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Complementation and characterization of the *Pneumocystis carinii* MAPK, PCM

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Abstract **Mitogen-activated protein kinase (MAPK) pathways transfer environmental signals into intracellular events such as proliferation and differentiation. Fungi utilize a specific pheromone-induced MAPK pathway to regulate conjugation, formation of an ascus, and entry into meiosis. We have previously identified a MAPK, PCM, from the fungal opportunist *Pneumocystis*, responsible for causing severe pneumonia in patients with AIDS. In order to gain insight into the function of PCM, we expressed it in *Saccharomyces cerevisiae* deficient in pheromone signaling and tested activation and inhibition of this MAPK pathway. PCM restored pheromone signaling in *S. cerevisiae fus3*Δ *kss1*Δ mutants with α-factor pheromone (six-fold increase) and was not activated by osmotic stress. Signaling through this pathway decreased 2.5-fold with 10 μM U0126, and was unaffected with SB203580. We evaluated the conditions for native PCM kinase activity isolated from *Pneumocystis carinii* organisms and found that 0.1 mM $MgCl_2$, pH 6.5, temperature 30–35°C, and 10 μM ATP were optimal. The activity of PCM is significantly elevated in *P. carinii* trophic forms compared to cysts, implicating a role for PCM in the life cycle transition of *P. carinii* from trophic forms to cysts.**

Key words: Pheromone; U0126; SB203580; Differentiation

1. Introduction

Mitogen-activated protein kinase (MAPK) signal transduction cascades are used by fungi to regulate cellular responses for mating, environmental stress, and cell wall integrity [1,2]. The ascomycetous yeast *Saccharomyces cerevisiae* activates a MAPK pathway for mating upon exposure to pheromones, which results in the conjugation of yeast cells. Subsequent events include the formation of an ascus and entry into meiosis [3–7]. *Pneumocystis*, a fungal opportunist grouped phylogenetically within the ascomycetous yeasts, is responsible for causing severe pneumonia in patients infected with the human immunodeficiency virus. Patients receiving chronic immunosuppression or those with other immune system impairments are also at high risk for infection [8–11]. *Pneumocystis carinii* has two life cycle stages which are identifiable by light microscopy, the trophic form and the cyst [12–18]. Unfortunately, it is not possible to culture *P. carinii* outside the infected host [19]. Further, methods for gene transfer or disruption are not available for *P. carinii*. Therefore the mechanisms regulating life cycle events, such as the transition from trophic forms to cysts, are unknown.

The pheromone-induced MAPK pathway has been extensively studied in *S. cerevisiae*, where haploid cells conjugate after the secretion of peptide mating pheromones. A heterotrimeric G protein coupled to the pheromone receptor propagates the mating signal to a module of serine-threonine protein kinases, MEKK, MEK, and MAPK, each of which becomes activated by sequential phosphorylation. Activated MAPK induces a controlled exit from the mitotic cell cycle to allow cellular synchronization prior to meiosis, and the expression of a number of molecules required for meiosis and formation of the ascus [3–5,20]. In some pathogenic fungi, this same MAPK pathway regulates mating but also has additional roles in regulating fungal virulence [21–23]. MAPK molecules from *Giardia* have recently been shown to be important regulators of life cycle transition within these parasites. The *Giardia* MAPKs control the encystation of trophozoites under poor nutrient conditions, which is similar to the signals which activate this pathway in *S. cerevisiae* to prompt pheromone release [24].

We have previously cloned a MAPK from *P. carinii*, PCM, which has significant sequence homology to *S. cerevisiae* FUS3 [25]. Here we report that expression of PCM in *S. cerevisiae* complements pheromone signaling in these yeast, and that this response can be inhibited by U0126, which is known to block MEK activation of MAPK. PCM isolated from *P. carinii* directly from infected lungs reveals unique requirements of pH, temperature, and divalent cation concentration for optimal kinase activity. We investigated PCM mRNA expression and kinase activity in *P. carinii* trophic forms and cysts, and the elevated kinase levels present in trophic forms suggest that PCM may have a role in regulating the transition of trophic forms into cysts.

2. Materials and methods

2.1. Materials

S. cerevisiae α-factor pheromone and all chemical reagents were purchased from Sigma Chemical (St. Louis, MO, USA). *S. cerevisiae α-factor pheromone was dissolved in sterile water to produce a stock solution of 1 mg/ml. Restriction endonucleases, Taq* polymerase, Superscript II RT, and RNase-free DNase I were from Invitrogen. PHAS-I (phosphorylated heat- and acid-stable protein) was from Stratagene. [γ-^{32}P]ATP was obtained from ICN Pharmaceuticals. U0126 and the p38 inhibitor SB203580 were obtained from Promega. U0126 and SB20358 were dissolved in dimethyl sulfoxide (DMSO) to produce stock solutions of 10 mM. Fluorescein di-β-D-galactopyranoside

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(FDG) was obtained from Molecular Probes and made as a 10 mM stock solution in DMSO. The pT-E-pY antibody which detects dually phosphorylated MAPK was obtained from Sigma.

2.2. Yeast strains, plasmids, and transformation

S. cerevisiae EY2516 (*MAT***a** *sst1Δ ade2-1 hisΔ200 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ Gal+*) used in this study was a generous gift from Dr. Elaine Elion, Harvard Medical School [26]. The *S. cerevisiae* expression plasmid p426GPD (ATCC) containing the URA3 auxotrophic marker was used to express PCM (GenBank accession AF043941) under the constitutive GPD promoter [27]. The plasmid p426GPD-PCM was constructed as follows: PCM cDNA previously cloned into the plasmid pBluescript SK$^-$ was amplified by polymerase chain reaction (PCR) using the 5′ *Eco*RI primer: GAATTCATGACAGCTTCAAGTAGA and the 3′ *Xho*I primer: CTCGAGTCAATTGTTCATACTCATAAT with *Pfx* polymerase [25]. The PCR product was digested with *Eco*RI and *Xho*I, directionally cloned into p426GPD, and was sequenced completely to confirm that no PCR errors were introduced. *S. cerevisiae* FUS3 was expressed from the plasmid pYES2.1-GS (Invitrogen). EY2516 yeast were grown overnight in YEPD medium at 30°C to an OD_{600} of 1.0 and were electroporated with the plasmids p426GPD-PCM or pYES2.1-SCFUS3 as previously described [28]. Following electroporation, transformants were grown on glucose minimal medium lacking uracil at 30°C.

2.3. Fus1-lacZ *assays*

PCM complementation of pheromone-induced signaling was tested by measurement of the β-galactosidase reporter gene *Fus1-lacZ* in EY2516 using FDG as a substrate [29]. EY2516 yeast transformed with p426GPD-PCM or pYES2.1-SCFUS3 were grown in glucose liquid minimal medium lacking uracil at 30°C for 24 h. After an overnight growth, cultures were diluted to an OD_{600} of 0.1 in glucose (p426GPD-PCM) or galactose (pYES2.1-SCFUS3) minimal medium lacking uracil and grown to an OD_{600} of 0.3. Triplicate cultures were aliquoted into 96 well 'U' bottom microfluor plates (ThermoLabsystems). Cultures received U0126 (0–100 μM) or SB203580 (0–100 μM) for 30 min (final DMSO concentration for all dilutions was 1%), then α-factor pheromone (final concentration 10 μM) for 30 min. Additional control cultures received vehicle alone (1% DMSO), 500 mM NaCl, or 10 μM α-factor pheromone for 30 min. Total volume of all wells was 100 μl. FDG substrate solution was prepared by diluting the 10 mM FDG stock solution to 1 mM in 25 mM PIPES, pH 7.2, and mixing equal parts of this with 5% Triton X-100 in 250 mM PIPES (pH 7.2). 20 μl of the FDG substrate solution was aliquoted into each well and the plate was incubated in the dark for 60 min at 37°C. The fluorescent signal was read in a Fusion 3.5 multiplate fluormeter with an excitation filter of 485 and an emission filter of 530. In order to determine if the observed reduction in *Fus1-lacZ* activity with U0126 was due to yeast death, *S. cerevisiae* expressing PCM received 0–100 μM of U0126 for 1, 3, 7 or 24 h. Dilutions of control yeast and test yeast at each time point were plated in triplicate and colonies were counted.

2.4. Preparation of P. carinii *organisms*

All studies described in this report were approved by the institutional animal care and use committee (IACUC). *P. carinii* pneumonia was induced in Harlan Sprague–Dawley rats by immunosuppression with dexamethasone as previously reported [25,28,30,31]. Lungs from moribund rats were minced and homogenized in Hanks' balanced salt solution and *P. carinii* were purified from host lung cells by filtration through a 10 μm filter (Millipore). *P. carinii* organisms were confirmed by Wright-Giemsa staining and samples containing contaminating bacterial or fungal organisms were discarded. Separated life forms of *P. carinii* cysts and trophic forms were purified by differential filtration using 3 μm Nuclepore filters (which retain the cysts). The trophic forms are collected by centrifugation. The cysts are collected by washing and vortexing the 3 μm filter vigorously followed by centrifugation. To verify the purity of the separated trophic and cyst forms, we counted duplicate microscopy slides of each life form-enriched fraction which were stained with GMS or Diff-Quik.

2.5. Northern analysis

Total RNA was isolated from separated *P. carinii* trophic forms and cysts with Trizol reagent (Invitrogen), and 10 μg were separated on 1.0% formaldehyde-agarose gels [31–33]. RNA was transferred to Nytran Plus membranes (Schleicher and Schuell), and hybridization to the radiolabeled PCM probe was performed at 68°C using Express hybridization solution (Clontech). Following exposure to autoradiography film, the membranes were re-probed using a radiolabeled *P. carinii* actin probe.

2.6. Quantitative reverse transcription (RT)-PCR

Total RNA was isolated from separated *P. carinii* trophic forms and cysts with Trizol reagent (Invitrogen) as described above. 2 μg of total RNA from trophic forms and cysts was treated with RNase-free DNase I and cDNA was synthesized from these samples by priming with oligo$(dT)_{18}$ using the Superscript II RT. The PCR primers 5PCM (5′-CTTGGCACGATCTGCTGTTA-3′) and 3PCM (5′-GCTCTGCGAGACTTGATTCC-3′) amplify a 273 bp product of the PCM cDNA. A competitor mimic cDNA was prepared by PCR by amplifying a truncated portion of the target gene using the hybrid primer 5PCM1 (5′-CTTGGCACGATCTGCTGTTACTTCAAAGAATACACC-3′, which contains the 5PCM primer sequence and a downstream PCM sequence), and 3PCM. The resulting 197 bp PCR amplicon was subcloned into the pGEM-T Easy plasmid (Promega), propagated in *Escherichia coli* DH5α, and the plasmids purified with the QIAprep spin miniprep kit (Qiagen). The mimic cDNA was released from the plasmid by restriction endonuclease digestion with *Eco*RI, gel-purified using Geneclean spin (Bio 101), and quantified using a spectrophotometer. The mimic cDNA was initially diluted 10-fold ranging from 1 pmol/μl to 1 amol/μl and co-amplified with a constant amount of the trophic form or cyst cDNA sample in a 50 μl reaction that included 1×PCR buffer, 50 μM dNTPs, 0.4 μM each of the primers 5PCM and 3PCM, and 1.25 units of *Taq* polymerase. Samples were amplified for 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and the PCR products were subjected to electrophoresis on 2% agarose gels. After the initial screening using 10-fold serially diluted competitor mimic cDNA, the optimal range of competitor mimic cDNA used to quantify PCM was two-fold dilutions starting from 1×10^{-17}/μl to 6.25×10^{-19}/μl. The images were digitized and the intensities of bands in ethidium bromide-stained gels quantified using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and the ratio of PCM to mimic cDNA was plotted against the competitor numbers. Using linear regression, the equivalence point (log ratio = 0) was determined for the samples of trophic forms and cysts and reported as the number of copies of PCM/μg total RNA.

2.7. Immunoprecipitation kinase assays and Western blotting

A PCM antiserum was prepared by Bethyl Laboratories (Montgomery, TX, USA) by immunizing two rabbits with a peptide derived from the N-terminal PCM sequence (MTASSRNVRFNVSDD, residues 1–15) coupled to keyhole limpet hemocyanin [25]. The IgG fraction was purified from the antiserum using a protein A-Sepharose column, and tested by enzyme-linked immunosorbent assay (ELISA) against the cognate antigen to determine the optimal antibody titer compared to non-immune rabbit IgG. PCM peptide was coated on microtiter wells, reacted with dilutions of antibody, then with goat anti-rabbit IgG(h&l)/horseradish peroxidase, followed by TMB/H_2O_2 substrate. A PCM antibody dilution of 1:20 000 produced an OD of 1.0 in the ELISA with no reactivity with the non-immune antibody. A PCM antibody dilution of 1:10 000 was used for immunoprecipitation and Western blotting. EY2516 yeast transformed with p426GPD-PCM were grown in glucose minimal medium to an OD_{600} of 0.3. One half of the culture received α-factor pheromone (10 μM final concentration). Cultures were incubated for 30 min at 30°C, then cells were pelleted, washed in sterile water, and lysed in YPER reagent (Pierce) containing a protease/phosphatase inhibitor cocktail (1 μg/ml each of leupeptin, aprotinin, and pepstatin; 1 mM each of phenylmethylsulfonyl fluoride and sodium orthovanadate; and 50 mM sodium fluoride) for 20 min at room temperature. The protein concentration of the lysates was determined spectrophotometrically using the BCA method (Pierce) and equal protein (25 μg) was boiled in Laemmli buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membranes. The membranes were incubated with the pT-E-pY antibodies (1:1000), or PCM antibodies (1:10 000) for 2 h. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) following the procedures recommended by

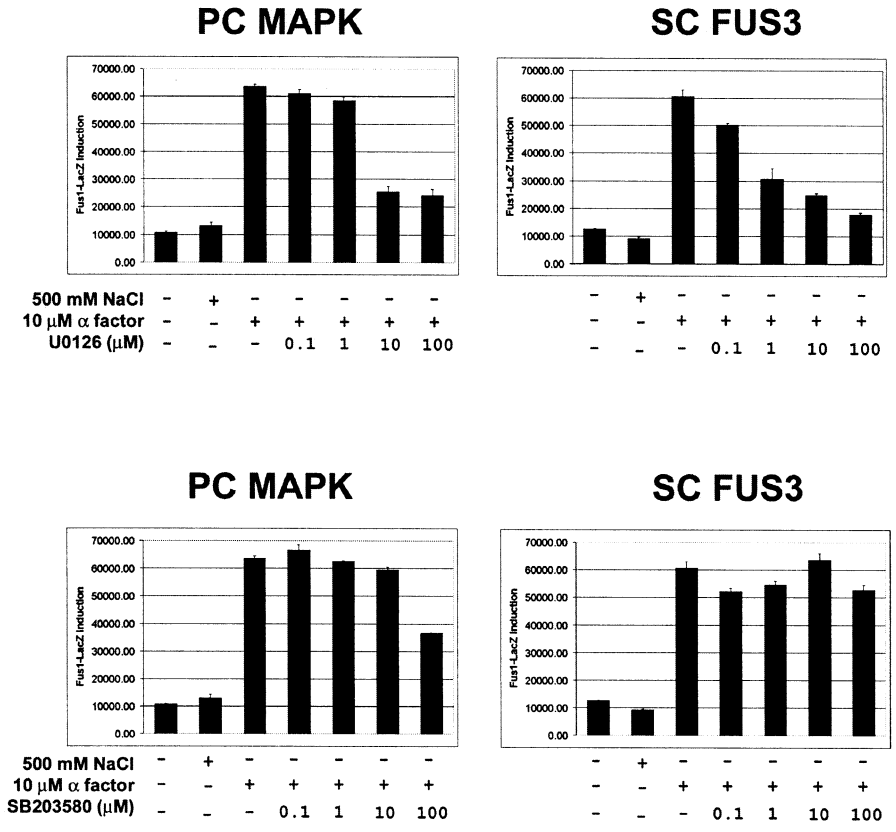

Fig. 1. *P. carinii* PCM complements pheromone signaling in yeast. *S. cerevisiae* EY2516 (*fus3*Δ *kss1*Δ) yeast transformed with either PCM or SCFUS3 were assayed for *Fus1-lacZ* activity in the presence of 10 μM α-factor, 500 mM salt, or the inhibitors U0126 and SB203580 (0–100 μM). Untreated yeast (−) received 1% DMSO as a vehicle control. Triplicate cultures were tested for each condition. *LacZ* units were calculated from the fluorescent intensity normalized for cell density and the mean of three individual experiments ± S.D. is shown.

the manufacturer. Purified *P. carinii* organisms were lysed in YPER reagent (Pierce) as above. Protein lysate (500 μg) was precleared with a 50% slurry of protein A-Sepharose at 4°C for 30 min, and PCM was immunoprecipitated at 4°C for 2 h using 1:10 000 dilution of PCM antibody. The immunocomplexes were captured with protein A-Sepharose and were washed twice in wash buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin, aprotinin, and pepstatin, and 25 mM β-glycerolphosphate) and twice in kinase buffer (50 mM HEPES pH 7.5, 1 mM dithiothreitol). The immunocomplexes were added to a 40 μl reaction containing 50 mM HEPES. We tested pH 6–9, $MgCl_2$ concentrations of 0–30 mM, PHAS-I substrate from 0 to 14 μg, and cold ATP from 5 to 200 μM. Each reaction was started by the addition of 10 μCi [γ-^{32}P]ATP and incubation in a shaking water bath for 30 min at temperatures ranging from 5 to 50°C. Kinase reactions were stopped with the addition of Laemmli buffer, boiled for 5 min, resolved by electrophoresis on 12% SDS–polyacrylamide gels, and subjected to autoradiography [25]. All kinase reactions were performed in triplicate from separate isolates of *P. carinii* organisms.

3. Results

3.1. P. carinii *PCM complements pheromone signaling in yeast*

We have previously shown that *P. carinii* PCM has 64% identity to the pheromone-induced MAPK *FUS3* from *S. cerevisiae* [25]. In order to determine if PCM is a functional kinase in *S. cerevisiae*, we used PCM to complement *S. cerevisiae fus3*Δ *kss1*Δ yeast treated with α-factor pheromone or salt. These yeast have a *Fus1-lacZ* reporter gene incorporated into their genome which is specifically activated upon exposure to α-factor pheromone if the pheromone-induced MAPK cascade is functional [26]. As shown in Fig. 1, PCM expressed in these yeast have a six-fold induction of *Fus1-lacZ* activity with α-factor pheromone, similar to the control yeast expressing wild-type FUS3. *Fus1-lacZ* induction is minimal in yeast given the vehicle control with 1% DMSO (designated −) or 500 mM NaCl. Specificity of the pheromone induction in

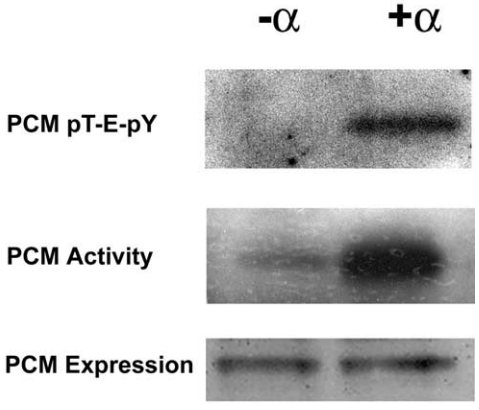

Fig. 2. *P. carinii* PCM kinase activity in yeast. PCM was immunoprecipitated from *S. cerevisiae* EY2516 (*fus3*Δ *kss1*Δ) yeast with and without exposure to α-factor and PCM activity was investigated by immunoblotting with a phosphoantibody to detect the dually phosphorylated motif pT-E-pY and by performing in vitro kinase assays.

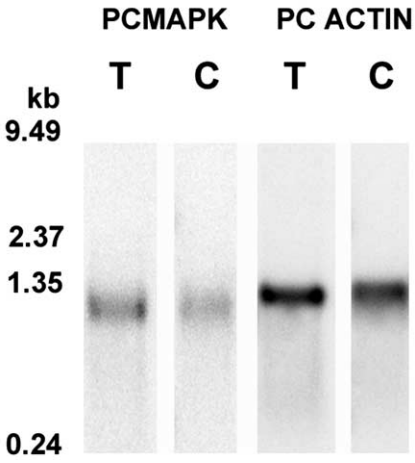

Fig. 3. PCM mRNA expression in trophic forms and cysts. *P. carinii* PCM mRNA expression was determined by Northern blotting using 10 μg of total RNA from separated trophic forms (T) and cysts (C). Hybridization was with PCM and *P. carinii* actin probes.

yeast expressing PCM with and without exposure to α-factor pheromone was verified by performing immunoprecipitation kinase reactions and immunoblotting (Fig. 2) using the phosphospecific antibody which detects the dually phosphorylated MAPK motif pT-E-pY, which is present in PCM and has been used previously to detect activated *S. cerevisiae* FUS3 [25,34].

3.2. U0126 inhibits pheromone-induced signaling in yeast

We attempted to decrease the observed α-factor pheromone induction in these yeast expressing PCM or wild-type FUS3 using inhibitors of MAPK signaling, U0126 and SB203580. U0126 inhibits the upstream kinases MEK1 and MEK2, thereby preventing activation of MAPK [35,36]. U0126 inhibits the mammalian extracellular signal-regulated kinase (ERK) pathway, which has closest homology to the pheromone-induced MAPK pathway in fungi. SB203580 is a specific inhibitor of the p38 HOG pathway and interacts directly with the active site in p38 to prevent its kinase activity. U0126 inhibits MEK1 and MEK2 with an IC_{50} of 0.5 μM in vitro, but effective in vivo doses range from 10 to 100 μM [35,36]. SB203580 is a specific inhibitor of the p38 HOG MAP kinase with an IC_{50} of 1.0 μM in vitro, and effective in vivo doses ranging from 1 to 10 μM [37–39]. As shown in Fig. 1, U0126 reduces *Fus1-lacZ* activity of *S. cerevisiae fus3*Δ *kss1*Δ yeast expressing PCM or FUS3 when treated for 30 min prior to induction with α-factor pheromone in a dose-dependent manner. Yeast expressing PCM had a reduction of 1.09-fold with

Table 1
Purity of separated *P. carinii* life cycle forms

Trophic form-enriched fraction	
Trophic forms (stained by Diff-Quik)	$(876.0 \pm 42) \times 10^6$ trophic forms/ml
Residual cystic forms (stained by GMS)	$(0.033 \pm 0.010) \times 10^6$ cystic forms/ml
Cyst form-enriched fraction	
Cystic forms (stained by GMS)	$(5.597 \pm 0.165) \times 10^6$ cystic forms/ml
Residual trophic forms (stained by Diff-Quik)	$(0.495 \pm 0.118) \times 10^6$ trophic forms/ml

Whole *P. carinii* organisms are separated from host lung cells by filtration through a 10 μm filter. Trophic forms are separated from cysts by further filtration through a 3 μm Nuclepore filter (which retains the cysts). The trophic forms are collected by centrifugation. The cysts are collected by washing and vortexing the 3 μm filter vigorously followed by centrifugation. We counted duplicate microscopy slides of each enriched fraction which were stained with GMS and Diff-Quik. Differential filtration yielded a trophic form fraction remarkably free from cysts, and cystic fractions were significantly enriched in thick-walled cyst forms of the organism. The trophic form fraction was greater than 99.99% pure trophic forms. The cyst fraction contained 92.6% cysts on a per organism basis.

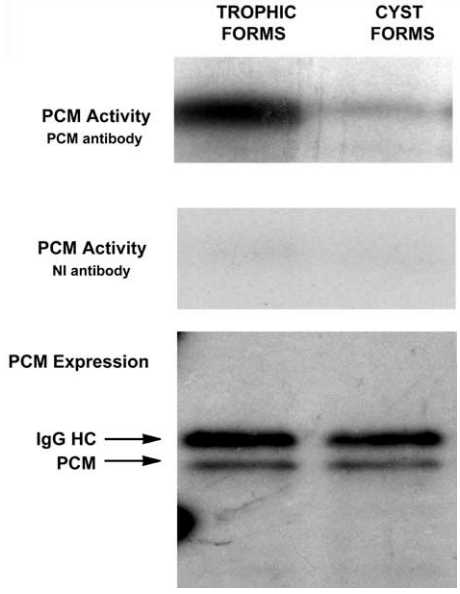

Fig. 4. PCM kinase activity in trophic forms and cysts. PCM was immunoprecipitated from *P. carinii* from infected rat lung using the specific PCM antibody (1:10 000 dilution) and tested in kinase assays. PCM activity is greatly augmented in trophic forms compared to cysts, with equal protein detection of PCM in both trophic forms and cysts. IgG heavy chains are indicated by the upper arrow. Control reactions using a non-immune antibody (1:10 000) does not demonstrate any significant kinase activity.

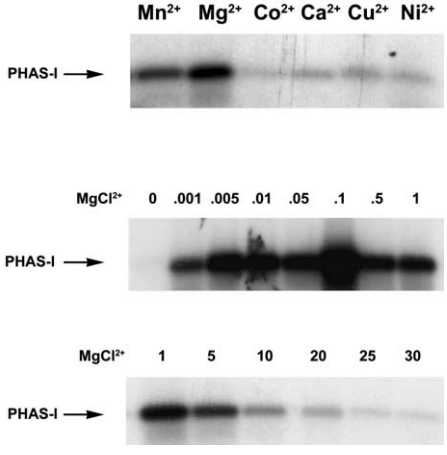

Fig. 5. Metal dependence of native PCM kinase. PCM was immunoprecipitated from *P. carinii* from infected rat lung and tested in kinase assays with various divalent cations (1 mM each). As shown in the upper panel, Mg^{2+} followed by Mn^{2+} are preferred cations for PCM. Next, the optimal concentration of Mg^{2+} was found to be 0.1 mM (middle panel). Concentrations above 10 mM inhibited kinase activity (lower panel).

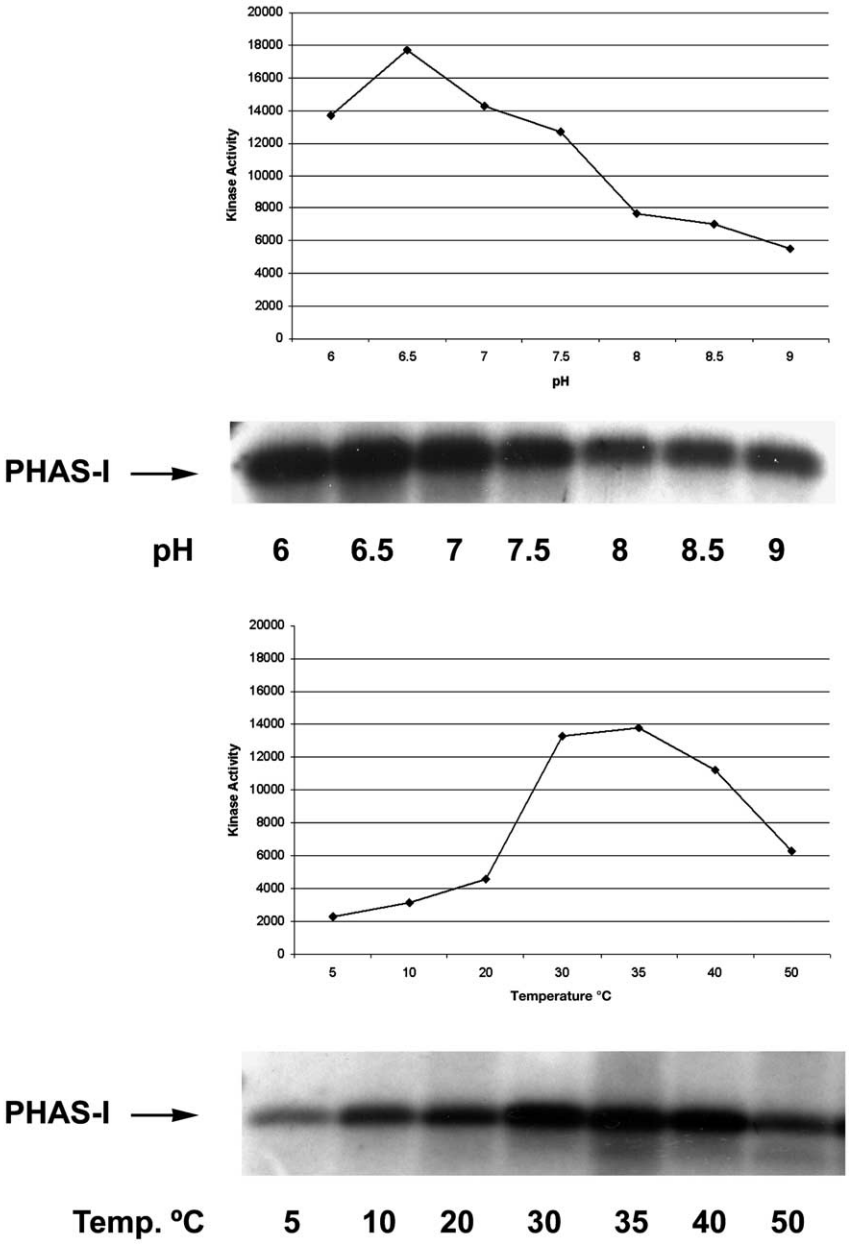

Fig. 6. Optimal pH and temperature for native PCM kinase activity. PCM was immunoprecipitated from *P. carinii* from infected rat lung and tested in kinase assays with $MgCl_2$ (0.1 mM) at pH 6–9 and at 5–50°C. As shown in the upper panel, pH 6.5 appears optimal for PCM kinase activity. In the lower panel, a temperature between 30 and 35°C is optimal for PCM kinase activity.

1 μM U0126, 2.5-fold with 10 μM U0126, and 2.6-fold with 100 μM U0126. Yeast expressing FUS3 had a reduction of 1.9-fold with 1 μM U0126, 2.5-fold with 10 μM, and 3.4-fold with 100 μM U0126. Yeast cultures expressing PCM or FUS3 had no significant reduction in *Fus1-lacZ* activity with the p38 inhibitor SB203580 in the range of 0.1–10 μM, however the yeast expressing PCM had a 1.7-fold reduction with 100 μM of SB203580 (Fig. 1). This dose of SB203580 is much higher than what is normally used for specific inhibition of p38 and the small observed reduction in *Fus1-lacZ* activity might represent non-specific inhibition of PCM. Since U0126 reduced *Fus1-lacZ* activity in these yeast, we grew these yeast expressing PCM for 24 h in 0–100 μM of U0126 and counted triplicate dilutions of treated and untreated colonies to determine if U0126 was causing cellular death of these yeast. *S. cerevisiae* expressing PCM and treated with U0126 had no reduction in colony-forming units; thus U0126 appears to specifically inhibit MAPK signaling without resulting in death of the yeast.

3.3. P. carinii *trophic forms exhibit increased PCM kinase activity*

Since MAPK molecules can be differentially regulated in certain life cycle stages of pathogenic fungi or protozoans, we sought to determine if the expression level and kinase activity of PCM was predominant in a single life cycle form of *P. carinii*. This may indicate that a single life cycle form uses this MAPK signaling pathway to regulate the transition from one life cycle form to another. First, we separated trophic forms and cysts and verified the purity of these popula-

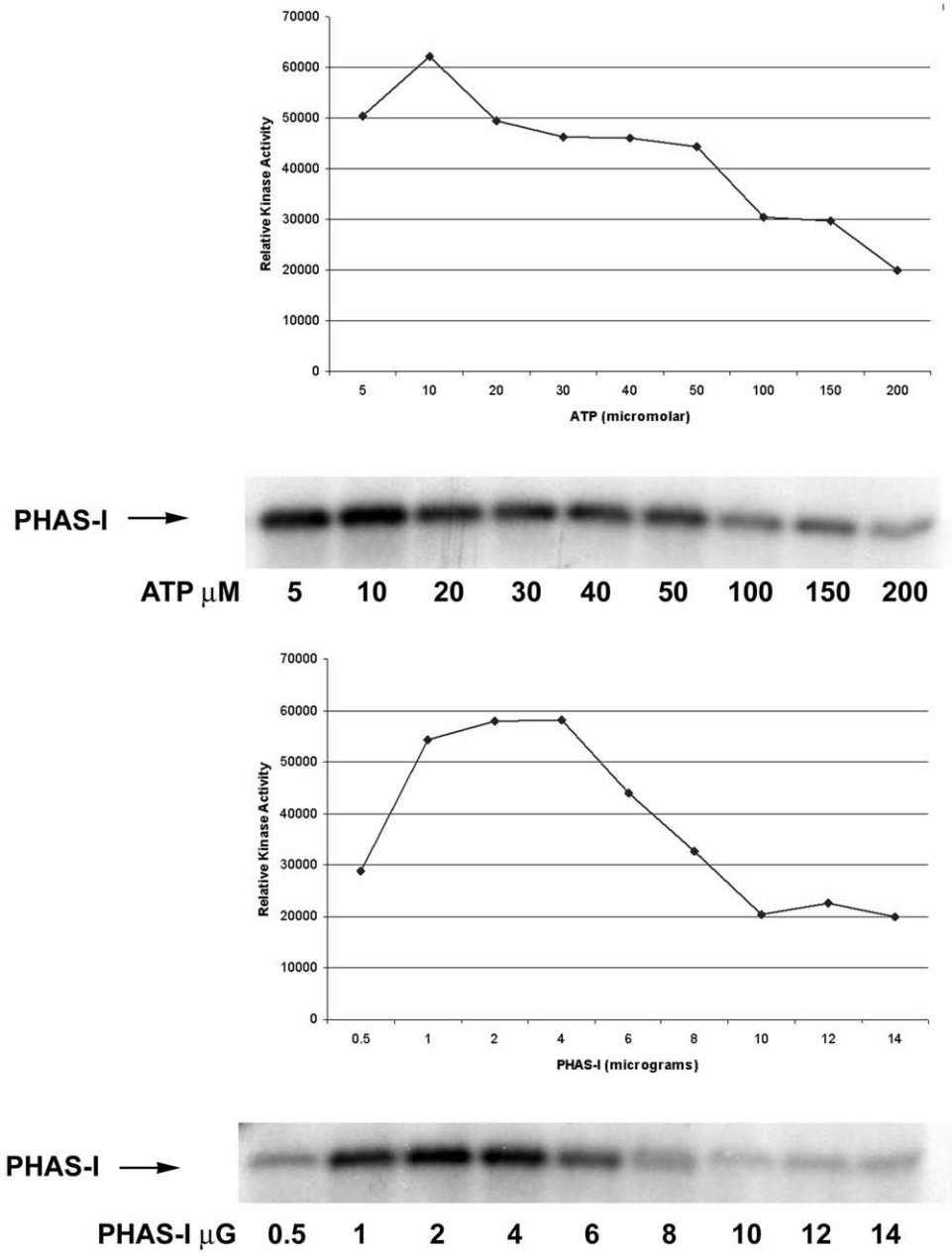

Fig. 7. Effect of ATP and substrate concentration on native PCM kinase activity. PCM in vitro kinase assays were performed as described with $MgCl_2$ (0.1 mM) at pH 6.5 and 35°C testing a range of ATP as a phosphate donor (upper panel) or PHAS-I as a substrate (lower panel). As shown, 10 μM ATP and 2–4 μg PHAS-I are optimal for PCM kinase activity.

tions. As shown in Table 1, trophic forms and cyst preparations were significantly enriched with little cross-contamination. These separated populations were used for PCM mRNA expression and kinase activity. Northern analysis using a radiolabeled PCM probe revealed that the level of expression of the PCM mRNA in both trophic forms and cysts was low compared to the actin gene (Fig. 3). We also evaluated PCM mRNA expression by competitive RT-PCR using a mimic system, where PCM expression by competitive RT-PCR was slightly increased in trophic forms (3.2×10^{-18} copies/μg RNA) compared to cysts (1×10^{-18} copies/μg RNA). PCM kinase activity from cellular lysates of purified trophic forms and cysts (500 μg each) was examined by immunoprecipitation kinase assay. PCM kinase activity from trophic forms is significantly increased compared to cysts (Fig. 4). Control immunoprecipitation experiments on these same samples of trophic forms and cysts using a non-immune antibody (1:10 000 dilution, same as that used for the PCM antibody) show minimal kinase activity, confirming that the observed kinase activity is derived from PCM. Western blotting with PCM in both samples reveals equal PCM protein was immunoprecipitated from trophic forms and cysts. These data indicate that PCM is activated in *P. carinii* trophic forms, and that the increased activity is not due to differences in the amount of PCM protein immunoprecipitated from trophic forms and cysts.

3.4. Characterization of PCM kinase activity

For partial biochemical characterization of PCM, we isolated native PCM from *P. carinii* by immunoprecipitation and performed in vitro kinase assays using PHAS-I as a phosphorylation substrate in the presence of [γ-^{32}P]ATP. Metal dependence with divalent cations for PCM activity was greatest with magnesium chloride followed by manganese chloride, while calcium chloride, copper chloride, and nickel chloride demonstrated minimal kinase activity (Fig. 5). Use of cobalt chloride in the reaction abolished kinase activity. Next, we tested a range of magnesium chloride concentrations for kinase activity and found 0.1 mM was optimal (Fig. 5). PCM kinase activity was significantly reduced with 10 mM or higher concentrations of magnesium chloride. We discovered that optimal PCM kinase activity occurred under acidic conditions at pH 6.5 (Fig. 6) and at elevated temperature (30–35°C). This is intriguing, since these conditions occur physiologically in the host during pneumonia. The optimal concentration of ATP as a phosphate donor is 10 μM, and the optimal concentration of PHAS-I substrate is between 2 and 4 μg (Fig. 7).

4. Discussion

Insight into the life cycle of *P. carinii* has come from microscopic analysis of organisms obtained from the lungs of infected animals or humans. The major limitation to a detailed analysis of the *P. carinii* life cycle has been the inability to culture and propagate *P. carinii* outside the lung. Because of this limitation, our accumulated knowledge of the *P. carinii* life cycle comes from observational studies. By light microscopy one can identify the small trophic forms (one μm) and the larger cysts (8 μm) [16–18]. We and others have demonstrated that the trophic forms are predominately haploid, but also exist in diploid forms, and that the cyst have complements of 2, 4 or 8 nuclei [40,41]. We hypothesize that the trophic forms use a MAPK signal transduction cascade to control the transition into cysts. In the present investigation we examine the function of *P. carinii* PCM in a heterologous fungal system and perform partial biochemical characterization of native PCM from *P. carinii*. We also investigate the expression and activity of PCM in separated populations of trophic forms and cysts in order to gain insight into the *P. carinii* life cycle.

Our experimental data show that PCM can functionally complement pheromone signaling in *S. cerevisiae*. PCM was able to restore pheromone signaling in these yeast to identical levels of the wild-type FUS3. Further, PCM is specific for the *S. cerevisiae* pheromone pathway, since induction occurred only with α-factor pheromone. There was no induction with 500 mM NaCl as an osmotic stress, nor any reduction using the p38 HOG inhibitor SB203580 in physiologically relevant doses. The upstream kinase MEK phosphorylates ERK and fungal pheromone-induced MAPKs on the threonine and tyrosine amino acids in the activation domain, and both phosphorylations are required for functional kinase activity [6]. ERK and fungal pheromone-induced MAPK molecules have a T-E-Y motif in the activation domain of the kinase, which is present in *S. cerevisiae* FUS3 and *P. carinii* PCM [25,34]. MAPKs which function in response to osmolar stress have T-G-Y in the activation domain, and cell wall integrity MAPKs have T-P-Y. Treatment of *S. cerevisiae* expressing PCM or FUS3 with U0126 prior to α-factor pheromone exposure significantly reduced *Fus1-lacZ* activity in a dose-dependent manner. Activated PCM was detected in yeast treated with α-factor pheromone both by immunoprecipitation kinase reactions and by using a MAPK phosphorylation-specific antibody which detects the dual phosphorylations on the motif pT-E-pY, confirming activation of PCM within the yeast pheromone-induced MAPK pathway.

MAPK molecules such as ERK and FUS3 have specific biochemical requirements for functional kinase activity. MAPKs utilize a divalent cation, usually Mg^{2+}, as a necessary co-factor for activity. The kinases also require ATP as a phosphate donor, and have an optimal range for temperature and pH to function properly. We investigated these properties of PCM isolated from *P. carinii* organisms during active infection. Similar to other MAPKs, PCM preferred Mg^{2+}, followed by Mn^{2+}, as the divalent cation. Interestingly, a lower than expected concentration of Mg^{2+} was optimal, 0.1 mM, which is much less than other MAPKs. Concentrations of Mg^{2+} above 10 mM inhibited kinase activity. PCM activity was maximal with 10 μM ATP as a phosphate donor and 2–4 μg of PHAS-I substrate. We also found PCM prefers pH 6.5 and elevated temperature, 30–35°C, for optimal kinase activity. These findings are in contrast to the optimal conditions for mammalian ERK (which uses 10 mM $MgCl_2$, pH 7.4 or 8, 50–100 μM ATP, and 30°C) [42,43], or *S. cerevisiae* FUS3 (which uses 20 mM $MgCl_2$, pH 7.4, 20 μM ATP, and 30°C) [5]. Elevated temperature and acidic pH are conditions which *P. carinii* encounters within the infected host during pneumonia, where the alveolar environment becomes more acidic in the setting of pneumonia, and fever is part of the host response to infection.

Differential expression and/or activity of MAPK molecules has been noted in pathogenic organisms [44–47]. We examined separated preparations of *P. carinii* trophic forms and cysts for PCM mRNA expression and kinase activity to determine if this were the case in *P. carinii*. What is the role of *P. carinii* PCM in regulating the life cycle of *P. carinii*? Although this will require the ability to isolate pure life cycle stages of the organism and maintain them in culture, which is currently not possible, our experimental data implicate a role for PCM in life cycle transition of *P. carinii*. Our observation of enhanced PCM functional kinase activity in the trophic forms compared to the cysts suggests that the trophic forms utilize PCM. We cannot conclusively exclude that the techniques for cell preparations of trophic forms and cysts affect the observed kinase activity, or that the lack of activity in the cyst may be due to a lack of a specific stimulus for this pathway within *P. carinii*; however, these observations of differential expression or activity of signaling molecules have also been recognized in the life cycle stages of other pathogens. For example, the growth-regulating MAPK Pf-Map2 from the protozoan parasite *Plasmodium falciparum* is expressed only in the gametocyte life cycle stage, which is formed by sexual differentiation of merozoites after invasion of the red blood cell. Further, the authors speculate that the kinase activity of the gametocyte MAPK keeps the gametocyte mitotic cell cycle arrested while in the blood of the human host, and that the cell cycle block is relieved when the parasites are transferred back to a mosquito [22]. Another example comes from the protozoan parasite *Leishmania mexicana*, which has two major life cycle forms, the promastigotes which live in insects and the amastigotes which replicate in mammalian macrophages. Although not

implicated in life cycle transition, the LMPK MAPK is essential for the amastigote survival in an infected host. LMPK mRNA is found in both promastigotes and amastigotes, but LMPK kinase activity is only found in amastigotes. Deletion of LMPK in these organisms results in the parasites' inability to grow as amastigotes and thus a loss of infectivity [23]. If PCM is used for life cycle transition, finding activated PCM in trophic forms makes sense, since following conjugation the stimulus and requirement for an activated PCM would not be needed in cysts. Within *Giardia lamblia*, a protozoan parasite which can be cultured and manipulated, MAPK molecules play a critical role in the trophozoite differentiation into cysts under conditions of nutrient deprivation [24]. Once the process of encystation occurs, MAPK activity becomes attenuated in these organisms and reaches its lowest catalytic activity 24 h after the trophozoites are stimulated to transform into cysts, which correlates with the maximum number of cysts identified in the culture [24]. Unfortunately, the ability to transform *P. carinii* with plasmid DNA or to disrupt its genes has not yet been perfected, and the inability to culture *P. carinii* makes analogous studies currently impossible. We believe, however, that our observation of increased PCM kinase activity in the *P. carinii* trophic forms, combined with PCM functional complementation of the pheromone signaling MAPK pathway in yeast, suggests that PCM has a role in regulating the life cycle transition in *P. carinii*.

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