Cryptosporidium parvum appears to lack a plastid genome

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Surprisingly, unlike most Apicomplexa, Cryptosporidium parvum appears to lack a plastid genome. Primers based upon the highly conserved plastid small- or large-subunit rRNA (SSU/LSU rRNA) and the tufA-tRNAPhe genes of other members of the phylum Apicomplexa failed to amplify products from intracellular stages of C. parvum, whereas products were obtained from the plastid-containing apicomplexans Eimeria bovis and Toxoplasma gondii, as well as the plants Allium stellatum and Spinacia oleracea. Dot-blot hybridization of sporozoite genomic DNA (gDNA) supported these PCR results. A T. gondii plastid-specific set of probes containing SSU/LSU rRNA and tufA-tRNAPhe genes strongly hybridized to gDNA from a diverse group of plastid-containing organisms including three Apicomplexa, two plants, and Euglena gracilis, but not to those without this organelle including C. parvum, three kinetoplastids, the yeast Saccharomyces cerevisiae, mammals and the eubacterium Escherichia coli. Since the origin of the plastid in other apicomplexans is postulated to be the result of a secondary symbiogenesis of either a red or a green alga, the most parsimonious explanation for its absence in C. parvum is that it has been secondarily lost. If confirmed, this would indicate an alternative evolutionary fate for this organelle in one member of the Apicomplexa. It also suggests that unlike the situation with other diseases caused by members of the Apicomplexa, drug development against cryptosporidiosis targeting a plastid genome or metabolic pathways associated with it may not be useful.

Keywords: Cryptosporidium parvum, plastid genome, apicoplast, chloroplast

INTRODUCTION

A highly conserved circular 35 kb DNA has been recently identified from several genera within the phylum Apicomplexa (Denny et al., 1998; Wilson & Williamson, 1997), including the coccidians Eimeria (Dunn et al., 1998), Sarcocystis (Hackstein et al., 1995) and Toxoplasma (Beckers et al., 1995), as well as the haematozoans Babesia bovis (Gozar & Bagnara, 1995) and Plasmodium spp. (Wilson et al., 1996; Yap et al., 1997). The entire plastid genomes of both Toxoplasma gondii and Plasmodium falciparum have been sequenced (GenBank U87145 and cited in Fichera & Roos, 1997; Kohler et al. 1997; Wilson et al., 1996). This DNA resides in an organelle called the apicoplast, the origin of which is hypothesized to be either a red or a green alga (Blanchard & Hicks, 1999; Kohler et al., 1997; Williamson et al., 1994). In T. gondii, inhibition of apicoplast DNA synthesis blocked replication of the parasite, thus suggesting that the organelle plays a critical role in its survival (Fichera & Roos, 1997). Because neither humans nor other mammals contain chloroplast-derived organelles, the genome and/or the functions of the apicoplast might serve as parasite-specific targets for drug development against Apicomplexa.

Cryptosporidium parvum is an emerging pathogen that causes one of the opportunistic infections in AIDS patients. Although C. parvum is currently assigned to the class Coccidia, evidence based upon SSU rRNA sequences indicates that Cryptosporidium does not form a monophyletic clade with either intestinal or cyst-forming coccidia, and that the genus is instead monophyletic to the Coccidia + Haematozoa (Escalante & Ayala, 1995; Gajadhar et al., 1991; Van de Peer & De

Abbreviations: FAS, fatty acid synthase; gDNA, genomic DNA; LSU, large subunit; SSU, small subunit.
Wachter, 1997). In fact, others have shown that the class Coccidia is monophyletic only if the genus Cryptosporidium is excluded (Morrison & Ellis, 1997), and that the genus can even be used as an outgroup to determine the phylogenetic position of eucoccidia (Barta et al. 1997).

In addition to its elusive phylogenetic position, several fundamental distinctions between C. parvum and other coccidia have been described, including: (1) the extracytoplasmic, but intracellular, location of the parasite just beneath the enterocyte apical membrane (Fayer et al., 1997); (2) polyamine synthesis by arginine rather than ornithine decarboxylation (Keithly et al., 1997); (3) absence of introns in most genes; (4) insensitivity to anticoccidial drugs (Coombs, 1999); and (5) sporulation of oocysts within the gut lumen, resulting in enterocyte Reinvasion and prolonged, life-threatening infection in patients who are immunocompromised. Since there is currently no effective treatment against cryptosporidiosis, considerable effort has been and is still being expended toward the discovery of cytosolic or organellar metabolic pathways which could serve as leads for therapy (Coombs, 1999).

Previously, others had observed both a plastid-like organelle in sporozoites of C. parvum (Terley et al., 1998) and a low-molecular-mass band in pulse-field gels prepared from them (Blunt et al., 1997). However, in contrast to work with P. falciparum and T. gondii, neither these nor other studies provided direct biochemical or molecular evidence for the presence of an apicoplast or its genome in C. parvum. Therefore, in this study both intracellular stages and free sporozoites of C. parvum were tested for the presence of a plastid genome. Specifically, gDNA from intracellular stages was amplified by PCR using primers specific for plastid LSU/SSU rRNA and tufA-tRNA
\(^{\text{Phe}}\) genes, whereas gDNA of sporozoites was tested by dot-blot hybridization using T. gondii LSU/SSU rRNA and tufA-tRNA
\(^{\text{Phe}}\) plastid genes as probes. A number of organisms with or without a plastid were used as controls for both experiments. Unexpectedly, no plastid genome was detected in C. parvum, whereas its presence in plants, a euglenoid and other apicomplexans used as positive controls was uniformly confirmed. This finding not only provides new molecular evidence for the divergence of C. parvum from its nearest relatives, but suggests that the plastid genome may be unavailable as a drug target in this opportunistic pathogen.

METHODS

Organisms and DNA. Genomic DNA was obtained from cells or tissues representative of a wide range of eukaryotes, including the apicomplexans Cryptosporidium parvum KSU-1 and Iowa strains, Toxoplasma gondii RH strain and Plasmodium falciparum strain C10; kinetoplastids of Leishmania amazonensis MHOM/BR/73/M2269 (ATCC 50131), Trypanosoma brucei brucei EATRO 110 and Crithidia fasciculata SH Hutner strain (ATCC 11745); the euglenoid Euglena gracilis SH Hutner strain Z (ATCC 12716); leaves of the plants Allium stellatum (wild scallions) and Spinacia oleracea (spinach); mammals Homo sapiens (human adenocarcinoma HCT-8 cells, ATCC CCL244) and Mus musculus C57Bl/6J (mouse); the yeast Saccharomyces cerevisiae strain1513; and the eubacterium Escherichia coli XL-1 Blue. Fresh oocysts for obtaining sporozoites by excystation were the Iowa strain (Pleasant Farms, Troy, ID, USA), whereas intracellular parasites in HCT-8 cells were the KSU-1 strain. Uninfected HCT-8 cells served as controls. DNA was isolated from both stages of C. parvum, and from other organisms, using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA), whereas T. gondii and P. falciparum DNA were provided by Drs D. Roos and J. Feagin (University of Pennsylvania and Seattle Biomedical Research Institute, respectively). Concentrations were determined by electrophoresis through 0.8% agarose using 2 DNA standards.

Detection by PCR. Six pairs of degenerate, plastid-specific primers were used to amplify several highly conserved regions of known organellar genes, including three pairs of the LSU rRNA and two pairs of the SSU rRNA or tufA-tRNA
\(^{\text{Phe}}\) genes (Table 1). One pair of primers specific to a C. parvum P-type ATPase (Zhu & Keithly, 1997) served as the positive control in all experiments. The DNA tested included that isolated from uninfected and C. parvum-infected HCT-8 cells, or that from the known plastid-containing eukaryotes T. gondii, Eimeria bovis, A. stellatum and S. oleracea. Each 50 µl PCR reaction consisted of hot-start TaqBezd PCR reagents (Promega) plus 0.2 µM each of the primers and other appropriate reagents. Additional conditions of amplification are detailed in Table 1.

Dot-blot analysis. Duplicate samples containing 1 µg gDNA from each organism were denatured in 0.4 M NaOH/20 mM EDTA by heating at 95°C for 10 min, and were aliquoted into two wells for transfer onto Zeta-probe nylon membranes according to the manufacturer’s instruction (Bio-Rad). Membranes were rinsed with 2×SSC, air-dried for 30 min, baked under vacuum at 80°C for 45 min, and prehybridized for 2 h at 42°C in a 2×SSC solution containing 50% formamide and other standard reagents. Two probes were used for hybridization: (1) the 7-8 kb C. parvum SSU/LSU rRNA genes (GenBank AF040725), which are also highly conserved among all Apicomplexa; and (2) 1 approximately 6.2 kb T. gondii plastid gDNA containing a mixture of SSU/LSU rRNA and tufA-tRNA
\(^{\text{Phe}}\) plastid genes, the gene content and organization of which is remarkably conserved in all apicoplast genomes (see Fig. 1 in Denny et al., 1998). Both probes were labelled with [\(^{32}\)P]ATP using the random-primer method and denatured by incubation in 0.1 M NaOH for 10 min, then 4×105 c.p.m. of each probe was added to the blots for hybridization at 42°C for 18 h. Blots were each washed for 30 min twice in 2×SSC/2% SDS at 55°C and once at 65°C, and then analysed by autoradiography after 4–17 h exposure of Kodak X-Omat AR film.

RESULTS AND DISCUSSION

Although it was assumed that all extant lineages within the phylum Apicomplexa would possess plastid DNA, it now appears that one member of the phylum may not. This should not be too surprising, since the genus Cryptosporidium has always been something of an enigma among apicomplexans in its life style, metabolic pathways, and sensitivity to drugs (Coombs, 1999; Fayer et al., 1997; Keithly et al., 1997). Furthermore, molecular and phylogenetic evidence has been accumu-
lating for some time that this organism may have diverged quite early from other members of the Apicomplexa (Barta et al., 1997; Escalante & Ayala, 1995; Morrison & Ellis, 1997; Van de Peer & De Wachter, 1997). If the hypothesis is accepted that all plastids within the Apicomplexa were acquired through secondary symbiogenesis of either a red or a green alga (Blanchard & Hicks, 1999; Denny et al., 1998; Williamson et al., 1994), then the most parsimonious explanation for the lack of an apicoplast genome in *Cryptosporidium* is that this genus may have emerged as an early branch of the Apicomplexa. This early event would permit the accumulation of lineage-specific differences, which might include loss of the plastid and/or its genome during a gradual adaptation to intracellular life. Our preliminary data are consistent with this interpretation.

**Analysis by PCR indicates that *C. parvum* has no plastid genes**

No plastid product was amplified from *C. parvum* regardless of the primers used (Fig. 1F). As expected, however, all six pairs of primers amplified products from the apicomplexans *T. gondii* and *E. bovis* (Fig. 1A, B). To confirm their identity as plastid genes, one amplicon each of the *E. bovis* LSU, SSU and *tufA*-tRNA*Phe* genes was cloned into a pCR2.1 vector using a TA-cloning kit (Invitrogen) and sequenced. Further support was obtained when an additional four pairs of primers amplified LSU and SSU genes from the plants wild scallions and spinach (Fig. 1C, D). Together these data suggest that degenerate primers, especially those for rRNA, can be used to amplify plastid genomes from a wide range of eukaryotes. That some non-specific priming of organellar rRNA genes can occur is shown by the single SSU rRNA product amplified from uninfected and *C. parvum*-infected HCT-8 cells (lane 6 in Fig. 1E, F). When this band was cloned and sequenced it was shown to be a human mitochondrial, rather than a plastid, rRNA gene. This is not especially surprising since it is well known that some regions of mitochondrial DNA have homologous regions within plastid DNA that can cause apparent cross-hybridization (Supplick et al., 1988). The failure to amplify apicoplast genes was not due to denatured gDNA since consistent amplification of a *C. parvum*-specific calcium transporter (Zhu & Keithly, 1997), which could not be amplified by PCR from a wide variety of organisms (Fig. 1A–E), always occurred. Therefore, we propose that the most logical explanation is the absence of an organellar template for the rRNA and *tufA*-tRNA*Phe* genes in *C. parvum*.

**Plastid probes do not hybridize to *C. parvum* gDNA**

To further test the hypothesis that there is no plastid genome in *C. parvum*, the gDNA of a representative group of eukaryotes with and without a plastid, as well as a eubacterium, were examined by dot-blot hybridization using both a *C. parvum* nucleus-encoded rRNA and several *T. gondii* plastid genes as probes. As

### Table 1. PCR primers and conditions

<table>
<thead>
<tr>
<th>PCR no.</th>
<th>Gene</th>
<th>Specificity&quot;</th>
<th>Product size (bp)†</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CpATPase1</td>
<td>+  -  -</td>
<td>773</td>
<td>Sense: TATATCTCCACAGGTAATG  Antisense: CAGAAGGGACCCCTTAATAC</td>
</tr>
<tr>
<td>2</td>
<td>LSU rRNA</td>
<td>-  +  -</td>
<td>870</td>
<td>Sense: CTGAATCATCTTAGTACTCAAAG  Antisense: A(A/G)TGAGCT(T/A)TTACGCACTCTT</td>
</tr>
<tr>
<td>3</td>
<td>LSU rRNA</td>
<td>-  +  +</td>
<td>880</td>
<td>Sense: ATT(T/G)TCAAA(G/A)AGGAAACAGCC  Antisense: TTACACCTTTCC(G/A)TGCGG(A/G)GGTC</td>
</tr>
<tr>
<td>4</td>
<td>LSU rRNA</td>
<td>-  +  +</td>
<td>720</td>
<td>Sense: GCGAATTCTCTGGTCCGGAATGCC  Antisense: TTT(C/T)GAGTTGTACCTGC</td>
</tr>
<tr>
<td>5</td>
<td>SSU rRNA</td>
<td>-  +  +</td>
<td>1400</td>
<td>Sense: AGAGTTGTAGCTGACTG  Antisense: TACCTTGTAGGACTT</td>
</tr>
<tr>
<td>6</td>
<td>SSU rRNA</td>
<td>-  +  +</td>
<td>730</td>
<td>Sense: AGGATTAGATACC  Antisense: TACCTTGTAGGACTT</td>
</tr>
<tr>
<td>7</td>
<td>tufA-rRNA</td>
<td>-  +  -</td>
<td>1400</td>
<td>Sense: CATGT(A/T)GATCATGG(A/T)AAAC  Antisense: GGTAGACAAATGGATTGAAG</td>
</tr>
</tbody>
</table>

* Specificity: Cp, *C. parvum*, Api, apicoplast (apicomplexan plastid); Chl, chloroplast; +, yes; −, no.
† Expected size of product is approximate due to length variation between species.
‡ The sense primer is within the *tufA* gene, whereas the antisense primer occurs within the tRNA*Phe* gene (Wilson et al., 1996).
G. ZHU, M. J. MARCHEWKA and J. S. KEITHLY

Fig. 1. PCR detection of plastid genes in *T. gondii* (A), *E. bovis* (B), *Allium* (C), *S. oleracea* (D), HCT-8 cells (E) and *C. parvum* (F) using primers specific to *C. parvum* CpATPase1 (lane 1), plastid LSU (lanes 2–4) and SSU (lanes 5–6) rRNA, and *tufA*-tRNA genes. Detailed information on primers is given in Table 1. Primers that amplified plastid genes from apicomplexans (A, B) and/or plants (C, D) showed no plastid product in *C. parvum*. A mitochondrial gene fragment was amplified from HCT-8 cells (lane 6 in panels E and F), as discussed in the text. L, 1 kb DNA standards.

expected, the 7-8 kb *C. parvum* rRNA probe hybridized strongly to the gDNA samples of all Apicomplexa, less strongly to those of a euglenoid, kinetoplastids and yeast, slightly to those of mammals, and not at all to the prokaryote *E. coli* (Fig. 2). On the other hand, the *T. gondii* plastid probe containing >6.2 kb rRNA and *tufA*-tRNA<sup>Phe</sup> genes strongly hybridized to gDNA isolated from all plastid-containing organisms, including the apicomplexans *E. bovis*, *P. falciparum* and *T. gondii*, the plants *A. stellatum* and *S. oleracea* and the euglenoid *E. gracilis*, but not to those without plastids (mammals, yeast, kinetoplastids and *E. coli* [except for a slight hybridization in HCT-8 cells and *E. coli*]) (Fig. 2). Thus, these observations support the PCR data showing the failure of plastid probes to hybridize with *C. parvum* gDNA, and suggest that *C. parvum* indeed lacks the apicoplast homologues for rRNA and *tufA*-tRNA<sup>Phe</sup> genes.

Because both the plastid-encoded rRNA and *tufA* genes are highly conserved in apicomplexans, a euglenoid and plants, their apparent absence in *C. parvum* (Figs 1 and 2) was surprising. Using the cumulative data for amitochondrial protists as a paradigm (Embley & Hirt, 1998; Martin & Muller, 1998), the simplest explanation for the absence of a plastid genome is that it was present in an ancestor common to the Apicomplexa, but has been secondarily lost in *C. parvum*. For example, it is well known that some ancestral, plastid-encoded genes for ribosomal proteins S9 and L28, and a type II fatty acid synthase (FAS) acyl carrier protein, in the cyst-forming coccidian *T. gondii* and the haematozoon *P. falciparum* have been transferred to the nucleus, possibly during the original endosymbiotic event, and that they are encoded there. These proteins are then synthesized on polyribosomes and finally targeted back to the apicoplast (Waller <i>et al.</i> 1998). As yet, however, there is
specific probes. C. parvum (KS) (Waller including acyl carrier protein and two ketoacyl synthases harbours several nucleus-encoded type II FAS enzymes, mycin, markedly inhibited the shown that a type II KS-specific inhibitor, thiolactomycin, markedly inhibited the apparent absence of a plastid, or remnants of its genome. Furthermore, although 100–300 μg ml⁻¹ doses of ciprofloxacin, the mode of action of which is usually associated with a gyrase encoded by a supercoiled, circular genome, can inhibit the intracellular growth of C. parvum up to 95% (Woods et al., 1996), the effective dose was >1000 times that needed to inhibit the growth of P. falciparum in vitro (Yeo & Rieckmann, 1994). Therefore, the significance of this observation for C. parvum is as yet unknown.

Using sporozoites of C. parvum, molecular, ultrastructural and drug-testing data suggest that C. parvum has a mitochondrion (Riordan et al., 1999), thus supporting the hypothesis that no pre-mitochondrial eukaryotic species is extant today (Martin & Muller, 1998). First, our data have shown that three signature genes for mitochondrial function are encoded by the nuclear genome of C. parvum, including the genes for adenylate kinase 2 (CpAK2), valyl-tRNA synthase and chaperonin 60. Phylogenetic analysis of CpAK2 robustly places this gene within the clade of organisms possessing mitochondria (Riordan et al., 1999). Secondly, an unusual, ribosome-studded double-membrane-bound, acristate organelle has been identified posterior to the nucleus which resembles other prokaryot mitochondria. Unlike others (Tetley et al., 1998), we think it is unlikely this organelle is an apicoplast since it is located posterior to the nucleus and lacks the four enveloping membranes characteristic of plastids (Fichera & Roos, 1997; Kohler et al., 1997). Third, drug-testing indicates that this organelle may be a functional mitochondrion, since micromolar concentrations of naphthoquinone drugs known to inhibit respiration in other apicomplexans (Srivastava et al., 1997) prevent growth of C. parvum in vitro.

It is now generally accepted that eukaryotes which have secondarily lost mitochondria and/or plastids once had these organelles, and that remnants of these symbiogenetic events persist in their genomes (Hashimoto et al., 1998; Muller, 1998; Waller et al., 1998). If confirmed, the loss of a plastid genome and its structure in C. parvum would be exciting, and could possibly indicate a second evolutionary fate for this organelle in the Apicomplexa. If, on the other hand, evidence for a plastid genome is eventually discovered, it might help determine whether the phylogenetic position of Cryptosporidium is truly ancestral to the Apicomplexa, and perhaps more nearly related to the dinoflagellates as some have proposed (Gajadhar et al., 1992; Van de Peer & De Wachter, 1997). The discovery that dinoflagellates have plastid genomes whose organization differs radically from those of their nearest relatives indicates that there may be two distinct types of plastid within the group Alveolata (Zhang et al., 1993), which includes apicomplexans, ciliates and dinoflagellates. If C. parvum contains the ‘one gene-one circle’ plastid, and perhaps an unusually reduced plastid genome like the dinoflagellates, this might help elucidate both its phylogenetic position within the Apicomplexa and among the

no evidence to suggest a similar phenomenon in C. parvum. In fact, our drug-testing data for some of these same enzymes support the apparent absence of a plastid and its genome. As mentioned, it is known that the apicoplast of T. gondii and P. falciparum specifically harbours several nucleus-encoded type II FAS enzymes, including acyl carrier protein and two ketoacyl synthases (KS) (Waller et al., 1998). These authors have also shown that a type II KS-specific inhibitor, thiolactomycin, markedly inhibited the in vitro growth of P. falciparum (Waller et al., 1998). Unlike these apicomplexans, however, C. parvum encodes and expresses a cytosolic type I FAS (GenBank AF082993), rather than plastid type II FAS, and is completely insensitive to inhibition by thiolactomycin (G. Zhu, M. J. Marchewka, K. M. Woods, S. J. Upton & J. S. Keithly,
Alveolata, as well as its lack of sensitivity to some antiplastid and most anticoaccidial drugs. It is clear that the absence or presence of a plastid genome in \textit{C. parvum} has valuable phylogenetic and therapeutic implications. The elucidation of these implications awaits further biochemical, molecular and phylogenetic analyses, which will require more inclusive datasets of genera within the Alveolata.

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