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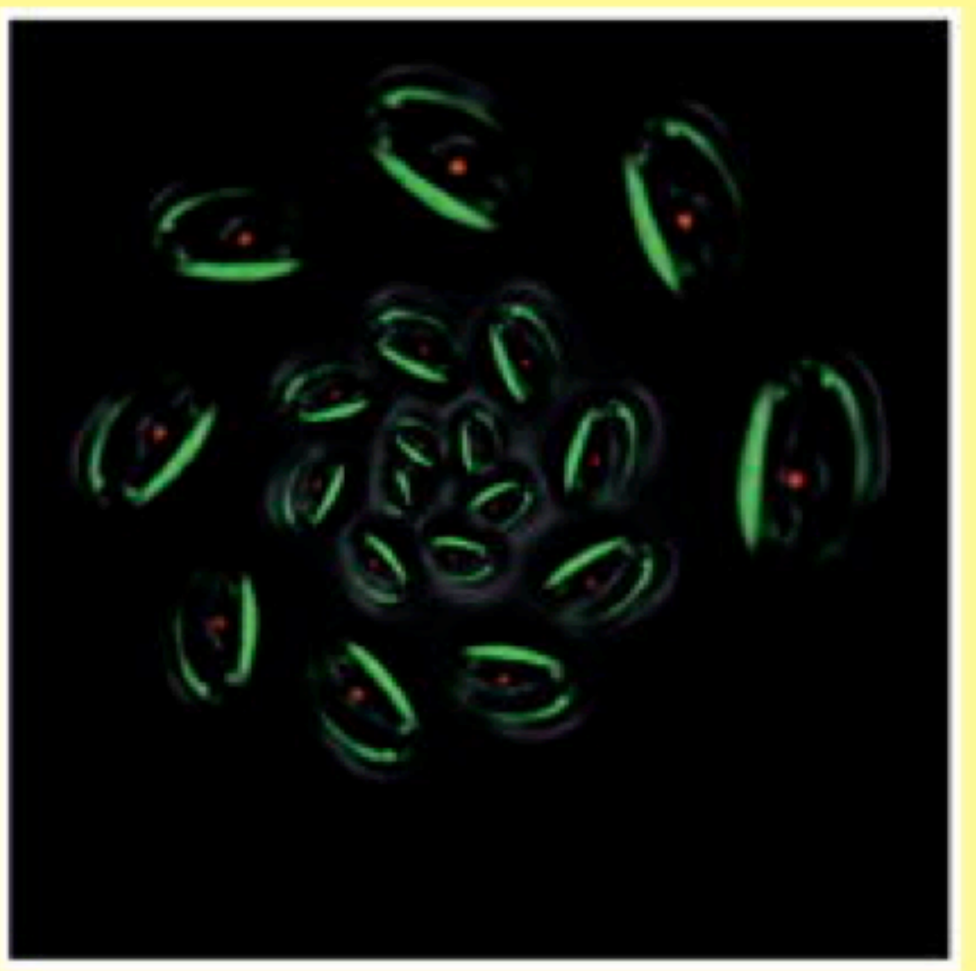

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N-terminal positively charged amino acids, but not their exact position, are important for apicoplast transit peptide fidelity in *Toxoplasma gondii*

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Abstract

The non-photosynthetic plastid – or apicoplast – of *Toxoplasma gondii* and other apicomplexan parasites is an essential organelle and promising drug target. Most apicoplast proteins are encoded in the nucleus and targeted into the organelle through the apicoplast's four membranes courtesy of a bipartite N-terminal leader sequence comprising of an endomembrane signal peptide followed by a plastid transit peptide. Apicoplast transit peptides, like plant plastid transit peptides, have no primary consensus, are variable in length and may be distinguishable only by a relative depletion of negative charged residues and consequent enrichment in basic residues. In this study we examine the role of charged residues within an apicoplast transit peptide in *T. gondii* by point mutagenesis. We demonstrate that positive charged residues, combined with the absence of negatively charged amino acids, are essential for apicoplast transit peptide fidelity, as also observed in *P. falciparum*. Furthermore, we show that positive charge is more important at the transit peptide's N-terminus than its C-terminus, and that the nature of the positive residue and the exact position of the N-terminal positive charge are not important. These results suggest that a simple, rule-based prediction for *T. gondii* transit peptides, similar to that successfully implemented for *P. falciparum* should help to identify apicoplast proteins and facilitate the identification of drug targets in this important human pathogen.

Keywords: *Toxoplasma gondii*; Apicomplexa; Protein targeting; Apicoplast; Transit peptide; Parasitophorous vacuole; Endoplasmic reticulum

1. Introduction

Toxoplasma gondii is a ubiquitous obligate intracellular parasite of significant medical importance in AIDS patients, pregnant women and immuno-compromised individuals. Like most apicomplexan species, including the *Plasmodium* parasites, *T. gondii* contains a vestigial plastid – referred to as the apicoplast – which has attracted attention due to its potential target for selective anti-parasitic drugs [1,2].

Plastids arose by endosymbiosis, when a nucleated heterotrophic host cell engulfed a free-living photosynthetic organism [2]. Primary endosymbiosis gave rise to plastids with two membranes (land plants, green algae and red algae), and massive transfer of genetic material from the plastid to the nucleus has occurred during the plastid's tenure as an endosymbiont [3]. Products of these nuclear-encoded genes are typically targeted back into the plastid across its two bounding membranes courtesy of an N-terminal targeting sequence referred to as a transit peptide. Plastid transit peptides are variable in length, have no primary consensus sequence and are only distinguished by positive charged residues and an abundance of hydroxylated residues [4]. Select plant chloroplast transit peptides form amphipathic alpha-helices in lipid environments similar to that of the outer plastid envelope [5], but it is not known if this is a universal characteristic, and some have argued that transit peptides lack any structure [6]. How transit peptides are recognised is not well understood, but it is clear that some type of receptor resides within a multimeric protein complex known as translocon of outer chloroplast (TOC) that sits within the outer of the two plastid membranes. In addition to one or more receptors, the TOC complex includes a channel (Toc75) through which the transit peptide-bearing proteins are translo-

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cated to another complex – translocon of inner chloroplast (TIC) – that mediates further translocation across the inner plastid membrane. Once inside the plant plastid, the transit peptide has fulfilled its role and is removed by a stromal processing protease [7].

The apicoplast, like some other plastids, is bounded by four (although some have suggested three [8,9]) membranes, and arose by secondary endosymbiosis [10,11]. Secondary endosymbiosis refers to the engulfment and stable acquisition of a photosynthetic eukaryote (already equipped with its own two-membrane plastid) by another (unrelated) nucleated heterotrophic cell. Just as the majority of plastid protein genes have relocated to the host nucleus in primary endosymbiotic plastids, most apicoplast protein genes have transferred to the apicomplexan nucleus [11,12]. However, the extra membranes around the apicoplast require a more complex targeting system for nucleus-encoded proteins to enter the apicoplast, and transferred genes have acquired an additional N-terminal targeting domain – a classical endomembrane signal peptide – that together with the transit peptide is able to mediate passage across all four membranes [11,13]. Apicoplast protein targeting therefore requires both a endomembrane signal peptide and a plant-like transit peptide to traffic proteins into the stromal compartment [13,14]. Similar bipartite leaders also occur in other organisms with secondarily derived plastids [15–19].

In the first stage of targeting to the apicoplast, the signal peptide directs proteins across the endoplasmic reticulum (ER) membrane and into the parasite's secretory pathway; cleavage by signal peptidase then exposes the transit peptide, which is responsible for all remaining transport steps [14]. Apicoplast transit peptides are somewhat similar to primary plastid transit peptides in that they are variable in length, have no consensus sequence and are only distinguished by a depletion of negatively charged residues and the presence of positively charged residues. Furthermore, species-specific nucleotide biases influences the amino acid bias of transit peptides in different organisms, making them difficult to compare at a primary sequence level [20]. In this report we demonstrate that a net positive charge in the transit peptide of *T. gondii* is important for apicoplast targeting, corroborating similar findings in *P. falciparum* [21]. Moreover, we show that positive charges are more influential in the N-terminal portion of the transit peptide, that arginine and lysine are equally suitable, and that the exact position of these charges is not important.

2. Materials and methods

2.1. Prediction of ACP transit peptide from a range of apicomplexan species

The transit peptide of acyl carrier protein (ACP) from *P. falciparum* [accession number AAC63959] [11], *T. gondii* [AAC63956] [11] and *P. berghei* [CAI04930] were downloaded from GenBank, and ACP from *Theileria annulata* from GeneDB.org. Transit peptide boundaries have only been deter-

Table 1
Constructs engineered for this study

Construct name	Template[a]	Primers[b]	Amino Acids at position							Figure
			31	35	37	41	73	74	79	
ACP(l)-eYFP	*Tg*ACP(l)-eYFP parent	None	V	L	R	R	R	R	R	Figs. 2A and 3A
ACP(s)-eYFP	*Tg*ACP(l)-eYFP parent	1 + 2	–	–	–	–	–	–	–	Fig. 2B
ACP(l)R41D-eYFP	*Tg*ACP(l)-eYFP parent	4 + 9; 3 + 9	V	L	R	D	R	R	R	Fig. 2C
ACP(l)R37D-eYFP	*Tg*ACP(l)-eYFP parent	5 + 9; 3 + 9	V	L	D	R	R	R	R	Fig. 2D
ACP(l)R37D + R41D-eYFP	*Tg*ACP(l)-eYFP parent	6 + 9; 3 + 9	V	L	D	D	R	R	R	Fig. 2E
ACP(l)R37K + R41K-eYFP	*Tg*ACP(l)-eYFP parent	7 + 9; 3 + 9	V	L	K	K	R	R	R	Fig. 2F
ACP(l)R37A-eYFP	*Tg*ACP(l)-eYFP parent	10 + 11	V	L	A	R	R	R	R	Fig. 3B
ACP(l)R37A + R41A-eYFP	*Tg*ACP(l)-eYFP parent	8 + 9; 3 + 9	V	L	A	A	R	R	R	Fig. 3C
ACP(l)R37A + R41A + R73A-eYFP	above[a]	12 + 13	V	L	A	A	A	R	R	Fig. 3D
ACP(l)R37A + R41A + R73A + 74A-eYFP	above[a]	14 + 15	V	L	A	A	A	A	R	Fig. 3E
ACP(l)R37A + R41A + R73A + 74A + 79A-eYFP	above[a]	16 + 17	V	L	A	A	A	A	A	Fig. 3F
ACP(l)V31R + R37A + R41A + R73A + 74A + 79A-eYFP	above[a]	18 + 19	R	L	A	A	A	A	A	Fig. 4A
ACP(l)V31R + R35R + R37A + R41A + R73A + 74A + 79A-eYFP	above[a]	20 + 21	R	R	A	A	A	A	A	Fig. 4B

Yellow indicates charged amino acids in the plastid transit peptide of wild-type TgACP (all are positive). Mutagenesis was used to introduce negative charges (green), positive charges (red), and/or neutral amino acids (magenta).

[a] 'above' indicates that the clone shown on the previous line of this table was used as a template for mutagenesis.

[b] See Table 2 for primer sequences and descriptions.

mined for *P. falciparum* [22] but cleavage sites were predicted for the other leaders. The signal peptide-transit peptide cleavage point was determined using the hidden Markov models and neural networks of SignalP (www.cbs.dtu.dk/services/SignalP/). The transit peptide-mature protein boundary was estimated by aligning the four ACP proteins using ClustalW [23] and hypothesizing that the region at which the mature ACP proteins begin to exhibit identity corresponds to be the end of the transit peptide and the start of the mature protein.

2.2. Creation of T. gondii N-terminal transit peptide charge mutants

To test the importance of N-terminal charge in transit peptides of *T. gondii*, we engineered a series of ACP transit peptide mutants similar to those previously constructed for *P. falciparum* [21], as shown in Table 1 (primers in Table 2). The first two charged amino acids in *Tg*ACP(l) are arginines (R) at positions 37 and 41 relative to the methionine initiation codon, corresponding to transit peptide amino acids 8 and 12. These codons were mutagenized using long oligonucleotides in two sequential PCR reactions: the first amplifying the transit peptide (with desired mutations) from plasmid *p*tub-*Tg*ACP(l)-eYFP (kindly provided by M. Crawford, University of Pennsylvania), and the second reaction adding the signal peptide. We also created a negative control containing only the signal peptide. All PCR products were digested with *Avr*II and *Bgl*II and ligated into the corresponding sites of the *T. gondii* transfection vector *p*tub-eYFP. *ptub-Tg*ACP(1)-hcRED was created by cutting out *Tg*ACP(l) from *ptub-Tg*ACP(l)-eYFP with *AvrII/BglII* and replacing *Tg*FNR(1) in *ptub-Tg*FNR(l)-hcRED (kindly provided by O. Harb, University of Pennsylvania).

2.3. Sequential removal of positive charge from the T. gondii ACP transit peptide

The *T. gondii* ACP leader (*Tg*ACP(l)) contains five basic (and no acidic) amino acids. To assess their role in apicoplast targeting we undertook alanine mutagenesis using the Quickchange mutagenesis kit (Stratagene; La Jolla, CA). Briefly, two overlapping primers were designed to anneal to opposite DNA strands and contain a single mutation of interest. With *ptub-Tg*ACP(l)-eYFP as a starting template, long range PCR was used to amplify around the full plasmid. PCR reactions were then digested with *Dpn*I to remove any methylated PCR template, transformed into *E. coli*, and colonies sequenced to confirm presence of the desired mutation(s). Sequential application of different primers to various templates permitted systematic mutation of all of the five arginines, as indicated in Table 1. In order to further explore

Table 2
PCR primers employed for mutagenesis and plasmid construction

ID#	Strand	Description	Sequence[a]
1[b]	Sense	Upstream of signal peptide	5′-cacc*agatct*ATGGAGATGCATCCCCGC-3′
2[b]	Antisense	Downstream of signal peptide	5′-gtc*cctagg*TGGTGACACAAAACCGTAGG-3′
3[b,c]	Sense	Entire signal peptide	5′-*agatct*aaaATGGAGATGCATCCCCGCAACGCCGGCAGGAAAACACTCCT TGCCCTGGCCCTGTTTATGGCGACATCCATTGCTTCTTCCTACGGT-3′
4[c]	Sense	R37D	5′-TTTATGGCGACATCCATTGCTTCTTCCTACGGTTTTGTGTCACCA-GGCCTGATC**GAC**TTTAATTACCGATATGGCACT-3′
5[c]	Sense	R41D	5′-TTTATGGCGACATCCATTGCTTCTTCCTACGGTTTTGTGTCACCAGG-CCTGATCAGGTTTAATTAC**GAT**TATGGCACT-3′
6[c]	Sense	R37D + R41D	5′-TTTATGGCGACATCCATTGCTTCTTCCTACGGTTTTGTGTCACCAGGCC-TGATC**GAC**TTTAATTAC**GAT**TATGGCACT-3′
7[c]	Sense	R37K + R41K	5′-TTTATGGCGACATCCATTGCTTCTTCCTACGGTTTTGTGTCACCAGGCC-TGATC**AAG**TTTAATTAC**AAA**TATGGCACT-3′
8[c]	Sense	R37A + R41A	5′-TTTATGGCGACATCCATTGCTTCTTCCTACGGTTTTGTGTCACCAGGCC-TGATC**GCG**TTTAATTAC**GCA**TATGGCACT-3′
9[b]	Antisense	Downstream of transit peptide	5′-agtg*cctagg*CCGATCATCAGAACTCGCCTCGT-3′
10	Sense	R37A	5′-GTGTCACCAGGCCTGATC**GCG**TTTAATTACCGATAT-3′
11	Antisense	R37A	3′-CACAGTGGTCCGGACTAG**CGC**AAATTAATGGCTATA-5′
12	Sense	R73A	5′-CCCGCAGGAACTGTTGCA**GCC**CGCCCAGGACCGTTTC-3′
13	Antisense	R73A	3′-GGGCGTCCTTGACAACGT**CGG**GCGGGTCCTGGCAAAG-5′
14	Sense	R73A + R74A	5′-CCCGCAGGAACTGTTGCA**GCCGCC**CCAGGACCGTTTC-3′
15	Antisense	R73A + R74A	3′-GGGCGTCCTTGACAACGT**CGGCGG**GGTCCTGGCAAAG-5′
16	Sense	R73A + R74A + R79A	5′-**CCGCC**CCAGGACCGTTT**GCA**AGTGTTAGCGCAAATG-3′
17	Antisense	R73A + R74A + R79A	3′-**GGCGG**GGTCCTGGCAAA**CGT**TCACAATCGCGTTTAC-5′
18	Sense	V31R	5′-GCTTCTTCCTACGGTTTT**AGG**TCACCAGGCCTGATC-3′
19	Antisense	V31R	3′-CGAAGAAGGATGCCAAAA**TCC**AGTGGTCCGGACTAG-5′
20	Sense	V31R + L35R + R37A	5′-CGGTTTT**AGG**TCACCAGGC**AGG**ATC**GCG**TTTAATTAC-3′
21	Antisense	V31R + L35R + R37A	3′-GCCAAAA**TCC**AGTGGTCCG**TCC**TAG**CGC**AAATTAATG-5′

[a] Codon changes are underlined in bold.
[b] *Avr*II and *Bgl*II restriction sites are underlined and italicized.
[c] Overlap between primers 3 & 4–8 are underlined.

the importance of positive charge, the Quickchange method was also used to introduce arginines at different positions in a transit peptide template lacking positive charge due to alanine replacement (bottom two rows of the table).

2.4. Parasite transfection and microscopy

Parasite cultures were grown and transfected as previously described [24]. Briefly, RH strain *T. gondii* tachyzoites were propagated in human foreskin fibroblasts (HFF) in 25-cm^2 plastic culture flasks (Falcon) and on glass slides in 6-well culture dishes (Falcon). HFF were first grown to confluence in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% cosmic calf serum (HyClone), followed by infection with *T. gondii* in DMEM supplemented with 1% foetal calf serum (Gibco).

Transfection was conducted by mixing 10^7 freshly lysed-out tachyzoites with 50 μg plasmid DNA and resuspending in incomplete cytomix. DNA–parasite suspensions were then electroporated at 25 μF and 1.5 KeV in a 4 mm gap cuvette as previously described [24] and immediately inoculated into confluent HFF cell cultures. Parasites were analyzed ~24–30 h post-transfection by fluorescence and phase contrast microscopy, on a Ziess inverted microscope equipped with AxioVision v4.5. Raw image stacks were deconvolved using the inverse filter algorithm. Each image represents a single optical section.

3. Results

3.1. N-terminal positive charge and the absence of negative charge is important for transit peptide fidelity in protein trafficking to the T. gondii apicoplast

Apicoplast transit peptides of *T. gondii*, like those of *P. falciparum* [21] (and plant plastids), contain positively charged amino acids but are depleted in negatively charged amino acids, especially at their N-termini [20]. To gain a wider perspective into the significance of this observed trend, we introduced a series of point mutations into the *T. gondii* ACP transit peptide, analogous to those previously studied in *P. falciparum* [21]. As shown in Fig. 1, the *T. gondii* ACP transit peptide exhibits no significant sequence identity to the ACP transit peptide from other apicomplexan parasites [21,14]. The *Tg*ACP transit peptide is ~64 amino acids in length, versus 24–29 in *Plasmodium* and *Theileria* species. Net positive charge is maintained however, as the *Tg*ACP transit peptide contains five positive and no negative charges (5 + 0), versus 6 + 1 in *P. falciparum*, 5 + 2 in *P. berghei* and 4 + 1 in *T. annulata*. These observations further suggest the importance of positive charge over primary sequence in apicoplast transit peptides [20].

To investigate the role of apicoplast transit peptide charge in greater detail, we created a series of mutant leader-eYFP fusions and introduced these constructs into *T. gondii* parasites in parallel with the validated apicoplast marker *Tg*ACP(l)-

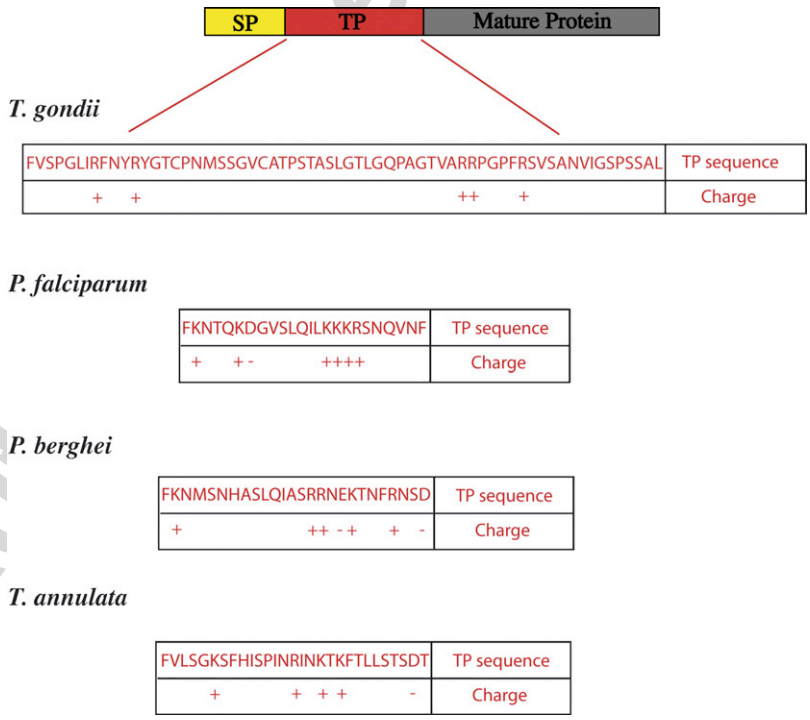

Fig. 1. Sequence and charge profile of ACP transit peptides in apicomplexan parasite species. The apicoplast transit peptide (TP, red) is the second domain of the bipartite leader sequence found on most nuclear-encoded apicoplast proteins, and typically lies immediately downstream of the secretory signal peptide (SP, yellow). A comparison of the acyl carrier protein (ACP) transit peptides from four Apicomplexa shows no conservation in overall length or primary sequence, but a similar net positive charge, attributable to a depletion of negatively charged amino acids and a consequent abundance of positive charged residues relative to other coding sequence in these species.

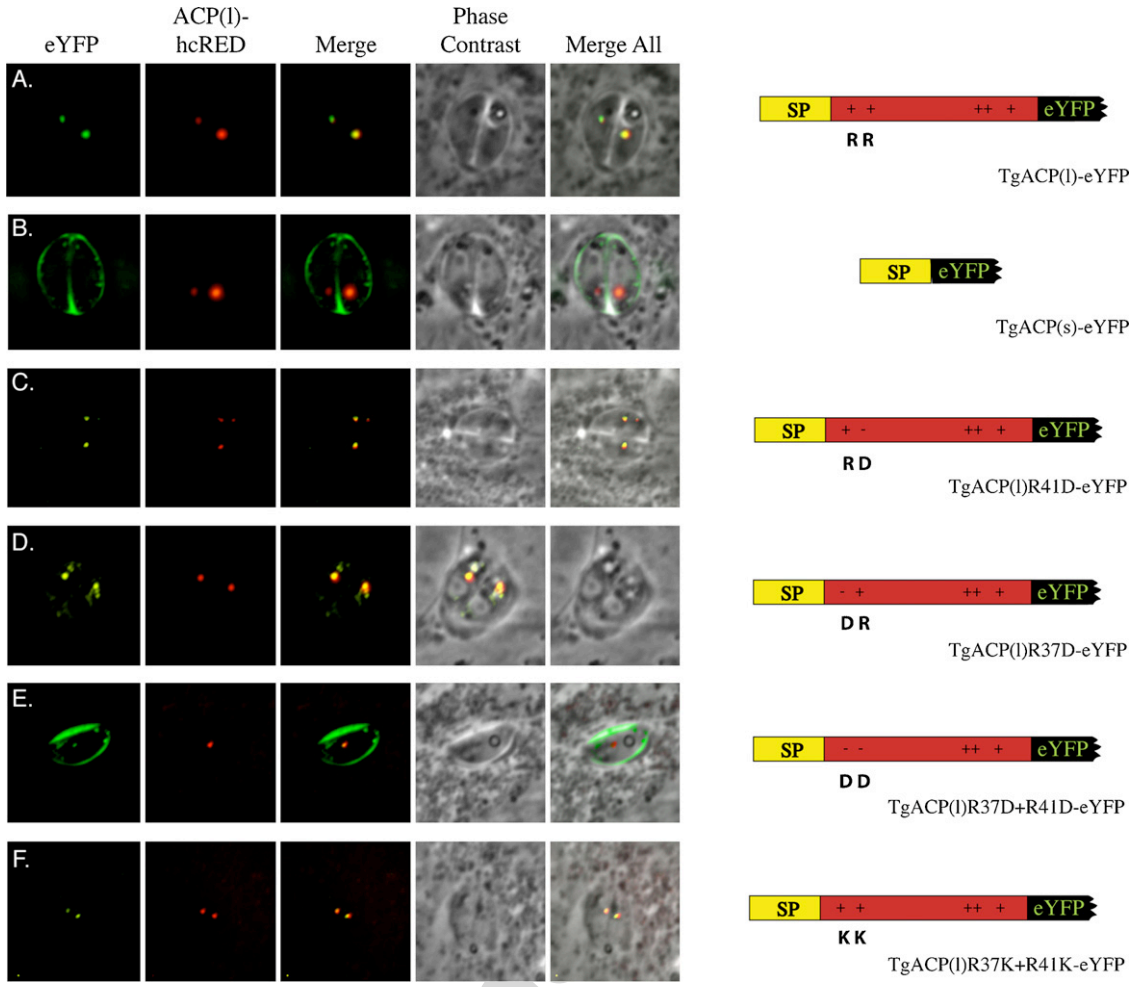

Fig. 2. N-terminal positive charge is important for high fidelity trafficking to the *T. gondii* apicoplast. A series of *Tg*ACP transit peptide charge mutants were fused to eYFP and transiently co-transfected into *T. gondii* tachyzoites in combination with plasmid *Tg*ACP(l)-hcRED, in which the wild-type *Tg*ACP leader directs hcRED to the apicoplast. In the schematic diagrams at right, the signal peptide (SP) is shown in yellow, transit peptide (TP) in red, and the sequence, position, and charge of each mutation is indicated: +, positive charge; −, negative; R, arginine; D, aspartic acid; K, lysine. Relative to wild-type controls (A), replacing the second positively charged arginine with a negatively charged aspartic acid in mutant R37D has little to no effect (C), whereas changing the first arginine to aspartic acid reduces the efficiency of apicoplast targeting slightly (D), as some protein remains in an internal structure resembling the endoplasmic reticulum. The R37D + R41D double mutant dramatically inhibits targeting to the apicoplast (E), leaving most protein to be secreted out into the PV via the dense granules similar to that observed when the transit peptide is eliminated altogether (B). Swapping two positively charged lysines for the two arginines has no effect on targeting (F).

hcRED, as shown in Fig. 2. Fusion of the intact wild type *Tg*ACP leader to eYFP (*Tg*ACP(l)–eYFP) results in accumulation of fluorescence within the apicoplast (Fig. 2A), whereas omission of the transit peptide (leaving just the signal peptide component) results in secretion of eYFP into the parasitophorous vacuole (PV) (Fig. 2B)—the default pathway for signal peptide-bearing proteins [25]. These observations are consistent with findings reported for *P. falciparum* [14]. Changing one of the two amino-terminal domain arginines (at position 37 and 41) to negative amino acids (aspartic acid) produced variable effects: the R41D mutant does not noticeably disrupt apicoplast targeting (Fig. 2C), but R37D causes partial mistargeting of the eYFP fusion, where a portion of which remains stuck in the ER (Fig. 2D). Replacing both amino acids (*Tg*ACP(1)R37D + R41D) directs the majority of the eYFP fusion into the PV; only a very small amount reaches the apicoplast (Fig. 2E). Conservative changes, such as converting both R37 and R41 to lysines does not impede accurate apicoplast targeting, however (Fig. 2F), consistent with preliminary findings in *P. falciparum* (CJT and GIM unpublished).

3.2. Overall transit peptide positive charge is important for apicoplast targeting fidelity

To further investigate the importance of transit peptide charge, we created a series of mutants in which all five arginines found in the *Tg*ACP(l) transit peptide (at amino acid positions 37, 41, 73, 74 and 79, corresponding to transit peptide amino acids 8, 12, 44, 45 and 50) were sequentially replaced by the uncharged amino acid alanine, proceeding from the N-terminus to the C-terminus (Figs. 1 and 3). Each mutant was fused to eYFP and transit peptide fidelity monitored by comparison with co-transfected *Tg*ACP(l)-hcRED. Compared to *Tg*ACP(l) (Fig. 3A)

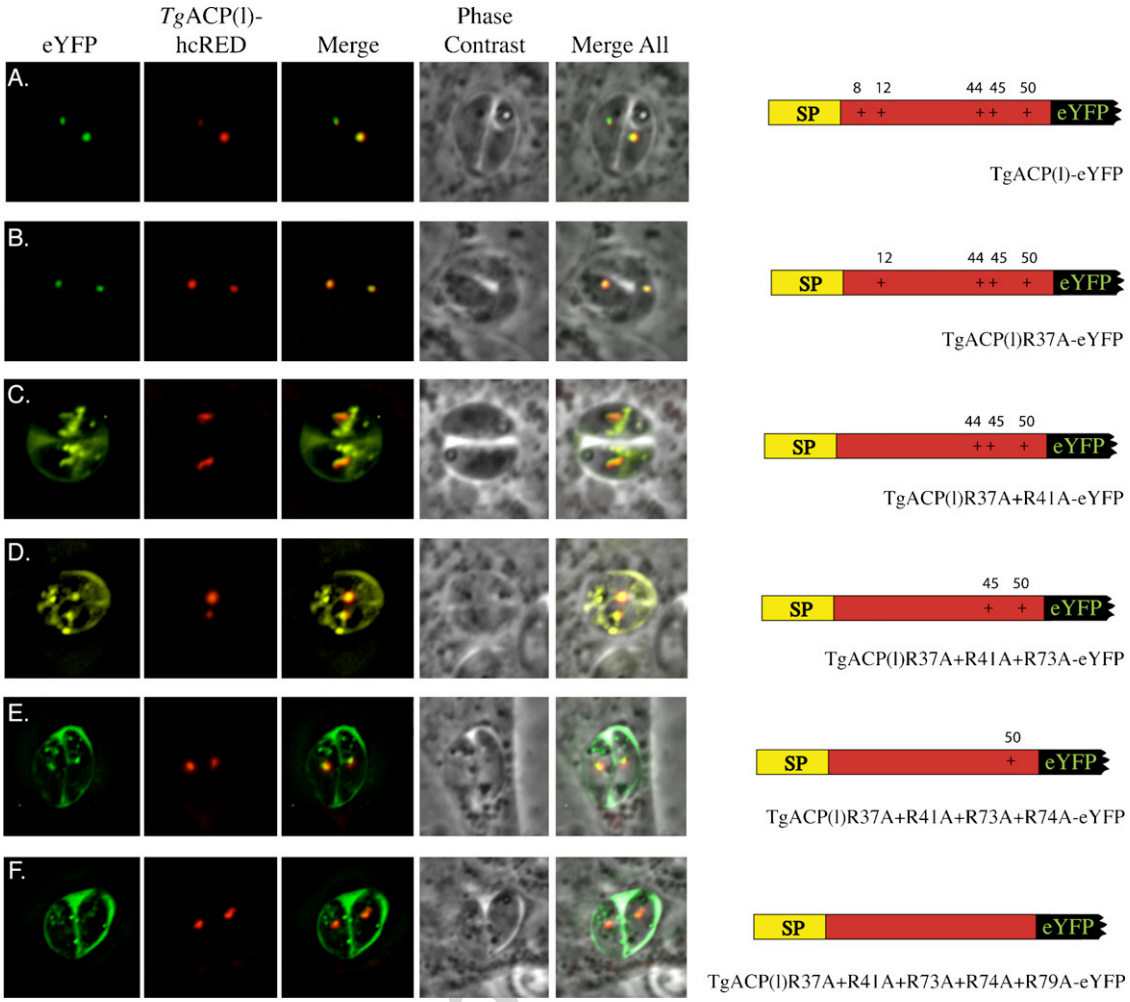

Fig. 3. Progressive removal of positively charged amino acids throughout the ACP transit peptide. *Tg*ACP(l) contains five positive charges at amino acids 8, 12, 44, 45, 50 of the mature transit peptide. Mutations were introduced progressively, from the N-terminus to the C-terminus of the transit peptide, replacing each charged arginine with a neutral alanine. Single mutant R37A has no discernable effect on targeting to the apicoplast (B), based on comparison with a parental control containing the entire intact ACP transit peptide (A). The double mutant R37A + R41A shows labelling of the apicoplast, and other internal secretory structures, and staining of the PV (C). The triple mutant R37A + R41A + R73A (D) and quadruple mutant R37A + R41A + R73A + R74A (E) show progressively less apicoplast staining, and more secretion into the PV. In the quintuple mutant (F), virtually no eYFP was targeted to the apicoplast. See Fig. 2 legend for a description of methods and schematic diagrams (A, alanine).

removal of the first arginine (*Tg*ACP(l)R37A) has no discernable effect on targeting of eYFP to the apicoplast (Fig. 3B). Note that introduction of a negative charge at this position reduced apicoplast targeting (Fig. 2D). Further replacement of the second arginine at position 41 with alanine (*Tg*ACP(l)R37A + R41A) results in a portion of the eYFP being targeted into the PV (Fig. 3C). A significant amount of eYFP is also trapped in other internal secretory locations [26]. Replacement of the third, fourth and fifth arginines (Fig. 3, panels D, E and F, respectively) yields even stronger effects, with progressively greater loss of eYFP from the apicoplast (see loss of yellow colocalization in the 'merge' panels), and more intense secretion into the PV. Virtually no eYFP is targeted to the apicoplast in the quintuple mutant *Tg*ACP(l)R37A + R41A + R73A + R74A + R79A, although some internal fluorescence is visible within the parasite, most likely reflecting dense granules en route to release their cargo into the PV.

3.3. The precise position of N-terminal positive charge is not important for transit peptide fidelity

In order to assess the importance of specific charged amino acid position on apicoplast transit peptide function, 'add-back' mutants were engineering to reintroduce positively charged amino acids at novel sites in charge-depleted mutant leader *Tg*ACP(l)R37A + R41A + R73A + R74A + R79A. These mutants were created by sequentially adding back arginines within the N-terminal domain of the *Tg*ACP transit peptide, but at different positions from the native arginine sites. Despite the ablation of apicoplast targeting in the quintuple-alanine mutant background (Fig. 3F), the introduction of a single arginine at position 31 (corresponding to transit peptide amino acid 2) was sufficient to partially restore eYFP targeting to the apicoplast (Fig. 4A). Some eYFP can be seen in internal endomembrane locations, as with many of our other mutants,

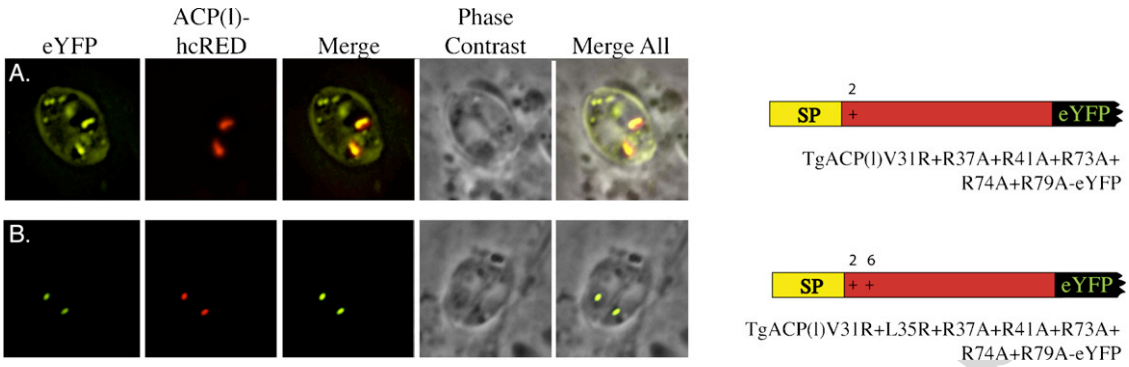

Fig. 4. The precise position of transit peptide positive charge is not important. The effect of position of N-terminal positively charged residues within the transit peptide was assayed by sequentially adding back two arginines into the charge-depleted mutant ACP transit peptide (see Table 1 and Fig. 3F). Introducing arginine in place of valine at position 31 (V31R) showed reduced targeting to the PV, and some targeting to the apicoplast (A). Introduction of a second arginine in the construct *Tg*ACP(l)V31R + L35R + R37A + R41A + R73A + R74A + R79A-eYFP restored effectively complete apicoplast targeting (B; compare with Figs. 2A and 3A). See Fig. 2 legend for a description of methods and schematic diagrams (V, valine; L, leucine).

and reasons why this might occur are unknown. It is possible that our manipulation of the ACP transit peptide has caused prolonged interactions with cognate receptors, delayed translocation through membranes or many other non-native interactions (has also been seen in other studies [27]). Subsequent addition of a second positive charge at position 35 (transit peptide position 6) (*Tg*ACP(1)V31R + L35R + R37A + R41A + R73A + R74A + R79A) completely restored apicoplast targeting (Fig. 4B). It is informative to compare this experiment (harbouring positively charged arginines at positions 2 and 6 of the transit peptide) with the R37A + R41A + R73A triple mutant, which also retains a net charge of + 2 due to arginines at amino acids 74 and 79 (positions 45 and 50 of the transit peptide). The former (Fig. 4B) exhibits far more effective targeting of eYFP to the apicoplast than the latter (Fig. 3D). Thus, while the precise position of positively charged amino acids is unimportant for transit peptide fidelity, positive charges in the N-terminal domain of the transit peptide appear to be more effective in directing proteins into the apicoplast.

4. Discussion

We have demonstrated that the presence of positively charged amino acids, and the relative absence of negatively charged amino acids, is critical for transit peptide efficacy in targeting to the apicoplast of *T. gondii*. By co-transfecting wild-type transit peptide fused to a red reporter protein and mutant transit peptide fused to a yellow reporter protein we were able to visualise the specific effect of point mutations within the same cell. Site-directed mutagenesis indicates that specific amino acid identity (e.g. arginine versus lysine), and even their exact position, are not critical as long as a majority of positive charges are located within the more N-terminal portion of the transit peptide. These findings corroborate and extend previous studies on the transit peptide of the ACP homolog in *P. falciparum*, where charge was also found to be a vital feature of transit peptides [21]. It should be noted however, that point mutations introduced into the N-terminus of *Tg*ACP transit peptide were less severe (that is less apicoplast mistargeting) than the corresponding mutations *Pf*ACP, and reasons for this difference remain unknown. A factor that likely influences the comparative severity of the *Pf*ACP mutants is the presence of an endogenous aspartic acid directly after the second lysine [21], which would result in a more severe change in transit peptide fidelity upon the mutation of the first two positive charged residues.

*Pf*ACP and *Tg*ACP differ significantly in their length, amino acid sequence, and amino acid composition, and charge profiles (Fig. 1). Nevertheless, they clearly must follow similar rules for plastid targeting, as apicoplast transit peptides have been shown to function properly across species boundaries [14,28]. Detailed bioinformatics analysis of a large collection of plastid transit peptides has identified positive charge combined with overall hydrophilicity as a general characteristic of all systems, from plants to parasites [20]. While differing nucleotide composition and codon biases impose skewed amino acid compositions on proteins from various organisms, transit peptides that are remarkably different at the primary sequence level still retain high positive charge and hydrophilicity [20]. This trend is apparent in comparisons between *T. gondii* (which exhibits no unusual nucleotide or amino acid compositional bias) and *P. falciparum* (where an extremely A/T-rich genome leads to an abundance of lysine and asparagine). Their apicoplast leader sequences are nevertheless similar, with positive charge generally attributable to arginine in *T. gondii* versus lysine in *P. falciparum*; additional influences on hydrophilicity come from serine in *T. gondii* and asparagine in *P. falciparum*. We have shown that in at least one case, lysine residues can effectively substitute for arginine residues in a *T. gondii* transit peptide (Fig. 2F).

How apicoplast transit peptides are recognised is not known. It is tempting to speculate that the charge effects noted above may be an important component for plastid transit peptide recognition in general, indeed, it will be interesting to conduct similar studies on plant transit peptides where the criticality of charge has not been thoroughly explored. In plants, galactolipids exclusive to the outer plastid membrane may play a role in recognizing hydrophilic amino acids (particularly serine) in transit peptides, inducing amphipathic alpha-helical organization of the transit peptide [4,5,29–33]. Phosphorylation of serine or threonine residues may also be important in discriminating between plastid and mitochondrial transit peptides [34,35]. Whether secondary

structure or phosphorylation plays a role in apicoplast targeting is unknown, but it is interesting that galactolipids have been reported in *T. gondii* and *P. falciparum* [36], although whether they are located in the apicoplast membranes is not known. On the other hand, the fact that the precise position of charge within the transit peptide is relatively unimportant (Fig. 4) suggests that secondary structure may not be a critical feature of apicoplast transit peptides. In comparing targeting to primary versus secondary endosymbiotic plastids, it is worth noting that whereas the former reside in the cytoplasm, the latter (including apicoplasts) lie within the endomembrane system, away from the mitochondrion, and this physical separation may be sufficient to avoid mistargeting and therefore might remove the need for transit peptide phosphorylation [20].

Plant transit peptides are recognised and translocated through the plastid membranes by the TOC/TIC machinery. It is interesting to note that a search of the available apicomplexan genomes shows that this group of parasites lack most identifiable homologues, including the translocation pore itself—Toc75. A Tic22 homologue, however, is present and this localises to the apicoplast in *P. falciparum* (CJT and GIM unpublished), however, its role (if any) in protein translocation is still to be demonstrated. It is therefore unknown what recognises apicoplast transit peptides and how membrane translocation takes place.

Other studies have undertaken deletion analysis of apicoplast transit peptides with the aim of identifying critical elements empirically [13,27,37,38]. The most comprehensive of these deletion analyses dissected the extremely long apicoplast transit peptide of ferredoxin NADP + reductase from *T. gondii* (*Tg*FNR) [27]. Harb et al. concluded that the large transit peptide of FNR had multiple, functionally redundant targeting domains that could independently mediate apicoplast targeting [27]. Other groups examining the apicoplast transit peptide for the ribosomal small subunit protein S9 reached similar conclusions [13,37,38]. In all cases a signal peptide was found to be an essential first component of an apicoplast leader in order to direct the protein into the ER. Tampering with the transit peptide component had various results, sometimes directing the protein to the apicoplast but also resulting in reporter protein going to other destinations such as the apicoplast periphery, apical organelles and PV. Overall these analyses suggest that certain regions of the apicoplast transit peptide mediate steps such as transport through the ER and then through the apicoplast membranes [13,27,37,38]. Interestingly, we were unable to find any sequence conservation between the important regions identified of the FNR and S9 transit peptides making it difficult to define what properties of the leader confer these targeting features. However, we do observe correlation between the charge profile of those truncated leaders able to successfully target reporter protein to the interior of the apicoplast and our general hypothesis that net positive charge is an essential feature. Both the FNR and S9 transit peptides are much longer than the ACP transit peptide, and transit peptides in general vary considerably in length [20], but our mutagenesis data indicate that the critical portion for recognition is clustered in the more N-terminal region immediately downstream of the signal peptidase cleavage site. Suitably charged domains could also affect apicoplast targeting in the appropriate context, and the longer transit peptides may have redundant regions that fit these characteristics. Thus, although our data does not rule out the existence of other important domains within apicoplast transit peptides such as a cleavage site for the stromal processing peptidase that removes the transit peptide within the apicoplast [22,27], we propose a much simpler model of transit peptide recognition for apicoplasts that is based on the presence of sufficient numbers of positively charged residues within an overall hydrophilic context.

The apicoplast is an attractive drug target, and a numerous existing antibiotics and herbicides have been shown to kill parasites *in vitro* and/or *in vivo* [11,28,39–41]. Doxycycline and rifampicin, believed to target apicoplast translation and transcription (respectively) are effective as anti-malarials, and drugs that target synthesis of proteins (clindamycin) and isoprenoids (fosmidomycin) within the apicoplast have been used in field trials against *P. falciparum* [42]. Numerous apicoplast functions are now known, and we have provided a comprehensive metabolic pathway map for the organelle in *Plasmodium* [1], but a complete definition of potential apicoplast targets requires better methods for identifying nuclear-encoded apicoplast proteins. We have recently shown that the apicoplast proteome of *P. falciparum* can be predicted using a simple rule-based algorithm based principally on transit peptide N-terminal net positive charge [21]. The present study suggests that a similar algorithm to identify bipartite apicoplast leaders could help predict the apicoplast proteome from *T. gondii*, elucidating the function of this intriguing organelle and helping to identify potential new drug targets for toxoplasmosis.

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