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Expression analysis of PCSTE3, a putative pheromone receptor from the lung pathogenic fungus *Pneumocystis carinii*

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Abstract

The fungal pathogen *Pneumocystis carinii* remains the most prevalent opportunistic infection in patients infected with HIV. Fungal pheromone receptors are seven transmembrane domain G-protein-coupled receptors which are expressed on specific mating types, and have ligand-binding extracellular domains for specific pheromones from cells of the opposite mating type. We have cloned and characterized PCSTE3 from *P. carinii*, which encodes a seven transmembrane domain protein orthologous to the *Saccharomyces cerevisiae* pheromone receptor Ste3. We detect PCSTE3 by indirect immunofluorescence using antibodies designed to extracellular domains of the receptor in yeast expressing the protein. Using a downstream *Fus1-lacZ* reporter gene, we determined that PCSTE3 does not recognize a- or α-factor pheromones as ligands for the receptor. We isolated *P. carinii* life cycle stages and examined PCSTE3 expression by immunofluorescence microscopy and flow cytometry, and found PCSTE3 expression exclusively on a population of trophic forms. PCSTE3 receptor expression was not found on cysts.

Keywords: MAPK; AIDS; Opportunistic infections; Pheromone

People who are infected with HIV are at significant risk for developing opportunistic infections, especially pneumonia. The fungal pathogen *Pneumocystis carinii*, which has been recently renamed *Pneumocystis jiroveci* for the strain which infects humans [1,2], remains the most prevalent opportunistic infection resulting in severe pneumonia (PCP) in HIV-infected patients [3,4]. Additionally, patients with underlying inflammatory conditions requiring treatment with chronic immunosuppressive medications, patients with weakened immunity due to malignancy, and those with inherited or acquired immune system defects are at high risk for developing PCP [5,6]. The inability to achieve sustained propagation of *P. carinii* outside of the host lung remains the major obstacle to studying this pathogen's cell biology [7,8]. Indeed, without an effective method for cultivation, genetic studies such as gene deletions or insertions, and genetic crosses, are not feasible. Given these limitations, we and others have studied *P. carinii* proteins in the closely related yeasts *Saccharomyces cerevisiae and Schizosaccharomyces pombe*.

The pheromone-responsive mitogen-activated protein kinase (MAPK) cascade has been well studied in the non-pathogenic ascomycete *S. cerevisiae*, where pheromones bind to the extracellular domains of their respective pheromone receptors and trigger signaling events through a MAPK pathway [9–11]. The fungal pheromone receptors are structurally similar to the seven transmembrane domain GTP-coupled receptor family, which includes rhodopsin and the β2 adrenergic receptor [12,13]. In *S. cerevisiae*, conjugation of haploid cells to form zygotes is initiated by the mutual secretion of peptide mating pheromones by each of the two haplotypes [14]. Nutrient deprivation is the major stimulus for pheromone secretion, where *Mat* α cells secrete α-factor pheromone and express the Ste3 pheromone receptor (which recognizes a-factor pheromone), and *Mat* a cells secrete a-factor pheromone and express the Ste2 pheromone receptor (which recognizes α-factor

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pheromone). The extracellular domains of each receptor carry determinants for pheromone binding which are specific for that particular ligand [15], while the 3rd cytoplasmic loop of the pheromone receptors is implicated in signal transduction by activation of a heterotrimeric G-protein complex [16].

Following pheromone binding to its respective receptor, dissociation of the Gα and Gβγ subunits occurs through exchange of Gα bound GDP with GTP [17]. This triggers activation of the MAPK cascade which culminates in a number of cellular events, including mitotic cell cycle arrest, the expression of differentiation genes to allow formation of an ascus, and meiosis [18–20]. In the pathogenic fungi, the pheromone MAPK pathway is additionally important for pathogenicity. In the ascomycete phytopathogens *Cochliobolus heterostrophus* and *Magnaporthe grisea*, disruption of pheromone MAPK signaling results in a loss of pathogenicity associated with poor fungal development, the inability to mate, and altered cell morphology [21,22]. *Cryptococcus neoformans*, which causes pneumonia and meningitis in immunocompromised patients, exists as a haploid yeast which goes through meiosis and sporulation with nutrient deprivation or desiccation [23]. Recently, the *MAT**a*** pheromone receptor of *C. neoformans* has been found to be important for virulence as well as mating [24].

Relatively little is known about the life cycle of *P. carinii*, in part due to the difficulty propagating these organisms. Although phylogenetically classified as an ascomycete, thus being related to *S. cerevisiae* and *S. pombe*, *P. carinii* has features atypical of these yeast [25,26]. *P. carinii* has a unique tropism for the lung, where it attaches tightly to the alveolar epithelium without invading the host [27,28]. Morphologically, *P. carinii* has two predominant life cycle stages, a small trophic form (1–4 μm) and a larger cyst form (8 μm) [29]. Trophic forms are haploid or diploid, while the cyst contains 2, 4 or 8 nuclei [30,31]. These subsequently leave the cyst as trophic forms to repeat the life cycle. The focus of our laboratory is the investigation of signal transduction molecules from *P. carinii* which might be involved in life cycle regulation. Here we describe the cloning and expression of the PCSTE3 receptor, which is orthologous to the *S. cerevisiae* Ste3 pheromone receptor, to better understand its function in *P. carinii*.

Materials and methods

Preparation of P. carinii. *Pneumocystis carinii* pneumonia was induced in rats as previously reported [32–34]. *P. carinii* was purified from host lung cells by homogenization, filtration through 10-μm filters, and centrifugation. Samples containing contaminating microorganisms were discarded. Separated life cycle stages of *P. carinii* trophic forms and cysts were purified by filtration and centrifugation [34].

Cloning and yeast expression of PCSTE3. Two degenerate primers, PR-1 (5′-GTWGATTCWGCWATWTGGT-3′) and PR-2 (5′-AAYAA NCGYAARAANCG-3′), were designed based on an alignment of fungal pheromone receptors. PCR was performed using 1 μM of primers PR-1 and PR-2 with 500 ng *P. carinii* DNA under the following conditions: 30 cycles of 94 °C for 30 s, 54 °C for 60 s, and 72 °C for 60 s. A 609-bp amplicon was generated and used to screen a rat-derived *P. carinii* cDNA library as previously described [32]. The *PCSTE3* cDNA open-reading frame was amplified by PCR using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) with primers PCSTE3-1 (5′-ATG-GGA-GAA-GCG-TTT-TAT-ATA-TTT-3′) and PCSTE3-2 (5′-CCT-GCC-TAA-AGA-TTT-TTC-AAA-ACT-3′), ligated into the galactose-regulated yeast expression plasmid pYES2.1 (Invitrogen, Carlsbad, CA, USA), and then sequenced to confirm the absence of PCR errors and correct orientation. *S. cerevisiae STE3* was amplified by PCR from *S. cerevisiae* DNA using *Pfx* and primers SCSTE3-1 (5′-ATG-TCA-TAC-AAG-TCA-GCA-ATA-ATA-3′) and SCSTE3-2 (5′-AGG-GCC-TGC-AGT-ATT-TTC-TGAA-3′) and ligated into pYES2.1. *S. cerevisiae* SY2011 (*MAT-α ura3-52 leu2-3,112 ade1 ste3Δ ste2Δ mfa1Δ mfa2::FUS1-lacZ*) used in this study was a generous gift from Dr. N. Davis, Wayne State University [35]. SY2011 was transformed with pYES2.1/PCSTE3 or pYES2.1/SCSTE3 as previously reported and grown in galactose minimal media lacking uracil at 30 °C to induce protein expression [36].

Antibodies and membrane isolation. Antibodies to the extracellular domains of PCSTE3 were prepared (Bethyl Laboratories, Montgomery, TX, USA) by immunizing rabbits with peptides derived from the following sequences: ED1:(WYNGSETSYRGDLYC); ED2:(PAR YVIDGTSGCMPWTDRSW). The IgG fractions were purified with a protein A–Sepharose column. Horseradish peroxidase-conjugated anti-rabbit antibodies from goat were used as secondary reagents to detect the polyclonal ED1 and ED2 antibodies by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Membrane preparations from yeast and *P. carinii* were prepared by lysing the cells in YPER (Pierce Biotechnology, Rockford, IL, USA) containing a protease cocktail (1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM each of PMSF and sodium orthovanadate) followed by brief sonication, centrifugation at 5000*g* for 10 min at 4 °C to remove cellular debris, and ultracentrifugation at 100,000*g* for 2 h at 4 °C to isolate the membranes.

Immunofluorescence microscopy and flow cytometry. *Pneumocystis carinii* trophic forms and cysts, and *S. cerevisiae* expressing PCSTE3, were washed in sterile PBS, resuspended in 5% normal goat serum (NGS)/PBS/BSA (1 mg/ml), incubated with the ED1 or ED2 primary antibodies (1:100 dilution) at room temperature for 30 min, washed three times with 5% (NGS)/PBS/BSA (1 mg/ml), and then incubated with a fluorescein anti-rabbit IgG (H + L) antibody (1:100 dilution) from goat (Molecular Probes, Eugene, OR, USA) or a Texas Red anti-rabbit IgG (H + L) antibody (1:100 dilution) from goat (Molecular Probes, Eugene, OR, USA) for 30 min at RT. Samples were washed three times with PBS and examined by fluorescence microscopy using an Olympus IX70 (Olympus, Melville, NY, USA). Image analysis was performed using the Metamorph image processing software (Universal Imaging, Downingtown, PA, USA). *P. carinii* samples were also analyzed by flow cytometry (Becton–Dickinson FACScan, BD Bioscience, San Jose, CA, USA) using these identical methods with the addition of a pre-wash with 0.5% glutathione to prevent *P. carinii* clumping.

Receptor signaling assays. PCSTE3 signaling was tested by measurement of the β-galactosidase reporter gene Fus1-lacZ in *S. cerevisiae* SY2011 using fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes, Eugene, OR, USA) as a substrate [34,37]. SY2011 yeast expressing PCSTE3 or *S. cerevisiae* Ste3 were grown in galactose minimal media lacking uracil at 30 °C for 24 h, diluted to an OD_{600} of 0.1, and then grown to an OD_{600} of 0.3. Triplicate cultures were aliquoted into 96-well microfluor plates. Cultures received a-factor or α-factor pheromone (Sigma–Aldrich, St. Louis, MO, USA) at a final concentration of 10 μM for 30 min prior to the addition of FDG substrate solution [34]. The fluorescent signal was read in a Fusion 3.5

Alpha multiplate fluorometer (Packard Biosciences-Perkin–Elmer Life and Analytical Sciences, Boston, MA, USA) with an excitation filter of 485 and an emission filter of 530.

Results

Cloning of PCSTE3

We employed a degenerate PCR technique followed by conventional library screening to clone the *P. carinii* STE3 gene. By degenerate PCR, using oligonucleotide primers designed from the conserved regions of amino acids of fungal pheromone receptors, we obtained a 609-bp product. After verifying that this PCR amplicon was unique and homologous to the *S. cerevisiae* Ste3 receptor by GenBank BlastX database analysis, we used it as a probe to screen a *P. carinii* cDNA library in the bacteriophage Uni-Zap XR to obtain a 1242-bp full-length cDNA (GenBank Accession No. AY007236). Subsequently, PCSTE3 has been identified in the

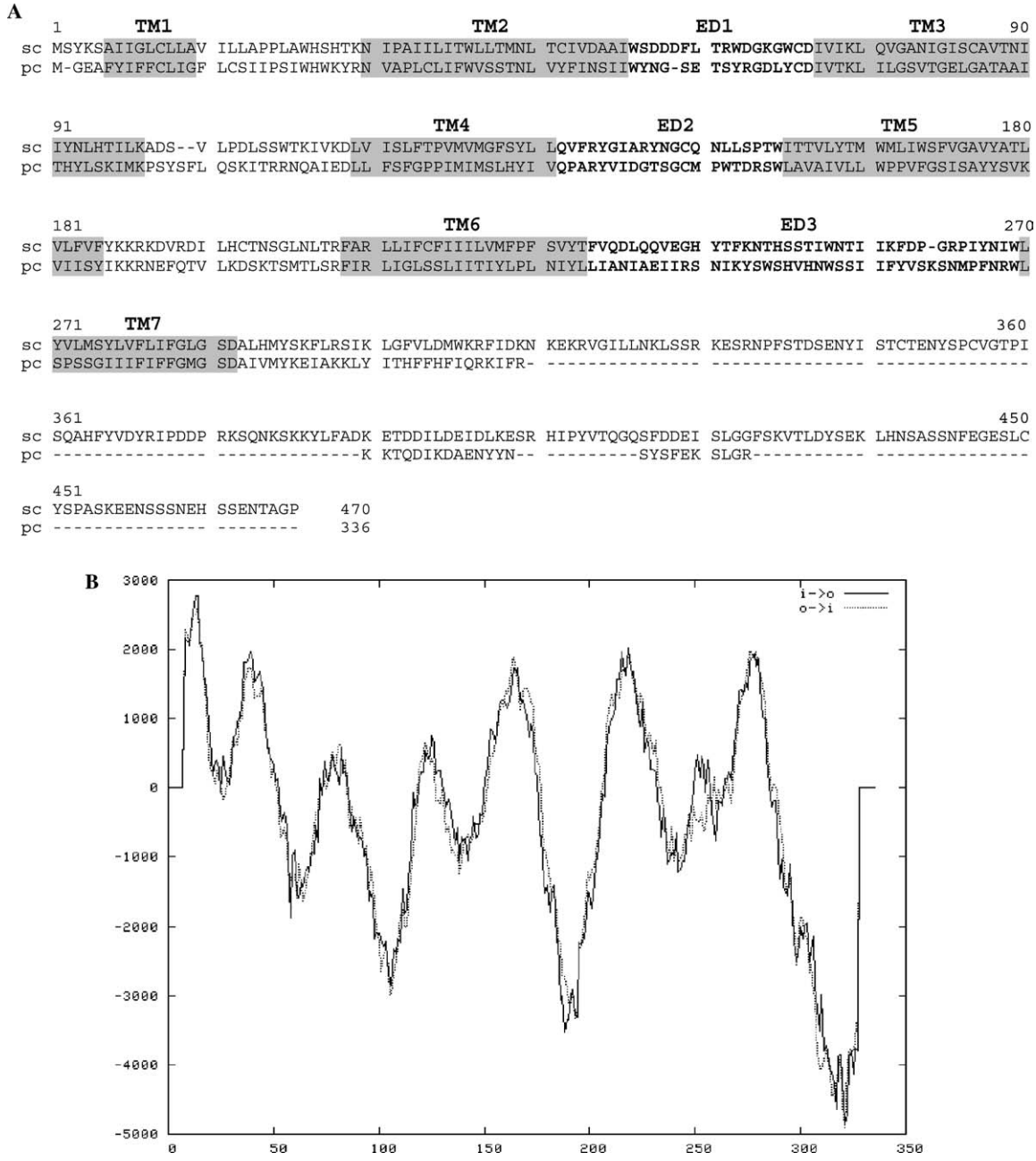

Fig. 1. Analysis of PCSTE3. PCSTE3 was compared to *S. cerevisiae* Ste3 by computer modeling. The transmembrane domains (TMs) are shaded in grey (A), and the extracellular domains (EDs) are bolded. Although PCSTE3 has 23% identity to *S. cerevisiae* Ste3 over the entire length of the protein, most of the homology is in the transmembrane domains. Peptides for antibody generation were from ED1 and ED2 of PCSTE3. The location of the transmembrane domains was determined by computer modeling of PCSTE3 using the Tmpred application (B).

P. carinii genome with a cluster of signaling molecules as part of the *P. carinii* genomic project [38].

PCSTE3 is a seven transmembrane domain protein

Computer modeling and database analysis of the PCSTE3 cDNA revealed a number of interesting findings. First, the translated open-reading frame (1011-bp), which encodes a protein of 336 amino acids and predicted molecular mass of 38.9 kDa, was most homologous to the pheromone receptor from the basidiomycete fungus *Coprinopsis cinerea* (36% identity by NCBI BlastX, GenBank Accession No. AAO17256). PCSTE3 has 23% identity to the *S. cerevisiae* Ste3 receptor (GenBank Accession No. M12239). Next, we performed an alignment of the protein sequences of PCSTE3 with *S. cerevisiae* Ste3 (Fig. 1A) to gain insights into the location of transmembrane domains (TMs) and extracellular domains (EDs) for PCSTE3. These regions are well established for *S. cerevisiae* Ste3 and have not been determined for other fungal receptors. As shown in Fig. 1A, there are conserved regions in the transmembrane domains of the PCSTE3 receptor and *S. cerevisiae* Ste3. Additional computer analysis was used to verify the presence of 7 transmembrane domains using the Tmpred application (http://www. ch.embnet.org/software/TMPRED_form.html, Fig. 1B).

PCSTE3 localizes to cell membranes

Synthetic peptides from the putative extracellular domains of PCSTE3, designated ED1 and ED2 (Fig. 1A), were constructed and used as immunogens for antibody production. We expressed PCSTE3 in *S. cerevisiae* SY2011 yeast which have deletions in the genes for both Ste3 and Ste2 receptors to better understand its function. First, we examined cell surface staining of PCSTE3 expressed in SY2011 yeast by immunofluorescence microscopy (Fig. 2). As shown in Figs. 2A and B, we can detect PCSTE3 using the ED1 or ED2 primary antibodies with a fluorescein-labeled secondary antibody. Fluorescence is not detected using a non-immune primary antibody with a fluorescein-labeled secondary antibody (Fig. 2C), nor with the secondary fluorescein antibody alone (Fig. 2D). Next, we isolated the membranes from SY2011 yeast, SY2011 yeast expressing PCSTE3, or from *P. carinii* organisms, and performed Western blotting using the ED1 and ED2 antibodies. As shown in Fig. 3, we detect PCSTE3 in the cell membranes of the yeast expressing PCSTE3 and from *P. carinii*, but not from untransformed yeast. We found these results consistently using the ED2 antibody, however with the ED1 antibody, we noted increased non-specific detection of proteins (data not shown), which we believe is due to the relatively short amino acid sequence used to create ED1. Antibodies directed

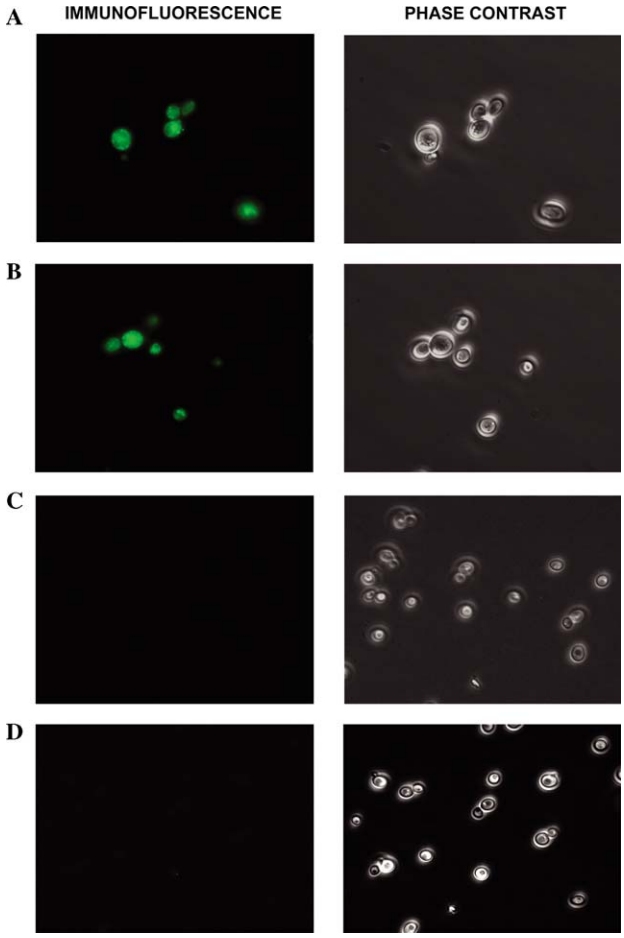

Fig. 2. Yeast expression of PCSTE3. Cell surface staining of PCSTE3 expressed in *S. cerevisiae* SY2011 was detected by immunofluorescence microscopy using the ED1 or ED2 primary antibodies with a fluorescein-labeled secondary antibody (A and B, respectively). Fluorescence is not detected using a non-immune primary antibody with a fluorescein-labeled secondary antibody (C), nor with the secondary fluorescein antibody alone (D). Phase contrast microscopy was performed on the identical field to show the presence of the yeast.

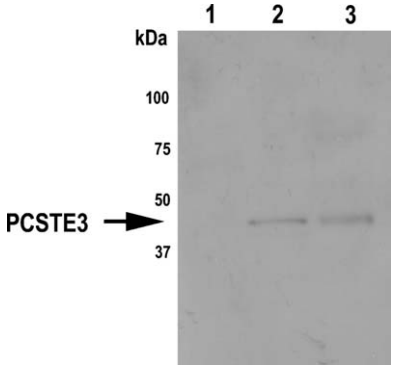

Fig. 3. PCSTE3 is detected in cellular membranes. Membranes were prepared by ultracentrifugation of *S. cerevisiae* SY2011 (lane 1), *S. cerevisiae* SY2011 expressing PCSTE3 (lane 2), or *P. carinii* organisms (lane 3). Antibodies to PCSTE3 ED2 detect a protein of the correct molecular mass in lanes 2 and 3, but not in the control condition (lane 1).

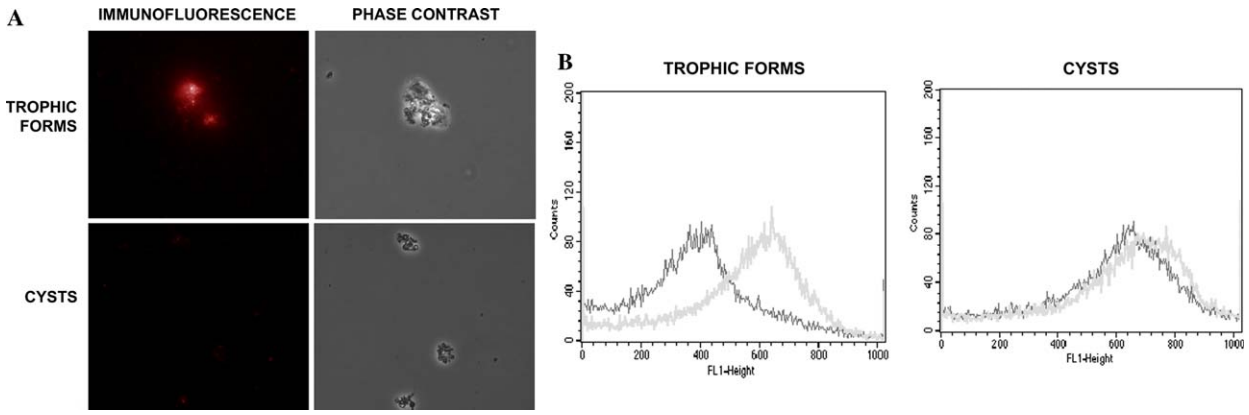

Fig. 4. Expression of PCSTE3 on *P. carinii* life cycle forms. We examined PCSTE3 expression on isolated life cycle stages of *P. carinii* trophic forms and cysts by immunofluorescence microscopy and flow cytometry. PCSTE3 was found on a subpopulation of trophic forms using the ED2 antibody as a primary antibody and a TR-labeled secondary antibody (A). PCSTE3 receptor expression was not found on cysts by immunofluorescence. Likewise, we examined PCSTE3 expression on separated *P. carinii* trophic forms and cysts by flow cytometry. Again, a population of trophic forms expresses the PCSTE3 receptor (B), while PCSTE3 receptor expression was not detected on cysts.

against ED2 were used exclusively for the remainder of the expression studies.

PCSTE3 does not recognize yeast pheromones

We investigated whether PCSTE3 expressed in the receptor mutant yeast would recognize yeast pheromones. The SY2011 yeast have a *Fus1-lacZ* reporter gene in their genome. This reporter gene is activated upon exposure to pheromone only if all of the components of the pheromone-induced MAPK pathway are functional. Using a sensitive detection method with fluorescein di-β-D-galactopyranoside (FDG) as a substrate for the reporter gene and a multiplate fluorometer for detection, we found that neither the *S. cerevisiae* a-factor pheromone nor α-factor pheromone would act as a ligand for the PCSTE3 receptor (data not shown). We verified that this experimental system was functional by expressing the wild-type *S. cerevisiae* Ste3 receptor in the SY2011 yeast in an identical manner for PCSTE3. The wild-type *S. cerevisiae* Ste3 receptor expressed in these yeast signal appropriately with a-factor pheromone as the ligand for the Ste3 receptor, and as expected, do not signal with α-factor pheromone. It is highly plausible that the conformations of the PCSTE3 extracellular domains are sufficiently different from those of the *S. cerevisiae* pheromone receptors, therefore the *S. cerevisiae* pheromones are not recognized as ligands for the PCSTE3 receptor. These same differences are present in the *S. cerevisiae* Ste2 and Ste3 receptors, preventing cross-stimulation by the inappropriate pheromone.

Detection of PCSTE3 expression on P. carinii trophic forms

Pneumocystis carinii has two major life cycle stages, the trophic forms and the cysts. We examined PCSTE3 expression on isolated life cycle stages by immunofluorescence microscopy and flow cytometry. Detection of PCSTE3 was found on a population of trophic forms using the ED2 antibody as a primary antibody and a TR-labeled secondary antibody (Fig. 4A). Additionally, there are trophic forms where we could not detect expression of the PCSTE3 receptor. PCSTE3 receptor expression was not found on cysts by immunofluorescence. We used a TR-labeled secondary antibody instead of a fluorescein-labeled secondary antibody to reduce green autofluorescence from the *P. carinii*. We confirmed these results by performing flow cytometry on separated *P. carinii* trophic forms and cysts, and a population of trophic forms expresses the PCSTE3 receptor (Fig. 4B). Again, receptor expression was not detected on cysts.

Discussion

The main finding of this study is the expression of the putative pheromone receptor, PCSTE3, on a population of *P. carinii* trophic forms, and lack of expression on cysts. We cloned PCSTE3 using molecular techniques of degenerate PCR and cDNA library screening. Our work also shows the surface expression of PCSTE3 in yeast as detected by antibodies to the extracellular domains of the molecule, and lack of signaling of PCSTE3 by *S. cerevisiae* pheromones. Subsequent to our reporting PCSTE3 in GenBank, Smulian and colleagues reported the same gene as part of a large complex of signaling molecules identified from the *P. carinii* genome sequencing project (GenBank Accession No. AF309805), where they determined the chromosomal location of PCSTE3 [38].

The budding yeast *S. cerevisiae* responds to conditions of nutrient deprivation by a complex process

where haploid cells mate with cells of the opposite mating type through the production of peptide pheromones which bind to pheromone receptors on cells of the opposite mating type. Pheromone binding to its receptor activates a MAPK pathway which results in conjugation, the formation of an ascus, and differentiation events such as meiosis. The majority of *P. carinii* trophic forms are haploid [39,40], and the *Pneumocystis* cyst, which is likely formed by mating of the trophic forms, contains 2, 4, or 8 intranuclear bodies as it matures, which ultimately exit the cyst as trophic forms to complete the life cycle [29].

Our finding of PCSTE3 expression on a subpopulation of trophic forms is consistent with the expression of pheromone receptors from other fungal organisms. In *S. cerevisiae* and *S. pombe*, each haplotype expresses a unique pheromone receptor with unique specificity for pheromones produced from the opposite mating type. Thus, it is not surprising that PCSTE3 does not recognize *S. cerevisiae* pheromones as ligands. We suspect that *P. carinii* has at least two pheromone receptors, and our methods of indirect immunofluorescence and flow cytometry detect PCSTE3 on a subpopulation of trophic forms.

Why is PCSTE3 not expressed on cysts? The *Pneumocystis* cysts, which are morphologically very different from the trophic forms, have a thick cell wall composed of complex carbohydrates, which include chitins, glucans, and mannoproteins [41]. In fungi related to *P. carinii*, the pheromone-induced MAPK pathway is instrumental in regulating the differentiation events in the haploid forms but not in the ascus. Furthermore, after the pathway is initiated, the signal is attenuated. If this pathway in *P. carinii* functions in an analogous manner to other fungi, then activation of the pheromone MAPK pathway is important in the trophic forms and not the cysts, since the events which culminate in the formation of the cyst come from the differentiation of trophic forms.

The yeast assay we have established to study the function of PCSTE3 in the *S. cerevisiae* SY2011 receptor mutant yeast has the potential for identifying ligands which would specifically bind to PCSTE3. It is currently unknown whether *P. carinii* has pheromones. Using this system with a microtiter plate and highly sensitive fluorescent substrate for the reporter gene, one could screen a number of potential ligands. *P. carinii* pheromones might be secreted from trophic forms under various environmental conditions including nutrient deprivation, or during infection if the pheromone-induced MAPK pathway is linked to virulence. It is also possible that a host protein or growth factor present in the lung alveolar lining fluid could bind PCSTE3 and activate the signaling pathway. Interrupting life cycle transition in *P. carinii* by interfering with pheromone signaling may have important implications for organism virulence or as a potential therapeutic modality.

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