Pneumocystis carinii Inhibits Cyclin-dependent Kinase Activity in Lung Epithelial Cells

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Abstract

Pneumocystis carinii remains an important cause of pneumonia in patients with AIDS. Attachment of the organism to epithelial cells is a central event in establishing infection, impairing the growth potential of lung epithelial cells and thereby slowing repair. In light of investigations documenting a central role for cyclin-dependent kinases in controlling the cell cycle, we addressed the hypothesis that P. carinii inhibits epithelial cell growth by interfering with host epithelial cyclin-dependent kinase (cdk) activity. We observed that P. carinii significantly impaired growth of cultured mink lung epithelial cells, with effects observed after 48–72 h of treatment. However, the kinase activity associated with p34cdc2 or p33cdc2 was maximally inhibited as early as 24 h after P. carinii exposure. The inhibitory effect on cyclin-dependent kinase activity was mediated by the trophozoite form of P. carinii, in that highly purified trophozoites exerted marked inhibition of p34cdc2 activity. Growth impairment was similarly preceded by P. carinii–induced alteration in the state of epithelial cell p34cdc2 phosphorylation, with no change in p34cdc2 or p33cdc2 protein levels. These data strongly suggest that the antiproliferative activity of P. carinii on respiratory epithelium is mediated in part through modulation of the host cell cycle machinery. (J. Clin. Invest. 1998. 101:1148–1155.) Key words: Pneumocystis carinii • epithelium • cyclin-dependent kinase • cell cycle

Introduction

Pneumocystis carinii remains an important cause of life-threatening pneumonia in immuno-compromised patients, particularly in those with AIDS, hematological or solid malignancies, organ transplantation, or inflammatory conditions treated with immunosuppressive agents or chronic corticosteroid therapy (1–5). Severe P. carinii pneumonia in patients with AIDS is associated with diffuse lung injury, representing the most frequent cause of acute respiratory failure requiring admission to the intensive care unit in these patients (6–8). In addition, recovery from P. carinii pneumonia is frequently prolonged, reflecting impairment of normal lung reparative mechanisms during this disorder. Despite this fact, pathogenic mechanisms of lung injury and suppressed lung repair remain poorly understood during this infection.

Recent investigations indicate that attachment of P. carinii to lung epithelial cells is a central component of the organism’s life cycle (9–12). P. carinii trophozoites attach to lung epithelial surfaces by closely approximating their membranes with the host cell surface, without fusion or internalization of the organism (12, 13). In vitro experiments indicate that attachment of P. carinii to host cells promotes life cycle completion and proliferation of the organism (14, 15). Several cell lines have been reported to support P. carinii proliferation in culture, including Mv1Lu mink lung epithelial cells, A549 lung carcinoma cells, and HEL299 lung fibroblasts (16–19). Additional work in our laboratory further indicated that P. carinii attachment impairs the growth potential of cultured A549 lung epithelial cells in a process requiring cytoskeletal rearrangement of the organism (9). Similar impairment of lung cell proliferation in vivo may retard reepithelialization, and hence slow lung repair of denuded alveolar surfaces during severe P. carinii pneumonia.

The mechanisms by which P. carinii impairs lung cell proliferation are largely unknown, but very likely involve alterations in expression and/or activity of the host cell cycle machinery. Of particular importance are the cyclin-dependent kinases (cdk)1 p34cdc2 and p33cdc2, serine/threonine kinases required for the transition from G2 to M and for entry into S phase from G1 (20, 21). Regulated cdk activity mediates normal cell cycle progression, and has been reported to mediate cell cycle arrest induced by a number of cytokines such as TGF-β (22–24). Mink lung epithelial cells (Mv1Lu, CCL64) have proven to be a particularly useful cell line to evaluate regulation of cdk activity (22, 25). The activity of p34cdc2 and p33cdc2 is carefully regulated by specific activating and inhibitory phosphorylations (26–29). For instance, phosphorylation of p34cdc2 at threonine 161 by a cdk-activating kinase (CAK) is required for activation, while phosphorylation at threonine 14 or tyrosine 15 is inhibitory (28, 29). In that regard, associated alterations of epithelial cell cdk activity during P. carinii pneumonia have not been previously evaluated.

This investigation, therefore, was undertaken to address the following issues: (a) to determine the extent to which P. carinii inhibits proliferation of mink lung epithelial cells in culture; (b) to evaluate whether P. carinii–induced inhibition of lung epithelial growth is preceded by alterations in host cell cyclin-dependent kinase activity; and (c) to investigate potential mechanisms associated with any alteration in lung epithelial cell cdk activity. We report herein that P. carinii impairs...
growth of mink lung epithelial cells after 48–72 h of treatment. This impairment of growth is temporally preceded by a reduction in p34cdc2 and p33cdc2 kinase activity mediated by the trophozoite form of \textit{P. carinii}. We further provide evidence that this reduction in p34cdc2 activity occurs through modification in p34cdc2 phosphorylation with no detected change in steady-state protein levels.

**Methods**

**Materials.** All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Mink lung epithelial cells (Mv1Lu, CCL64) were obtained from the American Type Culture Collection (Rockville, MD) and grown at 37°C in DMEM supplemented with 10% FBS (Summit Biotechnologies, Ft. Collins, CO). [3H]Thymidine, [$^{32}$P]Orthophosphate were purchased from NEN Research Products (Boston, MA). A rabbit polyclonal antibody to the COOH terminus of the cdc2 kinase (p34cdc2) was generated as previously reported (23). Rabbit polyclonal antibody to p33cdc2 was the generous gift of Dr. W.J. Pledger (H. Lee Moffitt Cancer Center Research Institute, Tampa, FL.) Ciprofloxacin was kindly provided by Miles Pharmaceuticals, Inc. (West Haven, CT).

**Preparation of Pneumocystis carinii.** All investigations were approved by the institutional animal care and use committee. Harlan Sprague-Dawley rats were immunosuppressed with dexamethasone and pneumonia produced by transtracheal injection with \textit{P. carinii} (9, 30). Pathogen-free rats were given drinking water containing dexamethasone (2 mg/liter), tetracycline (500 mg/liter), and nystatin (200,000 U/liter) and fed a low-protein diet (8%; Teklad Premier Laboratory Diets, Madison, WI). Each week, the animals received oral ciprofloxacin (0.45 g/liter) on two consecutive days to reduce the incidence of contamination, purified at a ratio of 9:1 (trophozoite/cyst). The residual cyst populations are more than 40-fold enriched for trophozoites. The postinfection material was discarded. These preparations contain a mixture of trophozoite and cyst forms in a typical ratio of 9:1 (trophozoites/cysts). To exclude further the possibility of endotoxin contamination, purified \textit{P. carinii} isolates were also assayed for endotoxin using a modified Limulus amebocyte lysate assay (Whittaker M.A. Bioproducts, Inc., Walkersville, MD) with a lower limit of sensitivity of 0.125 U/ml. \textit{P. carinii} preparations shown to inhibit epithelial cell growth were also free of soluble endotoxin using this assay.

**Assessment of epithelial cell cyclin-dependent kinase activity.** To determine the effects of \textit{P. carinii} on cyclin-dependent kinase activity in host epithelial cells, in vitro kinase assays were performed after culture in the presence or absence of the organism. Mink lung epithelial cells (3 × 10⁶) were plated in 4 ml of DMEM containing 10% FBS in 60-mm tissue culture dishes (22 cm²) for 24 h. \textit{P. carinii} were added at the indicated concentrations, and the cells were incubated for an additional 24–48 h. Subsequently, the cultures were harvested by trypsinization (37°C for 10 min), and the cells were pelleted by low-speed centrifugation (400 g for 5 min at 4°C). Under these conditions, the pellet contains the host epithelial cells, while the \textit{P. carinii} remain suspended (9). Cyclin-dependent kinase activity was determined on the cellular pellet as previously described (23, 33). In brief, the cell pellet was lysed on ice in 50 mM Tris (pH 7.4) containing 500 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1% Triton X-100, 50 μg/ml PMSF, 0.1 mM sodium vanadate, and 1 μg/ml leupeptin (final volume of 500–700 μl). The insoluble material was pelleted at 15,840 g for 10 min at 4°C, and total protein concentration was determined using the bichinchoninic acid method (BCA reagent; Pierce Chemical Co., Rockford, IL). 50 μg of total cellular lysate was preclarified for 20 min at 4°C with formalin-fixed Staph A before immunoprecipitation at 4°C for 2 h with a rabbit polyclonal antibody to the COOH terminus of the Cdc2 (p34cdc2) or Cdc2 (p33cdc2) kinase. Antibody complexes were precipitated with protein A sepharose, and in an in vitro kinase assay was performed in a 50-μl reaction consisting of kinase buffer (50 mM Tris, 10 mM MgCl₂, 1 mM DTT, pH 7.4) supplemented with 5 μM cold ATP, 5 μCi [$^{32}$P]ATP, and 100 μg/ml histone H1 for 5 min at 30°C. The kinase reaction was stopped by adding 2X Laemmli buffer, and was boiled for 5 min. Phosphorylated histone H1 was resolved on 10% SDS-PAGE and visualized by autoradiography.

**Determination of p34cdc2 phosphorylation.** The state of activation of cyclin-dependent kinases is carefully regulated by the presence of activating and inhibiting phosphorylations (26–29). Accordingly, additional experiments were performed to evaluate whether interactions of \textit{P. carinii} with epithelial cells resulted in alterations of host p34cdc2 kinase phosphorylation. Mink lung epithelial cells (3 × 10⁶) were plated in DMEM containing 10% FBS for 24 h. \textit{P. carinii} were added, and the cells were cultured for an additional 24 h at 37°C. During the final 4 h of culture, the media were removed and replaced with 1 ml of phosphate-free medium containing 10% dialyzed FBS and 500 μg/ml [$^{32}$P]Orthophosphate. Cells were harvested, and 200 μg of protein was immunoprecipitated with antibodies to p34cdc2 as described above, except that cellular lysis and washes were performed in RIPA buffer (50 mM Tris pH 8.0, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 0.1 mM Na vanadate, 75 μg/ml PMSF, 1149
0.1 TIU/ml aprotinin, 1 μg/ml leupeptin, and 8 mM iodoacetamide in PBS). The phosphorylated p34\textsuperscript{cdk2} was resolved on 10% SDS-PAGE and visualized by autoradiography.

Statistical analysis. All data are expressed as the mean±SEM from multiple experimental runs. Differences in measured variables from experiments containing multiple treatment groups were first assessed using ANOVA. Subsequently, differences among individual data groups were evaluated using Student’s t test. Statistical testing was performed on the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA) using a Macintosh Ici personal computer. Statistical differences were considered to be significant if P was < 0.05.

Results

P. carinii inhibits growth of cultured mink lung epithelial cells. P. carinii infection results in denudation of the epithelial lining in the alveolar space (11, 13). It has been proposed that infection results in denudation of the epithelial cells. P. carinii inhibits growth of cultured mink lung epithelial cells. In that regard, previous studies have shown a similar trend in cellular growth. Moreover, once a cell ratio of 100:1 was attained, a statistically significant decrease in host cellular proliferation was observed. P. carinii causes similar degrees of growth impairment for A549 human lung epithelial cells (9).

P. carinii inhibits p34\textsuperscript{cdk2} and p33\textsuperscript{cdk2} kinase activity and protein level. After overnight incubation, mink lung epithelial Mv1Lu cells (3×10\textsuperscript{5} cells) were cultured in the absence (–) or presence (+) of P. carinii at a ratio of 100:1 for 24 h. The top represents equivalent amounts of cellular protein extracted from P. carinii-treated and –untreated Mv1Lu cell cultures immunoprecipitated with antibodies to p34\textsuperscript{cdk2} (labeled Cdc2) or p33\textsuperscript{cdk2} (labeled Cdk2). The kinase activity associated with the immunoprecipitated products was determined by its ability to phosphorylate the substrate H1 histone. On the bottom, parallel plates were treated as described above, except that total p34\textsuperscript{cdk2} (Cdc2) or p33\textsuperscript{cdk2} (Cdk2) protein levels were examined by SDS-PAGE (10 μg protein each) and immunoblot analysis using antibodies specific for p34\textsuperscript{cdk2} (Cdc2) or p33\textsuperscript{cdk2} (Cdk2) proteins, respectively.

Figure 2. Effect of P. carinii on lung epithelial cell p34\textsuperscript{cdk2} and p33\textsuperscript{cdk2} kinase activity and protein level. After overnight incubation, mink lung epithelial Mv1Lu cells (3×10\textsuperscript{5} cells) were cultured in the absence (–) or presence (+) of P. carinii at a ratio of 100:1 for 24 h. The top represents equivalent amounts of cellular protein extracted from P. carinii-treated and –untreated Mv1Lu cell cultures immunoprecipitated with antibodies to p34\textsuperscript{cdk2} (labeled Cdc2) or p33\textsuperscript{cdk2} (labeled Cdk2). The kinase activity associated with the immunoprecipitated products was determined by its ability to phosphorylate the substrate H1 histone. On the bottom, parallel plates were treated as described above, except that total p34\textsuperscript{cdk2} (Cdc2) or p33\textsuperscript{cdk2} (Cdk2) protein levels were examined by SDS-PAGE (10 μg protein each) and immunoblot analysis using antibodies specific for p34\textsuperscript{cdk2} (Cdc2) or p33\textsuperscript{cdk2} (Cdk2) proteins, respectively.

Figure 1. Growth impairment index of mink lung epithelial cells cultured in the presence of P. carinii. Mv1Lu cells were plated in six-well culture dishes at 2×10\textsuperscript{5} cells/well in DMEM containing 10% FBS. After an overnight incubation at 37°C, P. carinii was directly added to the culture to the indicated ratio of P. carinii/cells. Triplicate wells were counted over the next 3 d. *Cell counts that are significantly different (P < 0.05) from the control wells containing no P. carinii at the same time point.
It is possible that *P. carinii* might suppress cyclin-dependent kinase activity through a generalized toxic effect of the organisms on the lung epithelial cells rather than by a specific effect on the epithelial cell cycle regulatory machinery. Several lines of evidence strongly argue against this possibility. First, although *P. carinii* induced a significant decrease in p34\(^{cd2}\) or p33\(^{cd2}\) activity, the organism did not decrease the levels of either of these proteins; only their functional status (Fig. 2). This result indicates that generalized cellular processes including protein synthesis are intact in cells challenged with *P. carinii*. Furthermore, mink lung cells continue to divide in the presence of *P. carinii*, albeit at a slower rate, providing further evidence that the cells are not diffusely injured by *P. carinii*. The previous data (Figs. 1 and 2) clearly document an association between decreased p34\(^{cd2}\) and p33\(^{cd2}\) kinase activity and cellular growth arrest. To begin investigating both the causal relationship as well as the associated mechanism(s) mediating this response, we initially focused our studies on the effect of *P. carinii* on host p34\(^{cd2}\) activation because of its fundamental role in cell cycle traverse (26, 28, 35). Since Fig. 1 showed a decrease in Mv1Lu cell growth with increasing amounts of *P. carinii*, we next determined whether a similar *P. carinii* dose dependence would be observed on p34\(^{cd2}\) kinase activity (Fig. 3). At p34\(^{cd2}\) to epithelial cell ratios of < 50:1, no appreciable effect on p34\(^{cd2}\) kinase activity was seen. However, when those ratios exceeded 50:1 (e.g., 75–100:1), a significant decrease in H1 kinase activity was observed. Although these experiments do not show a direct causal relationship, it is of interest that the inhibitory effect of *P. carinii* on p34\(^{cd2}\) kinase activity has a similar dose-dependence as that seen for host cell growth (Figs. 1 and 3).

Attachment of *P. carinii* to lung epithelial cells is necessary for inhibition of epithelial p34\(^{cd2}\) kinase activity. Our prior studies indicate that *P. carinii* attachment to lung epithelial cells is an integral component in establishing infection. Therefore, we next questioned whether *P. carinii* binding to mink lung epithelial cells was necessary for *P. carinii* to suppress epithelial cell p34\(^{cd2}\) kinase activity. We have recently shown that *P. carinii* can be cocultured with lung epithelial cells on the upper chamber of a permeable Transwell membrane (Becton Dickinson, Franklin Lakes, NJ), impairing growth of the organism (14, 15). Although the *P. carinii* organisms are not permitted to bind to the lung cells, they share the same media as the lung cells, and soluble components can pass freely through the membrane (0.4-μM pore size). Therefore, we used this system to address whether binding of *P. carinii* to host cells was necessary for inhibiting mink lung epithelial cell p34\(^{cd2}\) activity. *P. carinii* were cultured either directly on epithelial cell monolayers, or in the upper chamber of a Transwell tissue culture insert that prevents organism attachment to the cells. After 24 h, the epithelial cells were removed and extracted. Equivalent amounts of cellular protein extracted from *P. carinii*-treated and –untreated Mv1Lu cell cultures (100 μg) were immunoprecipitated with antibodies to p34\(^{cd2}\). The kinase activity associated with the immunoprecipitated products was determined by its ability to phosphorylate the substrate H1 histone. *P. carinii*, permitted to directly interact with the cells, caused marked suppression of p34\(^{cd2}\) kinase activity. In contrast, *P. carinii* that were prevented from attaching to the cells by the permeable Transwell membrane had no appreciable effect on mink lung epithelial cell p34\(^{cd2}\) activity.

**Figure 4.** Attachment of *P. carinii* to epithelial cells is necessary for inhibition of epithelial cell p34\(^{cd2}\) kinase activity. Mv1Lu epithelial cells were plated in DMEM supplemented with 10% FBS on 6-well plates. After 24 h of incubation at 37°C, isolated Pneumocystis carinii were added (100:1; *P. carinii* to Mv1Lu cells) either directly onto the cell monolayers or to the upper chamber of a Transwell tissue culture insert that prevents organism attachment to the cells. After 24 h, the epithelial cells were removed and extracted. Equivalent amounts of cellular protein extracted from *P. carinii*-treated and –untreated Mv1Lu cell cultures (100 μg) were immunoprecipitated with antibodies to p34\(^{cd2}\). The kinase activity associated with the immunoprecipitated products was determined by its ability to phosphorylate the substrate H1 histone. *P. carinii*, permitted to directly interact with the cells, caused marked suppression of p34\(^{cd2}\) kinase activity. In contrast, *P. carinii* that were prevented from attaching to the cells by the permeable Transwell membrane had no appreciable effect on mink lung epithelial cell p34\(^{cd2}\) activity.
Isolated \textit{P. carinii} trophozoites mediate inhibition of mink lung epithelial cell p34\(^{cd2}\) kinase activity. Previous studies in human and animal tissues have revealed that the life cycle of \textit{P. carinii} consists of both the more numerous trophozoite forms (1–2 \(\mu\)m), and larger cystic forms (8 \(\mu\)m), containing a characteristic \(\beta\)-glucan–rich wall (11, 13). Adherence of the trophozoite form to the host cell membrane is necessary for completion of the fungal life cycle and subsequent effects on the lung epithelium (9, 10, 14, 15). Our earlier studies indicate that trophozoites adhere to lung epithelial cells by interdigitation of their cellular membranes with those of the host cell (9). Figs. 1–4 document that mixed isolates of trophozoites and cyst forms of \textit{P. carinii} (in a ratio of roughly 9:1 trophozoites/cysts) suppress host cyclin-dependent kinase activity and epithelial cell growth. Next, we further examined whether purified preparations containing essentially only trophozoites could also mediate this process. To investigate this, \textit{P. carinii} isolates were further separated into isolated trophozoite preparations by differential filtration. Examinations of \textit{P. carinii} preparations before and after differential filtration using modified Wright-Giemsa and methenamine silver stains revealed the trophozoite preparations to contain > 99.5% trophozoites.

Subsequently, the effects of isolated trophozoites on p34\(^{cd2}\) activity were determined (Fig. 5). As shown in Fig. 5A, as the ratio of trophozoites to host cells was increased, there was a dose-dependent inhibition in host cell p34\(^{cd2}\) kinase activity on histone H1 substrate in a manner similar to that observed with unfractionated \textit{P. carinii} (Fig. 3). In fact, the inhibitory response with purified trophozoites was approximately 2–3 fold more sensitive when compared with the mixed \textit{P. carinii} cultures containing both cysts and trophozoites (Fig. 3 and Fig. 5, A and B). Again, similar to that observed in Fig. 2 with unfractionated \textit{P. carinii}, the isolated \textit{P. carinii} trophozoite preparations had no detectable effect on total p34\(^{cd2}\) protein levels, even at higher concentrations (Fig. 5 C). Thus, we observed that highly purified \textit{P. carinii} trophozoites exhibit marked inhibition of lung epithelial cell p34\(^{cd2}\) activity, but have no effect on the corresponding level of protein expression.

\textit{P. carinii} alter the phosphorylation state of lung epithelial cell cdc2 kinase. The activity of p34\(^{cd2}\) is regulated by a number of post-translational modifications (22–29, 35). For instance, while phosphorylation of p34\(^{cd2}\) by a CAK on threonine 161 is required for activity (27, 35), phosphorylation on tyrosine 15 is inhibitory (29). Since the previous data (Figs. 2–4) showed an inhibition in kinase activity with no associated change in total protein, this suggested that \textit{P. carinii} might inhibit cdk activity through a post-translational mechanism. To address that question, Mv1Lu cells were metabolically labeled with \(\text{[}^{32}\text{P}\text{]}\)orthophosphate after 24-h treatment in the presence or absence of \textit{P. carinii}, and the effect on p34\(^{cd2}\) phosphorylation was determined (Fig. 6). A shift in p34\(^{cd2}\) protein to a less

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5}
\caption{Effect of isolated \textit{P. carinii} trophozoites on mink lung epithelial cell p34\(^{cd2}\) kinase activity. Mv1Lu epithelial cells were plated as described in Fig. 3 in DMEM supplemented with 10% FBS. After 24 h of incubation at 37°C, highly purified \textit{P. carinii} trophozoites, prepared as described in Methods, were added at the indicated \textit{P. carinii} to Mv1Lu epithelial cell ratio. (A) The p34\(^{cd2}\) (cdc2) kinase activity of the mink lung epithelial cells was assessed after 24 h of \textit{P. carinii} treatment by determining the ability of immunoprecipitated mink lung epithelial cell extracts to phosphorylate the substrate H1 histone. Lane 1 is untreated cycling cultures, lanes 2–6 contain epithelial cells cultured with \textit{P. carinii} trophozoites at \textit{P. carinii}/epithelial cell ratios of 100, 50, 25, 10, and 5:1, respectively. (B) Densitometric analysis of the presented autoradiogram from A with the kinase activity seen in untreated cycling cultures reported as 1.0. (C) Total p34\(^{cd2}\) (labeled Cdc2) protein in samples from A was determined by immunoblot analysis. Lanes a, b, c, and d demonstrate the level of Mv1Lu epithelial cell cdc2 protein level after culture with \textit{P. carinii} trophozoites at ratios of 100, 50, 25, and 5:1, respectively.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Phosphorylation of mink lung epithelial cell p34\(^{cd2}\) is altered by \textit{P. carinii}. Cells were plated as described in Fig. 3 and Methods. After a 24-h incubation in the absence (lane B) or presence (lane C) of a 100:1 ratio of \textit{P. carinii} to Mv1Lu epithelial cells, the culture was metabolically labeled with \(\text{[}^{32}\text{P}\text{]}\)orthophosphate during the last 4 h of culture, and harvested. Equal cellular protein was immunoprecipitated with antibodies to p34\(^{cd2}\) and analyzed by SDS-PAGE and autoradiography. The more mobile bands (lane B) indicate phosphorylation of threonine 161 associated with active cdc2, while the less mobile band (lane C) is consistent with phosphorylation of tyrosine 15 known to inactivate cdc2 kinase activity. Lane A contains a molecular weight standard at 30 kDa.}
\end{figure}
mobile form was observed after treatment with *P. carinii* organisms. This decrease in p34\(^{cd2}\) migration has been previously shown to correspond with the inactive tyrosine 15 phosphorylated form of the protein (29), and is consistent with the lack of functional kinase activity observed after *P. carinii* treatment (Figs. 2–5).

**Discussion**

*P. carinii* causes diffuse alveolar damage and respiratory failure with prolonged lung repair in a subset of patients with pneumonia. Mechanisms mediating this lung injury are not fully known, but likely incorporate both direct effects from the organisms as well as the adverse effects of neutrophilic lung inflammation that accompanies severe infection (1, 13, 36). Recovery and repair of lung injury in these patients is frequently prolonged, requiring several weeks of intensive therapy (6). *P. carinii*–induced impairment of lung epithelial cell growth may be responsible for the sluggish repair of clinically observed lung injury during *P. carinii* pneumonia. Further understanding of *P. carinii*–induced alterations in lung cell proliferation may yield additional new insights to enhance lung repair during this infection. To that end, the data in the present manuscript indicate that *P. carinii* inhibits proliferation of mink lung epithelial cells. Furthermore, epithelial growth inhibition is preceded by *P. carinii*–induced alterations in cyclin-dependent kinases that regulate cell cycle traverse. As early as 12–24 h after addition of *P. carinii*, mink lung epithelial cells exhibited alterations in p34\(^{cd2}\) and p33\(^{cd2}\) kinase activity and p34\(^{cd2}\) phosphorylation necessary for normal progression through the cell cycle. As anticipated, molecular inhibition of cell cycle progression occurred before the observed reduction in cell growth occurred between 48 and 72 h. These findings strongly suggest that alterations of cyclin-dependent kinase activity represent a potential molecular target mediating the antiproliferative activity of *P. carinii* on host respiratory epithelia.

It is of interest that the observed effect of *P. carinii* on lung epithelial cell cyclin-dependent kinase activity exhibited a threshold effect. At *P. carinii*/epithelial cell ratios of \(<50:1\), no appreciable effect on p34\(^{cd2}\) kinase activity was seen. However, when those ratios exceeded 50:1, a significant decrease in H1 kinase activity was observed. A similar dose dependence was documented for host cell growth. Stepwise inductions of cell activation have been observed in other studies of *P. carinii* (37). It should be noted that *P. carinii* trophozoites are quite small (\(\sim 1–2\) \(\mu\)m in diameter), while the mink lung epithelial cells are appreciably larger (\(\sim 30\) \(\mu\)m in diameter). Our prior studies indicate that 35–40% of *P. carinii* bind to epithelial cells in culture (9). These data also indicate that direct attachment of *P. carinii* to lung cells is necessary for the observed reduction in mink lung epithelial cell cdk activity (Fig. 4). Therefore, a concentration of 50 *P. carinii* to each lung epithelial cell is consistent with the majority of the epithelial cell surface–binding *P. carinii* organisms.

Previous studies have supported the contention that the attachment of *P. carinii* to host epithelial cells is of differential consequence to the host and the organism. Our prior studies in A549 cells similarly indicated that *P. carinii* could impair the growth of a transformed lung epithelial cell line in culture (9). That study further demonstrated that the effect of *P. carinii* on the host required direct contact of the organism, and was not transmissible by conditioned media. Furthermore, prior inhibition of *P. carinii* with agents that inhibited cytoskeletal functions prevented binding of the organisms to the lung cells, and reversed epithelial growth impairment (9, 11). Unfortunately, cytoskeletal agents themselves alter basal levels of epithelial cell cdk activity, and were therefore not testable in the current study. Previous ultrastructural studies have further suggested that lung epithelial cells display vacuolization in regions of *P. carinii* attachment (12, 13). Taken together, these studies indicate that binding of *P. carinii* to lung epithelial cells exerts substantive deleterious effects upon the host.

In contrast to the previously described deleterious effects on the host, most investigations indicate that attachment of *P. carinii* to the alveolar epithelium is of net benefit to the organism. Binding of *P. carinii* to host epithelial cells has been consistently observed in lungs from both humans and animals with *P. carinii* during initiation of pneumonia (12, 13). In addition, proliferating *P. carinii* closely binds to epithelial cells in tissue culture in a manner closely mimicking that observed in vivo (10, 16). Recent studies additionally demonstrate that binding of *P. carinii* to epithelial cells promotes life cycle completion and organism proliferation (14, 15). What the organism derives from this intimate interaction with host cells is currently a matter of conjecture, although electron micrographic analyses have suggested that the epithelium provides a surface on which *P. carinii* conjugation and genetic exchange may occur (38). Moreover, the alveolar epithelial surface is particularly rich in host proteins and surfactant components that may provide unique moieties absorbed and used by the organisms (39–44). An additional possibility is suggested by the recent report that *Trypanosoma cruzii* uses host cell signal transduction pathway intermediates (45). Whether *P. carinii* uses a similar signaling mechanism is presently unknown. However, we have performed preliminary studies examining whether *P. carinii* uses a signaling pathway similar to that activated by the growth inhibitory cytokine TGF\(\beta\). In contrast to the results observed for *Trypanosoma cruzii*, adding *P. carinii* to Mv1Lu cells suppressed luciferase activity from a TGF\(\beta\)-regulated reporter construct (unpublished observations). Clearly, additional investigations will be necessary to explore these hypotheses further.

Our current study reveals a potential mechanism by which *P. carinii* binding inhibits cell cycle progression in the host epithelium. The eukaryotic cell cycle is precisely regulated by a number of cell division cycle control proteins of which p34\(^{cd2}\) is of primary importance. This protein serine/threonine kinase is needed for the transition from G2 to M, and for entry into S phase from G1 at a site known as START in fungi. Activity of the p34\(^{cd2}\) protein is sequentially controlled by a variety of positive- and negative-acting signals. For instance, activation of the kinase requires association with a regulatory cyclin molecule as well as both removal of negative-acting phosphorylations (e.g., tyrosine 15) and addition of positive-acting phosphorylations (e.g., threonine 161/167; 29). Cdc25, a tyrosine phosphatase, is responsible for removal of inhibitory phosphorylation on tyrosine 15 (29, 46, 47). Our current study indicates that attachment of *P. carinii* to respiratory epithelial cells alters both the phosphorylation state (and hence the protein kinase activity of lung epithelial cell p34\(^{cd2}\)) and the kinase activity of p33\(^{cd2}\) (Figs. 2 and 6). Most importantly, these modifications occur independently of any change in total cdk protein level without any decrease in epithelial cell viability, and occur significantly earlier than the observed suppression in lung cell.
growth (Figs. 1 and 2). The finding that the inhibitory effects
on cdk activity precedes the observed decrease in lung cell
growth by 24 h indicates (a) that the lack of cyclin-dependent
kinase activity is not just a reflection of the growth inhibition
state; and (b) that the inhibitory effects on these cell cycle con-
trol proteins may be the biochemical targets with which P. carinii
modulates proliferation of the host epithelium.

The exact molecular mechanisms by which P. carinii
decreases epithelial cell cyclin–dependent kinase activity and
proliferation are not yet fully known. Our data indicate in part
that alteration of p34cdc2 activity is accompanied by changes in
its state of phosphorylation. Other studies also indicate that
cyclin-dependent kinase activity can be regulated by potent in-
hibitory proteins, most notably the Cip/Kip and Ink4 families
of inhibitors (48–50). Information is not yet available as to the
expression and activity of such cyclin-dependent kinase inhibi-
tory proteins in lung epithelial cells after interaction with P. carinii.

Our findings of the suppressive effects of P. carinii on lung
epithelial cell cycle progression through decreased epithelial
 cell p34cdc2 activity may further explain the sluggish repair of
injured pulmonary tissue and slow restoration of respiratory
function that has been observed in severe Pneumocystis pne-
umonia in man (6). Despite effective drugs, severe P. carinii infec-
tion can be associated with diffuse alveolar damage, wide-
spread loss of respiratory epithelium, and markedly delayed
recovery of normal gas exchange. Ultrastructural studies indi-
cate that P. carinii binds predominantly to Type I cells that
cover the majority of the alveolar epithelial surface area (51).
Additional studies also reveal that P. carinii adhere in a similar
manner to progenitor type II cells (12). Type I cells are termi-
nally differentiated cells incapable of further division. Reepi-
thelialization of injured alveolar surfaces, therefore, requires
proliferation and differentiation of Type II cells. Binding of P. carinii
to proliferating alveolar epithelial cells with impairment of
cell cycle progression would therefore impede restoration of
a functioning epithelial surface and return of normal gas ex-
change.

Finally, we observed that the inhibitory effects of P. carinii
on cdk activity were mediated by the isolated trophozoite form
of the organism’s life cycle (Fig. 4). This result supports earlier
observations that trophozoites are the life form that actively
attaches to alveolar epithelium through membrane reorganiza-
tion (9, 11, 13). Additional investigations indicate that P. carinii
trophozoites themselves contain greater organismal Cdc2 activity than do cysts, and that trophozoite binding to epithe-
elial cells promotes P. carinii proliferation (10, 14–16, 52).
Thus, P. carinii trophozoites appear to represent the more
metabolically active replicative form of the organism.

In summary, we have demonstrated that P. carinii signifi-
cantly inhibits the growth of a cultured lung epithelial cell line.
Growth inhibition of mink lung epithelial cells was temporally
preceded by decreases in the protein kinase activity of p34cdc2
and p33cdc2 independently of changes in epithelial cell viability
or total cdk protein levels. Furthermore, a potential mecha-
nism for the decrease in p34cdc2 kinase activity is suggested by
the apparent increase in the inhibitory phosphorylation at ty-
rrosine 15 after P. carinii treatment. This ability of P. carinii to
impair lung epithelial growth may further delay healing and re-
pair of alveolar injury during severe infection. Understanding the
mechanisms that control epithelial cell growth suppression may
ultimately yield new potential P. carinii pneumonia therapies.

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