Differential Regulation of Growth and Checkpoint Control Mediated by a Cdc25 Mitotic Phosphatase from Pneumocystis carinii

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Summary

*Pneumocystis carinii* is an opportunistic fungal pathogen phylogenetically related to the fission yeast *Schizosaccharomyces pombe*. *P. carinii* causes severe pneumonia in immunocompromised patients with AIDS and malignancies. Although the life cycle of *P. carinii* remains poorly characterized, morphologic studies of infected lung tissue indicate that *P. carinii* alternate between numerous small trophic forms and fewer large cystic forms. To further understand molecular mechanisms which regulate progression of *P. carinii*’s cell cycle, we have sought to identify and characterize genes in *P. carinii* that are important regulators of eukaryotic cell cycle progression. In this study, we have isolated a cDNA from *P. carinii* that exhibits significant homology, but unique functional characteristics, to the mitotic phosphatase Cdc25 found in *S. pombe*. *P. carinii* Cdc25 was shown to rescue growth of the temperature-sensitive *S. pombe cdc25*-22 strain and thus provides an additional tool to investigate the unique *P. carinii* life cycle. While *P. carinii* Cdc25 could also restore the DNA damage checkpoint in *cdc25*-22 cells, it was unable to fully restore the DNA replication checkpoint. The dissociation of checkpoint control at the level of Cdc25 indicates that Cdc25 may be under distinct regulatory control in mediating checkpoint signaling.
Introduction

Despite advances in prophylaxis and treatment, Pneumocystis carinii remains an important cause of life-threatening pneumonia in patients with impaired immunity. Patients with AIDS, organ transplantation, and those receiving chemotherapy are particularly vulnerable to P. carinii pneumonia. The case fatality rate of P. carinii pneumonia ranges from approximately 10 to 40%, being substantially higher in infected immunocompromised patients without AIDS (1-3). Unfortunately, a considerable number of patients are intolerant of the currently available agents used to prevent and treat this infection, such as sulfamethoxazole and pentamidine. The development of new chemotherapeutic agents to treat P. carinii pneumonia has been hampered by a limited understanding of the P. carinii life cycle.

Morphological and ultrastructural studies of infected lung tissues indicate that P. carinii alternates between numerous diminutive trophic forms and fewer larger cyst forms (4). Trophic forms are known to preferentially bind Type I alveolar epithelial cells and eventually develop into mature cysts characterized by a thickened cell wall (5,6). The mature cyst, containing eight intracystic bodies, ruptures releasing these bodies as haploid trophic forms (4). While the proliferation of P. carinii in the lung is thus enhanced by the attachment of trophic forms to lung epithelium, only recently have there been initial reports of limited success with in vitro cultivation of P. carinii in the absence of feeder cells.
However, long term in vitro propagation P. carinii has perennially remained a key obstacle in the investigations of the organism’s life cycle.

Based on DNA sequence analysis, P. carinii has been classified as a member of the fungi and is phylogenetically related to the fission yeast Schizosaccharomyces pombe (10-12). The life cycle and the cell cycle of S. pombe have been well characterized and utilized to identify cell-division-cycle (cdc) genes in other organisms with obligate roles in cell proliferation (13-15). Despite the fact that these cell-division-cycle genes are well conserved, it is known that diverse eukaryotic organisms differentially regulate their cell cycle machinery in response to environmental stimuli and other internal signaling pathways (16).

In order to better understand molecular mechanisms which regulate progression of P. carinii’s cell cycle, we have sought to identify and characterize genes in P. carinii that are important regulators of cell cycle progression in related organisms. Accordingly, we began our investigations by demonstrating that P. carinii utilizes a cyclin dependent kinase, Cdc2, over its life cycle (17). Activated Cdc2, in association with cyclin B (cdc13), phosphorylates a variety of targets, such as histones and nuclear laminins, to initiate mitosis (18,19). The kinase activity of Cdc2 is regulated by both inhibitory and stimulatory phosphorylations (20). One important positive regulator is the Cdc25 phosphatase which activates Cdc2 by removing the inhibitory phosphoryl group...
on Tyr$^{15}$ of Cdc2 (21,22). In fission yeast, Cdc25 protein levels and activity rise in the G2 phase of the cell cycle (23). Therefore, at the G2/M border Cdc2 is able to phosphorylate and activate Cdc25, initiating an autofeedback loop resulting in a rapid entry into mitosis (24,25). In addition to mitotic control, Cdc25 has roles in regulating meiosis and imposing a checkpoint arrest in the DNA repair and DNA replication processes (26-31). Thus, Cdc25 represents an integral component of eukaryotic cell cycle regulation.

Current models state that the phosphorylation of the Tyr$^{15}$ residue of Cdc2 is a key component of maintaining the S/M and G2/M checkpoints (32,33). DNA damage or unreplicated DNA activates the appropriate checkpoint pathway which propagates signals that lead to the removal of Cdc25 from the nucleus and/or inactivation of Cdc25 activity, thereby maintaining the Tyr$^{15}$ phosphorylated state of Cdc2 (26,31,34-37). In this study, we have identified and characterized a cDNA and corresponding genomic clone from *P. carinii* that is structurally and functionally homologous to the essential Cdc25 mitotic regulator. In the context of a heterologous fungal system, this homolog rescues growth in Cdc25 temperature-sensitive mutants and restores the DNA damage checkpoint. However, it is unable to fully restore the DNA replication checkpoint following hydroxyurea treatment. These findings (i) provide evidence for distinct molecular mechanisms regulating G2/M and S/M checkpoints previously thought to be under similar control; and (ii) identify a
key regulatory molecule involved in *P. carinii* life cycle progression.
MATERIALS AND METHODS

Materials-Nitrocellulose membranes containing separated *P. carinii* chromosomes were kindly provided by Dr. Melanie T. Cushion, University of Cincinnati College of Medicine (38). A *P. carinii* cDNA library was obtained from the NIH AIDS Research and Reference Reagent program. The temperature-sensitive *Schizosaccharomyces pombe cdc25-22* mutant strain and the pREP41 vector were the kind gifts of Dr. Kathleen Gould, Vanderbilt University. Dr. Barbara Painter generously provided Ciprofloxacin (Miles Pharmaceuticals, Inc., West Haven, CT). All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

*P. carinii* isolation-All animal studies were approved by the Mayo Institutional Animal Care and Utilization Committee. Harlan Sprague-Dawley rats were immunosuppressed with dexamethasone and subsequently challenged with *P. carinii* sp. f. (special form) *carinii* via transtracheal injection to induce *P. carinii* pneumonia (39,40). *P. carinii* were purified by differential filtration through 10 µM filters as we have previously reported (17).

Cloning of the *P. carinii* Cdc25 Mitotic Phosphatase-*P. carinii* genomic DNA was prepared (17,41,42) and degenerate oligonucleotide primers synthesized to the conserved CH2A and CH2B domains of Cdc25 family members. The following primers were used for amplification: 5´-ATW ATW GAT TGT CGS TTG-3´ and
5’-WGG ATA ATA SAA AAA WGG ATA-3’. PCR was performed as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 5 min. A single 280-bp fragment was amplified, subcloned into the pGEM-Teasy vector (Promega, Inc.) and sequenced. The amplicon was radiolabeled with [32P]-α-dATP (Amersham-Pharmacia) with the RadPrime DNA Labeling System (Gibco Life Technologies, Inc., Gaithersburg, MD) and hybridized to a nitrocellulose membrane containing *P. carinii* chromosomes. The *P. carinii* chromosomes were separated by contour-clamped homogenous field electrophoresis (CHEF) and transferred to nitrocellulose (38). After 30 min of prehybridization in ExpressHyb (Clontech Laboratories, Inc., Palo Alto, CA), the CHEF blot was incubated with probe (1 x 10^6 cpm/mL) at 68°C for 60 min, washed four times at room temperature in 2X SSC/0.05% SDS buffer, washed twice at 50°C for 40 min in 0.1X SSC/0.1% SDS, and visualized by autoradiography. In addition, the probe was used to screen a rat-derived *P. carinii* cDNA library under similar conditions. Clones were isolated after plaques were purified to homogeneity. A 2.1-kb clone was purified and sequenced (GenBank Accession Number AF098935). The genomic sequence (GenBank Accession Number AF098936) was determined by PCR

1 The abbreviations used are: bp, base pair; CHEF, contour clamped homogeneous field electrophoresis; DAPI, 4,6-diamidino-2-phenylindole; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; GST, glutathione-S-transferase; Gy, gray; HU, hydroxyurea; IPTG, isopropylthio-β-galactosidase; IR, ionizing radiation; p-NPP, p-nitrophenylphosphate; PBS, phosphate buffered saline; PCR polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBE, tris-borate-EDTA.
amplification using freshly isolated *P. carinii* genomic DNA as the template. Primers made from the 5′ end and the 3′ end of the cDNA used for amplification are as follows: 5′-CATATATGGATACTTCACCTCTTG-3′ and 5′-CGGACGT TACTCACATCTTTTGAG-3′. PCT reactions were carried out as previously mention.

Amplification of Cdc25 from *P. carinii*, rat, and *S. pombe*-Total RNA was isolated from uninfected rat lung, *P. carinii* infected rat lung, isolated *P. carinii*, and *S. pombe* via the TRIZOL® (Gibco Life Technologies, Inc., Gaithersburg, MD) method. Total RNA from each sample was used to make cDNA with the Advantage" RT-for-PCR Kit (Clonetech Laboratories, Inc., Palo Alto, CA). Cdc25 primers for polymerase chain reaction amplification were used as follows: *P. carinii* specific primers at nucleotide 108 from the beginning of the coding region (5′-GGGAAGAAATATACTGATAAGAATAATAAATTCCGG-3′) and at nucleotide 748 (5′-GCAATATCAGTATCAGTATCTTTTTATCTG-3′); rat specific primers at nucleotide 39 (5′-CTCTCAGTGCTAGCCCATCCC-3′) and nucleotide 691 (5′-CATCATTTCTCTGATTTCTCCATCCAG-3′); and *S. pombe* specific primers at nucleotide 295 (5′-CTTGATCTGTAGAAACCCCTCTCAGC-3′) and at nucleotide 899 (5′-GAATTATCATGTGCAAGCCACAAATACACTG-3′). PCR components except primers were added and split into three tubes for each template. Specific primers were then added. Amplification conditions were the same in all reactions as discussed previously and the products electrophoresed on a 2% agarose TBE (100 mM Tris, 90 mM Boric acid, 1 mM
EDTA) gel.

Production of Cdc25 Proteins in Bacteria and p-NPP assays-The full length cDNAs for the *P. carinii* Cdc25 homolog, an inactive mutant (C432A), and the *S. pombe* cdc25 were subcloned into the pGEX-2T vector (Amersham-Pharmacia Biotech, Piscataway, NJ) and transformed into competent *E. coli* BL21 cells. The bacteria were grown to an OD$_{600}$ of 0.5 at 27°C in LB containing 100 µg/ml ampicillin. IPTG was added for 4 h at a final concentration of 1 mM for BL21 cells harboring *P. carinii* Cdc25 and C432A expression plasmids and 0.2 mM for BL21 cells containing the *S. pombe* Cdc25 expression plasmid. The pelleted cells were suspended in PBS containing 10 µg/ml leupeptin, pepstatin, aprotinin, and PMSF. A final concentration of 100 µg/ml of lysozyme and 1% Triton was added, incubated on ice for 15 min, lysed by mild sonication, and centrifuged to remove insoluble debris. The GST fusion proteins were purified from the soluble fraction using glutathione sepharose beads and eluted with 10 mM reduced glutathione/50 mM Tris pH 8.0. The inactivated *P. carinii* Cdc25 was created by mutating the Cys432 residue in the active site to an Ala residue with the Stratagene Quikchange Site-Directed Mutagenesis Kit®. The following primers were used: C432A-5´: (5´-GTTTGATTATTTTTCATGCTGAATATAGTTCACTCGTGC-GCCA-3´); and C432A-3´: (5´-GGCGCAGATGTGAACTATATTCCAGCATG-AAAAATAATCAAAC-3´) with
the mutated codon underlined. DNA sequencing verified the presence of the C432A mutation and the absence of other mutations. Reactions for p-NPP assays were performed as described by Dunphy and Kumagai (43). For GST-PcCdc25 purification, the eluted protein was loaded onto a Superose 12 (Amersham-Pharmacia) gel filtration column, fractions collected, and visualized by Coumassie-stained SDS-PAGE. Fractions containing the fusion protein were subsequently loaded onto a Mono Q (Amersham-Pharmacia) anion exchange column. Two buffers consisting of 25 mM Tris pH 8.2, 1 mM DTT with and without 1 M NaCl were used to create a 40 ml gradient to elute the protein.

Complementation of a S. pombe cdc25 Temperature-Sensitive Mutant Using P. carinii cdc25-A NdeI-BamHI fragment encoding the entire 1614-bp open reading frame encoding the P. carinii Cdc25 homolog was subcloned into NdeI-BamHI-digested pREP41 (containing a leu2 marker). The resulting construct contained the P. carinii gene in sense orientation under control of the thiamine repressible nmt promoter. A 7kb SalI genomic clone of the S. pombe Cdc25 was subcloned into SalI-digested pREP41 in an analogous fashion. The constructs, including pREP-C432A and pREP alone, were transformed into the S. pombe h+ cdc25-22 leu1-32 ura4-218 ade6-M210 strain as described (44) and selected on leucine and thiamine deficient media. Leucine positive clones that grew at the permissive temperature (25°C) were then tested for growth at the restrictive
temperature (35°C). The expression of the *P. carinii* Cdc25 was repressed by the addition of 25 µM thiamine.

**DNA damage and DNA replication checkpoint analysis with PcCdc25 complemented cdc25-22 cells**—For the DNA replication checkpoint studies, 12 mM hydroxyurea was added to asynchronous yeast cultures (1x 10^6/ml) and fixed with 3% formaldehyde at selected time points, then stained with DAPI (45). Cells were analyzed by fluorescence microscopy and counted for cells containing mitotic cuts. For the DNA damage checkpoint studies, cells were synchronized in G1 in media lacking nitrogen, released, and irradiated with 200Gy from a ^{137}Cs source. Following fixation and DAPI staining, images were taken on an Olympus AX-70 fluorescence microscope and analyzed using the Metamorph software program (Universal Imaging Corporation, West Chester, PA).
RESULTS

Cloning of the P. carinii Cdc25 Mitotic Phosphatase - A degenerative PCR approach was taken to determine whether P. carinii contained a Cdc25 homolog. Because the active site of Cdc25 family members is highly conserved across species, degenerate primers were designed from the CH2A and CH2B domains flanking the catalytic site. To optimize the efficiency of the degenerate primers, codon bias was used to reflect the adenine/thymine rich (>65%) P. carinii DNA (46). A 280 base-pair product was amplified from P. carinii genomic DNA. Sequencing the product revealed that the 280 base-pair fragment was unique but homologous to Cdc25 genes from other organisms (data not shown). To confirm the P. carinii-origin of the amplicon, it was hybridized to rat-derived P. carinii chromosomes separated by contour-clamped homogenous field electrophoresis (CHEF). Under high stringency, the amplicon hybridized to a single chromosome from P. carinii and to a 2.3-kb P. carinii RNA transcript (Fig. 1 and data not shown). Subsequent screening of a P. carinii cDNA library identified a 2.1-kb fragment which was isolated and characterized. Based on the northern data, this cDNA clone appears to contain the entire coding region and most of the surrounding regulatory regions. The genomic clone was isolated by PCR amplification from freshly isolated P. carinii genomic DNA. Comparison of the cDNA and genomic sequences indicated that the coding region is interrupted by five introns that span 40-50 base-pairs in length and contain the GT/AG
intron/exon junction sequence (GenBank Accession numbers AF098935 and AF098936, respectively). Figure 2 shows the predicted amino acid alignment of *P. carinii* Cdc25 with Cdc25 proteins from other eukaryotic species. Consistent with other Cdc25 family members, PcCdc25 exhibits a highly conserved C-terminus containing the catalytic site and a variable N-terminus. The PcCdc25 protein was found to be most closely related to fission yeast, being 61.2% homologous and 40.3% identical to the *Schizosaccharomyces pombe* Cdc25. The PcCdc25 homolog contained the HCXXXXXR consensus sequence (amino acids 431-438) and the conserved DCR motif at amino acids 389-391 unique to Cdc25 phosphatases (47). Although the homolog does not contain a LIGD motif found in higher eukaryotes, it has three putative Cdc2 consensus phosphorylation sites on the N-terminus at amino acids 91-93, 112-114, and 141-143 (47). These results demonstrate that the isolated cDNA is from *P. carinii* and exhibits significant homology to other Cdc25 species.

*The P. carinii cdc25 Transcript is Specific for P. carinii*- One of the major concerns in cloning genes from *Pneumocystis* is host cell or other microbial contamination during the isolation of the organism. While the CHEF blot provides substantial evidence for *P. carinii* origin, these preparations likely contain minor amounts of host material. As such, to ensure specificity, a PCR-based approach was taken to determine whether specific primers designed from one Cdc25 gene would amplify a Cdc25 gene from another organism. Primers
were designed near the 5’ end of the putative P. carinii cdc25+ homologue, rat cdc25+, and S. pombe cdc25+ genes, reflecting the region of the proteins that are the least-conserved (see Materials and Methods). RT-PCR was performed with RNA isolated from normal rat lung, P. carinii-infected rat lung, isolated P. carinii, and S. pombe. The P. carinii cdc25+ primers amplified products from only P. carinii-infected lungs and isolated P. carinii (Fig. 3A). Rat cdc25+ primers only generated a product from rat lung and P. carinii-infected rat lung, while S. pombe cdc25+ primers specifically produced a transcript from S. pombe cDNA. Having verified the origin of the cDNA, we wished to determine the gene expression pattern in the two major life forms of P. carinii. From northern analysis, the P. carinii cdc25+ transcript was expressed in both the trophic and cyst form, with a moderate increase in expression in the cysts (Fig. 3B). These results, in addition to the CHEF hybridization and nucleotide and amino acid sequence differences, clearly demonstrate the Cdc25 gene product is from P. carinii and is expressed in both life cycle forms.

PcCdc25 Exhibits In Vitro Phosphatase Activity-Cdc25 proteins are dual specific phosphatases known to catalyze the transition from various cell cycle checkpoints (25,28,48,49). In order to determine whether the P. carinii Cdc25 homolog had similar enzymatic activity as that seen for other Cdc25 proteins, we expressed the protein fused to a glutathione S-transferase (GST) domain at the
N-terminus in *Escherichia coli*. Phosphatase activity was measured by the ability to dephosphorylate *p*-nitrophenolphosphate and compared to GST fusion proteins of human Cdc25C and *S. pombe* Cdc25. As shown in figure 4, the kinetic parameters of the partially purified GST-PcCdc25, GST-SpCcdc25, and the GST-HuCdc25C were similar, with *K*<sub>m</sub> values of 17, 26, and 35 mM, and *V*<sub>max</sub> values of 6, 3, and 31 nmoles·min<sup>-1</sup>·mg<sup>-1</sup>, respectively. Similar kinetics have been reported for *Xenopus leavis* Cdc25 with a *K*<sub>m</sub> of 50 mM and *V*<sub>max</sub> of 56 nmoles·min<sup>-1</sup>·mg<sup>-1</sup> (21).

To ensure that the phosphatase activity observed in figure 4 reflected an enzymatic activity of GST-PcCdc25 and not some bacterial contaminant, a 432CysàAla mutation was introduced in the predicted catalytic domain of GST-PcCdc25. This mutation abolishes phosphatase activity by deletion of the active site nucleophile (43). As shown in figure 4, the introduced mutation abolished *P. carinii* Cdc25 activity. In addition, the GST-PcCdc25 fusion protein was purified to >80% purity using a gel filtration and ion exchange (Mono Q) chromatography (data not shown). The resulting purification resulted in a seven fold increase in the specific activity of the protein. Moreover, the *K*<sub>m</sub> of the Mono Q purified enzyme (average of two preparations) remained very close to the partially purified protein (15 vs. 17 mM, respectively) but the *V*<sub>max</sub> increased from 6 to
15 nmoles·min\(^{-1}\)·mg\(^{-1}\). These data not only documented that the phosphatase activity was due to the added fusion protein, but strongly suggested that GST-PcCdc25 has a similar active site to other Cdc25 proteins.

**P. carinii Cdc25 Rescues cdc25-22 Temperature-Sensitive Mutants Thereby Restoring Growth**—The absence of defined genetics and culture systems makes it difficult to do genetic manipulations in *P. carinii*. As such, it is presently not possible to directly examine the role of the *P. carinii* Cdc25 homolog in *P. carinii* proliferation. However, to overcome that limitation, we determined whether the *P. carinii* Cdc25 cDNA would complement a temperature sensitive Cdc25 *S. pombe* mutant (*cdc25-22*). This strain grows normally at a permissive temperature of 25°C, but at the restrictive temperature of 35°C the thermo-labile endogenous Cdc25 is no longer active and the strain arrests at the G2/M phase border (48). The PcCdc25 cDNA, the inactive PcCdc25(C432A) mutant, and the SpCdc25 genomic clone were subcloned downstream of a thiamine repressible *nmt* promoter (50) and transformed into *cdc25-22* cells. Individual clones containing the pREP expression vector were then selected on leucine deficient minimal media plates and transformants shifted to the restrictive temperature to measure their ability to restore growth. As shown in figure 5, while the pREP vector alone and the PcCdc25(C432A) mutant did not restore growth at the restrictive temperature of 35°C, complementation was observed in the *S. pombe*
clone containing the PcCdc25 homolog (Fig. 5C). Moreover, clones grown in the presence of thiamine, which represses expression of the PcCdc25 cDNA, were unable to grow (Fig. 5D). The growth of pREP-SpCdc25 in the presence of thiamine (Fig 5D, panel 2), reflects the intact promoter activity upstream of the genomic S. pombe Cdc25 sequence. Therefore, in the context of a heterologous fungal system, the P. carinii Cdc25 homologue is able to initiate mitosis and support fungal growth.

PcCdc25-complemented cdc25-22 Cells Restores the DNA Damage Checkpoint But Not the DNA Replication Checkpoint Pathway-Checkpoint pathways in eukaryotic cells ensure that genomic integrity is maintained in the response to environmental and genotoxic stress (51). In fission yeast, the entry into mitosis is blocked when DNA synthesis is incomplete or when DNA is damaged by such agents as ionizing radiation (52). The G2/M checkpoint is imposed by a signal transduction system that ultimately leads to the sequestration of the Cdc25 protein into the cytoplasm (mediated by Rad24, a 14-3-3 protein) and/or inactivation of Cdc25 activity. These events prevent the phosphatase from activating Cdc2 (34-36,49). S. pombe Cdc25 has three canonical 14-3-3 binding sites, RSL599CT, RRTQS5359MF, and RRSRS192SG, that are phosphorylated by Chk1 and Cds1 in response to DNA damage and incomplete DNA replication (26,36,49). In contrast to S. pombe Cdc25, we observed that the P. cariniiCdc25 contained only one similar consensus 14-3-3 binding site at serine 314
(RRTQS314LY). As such, it was unclear whether a complete checkpoint response could be provided by PcCdc25. To that end, we subjected PcCdc25-complemented cdc25-22 cells to ionizing radiation (IR) in order to determine whether the P. carinii Cdc25 restored the DNA damage checkpoint. The DNA damage checkpoint was measured by determining the number of cells passing mitosis following irradiation. G1-synchronized cells were exposed to 200Gy of ionizing radiation and harvested over the next 12 hours. As shown in figure 6A, SpCdc25 and PcCdc25-complemented yeast show the characteristic G2-arrested elongated phenotype following exposure to IR. When the number of septated cells (i.e., cells that have past mitosis and thus non-arrested) were quantified, SpCdc25-complemented cultures showed a significant decrease in the number of septated cells starting at four hours and recovering around 12 hours (Fig. 6B). A similar result held true for the PcCdc25-complemented yeast in that a delay into mitosis was observed. Although the kinetics of the delay were somewhat slower, the results demonstrate that in response to ionizing radiation, PcCdc25-complemented yeast arrest similar to that observed with the endogenous S. pombe Cdc25 protein. Thus, the DNA damage checkpoint is intact in cells harboring PcCdc25.

Since the DNA damage and DNA replication checkpoints are proposed to signal through Cdc25 by Chk1 and Cds1 phosphorylation, we determined whether PcCdc25-complemented clones would similarly maintain a checkpoint arrest in response to inhibitors of DNA replication. DNA synthesis was inhibited
by treating cultures with hydroxyurea (HU), an agent that inhibits the function of ribonucleotide reductase. Yeast that cannot maintain the DNA replication checkpoint enter mitosis with incompletely replicated DNA. This results in an unequal distribution of DNA as cells pass through mitosis and septate, therefore generating cells that contain little or no DNA (referred to as mitotic ‘cut’ cells). As shown in figure 7A, SpCdc25-complemented yeast grown in 12 mM HU maintained the replication checkpoint and generated very few cells exhibiting the mitotic cut phenotype. However, unlike HU treated SpCdc25-complemented yeast, a significant fraction of the PcCdc25-complemented cells grown in 12 mM HU bypassed the S/M checkpoint and entered into mitosis. Quantification of mitotic cut cells showed that SpCdc25-complemented cells grown in HU maintained the DNA replication checkpoint in that only 1% of cells exhibited the mitotic cut phenotype at 6 hours (Fig. 7B). This is contrasted by the PcCdc25-complemented cells grown in HU where the number of cut cells increased to 8.0% at eight hours. This result is similar to the 9 fold-induction of mitotic cut cells observed with SpCdc25-S3 yeast that have a diminished replication checkpoint response due to serine mutations in the three 14-3-3 binding sites (36). We therefore conclude that PcCdc25 does not restore the DNA replication checkpoint. While the SpCdc25-S3 mutation is unable to complement either the DNA replication or DNA damage checkpoints (34,36), we demonstrate that the PcCdc25 protein differentially responds to these genotoxic assaults. The data indicate that the pathways which eukaryotic organisms
employ in response to DNA damage and incomplete DNA synthesis can be
dissociated at the level of Cdc25 regulation.
DISCUSSION

Our laboratory has previously characterized roles for *P. carinii* Cdc2 and its cognate partner Cdc13 in cell cycle regulation. In this report, we have continued our approach to define key mediators of this system in *P. carinii*. A 2.1 kb cDNA and corresponding genomic clone has been isolated from *P. carinii* that shows greatest homology to the *S. pombe* Cdc25 gene (Figs. 1-2). The predicted amino acid sequence contains a consensus HCXXXXXR motif in the active site and many other consensus sites found in the Cdc25 family (47). We show that the cdc25+ transcript is specific for *P. carinii* and is expressed in both the trophic and cyst life cycle forms (Fig. 3). The Cdc25 homolog contains *in vitro* phosphatase activity and demonstrates similar kinetic parameters to other Cdc25 homologs (Fig. 4). Since molecular genetics in *P. carinii* is currently not feasible, we examined the function of PcCdc25 in a heterologous fungal system with a temperature sensitive deficiency of endogenous Cdc25. The results demonstrate that the PcCdc25 cDNA rescues the defect and supports growth of the cdc25-22 strain (Fig. 5). Therefore, in fission yeast, PcCdc25 provides the necessary function(s) for complete cell cycle progression. Interestingly, yeast cells containing the *P. carinii* Cdc25 homolog demonstrated differential responses to the G2/M and S/M checkpoints. Although the PcCdc25-complemented cdc25-22 yeast maintained the G2/M checkpoint in response to damaged DNA by IR (Fig. 6), the S/M DNA replication checkpoint, as tested with HU, was impaired (Fig. 7). Thus, PcCdc25 is capable of dissociating the signals regulating normal...
proliferation and checkpoint control.

Although the *P. carinii* life cycle has been well characterized morphologically, the molecular events regulating life cycle progression have only recently begun to be defined (17,53-55). It is evident that while cell division cycle molecules have key roles in the proliferation of *P. carinii*, the unique requirements of *P. carinii* for life cycle progression suggest other regulatory mechanisms. For instance, *P. carinii* attachment to Type I pneumocytes promotes proliferation while *C. albicans*, *S. cerevisiae*, and *S. pombe* propagate independent of binding to a substrate. Second, *P. carinii* progresses through a cyst form which is critical for survival and necessary for life cycle progression (54,56). As such, when *P. carinii*-infected rats are treated with β-glucan synthesis inhibitors to prevent cyst formation, the infection is eliminated (56). This is in contrast to budding and fission yeast haploid forms which utilize a mitotic cycle independent of forming an ascus, unless environmental conditions are unfavorable (57). Although these differences need to be considered as the *P. carinii* life cycle is investigated, a common feature found in all eukaryotic proliferation is a central regulatory role for cell-division-cycle gene products (16). Therefore, it is very likely that these molecules are intimately involved in similarly coordinating *P. carinii* life cycle progression.

In a cell’s response to environmental stimuli, signal transduction
pathways are activated which eventually impact on the cell cycle machinery. Cdc25 is a key regulator of several cellular processes including regulating entry into mitosis, meiotic phase transitions, and maintaining G2/M and S/M checkpoints in the response to DNA damage and incomplete DNA replication (26-31). The protein complexes that sense DNA damage or stalled DNA replication forks transmit signals that ultimately lead to the inactivation of Cdc2 (32,33,37). One such mechanism includes the phosphorylation of Cdc25 by Chk1 and Cds1 kinases. Phosphorylation of Cdc25 results in the inactivation and/or sequestration of Cdc25 to the cytoplasm thereby maintaining Cdc2 in the inactive Tyr15-phosphorylated state (26,31,34-36).

The observation that PcCdc25 restored the DNA damage (Fig. 6) but not the DNA replication checkpoint (Fig. 7) indicates a dissociation of checkpoint pathways at the level of Cdc25 regulation. In response to IR, PcCdc25-complemented yeast exhibited a decrease in the number of cells passing mitosis and an increase of cells in G2 (elongated phenotype). While the DNA damage checkpoint is dampened in fission yeast harboring a S99A mutation in Cdc25 (34), PcCdc25 restored the checkpoint even though it lacks the appropriate context of this regulatory site found in S. pombe Cdc25. Although PcCdc25 can restore the checkpoint response to IR (Fig. 6), following treatment with HU, PcCdc25-complemented yeast exhibited an 8-fold increase in mitotic cut cells when compared to untreated cells. The impaired S/M checkpoint elicited by PcCdc25
in response to inhibitors of DNA replication is consistent with that observed in yeast containing a Cdc25 protein in which the 14-3-3 binding/Chk1 and Cds1 phosphorylation sites have been mutated (SpCdc25-S3) (31). Although Chk1 appears to be the major kinase involved in the response to DNA damage and Cds1 in the incomplete DNA replication checkpoint, they both appear to phosphorylate Cdc25 at the same residues (S99, S192, S359) in fission yeast (34).

The definitive role of the three Chk1/Cds1 phosphorylation sites (S99, S192, S359) has yet to be clarified. While Zeng et al. (1998) (36) analyzed the HU-induced S/M checkpoint with a Cdc25 containing Ser→Ala mutations of the three sites (SpCdc25-S3), they did not dissect the role of each site individually in maintaining the arrest nor was the response to IR examined. Although a SpCdc25-S99A mutant generated by Furnari et al. (1999) (34) was found to impair both the S/M and G/2 checkpoints, the role of the other two mutations was not determined. Moreover, results from both studies suggest that there may be other Cdc25 sites weakly phosphorylated by Cds1 or Chk1. It remains unclear whether these additional sites have functional significance. Finally, the finding that (i) these Cdc25 mutations do not completely abolish the checkpoints; and (ii) Mik1 and Wee1 kinases phosphorylate and inhibit Cdc2 activity in response to DNA damage and unreplicated DNA (36,58,59), indicates that multiple mechanisms cooperate with Cdc25 to maintain genomic integrity.
It is surprising that PcCdc25 would only restore one checkpoint pathway since cell cycle proteins are highly conserved and can function normally in heterologous systems. Since PcCdc25 complements growth in fission yeast, the host machinery must recognize sites of control in the primary sequence and secondary structure of PcCdc25. Furthermore, this differential control cannot easily be explained by the fact that PcCdc25 is over-expressed in yeast (due to the nmt promoter) in that cdc25-22 yeast containing over-expressed SpCdc25 maintain both checkpoints. The data provide initial evidence for distinct domains on Cdc25 family members or other unknown checkpoint regulatory sites which differentially regulate the response to IR and HU. Further studies will identify the sequence(s) controlling this differential response in checkpoint control and determine the manner which Chk1, Cds1 and/or Rad 24 interact with PcCdc25.

Our current studies continue to be focused on the role of the P. carinii Cdc25 homolog in regulating the cyst/trophic form transitions. The data presented (Fig. 3B) show that PcCdc25 is expressed in both life forms. We hypothesize that PcCdc25 will have a vital role in regulating organism replication and life cycle transitions. Furthermore, Cdc25 gene expression in the trophic forms provides evidence for the model in which trophic forms might undergo their own mitotic cycle. Although these studies are limited by the ability to genetically manipulate the organism, the further development of an
axenic culture will be necessary to address this problem. It is anticipated that further studies of the *P. carinii* Cdc25 homolog in the context of life cycle progression will provide new insights to understanding *P. carinii* pathogenesis and Cdc25 biology in checkpoint control.
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Fig. 1. **PcCdc25 PCR product hybridizes to Pneumocystis carinii chromosomes.** Lanes 1-4: Four preparations of *P. carinii* chromosomes separated by CHEF. *A*, Ethidium bromide stained agarose gel of *P. carinii* chromosomes. *B*, Autoradiograph of the corresponding nitrocellulose blot hybridized with the 280 bp product amplified from *P. carinii* genomic DNA.

Fig. 2. **Comparison of the predicted amino acid sequence from PcCdc25 with other Cdc25 family members.** Sequences from the *P. carinii* Cdc25 cDNA (Accession #AF098935), *S. pombe* Cdc25 (#M13158), *S. cerevisiae* Cdc25 homolog, MIH1 (#J04846), Human Cdc25C (#4502706), and rat Cdc25B (#D16237) were obtained from the GenBank database. Alignments were performed with the Baylor College of Medicine Boxshade program. Block boxes indicate conserved identical amino acids and gray boxes indicate homologous amino acids.

Fig. 3. **Pc cdc25+ transcript is specific for Pneumocystis carinii.** A, RT-PCR was performed with primers made near the 5 end of *P. carinii* cdc25+, rat cdc25+, and *S. pombe* cdc25+. Templates are as follows: (U) uninfected rat lung, (I) *P. carinii*-infected rat lung, (Pc) isolated *P. carinii*, and (Sp) *S. pombe*. The cDNA templates were made from 1 µg of total RNA primed with oligo d(T) primers. The same template was used for each of the different primer sets and the appropriate size fragment was amplified from each condition. *P. carinii* cdc25+, 640 bps; rat cdc25+, 652 bps; and *S. pombe* cdc25+,
604 bps. B, Northern blot analysis of 5 µg total RNA isolated from the trophic and cyst forms of P. carinii. The ethidium bromide stained gel (EtBr) shows the ribosomal subunits of P. carinii. The blot was hybridized with the radiolabeled 280 bp amplicon.

**FIG. 4. PcCdc25 exhibits phosphatase activity.** The velocities of PcCdc25 ( ), PcCdc25-C432A catalytic site mutant ( ), SpCdc25 ( ), and HuCdc25C (inset) were compared in a standard V versus [S] plot with p-nitrophenol phosphate as the substrate. \( V_{\text{max}} \) and \( K_m \) values were obtained from fitting the data to the Michaelis-Menten equation. The data represent the mean and +/- standard error of samples from two different GST preparations each analyzed in duplicate.

**FIG. 5. PcCdc25 rescues the S. pombe temperature-sensitive Cdc25 mutant.** *S. pombe* cdc25-22 cells were transfected with the thiamine repressible vector alone (1), pREP-SpCdc25 (2), pREP-PcCdc25 (3), or pREP-PcCdc25-C432A (4). An equal number of cells were plated on all conditions. A, Transformants grown at the permissive temperature of 25°C. B, Transformants grown at the permissive temperature with the addition of 25µM thiamine. C, Transformants grown at the restrictive temperature of 35°C. D, Transformants grown at the restrictive temperature with the addition of 25µM thiamine.

**FIG. 6. The DNA damage checkpoint is restored in PcCdc25 complemented cdc25-22**
cells. A, Photomicrographs of the cdc25-22:SpCdc25 cells exposed to 200Gy of radiation, unexposed cdc25-22:PcCdc25 cells, and cdc25-22:PcCdc25 cells exposed to 200Gy of radiation. After G1 release, cells were exposed to IR versus unexposed, fixed after eight hours, and stained with DAPI for fluorescence microscopy analysis. Each panel was taken at 1000X magnification and the white bars represent 10 microns. The arrows indicate septated cells which represent dividing, non-arrested cells. B, Quantitative analysis measuring the number of septated cells following radiation exposure or no treatment. At indicated time points, the cells were fixed and mounted. The graph on the right represents PcCdc25-complemented clones and the graph on the left represents SpCdc25-complemented clones. The % septated cells were determined in unexposed cultures ( ) and IR-exposed cultures ( ). Numbers were derived from two clones analyzed in four separate experiments with at least 100 cells counted per time point.

Fig. 7. PcCdc25 does not restore the DNA replication checkpoint. A, Photomicrographs of the cdc25-22:SpCdc25 cells grown in the presence of 12mM hydroxyurea (HU), untreated and HU-treated cdc25-22:PcCdc25 cells. Asynchronous cultures were grown in either the presence or absence of 12mM hydroxyurea, fixed after eight hours, and stained with DAPI. The arrows indicate yeast that exhibit the mitotic cut phenotype representing non-arrested cells. B, Quantitative analysis measuring the number of mitotic cut cells in the presence HU. The white bar represents PcCdc25-complemented clones and the black bar represents SpCdc25-clones. At the indicated time points, the
cells were fixed and mounted for fluorescence microscopy. The % mitotic cut cells were quantified from cells from HU-treated $\textit{cde25-22: PcCdc25}$ and $\textit{cde25-22: SpCdc25}$. Four separate experiments were performed from two clones from each group with at least 100 cells counted at each time point.
Figure 2
Figure 3

A

<table>
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<tr>
<th></th>
<th>P. carinii cdc25+ specific primers</th>
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B

- Cyst
- Troph

Blot
EtBr
Figure 4
Figure 6

A

SpCdc25 + IR  PccCdc25  PccCdc25 + IR

B

% Separated


Differential regulation of growth and checkpoint control mediated by a Cdc25 mitotic phosphatase from Pneumocystis carinii

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