Apicomplexan parasites contain a single lipoic acid synthase located in the plastid

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Abstract Apicomplexan parasites contain a vestigial plastid called apicoplast which has been suggested to be a site of [Fe–S] cluster biogenesis. Here we report the cloning of lipoic acid synthase (LipA) from *Toxoplasma gondii*, a well known [Fe–S] protein. It is able to complement a LipA-deficient *Escherichia coli* strain, clearly demonstrating that the parasite protein is a functional LipA. The N-terminus of *T. gondii* LipA is unusual with respect to an internal signal peptide preceding an apicoplast targeting domain. Nevertheless, it efficiently targets a reporter protein to the apicoplast of *T. gondii* whereas co-localization with the fluorescently labeled mitochondrion was not detected. In silico analysis of several apicomplexan genomes indicates that the parasites, in addition to the presumably apicoplast-resident pyruvate dehydrogenase complex, contain three other mitochondrion-localized target proteins for lipoic acid attachment. We also identified single genes for lipoyl (octanoyl)-acyl carrier protein:protein transferase (LipB) and lipoate protein ligase (LplA) in these genomes. It thus appears that unlike plants, which have only two LipA and LipB isoenzymes in both the chloroplasts and the mitochondria, Apicomplexa seem to use the second known lipoylating activity, LplA, for lipoylation in their mitochondrion.

Key words: Apicoplast; Iron–sulfur protein; Organellar targeting; *Toxoplasma gondii*; *Plasmodium falciparum*

1. Introduction

Apicomplexan parasites are obligate intracellular protists and comprise several important pathogens of humans or economically important animals, e.g. *Plasmodium* spp. (causative agent of malaria), *Toxoplasma gondii* (toxoplasmosis), *Cryptosporidium parvum* (cryptosporidiosis) and *Eimeria* spp. (coccidiosis). Nearly all Apicomplexa possess a unique organelle derived from plastids, called apicoplast, which was acquired by a secondary endosymbiotic event (for reviews see [1–4]). Since its description several years ago the apicoplast has received great attention because it has been shown to contain metabolic pathways unique to plants and it is thus a prime candidate for the design of novel therapeutic approaches (reviewed in [5,6]). Proteins to be imported into the organelle have been shown to possess a characteristic N-terminal bipartite targeting sequence, consisting of a signal peptide followed by a targeting sequence of variable length and amino acid composition, which is both necessary and sufficient to transport these proteins into the apicoplast [7–11]. This distinct sequence feature has been very instrumental in the identification of proteins assumed to be localized in this organelle [7,8,12,13]. Consequently, these general rules have been incorporated into two predictive algorithms [11,14] which allowed the assembly of a provisional metabolic map of this organelle by analysis of the recently finished whole genome of *Plasmodium falciparum* [15].

We and others have recently proposed that the apicoplast is probably the second site (besides the mitochondrion) for the synthesis of [Fe–S] clusters in the parasite [16,17]. This assumption is based on the presumed or experimentally verified localization in the apicoplast of several key proteins known to be required for the biogenesis of these essential building blocks identified by in silico analysis of different apicomplexan genomes. In addition, we had shown previously that this organelle harbors a nuclear-encoded [2Fe–2S] ferredoxin [18], and recently two other plastid-localized proteins involved in isoprenoid biosynthesis have been shown to be [Fe–S] proteins, namely GcpE and LytB [19,20]. Since [Fe–S] clusters in general are essential for the enzymatic function of these proteins, disruption of their biogenesis in the plastid might be exploited as a future drug target [6].

In this article we describe the initial characterization of the [Fe–S] protein lipoic acid synthase [21,22] from *T. gondii* (TgLipA). Lipoic acid (LA) is an essential co-factor for most organisms and can be synthesized from octanoyl-acyl carrier protein (ACP) by LipA [23]. This protein and its biosynthetic pathway have been best studied in bacteria [24], but LipA homologues were also shown to be present in mitochondria of eukaryotes and, more recently, in plastids of plants [25]. Subsequent to its synthesis LA is then covalently attached to several proteins by an enzyme called lipoyl (octanoyl)-acyl carrier protein:protein transferase, or LipB. This so-called lipoylation is essential for several enzymes involved in the citric acid cycle whereby LA serves as a swinging arm in the transfer of an acyl group during acyl-CoA derivative synthesis [26,27].

We provide evidence for the confined cellular distribution

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Abbreviations: ACP, acyl carrier protein; BCDC, branched-chain 2-oxo acid dehydrogenase complex; EST, expressed sequence tag; GDC, glycine decarboxylase complex; GFP, green fluorescent protein; LA, lipoic acid; LipA, lipoic acid synthase; N-LipA/mycGFP, N-terminal TgLipA fused with myc-tagged GFP; OGDC, 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; TgLipA, *Toxoplasma gondii* LipA

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of LipA to the apicoplast of *T. gondii*. We also show by in silico analysis that LA-modified proteins are presumably present in both the single mitochondrion and the apicoplast of the parasite, and discuss how lipoylation in this organelle most likely occurs.

2. Materials and methods

2.1. Cloning of T. gondii LipA

Starting from a genomic contig (TGG7157; http://www.toxodb.org) primers were designed which allowed the individual amplification of the presumed N-terminus of TgLipA (primers #1, GCGAATTCCCTTTTTCGACAAAATGGCGTATTTCTTTGACTTCC and #2, CGCGTTTCGCCGGGTGTTGC*GAGCAGAAGCTCATCTCCGAGGAGGAC*AGATCTCTGCAGCAATTGTTAATTAACATGCATGCA) and its preceding catalytic domain with primers #3, GGAATTCGGATCCCAGGGCCGGTCTTGCG and #4, GGAATTCTCACGCCTCTTTCTCGTTTACTTG). Underlined sequences are recognition sites for restriction endonucleases used for cloning. The sequence in italics in primer 2 encodes the myc epitope. Reverse transcription polymerase chain reaction (RT-PCR) with total RNA isolated from RH strain using the cycling conditions of the manufacturer (RobusT I Kit, Finnzymes, Espoo, Finland) and the respective primers resulted in a single band in each case. They were cloned into pCR2.1 using the TOPO cloning kit (Invitrogen) and fully sequenced. The resulting composite nucleotide sequence has been deposited in the GenBank/EMBL database under accession number AJ556158.

2.2. Computational analysis

The different LipA and E2 sequences were extracted using appropriate BLAST searches from PlasmoDB (http://www.plasmodb.org) and ToxoDB (http://www.toxodb.org). Some sequences were verified or assembled from overlapping expressed sequence tag (EST) sequences published in the dbEST database or from genomic contigs and

Table 1
Prediction of targeting sequences of apicomplexan lipoyl acceptor proteins and LipA, LipB and LplA using different algorithms

	Mitoprot[a]	TargetP[b]	iPSORT[c]	Predotar[d]	PlasmoAP[e]	PATS[f]
BCDC-E2						
Tg	+	mt	mt	mt	na	na
Pf	+	mt	none	mt	na	na
Pv	+	mt	mt	mt	na	na
Py	+	mt	none	mt	na	na
OGDC-E2						
Tg	+	mt	mt	mt	na	na
Pf	+	mt	none	mt	na	na
GDC-H						
Tg	+	mt	mt	mt	na	na
Pf	+	none	sp	mt	na	na
Pk	+	mt	mt	mt	na	na
Py	+	mt	mt	mt	na	na
PDC-E2						
Tg	+	sp	pl	pl	na	na
Pf	+	sp	pl	pl	+	+
Py	+	sp	pl	mt	+	+
LipA						
Tg	−	none (sp[g])	none	pl	na	na
Pb	−	sp	sp	mt	−	+
Pf	+/−	sp	sp	pl	+	+
Pk	+/−	sp	sp	mt	+	+
Py	−	sp	sp	mt	−	+
LipB						
Tg[h]	+/−	mt (sp[g])	mt	mt	na	na
Pf	+	none	none	mt	+	−
Py	+	sp	sp	mt	+	−
LplA						
Tg	+	mt	mt	none	na	na
Pf	+	mt	mt	mt	−	−
Py	+/−	none	none	mt	+	−

TargetP and iPSORT are designed to predict all cellular localizations whereas Mitoprot is restricted to mitochondria. The neural network Predotar has been trained on plant proteins and the results should therefore be interpreted with this in mind. However, it performs like Mitoprot with well known apicomplexan mitochondrial proteins and has therefore been included. For a comparison of the different methods see [36]. Apicoplast localization for PDC-E2 was predicted using PATS and PlasmoAP. Due to their training set with *P. falciparum* proteins their predictive power for other Plasmodia and for *T. gondii* is currently undefined.
na, not applicable; mt, mitochondrial targeting; pl, plastid; sp, signal peptide. Pb, *P. berghei*; Pf, *P. falciparum*; Pk, *P. knowlesi*; Pv, *P. vivax*; Py, *P. yoelii*; Tg, *T. gondii*.
[a] http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter [47].
[b] http://www.cbs.dtu.dk/services/TargetP/ [34].
[c] http://www.HypothesisCreator.net/iPSORT/ [35].
[d] http://www.inra.fr/Internet/Produits/Predotar/.
[e] http://www.plasmodb.org/restricted/PlasmoAPcgi.shtml [11].
[f] http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php [14].
[g] Predicted with SignalP 2.0.
[h] Putative N-terminus.

removal of introns (where necessary) by visual inspection and comparison with known protein sequences. Sequence alignments were performed using CLUSTAL X and optimized by visual inspection. Details are available upon request. Prediction of signal peptides was performed using the SignalP V2.0 server at http://www.cbs.dtu.dk/services/SignalP-2.0/. All other targeting predictions were done using the servers mentioned in the legend to Table 1.

2.3. Complementation of a ΔlipA Escherichia coli strain

TgLipA was cloned into the expression plasmid pS1 which yields recombinant proteins with an N-terminal hexahistidine tag [28]. For complementation of the Δ*lipA E. coli* strain KER176 [29] the following medium was used: 1 g/l vitamin-free casein hydrolysate, 7 g/l K_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l Na-citrate·$3H_2O$, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l ammonium sulfate, 50 mM Na-succinate, 2 mg/l vitamin B1, 50 mg/l ammonium-ferric citrate, 100 mg/l ampicillin and 50 mg/l kanamycin. Controls received 10 μg/l DL-α-LA. 2 ml liquid cultures inoculated with the respective transformed bacteria were then cultivated at 37°C with constant shaking and their OD at 600 nm was determined after 40 h of growth.

2.4. Generation of transgenic parasites and immunofluorescence assays

Transient transgenic expression of epitope-tagged proteins in *T. gondii* was achieved using the inducible expression system described by Meissner et al. [30]. To evaluate if the N-terminus of TgLipA is able to direct a reporter into the apicoplast, its sequence was excised from pCR2.1 by *Eco*RI and *Nsi*I digestion and cloned into the appropriately cut vector p5RT70Tet4GFP, resulting in a fusion of the TgLipA targeting domain and a myc-tagged green fluorescent protein (N-LipA/mycGFP). The resulting protein could be detected in transgenic tachyzoites of the RH Rep1/2 strain grown for 8 h in the presence of anhydrotetracycline using a mouse anti-myc monoclonal antibody (9E10; diluted 1:1000) and a Cy3-coupled secondary antibody (diluted 1:300, goat anti-mouse, Jackson ImmunoResearch). Specific visualization of the mitochondrion was achieved by transfection with plasmid pCAT S9(33–159)-GFP described earlier which encodes GFP with a mitochondrial targeting sequence [10]. All images were acquired with a Leica TCS SP2 confocal laser scanning microscope. Image processing was performed with either Leica LCS software or Adobe Photoshop 6.

3. Results and discussion

3.1. Characteristics of the T. gondii *LipA sequence*

BLAST searches of the ongoing *T. gondii* genome project with bacterial LipA sequences as query resulted in the identification of a genomic contig (TGG7157) showing high similarity to LipA. Its cDNA was subsequently cloned by RT-PCR as described in Section 2. The gene for LipA in *T. gondii*

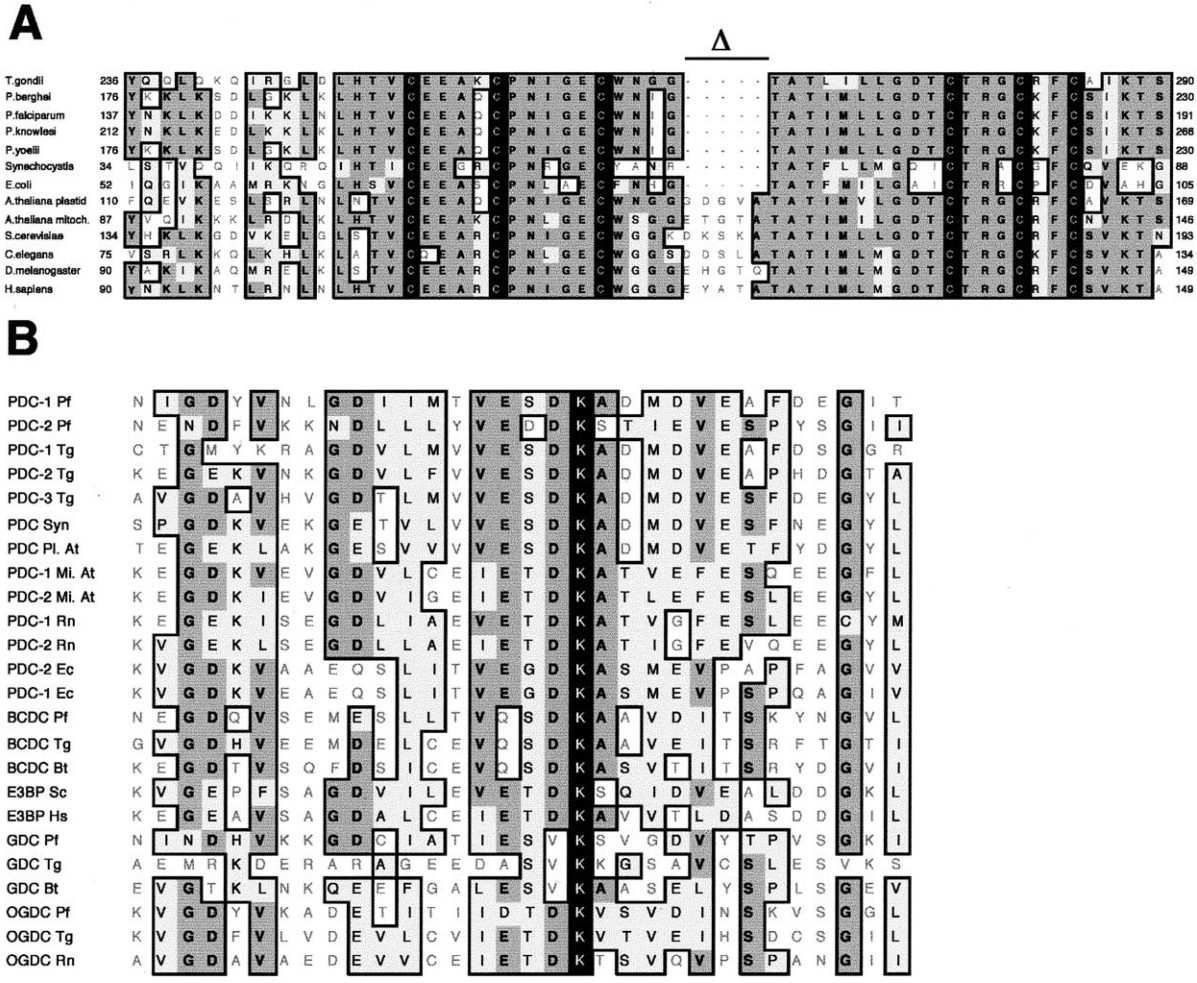

Fig. 1. Sequence alignments of parts of LipA proteins (A) and of the lipoyl attachment sites of E2 proteins (B). In A amino acids around the six cysteines conserved in all LipA proteins (black shading) are aligned. The five amino acid deletion characteristic for bacterial and apicomplexan LipA is indicated (Δ). In B the core regions of the lipoyl attachment site of different E2 proteins from *T. gondii* and *P. falciparum* are compared with those of other organisms. The lysine residue which is lipoylated is indicated by black shading. Dark gray shading indicates identical residues, light gray shading indicates similar amino acids. E3BP, E3 binding protein; Mi., mitochondrion; Pt., plastid. Species are abbreviated as follows: At, *A. thaliana*; Bt, *Bos taurus*; Ec, *E. coli*; Hs, *Homo sapiens*; Pf, *P. falciparum*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Syn, *Synechocystis* sp.; Tg, *T. gondii*.

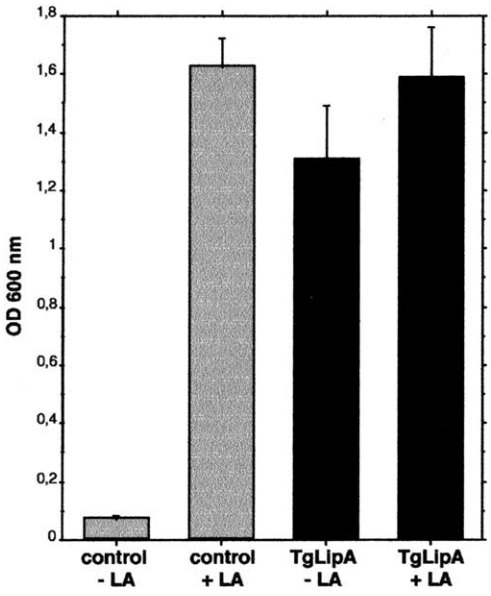

Fig. 2. Complementation of LipA-deficient *E. coli* KER176 cells with TgLipA. Growth of plasmid-transformed cells in the absence or presence of 10 ng/ml LA was monitored by measurement at 600 nm. The vector pS1 alone served as control.

consists of four exons and three introns, comprising 3375 bp. The complete assembled cDNA is 1632 bp long, encoding 543 amino acids. The putative translational start site of this gene conforms well to the consensus sequence for this organism [31].

A multiple sequence alignment illustrates the high conservation between the apicomplexan LipAs and other selected LipA proteins from different organisms (Fig. 1A and data not shown). They all contain six conserved cysteine residues found in all lipoate synthases, of which the latter three conform to the CX_3CX_2C motif also found in other adenosylmethionine-dependent iron–sulfur enzymes and which are assumed to be the [Fe–S] cluster ligands (reviewed in [32,33]). It is of note that all eukaryotes, including fungi and Kinetoplastidae, for which LipA sequences are known, contain an insertion of 4–6 amino acids between these two cysteine motifs (Fig. 1A and data not shown) whereas almost all known sequences from bacteria have no such insertions. Interestingly, all five LipA sequences from Apicomplexa, like bacteria, do not possess this insertion. The significance of this indel for the phylogenetic origin of this protein remains to be determined. It should be noted that many of the predicted apicoplast proteins of *P. falciparum* are likely not of plastid origin [15].

3.2. *TgLipA complements an* E. coli *LipA-deficient strain*

To prove that the TgLipA protein functions as predicted the coding sequence starting with amino acid 180 was expressed as a hexahistidine-tagged recombinant protein (6H-TgLipA) in *E. coli*. This plasmid was then used to complement a LipA-deficient *E. coli* strain, KER176 [29]. After 40 h of growth in LA-deficient medium the 6H-TgLipA-containing bacteria reached almost wild-type level of cell mass, whereas the control culture containing the expression vector alone did not grow at all (Fig. 2). This demonstrates the absolute requirement of LA for cell growth [29] and the synthesis of this compound by TgLipA. This experiment clearly demonstrates that TgLipA is a functional LipA.

3.3. *TgLipA has an unusual N-terminal targeting domain*

The putative N-terminus of the protein possesses an unusual N-terminal targeting domain which could be easily missed when applying the popular TargetP algorithm [34] for the prediction of cellular localization since no signal peptide is detected (Table 1). The same is true for another similar server, iPSORT [35]. However, using SignalP 2.0 instead [36], a possible cleavage site between amino acids 63 and 64 is predicted (Fig. 3A). Inspection of a hydrophilicity plot shows that starting at around amino acid 40 the sequence indeed looks much more like a typical signal peptide, with a short positive region and a central hydrophobic segment (Fig. 3A). It is then followed by a sequence of ca. 120 amino acids which is rich in serine (19%) and basic amino acids (8.5%). This would be indicative of a bipartite targeting domain in this organism, directing this protein into the apicoplast [10]. The genomes of several related Plasmodia also contain LipA proteins with a clearly predicted signal peptide using all methods tested (Table 1). Applying the two predictive algorithms for apicoplast targeting domains, PATS [14] and PlasmoAP [11], to the plasmodial sequences indicates that they all are presumably localized in the apicoplast, although the predictions differ for some proteins (Table 1). Taken together, all five presently known apicomplexan LipA protein sequences are likely preceded by a putative N-terminal apicoplast targeting domain, which in the case of *T. gondii*, however, is unusual with respect to the internal signal peptide.

3.4. *The N-terminal targeting domain of TgLipA transports a reporter to the apicoplast*

To experimentally verify the localization of the TgLipA protein within the parasite, the N-terminal part of the protein spanning amino acids 1–179 was fused with myc-tagged GFP (N-LipA/mycGFP) and introduced into tachyzoites on a tetR regulated promoter plasmid [30] (see Section 2). Upon induction of transgenic parasite cultures a distinct red fluorescence was observed when a Cy3-labeled antibody was used to detect the myc epitope in N-LipA/mycGFP (Fig. 3B, I). This signal co-localized with a previously shown apicoplast-resident protein, ferredoxin-$NADP^+$ reductase [13] (data not shown). This result clearly demonstrates that the unusual signal peptide of TgLipA together with its targeting domain still serves as an efficient means to transport a reporter to the apicoplast. Decoration of the single mitochondrion of *T. gondii* with a mitochondrion-targeted GFP [10] (Fig. 3B, II) was used to assess the possible co-localization of TgLipA to this organelle where LipA activity, like in plants, might also be expected. However, there were no indications of co-targeting of N-LipA/mycGFP at this level of sensitivity to the mitochondrion (Fig. 3B, I and III). Note that the observable yellow signal in the apicoplast in Fig. 3B, III is due to the dual detection of N-LipA/mycGFP (green from GFP and red from the anti-myc/Cy3 antibody). Taken together, both, bioinformatics and in vivo targeting experiments provide strong evidence for the localization of apicomplexan LipA in the apicoplast. Since this organelle has been shown to be a site for fatty acid synthesis (reviewed in [37]) all necessary precursors for LA biosynthesis are likely to be present.

3.5. *Evidence for the requirement of LA in the mitochondrion and the apicoplast*

In *Arabidopsis thaliana* the biosynthetic pathway for LA is

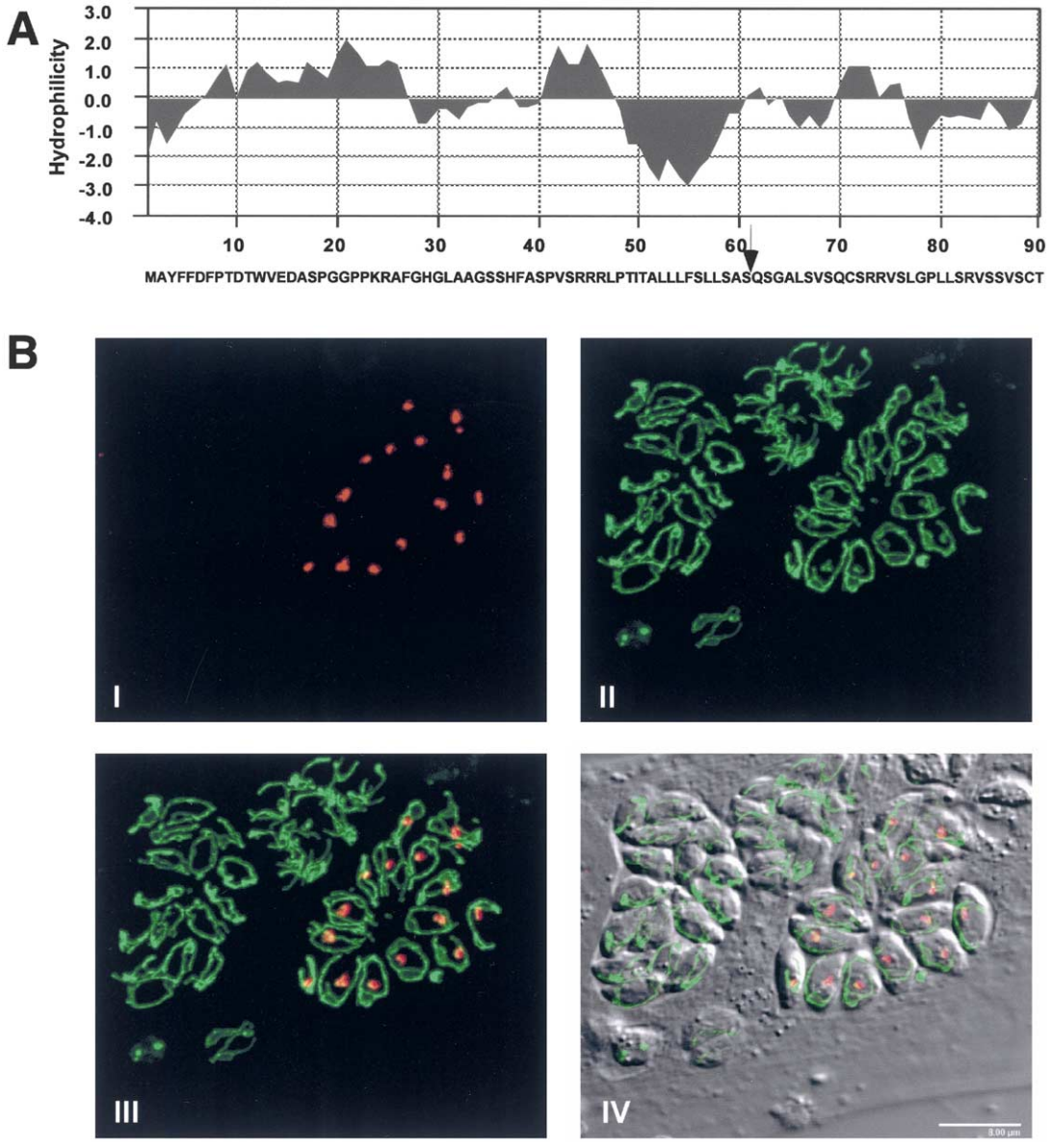

Fig. 3. Targeting of the N-terminal TgLipA sequence fused to myc-tagged GFP to the apicoplast. A: Kyte–Doolittle plot of the first 90 amino acids of TgLipA showing at around amino acid 40 the usual hydrophilicity profile of a signal peptide. The predicted cleavage site is indicated by a vertical arrow. B: Detection of N-LipA/mycGFP by anti-myc antibody in anhydrotetracycline-induced (0.75 μg/ml for 8 h) transfected *T. gondii* cultures (I). The GFP-tagged single mitochondria of the same parasites are shown in II. The overlay of I and II shows that both labels are in distinct locations (the observable yellow signal is due to the dual color of the apicoplast (green from the GFP part of N-LipA/mycGFP and red from the anti-myc/Cy3 detection) and no anti-myc reactivity is seen in the mitochondrion (III). Note that due to the transient transfection no labeling of the apicoplast is observed in the two vacuoles on the left. For better orientation the fluorescent signals are overlaid with the Nomarski image of the cells (IV). The white bar represents 8 μm.

present in plastids and mitochondria, both of which contain distinct genes for these different isoenzymes [25]. This is not surprising since in both organelles proteins reside which require LA modification for enzymatic activity [38]. These are the E2 subunits of the following mitochondrial 2-oxo acid dehydrogenase complexes: pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC), branched-chain 2-oxo acid dehydrogenase complex (BCDC) [26,27], the so-called H-protein of the glycine decarboxylase system (GDC) in mitochondria [39], together with the E2 subunit of PDC in chloroplasts [38].

In contrast, the *P. falciparum* and *Plasmodium yoelii* genome databases, respectively, contain only a single LipA gene each. Both genomes are considered to be completely known at a level of >99% [15,40]. Also, the available *T. gondii* genome sequences (currently at 4× coverage) and Southern blot analysis give no indication for a second LipA gene in this organism (data not shown). In addition, it has been noted previously that *P. falciparum* seems to possess only a single PDC which is predicted to be transported to the apicoplast; this raises some interesting questions about the functionality of the trichloroacetic acid cycle in the mitochondrion of *P. falciparum* [15]. If PDC-E2 were the only LA-modified protein in the parasite then LipA would not be required in the mitochondrion. However, we could clearly identify the above mentioned additional mitochondrial target pro-

teins for lipoylation in the plasmodial DNA databases and that of *T. gondii* (Table 1 and Fig. 1B). This analysis revealed that in the case of *T. gondii* the putative N-terminus of the PDC-E2 protein (which is predicted to constitute an apicoplast targeting domain, Table 1 and data not shown) contains three potential lipoyl attachment sites instead of the usual two domains in the plasmodial or other eukaryotic PDC-E2 proteins (Fig. 1B) [27,38]. We also identified OGDC-E2 and BCDC-E2, both having single lipoyl domains, and a putative H-protein homolog of the glycine cleavage system in both *T. gondii* and Plasmodia (see Fig. 1B, and data not shown). All three proteins from these Apicomplexa have clearly identifiable N-terminal mitochondrial targeting sequences (Table 1), and the presence of ESTs and/or microarray data published in the databases for all of them indicates that they are expressed (data not shown). This strongly suggests that LA is also required in the mitochondrion of *T. gondii* and Plasmodia.

3.6. Implications of the TgLipA localization for LA transport

Since LipA is probably only present in the apicoplast, the interesting question is how lipoylation takes place in the mitochondrion. In bacterial and mammalian mitochondria a second system exists which allows the uptake of free LA from exogenous sources. In this case LA is energetically activated by ATP and the resulting LA-AMP is then transferred to acceptor proteins by a single distinct enzyme called lipoate protein ligase (LplA) in *E. coli* [41]. In mammals the first reaction is catalyzed by a so-called lipoate-activating enzyme [42] and the resulting LA-AMP is then used for lipoylation by a LplA homolog [43]. Plants differ in this respect, since they contain LipA isoforms in both chloroplasts and mitochondria [25], but no LplA homologs can be found in the genomes of *A. thaliana* and rice (data not shown), implying that plants might rely solely on LA biosynthesis from endogenous sources. In contrast, when we searched the apicomplexan databases for both LipB and LplA homologs, significant matches for both of them were found for Plasmodia and *T. gondii* (see Table 1, and data not shown). Analysis of their N-termini suggests that LipB is apicoplast-localized whereas LplA probably targets to the mitochondrion (Table 1). Taken together, these preliminary data are consistent with a scenario where, unlike plants, the parasites possess two distinct lipoylation pathways, LipA/LipB in the apicoplast and LplA in the mitochondrion. If correct, this would also imply that the mitochondrion might receive LA directly from the host cell. It is known that *T. gondii* has access to host cell lipids and their precursors [44], and these pathways might also be a source for mitochondrial LA. On the other hand, the close physical association of apicoplast and mitochondrion has been documented [45], and one could therefore imagine that some LA might also come from the apicoplast. Although LA synthesized by LipA is most likely bound to ACP [24,46], it has been shown that LplA from *E. coli* can also use LA–ACP as lipoyl donor, although very inefficiently [23]. This would, however, require the LA–ACP complex to be translocated to the plastid through five or six membranes for which there is no precedent yet. Taken together, this discussion highlights a number of interesting questions with regard to the lipoylation pathway in these unique intracellular parasites.

3.7. Conclusions

We have shown (i) that *T. gondii* contains a functional LipA which is transported to the apicoplast by an unusual N-terminal targeting sequence, (ii) that this single gene is present in Plasmodia as well and that the deduced LipA proteins are predicted to contain an apicoplast targeting domain, and (iii) that the presence of several vital lipoyl acceptor proteins with a mitochondrial targeting domain in all examined Apicomplexa suggests that LA is also needed in this second organelle. Unlike plants however, which have two LipA and LipB isoenzymes in both the plastids and the mitochondria, the parasites seem to use LplA for lipoylation in their mitochondrion.

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