Characterization of variants of the gene encoding the p55 antigen in *Pneumocystis* from rats and mice

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Variants of the *p55* gene in rat-derived *Pneumocystis carinii* have been identified and its counterpart in mouse-derived *P. carinii* f. sp. *muris* has been cloned. By PCR amplification of *P. carinii* genomic DNA, five variants were identified that differed from each other in size and sequence, primarily in the number and size of encoded amino acid repeats. For *P. carinii* f. sp. *muris*, a single PCR fragment (471 bp) was obtained, which contained an incomplete ORF encoding a 157 aa protein that was most similar to a p55 variant in *P. carinii*, with nucleotide and amino acid sequence identity of 79 and 68 %, respectively. Southern blot analysis revealed the presence of more than one copy of the *p55* gene in both *Pneumocystis* species. Thus, like other *Pneumocystis* antigens, p55 exhibits polymorphism that could potentially benefit the organism in host interactions.

Received 20 November 2002

Accepted 3 July 2003

INTRODUCTION

Pneumocystis is an important opportunistic pathogen that causes severe pneumonia in patients with AIDS and other immunocompromising conditions. Two major groups of antigens have been identified in this organism by immunoblotting. One group, which is present as a broad band of 90–120 kDa, has been well-characterized as the major surface glycoprotein (MSG) in *Pneumocystis* organisms derived from rats, humans, mice, ferrets and rabbits (Graves *et al.*, 1986; Walzer & Linke, 1987; Gigliotti *et al.*, 1988; Kovacs *et al.*, 1989, 1993; Bauer *et al.*, 1993; Kutty *et al.*, 2001). The second group, which appears as a broad band of 45–55 kDa in *P. carinii* and 35–45 kDa in human-derived *Pneumocystis jiroveci* (Stringer *et al.*, 2002), is less well-studied (Walzer & Linke, 1987; Smulian *et al.*, 1992, 1993, 2000). The gene that encodes a 45–55 kDa protein of *P. carinii*, designated p55, has been cloned (Smulian *et al.*, 1992, 1993). This gene is characterized by the presence of 10 repeats of a heptapeptide motif that is rich in glutamic acids near the C-terminus. Southern blot analysis has shown that this gene is present as a single copy. Unlike MSG, which resides on the outer surface of *P. carinii*, p55 is located within the cell wall (Broomall *et al.*, 1998). Currently, the role of p55 in the biology and pathogenesis of *Pneumocystis* infection remains unclear. Theus *et al.* (1994) demonstrated a cell-mediated immune response to p55 in animals exposed previously to *P. carinii*. A

more recent study has shown that immunization with recombinant *P. carinii* p55 can afford partial protection against infection (Smulian *et al.*, 2000).

Whilst screening a *P. carinii* cDNA library, we identified a DNA fragment that was highly homologous to the *P. carinii* *p55* gene but lacked the heptapeptide repeats. We undertook to further characterize the *p55* gene in *P. carinii* and to clone the *p55* gene in mouse-derived *P. carinii* f. sp. *muris*.

METHODS

***Pneumocystis* organisms and DNA/RNA extraction.** *P. carinii* organisms were isolated from the lungs of immunosuppressed rats, provided under contract by Indiana University (Indianapolis, IN, USA). *P. carinii* f. sp. *muris* organisms were isolated from the lungs of severe combined immune deficient (SCID) mice in our laboratory. Genomic DNA was extracted by treatment with proteinase K followed by phenol/chloroform extraction, as described previously (Ma *et al.*, 1999). Total RNA was extracted by using a RNeasy B Isolation kit (Tel-Test). Experimentation guidelines of the US Department of Health and Human Services and the NIH were followed in the conduct of this research.

PCR and DNA sequencing. Based on regions conserved between the previously reported *p55* sequence (Smulian *et al.*, 1992, 1993) and a DNA fragment that we identified by sequencing randomly picked clones from a *P. carinii* cDNA library (Edman *et al.*, 1989) (GenBank accession no. AF494450), we designed the primer pair PU947 and PD1637 (Table 1). PCR mixture (50 µl) contained 100 ng genomic DNA, 0.5 µM each primer, 0.2 mM dNTPs, 1× PCR buffer (10 mM Tris/HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl₂) and 2.5 U AmpliTaq Gold DNA polymerase (PerkinElmer). Amplification was performed in a PTC-100 programmable thermal controller (MJ Research) with a touchdown cycling protocol as follows: 95 °C for 8 min, then 10 cycles of 1 min at 95 °C, 2 min at 65 °C (with a decrease by 1.5 °C every cycle to reach 50 °C in the last cycle) and 2 min at 72 °C, followed by 30 cycles of 1 min

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Abbreviation: MSG, major surface glycoprotein.

The GenBank/EMBL/DBJ accession numbers for the sequences of *Pneumocystis* *p55* variant genes are AF494448–AF494452.

Table 1. Oligonucleotide primers and probes used in this study

Oligonucleotide	Sequence (5'→3')	Description
PU947	GCAAAGACATTTTAGCAGAATCGTG	Corresponding to positions 280–304 of <i>p55</i> variant v3 (GenBank accession no. AF494450)
PD1637	ACTCCTAATGTCATTCCCTATAATTGCAC	Complementary to positions 769–796 of <i>p55</i> variant v3
<i>cp</i>	GCAAAGACATTTTAGCAGAATCGTGTAAGGAGTGCA	Corresponding to positions 280–316 of <i>p55</i> variant v3
<i>v0p</i>	GATGAAAAGGAAAAGCCTACTGTAGAAGGGGGAGAA	Corresponding to positions 382–417 of <i>p55</i> (M77242)
<i>c1r</i>	TGCACTTTTTTACACGATTCTGCTAAAATGTCTTTGC	Complementary to <i>cp</i>
<i>v01r</i>	TCAGGAGGAGGAGAAGGATGAGGACCAGGAAGACCTGCAC	Complementary to positions 14–62 of <i>p55</i>
<i>v23r</i>	TCAGTAGGCCCTGGTTCTTCAGTAGGTGCTGGTTTTTCAGTAGTCGCTGGCTC	Complementary to positions 525–577 of <i>p55</i> variant v3
M39	GAGAGCTGCTCAAAATTTAGTGATAGTTCTACCTTTACA	Corresponding to 14–52 of <i>p55</i> (AF494452)
M472	TCTACACGAACACCGTGACCTT	Complementary to 426–447 of <i>p55</i> (AF494452)

at 95 °C, 2 min at 50 °C and 2 min at 72 °C. Each experiment included a negative control without template DNA. PCR products were sequenced either directly or after subcloning into vector pCR 2.1 as described previously (Ma *et al.*, 1999). Sequence analysis was performed by using MacVector 7.0 software (Oxford Molecular). Nucleotide sequences obtained in this study are available from GenBank under accession numbers AF494448–AF494451 for *P. carinii* *p55* variant genes and AF494452 for the *P. carinii* f. sp. *muris* *p55* gene.

Southern blot analysis with *P. carinii* DNA. Based on the sequence alignment of the five *p55* gene variants in *P. carinii* (Fig. 1), we designed conserved and variant oligonucleotide probes, *cp* and *v0p* (Table 1). Probes were labelled with digoxigenin-dUTP by using the DIG Oligonucleotide Tailing kit (Boehringer Mannheim). Genomic DNA samples (2.5 µg) were digested with *Ssp*I, *Ase*I, *Xba*I or *Eco*RI. These enzymes recognize AT-rich sequences and have been found to cut *Pneumocystis* genomic DNA more frequently than other enzymes (Ma

Fig. 1. Alignment of deduced amino acid sequences of *p55* variants in *P. carinii* (rPC) and *P. carinii* f. sp. *muris* (mPC). Identical residues are shaded and boxed. The sequence of variant v0 is identical to that reported previously (Smulian *et al.*, 1992). Lines above the sequences indicate heptapeptide repeats and lines below the sequences indicate hexapeptide repeats; the regions from which the sequences of probes *cp* and *v0p* were derived for use in Southern blotting are shown. GenBank accession numbers for the sequences of variants v1–v4 are AF494448–AF494451.

et al., 1999; Kutty *et al.*, 2001). No restriction sites for these enzymes were present in the sequences of probes used in this study. Digested DNA was separated by 1 % agarose gel electrophoresis and transferred to a Nytran membrane (Schleicher & Schuell). Blots were hybridized with probes overnight at 42 °C by using DIG Easy Hyb hybridization solution (Boehringer Mannheim), washed twice in 2× SSC and 0.1 % SDS at room temperature for 5 min each, then twice in 1× SSC and 0.1 % SDS at 42 °C for 15 min each. Hybridization signal was detected by using a DIG Luminescent Detection kit (Boehringer Mannheim). Blots were stripped by incubating twice for 30 min each at 80 °C in stripping buffer that contained 50 % deionized formamide, 5 % SDS and 50 mM Tris/HCl (pH 7.5) before rehybridizing with a different probe.

Southern blot analysis with *P. carinii* f. sp. *muris* DNA. A DNA fragment was amplified from *P. carinii* f. sp. *muris* genomic DNA by PCR with primers M39 and M472 (Table 1) and a PCR DIG Probe Synthesis kit (Boehringer Mannheim). Genomic DNA samples (9.4 µg) were digested with *EcoRV*, *HindIII*, *HpaI* or *XbaI*. Subsequent procedures were done under the same conditions as described above, except that high-stringency washing was performed in 0.16× SSC and 0.1 % SDS at 65 °C for 15 min each.

Northern blot analysis with *P. carinii* RNA. Total RNA was separated by 1.2 % agarose gel electrophoresis in the presence of formaldehyde, transferred to a Nytran membrane (Schleicher & Schuell) and hybridized at 42 °C overnight with probes *c1r*, *v01r* or *v23r*, which were designed from conserved regions or specific variants. Washing and detection conditions were the same as for Southern blotting with *P. carinii* DNA.

RESULTS AND DISCUSSION

p55 in *P. carinii*

Whilst screening a *P. carinii* cDNA library, we identified a 906 bp fragment (GenBank accession no. AF494450), which contained a single incomplete ORF encoding a 269 aa protein that was homologous to the previously reported *P. carinii* p55 gene and protein (Smulian *et al.*, 1992, 1993), with respectively 76 and 60 % sequence identity in overlapping regions. Given that MSG and another *P. carinii* surface protein, PRT-1, are encoded by multi-copy gene families (Kovacs *et al.*, 1993; Lugli *et al.*, 1997; Russian *et al.*, 1999), we wanted to determine whether p55-related proteins are encoded by multiple genes. We amplified *P. carinii* genomic DNA from a single rat isolate by PCR with primers (PU947 and PD1637; Table 1) that were based on regions conserved between these two sequences. PCR amplification resulted in two distinct bands. After subcloning of amplified products, we obtained 12 clones, which were subsequently sequenced. Alignment of these sequences identified closely related but unique p55 variants, designated v0–v4, which consisted of five, one, one, three and two clones, respectively. Replicate clones of each variant were identical, except that two of the five clones of variant v0 contained a single nucleotide change at unique positions, which may be PCR artefacts. Fig. 1 shows an alignment of deduced amino acid sequences of the five variants. The sequence of variant v0 is identical to the published p55 sequence (Smulian *et al.*, 1992) and contains 10 repeats of a heptapeptide motif near the C-terminus. Compared to v0, the other four variants (v1–v4) differed

primarily in the number and size of heptapeptide repeats, although sporadic single nucleotide polymorphisms were identified. Variant v1 differs from v0 by the presence of only two repeats of the same heptapeptide motif. Variants v2, v3 and v4 lack a 10 aa sequence near the N-terminus compared to v0 and contain one or five repeats of a hexapeptide, rather than heptapeptide, motif. Variants v2 and v3 differ by nine amino acids in the C-terminus; v3 is identical to the sequence we identified initially. Variant v4 is the shortest sequence and contains only one hexapeptide motif. Additional clones were obtained by subcloning the PCR product from another rat isolate; they were found to correspond to the five variants, with no additional sequences being identified.

Southern blotting with *P. carinii* genomic DNA and a probe (*v0p*) that was specific for variant v0 showed a single band with all four enzymes (Fig. 2), consistent with previous reports (Smulian *et al.*, 1992). When the same blot was stripped and hybridized with a probe (*cp*) that corresponded to a region conserved among all five p55 variants, two bands (one of which corresponded to the single band in Fig. 2a) were seen for all enzymes except for *EcoRI*, which showed a single, high-molecular-mass band. These findings show that *P. carinii* has at least two copies of the p55 gene and may have additional copies or allelic variants.

To determine whether the different variants were transcribed, Northern blotting was performed by using *P. carinii* total RNA (Fig. 3). Hybridization with a probe (*c1r*) from a region conserved among all five variants showed two bands of ~1.5 and 1.7 kb in size (Fig. 3). When the same blot was stripped and hybridized with a probe that corresponded to a region conserved between variants v1 and v0 (probe *v01r*) or between variants v2 and v3 (probe *v23r*), only one band was observed. Probe *v01r* hybridized to the higher band and *v23r* hybridized to the lower band. As only partial sequences were available for variants v1–v4, precise size of the corresponding mRNA could not be predicted. It is unclear whether individual bands present on the blot represent single variants. Given that available sequences for variants v2 and v3 are identical in size (487 bp) but differ in nucleotide sequence, the single band that hybridized to probe *v23r* is probably a mixture of two transcripts of the same size. As the available DNA sequence of variant v1 is only 147 bp shorter than that of variant v0 in the overlapping region, they may not be separated well by the gel; thus, the single band detected with probe *v01r* may also represent a mixture of two transcripts. However, the possibility that RNA expression levels differ among variants cannot be ruled out.

p55 in *P. carinii* f. sp. *muris*

PCR amplification of *P. carinii* f. sp. *muris* genomic DNA with primers PU947 and PD1637 (Table 1) yielded a single band. Direct sequencing revealed a 471 bp fragment (GenBank accession no. AF494452) with an incomplete ORF encoding a 157 aa protein, which was most similar to variant v3 of p55 *P. carinii* (Fig. 1) with 79 and 68 % identity in nucleotide and amino acid sequences, respectively. Southern

Fig. 2. Southern blot analysis of *P. carinii* genomic DNA digested with four restriction enzymes (indicated above each lane). (a) Hybridization with a probe (v0p) that is specific for *p55* variant v0; (b) the blot in (a) was stripped and hybridized with a probe (cp) derived from a region conserved among five *p55* variants. Size markers are in lane M; DNA sizes (bp) are given on the left.

Fig. 3. Northern blot analysis of *P. carinii* RNA. Lanes 1 and 2, hybridization with probe *c1r*, which is complementary to a region shared among all five *p55* variants. Lanes 1 and 2 contained different amounts of *P. carinii* RNA, obtained from a single rat isolate. The blot was then stripped and hybridized with probe *v01r* (lanes 3 and 4) or *v23r* (lanes 5 and 6) derived from a region shared between variants v0 and v1 or between v2 and v3, respectively.

blotting with *P. carinii* f. sp. *muris* genomic DNA and a probe amplified by using primers M39 and M472 (Table 1) showed variable numbers of bands for four restriction enzymes (Fig. 4). Whilst we did not perform subcloning of the PCR product to obtain individual clones, variation of the number of bands in the Southern blot may reflect sequence variation or a complicated organization of multiple gene copies, which suggests that, like *P. carinii*, *P. carinii* f. sp. *muris* may have multiple copies or allelic variants of the *p55* gene.

These studies expand our understanding of the molecular organization of *p55*, which is one of the few antigens of *Pneumocystis* that has been characterized. Although the function of *p55* is unknown, several studies have shown that *p55* is capable of eliciting strong cellular and humoral immune responses in animals naturally infected with *P. carinii* (Smulian *et al.*, 1992, 1993). Active immunization studies with recombinant *p55* antigen have demonstrated partial protection against subsequent *P. carinii* infection in immunosuppressed rats (Smulian *et al.*, 2000). Hence, *p55* appears to play an important role in host–organism interactions. In the present study, we identified multiple variants of the *p55* gene, which are closely related but differ clearly. Whilst PCR or subcloning artefacts could have generated some of the variants that we have identified, identification of the same variants in organisms obtained from two separate animals strongly suggests that all variants are real. Whilst additional studies are needed to better characterize these variants, to try to identify additional variants and to localize the variants on chromosomes, it seems likely, based on variable sequence data as well as Southern and Northern blot studies, that *p55* is encoded by multiple genes, similar to two other surface proteins, MSG and PRT-1 (Kovacs *et al.*, 1993; Lugli *et al.*, 1997; Russian *et al.*, 1999). The presence of multiple *p55* genes supports a hypothesis that was derived from studies of MSG (Angus *et al.*, 1996; Kovacs *et al.*, 1993), i.e. that *Pneumocystis* has the potential for antigenic variability, which could play a role in immune evasion. Previous studies of rats exposed naturally to *P. carinii* have demonstrated that the repeat domain in the carboxyl portion of the *p55* molecule is the target of both cellular and humoral immune responses, whereas the amino portion of this molecule is immunologically silent (Theus *et al.*, 1994). In the current study, we found that *p55* variants differed from each other primarily in the number and size of repeats, suggesting that *Pneumocystis* uses variation in the repeat region to evade host immune defences.

Fig. 4. Southern blot analysis of *P. carinii* f. sp. *muris* genomic DNA digested with four restriction enzymes (indicated above each lane). The probe was a DNA fragment amplified by PCR with primers M39 and M472. DNA sizes (bp) are depicted on the left.

In *P. jiroveci*, the antigen complex of 35–45 kDa is the most common antigen found in lungs and bronchoalveolar fluid of patients with *Pneumocystis* pneumonia and is recognized strongly by serum antibodies of infected patients (Walzer & Linke, 1987; Smulian *et al.*, 1992, 1993). Thus, it would be of great interest to clone the p55 counterpart in *P. jiroveci*. Previous studies have demonstrated that affinity-purified antibodies to the recombinant rat-derived *P. carinii* p55 antigen react with the 35–45 kDa band of human-derived *P. jiroveci*, suggesting the presence of shared epitopes (Smulian *et al.*, 1992). We attempted to amplify *P. jiroveci* genomic DNA by PCR with a number of different primer pairs that were designed from regions conserved among the six sequences shown in Fig. 1. However, no specific sequences were identified. In addition, no hybridization signal was observed in Southern blot analysis with *P. jiroveci* genomic DNA and a variety of probes (either oligonucleotides or PCR products), even under conditions of low stringency (data not shown). These results suggest that *P. jiroveci* does not have a homologue of p55 or that it has evolved substantially and does not have adequate homology to permit identification by these methods.

In conclusion, we have found five variants of the gene that

encodes the p55 antigen in *P. carinii*. Our results suggest the presence of polymorphism in the p55 gene and that at least two copies of this gene are present in the *P. carinii* genome. The p55 gene in *P. carinii* f. sp. *muris* shows a high degree of homology to the *P. carinii* p55 gene, but lacks the heptapeptide repeats. Novel approaches are needed to characterize the p55 gene counterpart in *P. jiroveci*.

ACKNOWLEDGEMENTS

We would like to thank Rene Costello and Howard Mostowski for their assistance. Part of this work was presented at the 7th International Workshops on Opportunistic Protists, Cincinnati, Ohio, 13–16 June 2001, abstract no. PO16.

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