°C . Genomic DNA (10 μg) from normal rat lung tissue (BioChain, Hayward, CA) was also digested under the same conditions as controls. The digested genomic DNA was then electrophoresed through a 1% agarose gel in Tris base, acetic acid and EDTA (TAE) buffer and transferred onto a Nytran Supercharge nylon membrane with downward capillary flow (TurboBlotter; Whatman, Piscataway, NJ) of $20\times$ saline-sodium citrate (SSC) buffer overnight at room temperature, followed by ultraviolet cross-linking. For CHEF blot analysis, *P. carinii* genomic DNA blots separated by CHEF were generously provided by M.T. Cushion (University of Cincinnati College of Medicine, Cincinnati, OH) (35). Radiolabeled probes were hybridized to the membranes with ExpressHyb Hybridization solution (Clontech, Mountain View, CA) at a concentration of 1.0×10^6 cpm/ml. Prehybridization, hybridization, and washing were done according to manufacturer's protocol. The membrane was then exposed to X-ray film with two intensifying screens at -70°C for 48 hours.

Assessment of *PCEng2* mRNA Expression during the Life Cycle of *Pneumocystis*

To begin to understand expression of *PCEng2* over the life cycle of *Pneumocystis*, RNA expression was evaluated in separated Pc cysts and trophic life cycle forms. Whole *P. carinii* isolates were separated into cyst- and trophic-form populations by differential filtration as, described previously here. Total RNA was extracted from these isolated populations by resuspending the cystic- and trophic-form pellets in 1 ml Trizol reagent (Invitrogen) and collecting the aqueous phase. The RNA was then precipitated with isopropanol, washed with 70% ethanol, and resuspended in nuclease-free water. Subsequently, 10 μg of RNA were loaded on each lane of the gel. Samples were then electrophoresed through a 1% agarose gel in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and transferred onto a nylon membrane. A specific *PCEng2* radiolabeled probe was generated and hybridized to these membranes, as described previously here, with a probe concentration of 2.0×10^6 cpm/ml. Bound probe was detected by X-ray film exposure. As a specific loading control, the blots were repeated with a specific *P. carinii* β -actin loading control.

Localization of Protein Expression of the Putative *PCEng2* Glucanase in *P. carinii* by Electron Microscopy

We next sought to determine the regional protein expression of the *PCEng2* protein in *Pneumocystis* life cycle forms. Peptide from the complete coding region of *PCEng2p* was used to generate a gene-specific antibody in rabbits (Bethyl Laboratories, Inc., Montgomery, TX). A suitable antigenic peptide region was selected with the antigenic index (MacVector; Invitrogen), an algorithm based on Kyle-Doolittle hydrophobicity, surface probability, protein flexibility, and Chou-Fasman and Robson-Garnier secondary structure analysis. The peptide sequence used for antibody generation was: NH₂-YSPSG-KEPSYSEEAKEAIKK-COOH. Next, immunoelectron microscopy was performed with this antibody to identify the subcellular localization of *PCEng2p* in freshly isolated *Pneumocystis* organisms (34). *Pneumocystis* cyst and trophic forms were isolated and fixed in 4% formaldehyde and 0.2% glutaraldehyde in phosphate buffer over 16–24 hours. Next, the specimens were rinsed in PBS and dehydrated through a series of graded ethyl alcohol solutions, while progressively lowering the temperature to -20°C . Finally, the specimens were infiltrated with a 1:1 ratio of ethanol:LR White resin overnight and exposed to fresh LR White resin for 60 minutes, then brought to room temperature and embedded in LR White resin. These specimens were polymerized at 55°C for 2–3 days. Ultrathin sections (5 μm) were mounted on nickel grids and dried overnight. Nonspecific binding sites were next blocked with aqueous 1% glycine solution, followed by PBS and 0.05% Tween 20 (PBST) with 1% normal serum. The primary antibody was diluted in PBST with 1% normal serum, and sections were incubated with primary antibody solution (100 $\mu\text{g}/\text{ml}$) for 2 hours at room tempera-

ture. Prepared grids were then rinsed in PBST and further incubated with goat anti-rabbit Ig conjugated to 10-nm colloidal gold for 60 minutes (34). After incubation, all grids were rinsed thoroughly in PBST and finally stained with uranyl lead. The sections were washed, and then analyzed with a transmission electron microscope (Model 6400; JEOL USA Inc., Peabody, MA). As a control, sections were identically processed and treated with the detection reagents, including the anti-rabbit Ig conjugated to 10-nm colloidal gold, but in the absence of anti-PCEng2p antibody.

Heterologous Expression of *PCEng2*

Because *Pneumocystis* species cannot be continuously cultured or genetically manipulated to test gene function, we next sought to address the potential functionality of PCEng2p with heterologous expression in *S. cerevisiae*. To accomplish this, the *PCEng2* open reading frame was amplified by PCR from the sequencing vector with the following sets of primers: (1) for cloning in frame with the V5-His tag, the forward primer was 5'ATGGGGATAGGGAGGGAACTTCTTAGG3', and the reverse primer was 5'TTATGCCGAATTTAGAAAAGCAGATGAG3'; and (2) for cloning without the V5-His tag, the forward primer was 5'ATGGGGATAGGGAGGGAACTTCTTAGG3', and the reverse primer was 5'TGCCGAATTTAGAAAAGCAGATGAG3'. The amplicons were ligated into pYES2.1/V5-His-TOPO (Invitrogen), a galactose-inducible yeast expression vector containing ampicillin resistance and *URA3* genes for selective colony growth. The plasmids were amplified in TOP10 electrocompetent *Escherichia coli* and purified with Qiagen (Valencia, CA) midi-prep with elution in Tris buffer. Plasmids were stored at -20°C until needed.

Various strains of *S. cerevisiae* were transformed with the *PCEng2* expression vectors for either protein expression and purification or gene complementation studies (Table 1). The strain used for *PCEng2* expression and purification was a diploid strain (INVSc1), and the strains used in complementation studies were *MATα* haploids that were either wild type (BY4742) or contained the yeast *Eng2* gene deletion (BY4742-*Eng2*). Cultures of yeast extract peptone dextrose media (10 ml) were inoculated with a colony of each yeast strain, and incubated overnight at 30°C. The overnight cultures were diluted to an optical density of 600 nm (OD₆₀₀) 0.4 in 25 ml yeast extract peptone dextrose, grown an additional 2–4 hours, then recovered by centrifugation, washed in 1× 10 mM Tris-Cl, pH 7.5, 1 mM EDTA buffer and resuspended in 1 ml LiAc/0.5× TE buffer. After incubation at room temperature for 10 minutes, 50 µl of each strain were inoculated with 0.5 µg plasmid DNA, 100 µg denatured sheared salmon sperm DNA, and 350 µl LiAc/40% PEG-3350/1× TE, and incubated at 30°C for an additional 30 minutes. DMSO (44 µl) was then added to the transformation mixtures and heat shocked at 42°C for 7 minutes. Cells were washed in 1× TE, resuspended in 100 µl 1× TE, and plated on drop out base with complete supplement media without uracil (DOB/CSM-URA3)-selective plates.

Purification and Detection of PCEng2p after Expression in Yeast

Transformed yeast strains were incubated in 5 ml of selective growth media (DOB/CSM-URA) at 30°C in a shaker for 24–48 hours. An aliquot of each culture was recovered by centrifugation at 3,000 × g at 4°C for 5 minutes, resuspended in 30 ml of inducing media (DOB/CSM-URA plus galactose) to yield an OD₆₀₀ of 0.4, and then incubated at 30°C in a shaker for an additional 24 hours. An aliquot of cells was also grown in noninducing growth media lacking galactose. After

incubation, the cells were recovered and the growth media supernatant saved for further analysis. Cell pellets were resuspended in 2.5 ml yeast protein extraction reagent lysis buffer (Pierce, Inc., Rockford, IL), containing protease inhibitors (Complete Mini Cocktail; Roche, Inc.) and 10 mM phenylmethanesulfonyl fluoride per gram of cells and incubated at room temperature for 20 minutes. Acid-washed glass beads were added to half volume, and the cells were vortexed for 30 seconds, followed by a 30-second incubation on ice. This was repeated four times. Lysates were centrifuged at 13,000 × g at 4°C for 2 minutes to remove the cellular walls, membranes, and debris. The resulting yeast lysates and growth media were immunoprecipitated with anti-V5 antibody-conjugated agarose beads (Sigma, St. Louis, MO) with incubation at 4°C for 16–20 hours. The agarose beads were then recovered and washed three times by centrifugation at 13,000 × g for 2 minutes at 4°C, followed by resuspension in PBS. After the final wash, the pellets were resuspended in 30 µl of 2× Laemmli sample buffer containing 710 mM β-mercaptoethanol, and placed in a boiling water bath for 10 minutes. Large-scale yeast cultures were prepared for detection with the PCEng2p-specific synthetic peptide antibody. Cells were induced and grown in 1.5 L culture and lysed under the same conditions as previously described here. PCEng2p was isolated by nickel affinity column purification (Pierce, Inc.) and dialyzed against 100 mM potassium phosphate buffer (pH 6.5) containing 10% glycerol.

Immunoprecipitated samples were loaded onto 4–15% Tris-HCl SDS-PAGE gels (Bio-Rad, Hercules, CA) and separated by electrophoresis at 100 V for approximately 1.5 hours. The proteins were transferred onto nitrocellulose membranes and incubated in blocking buffer (Tris-buffered saline, 0.1% vol/vol Tween20, 10% wt/vol nonfat dry milk) overnight on a shaker at 4°C. The membranes were then probed with either a 1:1,000 dilution of mouse anti-V5 IgG-horseradish peroxidase (Invitrogen) in TBST buffer (Tris-buffered saline, 0.1% Tween20) or a similar dilution of the synthetic peptide anti-PCEng2p antibody overnight on shaker at 4°C, followed by three washes in TBST buffer for 10 minutes each at room temperature. Bound antibodies were detected by incubation in ECL detection reagents (GE/Amersham, Piscataway, NJ) for 1 minute, followed by exposure to autoradiography film for 1 hour.

Complementation of *Eng2*-Deficient *S. cerevisiae* with *PCEng2*

To further evaluate the function of PCEng2p, we next evaluated morphologic characteristics of *Eng2*-deficient *S. cerevisiae* complemented with *PCEng2*. *Eng2*-deficient yeast strains (BY4742-*Eng2*-pYES2.1/*LacZ*) demonstrate clumping during growth. *Eng2*-deficient yeast was transformed with either *PCEng2* or control vector, and compared with wild-type yeast. One colony of each strain was cultured in the presence of galactose. The cultures were recovered by centrifugation, resuspended in 4% paraformaldehyde and incubated for 90 minutes at room temperature. The cells were then washed three times with 0.1 M potassium phosphate (pH 6.5) and resuspended in 1.2 M sorbitol, 0.12 M K₂HPO₄, and 33 mM citric acid (pH 5.9). The cell suspension were applied to poly-L-lysine-coated microscope slides and visualized under phase microscopy. These complementation data were quantified by direct counting of the number of organisms contained in each cluster of yeast in randomly selected consecutive fields, enumerating at least 50 consecutive organism clusters under each condition.

Yeast Sedimentation Assay

Next, a sedimentation assay was developed to further assess the effects of cell wall regulation on the clumping of *Eng2*-deficient and *PCEng2*

TABLE 1. YEAST STRAINS USED IN EXPRESSION AND COMPLEMENTATION STUDIES

Strain	Genotype
INVSc1-pYES2.1/PCEng2*	MATα his3D1 leu2 trp1-289 ura3-52 MATα his3D1 leu2 trp1-289 ura3-52 pYES2.1-PCEng2-URA3
BY4742-pYES2.1/LacZ†	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pYES2.1-LacZ-URA3
BY4742- <i>Eng2</i> -pYES2.1/LacZ†	MATα <i>Eng2Δ</i> KanMX3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pYES2.1-LacZ-URA3
BY4742- <i>Eng2</i> -pYES2.1/PCEng2†	MATα <i>Eng2Δ</i> KanMX3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pYES2.1-PCEng2-URA3

* Parent strains provided by Invitrogen.

† Parent strains provided by D. J. Katzmann and colleagues, *Saccharomyces* Genome Deletion Project.

complemented yeast. Aliquots of selective growth media (5 ml, DOB/CSM-URA) were inoculated with each colony of the transformed yeast strains. Cultures were grown at 30°C with shaking for 24–48 hours, then diluted in inducing growth media (DOB/CSM-URA plus Galactose) to yield an OD₆₀₀ of 0.4. The cultures were then incubated at 30°C with shaking for an additional 24 hours. Samples of each culture in triplicate were normalized to an OD₆₀₀ of 1.0 (± 0.03 ; SD) by dilution in inducing growth media, then placed in an optical cuvette within a spectrophotometer and left undisturbed. The OD₆₀₀ was measured every minute over a period of 1 hours as an assessment of the yeast clumping and sedimenting.

RESULTS

Identification of a Gene Sequence *PCEng2*

An initial *in silico* search of the partially complete *P. carinii* genome database (<http://pgp.cchmc.org/>) identified a 600-bp region with considerable homology to various β -glucanases present in other fungi. Using this starting sequence, the complete 2.2-kb coding region of *P. carinii* endo- β -1,3-glucanase gene was obtained by RACE and redundantly sequenced (GenBank accession no. EU814521). The archived *P. carinii* genome project sequence represented only approximately 27% of the total 2.2-kb coding sequence. The remainder of the coding sequences, as well as the 5' and 3' untranslated regions, were obtained directly from our RACE extension and coding strategies. The 5' untranslated region (GenBank accession no. GQ249166) and the 3' UTR (GenBank accession no. GQ249167) were also fully sequenced and analyzed. The predicted conserved amino acid domains of the coding regions classified the putative glucanase gene product as a glycoside hydrolase (GH family 81), with closest homology to *S. cerevisiae* and *S. pombe* endo- β -1,3-glucanases, Eng1 and Eng2 (36–39) (Figure 1A). Due to the absence of a recognizable signal sequence and a serine/threonine rich region, the *P. carinii* glucanase more closely resembles the yeast Eng2p rather than Eng1p. The predicted *Pneumocystis* PCEng2p revealed 43% homology to *S. pombe* Eng2p after BLASTX analysis (National Center for Biotechnology Information, Bethesda, MD). Analysis with VectorNTI protein AlignX (Life Tech, Carlsbad, CA), revealed the sequence to have 33.8% identical homology to *S. cerevisiae*, and 41.2% identical homology to *S. pombe*.

A closer look at the amino acid alignment of the predicted PCEng2p with *S. cerevisiae* and *S. pombe* Eng1 and Eng2 molecules revealed several identical and strongly conservative regions (Figure 1B, indicated in gray highlights). Aspartic and glutamic acid residues (enclosed in black boxes) are predicted to be potential catalytic nucleophiles required for the hydrolysis of glycosidic bonds within carbohydrate polymers (31). Other studies in *S. cerevisiae* and *S. pombe* have identified, by site-directed mutagenesis, that four of these residues are essential for enzymatic activity (33, 39, 40). Additional sequencing of the genomic region of *PCEng2* (GenBank accession no. EU938375) and our previously published splicing rules revealed that four intronic regions are present within this gene (41).

Detection of *PCEng2* within the *P. carinii* Genome

We next sought to verify that the *PCEng2* sequences were represented within the *Pneumocystis* genome. Such studies are necessary to verify that sequences amplified from *P. carinii* nucleic acids do not represent inadvertent amplification of contaminating host rat DNA sequences. Therefore, to accomplish this, a radioactive nucleic acid probe was produced from the original *PCEng2* partial sequence by PCR. Hybridization of this probe to *P. carinii* genomic DNA separated by CHEF (35,

42) revealed the presence of a *PCEng2* gene in only one chromosome of *P. carinii* isolated from the lungs of two separate rats (Figure 2).

Next, we assayed for the presence of the putative *PCEng2* endo- β -1,3-glucanase gene in *P. carinii* genomic DNA by Southern blot analysis (Figure 3). *P. carinii* genomic DNA was digested with various restriction enzymes (*HindIII* and *EcoRI*) and hybridized with the 600-bp *PCEng2* radiolabeled probe. Genomic DNA from normal rat lung was also assayed under the same digestion conditions as a negative control. The *PCEng2* probe hybridized to the digested *P. carinii* DNA as single band under all conditions. Because the probe was produced from a region of the gene that did not contain *HindIII* and *EcoRI* sites, this single band confirms that the gene is present in one location of the genome. No hybridization was observed with the rat DNA, confirming the specificity of the *PCEng2* probe.

Steady-State *PCEng2* mRNA Expression Is Largely Restricted to *P. carinii* Cysts and Is Not pH Responsive

We next sought to determine the relative expression of *PCEng2p* in the two major morphological forms, namely, cysts and trophic forms. Accordingly, we performed Northern analysis with RNA samples obtained from isolated cyst- and trophic-form populations (Figure 4A). In three independent determinations, the *PCEng2* probe hybridized specifically to RNA (~3-kb size) obtained from cyst form, with minimal expression in trophic forms. Reprobing the membrane with a specific *Pneumocystis* β -actin confirmed equal loading of the RNA.

In addition, our prior studies have indicated that certain genes responsible for *Pneumocystis* cell wall integrity exhibit differential expression under various environmental pH conditions (22). We next addressed steady-state *PCEng2* expression in freshly isolated *Pneumocystis* organisms exposed over 2 hours to varying environmental pH before harvest of the total RNA (Figure 4B). Whereas freshly isolated *Pneumocystis* organisms expressed abundant mRNA representing *PCEng2*, the transcription of the putative *PCEng2* β -1,3-endoglucanase gene was not substantially influenced by pH (Figure 4). Thus, unlike *PCPhr1*, which is similarly engaged in regulation of *Pneumocystis* cell wall integrity, *PCEng2* is not significantly influenced by environmental pH conditions (22).

Regional Expression of *PCEng2p* Protein in *P. carinii* as Assessed by Electron Microscopy

To begin to address the regional protein expression of *PCEng2p* protein over the life cycle of *Pneumocystis*, a *PCEng2p*-specific synthetic peptide antibody was generated. To verify the reactivity of this antibody, we first tested its reactivity against *PCEng2p* expressed in yeast (Figure 5A). The *PCEng2* open reading frame was cloned into an inducible yeast expression vector in frame with a V5 epitope and 6 $\times$ histidine tag. *S. cerevisiae* was transformed, and then induced to express the gene. The 75-kD protein was detected in the yeast lysate, but not in the culture media, indicating that this enzyme remained in the cytosol and was not secreted. Large-scale expression and purification by nickel-affinity column also detected a 75-kD protein, as predicted. Nonimmune rabbit IgG was used as a negative control, confirming the specificity of the anti-*PCEng2p* antibody.

Next, the *PCEng2p*-specific antibody was used to detect the ultrastructural regional expression in *P. carinii* organisms by gold particle labeling by electron microscopy (Figure 5B). As expected from the Northern expression analysis, *PCEng2p* was detected in focal location on the cyst walls (Figure 5B, arrows)

Figure 1. Eng conserved domains in *Pneumocystis carinii* (Pc), *Saccharomyces cerevisiae* (Sc), and *S. pombe* (Sp). (A) Protein annotation and domain identification by interactive software (MyHits). Glycosyl hydrolase family 81 (GH81), serine- and threonine-rich region (Ser/Thr), signal sequence (black boxes). (B) Amino acid sequence alignment (AlignX) illustrating identical and strongly conservative regions (gray highlights). Identical residues of aspartic acid and glutamic acid (black boxes) are potential catalytic sites, where four of these residues have been identified in other studies by site-directed mutagenesis in Sc and Sp (asterisks).

and also within the intracytic structures. However, reactivity was detected also within the trophic forms, suggesting that the protein may be relatively long lived once PCEng2p is expressed. Nonimmune control sections were negative for localization of gold particles.

Assessment of PCEng2p Function by Complementation of Eng2-Deficient *S. cerevisiae*

To assess the potential function of PCEng2p, we performed complementation studies of *Eng2Δ* *S. cerevisiae* strains. Whereas wild-type yeast exhibit normal cell wall separation morphology during budding, *Eng2*-deficient strains exhibit a defect in cell separation, causing clumping of cells. Induced expression of PCEng2 in the *Eng2*-deficient yeast resulted in

restoration of the wild phenotype, showing markedly less clumping (Figure 6A). All strains were transformed with pYES2.1 galactose-inducible expression plasmid; the wild-type and *Eng2*-deleted strains served as controls that were induced to express a nonspecific *LacZ* gene. The respective controls did not show restoration of the wild phenotype. The various complementation conditions were quantified by direct counting of the number of organisms contained in each cluster of yeast in randomly selected consecutive fields. These studies revealed significantly greater numbers of yeast cells per cluster in the *Eng2*-deficient yeast (5.180 ± 0.439 yeast/cluster; mean $\pm$ SEM) compared with wild-type control (1.960 ± 0.169 yeast/cluster; mean $\pm$ SEM). This abnormal phenotype was successfully rescued by PCEng2 complementation (2.540 ± 0.286 yeast/cluster).

Figure 2. *P. carinii* β -1,3-endoglucanase (*PCEng2*) is present on a single *P. carinii* chromosome. *Left panel:* Molecular weight markers. *Middle panel:* *P. carinii* chromosomal DNA separated by contour-clamped homogeneous electrophoresis field (CHEF). Each lane represents *P. carinii* isolated from a single rat. *Right panel:* hybridization with a 600-bp *PCEng2*-specific radiolabeled probe.

ANOVA showed significant differences between all groups ($P < 0.001$). Furthermore, the *PCEng2* complementation cluster counts were not significantly different than wild-type controls ($P > 0.05$, by unpaired *t* test). Thus, these complementation studies verify that the *PCEng2p* can function to partially restore the cell wall defect present in yeast lacking functional *Eng2p*.

We further developed a sedimentation assay to quantify the effect of complementation on the cell separation phenotype (Figure 6B). The *Eng2*-deficient yeast containing only the control plasmid, which we had demonstrated to exhibit a greater degree of organism clumping, demonstrated a greater degree of sedimentation than the *PCEng2p*-expressing cells that demonstrated less clumping and more cell separation. The differences

Figure 3. *PCEng2* is detected in genomic *Pneumocystis* DNA by Southern analysis. *P. carinii* genomic DNA was digested with *HindIII* and *EcoRI*, separated by agarose gel electrophoresis, transferred onto nitrocellulose membrane, and hybridized with the 600-bp *PCEng2*-specific radiolabeled probe. Genomic DNA from normal rat lung was similarly analyzed as a control.

Figure 4. *PCEng2* transcript detection by Northern analysis. (A) RNA was isolated from *P. carinii* cysts and *P. carinii* trophs and hybridized with *PCEng2* radiolabeled probe in two separate blots; transcript was present in cyst isolates, but not in the trophic-form isolates. (B) RNA was isolated from total *P. carinii* that was exposed to pH 4.0–8.0 at 37°C over 2 hours; transcript was present in all conditions at relatively equal amounts. PC ribosomal subunits (rRNA) were used as loading control.

in sedimentation ($P < 0.05$) were evident as early as 30 minutes and continued throughout the 2-hour measurement period.

DISCUSSION

PcP remains an important opportunistic fungal lung infection that greatly impacts the health outcomes of immunosuppressed patients, such as patients with AIDS and malignancy (7–9). Understanding the *Pneumocystis* life cycle is important for the development of new strategies to prevent and to treat this often-lethal infection. Although *Pneumocystis* organisms were discovered approximately 100 years ago, this fungal species still cannot be grown and studied in culture outside of an infected host lung (43). Therefore, the central mechanisms of its life cycle and, accordingly, many factors underlying its viability and infectivity still remain to be elucidated. Although studies have characterized the major *Pneumocystis* life cycle stages, in which trophic forms attach to lung alveolar cells, and then conjugate and multiply within a maturing cystic phase, the process of cell wall regulation during excystation of the trophic forms from the cysts to complete the life cycle has not yet been studied.

Studies of glucanases in other fungal species have shown them to be important enzymes in regulating the organism's cell wall dynamics and the overall ability to proliferate (31–34). Accordingly, the current studies were undertaken to identify a gene with β -1,3-endoglucanase homologies, *PCEng2*, within the *Pc* genome, and determine its activity in cell wall regulation in the organism. Our studies have demonstrated the chromosomal location of this sequence, and have indicated that this cognate protein is expressed in the organism as well. We have further shown the activity of the *PCEng2p* in restoring the cell wall separation phenotype in *Eng2* Δ yeast. Thus, we propose that *PCEng2* likely serves parallel cell wall regulatory function in *Pneumocystis*.

Although the *PCEng2* homolog may not exhibit as high a homology as other molecules that we have cloned, the

Figure 5. PCEng2 Expression in *S. cerevisiae* and localization in *P. carinii*. (A) Detection of inducible protein expression of PCEng2 by V5 epitope in yeast lysate and growth media supernatant. Large scale PCEng2 expression was in 1.5-L yeast culture, followed by nickel column purification. The Western blot detected the approximately 75-kD band from large-scale yeast expression with PCEng2-specific antibody produced in rabbit. Negative control with nonimmune rabbit IgG revealed no specific reactivity at 75 kD. (B) Electron micrographs showing cysts and trophic forms labeled with PCEng2-specific antibody and gold particles. Nonimmune controls were devoid of staining, further confirming the specificity of the anti-PCEng2p antibody.

homology is still considerable (9, 24). We have further scanned the *P. carinii* genome database to detect other orthologs of greater homology. Despite an exhaustive search, no additional sequences have been identified. It should be noted, however, that the *Pneumocystis* genome database only provides approximately 50% coverage of the genome. Indeed, the available sequence found in the genome database only represented roughly 27% (i.e., 0.6 kb of the final total 2.2-kb coding region sequence) that we eventually cloned. It does, however, remain possible that other Eng-related molecules are present with *Pneumocystis*, and remain to be discovered. We do believe that PCEng2 represents one such molecule participating in cell wall regulation, and hence in life cycle control. To further verify that such a molecule might exist in other species of *Pneumocystis*, initial PCR-based studies were recently undertaken, revealing the presence of a mouse-derived *P. murina* endo- β -1,3-glucanase mRNA (partial 942-bp mRNA, GenBank accession no. GQ403793) with 84% identical homology to PCEng2 (NCBI BLAST-N analysis), and a human-derived *P. jirovecii* endo- β -1,3-glucanase gene (partial 86-bp DNA, GenBank accession no. GQ403794) with 78% identical homology to PCEng2 (NCBI BLAST-N analysis). These data provide further support

Figure 6. Complementation of PCEng2 in *S. cerevisiae* Eng2-deleted strains. (A) Eng2-deleted yeast strain transformed with control expression vector showed defect in cell separation, whereas transformation with PCEng2 expression vector showed restoration of the wild type phenotype. (B) Sedimentation profiles of Eng2 knockout yeast strain (closed circles) and complementation with PCEng2 expression (open circles).

for the generally conserved nature of the PCEng2 gene across *Pneumocystis* species.

Sequencing the complete coding regions and 5' untranslated sequence of *P. carinii* PCEng2 has provided interesting functional insight after amino acid motif analysis, and potential enzyme classification based on sequence homology of related fungi. The amino acid signature pattern classifies PCEng2p as a glycosyl hydrolase within family 81 (GH81), the family in which *S. cerevisiae* and *S. pombe* Eng1 and Eng2 are also classified (44–48). The lack of certain predicted motifs, such as

the serine/threonine-rich domains and a signal sequence, indicate that this enzyme is more functionally related to *S. pombe* Eng2 rather than Eng1, and likely to additionally exhibit cytosolic functions, such as actin cytoskeletal regulation, as well as cell wall β -glucanase activity (49). Further functional characterizations, such as specific glycosidic linkage sites of hydrolase activity and substrate specificity, will be required to formally classify this enzyme, according to the International Union of Biochemistry and Molecular Biology, as a eukaryotic β -endoglucanase (33).

Based on this predicted sequence, we were successful in generating a PCEng2p-specific antibody to undertake immune localization in *Pneumocystis* cysts and trophic forms. Interestingly, these studies suggest that, in addition to expression in cysts, PCEng2p is also expressed within the trophic-form cytoplasm. Expression of recombinant PCEng2p with a yeast expression system not only demonstrated the specificity of the antibody, but also that the enzyme was expressed within the yeast cytosol, and not secreted into the culture media, in these heterologous fungi. These observations support the contention that PCEng2p is likely to possess cytoplasmic activity within both *Pneumocystis* trophic forms and cysts. For instance, in yeast, PCEng2p exhibits additional functions in regulating actin filament formation (49). In contrast, Eng1 in *S. cerevisiae* and *S. pombe* has been shown to contain a signal sequence and to be secreted into culture media. Thus, it can be hypothesized that a secreted PCEng1 homolog may also exert a compounding effect on cyst wall degradation. Accordingly, studies are currently underway to identify and characterize any potential PCEng1 sequences in *P. carinii*.

Studies of gene functionality in *Pneumocystis* itself are limited by the inability to culture these organisms *in vitro* and the consequent inability to transform and select genetically manipulated *Pneumocystis* organisms. To circumvent these issues, we have adopted a strategy of assessing gene function with heterologous expression in related tractable fungal species (16). Based on our earlier studies of complementing cell wall regulatory enzymes, we selected *S. cerevisiae* as a suitable heterologous model system (16, 18). In *S. cerevisiae*, Eng1 and Eng2 deletions result in altered phenotype related to cell morphology and growth. In our studies, phase-contrast microscopy revealed that a clumping phenotype was present in the *MAT α* haploid Eng2-deleted mutants, and introduction of the PCEng2 gene demonstrated restoration of the wild-type cell separation phenotype. These complementation studies indicate that PCEng2p is an important enzyme in cell wall regulation during organism growth and proliferation.

The AT-rich genome of *Pneumocystis* renders protein expression of many *Pneumocystis* genes in yeast or bacteria extremely problematic (50). Indeed, immunoprecipitation was even required to generate enough recombinantly expressed PCEng2p for detection by Western analysis. Such reduced protein expression, to date, has limited our ability to directly study enzymatic function of PCEng2p on β -glucan degradation. Therefore, novel expression approaches will be required to fully characterize the enzymatic mechanisms of PCEng enzymes on the *Pneumocystis* cyst wall. Our immunoelectron micrographs did localize some PCEng2p to foci in the cysts wall. Enzymatic proteins, such as PCEng2p, are less abundant and more difficult to detect with immune reagents compared with structural proteins. Notwithstanding, we have recently also successfully detected the PCran1p kinase by this immunoelectron microscopy approach (50). Thus, these observations on the localization of PCEng2p further suggest a role for PCEng2p in cyst wall regulation.

Nonetheless, a more complete understanding of *Pneumocystis* cell wall regulation, β -glucan degradation, and trophic excystment will be essential for unraveling the elusive life cycle of this fascinating and important opportunistic fungal pathogen. These studies have demonstrated the potential role of PCEng2p in regulating cell wall dynamics in this organism. Moreover, the generation and degradation of the β -glucan cell wall represents an important target for the prevention and treatment of PcP (25).

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