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Characterization of *Pneumocystis carinii PHR1*, a pH-Regulated Gene Important for Cell Wall Integrity

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Pneumocystis carinii **remains an important opportunistic fungal pathogen causing life-threatening pneumonia in patients with AIDS and malignancy. Currently, little is known about how the organism adapts to environmental stresses and maintains its cellular integrity. We recently discovered an open reading frame approximately 600 bp downstream of the region coding *GSC-1*, a gene mediating β-glucan cell wall synthesis in *P. carinii*. The predicted amino acid sequence of this new gene, termed *P. carinii PHR1,* exhibited 38% homology to *Saccharomyces cerevisiae GAS1*, a glycosylphosphatidylinositol-anchored protein essential to maintaining cell wall integrity, and 37% homology to *Candida albicans PHR1*/*PHR2*, pH-responsive genes encoding proteins recently implicated in cross-linking β-1,3- and β-1,6-glucans. In view of its homology to these related fungal genes, the pH-dependent expression of *P. carinii PHR1* was examined. As in *C. albicans*, *P. carinii PHR1* expression was repressed under acidic conditions but induced at neutral and more alkaline pH. *PHR1*-related proteins have been implicated in glucan cell wall stability under various environmental conditions. Although difficulties with *P. carinii* culture and transformation have traditionally limited assessment of gene function in the organism itself, we have successfully used heterologous expression of *P. carinii* genes in related fungi to address functional correlates of *P. carinii*-encoded proteins. Therefore, the potential role of *P. carinii PHR1* in cell wall integrity was examined by assessing its ability to rescue an *S. cerevisiae gas1* mutant with absent endogenous Phr1p-like activity. Interestingly, *P. carinii PHR1* DNA successfully restored proliferation of *S. cerevisiae gas1* mutants under lethal conditions of cell wall stress. These results indicate that *P. carinii PHR1* encodes a protein responsive to environmental pH and capable of mediating fungal cell wall integrity.**

Pneumocystis carinii remains an important fungal agent causing life-threatening pneumonia in patients with impaired immunity (19). Other fungi such as *Aspergillus nidulans* and *Candida albicans* alter gene expression as an adaptive response to environmental pH changes in order to maintain cell wall integrity and promote viability under various conditions (6, 11, 21, 23, 32–35). Specifically, it has been demonstrated that *C. albicans* expresses a unique family of pH-regulated genes required for virulence. These genes nominally include *PHR1*, a gene expressed maximally at pH 5.5 to 8.0 which encodes a protein promoting systemic infection of mice. Alternatively, *C. albicans* also expresses *PHR2,* whose transcription is greatest at acidic pH (4 to 5) values (24). If *PHR2* is rendered inactive, *C. albicans* mutants exhibit decreased pathogenesis in a mouse model of vaginal infection (6, 24, 32).

Recent studies indicate that Phr1p and Phr2p act on β-1,3-glucans of the *C. albicans* cell walls, elongating the β-1,3 polysaccharide backbone and potentially mediating the attachment of β-1,6 glucosyl side chains and β-1,6-glycosylated mannoproteins (8, 23). The role of glucan cell wall structure in pathogenesis is not completely understood, but has been postulated to participate in maintenance of cell wall integrity during environmental stress.

The mechanisms by which *P. carinii* assembles its cell wall have only recently been elucidated. Initial studies largely focused on the prominent surface glycoprotein complexes termed glycoprotein A or major surface glycoproteins, which have been demonstrated to participate in *P. carinii* attachment to type I alveolar epithelial cells and alveolar macrophages (4, 12, 19). More recently, our group has focused on generation of β-glucan cell wall components, which represent major structural constituents of the cystic form. The cystic form has been postulated to represent a transmissible agent capable of surviving the harsh environmental conditions outside of the mammalian host (3).

To this end, we recently characterized *P. carinii GSC1*, a gene responsible for assembly of the 1,3-β-D-glucan core carbohydrate of the cyst cell wall (16). During screening of the *P. carinii* genomic DNA library for *GSC1*, we isolated an open reading frame downstream of *P. carinii GSC1* with considerable homology to *C. albicans PHR1*/*PHR2* and *Saccharomyces cerevisiae GAS1*. Here we report the identification and initial characterization of this gene, termed *P. carinii PHR1*. We demonstrate pH-dependent expression of *P. carinii PHR1* with greatest expression under physiologic (pH 7.0 to 7.5) conditions present in the lung. We further show that *P. carinii PHR1* participates in the maintenance of fungal cell wall integrity, as assessed by the ability of *P. carinii PHR1* to complement growth of *S. cerevisiae gas1* mutants with defective Gas1p (Phr1p-like) activity.

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MATERIALS AND METHODS

Materials. All reagents were from Sigma Chemical Company (St. Louis, Mo.) unless otherwise specified. Restriction endonucleases were obtained from Gibco-BRL (Life Technologies, Rockville, Md.), and *Pfu* polymerase was from Strat-

agene (La Jolla, Calif.). [α-^{32}P]ATP was obtained from ICN Pharmaceuticals (Costa Mesa, Calif.). The WB2d *Saccharomyces cerevisiae* strain expressing a mutated and ineffective Gas1p (YEp-*gas1* mutant) was the generous gift of Marina Vai, Università degli Studi di Milano, Milan, Italy (38). The plasmid YEp-*GAS1,* containing the wild-type *GAS1* gene used as a complementation control, was also obtained from M. Vai. The yeast expression plasmid p425GAL used in the generation of the p425GAL-*PHR1* construct was obtained from the American Type Culture Collection (Rockville, Md.).

Preparation of *P. carinii* organisms. *P. carinii* pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone, as reported previously (5, 15, 36). Rats received drinking water containing dexamethasone ad libitum (2 mg/liter). After 1 week of immune suppression, rats were anesthetized with ether and inoculated with ~10^6 *P. carinii* organisms intratracheally. After 6 weeks of additional dexamethasone treatment, the animals were sacrificed, and *P. carinii* was harvested. Lungs from the rats were minced in Hanks' balanced salt solution and homogenized in a stomacher microbiological blender (Tekmar Inc., Cincinnati, Ohio) for 5 min. *P. carinii* organisms were passed through a 10-μm filter (Millipore), which retains lung cells but allows passage of *P. carinii*. These preparations were stained with Wright-Giemsa to confirm that *P. carinii* organisms were present (19). Preparations containing detectable bacterial or fungal contamination were discarded.

Identification of putative *P. carinii PHR1* gene. In the course of screening a *P. carinii* λgt11 genomic library for the *P. carinii* 1,3-β-glucan synthetase (*GSC1*), an open reading frame was identified ≈600 bp downstream of the *GSC1* clone that contained 465 bp of coding sequence. Translation of the open reading frame (BlastX; EMBL) revealed a partial sequence clone with substantial sequence homology to the *C. albicans* pH-responsive gene *PHR1* and containing a start site for the putative *P. carinii PHR1* gene. In light of our interest in *P. carinii* cell wall assembly and because of the reported roles of *PHR* genes in generation of fungal glucans, we sought to fully identify this new *P. carinii* gene and characterize its potential functions.

To obtain the remaining 3′ end of *P. carinii PHR1,* a modified 3′ rapid amplification of genomic ends (RAGE) procedure was used as follows. The λgt11 phage clones that contained the partial *P. carinii PHR1* sequences were plaque purified to homogeneity. This λgt11 insert was used as the PCR template. The primers used were 5′-TCGCTTATCAGCCTCCGTTAAG-3′ (gene-specific primer) and 5′-CCAACTGGTAATGGTAGCGACC-3′ (3′ λgt11-specific primer). An initial 5-min hot start at 94°C was followed by 30 cycles of 94°C for 30 s, 60°C for 60 s, 72°C for 90 s, and a final 72°C 15-min extension. A single amplicon of approximately 2.3 kb was generated, subcloned into pGEM-T Easy vector (Promega, Madison, Wis.), and sequenced on both forward and reverse strands. Sequence comparisons to GenBank were performed using the Blast genetic analysis program (NCBI). Sequence analysis was undertaken with MacVector software (Kodak, IBI, New Haven, Conn.). Homology comparisons were conducted with the BlastX (EMBL) algorithms. To perform multiple sequence alignments, the PileUp program of the Genetics Computer Group was used (7).

Confirmation of *PHR1* sequences in *P. carinii* genomic DNA. To confirm that the *PHR1* gene was truly represented within the *P. carinii* genome and not related to host or other microbial contamination, Southern hybridization was performed using the *PHR1* gene as a probe against freshly isolated *P. carinii* genomic DNA. Genomic DNA from *P. carinii* was prepared with the IsoQuick nucleic acid extraction kit (Orca Research Inc., Bothell, Wash.). A ^{32}P-labeled *P. carinii PHR1* probe was generated using the random primer method (RadPrime; Amersham Pharmacia, Piscataway, N.J.). Twenty micrograms of genomic DNA was digested with either *Eco*RI or *Hin*dIII and separated on a 1% agarose gel. Transfer and hybridization were performed as described (31).

Expression of *P. carinii PHR1* mRNA in response to environmental pH. In light of the strong homology of *P. carinii PHR1* to pH-regulated genes in other fungi, we next evaluated the expression of *P. carinii PHR1* mRNA in response to environmental pH. To address this, *P. carinii* organisms were placed in 1.0 ml of Ham's F-12 tissue culture medium supplemented with 10% fetal bovine serum at the indicated pH ranges for 1.0 h at 37°C. Total RNA was isolated with hot acidic phenol and separated on 1.0% formaldehyde–agarose gels (1). RNA was transferred to Nytran Plus membranes (Schleicher & Schuell, Keene, N.H.), and hybridization to the radiolabeled *PHR1* probe performed at 68°C using Clontech Express hybridization solution (Clontech, Inc., Palo Alto, Calif.). Total RNA loading was verified by ethidium bromide staining and by further stripping the final blots and repeat probing with a *Pneumocystis* actin probe (16, 17).

Role of *P. carinii PHR1* in maintaining fungal cell wall integrity. Studies of *P. carinii* gene function have long been hindered by the inability to culture and transform the organism. To circumvent these obstacles, we have recently undertaken analysis of *P. carinii* gene function by heterologous expression of *P. carinii* proteins in phylogenetically related fungi which lack endogenous homologues under selective conditions (17). Using such a strategy, we analyzed the ability of *P. carinii* Phr1p to restore cell wall integrity by transforming Gas1p-deficient *S. cerevisiae* mutants with *P. carinii PHR1* DNA. *S. cerevisiae gas1* mutants have increased cellular fragility in the presence of osmotic destabilizing agents such as sodium dodecyl sulfate (SDS), a stringent assessment of cell wall stability. Previous studies have shown that *S. cerevisiae gas1* mutants are unable to grow in the presence of 0.01% SDS (38).

P. carinii PHR1 DNA was excised from pGEM-T Easy by digestion with *Hin*dIII and *Nde*I and directionally cloned into the yeast expression vector p425GAL. The p425GAL plasmid has a *LEU2* gene that permits growth on medium lacking leucine (25). *S. cerevisiae gas1* mutants were grown to mid-log phase in YPD broth at 30°C and transformed by electroporation using 10.0 ng of p425GAL/*P. carinii PHR1* DNA or 10.0 ng of p425GAL vector alone without insert (31). Transformed *S. cerevisiae* cells were plated onto minimal medium plates deficient in leucine at 30°C. After 48 h, yeast transformants were streaked onto minimal medium leucine-deficient plates containing 0.005% SDS, placed at 30°C, and assessed for growth. Transformed yeast colonies proliferating at these conditions were cultured to mid-log phase in leucine-deficient broth, and plasmid DNA was extracted and sequenced to confirm the presence of *P. carinii PHR1* DNA.

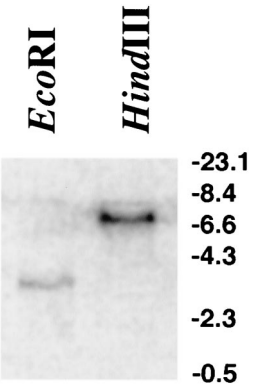

FIG. 1. *P. carinii PHR1* homologue is represented in the organism's genome. The full-length *P. carinii PHR1* cDNA homologue was radiolabeled and hybridized to *P. carinii* genomic DNA digested with *Eco*RI or *Hin*dIII, as indicated. *PHR1* was present in one location on each Southern analysis.

RESULTS AND DISCUSSION

***P. carinii* contains a putative pH-responsive gene, *PHR1*.** The mechanisms through which *P. carinii* responds to alterations in the environment remain largely unknown. In an effort to better understand the mechanisms by which *P. carinii* assembles its cyst wall and maintains integrity under various environmental conditions, we fully cloned and sequenced the putative *P. carinii PHR1* gene. The initial 465-bp partial *P. carinii PHR1* open reading sequence revealed a substantial degree of homology to its *C. albicans* pH-responsive counterpart (BlastX, 50%). After redundant sequencing of this partial clone to its putative start site, a modified 3′ RAGE procedure was used to obtain the remaining portion of the gene by using a 5′ *PHR1* gene-specific primer and a 3′ primer matching the λgt11 cloning site of the *P. carinii* genomic library. The DNA template used for PCR was obtained by purifying λgt11 plaques that initially hybridized with *GSC1* sequences.

After screening a number of colonies obtained by this strategy, a clone containing the remaining genomic *P. carinii PHR1* sequence was isolated. To verify that the DNA sequence obtained by PCR was not a result of amplification of host cell DNA or other foreign DNA and was specifically represented

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P. carinii Phr1p     1  MHLNYHVFYGIIYLYIIGSAYAR--IQTIYAYGNKLFYQN-GTQFFIAG  46
S. cere. Gas1p       1 MLFKSLSKLATAAAFFAGVATADD--VPAIEVVGNKFFYSNNGSQFYIRG  48
C. albicans Phr1p    1  MYSLIKSLATFATLFSLTLAKFESSTPPVEVVGNKFYFSNNGSQFLIRG  49
C. albicans Phr2p    1 MLLKSLFPSILAATSFVSSVAAED--LPAIEIVGNKFFYSNNGSQFYIKG  48
C. maltosa Epd1p     1 MLLNSLFPSILAAATFVTSAAAED--LPPIEIVGNKFFYSNNGSQFYIKG  48

P. carinii Phr1p    47 VAYQPPLRGDAD---------HYKNPLADPASCERDLRYFLELGIN--TI  85
S. cere. Gas1p      49 VAYQADTANETSG-------STVNDPLANYESCSRDIPYLKKLNTN--VI  89
C. albicans Phr1p   50 IAYQQDAAGSVSSGYDADPNRKYNDPLADADACKRDVKYFKESNTN--TL  97
C. albicans Phr2p   49 IAYQQNNLDSNE---------SFVDPLANPEHCKRDIPYLEAVDYDSNVI  89
C. maltosa Epd1p    49 IAYQQNNLDSNS---------TFVDPLADADNCKRDIPYLEQVDTN--VI  87

P. carinii Phr1p    86 RVYTVNPENNHDYCMRLFENSGIYVLLDLSEPRNSIISTDPSWNVRLFWR 135
S. cere. Gas1p      90 RVYAINTTLDHSECMKALNDADIYVIADLAAPATSINRDDPTWTVDLFNS 139
C. albicans Phr1p   98 RVYAIDPDKDHEECMKIFSDAGIYIVADLSEPTVSINRNNPEWNLDLYKR 147
C. albicans Phr2p   90 RVYALDTSQDHTECMQMLQDAGIYVIADLSQPDESINRDDPSWDLDLFER 139
C. maltosa Epd1p    88 RVYALDVTQDHTECMQMLQDAGIYIIADLSQPDESINRNDPQWNLDLFER 137

P. carinii Phr1p   136 YSKVIDNMHKYPNLLGFFAGNEVILDTENTHSAAYVKAAVRDVKSYMRSK 185
S. cere. Gas1p     140 YKTVVDTFANYTNVLGFFAGNEVTNNYTNTDASAFVKAAIRDVRQYISDK 189
C. albicans Phr1p  148 YTKVIDKMQEYSNVLGFFAGNEVTNNRSNTDASAFVKAAIRDMKKYIKES 197
C. albicans Phr2p  140 YTSVVDLFHNYTNILGFFAGNEVTNKKSNTDASAFVKAAIRDTKAYIKSK 189
C. maltosa Epd1p   138 YTSVVDKFHNYTNVLGFFAGNEVTNNVSNTDASAFVKAAIRDTKAYIKAK 187

P. carinii Phr1p   186 GYRKILVGYAANQHEHTPIPSANYFACGKFCIKLVIFLGSICNIYFLCLK 235
S. cere. Gas1p     190 NYRKIPVGYSSNDDEDTRVKMTDYFACG----------DDDVKADFYGIN 229
C. albicans Phr1p  198 DYRQIPVGYSSNDDEEIRVAIADYFSCG----------SLDDRADFFGIN 237
C. albicans Phr2p  190 GYRSIPVGYSANDDSAIRVSLADYFACG----------DEDEAADFFGIN 229
C. maltosa Epd1p   188 GYRTIPVGYSANDDSDIRVSLARYFACG----------DEDESADFFGMN 227

P. carinii Phr1p   236 NIYLLHFSYEWCDPTSYETSGYRDRVNDFRNYNVPIFFSEYGCNIVNGKI 285
S. cere. Gas1p     230 -------MYEWCGKSDFKTSGYADRTAEFKNLSIPVFFSEYGCNEVTPRL 272
C. albicans Phr1p  238 -------MYEWCGKSTFETSGYKDRTEEIKNLTIPAFFSEYGCNANRPRL 280
C. albicans Phr2p  230 -------MYEWCGDSSYKASGYESATNDYKNLGIPIFFSEYGCNEVRPRK 272
C. maltosa Epd1p   228 -------MYEWCGSSSFKASGYESATDDYKNLGIPIFFSEYGCNEVTPRK 270

P. carinii Phr1p   286 GVRSFSQVPHIYSEKMTDVFSGGIVYEWFQNVNNYGLVNLLPDNTISVRQ 335
S. cere. Gas1p     273 ----FTEVEALYGSNMTDVWSGGIVYMYFEETNKYGLVSID-GNDVKTLD 317
C. albicans Phr1p  281 ----FQEIGTLYSDKMTDVWSGGIVYMYFEEANKYGLVSVD-GNSVKTLS 325
C. albicans Phr2p  273 ----FTEVATLFGDQMTPVWSGGIVYMYFEEENNYGLVSIK-DNTVSTLK 317
C. maltosa Epd1p   271 ----FQEVGTLFGSDMTDVWSGGIVYMYLQEENNYGLVSVS-GSSVSTLQ 315

P. carinii Phr1p   336 DFLNLREQLRRINPKAIQRSTYTPR---NGPPECPAIGQY-WSSSTLLPP 381
S. cere. Gas1p     318 DFNNYSSEINKISPTSANTKSYSAT---TSDVACPATGKY-WSAATELPP 363
C. albicans Phr1p  326 DYNNYKSEMNKISPSLAHTSTLSSSDA-SKTLQCPGTAASTWKAATNLPP 374
C. albicans Phr2p  318 DYSYYSSEIKDIHPSSAKASAESAS--SISRTTCPTNTNN-WEASTNLPP 364
C. maltosa Epd1p   316 DFNSYKSEILDISPSSVQASAESAS--GVSRTSCPTNTDN-WEASTELPP 362

P. carinii Phr1p   382 IPNSELCACASRASSCIAVNDITDAEIAEIFSYICGEISCKAVSKDSKIG 431
S. cere. Gas1p     364 TPNGGLCSCMNAANSCVVSDDVDSDDYETLFNWICNEVDCSGISANGTAG 413
C. albicans Phr1p  375 TPDESYCDCISKSLECVVADDVDKEDYGDLFGQVCGYIDCSAISADGSKG 424
C. albicans Phr2p  365 TPDKEVCECMSASLKCVVDDKVDSDDYSDLFSYICAKIDCDGINANGTTG 414
C. maltosa Epd1p   363 TPDKDICDCMSSSLKCVVADNVSTDDYSDLFDYVCAKIDCSGINANATTG 412

P. carinii Phr1p   432 LYGAFSVCEPIDQLNVILNLYYNKHHRQESACNFKGLAYVVTSETSKTCS 481
S. cere. Gas1p     414 KYGAYSFCTPKEQLSFVMNLYYEKSGGSKSDCSFSGSATLQTATTQASCS 463
C. albicans Phr1p  425 EYGVASFCSDKDRLSYVLNQYYLDQDKKSSACDFKGSASINSKASASGSC 474
C. albicans Phr2p  415 EYGAYSPCHSKDKLSFVMNLYYEQNKESKSACDFGGSASLQSAKTASSCS 464
C. maltosa Epd1p   413 DYGAYSPCGAKDKLSFVLNLYYEEQNESKSACDFSGSASLQSASTASSCA 462
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FIG. 2. Alignment of the predicted amino acid sequences of *P. carinii* Phr1p and related proteins derived from heterologous fungi. *S. cere*., *S. cerevisiae.*

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P. carinii Phr1p  482 SLLQIVGIDGSGTITGPPVATG-------FGKEKDSR---------DGKE 515
S. cere. Gas1p    464 SALKEIGSMGTNSASGSVDLGSGTESSTASSNASGSSSKSNSGSSGSSSS 513
C. albicans Phr1p 475 KAVSGVATGKASSSGGSSKSGS------SSASASGSS---------SSST 509
C. albicans Phr2p 465 AYLSSAGSSGLGTVSGTVRTDT---SQSTSDSGSGSS--------SSSSS 503
C. maltosa Epd1p  463 AYLSSAGVSGLGTVQGSVRTDT---SEATTDSGSGSSN---SGSASSSKS 506

P. carinii Phr1p  516 SLGK--AIYPDWR--------------LIFGIMTYFFGIIIVIAN    544
S. cere. Gas1p    514 SSSSSASSSSSSKKNAATNVKANLAQVVFTSIISLSIAAGVGFALV   559
C. albicans Phr1p 510 SSGS--SSSSGVK------ATQQMSMVKLVSIITIVTAFVGGMSVVF  548
C. albicans Phr2p 504 SSSSSSSGSSGSK-----SAASIVSVNLLTKIATIGISIVVGFGLITM 546
C. maltosa Epd1p  507 TSSSTSSGSSGSK-----SAATAVTVTTLTKIAAVGVSIIVGFGLITM 549
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FIG. 2—*Continued.*

within the *P. carinii* genome, the full-length *PHR1* sequence was used as a probe for Southern hybridization studies against digested *P. carinii* genomic DNA. The full-length *P. carinii PHR1* probe strongly hybridized to a single band on both the *Eco*RI and *Hin*dIII digests, indicating its presence in the *P. carinii* genome (Fig. 1). As anticipated, an identical pattern of hybridization had previously been observed with the adjacent *P. carinii GSC1* DNA sequences (16).

Thus, a 2.3-kb genomic DNA fragment containing the complete *PHR1* gene was available for further study. Similar to *C. albicans PHR1,* a single uninterrupted open reading frame was identified in the *P. carinii* sequence (32). This open reading frame of 1,632 bp was predicted to encode a 544-amino-acid peptide. The deduced amino acid sequence of *P. carinii PHR1* is shown in Fig. 2 (GenBank accession no. AF191097). Computer analysis of the GenBank database and *P. carinii* genome project website (http://www.uky.edu/Projects/Pneumocystis/) revealed the entire *PHR1* sequence to be unique and yet most homologous to a protein encoded by *S. cerevisiae GGP1*/*GAS1* (38%; BlastX) and to the *C. albicans* pH-responsive gene products PHR1 and PHR2 (37% each) (24, 32, 37). All three proteins are thought to encode major glycoproteins localized to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor and important for the maintenance of cell wall integrity (28, 30, 32, 38).

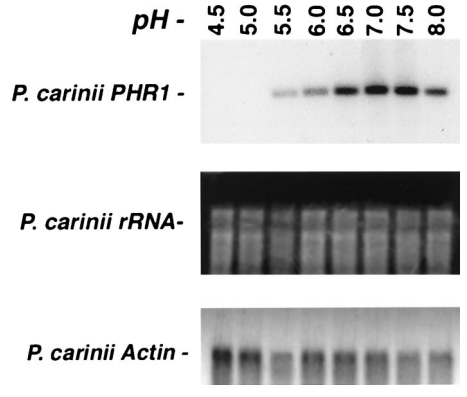

FIG. 3. *P. carinii PHR1* exhibits pH-regulated mRNA expression. To examine whether *P. carinii PHR1* expression responded to environmental pH, organisms were freshly isolated and maintained in medium at the indicated pH prior to isolation of RNA and Northern analysis. Optimal steady-state mRNA expression of *P. carinii PHR1* was observed at physiological pH of 7.0 to 7.5. RNA loading was verified by ethidium bromide staining and reprobing of the blot with *P. carinii* actin.

The predicted *P. carinii* Phr1p protein contains a potential GPI attachment site (G^{518}) followed by a polar region at amino acid residues 519 to 526 (KAIYPDWR) and finally a hydrophobic carboxy terminus encompassing residues 527 to 543 (LIFGIMTYFFGIIIVIA) (2, 13, 18, 22, 27). The putative *P. carinii PHR1* exhibited substantial similarity to *S. cerevisiae GGP1*/*GAS1* and *C. albicans PHR1*/*PHR2* over the majority of its length, including conservation in two of the three N-glycosylation sites and 11 of 13 conserved cysteine residues. The greatest divergence of the predicted *P. carinii* Phr1p to the three previously described proteins was noted in both the amino- and carboxyl-terminal regions. Alignments of fungal GPI-anchored proteins with homology to *P. carinii PHR1,* including *EPD1*, which encodes a protein thought to be involved in pseudohyphal growth of *Candida maltosa* (26), are also demonstrated in Fig. 2.

***P. carinii PHR1* expression is regulated by environmental pH.** In light of the substantial homology of *P. carinii PHR1* to genes responsive to environmental pH in *C. albicans,* we further evaluated whether expression of this *P. carinii* gene was similarly responsive to changes in the ambient pH. To test this, *P. carinii* organisms were removed from rat lung and placed in medium at a range of ambient pHs for 1 h. We have previously used such a short-term medium exposure system to monitor gene expression of *P. carinii GSC1* (16). Parallel to *C. albicans*, differential expression of *P. carinii PHR1* steady-state mRNA was observed on Northern analysis (Fig. 3). *P. carinii PHR1* mRNA was virtually absent at pHs ranging between 4.5 and 5.0. Over the pH range of 5.5 to 7.5, incremental increases in *P. carinii PHR1* mRNA were seen, with optimal expression at physiological pH (7.0 to 7.5). This pH range has also been reported previously to induce expression of *C. albicans PHR1* (32). Such a pH is compatible with the environmental pH of the alveolar space under basal and stress conditions (9). Environmental pH may provide one signal to *P. carinii* organisms that the milieu is conducive for life cycle progression and proliferation (20). To our knowledge, this represents the first description of an environmentally regulated gene in this important opportunistic fungus.

Recent investigations in *C. albicans* document the importance of *PHR* activity in disease pathogenesis (6, 8, 11). *C. albicans* deleted of *PHR1* exhibits aberrant morphology and is less virulent than *PHR1*$^{+}$ strains in mice with disseminated

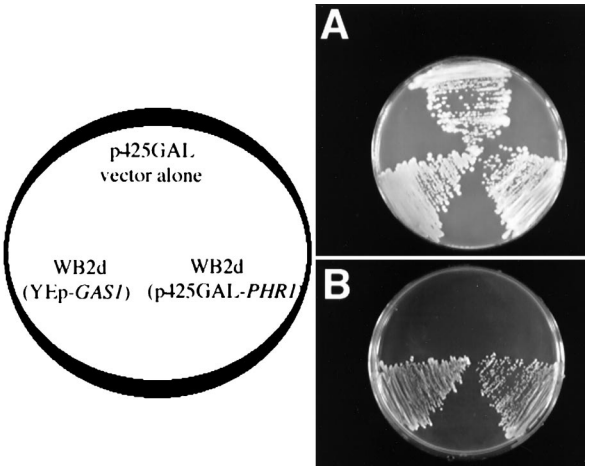

FIG. 4. *P. carinii PHR1* complements growth of *S. cerevisiae gas1* mutants in the presence of SDS. *S. cerevisiae gas1* mutants lack the ability to grow in the presence of the osmotic destabilizing agent SDS due to cell wall instability. The Gas1p-deficient mutants were cultured following transformation with vector alone, with the wild-type gene present in YEp-*GAS1* (positive control), or with the vector containing *P. carinii PHR1* (p245GAL-*PHR1).* Identical plates were cultured in (A) selective minimal medium or (B) minimal plates supplemented with 0.005% SDS. *P. carinii PHR1* successfully rescued the cell wall instability of the yeast mutant and restored growth in the presence of SDS.

systemic candidiasis. Specifically, the growth of *C. albicans PHR1*$^+$ strains is favored in the neutral to slightly alkaline pH of the bloodstream (11). Whether *P. carinii* similarly expresses other pH-related genes under alternative conditions is an attractive hypothesis, which merits further investigation. Review of the evolving *P. carinii* project genome database (http://www.uky.edu/Projects/Pneumocystis/) has not yet revealed any additional candidate pH-responsive genes. Identification of genes active under alternative environmental conditions might provide important insights into alternative niches used by this intractable organism.

P. carinii* PHR1 DNA confers cell wall integrity on Gas1p-deficient *S. cerevisiae. *PHR* class genes have been implicated in cell wall generation, cross-linking, and stability under adverse conditions. Heterologous expression of *P. carinii* genes in culturable fungal species has recently proven a useful experimental approach to infer *P. carinii* gene function (10, 17, 36). Accordingly, we evaluated the ability of *P. carinii PHR1* to growth complement an *S. cerevisiae* mutant strain deficient in the *PHR1* analogue *GAS1* in the presence of a potent osmotic destabilizing agent, SDS (38).

P. carinii PHR1 sequences were subcloned into the *S. cerevisiae* expression vector p425GAL (25). *S. cerevisiae gas1* mutants susceptible to growth inhibition by SDS were transformed with *P. carinii PHR1* DNA in pGAL425 and selected by proliferation at 30°C on medium containing 0.005% SDS. A number of colonies were isolated in which the *P. carinii PHR1* DNA strongly restored proliferation of the *S. cerevisiae* mutant parent (Fig. 4). Complemented colonies were propagated at 30°C under the selective conditions, and plasmid DNA from *P. carinii PHR1*-complemented colonies was isolated to verify the presence of the *P. carinii PHR1* gene in the selected transformants. *S. cerevisiae gas1* mutant strains transformed with the pGAL425 vector alone failed to grow on selective medium containing 0.005% SDS. In contrast, both the *S. cerevisiae* strains transformed with either the *GAS1* gene (37) or the *P. carinii PHR1* gene displayed normal growth in the presence of SDS. Thus, *P. carinii PHR1* is fully capable of functioning in fungal cell wall integrity when expressed heterologously in this tractable fungal species.

Biochemical analyses in *C. albicans* reveal that Phr1p strongly influences the stability and solubility of glucans under alkaline conditions. Specifically, Phr1$^+$ strains exhibit 50% reduction in alkaline-insoluble glucan at pH 8.0 compared to null mutant strains, likely related to inefficient β-1,3–β-1,6 cross-linking (29). This defective cell wall structure may play a role in the decreased virulence of the *PHR*-deleted mutants. Furthermore, mutations of the homologous *GAS1* gene in *S. cerevisiae* have been demonstrated to cause rounded, abnormal budding, resulting in decreased fungal proliferation and decreased cell wall integrity, with loss of β-1,3-glucans from the cell wall into the surrounding environment (28, 30, 38).

Additional biochemical activities of this family of proteins have recently been defined by Mouyna and colleagues, who characterized a novel 1,3-beta-glucanosyltransferase from *Aspergillus fumigatus* and cloned its corresponding gene, *GEL1* (23). This enzyme mediates internal cleavage of the β-1,3-glucan chain and transfers the resulting reducing end to the nonreducing end of another β-1,3-glucan molecule, thereby elongating the polysaccharide. The predicted amino acid sequence of Gel1p was homologous to Gas1p from *S. cerevisiae*, Phr1p of *C. albicans*, and Epd from *C. maltosa* (23). Recombinant Gas1p, Phr1p, and Phr2p were also shown to have similar 1,3-beta-glucanosyltransferase activity in vitro. Additionally, Gel1p appears to be similarly attached to the membrane via a GPI anchor.

It is an attractive hypothesis that *P. carinii PHR1* may have similar activities in *P. carinii*, maintaining proper cell wall glucan structure in the host's lung where a pH of 7 to 7.5 predominates. It is again notable that *P. carinii PHR1* is located immediately downstream of the *GSC1,* gene which directs synthesis of the β-1,3-glucan cyst wall (16). Substantial investigation indicates that generation and maintenance of the β-glucan cell wall of *P. carinii* are essential for establishment of infection and represent a major target of host recognition and inflammatory response to the organism (14, 34, 39). Further studies aimed at determining the mechanisms by which β-glucan is generated and remodeled under various environmental conditions should further illuminate the life cycle of this intriguing fungus, which afflicts immunocompromised individuals.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology, vol. 2, p. 13.12.1–13.12.5. John Wiley & Sons, Inc., New York, N.Y.
2. **Berger, J., A. D. Howard, L. Brink, L. Gerber, J. Hauber, B. R. Cullen, and**

S. Udenfriend. 1988. COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan anchored membrane protein. J. Biol. Chem. **263:**10016–10021.
3. **Chin, K., T. D. Luttrell, J. D. Roe, S. Shadzi, M. A. Wyder, and E. S. Kaneshiro.** 1999. Putative *Pneumocystis* dormant forms outside the mammalian host, and long-term culture derived from them: initial characterizations. J. Eukaryot. Microbiol. **46:**95S–99S.
4. **Cushion, M. T., A. Harmsen, Y. Matsumoto, J. R. Stringer, A. E. Wakefield, and M. Yamada.** 1994. Recent advances in the biology of *Pneumocystis carinii*. J. Med. Vet. Mycol. **32:**217–228.
5. **Cushion, M. T., M. Kaselis, S. L. Stringer, and J. R. Stringer.** 1993. Genetic stability and diversity of *Pneumocystis carinii* infecting rat colonies. Infect. Immun. **61:**4801–4813.
6. **De Bernardis, F., F. A. Muhlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. **66:**3317–3325.
7. **Feng, D. F., and R. F. Doolittle.** 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. **25:**351–360.
8. **Fonzi, W. A.** 1999. *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans. J. Bacteriol. **181:**7070–7079.
9. **Forster, H. V., J. A. Dempsey, and L. W. Chosy.** Incomplete compensation of CSF (H^+) in humans during acclimitization to high altitude (4300 m). J. Appl. Physiol. **38:**1067–1072.
10. **Fox, D., and A. G. Smulian.** 1999. Mitogen-activated protein kinase Mkp1 of *Pneumocystis carinii* complements the *slt2*Δ defect in the cell integrity pathway of *Saccharomyces cerevisiae*. Mol. Microbiol. **34:**451–462.
11. **Ghannoum, M. A., B. Spellberg, S. M. Saporito-Irwin, and W. A. Fonzi.** 1995. Reduced virulence of *Candida albicans PHR1* mutants. Infect. Immun. **63:** 4528–4530.
12. **Guadiz, G., C. G. Haidaris, G. N. Maine, and P. J. Simpson-Haidaris.** 1998. The carboxyl terminus *of Pneumocystis carinii* glycoprotein A encodes a functional glycosylphosphatidylinositol signal sequence. J. Biol. Chem. **273:** 26202–26209.
13. **Hemperly, J. J., G. M. Edelman, and B. A. Cunningham.** 1986. cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate. Proc. Natl. Acad. Sci. USA **83:**9822–9826.
14. **Hoffman, O. A., J. E. Standing, and A. H. Limper.** 1993. *Pneumocystis carinii* stimulates tumor necrosis factor-alpha release from alveolar macrophages through a beta-glucan-mediated mechanism. J. Immunol. **150:**3932–3940.
15. **Hong, S. T., P. E. Steele, M. T. Cushion, P. D. Walzer, S. L. Stringer, and J. R. Stringer.** 1990. *Pneumocystis carinii* karyotypes. J. Clin. Microbiol. **28:**1785–1795.
16. **Kottom, T. J., and A. H. Limper.** 2000. Cell wall assembly by *Pneumocystis carinii*: evidence for a unique GSC-1 subunit mediating beta 1,3-glucan deposition. J. Biol. Chem. **275:**40628–40634.
17. **Kottom, T. J., C. F. J. Thomas, K. K. Mubarak, E. B. Leof, and A. H. Limper.** 2000. *Pneumocystis carinii* utilizes a functional cdc13 B-type cyclin complex during its life cycle. Am. J. Respir. Cell. Mol. Biol. **22:**722–731.
18. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
19. **Limper, A. H., J. S. Hoyte, and J. E. Standing.** 1997. The role of alveolar macrophages in *Pneumocystis carinii* degradation and clearance from the lung. J. Clin. Investig. **99:**2110–2117.
20. **Limper, A. H., C. F. Thomas, Jr., R. A. Anders, and E. B. Leof.** 1997. Interactions of parasite and host epithelial cell cycle regulation during *Pneumocystis carinii* pneumonia. J. Lab. Clin. Med. **130:**132–138.
21. **Maccheroni, W., Jr., G. S. May, N. M. Martinez-Rossi, and A. Rossi.** 1997. The sequence of *palF*, an environmental pH response gene in *Aspergillus nidulans*. Gene **194:**163–167.
22. **Micanovic, R., L. D. Gerber, J. Berger, K. Kodukula, and S. Udenfriend.** 1990. Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase. Proc. Natl. Acad. Sci. USA **87:**157–161.
23. **Mouyna. I., T. Pontaine, M. Vai, M. Monod, W. A. Fonzi, M. Diaquin, L. Popolo, R. P. Hartland, and J. P. Latge.** 2000. Glycophosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. J. Biol. Chem. **275:**14882–14889.
24. **Muhlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. Mol. Cell. Biol. **17:**5960–5967.
25. **Mumberg, D., R. Muller, and M. Funk.** 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. **22:**5767–5768.
26. **Nakazawa, T., H. Horiuchi, A. Ohta, and M. Takagi.** 1998. Isolation and characterization of *EPD1*, an essential gene for pseudohyphal growth of a dimorphic yeast, *Candida maltosa*. J. Bacteriol. **180:**2079–2086.
27. **Nuoffer, C., P. Jeno, A. Conzelmann, and H. Riezman.** 1991. Determinants for glycophospholipid anchoring of the *Saccharomyces cerevisiae* Gas1 protein to the plasma membrane. Mol. Cell. Biol. **11:**27–37.
28. **Popolo, L., D. Gilardelli, P. Bonfante, and M. Vai.** 1997. Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *ggp1*Δ mutant of *Saccharomyces cerevisiae*. J. Bacteriol. **179:**463–469.
29. **Popolo, L., and M. Vai.** 1998. Defects in assembly of the extracellular matrix are responsible for altered morphogenesis of a *Candida albicans phr1* mutant. J. Bacteriol. **180:**163–166.
30. **Ram, A. F., J. C. Kapteyn, R. C. Montijn, L. H. Caro, J. E. Douwes, W. Baginsky, P. Mazur, H. van den Ende, and F. M. Klis.** 1998. Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta-1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. J. Bacteriol. **180:**1418–1424.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. **15:**601–613.
33. **Sarkar, S., M. X. Caddick, E. Bignell, J. Tilburn, and H. N. Arst, Jr.** 1996. Regulation of gene expression by ambient pH in *Aspergillus*: genes expressed at acid pH. Biochem. Soc. Trans. **24:**360–363.
34. **Schmatz, D. M., M. A. Romancheck, L. A. Pittarelli, R. E. Schwartz, R. A. Fromtling, K. H. Nollstadt, F. L. Vanmiddlesworth, K. E. Wilson, and M. J. Turner.** 1990. Treatment of *Pneumocystis carinii* pneumonia with 1,3-beta-glucan synthesis inhibitors. Proc. Natl. Acad. Sci. USA **87:**5950–5954.
35. **Sentandreu, M., M. V. Elorza, R. Sentandreu, and W. A. Fonzi.** 1998. Cloning and characterization of *PRA1*, a gene encoding a novel pH-regulated antigen of *Candida albicans*. J. Bacteriol. **180:**282–289.
36. **Thomas, C. F., R. A. Anders, M. P. Gustafson, E. B. Leof, and A. H. Limper.** 1998. *Pneumocystis carinii* contains a functional cell-division-cycle Cdc2 homologue. Am. J. Respir. Cell Mol. Biol. **18:**297–306.
37. **Vai, M., E. Gatti, E. Lacana, L. Popolo, and L. Alberghina.** 1991. Isolation and deduced amino acid sequence of the gene encoding gp115, a yeast glycophospholipid-anchored protein containing a serine-rich region. J. Biol. Chem. **266:**12242–12248.
38. **Vai, M., I. Orlandi, P. Cavadini, L. Alberghina, and L. Popolo.** 1996. *Candida albicans* homologue of *GGP1*/*GAS1* gene is functional in *Saccharomyces cerevisiae* and contains the determinants for glycosylphosphatidylinositol attachment. Yeast **12:**361–368.
39. **Vassallo, R., J. E. Standing, and A. H. Limper.** 2000. Isolated *Pneumocystis carinii* cell wall glucan provokes lower respiratory tract inflammatory responses. J. Immunol. **164:**3755–3763.