**Pneumocystis carinii** remains a persistent cause of severe pneumonia in immune compromised patients. Recent studies indicate that *P. carinii* is a fungal species possessing a glucan-rich cyst wall. Pneumocandin antagonists of β-1,3-glucan synthesis rapidly suppress infection in animal models of *P. carinii* pneumonia. We, therefore, sought to define the molecular mechanisms of β-glucan cell wall assembly by *P. carinii*. Membrane extracts derived from freshly purified *P. carinii* incorporate uridine 5'-diphosphoglucose into insoluble carbohydrate, in a manner that was completely inhibited by the pneumocandin L733-560, an antagonist of Gsc-1-type β-glucan synthetases. Using degenerative polymerase chain reaction and library screening, the *P. carinii* Gsc1 catalytic subunit of β-1,3-glucan synthetase was cloned and characterized. *P. carinii* gsc1 exhibited homology to phylogenetically related fungal β-1,3-glucan synthetases, encoding a predicted 214-kDa integral membrane protein with 12 transmembrane domain structure. Immunoprecipitation of *P. carinii* extracts, with a synthetic peptide anti-Gsc1 antibody, specifically yielded a protein of 219.4 kDa, which was also capable of incorporating 5'-diphosphoglucose into insoluble glucan carbohydrate. As opposed to other fungi, the expression of gsc-1 mRNA is uniquely regulated over *P. carinii*'s life cycle, having minimal expression in trophic forms, but substantial expression in the thick-walled cystic form of the organism. These results indicate that *P. carinii* contains a unique catalytic subunit of β-1,3-glucan synthetase utilized in cyst wall formation. Because synthesis of β-1,3-glucan is absent in mammalian cells, inhibition of the *P. carinii* Gsc1 represents an attractive molecular target for therapeutic exploitation.

**Classification of Pneumocystis carinii** as a fungus revolutionized study of this organism, which continues to cause life-threatening pneumonia in immune-compromised patients with AIDS, malignancy, and organ transplantation (1–5). Studies of *P. carinii* in infected lung indicate its life cycle alternates between smaller trophic forms and thick-walled cysts (6–8). The origin of *P. carinii* cysts remains controversial, but it has been postulated they arise from sexual conjugation, analogous to ascomycetous fungi (9, 10). The mechanisms of cyst wall assembly by *P. carinii* are not well known, although recent studies reveal that these walls are largely composed of β-glucans, glycoprotein A, and chitins (11–16).

β-Glucans are glucose homopolymers composed mainly of a β-1,3-linked carbohydrate core, with variable amounts of β-1,6- and β-1,4-linked glucose side chains (17–19). Glucans represent principal components of cell walls in fungi related to *P. carinii*. Ultrastructural investigations demonstrate an electron-lucent layer unique to the cystic form of *P. carinii*, which is specifically degraded by β-1,3-glucanases (16). Additional studies with specific β-1,3-glucan antiserum also localize glucan to the walls of cysts (20). β-1,3-Glucan has been detected in bronchoalveolar lavage from patients with *P. carinii* pneumonia (21). Furthermore, *P. carinii* β-glucans also represent important epitopes recognized by host cells (11, 22). *P. carinii* β-glucans interact with alveolar macrophages mediating phagocytic uptake of the organism and lung inflammatory responses (11). Subsequent glucan-mediated influx of neutrophils into the lung is an important contributor to respiratory impairment during this infection (23–25).

Fungal β-glucans are assembled by a multisubunit enzyme complex within the organism’s cell membrane. Gsc-1 proteins mediate the polymerization of uridine 5'-diphosphoglucose (UDP-Glc) into the insoluble β-1,3-glucan core required for cell wall assembly (26). Glucan synthetases are generally encoded by gsc-1 genes, which generate a 210-kDa catalytic protein in Saccharomyces cerevisiae and comparable proteins in other fungi (27–32). Glucan synthetase activity by Gsc-1-type proteins is specifically inhibited by pneumocandin and echinocandin class compounds (32).

Because mammalian cells do not possess glucan biosynthetic pathways, cell wall assembly represents an attractive target for the treatment of fungal infection. It is particularly noteworthy that pneumocandin inhibitors of β-glucan synthesis have been shown to rapidly inhibit *P. carinii* growth in rodent models (33–36). Such studies provide evidence of the importance of β-glucan generation during life cycle progression of this organism. Despite the considerable importance of β-glucan assembly in life cycle expression of this organism, in immune recognition during infection, and as a potential therapeutic target for pneumonia, the mechanisms of β-1,3-glucan assembly by *P. carinii* are not yet fully understood.

The current investigation was undertaken to accomplish the following: 1) to establish whether *P. carinii* cell wall assembly occurs through action of a Gsc-1 protein mediating β-1,3-glucan synthesis; 2) to clone and characterize the respective gsc-1
encoding this activity in *P. carinii*; and finally 3) to evaluate expression of *P. carinii* gsc-1 over the life cycle of the organism.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-733,560, a semisynthetic analog of pneumocandin B₀ was provided by Dr. H. Proffus-Juchelka of Merck Research Laboratories, Rahway, NJ. The *P. carinii* genomic DNA library in λgt11 bacteriophage was supplied by Dr. J. R. Stringer, University of Cincinnati. This rat-derived *P. carinii* library was derived from *P. carinii* t. sp. *carinii* (37). Nitrocellulose membranes containing *P. carinii* chromosomes separated by contour-clamped homogenous field electrophoresis were the gift of Dr. M. T. Cushion, University of Cincinnati (38). *P. carinii* Cell Wall and Membrane Isolation and Assessment of Glucan Synthetase Activity—*P. carinii* pneumonia was induced in Harlan Sprague-Dawley rats by immune suppression with dexamethasone and transtracheal inoculation, as we previously reported (39, 40). *P. carinii* were purified by homogenization and filtration through 15-μm filters to remove lung cells (41, 42). To determine whether *P. carinii* contained glucan synthetase activity, cell wall membrane fractions were isolated and assessed for incorporation of UDP-[14C]glucose into carbohydrate (43). One gram of *P. carinii* was suspended in 50 mM Tris-HCl, 150 mM NaCl buffer with 1 mM EDTA and sonicated on ice. The supernatant was centrifuged (55,000 *g* for 45 min) to separate membranes, and the pellet was washed once with 50 mM Tris-HCl buffer containing 1 mM EDTA and 1 mM mercaptoethanol (pH 7.5; Buffer A). The pellet was then resuspended in Buffer A containing 33% glycerol. A final assay mixture (40 μl of total volume) was prepared with 5 mM UDP-[14C]glucose (250,000 cpm/μmol), 7.5 mM Tris-HCl (pH 7.5), bovine serum albumin (1 mg/ml), 25 mM KH, 1 mM EDTA, 20 μM GTP, and enzyme (20–35 μl of *P. carinii* membrane suspension). The reaction mixtures were incubated for 4 h at room temperature. Aliquots were spotted onto glass filter discs, washed twice with 20% trichloroacetic acid, washed twice with acetone, and counted. The background cpm (buffer alone) was subtracted from the data. Additional assays were performed in the presence of the pneumocandin B₀ analog L-733,560 (0–10 μM), an agent that inhibits β-1,3-glucan synthetase through the proteins (34).

Cloning of *P. carinii* Genomic and cDNA Sequences Encoding Gsc-1—A degenerate PCR strategy was utilized to clone *P. carinii* sequences encoding the putative Gsc-1 synthetase (44–46). PCR amplification of *P. carinii* genomic DNA was performed using degenerate primers designed from conserved amino acid sequences found in β-1,3-glucan catalytic subunits from Aspergillus nidulans (fksA) and *S. cerevisiae* (FKS1, FKS2) (27, 28, 47–49). The codon bias for *P. carinii* was used to limit degeneracy in the third codon position (46). The primer sequences were: 5'-CA(CT)/GT GA(CT)/TG GA(CT)/TG TA(CT)/AT AA(CT)/TG GG(A/C/G/T) GG(A/C/G/T) GA-3' and 5'-AC(CT)/AG TA(CT)/TG TA(CT)/AG GG(CT)/GC GC(CT)/GC TC(A/G)/CT CCC GA(A)/G CA-3'. *P. carinii* genomic DNA served as the template. An initial 5-min hot start at 94 °C was followed by 35 cycles of 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s, for a total of 72 °C 15-min extension. A single 324-bp amplicon was generated. Sequence comparisons to GenBank™ were performed using the Basic Local Alignment Search Tool (BLAST) genetic analysis program (National Center for Biotechnology Information) (50). The 324-bp amplicon was used to obtain genomic DNA sequence by probing a rat-derived *P. carinii* genomic DNA library in λgt11 (37). The initial amplicon was represented near the 5′-region of Gsc-1. Sequences from the 3′-regions of these genomic sequences were used to further probe this library for four additional genomic clones. By identifying overlapping sequences from these four clones, the entire 6029-bp genomic region of the *P. carinii* gsc-1 gene was isolated. *P. carinii* gsc-1 cDNA sequences were subsequently isolated by reverse transcriptase-PCR of total RNA extracted from freshly purified *P. carinii* by guanidium isothiocyanate and using overlapping primer pairs covering the entire *P. carinii* gsc-1 genomic sequence. PCR amplification products were subcloned into pCRII and sequenced.

Southern and Chromosomal Hybridization of *P. carinii* Gsc-1—To verify that the PCR product was of *P. carinii* origin, the 324-bp ampli- con was hybridized both to digested *P. carinii* genomic DNA and sepa- rated *P. carinii* cell wall membrane isolates. The hybridization was labeled using [α-32P]ATP (by the random primer method (Rediprime System, Ame- rsham Pharmacia Biotech). 20 μg of genomic DNA was digested with the restriction enzymes specified, separated on a 1% agarose gel, and trans- ferred to nitrocellulose (51). The membranes were incubated with the probe (1.5 × 10⁶ cpm/ml) at 60 °C over 1 h, washed three times at room temperature for 40 min in 2× SSC buffer containing 0.05% SDS, washed twice at 50 °C for 40 min in 2× SSC buffer containing 0.1% SDS, and examined by autoradiography. In parallel, the 324-bp ampli- con was hybridized to *P. carinii* chromosomes separated by contour- clamped homogenous field (CHEF) electrophoreses blot as described previously (38).

**Antibody Generation to the Predicted *P. carinii* Gsc-1 Protein and Immunoblotting of *P. carinii* Extracts**—To determine whether a Gsc-1 protein was present in *P. carinii* membranes, a polyclonal antibody was generated to the predicted amino acid sequence of *P. carinii* Gsc-1. A 15-residue peptide (EEMTPTEESPYNPNE), corresponding to amino acids 1196–1210, was chosen for its high specificity and antigenic profile (CCG software, Oxford Molecular Co., Madison WI) (52). Polyclonal antiserum against this peptide were generated in rabbits, and the antibody was linked immunogen immobilized on to the activated IgG antibody was isolated by purification over protein A-Sepharose (45). To isolate the *P. carinii* Gsc-1 protein, *P. carinii* cell wall membrane isolates (~50 μg) were immunoprecipitated with either 100 μg/ml of the anti-Gsc-1 antibody or non-immune IgG using protein A-Sepharose. Precipitated proteins were subsequently separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellu- loss. The presence of *P. carinii* Gsc-1 was assessed by immunoblotting with anti-Gsc-1 or non-immune IgG (100 μg/ml) for 1 h. To assess the abundance of Gsc-1 over the life cycle of *P. carinii*, isolated organisms were further separated into cystic and trophic forms by differential filtration through 3-μm filters, which allow the passage of trophic forms, but retain cysts. This separation procedure yielded 99.5% pure trophic populations and preparations that were 40-fold enriched in *P. carinii* cysts (44). The separated populations were lysed and analyzed for incorporation of UDP-[14C]glucose as described previously (43).

**Assessment of *P. carinii* gsc-1 cDNA Expression over the Life Cycle of the Organisms**—*P. carinii* were separated into cystic and trophic forms by differential filtration through 3-μm filters, as detailed previously. RNA was extracted from the separated forms (Trizol Reagent, Life Technologies, Inc.) and equal RNA (5.0 μg) was separated by electrophoresis through 1.2% agarose in the presence of 2.2 μl formaldehyde. Separated RNA species were transferred to nitrocellulose. The 324-bp gsc-1 DNA partial clone was labeled with [α-32P]dCTP (Amer- sham Pharmacia Biotech) by a random primer method) using a hybridiza- tion solution (1 × 10⁶ cpm/ml), and incubated with the membranes for 1 h at 68 °C. After hybridization, the membranes were washed four times with 2× SSC solution (sodium citrate, where 1× solution contained 150 mM NaCl and 15 mM sodium citrate; pH 7.0) with 0.05% SDS at room temperature for 40 min followed by 0.1× SSC with 0.1% SDS solution at 50 °C for 40 min. The blots were visualized by autoradiography. For comparison to *P. carinii* gsc-1, membranes were rehy- bridized with a *P. carinii* actin probe (53).

**RESULTS**

*P. carinii* Cell Wall Membrane Isolates Incorporate UDP in a Fashion Inhibitable by the Pneumocandin B₀ Analog L-733,560—Isolated *P. carinii* cell wall membrane preparations incorporated significant UDP-[14C]glucose into insoluble carbohydrate over 4 h of incubation (Fig. 1; *p* = 0.0392 comparing UDP-Glc incorporation in the presence and absence of *P. carinii* membrane isolates). To further address whether *P. carinii* cell wall membrane isolates contained a Gsc-1 type β-1,3-glucan synthetase, parallel assays were performed in the presence of the pneumocandin analog L-733,560 (Merck, Inc.) (34). In a concentration-dependent manner, L-733,560 suppressed glu- can synthesis (Fig. 1; **p* < 0.05 comparing UDP-Glc incorporation in the presence of = 1.0 mM of L-733,560 versus its absence). Pneumocandin L-733,560 exhibited an IC₅₀ of ~0.30 μM against *P. carinii* cell wall membrane isolates, strongly
- **P. carinii**

Presence of the pneumocandin analog L-733,560, an agent which specifically inhibits Gsc-1-type proteins. In a concentration-dependent manner, complex carbohydrates were precipitated with trichloroacetic acid, washed, and counted. Purified and cell wall membrane preparations isolated and reacted with radiolabeled UDP-Glc as described under "Experimental Procedures." Significant incorporation of UDP-Glc (** denotes UDP-Glc in the presence and absence of L-733,560). 40630

UDP-[14C]glucose under these conditions. Normal rat lung protein (30 μg) only incorporated 98.8 ± 15.4 cpm, barely distinguishable above background, compared with a concurrent incorporation of 1327.5 ± 122.2 cpm resulting from an equal concentration (30 μg) of *P. carinii*-derived cell wall membrane isolates. Hence, the activity measured strongly represents the ability of *P. carinii* organisms, and not any host cell contaminant, to incorporate UDP-[14C]glucose into trichloroacetic acid-insoluble glucans utilizing a Gsc-1-type glucan synthetase.

**Molecular Cloning Reveals That *P. carinii* Contains a Unique gsc-1 β-1,3-Glucan Synthetase Gene**—We utilized a degenerative PCR cloning strategy based on amino acid similarities in other fungal glucan synthetases to clone *P. carinii* gsc-1 (26–28). Amplification of *P. carinii* genomic DNA with these degenerate primers yielded a single 324-bp product with homologies to related fungal gsc and fks genes. To verify that the PCR product was specifically represented within *P. carinii* and not an amplified contaminant, this amplicon was hybridized to genomic DNA of *P. carinii* and not any host cell contaminant, to incorporate UDP-[14C]glucose into trichloroacetic acid-insoluble glucans utilizing a Gsc-1-type glucan synthetase.

**Fig. 1.** *P. carinii* cell wall membrane preparations incorporate UDP-[14C]glucose into β-glucans, in a manner inhibited by the pneumocandin analog L-733,560. To assess whether *P. carinii* actively incorporates UDP-Glc into insoluble carbohydrates, organisms were purified and cell wall membrane preparations isolated and reacted with radiolabeled UDP-Glc as described under "Experimental Procedures." Complex carbohydrates were precipitated with trichloroacetic acid, washed, and counted. *P. carinii* cell wall membrane isolates mediated significant incorporation of UDP-Glc (** denotes *p* < 0.05 comparing *P. carinii*-mediated incorporation of UDP-Glc in the presence and absence of L-733,560).

To further verify that the activity was indeed derived from *P. carinii* membranes rather than from some host cell contaminant, parallel experiments were also performed in which equal volumes of rat lung from uninfected control animals were processed in an identical fashion. An equal amount of protein from uninfected control lung was assayed in parallel to uninfected control lung was assayed in parallel to *P. carinii* cell wall membrane isolates mediated incorporation of 1327.5 ± 122.2 cpm resulting from an equal concentration (30 μg) of *P. carinii*-derived cell wall membrane isolates. Hence, the activity measured strongly represents the ability of *P. carinii* organisms, and not any host cell contaminant, to incorporate UDP-[14C]glucose into trichloroacetic acid-insoluble glucans utilizing a Gsc-1-type glucan synthetase.

**Fig. 2.** The 324-bp *P. carinii* gsc-1 gene fragment specifically hybridizes to *P. carinii* genomic DNA. *P. carinii* was freshly isolated and genomic DNA isolated and digested with the indicated restriction endonucleases. The digestion products were separated by electrophoresis and transferred to nitrocellulose. The 324-bp gsc-1 amplicon was labeled and hybridized to the membrane showing specific interaction as a single band on the EcoRI and HindIII digestions. Thus, gsc-1 appears to represent a single locus within *P. carinii* genomic DNA.

Molecular cloning revealed that *P. carinii* contains a unique gsc-1 β-1,3-glucan synthetase gene. This gene is present as a single copy gene within the organism. To further verify that the activity was derived from *P. carinii* membranes rather than from some host cell contaminant, parallel experiments were performed in which equal volumes of rat lung from uninfected control animals were assayed in parallel to uninfected control lung. An equal amount of protein from uninfected control lung was assayed in parallel to *P. carinii* cell wall membrane isolates mediated incorporation of 1327.5 ± 122.2 cpm resulting from an equal concentration (30 μg) of *P. carinii*-derived cell wall membrane isolates. Hence, the activity measured strongly represents the ability of *P. carinii* organisms, and not any host cell contaminant, to incorporate UDP-[14C]glucose into trichloroacetic acid-insoluble glucans utilizing a Gsc-1-type glucan synthetase.

**Molecular Cloning Reveals That *P. carinii* Contains a Unique gsc-1 β-1,3-Glucan Synthetase Gene**—We utilized a degenerative PCR cloning strategy based on amino acid similarities in other fungal glucan synthetases to clone *P. carinii* gsc-1 (26–28). Amplification of *P. carinii* genomic DNA with these degenerate primers yielded a single 324-bp product with homologies to related fungal gsc and fks genes. To verify that the PCR product was specifically represented within *P. carinii* and not an amplified contaminant, this amplicon was hybridized to *P. carinii* genomic DNA. The 324-bp product was consistently represented within *P. carinii* and not an amplified contaminant, this amplicon was hybridized to *P. carinii* genomic DNA demonstrating strong localization as a single band following both EcoRI and HindIII digestions (Fig. 2). In addition, the 324-bp *P. carinii* gsc-1 sequence was hybridized to a CHEF blot of *P. carinii* chromosomes. The 324-bp probe consistently hybridized to a single *P. carinii* chromosomal location (Fig. 3). Together, these studies indicate that *P. carinii* gsc-1 is present as a single copy gene within the organism.

Subsequently, a *P. carinii* λgt11 genomic library was screened, and four clones were isolated based on hybridization with the 324-bp gsc-1 fragment. Clone G6 of ~3.5-kb size was fully sequenced. Comparison using BLAST-X analyses revealed this unique sequence to contain homologies to the amino-terminal domains of the β-1,3-glucan catalytic subunits of *A. nidulans*, *A. fumigatus*, and *S. cerevisiae*. Sequence from the 3′-coding region of clone G6 were used to further screen the library. Three additional clones were identified and found to contain additional sequence, including a putative stop codon.

Complete genomic DNA sequences of *P. carinii* gsc-1 have been deposited in GenBank® (accession number AF191096). Translation suggested three separate open reading frames encoding peptide sequences with homology to *A. nidulans* FksAp and *A. fumigatus* Fksp. An analysis for the presence of putative *P. carinii*-type introns, using our recently reported acceptor and donor consensus criteria, revealed the likelihood of three introns positioned at nucleotides 334–398, 2619–2695, and 5495–5546 of *P. carinii* gsc-1 (54). Predicted excision of these introns resulted in a mature protein with overall sequence homology comparable to other fungal β-1,3-glucan synthetases. To further confirm the intron/exon splicing sites, complete cDNA sequences for *P. carinii* gsc-1 (GenBank® accession number AF291999) were derived by reverse transcriptase-PCR.
of total RNA extracted from freshly isolated *P. carinii* using overlapping primer pairs covering the entire *P. carinii* gsc-1 genomic sequence. Comparison of the genomic and cDNA sequences confirmed that the *P. carinii* gsc-1 gene is comprised of four exons and three introns spliced at the sites predicted using the acceptor and donor consensus criteria. Minor variations in sequences derived from different sources of *P. carinii* organisms have been previously reported by our group and others (44–46). A 3.6% difference in nucleotide sequence was observed comparing the genomic clone, derived from the University of Cincinnati library, to the cDNA sequence generated from *P. carinii* RNA freshly obtained from our rat colony housed in Rochester, MN. This is comparable with previous reports for *P. carinii* cdc2, another similarly conserved single
copy gene, which demonstrated a 6.3% difference comparing nucleotide sequences obtained from \textit{P. carinii} strains obtained from Rochester, MN and Cincinnati, OH (44). These very minor differences between the genomic and the cDNA \textit{P. carinii gsc-1} sequences likely represent strain variations in the \textit{P. carinii} sources.

Upon contrasting the Gsc-1 peptide against these synthetases, the closest homology was with \textit{A. nidulans} FksAp (67% identity on BLAST-X analysis) followed by \textit{A. fumigatus} Fksp (66% identity). The predicted \textit{P. carinii} Gsc-1 protein has a predicted molecular mass of 214 kDa and pI of 9.17. Unlike \textit{A. nidulans}, \textit{A. fumigatus}, and \textit{S. cerevisiae} \textbeta-1,3-glucan synthetases that contain 16 transmembrane helices, hydropathy analysis of \textit{P. carinii} Gsc-1 predicts 12 transmembrane-spanning regions (Fig. 4) (26–28). Importantly, the predicted \textit{P. carinii} Gsc-1 protein was found to contain an ATP/GTP binding site motif (amino acid 1657–1664) required for activity.

Isolated \textit{P. carinii} Cell Wall Membrane Preparations Contain an Appropriately Sized Gsc-1 Protein—An antibody to a unique extracytoplasmic region of \textit{P. carinii} Gsc-1 was generated and used to immunoprecipitate \textit{P. carinii} membrane isolates and uninfected control rat lung. The anti-Gsc-1 antibody reacted specifically with a single \textit{P. carinii} protein of molecular mass 219.4 kDa on silver staining (arrow, Fig. 5A). In contrast, uninfected rat lung exhibited no specific reactivity. The additional material migrating at ~50 kDa is consistent with immunoglobulin heavy chain, and was present as expected in both the \textit{P. carinii} control lung precipitation. It is noteworthy that the observed molecular mass of 219.4 kDa correlated very closely to the 214-kDa size predicted from the gene sequences.

To further assess the presence of Gsc-1 over the life cycle of \textit{P. carinii}, isolated organisms were next separated into cysts and trophic forms. Previous immunohistochemical studies have demonstrated that \textbeta-glucans are prominent components of \textit{P. carinii} cysts and are relatively less prevalent in the walls of trophic forms (20). In contrast, recent work also suggests that pneumocandin Gsc-1 antagonists may exert some activity against trophic forms of the organism (36). To address this, trophic forms and cysts were separated by differential filtration, and their cell wall membrane isolates were analyzed by sequential immunoprecipitation and immunoblotting (Fig. 5B). Considerable Gsc-1 protein was present in \textit{P. carinii} cysts. In contrast, detectable, but rather limited amounts, of Gsc-1 were observed in isolated trophic forms. Thus, the Gsc-1 glucan synthetase, putatively active in cell wall assembly, is present at markedly different levels over the life cycle of the organism, with greatest expression found in \textit{P. carinii} cysts. As a further confirmation of the specificity of the anti-Gsc-1 antibody, an equal amount of uninfected rat lung protein was similarly processed as before and showed no reactivity with the anti-Gsc-1 antibody.

\textit{P. carinii} gsc-1 mRNA Expression Is Largely Restricted to Cystic Forms—Having observed differential abundance of Gsc-1 protein in isolated cyst and trophic forms of the organism, we next investigated whether \textit{gsc-1} gene expression was regulated over \textit{P. carinii}'s life cycle. Cysts and trophic forms were separated and \textit{gsc-1} mRNA expression evaluated by Northern analysis (Fig. 6). Again, \textit{P. carinii} \textit{gsc-1} mRNA was largely restricted to cysts. Repeat hybridization with \textit{P. carinii}-specific actin revealed that the marked abundance of \textit{gsc-1} in cysts was not the consequence of RNA loading. (In fact, greater actin mRNA was present in the trophic lane.) Lastly, we observed no cross-hybridization of either the \textit{P. carinii gsc-1} or \textit{actin} probes with RNA derived from uninfected rat lung, confirming the specificity of the \textit{P. carinii} sequences. Thus, the expression of \textit{gsc-1} is uniquely regulated over the life cycle of \textit{P. carinii}.

Antibody to the Predicted \textit{P. carinii} Gsc-1 Protein Immunoprecipitates a Molecule Catalyzing UDP-Glc Incorporation into Glucans—We next sought to directly link enzymatic activity to the genetic sequences to confirm that we have indeed cloned and characterized a Gsc-1-type protein from \textit{P. carinii}, capable of mediating glucan synthesis. This was investigated by performing immunoprecipitation with the specific anti-\textit{P. carinii} Gsc-1 antibody followed by UDP-[\textsuperscript{14}C]glucose incorporation of the precipitated products (Fig. 7). Anti-\textit{P. carinii} Gsc-1 antibody, but not non-immune IgG, precipitation of \textit{P. carinii} cell wall membrane isolates yielded a product that strongly incorporates UDP-[\textsuperscript{14}C]glucose into trichloroacetic acid-insoluble material, thus providing strong independent evidence that we have identified a Gsc-1 glucan synthetic enzyme from \textit{P. carinii} organisms. In contrast, uninfected rat lung proteins did not appreciably react with either the anti-Gsc-1 antibody or non-immune IgG, again confirming that the activity identified is from the organisms and not the result of host cell contamination.

**DISCUSSION**

\textit{P. carinii} membrane isolates possess the ability to incorporate UDP-[\textsuperscript{14}C]glucose into insoluble carbohydrate, which is
inhibited by the pneumocandin L-733,560 antagonist of Gsc-1-type β-1,3-glucan synthetases. Molecular cloning of the *P. carinii* gsc-1 gene predicts a mature protein with both similarities and unique differences to other fungal β-1,3-glucan synthetases. Specifically, the mRNA and protein expression of *P. carinii* gsc-1 are highly regulated over the life cycle of the organism being predominantly expressed by cystic forms of the organism. A protein corresponding to the predicted sequence of the cloned gsc-1 gene was present in *P. carinii* membrane isolates. Furthermore, immunoprecipitation of the putative *P. carinii* Gsc-1 protein with a synthetic peptide antibody yielded a product capable of mediating incorporation of UDP-Glc into trichloroacetic acid-insoluble material, consistent with glucan.

*P. carinii* Gsc-1 glucan synthetase exhibits several unique features. As discussed, structural differences were detected in the domain configuration of *P. carinii* Gsc-1 compared with *S. cerevisiae* and *Aspergillus* (26–28). Of further contrast, is the significant restriction of gsc-1 expression predominantly to the cystic forms of the *P. carinii*. Most other fungi, including ascomycetous fungi, exhibit cell wall assembly constitutively throughout the life cycle (55). Our immunoblot and Northern analyses are complementary to previous immune localization studies, which also indicate that β-1,3-glucan is largely found within cysts (6, 20).

Because mammalian hosts do not possess an equivalent to Gsc-1, inhibition of β-1,3-glucan synthase represents an attractive target for treatment of fungal infections. Echinocandins and pneumocandins are selective lipopeptide inhibitors that may expand our armamentaria for fungal infections, including those organisms resistant to standard agents (32). Schmatz and colleagues (33, 34) have shown rapid reduction of organisms in rat and mouse models of *P. carinii* pneumonia. One pneumocandin in particular, L-671,329 has shown remarkable activity in the *P. carinii* rat model with >98% of cysts being eliminated (33).

Concern had arisen that pneumocandins might only be effective against cystic forms of *P. carinii*, thereby limiting efficacy of such compounds during *P. carinii* pneumonia. The striking results of pneumocandins in animal models of *P. carinii* pneumonia strongly argue against this argument (34). These findings do suggest, however, that progression of trophic forms into cysts represents an essential component of life cycle progression in *P. carinii*, rather than an elective form utilized only under hostile conditions (7). Other investigators have also found some effect of echinocandins on trophic structure after in vitro exposure (36). Although our study demonstrates low levels of gsc-1 mRNA expression in trophic forms, small residual amounts of Gsc-1 protein were detected by Western analysis to be present in *P. carinii* using an antibody generated to the predicted Gsc-1 protein. The expression of *P. carinii* gsc-1 is regulated over the life cycle of the organisms. In view of its central role in assembly of the *P. carinii* cyst wall, the Gsc-1 β-1,3-glucan synthetase is an attractive therapeutic target for the treatment of *P. carinii* pneumonia.

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