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Sequential processing of the *Toxoplasma* apicoplast membrane protein FtsH1 in topologically distinct domains during intracellular trafficking

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ABSTRACT

FtsH proteins are hexameric transmembrane proteases found in chloroplasts, mitochondria and bacteria. In the protozoan *Toxoplasma gondii*, FtsH1 is localized to membranes of the apicoplast, a relict chloroplast present in many apicomplexan parasites. We have shown that although *T. gondii* FtsH1 lacks the typical bipartite targeting presequence seen on apicoplast luminal proteins, it is targeted to the apicoplast via the endoplasmic reticulum. In this report, we show that FtsH1 undergoes processing events to remove both the N- and C-termini, which are topologically separated by the membrane in which FtsH1 is embedded. Pulse-chase analysis showed that N-terminal cleavage precedes C-terminal cleavage. Unlike the processing of the N-terminal transit peptide of luminal proteins, which occurs in the apicoplast, analysis of ER-retained mutants showed that N-terminal processing of FtsH1 occurs in the endoplasmic reticulum. Two of four FtsH1 mutants bearing internal epitope tags accumulated in structures peripheral to the apicoplast, implying that FtsH1 trafficking is highly sensitive to changes in protein structure. These mutant proteins did not undergo C-terminal processing, suggesting that this processing step occurs after localization to the plastid. Mutation of the peptidase active site demonstrated that neither processing event occurs in *cis*. These data support a model in which multiple proteases act at different points of the trafficking pathway to form mature FtsH1, making its processing more complex than other FtsHs and unique among apicoplast proteins described thus far.

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1. Introduction

Apicomplexan parasites are responsible for considerable suffering worldwide, both directly in the case of the human pathogens and indirectly in the case of pathogens affecting farm animals. This phylum of obligate intracellular parasites includes *Toxoplasma gondii*, an important opportunistic pathogen of AIDS patients [1] and a causative agent of severe birth defects [2]. It also includes *Plasmodium* spp., which cause approximately two millions deaths each year from malaria [3]. The drugs currently available to treat these diseases are either not very effective, poorly tolerated, or facing resistance [4]. Thus there is an urgent need to identify new drugs and drug targets. The apicoplast is an organelle present in most medically important apicomplexans, with the exception of *Cryptosporidium parvum* [5], but it is absent in the animal host. The apicoplast is home to several metabolic pathways, including the type II fatty acid synthesis pathway [6] as well as part of the type II heme biosynthesis pathway [7]. The type II isoprenoid synthesis pathway is also apicoplast-localized in *P. falciparum* [8], although its presence in the apicoplast of *T. gondii* is not yet confirmed [9]. The apicoplast and the pathways it compartmentalizes have been shown to be essential in both *T. gondii* and *P. falciparum* [8,10,11], and hence are of considerable interest as potential drug targets.

The apicoplast is a remnant chloroplast that is thought to have been acquired by secondary endosymbiosis wherein an ancestral apicomplexan engulfed an alga and appropriated its chloroplast. The result is a plastid surrounded by four membranes [12]. The two inner membranes are thought to be homologues of chloroplast membranes, the third is proposed to be homologous to the plasma membrane of the algal cell, and the outermost membrane is thought to be derived from the endomembrane system of the apicomplexan [for review see [13]]. The apicoplast has its own genome, whose organization resembles that of chloroplasts, albeit highly reduced (~35 kb). It mainly encodes housekeeping genes such as rRNAs and tRNAs and thus most proteins required for the organelle's function are encoded in the nucleus. Those destined for

Abbreviations: CP, C-terminally processed; DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; mAb, monoclonal antibody; NP, N-terminally processed; TMD, transmembrane domain.

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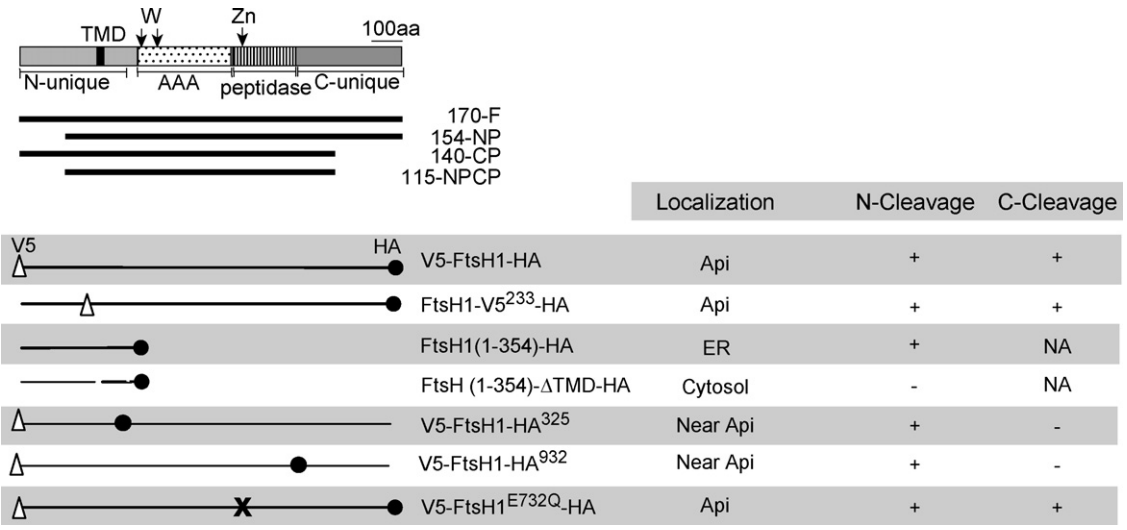

Fig. 1. Scale schematic of the FtsH1 protein and expression constructs. Unique regions at N-terminus (aa 1-347) and C-terminus (aa 903-1250) as well as AAA (aa 386-691) and peptidase (aa 698-902) domains are indicated. The transmembrane domain (TMD), Walker A and B motifs that function in ATP binding (W), and zinc binding (Zn) sites are marked. Beneath the schematic, the major isoforms discussed in the manuscript are shown as bars (the N-terminal cleavage site is approximate). The table below presents diagrams of the expression constructs described herein. V5 tags are indicated by triangles and HA tags are indicated by balls. Mutation at the peptidase active site residue is indicated by "X". Shown at right is the localization of the expressed proteins as revealed by anti-V5 mAb (except for the mutants that lacked a V5 tag): Api, apicoplast; ER, endoplasmic reticulum. Cleavage (+) or lack thereof (−) is indicated for each protein. Proteins which were not assessed for cleavage due to the position of the tag and truncated proteins that lack the cleavage site are marked "NA".

the apicoplast lumen are targeted there by virtue of an N-terminal targeting sequence. This consists of a signal sequence followed by a transit peptide, both of which are essential for proper localization [14–16]. The signal sequence is required for entry into the endoplasmic reticulum (ER), and is cleaved upon import. The exposed transit peptide then targets the protein to the apicoplast, where it too is cleaved, resulting in the formation of the mature protein [17]. Recently, we identified the first apicoplast membrane proteins in *Toxoplasma*: APT1 (apicoplast phosphate translocator 1) [18] and the zinc-dependent protease FtsH1 (so named for the filamentous phenotype observed in the original *E. coli* mutants) [19]. Both proteins lack the targeting sequences characteristic of luminal proteins, but appear to traffick to the organelle via the ER. Immunoelectron microscopy showed apparent residence of both proteins in multiple membranes of the apicoplast, as well as in vesicles that may serve to transport the molecules to the apicoplast. Furthermore, both proteins behaved as integral membrane proteins in biochemical studies. Recently, Tic20, a protein of the innermost membrane of apicoplast, was identified in *T. gondii* and shown to bear a signal and transit peptide [20]. The authors showed that Tic20 is essential for the survival of the parasite and that it likely plays an indirect role in import of proteins across the innermost membrane of the apicoplast.

FtsHs are ubiquitous proteins found in prokaryotes as well as in the mitochondria and chloroplasts of eukaryotes. All FtsHs described to date exist as hexamers that are either homo-oligomers or hetero-oligomers [21,22]. FtsHs are transmembrane metalloproteases that possess AAA domains (ATPase associated with several cellular activities), and require ATPase function for protease activity. The main function of FtsHs identified thus far is to maintain quality control by degrading misassembled and damaged membrane proteins. For example, chloroplast FtsHs degrade proteins damaged by photooxidation, and mutants are typically defective in photosynthesis [23]. Similarly, *Saccharomyces cerevisiae* lacking the mitochondrial FtsH Yta10p is defective in respiration [24]. *Escherichia coli* FtsH (HflB) as well as FtsH homologues in the mitochondrial inner membrane of *Saccharomyces cerevisiae*, Yme1 and the Yta10-12 complex, have additionally been shown to function as chaperones [25–27]. Recently, Yme1 was also shown to be required for import of polynucleotide phosphorylase to the intermembrane space of mitochondria [28]. FtsH is essential for division of *E. coli*, but the function of *T. gondii* FtsH1 and whether it is essential remain unknown.

T. gondii FtsH1 encodes a 1250 aa protein with a single transmembrane domain (TMD) (Fig. 1). It has a long N-terminal extension that does not show sequence homology to any predicted proteins. This region, which contains the TMD, is followed by the hallmark AAA and peptidase domains. The protein ends in another unique region. We have previously shown that both the TMD and the peptidase domain are required for proper targeting of FtsH1 to the apicoplast [19], although the protein's topology within the apicoplast membranes remains unknown. FtsH1 tagged at the N-terminus localizes to the apicoplast, and shows two bands on immunoblot analysis, which suggested that the protein undergoes either post-translational modification or processing at the C-terminus. Here we demonstrate that FtsH1 is proteolytically processed at the N-terminus as well as the C-terminus. In addition to being the first FtsH to undergo multiple processing events, *T. gondii* FtsH1 is also the first example of an apicoplast protein to exhibit processing at both termini.

2. Materials and methods

2.1. Cell culture and transfections

T. gondii strain RHΔhxgprt and its derivatives [29], including a line expressing apicoplast-targeted *Heteractis crispa* red fluorescent protein (S+T^{ACP}-HcRed) [18], were grown and maintained in human foreskin fibroblasts as described previously [30]. Plasmids digested with *NotI* [31] were transfected into these lines and stable transfectants were obtained using xanthine and mycophenolic acid as selection reagents. Limiting dilution was used to obtain clonal lines. In previous studies, a single V5-FtsH1 transfectant showed a weak band at 151 kDa on immunoblot analysis using anti-V5 mAb, in addition to the bands described in this report [19]. Since this band appeared in only one of many transfectants, it is likely an integration artifact, and so other transfectants were used for the current studies.

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