The Carboxyl Terminus of *Pneumocystis carinii* Glycoprotein A Encodes a Functional Glycosylphosphatidylinositol Signal Sequence*

(Received for publication, January 7, 1998, and in revised form, July 7, 1998)

Gayle Guadiz‡§, Constantine G. Haidaris‡§, Gabriel N. Maine§, and Patricia J. Simpson-Haidaris‡§**

From the Departments ‡Microbiology and Immunology, §Medicine, Vascular Medicine Unit, ¶Dental Research, and †Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

*This work was supported by Public Health Service Grants HL50615, HL49610, and AI07362 from the National Institutes of Health. The costs of publication of this article were defrayed in part by advertisement "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**

**To whom correspondence should be addressed: Vascular Medicine Unit, Dept. of Medicine, P.O. Box 610, University of Rochester, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 716-275-8267; Fax: 716-473-4314; E-mail: jj_simpsonhaidaris@urmc.rochester.edu.

---

*Pneumocystis carinii* pneumonia is a hallmark disease associated with AIDS. An abundant glycoprotein, termed gpA, on the surface of *P. carinii* is considered an important factor in host-parasite interactions. The primary structure of ferret *P. carinii* gpA contains a carboxyl-terminal sequence characteristic of a signal for glycosylphosphatidylinositol (GPI) anchors. Here we report the capacity for this gpA carboxyl sequence to direct attachment of a secreted protein, human growth hormone (hGH), to the membranes of COS cells. A control fusion protein (hGHDAF37) was obtained which, under the direction of the GPI signal from decay accelerating factor, directs hGH cell surface expression. A construct (phGH2-1A30) was created similar to hGHDAF37 by fusing hGH to the putative GPI signal sequence encoded in the terminal 30 residues from a ferret *P. carinii* gpA cDNA clone. By indirect immunofluorescent staining, hGH was detected on the surface of COS cells transfected with phGH2-1A30; this surface location was confirmed by confocal laser cytometry. Metabolic labeling with [3H]ethanolamine and subsequent immunopurification of hGH from cells transfected with phGH2-1A30 confirmed that a lipid moiety characteristic of a conventional GPI anchor was linked covalently to hGH, and cell surface hGH2-1A30 fusion protein was sensitive to enzymatic cleavage by phosphatidylinositol-phospholipase C. Furthermore, hGH2-1A30 recombinant protein cofractionated with 5'-nucleotidase, a classical GPI-anchored membrane marker. Together, these results indicate that the carboxyl-terminal residues of ferret *P. carinii* gpA constitute a biologically functional GPI consensus domain, thus providing a potential mechanism for antigenic variation of *P. carinii* gpA during *P. carinii* pneumonia.

Immunocompromised hosts, such as those suffering from AIDS, cancer, malnutrition, and genetic immunodeficiencies, are susceptible to pneumonitis caused by the opportunistic pathogen *Pneumocystis carinii*. The pathogenesis of the infection is elusive, largely because of the lack of a continuous in vitro culture system and no knowledge of its reservoir in nature. Thus, animal models, particularly severe combined immunodeficient (SCID) mice and steroid-treated ferrets and rats, are used to isolate the organism and to study *P. carinii* pneumonia. Interestingly, the transmission of *P. carinii* from one host to another is restricted to within the same species, and *P. carinii* organisms isolated from different host species exhibit host species-specific karyotypes, genotypes (6–8), and antigens (9–11).

Much insight on the biology and pathogenicity of *P. carinii* has stemmed from studies of one antigen, glycoprotein A (gpA).1 Also termed major surface glycoprotein, gpA is an immunodominant surface antigen thought to facilitate attachment of *P. carinii* to type I alveolar epithelial cells (1, 12). *P. carinii* remains extracellular throughout *P. carinii* pneumonia, and the organism binds to several host adhesive glycoproteins and surfactant apoproteins via gpA (13–16). Passive immunoprophylaxis using anti-gpA monoclonal antibodies (mAbs) partially protects against *P. carinii* pneumonia; therefore, gpA may be a target of the humoral response in the prevention or recovery from infection (17, 18). Therapeutic approaches using anti-gpA antibodies are complicated, however, by the dramatic differences in gpA phenotypes among organisms isolated from various host species, as well as from a single host species. Although many gpA-specific mAbs have been characterized, only one mAb (5E12) recognizes a carbohydrate-containing epitope on gpA shared by *P. carinii* isolates from all host species studied (18). gpA genes are scattered in clusters throughout the *P. carinii* genome in highly polymorphic isoforms detected even within the same individual host (1, 6–8, 19). It remains inconclusive whether infection arises from multiple *P. carinii* organisms, each expressing single gpA isoforms, or from a single organism expressing polymorphic genes (20). Despite this ambiguity, the known characteristics of *P. carinii* gpA indicate some form of antigenic variation.

Although the life cycle of *P. carinii* is not fully defined, several developmental forms have been identified. These include pleomorphic trophozoites, thin walled precysts, and thick walled mature cysts containing up to eight intracytoplasmic bodies that, upon excystation, result in daughter trophozoites to repeat the cycle (2). All of these life cycle stages show surface staining for gpA using specific mAbs and polyclonal antibodies.
(pAbs) (2, 21). However, the mode of attachment of gpA to the P. carinii surface remains undetermined. The primary structure of gpA lacks a traditional hydrophobic transmembrane domain for insertion of the protein into a lipid bilayer (6–8). The absence of such a mechanism is consistent with the P. carinii cyst wall structure that, like yeast cell walls, consists of an inner plasma membrane surrounded by a thick layer of β-glucan (22, 23). Another mechanism for surface antigen attachment commonly employed by eukaryotes, including protozoa and yeast, is glycosylphosphatidylinositol (GPI) anchorage mediated by a characteristic carboxyl-terminal sequence. This sequence consists of a stretch of hydrophobic amino acids preceded by a short string of hydrophilic residues. Upstream of this domain is a trio of amino acids with small side groups that represent the cleavage and attachment site for GPI. One of the most well-characterized GPI-anchored proteins is the variant surface antigen (VSG) of Trypanosoma brucei. Cleavage via GPI-phospholipase C (GPI-PLC) and anchorage of a new isoform variant of VSG provide an efficient mechanism for antigenic variation and immune evasion (24–26).

Analyses of gpA cDNA clones isolated from ferret P. carinii revealed a carboxyl-terminal sequence with domains characteristic of GPI-anchored proteins (19). Deduced amino acid sequences from rat (8) and human (6) P. carinii gpA also display conserved regions indicative of signal sequences for GPI attachment. Although several standard methods exist for determining GPI anchorage, such procedures are hindered by the crude sources of P. carinii organisms, which must be isolated from infected animal lungs. These preparations inevitably contain host phospholipases and GPI-anchored proteins. To circumvent this limitation, we used a molecular approach to circumvent this limitation, we used a molecular approach to determine GPI anchorage, such procedures are hindered by the crude sources of P. carinii organisms, which must be isolated from infected animal lungs. These preparations inevitably contain host phospholipases and GPI-anchored proteins. To circumvent this limitation, we used a molecular approach to determine whether the 3'-terminus of a ferret gpA mediates attachment of a secreted protein, human growth hormone (hGH), to the surface of transfected COS cells via a GPI anchor.

**Experimental Procedures**

**Reagents—**Radiouclides, [3H]ethanolamine, and [35S]methionine were purchased from Amersham Pharmacia Biotech and NEK Life Science Products, respectively. Goat anti-hGH pAb and expression vector phGHDAF37 (27) containing hGH with a 37-amino acid carboxyl-terminal sequence containing the decay accelerating factor (DAF) GPI signal sequence were a kind gift from Genentech (South San Francisco, CA). Goat anti-hGH pAb and rabbit anti-hGH pAb (Dako, Carpinteria, CA) were used for immunopurification and indirect immunofluorescent staining. Fluorescein isothiocyanate-conjugated anti-goat IgG antibodies were obtained from Miles-Yeda (Israel), and BODIPY-conjugated goat anti-rabbit IgG was purchased from Molecular Probes Inc. (Eugene, OR). Protein G-Sepharose (Sigma) beads were used for immunopurification of metabolically labeled growth hormone. All tissue culture flasks, cell culture media, and media supplements were purchased from Life Technologies unless otherwise indicated, as were all DNA- and RNA-modifying enzymes. Kits were used per the manufacturers’ instructions: Wizard (Promega), Sephaglas, (Amersham Pharmacia Biotech), and Exsite (Stratagene).

**Isolation and Characterization of Ferret gpA cDNA Clone 2-1A**—A cDNA clone bank in λgt11 was constructed previously using mRNA isolated from ferret lungs heavily infected with P. carinii (8). Phase expressing gpA were isolated and mapped on the ferret gpA gene sequence. Determined previously by Wright et al. (19) using λgt11 primers coupled with ferret P. carinii gpA-specific primers (Table I) in the polymerase chain reaction (PCR) as described (19). One clone, designated 2-1A, was subcloned from λgt11 into the EcoRI site of pGem7Zf(−) (Promega) for further analysis. DNA sequencing was performed using the Perkin-Elmer Abirpism Dye Terminator method at the Nucleic Acid Core Facility at the University of Rochester. The nucleotide sequence of clone 2-1A is deposited in GenBank under accession number AF035226.

**PCR and Site-directed Mutagenesis—**Site-directed mutagenesis was conducted using gpA and hGH-specific oligonucleotides with XhoI and HindIII site adaptors to facilitate cloning the 30 carboxy-terminal amino acids containing the putative GPI signal of 2-1A-gpA and replace the 37 amino acids of the GPI signal of DAF in phGHDAF37 (Table I). The amplification reaction to obtain mutagenized 2-1A was performed for 30 cycles of 94 °C for 2 min, 50 °C for 2 min, and 72 °C for 4 min. The PCR product was purified (Promega Wizard kit) and then digested with XhoI and HindIII overnight at room temperature. The phGHDAF37 construct was mutagenized using the Stratagene pairs procedure (1 cycle of 94 °C for 4 min, 50 °C for 2 min, and 72 °C for 2 min; 12 cycles of 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 1 min; 1 cycle of 72 °C for 5 min). After amplification, tubes were cooled to 37 °C, and DpnI and PfuI polymerase were added and then incubated for 30 min at 37 °C and 30 min at 72 °C. PCR products were purified after agarose gel isolation using Sephaglas, and then the purified DNA was phosphorylated with T4 DNA kinase before ligation with T4 DNA ligase. The resulting constructs were designated phGH2-1A30 and phGHm1 as shown in Fig. 1.

**Transfection into COS Cells—**COS cells were transfected with plasmids containing the inserts hGHDAF37, hGH2-1A30, and the hGHm1 mutant lacking the DAF GPI signal sequence. Cells were plated onto glass coverslips, six well cluster plates, or T-25 flasks in complete medium consisting of Dulbecco’s modified Eagle’s medium/medium F-12 (DMEM/F-12) with 10% fetal bovine serum medium was added. Transfection continued for 5 h, and then an equivalent amount of DMEM/F-12 and 20% fetal bovine serum medium was added. Transfection proceeded for 24 h; transfection medium was then replaced with serum-free DMEM/F-12. The medium was then replaced with serum-free medium containing the DNA-LipofectAMINE complexes. Transfection proceeded for 5 h, and then an equivalent amount of DMEM/F-12 and 20% fetal bovine serum medium was added. Transfection continued for a total duration of 24–48 h. For metabolic labeling studies, transfections were carried out for 24 h; transfection medium was then replaced with complete medium containing [3H]ethanolamine at 50 Ci/ml and radiolabeled for 16 h. Immunoprecipitation was performed using goat anti-hGH pAbs and protein G-Sepharose beads (28). For immunofluorescent staining, cells were washed, fixed in 3.7% formaldehyde, then probed with goat anti-hGH antibodies and stained with fluorescein isothiocyanate-conjugated anti-goat IgG antibodies (Miles-Yeda) as described previously (28).

**PI-PLC Cleavage—**Transfected COS cells were cultured in six-well culture plates and treated with recombinant PI-PLC from Bacillus thuringiensis (Oxford Glycosystems, Rosedale, NY) to demonstrate the GPI phosphatidylinositol moiety. After a 24-h transfection with phGHDAF37, phGH2-1A30, or phGHm1, cells were radiolabeled continuously for 16 h with [35S]methionine in complete medium. Conditioned medium was removed and then pooled with three subsequent washes of Hanks’ balanced salt solution. PI-PLC (1 unit/ml) in buffer (RPMI 1640 supplemented with 0.5% bovine serum albumin, 50 μM β-mercaptoethanol, 10 mM HEPES, 0.1% sodium azide) was added directly to the wells (29); control cells received buffer only. Cells were

---

**Table I**

| Primer          | Sequence                          | Position | Template  |
|-----------------|-----------------------------------|----------|-----------|
| FgpA 307-10A 7 sense | 5’-CACA AAACACAGGGGAGGAC-3’       | 1380–1400 | p2-1A     |
| FgpA-GPI antisense | 5’-GGCTAATATCAGACCAACAC-3’       | 1906–1888 | p2-1A     |
| 2-1A30 sense     | 5’-GGCTAATATCAGACCAACAC-3’       | 1822–1839 | p2-1A     |
| 2-1A30 antisense | 5’-GGCTAATATCAGACCAACAC-3’       | 1914–1976 | p2-1A     |
| hGH sense       | 5’-GCTCAAAAGTCCTGCGGGTGGCACC-3’  | 2606–2621 | phGHDAF37 |
| hGH antisense   | 5’-CAATGCTTGAGGAGGCACAGCTGCC-3’  | 2491–2476 | phGHDAF37 |
incubated in a 37 °C CO₂ incubator for 1 h. Supernatants were collected and pooled with three subsequent Hanks’ buffered salt solution washes, cells were lysed, and then whole cell lysates and supernatants plus rinses were immunopurified to detect cell-associated or released hGH, respectively.

**Confocal Scanning Laser Cytometry (CSLC)—**To determine whether the recombinant fusion proteins encoded by phGHDAF37 and phGH2-1A30 were directed to the plasma membrane, transfected COS cells were analyzed by CSLC. Cells cultured on glass coverslips were transfected with 2.5 μg of plasmid DNA for 48 h, fixed with formalin, and permeabilized with Triton X-100. The treated cells were incubated with rabbit anti-hGH pAb (8 μg/ml) followed by incubation with BODIPY-conjugated goat anti-rabbit IgG (1:200). The cellular locale of fluorescence was determined by CSLC using a Meridian Ultima Adherent Cell Analysis System and its accompanying Data Analysis System Master Program V3.32 (Meridian Instruments, Inc., Okemos, MI). All samples were examined under oil immersion using a 100 × objective and scanned using an identical set of parameters so that results from different cells could be compared. Samples were excited by a 488 nm argon laser line, and emitted fluorescence was detected by a photomultiplier tube with an upper limit of 575 nm. Data were collected from serial, 1 μm-thick optical sections along the Z-axis (vertical axis) at steps of 0.5 μm. From the data points collected, false color digitized images of scanned cells were generated and then converted to gray scale for photographic reproduction.

**Cell Fractionation and 5’-Nucleotidase Activity Assay—**To confirm localization of hGH to the plasma membranes of transfected cells, whole cell lysates from 2 × 10⁶-cm² flasks were fractionated using Percoll (Amersham Pharmacia Biotech). COS cells were transfected with 75 μg of phGH2-1A30 for 40–48 h, then lysed and pooled in 2.4 ml of lysis buffer (0.25 M sucrose, 3 mM imidazole, 10 mM leupeptin, 200 units/ml aprotinin, and 5 mM EDTA in 10 mM Tris-HCl, pH 8.0) (30), and disrupted in a glass hand-held homogenizer (Wheaton, Millville, NJ) with 10 strokes. The cell suspension was centrifuged at 1,500 × g for 10 min. Supernatants were removed for further centrifugation, and pellets were resuspended in 200 μl of phosphate-buffered saline and frozen at −20 °C. Percoll (2.6 ml) was added to each supernatant, mixed gently by inversion, and then transferred to 13 × 51-mm Beckman ultracentrifuge tubes. The mixture was centrifuged in a Beckman ultracentrifuge using a SW 55Ti rotor at 70,000 × g for 15 min at 5 °C. Five fractions of 830 μl were removed from the top to bottom of each tube and then diluted in phosphate-buffered saline to 5 ml. Samples were centrifuged at 200,000 × g for 45 min at 5 °C to form a Percoll pellet. The top 4 ml was removed, and the remaining 1 ml was transferred to new tubes for further centrifugation at 200,000 × g for 45 min (30). Supernatants were removed carefully, saving the last 200 μl for analysis by gel electrophoresis and enzymatic activity measurement.

Equal volumes of each sample were analyzed, reduced on a 12.5% polyacrylamide gel, and then transferred onto nitrocellulose filters. A standard Western blot procedure utilizing chemiluminescence reagent was performed (NEN Life Science Products) using rabbit anti-hGH pAb followed by anti-rabbit IgG conjugated to horseradish peroxidase. Growth hormone was detected by exposure to autoradiographic film, and bands were quantitated by densitometric scanning using the NIH Image 1.59 program. In parallel, the enzymatic activity of a GPI-anchored plasma membrane marker, 5’-nucleotidase, was determined using a reagent kit from Sigma Diagnostics. The data were plotted as relative densitometric units of hGH and 5’-nucleotidase activity detected in each fraction.

**RESULTS**

A Gt11 cDNA library was constructed previously from mRNA isolated from *P. carinii*-infected ferret lungs (8). After induction of gene expression with isoprropyl β-D-galactopyranoside and screening with polyclonal anti-gpA, four positive plaques were isolated and mapped by PCR on the ferret gpA gene composite constructed previously by Wright et al. (19). One clone, designated 2-1A, was selected for further analysis because of its size (>1.9 kilobase pairs) and relative position at the 3’-end of the gpA gene. PCR amplification of the gpA 2-1A plasmid template with M13 universal forward and FgpA-GPI antisense primers (Table I) indicated that the cDNA clone included sequences encoding the putative GPI attachment site. Nucleotide sequencing confirmed that clone 2-1A encodes a stretch of amino acids characteristic of a signal for GPI attachment (Table II).

Direct analysis of *P. carinii* gpA attachment is problematic without an *in vitro* culture system. We utilized a eukaryotic expression system to determine whether the extreme 3’-sequence of cDNA clone 2-1A encoded amino acids that could direct attachment of hGH to the COS cell membranes. A construct (phGHDAF37) was obtained consisting of hGH fused to a sequence with the well-characterized GPI signal sequence from DAF (27). PCR site-directed mutagenesis was designed to delete the DAF37 sequence and to add a set of unique restriction sites (XhoI and HindIII) for forced cloning into this region downstream of hGH (Fig. 1); the resulting plasmid was designated phGHm1. DNA encoding the putative GPI signal sequence from gpA clone 2-1A, designated 2-1A30, was also isolated by PCR using primers that added the corresponding unique cloning sites XhoI and HindIII. The 2-1A30 sequence encoding the signal for GPI attachment of hGH was cloned into phGHm1. The constructs phGHDAF37, phGHm1, and phGH2-1A30 were expressed transiently in COS cells, and cell surface...
hGH expression was assessed by indirect immunofluorescent staining of nonpermeabilized cells. Cells transfected with phGHDAF37 expressed striking levels of hGH on the cell membrane, as detected with a specific pAb to hGH (Fig. 2A). Transfection with phGHm1, lacking the DAF signal sequence for GPI attachment, did not result in detectable cellular hGH (Fig. 2C), nor was any detectable on control cells (Fig. 2D). Importantly, transfection with phGH2-1A30 resulted in membrane hGH expression (Fig. 2B) comparable to that seen with phGHDAF37 (Fig. 2A), displaying intense cell-associated fluorescence. A standard feature present on all GPI-anchored proteins is an ethanolamine molecule bridging GPI to the carboxyl terminus of the protein through an amide linkage (31, 32). Hence, copurification of this moiety with a cell-associated hGH strongly indicates formation of a GPI lipid anchor mediating membrane attachment. COS cells were transfected with the constructs hGHDAF37, hGHm1, and hGH2-1A30 for 24 h, and then culture medium was replaced with fresh medium containing [3H]ethanolamine. Cell lysates were immunopurified with antibodies to hGH, and samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Control cells that underwent the same transfection conditions, but without plasmid DNA, did not express cellular hGH linked to [3H]ethanolamine (Fig. 3, No DNA). Similarly, expression of cellular hGH was not evident in hGHm1 transfectants (Fig. 3, hGHm1).

Immunopurification with anti-hGH pAb from phGHDAF37-transfected cells isolated a protein migrating to the expected molecular mass of hGH at ~24 kDa (Fig. 3, hGHDAF37). A similar pattern of hGH bands was also isolated from the lysate of phGH2-1A30-transfected cells (Fig. 3, hGH2-1A30), demonstrating that cell-associated hGH contains ethanolamine, indicative of a GPI anchor. The lipid moieties of GPI-anchored proteins render them susceptible to cleavage by exogenous, as well as endogenous, phospholipases (31, 32). PI-PLC is the most widely studied and utilized of the phospholipases in analyzing GPI attachment (31, 33). PI-PLC purified from bacterial extracts of Staphylococcus aureus and B. thuringiensis effectively cleave many eukaryotic GPI anchors, although some are resistant because of modified inositol molecules (33). To apply this method of GPI detection and characterization to our system, we transfected COS cells with phGHDAF37, phGH2-1A30, and phGHm1 and then treated the cells with PI-PLC derived from B. thuringiensis. Enzyme treatment released a detectable portion of the expressed hGH from cells into the supernatants of hGHDAF37 and hGH2-1A30 transfectants (Fig. 4).

Conventional fluorescence microscopy clearly demonstrated that COS cells transfected with phGH2-1A30 expressed hGH at levels comparable to cells transfected with phGHDAF37; the pattern of cell surface fluorescence (Fig. 2), together with the
biochemical analyses (Figs. 3 and 4), suggested localization on the cell membrane. To confirm that the recombinant fusion proteins encoded by phGHDAF37 and phGH2-1A30 were directed to the plasma membrane, transfected COS cells were analyzed by CSLC. This method allows visualization of optical sections of cells and permits precise localization of surface antigens. Cells transfected with each of the plasmids were stained for the expression of hGH using specific antibody and a fluorochrome-conjugated secondary antibody. Serial, 1-μm optical sections of transfected cells were scanned by CSLC, and the fluorescent signal was converted to gray scale, with white areas representing the most intense fluorescence. Serial scans of transfected cells indicated that the bulk of the immunostaining is localized to the cell membrane, demonstrating that the replacement of the DAF GPI signal sequence (Fig. 5A) with the \textit{P. carinii} gpa carboxyl-terminal peptide (Fig. 5B) resulted in functional anchoring of hGH on the COS cell surface. To confirm localization of the fusion protein hGH2-1A30 to the plasma membrane, transfected cells were fractionated by ultracentrifugation in a Percoll gradient. The enzymatic activity of 5'-nucleotidase, a GPI-anchored protein used as a standard marker of plasma membranes (34), was measured for comparison with the relative amounts of growth hormone in each fraction. The results are presented in Fig. 6 as relative densitometric units for hGH or units/liter of 5'-nucleotidase activity. The bulk of 5'-nucleotidase activity (>30 units/liter) and growth hormone was detected in the cell pellet (not shown), consistent with common techniques of isolating nuclei and plasma membrane sheets using this technique (35). 5'-Nucleotidase enzymatic activity, indicative of plasma membrane isolation, cofractionated with hGH in Fraction 2 (2.3 units/liter) and Fraction 3 (1.1 units/liter) (Fig. 6), whereas Fraction 1 likely corresponded to intracellular hGH. Recombinant hGHDAF37 also copurified in cell membrane fractions containing 5'-nucleotidase activity (not shown). These results demonstrate that the cellular fractions containing growth hormone indeed consisted of plasma membranes, verifying surface expression of hGH directed by the \textit{P. carinii} gpa-GPI anchor.

\textbf{DISCUSSION}

A significant area of study in \textit{P. carinii} pathogenesis has concentrated on characterizing gpa and understanding its impact on establishing disease. Recent studies have provided substantial evidence that gpa molecules isolated from different hosts exhibit genotypic, phenotypic, and antigenic polymorphism (6–8). Furthermore, individual infected hosts harbor organisms that express multiple divergent isoforms of gpa encoded by genes dispersed in clusters throughout the genome, suggesting that heterogeneity in this immunodominant antigen may be a considerable factor in the organism’s mode of survival (19, 36). Despite the marked differences in gpa gene expression, gpa homologs demonstrate several conserved properties, such as surface localization, molecular mass, a common carbohydrate epitope, and cysteine-rich domains (10, 11, 37). Wright et al. (19) also reported a novel property of ferret \textit{P. carinii} gpA C Terminus Forms a GPI Lipid Anchor

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{\textbf{[3H]Ethanolamine radiolabeling of COS cell transfectants.} COS cells were transfected with 10 μg of phGHDAF37 (first lane from left), phGH2-1A30 (second lane), or phGHm1 (third lane) for 24 h, then radiolabeled with 50 μCi/ml [3H]ethanolamine in complete medium for 16 h. The fourth lane is a control of cells not transfected but subjected to the same experimental conditions. Cells were washed in phosphate-buffered saline before lysis, and lysates were immunoprecipitated with goat anti-hGH pAb conjugated to protein G-Sepharose beads. Immunopurified samples were reduced and resolved in 12.5% polyacrylamide gels by SDS-electrophoresis and then analyzed by fluorography. Molecular mass markers (M) are, from top to bottom, in kDa: 200, 97, 68, 46, 30, 21, and 14. The solid arrow represents the migratory position of hGHDAF37, the dashed arrow represents the position of migration of the hGH2-1A fusion protein.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{\textbf{PI-PLC cleavage of hGH.} COS cells transfected with 5 μg of phGHDAF37 or phGH2-1A30 were treated with PI-PLC to determine whether membrane-bound hGH was susceptible to enzymatic cleavage. Samples of phGH2-1A30 transfectants represent 5-fold more cells than phGHDAF37 transfectants. hGH released by PI-PLC treatment and that remaining cell-associated were immunopurified and analyzed by electrophoresis on 12.5% acrylamide gels and fluorography. Each sample is designated with the transfection construct, (+) or (−) PI-PLC treatment, and either supernatant (sup) or cell lysate (cells). Molecular mass markers (M) are the same as indicated in Fig. 3. Open arrows designate GPI-linked hGH; closed arrows represent PI-PLC -cleaved hGH.}
\end{figure}
carinii gpA, namely the expression of a carboxyl-terminal domain conserved among eukaryotic GPI-anchored proteins. In our study, we sought to determine whether this putative GPI signal sequence could direct expression and membrane attachment of a secretory protein via a GPI moiety. Indeed, the presumed GPI signal sequence of an expressed gpA cDNA clone mediated hGH membrane attachment comparably to that of the well established mammalian DAF signal (27, 38). We determined conclusively by confocal scanning laser cytometry and cofractionation with another GPI-anchored membrane marker, 5'-nucleotidase, that hGH2-1A30 transfectants, as well as hGHDAF37 controls, expressed membrane-bound hGH. The likelihood that the carboxyl-terminal sequence of gpA is an efficient signal for GPI attachment in P. carinii is supported by the results of this study.

The molecular approach described in our study arose from the difficulty in analyzing P. carinii without an in vitro system to culture clonal populations. By utilizing a marker, such as a secreted protein or a reporter enzyme, one can ascertain definitively whether GPI membrane attachment is directed by a particular primary structure. For example, the mating factors a- and a-agglutinin of Saccharomyces cerevisiae are processed through a GPI-anchored intermediate before cross-linking into the β-glucan of the cell wall (39–42). A fusion protein consisting of the signal sequence of yeast invertase, α-galactosidase, and the carboxyl-terminal half of α-agglutinin to provide the

FIG. 5. Surface localization of hGH2-1A30 by CSLC of transfected COS cells. To localize hGH expression specifically in transfection COS cells, CSLC was performed. Cells cultured to ~50% confluence on glass coverslips were transfected with 2.5 μg of phGHDAF37 (panel A) or phGH2-1A30 (panel B). Monolayers were fixed, permeabilized, and stained with rabbit anti-hGH pAb followed by BODIPY-conjugated goat anti-rabbit IgG pAb. The value in the upper right corner of each panel represents the position of the optical section in μm relative to the initiation of scanning. Sequential sections starting from the cells’ basolateral surfaces demonstrate the specific location of fluorescence, which is indicative of surface localization of hGH.

FIG. 6. Cell fractionation and determination of 5'-nucleotidase (5'-NTase) activity. To ascertain that growth hormone was on the plasma membrane of phGH2-1A30 transfectants, cell lysates were fractionated in a Percoll gradient and then analyzed by Western blotting for detection of growth hormone. Enzymatic activity of a plasma membrane marker, 5'-nucleotidase, was also quantitated in the same samples using a kinetic assay (Sigma Diagnostics). Relative units of growth hormone, determined by densitometric scanning, were plotted on the same graph with the 5'-nucleotidase activity measured in units/liter.

GPI addition signal resulted in expression of the enzyme on the cell surface (43). The construct obtained from Genentech, hGH-DAF37, encodes a fusion protein that, despite a secretory signal sequence, anchors hGH to the mammalian cell membrane via the DAF carboxyl-terminal GPI sequence (27). However, fusions made between hGH and the GPI attachment sequence of T. brucei VSG failed to become membrane-bound (38). Mutational analyses identified the cleavage-attachment site between the first two residues of a trio of amino acids in the VSG GPI signal as responsible for the inability to anchor hGH. Various amino acid substitutions in this position restored the ability of the VSG sequence to mediate GPI-anchored hGH membrane expression (38). The upstream amino acid of this trio, termed ω, remains attached to the anchored protein after cleavage. A hierarchy of residues in the ω position facilitates anchorage in the order of: serine, asparagine, glycine, alanine, aspartate, cysteine, leucine, valine, and all others (44, 45). Another critical property appears to be the ω+2 position, which requires a residue with a small side chain. The native VSG attachment site, consisting of aspartate (ω), serine (ω+1), and serine (ω+2), is apparently less suitable using these criteria (44). From their studies, Moran and Caras (38) hypothesized that protozoan and mammalian cells exhibit similar but not identical prerequisites for GPI attachment. Such a disparity between lower eukaryotes and mammalian requirements was not evident in our study because the P. carinii carboxyl sequence encoded by phGH2-1A30 directed hGH membrane attachment. In fact, several factors strongly support our theory that gpA is GPI-linked. The putative cleavage-attachment site in clone 2-1A represents the optimal amino acids at the ω and ω+2 positions, respectively, serine and glycine (44). The overall characteristics for the carboxyl-terminal GPI signal sequence, particularly the hydrophobic tail, appear conserved among ferret (19), rat (8), mouse (46), and human (6) sources of P. carinii (Table III). Lipid domains, such as cholesterol, have been postulated to play a role in regulating the distribution of GPI-anchored proteins (47, 48). Because cholesterol is the major sterol synthesized by P. carinii, a similar regulation is possible (49). The phospholipid profile of P. carinii includes the presence of phosphatidyethanolamine and phosphatidylinositol (50–52), consistent with components of a GPI anchor (31, 32).

Based on our evidence that the carboxyl terminus of gpA can
function as a signal for GPI anchor addition in mammalian cells, it is tempting to speculate that at least a portion of the gpA molecules is anchored to the surface of *P. carinii* organisms in a similar manner. If so, it would have interesting implications for the biology of the organism and the pathogenesis of *P. carinii* pneumonia. By analogy to the African trypanosome *T. brucei*, which sheds its existing VSG coat via GPI-PLC cleavage during antigenic variation (24–26), *P. carinii* may use a similar mechanism to shed existing gpA isoforms so that they may be replaced by a different isoform. Furthermore, it is known that VSG expression in *T. brucei* is regulated by duplicative transposition of a VSG gene from a storage copy elsewhere in the genome into a telomeric expression site. There is substantial evidence that gpA expression by *P. carinii* is regulated in a similar manner (36, 53), in which a genomic storage copy of gpA recombines into a telomeric expression site. Although it is unknown whether more than one isoform of gpA is expressed by a single organism, a population of *P. carinii* from an individual infected lung expresses multiple isoforms of gpA, and the large number of copies of gpA genes in the genome assures the capacity for extensive heterogeneous in the antigenic repertoire (1, 8, 19, 54). An endogenous PI-PLC in *P. carinii* could facilitate the replacement of gpA isoforms on the organism surface. If such a mechanism of antigenic variation exists in *P. carinii*, it is not as efficient as that seen in *T. brucei*. Unlike the African trypanosome, *P. carinii* is not an overt pathogen, and any capacity for antigenic variation of gpA is dealt with adequately by the immunocompetent host. However, as the immune system wanes during immunodeficiency states, a capacity for variation in its immunodominant surface antigen may give the opportunist *P. carinii* the edge it needs to establish infection in the lung.

The structure of the *P. carinii* cyst wall strongly mirrors the composition of fungal cell walls, particularly *S. cerevisiae* and *Candida albicans* (22, 23, 55). GPI-anchored proteins are abundant on and within the thick β-glucan layer encompassing the plasma membrane of *S. cerevisiae* and *C. albicans*, and treatment with glucanase liberates these structural components (36, 55–59). Several studies of the *S. cerevisiae* mating factor α-agglutinin have suggested that the protein is anchored to the plasma membrane via GPI before traversing the β-glucan layer for exposure on the cell wall surface (41). The model is supported by evidence of α-agglutinin intermediates that, in their transport to the cell wall, may cross-link with β-glucan components (40, 41). Mutations to dolichyl phosphoryl mannose synthetase and phosphomannomutase, which prevent GPI synthesis, are lethal, suggesting a vital function for GPI-anchored proteins in fungal cell wall biosynthesis and cell survival (56). Other fungal GPI-anchored surface proteins include *S. cerevisiae* GPG1/GAS1 (58) and *C. albicans* PHR1 (60), proteins thought to be involved in cellular morphogenesis. Because GPI-anchored glycoproteins may be important in yeast cell wall composition, function, and morphology, it is possible that GPI-anchored gpA expressed on the different developmental forms during the life cycle of *P. carinii* may relate to morphogenesis as well. The major life forms of *P. carinii* are the trophozoite and cyst, with an intermediate precystic stage (2). Our laboratory demonstrated previously that most gpA transcription occurs in the actively replicating forms of *P. carinii*, the trophic and precystic forms, whereas the majority of mature cysts express minimal gpA mRNA (61). Cyst walls thicken during transition from the trophic and precystic forms (2); therefore, *P. carinii* morphogenesis may be related to the expression of GPI-linked gpA and its ability to promote attachment to lung epithelium and adhesiveness in alveolar exudate. GpA is abundant on the plasma membrane in the trophozoite (61), and a reported outer surface membrane on *P. carinii* cysts (22) suggests that a GPI anchor could mediate surface attachment of gpA on cysts. Alternatively, the GPI anchor may be part of an intermediate step, as described for α-agglutinin *S. cerevisiae* (40), and mature cyst walls may not contain detectable GPI-anchored gpA. In support of a model consisting of an intermediate GPI attachment, gpA is not readily released from cysts without stringent glucanase treatment (1, 23). Although these mechanisms remain elusive before the development of an *in vitro* cultivation system, the demonstration of a functional GPI signal sequence on gpA heralds compelling new implications for its role in *P. carinii* physiology and pathogenesis.

Acknowledgments—We thank Dr. Irene Caras and Genentech for graciously providing pGHDASF37 expression vector and polyclonal anti-human growth hormone antiserum and Dr. Karen Norris from the University of Pittsburgh School of Medicine for helpful suggestions regarding experimental design. From the University of Rochester we thank Dr. Mary-Anne Courtsey for assisting with nucleotide sequencing and Drs. Francis Gigiotti and Terry Wright for critical reading and helpful comments regarding the manuscript.

REFERENCES

1. Cushion, M. T., Harmsen, A., Matsumoto, Y., Stringer, J. R., Wakefield, A. E., and Yamada, M. (1994) *J. Med. Vet. Mycol.* 32, 217–228.
2. Hopkins, J. M. (1991) in *Pneumocystis carinii*. (Hopkin, J. M., ed) pp. 1–27, Oxford University Press, New York.
3. Armstrong, M. Y. K., and Cushion, M. T. (1994) in *Pneumocystis carinii* *Pneumonia* (Walzer, P. D., ed) pp. 189–222, Marcel Dekker, New York.
4. Chen, W., Mills, J. W., and Harmsen, A. G. (1992) *Int. J. Exp. Pathol.* 73, 99–120.
5. Hong, S.-T., Steele, S. E., Cushion, M. T., Walzer, P. D., Stringer, S. L., and Stringer, J. R. (1990) *Clin. Microbiol.* 28, 1785–1785.
6. Garber, T. R., and Stringer, J. R. (1994) *Infect. Immun.* 62, 3092–3101.
7. Haidaris, P. J., Wright, T. W., Gigiotti, F., and Haidaris, C. G. (1992) *J. Infect. Dis.* 166, 1113–1121.
8. Kovaecs, J. A., Powell, F., Edman, C. J., Lundsgren, B., Martinez, A., Drew, B., and Angus, C. W. (1993) *J. Biol. Chem.* 268, 6034–6040.
9. Bauer, N. L., Paulsrud, J. R., Bartlett, M. S., Smith, J. W., and Wilde, C. A. (1993) *Infect. Immun.* 61, 1315–1319.
10. Gigiotti, F. (1992) *J. Infect. Dis.* 165, 329–336.
11. Walzer, P. D., and Rutledge, M. E. (1980) *J. Infect Dis.* 142, 449.
12. Yoneda, K., and Walzer, P. D. (1980) *J. Biol. Chem.* 255, 709–720.
13. Limper, A. H., Potratz, S. T., and Martin, W. J. (1991) *J. Lab. Clin. Med.* 118, 492–499.
14. Limper, A. H., Stringer, J. E., Hoffman, O. A., Castro, M., and Neese, L. W. (1995) *Infect. Immun.* 63, 4302–4309.
15. Potratz, S. T., Paulsrud, J. S., and Martin, W. J. (1991) *J. Clin. Invest.* 88, 403–407.
16. Zimmerman, P. E., Voelker, D. R., McCormack, F. X., Paulsrud, J. R., and Martin, W. J. (1992) *J. Clin. Invest.* 89, 145–149.
17. Fisher, D. J., Gigliotti, F., Zauderer, M., and Harmsen, A. G. (1991) *Infect. Immun.* 59, 3372–3376.
18. Gigiotti, F., and Hughes, W. T. (1988) *J. Clin. Invest.* 81, 1666–1668.
19. Wright, T. W., Bissonodil, T. Y., Haidaris, C. G., Gigiotti, F., and Simpson, Haidaris, P. J. (1995) *DNA Res.* 2, 77–88.
20. Gigiotti, F., Garvy, B. A., and Harmsen, A. G. (1996) *Infect. Immun.* 64, 1592–1599.
21. Gigiotti, F., Stokes, D. C., Cheatham, A. B., Davis, D. S., and Hughes, W. T. (1986) *J. Infect. Dis.* 154, 315–322.
22. De Stefano, J. A., Cushion, M. T., Sleight, R. G., and Walzer, P. D. (1990)
23. Matsumoto, Y., Matsuda, S., and Tegoshi, T. (1989) J. Protozool. 36, 21S–22S

24. Bangs, J. D., Hereld, D., Krakow, J. L., Hart, G. W., and Englund, P. T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3207–3211

25. Ferguson, M. A. J., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) Science 239, 753–759

26. Hereld, D., Krakow, J. L., Bangs, J. D., Hart, G. W., and Englund, P. T. (1986) J. Biol. Chem. 261, 13813–13819

27. Caras, I. W., Weddell, G. N., and Williams, S. R. (1989) J. Cell Biol. 108, 1387–1396

28. Guadiz, G., Sporn, L. A., and Simpson-Haidaris, P. J. (1997) Blood 90, 2644–2653

29. Bergelson, J. M., Chan, M., Solomon, K. R., St. John, N. F., Lin, H., and Finberg, R. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6245–6248

30. Cezanne, L., Navarro, L., and Tocanne, J.-F. (1992) Biochim. Biophys. Acta 1112, 205–214

31. Cross, G. A. M. (1990) Annu. Rev. Cell Biol. 6, 1–39

32. Englund, P. T. (1993) Annu. Rev. Biochem. 62, 121–138

33. Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A., and Kincade, P. W. (1988) J. Immunol. Methods 113, 101–111

34. Aronson, N., and Touster, O. (1974) Methods Enzymol. 31, 90–102

35. Spector, D. L., Goldman, R. D., and Leinwald, L. A. (1998) Cells: A Laboratory Manual, vol. 1, 34.1–34.9 Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

36. Sunkin, S. M., and Stringer, J. R. (1996) Mol. Microbiol. 19, 283–295

37. Wright, T. W., Simpson-Haidaris, P. J., Gigliotti, F., Harmsen, A. G., and Haidaris, C. G. (1994) Infect. Immun. 62, 1515–1519

38. Moran, P., and Caras, I. W. (1994) J. Cell Biol. 125, 333–343

39. Conzelmann, A., Fankhauser, C., Pusti, A., and Desponds, C. (1991) Cell Biol. Int. Rep. 15, 863–873

40. Lu, C.-F., Montijn, R. C., Brown, J. L., Klis, F., Kurjan, J., Bussey, H., and Lipke, P. N. (1995) J. Cell Biol. 128, 333–340

41. Lu, C.-F., Kurjan, J., and Lipke, P. N. (1994) Mol. Cell. Biol. 14, 4825–4833

42. Wojciechowicz, D., Lu, C.-F., Kurjan, J., and Lipke, P. N. (1993) Mol. Cell. Biol. 13, 2554–2563

43. Schreuder, M. P., Brekelmans, S., van den Ende, H., and Klis, F. M. (1993) Yeast 9, 399–409

44. Rosse, W. F., and Ware, R. E. (1995) Blood 86, 3277–3286

45. Kodukula, K., Gerber, S. D., Amthauer, R., Brink, L., and Udenfriend, S. (1993) J. Cell Biol. 120, 657–664

46. Haidaris, C. G., Medzhiradsky, O. F., Gigliotti, F., and Simpson-Haidaris, P. J. (1998) DNA Res. 5, 77–85

47. Rothberg, K. G., Ying, Y.-S., Kamen, B. A., and Anderson, R. G. W. (1990) J. Cell Biol. 111, 2931–2938

48. Webster, P., Joiner, K., and Andrews, N. (1991) Cell Biol. Int. Rep. 15, 799–813

49. Florin-Christensen, M., Florin-Christensen, J., Wu, Y.-P., Zhou, L., Gupta, A., Rudney, H., and Kaneshiro, E. S. (1994) Biochem. Biophys. Res. Commun. 198, 236–242

50. Guo, Z., and Kaneshiro, E. S. (1993) Infect. Immun. 63, 1286–1290

51. Kaneshiro, E. S., and Sleigh, R. G. (1994) in Pneumocystis carinii Pneumonia (Walzer, P. D., ed) pp. 45–71, Marcel Dekker, New York

52. Pesanti, E. L. (1987) Infect. Immun. 55, 736–741

53. Wada, M., Sunkin, S. M., Stringer, J. R., and Nakamura, Y. (1995) J. Infect. Dis. 171, 1563–1568

54. Wada, M., and Nakamura, Y. (1996) DNA Res. 3, 55–64

55. Kapteyn, J. C., Montijn, R. C., Dijkstra, G. J. P., and Klis, F. M. (1994) Eur. J. Cell Biol. 65, 402–407

56. de Nobel, H., and Lipke, P. N. (1994) Trends Cell Biol. 4, 42–45

57. Muller, G., Schubert, K., Fiedler, F., and Bandlow, W. (1992) J. Biol. Chem. 267, 25357–25366

58. Vossen, J. H., Ram, A. F. J., and Klis, F. M. (1995) Biochim. Biophys. Acta 1243, 549–555

59. Nudler, E., Jeno, P., Conzelmann, A., and Riezman, H. (1991) Mol. Cell. Biol. 11, 27–37

60. Saporito-Irwin, S. M., Birse, C. E., Sypherd, P. S., and Fonzi, W. A. (1995) Mol. Cell. Biol. 15, 601–613

61. Haidaris, P. J., Wright, T. W., Gigliotti, F., Fallon, M. A., Whitbeck, A. A., and Haidaris, C. G. (1993) Mol. Microbiol. 7, 647–656