Review

Molecular Microbiology of *Pneumocystis carinii*

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Introduction

Pneumocystis carinii is an opportunistic pathogen which often causes fatal pneumonia in patients under immunosuppressed or immune deficient conditions due to AIDS, cancer chemotherapy or immunosuppressive therapy for organ transplantation¹⁾. Although *P. carinii* was first described by Chagas in 1909²⁾, it was not until the 1980's that people became seriously aware of *P. carinii* when it emerged as a leading cause of opportunistic pulmonary infection and mortality in AIDS patients. The clinical importance of the organism was accentuated by the fact that anti-*P. carinii* agents exhibited major problems of efficacy and toxicity. Solutions to these problems, however, were not easily found because basic research on *P. carinii* had so far been ignored. Nevertheless, in recent years since investigators succeeded in isolating nucleic acids from this organism in 1988^{3,4)}, there have been marked advances in the molecular biology of *P. carinii*, which led to a new stage of pathobiological and clinical studies^{5,6)}.

Life Cycle

P. carinii is a eukaryotic microorganism that can infect many mammalian hosts, including mice, rats, guinea pigs, rabbits, ferrets, dogs, and horses. The difficulty of continuous *in vitro* cultivation of *P. carinii* impeded the progress of the fundamental study of this organism. Therefore, most of our knowledge has emerged from ultrastructural studies of infected rodents or humans⁷⁾. *P. carinii* develops naturally in alveoli, and has two predominant forms in the life cycle: one, a vegetative form, i.e. a small unicellular

'trophozoite'; and the other, a resting form, i.e. 'cyst' containing eight intracystic bodies. Cysts are assumed to give rise to daughter trophozoites⁸⁾. Trophozoites are most likely responsible for the infection although there is no evidence of which form is infective.

According to the textbook view, inhalation of airborne organisms in early childhood exposes humans to *P. carinii*. It has been widely believed that the organism evades clearance and remains quiescent in the host for long periods of time despite intact immunity. Under conditions of immune deficiency or suppression, a latent infection is reactivated. On the other hand, evidence of miniepidemics of *P. carinii* pneumonia has recently raised the concern that *P. carinii* pneumonia is due to reinfection rather than relapse since the infection may be transferable from one immunosuppressed patient to another⁹⁾. Animal studies of *P. carinii* pneumonia have documented animal-to-animal transfer and seem to support this finding⁵⁾. Current application of PCR to diagnosis indicated that *P. carinii* disappears during the course of therapeutic treatment with anti-*P. carinii* drugs and is not detected in normal lungs or sputum^{10,11)}. Using animal models, it remains to be investigated whether *P. carinii* pneumonia is due to relapse or reinfection, though they may not be mutually exclusive.

Molecular Taxonomy

P. carinii was found more than 80 years ago and was traditionally thought to be a protozoan. This misconception was perpetuated by its response to anti-protozoan agents such as pentamidine and trimethoprim/sulfamethoxazole. However, a number of *P. carinii* genes have been cloned and sequenced, facilitating molecular taxonomy. Ribosomal RNA genes have been widely used to study the evolutionary relationships among microorganisms¹²⁾. Analysis of the 5S RNA or 18S

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RNA for rat *P. carinii* revealed that the organism is much more closely related taxonomically to fungi such as *Saccharomyces cerevisiae* or *Shizosaccharomyces pombe* than to protozoa^{4, 13, 14}). Analysis of other ribosomal RNA genes (5.8S, 26S) supported the close relationship to fungi^{15, 16}). This was further confirmed by the presence of the translation elongation factor 3 gene, which is specific to fungi, in *P. carinii* organisms¹⁷).

Although increasing evidence supports the close relationship to fungi, it is unknown to what degree this molecular taxonomy reflects the true nature of *P. carinii*. As described below, *P. carinii* has a genetic system to switch cell surface antigens, which is known for the protozoan *Trypanosoma*, but not for fungi. Several criteria may be required for determining the taxonomic status of *P. carinii*.

Molecular Biology

There have been striking advances in *P. carinii* research in recent years in the area of molecular biology and the related pathobiology^{5, 18}). As described above, several *P. carinii* genes encoding proteins, enzymes and rRNAs were cloned and sequenced. For taxonomic analysis as well as for the discovery of anti-*P. carinii* drugs, several metabolic enzyme genes were cloned, including those for dihydrofolate reductase (DHFR) and thymidylate synthase (TS) that are encoded on separate chromosomes^{19, 20}). Studies of the base composition of the genome of rat *P. carinii* have shown a high content of adenine (A) and thymine (T)¹⁹⁻²³). These studies not only significantly improved our basic understanding of *P. carinii*, but provided us with powerful technology for clinical application as will be described shortly.

The genome of *P. carinii* has been studied by electrophoretic karyotyping, i.e., the separation of chromosomes by pulse field radiant gel electrophoresis. This study showed that the haploid genome consists of about 13-16 chromosomes containing 700-1000 kb of DNA, which is approximately two-thirds the size of the *Saccharomyces* genome²⁴⁻²⁶). Differences in the karyotype pattern have appeared in *P. carinii* isolates from different rat strains and different commercial vendors²⁶⁻²⁸). The diversity of the karyotype pattern was also seen among human *P. carinii* isolates, which seem to consist of equivalent numbers of karyotype bands between 200 and 700 kb in size, as well as among *P. carinii* isolates from other animals²⁹), showing the heterogeneity of *P. carinii* isolates. Another work has also indicated that some laboratory rats can contain two different genetic variants of *P. carinii* that differ in electrophoretic karyotype²⁸).

Molecular Diagnosis

A definitive diagnosis of *P. carinii* pneumonia can only be made by detecting the organism in clinical specimens, such as sputum, bronchoalveolar lavage fluid and transbronchial biopsy samples. Conventionally, Grocott, toluidine blue O, and Giemsa stainings are usually performed to identify *P. carinii* in these materials. These histochemical methods, however, have an inherent lack of sensitivity and/or specificity and the organism can not always be detected, especially in non-invasive specimens. Therefore, due to the uncertainty of the diagnosis, a number of patients are treated with anti-*P. carinii* therapy coincident with ganciclovir and/or other antibiotic therapy. In contrast to the uncertainty of standard diagnosis methods, the polymerase chain reaction (PCR) method provides better sensitivity and specificity in detecting *P. carinii*^{10, 11, 30}). There have been increasing applications of PCR to detect *P. carinii* genes including ribosomal DNAs in the respiratory tract of patients and animal models with pneumocystosis³¹⁻³⁶). Improved diagnoses by PCR have indeed made bronchoscopy unnecessary in many cases.

P. carinii pneumonia in patients with AIDS is usually treated for 3 weeks and those with other types of immunodeficiency for 2 weeks, both determined empirically. In other words, therapy is continued based on the regimen but not on a valid index of morbidity, because there have been no reliable therapeutic markers. Nevertheless, PCR allows early diagnosis of *P. carinii* pneumonia and close therapeutic monitoring with non-invasive specimens such as sputum³⁷).

Surface Antigens and Pathobiology

P. carinii are coated by abundant surface proteins of great interest to investigators^{21, 22, 38-59}). The major protein components of both cysts and trophozoites are proteins called MSG (for major surface glycoprotein)^{5, 58, 59}), P115⁴⁶), gp120⁴⁸) or gpA^{56, 57}), with an apparent molecular mass ranging from 95 to 140 kDa, depending on host species, degree of glycosylation, and isolation procedures. The nomenclature for this antigen complex is somewhat confusing, and we refer to it here as MSG as it represents closely the nature of this antigen.

Upon *P. carinii* infection, the organism passed through the initial host defenses is deposited within alveoli. The initial event in the pathogenesis of *P. carinii* pneumonia is the preferential attachment of the organism to type I alveolar epithelial cells¹⁸). Although the mechanisms of attachment are not fully understood, MSG is

thought to play an important role in the interaction of *P. carinii* with host cells, involving other components such as cell adhesive proteins and lectins^{44, 60-68}). *P. carinii* trophozoites can bind to alveolar epithelial cells by using fibronectin as a ligand, and MSG has been implicated as the binding site for fibronectin. Other extracellular matrix adhesive proteins that may be involved include laminin and vitronectin. *P. carinii* may also bind to epithelial cell surface glycoproteins by means of a mannose-dependent mechanism⁶⁹). Thus, attachment to type-I alveolar epithelial cells may be mediated by multiple mechanisms.

There is evidence that *P. carinii* infection stimulates humoral and cellular immune responses in the host. For example, passive immunization with an anti-MSG monoclonal antibody partially protects against the progression of *P. carinii* pneumonia in animal models⁷⁰), and a specific T-cell response to MSG molecules occurs after immunization and natural infection^{71, 72}). Hyperimmune serum administered to SCID mice with *P. carinii* pneumonia results in clearing of the infection⁷³). Cases of pneumocystosis have also been described in patients and mice with B-cell defects⁷⁴⁻⁷⁶).

The alveolar macrophages play a central role in the clearance of microorganisms from the lung through migration, attachment and phagocytosis. Binding of *P. carinii* to macrophages is mediated by adhesive proteins such as fibronectin⁷⁷) and by mannose-dependent mechanisms in which the macrophage mannose receptor clearly targets the mannose moiety of MSG for uptake⁷⁸). Clearly, MSGs play a crucial role in pathobiology of *P. carinii* such as infection, host-parasite interaction and host defense.

Major Surface Glycoprotein

The frequent generation of anti-MSG monoclonal antibodies implicated the strong antigenic activity of MSG molecules, presumably, in part, because of their unusual abundance on the surface of *P. carinii*. Immunohistochemical analysis using anti-MSG monoclonal antibodies demonstrated that ferritin granules conjugated with these antibodies bound to the surface of the cyst as well as of the trophozoite of *P. carinii*⁴⁶). Immunoblotting analysis of *P. carinii* proteins separated by SDS-polyacrylamide gel electrophoresis clearly showed that a major part of the total protein is seen in both cysts and trophozoites at 105-120 kDa and that this major broad band is a common epitope to the anti-MSG monoclonal antibody^{46, 48}).

The *P. carinii* cyst has a thick cell wall composed of carbohydrates, consisting primarily of glucose, mannose, galactose, and lesser amounts of

N-acetyl-D-glucosamine, ribose, and sialic acid⁷⁹). MSG contains mannosyl, glycosyl, and N-acetylglucosamine residues, displays species-specific antigenic variation, and possesses collagenase sensitivity. Tanabe *et al.*⁴⁶) first demonstrated biochemically that MSGs are glycoproteins containing mannose-rich oligosaccharides.

They analyzed the monosaccharide composition of MSG by high-performance liquid chromatography after hydrolysis to monosaccharides, revealing that mannose is the main sugar component. Mammalian alveolar macrophages express a receptor that binds mannosylated glycoproteins⁸⁰⁻⁸²), and the mannose moiety of MSGs is attacked by the macrophage in the uptake of *P. carinii*⁷⁸).

Biochemical studies have revealed that MSGs are acidic glycoproteins that have several isoelectric points^{45, 46}). Deglycosylation with endoglycosidase F or α -mannosidase increased migration of MSG molecules in SDS polyacrylamide gel electrophoresis, and decreased isoelectric variation⁴⁶). Most monoclonal and polyclonal antibodies reacted with all of these isoelectric variants. However, these deglycosylated MSGs no longer, if at all, reacted with most antibodies against authentic MSGs, showing that the sugar moiety participates in the strong immunodeterminant structure of *P. carinii*. Limiting proteolysis of the isoelectric variants generated common major fragments, showing that they share a common polypeptide portion. Isoelectric variation, therefore, seems to be caused mainly by heterogeneity in the carbohydrate chains.

MSG cDNAs

MSG genes are highly polymorphic, preventing us from viewing them simplistically. After some years of investigation, three groups have succeeded in the cloning of MSG cDNAs. Haidaris *et al.*⁵⁷) have reported the partial cloning and sequence of a cDNA encoding a gpA antigen, analogous to MSG, of *P. carinii* from ferrets. Kovacs *et al.*⁵⁸) and Wada *et al.*⁵⁹) have reported the cloning and sequence of a cDNA encoding MSG of *P. carinii* from rats. Screening of the cDNA expression library of *P. carinii* using anti-MSG antibody has shown that the frequency of appearance of positive clones was unusually high ($10^{-2} \sim 10^{-3}$) reflecting the abundance of MSG in the vegetative cycle of *P. carinii*. These cDNA clones encoded a single open reading frame, shared identical or homologous sequences, and were classified to different subtypes.

Through these cDNA analyses, it became apparent that the MSG subtypes of rat *P. carinii* are homologous over the entire length of the

proteins except for some variable regions. The variable regions map to the middle of the proteins as well as to the C-terminal region⁸³⁾ except for the C-terminal end which is highly conserved⁸⁴⁾. The MSG family of rat *P. carinii* contains 65-80% identical amino acids among the subtypes and shares 32-36% identical amino acids with gpA of ferret-derived *P. carinii*.

The 5' upstream regions of MSG-cDNAs were investigated by the 5' RACE method⁸⁵⁾. The data revealed that 5' RACE products contained a highly conserved sequence in their 5' region (termed UCS for upstream conserved sequence). Northern blot hybridization showed that the UCS was present in the majority of the MSG transcripts. Sequence analysis of UCS-MSG transcripts showed them all to contain the same UCS⁸⁵⁾. MSG mRNAs initiate at one of two sites, P1 or P2. All the clones were identical in sequence from P2. Sequence analysis of these RACE clones revealed a translation start site, and initiation from this AUG would produce a putative signal peptide which may be used in deposition of MSGs on the cell surface.

Genomic Organization for MSG

Genomic hybridization analysis clearly indicated that the cDNA diversity reflects a multiple MSG-gene family in the *P. carinii* chromosome. An MSG cDNA probe hybridized to multiple restriction fragments, while the same probes did not hybridize to DNAs from uninfected rat lungs. Therefore, a complex group of MSG genes indeed exists in *P. carinii*. Moreover, PCR analysis demonstrated that at least some MSG genes cluster tandem in the chromosome⁵⁹⁾. In fact, several genomic fragments carrying two or three MSG genes have been cloned⁸⁶⁻⁸⁹⁾. Therefore, sequence diversity of MSG-cDNAs is likely caused by the diversity as well as the multiplicity of genomic MSG genes.

Each *P. carinii* cell has the potential to express a number of different MSG isoforms because the genome of *P. carinii* encodes at least a hundred different MSG genes, distributed among all of the 14-15 discernible chromosomes^{58, 59, 89)}. The multiplicity and diversity of MSG genes were first discovered in *P. carinii* from rats. Cloning and sequencing analyses using organisms from other animals such as ferrets, mice, and humans, have subsequently shown a similar sequence diversity and multiplicity of MSG^{29, 86, 90)}. The precise degree of multiplicity remains to be investigated.

Comparison of RACE clones to genomic clones⁸⁷⁻⁸⁹⁾ showed that they diverge just upstream of position 400. Southern blot hybridization clarified

the reason for this. The UCS probe hybridized to a single band (approximately 500 kb in size) in the electrophoretic karyotype, while an MSG probe hybridized to all the bands. Similarly, the UCS hybridized to relatively few restriction fragments of *P. carinii* DNA, as compared to an MSG probe. These experiments suggest that the UCS is located on one chromosome, in which case most MSG genes cannot be linked to the UCS.

Those MSG genes that were attached to a UCS were amplified by the PCR using an upstream sense primer of UCS, paired with a downstream antisense primer of the variable portion of MSG. The amplified products contained a mixture of DNA fragments that shared a UCS at one end, and contained a different MSG gene at the other^{85, 91)}. Some of these clones chosen at random were all different in the MSG region, suggesting that the population of *P. carinii* genomes contained many different MSG genes attached to the UCS. Although the sequence of UCS is highly conserved, extensive amplification of UCS sequences from rat *P. carinii* populations showed some slight polymorphism within the UCS sequence⁹¹⁾.

These results show that the most abundant MSG mRNAs are attached to UCS, a sequence that is found on only one of the *P. carinii* chromosomes resolved by electrophoresis. The UCS is found attached to MSG genes, suggesting that the UCS occupies a genome position permissive for MSG transcription. The most likely explanation is that the population is genetically heterogeneous at the UCS locus, and that this heterogeneity is brought about by DNA recombination, which installs different MSG coding sequences at a limited number of expression sites (perhaps only one) as will be discussed shortly. Initial hybridization data have shown that the UCS was present on only one band in an electrophoretic karyotype, suggesting that the UCS locus is unique⁸⁵⁾.

Telomeric Localization and Expression of MSG

Several lines of evidence showed that a UCS site is telomeric^{91, 92)}. A telomeric UCS DNA was cloned from a genomic library of *P. carinii*, and telomeric 11-kb UCS fragments were identified⁹²⁾. Sequence analysis of one of the clones, λ MW124, showed that it contained the UCS and one MSG-coding sequence, but no other significant open reading frames. This MSG sequence is highly homologous, but not identical, to the known MSG genes. At the 3' terminus, there were seven tandem hexanucleotide repeats with the sequence of TTAGGG oriented 5' to 3' to the chromosomal end. TTAGGG is the sequence of telomeric DNA in all vertebrates, the protozoan *Trypanosoma*, and

several slime molds and fungi, revealing that the UCS is telomeric. The subtelomeric regions immediately internal to the simple repeats consist of moderately repetitive sequences, called telomere-associated DNA.

The TTAGGG telomere sequence and subtelomeric sequences are not unique to the 0.5 megabase chromosome containing UCS, but seem to be common to all other *P. carinii* chromosomes. The 14-15 chromosome blot resolved by pulse-field gel electrophoresis hybridized to this hexamer sequence as well as to the subtelomeric sequences⁹². Telomeric fragments other than the UCS were cloned by screening with the (TTAGGG)₅-oligo DNA probe and the subtelomeric probe. Until now, two non-UCS telomeric clones were sequenced and shown to contain TTAGGG repeats at the termini as well as subtelomeric repetitive regions common to the UCS-MSG expression site. Moreover, these two clones contained MSG gene(s) being not associated with the UCS. Frequent occurrences of these telomeric MSG clones (3×10^{-4}) indicate that they represent silent MSG genes localized in the telomere regions.

The discovery of a UCS expression site within the telomere region suggests that MSG genes or batteries of them are translocated to the UCS expression site by means of reciprocal recombination or gene conversion. This seems to be true of the DNA rearrangement of the Variant Surface Glycoprotein (VSG) genes of the protozoan parasite *Trypanosoma brucei*⁹³⁻⁹⁵. Trypanosome VSG undergoes antigenic variation to evade the host immune system. There are many VSG genes on the chromosomes and minichromosomes, but the VSG expression sites are restricted to telomeric regions and silent VSG genes can be translocated to the expression site by DNA recombination⁹³⁻⁹⁵. The same telomere-associated recombination model for *P. carinii* MSGs has been proposed^{91,96}.

Antigenic Variation

MSGs play a crucial role in pathobiology of *P. carinii* such as infection, host-parasite interaction and host defense by T cells, alveolar macrophages or humoral immunity. Therefore, in immunocompromised states in man or experimental animals, it is likely that the surface structure of *P. carinii* may change by switching expression of the MSG genes to escape from cellular or humoral immunity. If recombination is involved in expression of specific MSG genes, it could take place through either of two mechanisms. One possibility is recombination mediated by sequence homology, as is thought to be the case in the rearrangement of trypanosome VSG genes⁹³⁻⁹⁵. Alternatively, a site

specific endonuclease (or recombinase) might be used. It is noteworthy that the 3' terminus of the UCS shares a 28-bp sequence with the 5' termini of silent MSG coding sequences (conserved recombination junction element, CRJE). Recombination at this site is suggested by most UCS-MSG junctions, and each showed sequence differences within 50 bp downstream of the UCS⁸⁵.

To our knowledge, *P. carinii* is the only fungus that uses a genetic system to switch cell surface determinants. The complexity of MSG gene expression implies that antigenic polymorphism and variability play an important role in *P. carinii* pathobiology. Antigenic variation may contribute to the prevalence of *P. carinii* pneumonia in AIDS patients with a low number of CD4+ T cells compared with other microbes. Alternatively, we speculate that antigenic variability is used to maintain opportunistic infection in a single animal host, rather than for relapses of this organism, because of moderate alterations (about 70% sequence conservation) in the MSG structures. As is true in other cases of recombination-mediated events, site-specific endonuclease(s) may be an important target for further investigation into genetic control of MSG switching.

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