

Theileria parva 104 kDa microneme–rhoptry protein is membrane-anchored by a non-cleaved amino-terminal signal sequence for entry into the endoplasmic reticulum

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Abstract

The 104 kDa microneme–rhoptry protein (p104) is the only known apical complex organelle-specific protein of *Theileria parva*. p104 exhibits striking structural similarities to circumsporozoite protein and sporozoite surface protein 2 of *Plasmodium yoelii*. Their primary sequences contain two hydrophobic segments, located at the amino- and the carboxy-terminus. The p104 amino-terminal hydrophobic region was suggested to be a signal peptide for entry into the endoplasmic reticulum and the extreme carboxy-terminal region to function as a membrane anchor. We have studied the biogenesis of p104 in a cell-free expression system and found that p104 is co-translationally transported into membranes derived from endoplasmic reticulum. The amino-terminal signal peptide is not cleaved off and anchors the protein in the membrane with the carboxy-terminal portion translocated into the lumen. We suggest that in vivo p104 is co-translationally integrated into the membrane of the endoplasmic reticulum, from where it is further transported to the microneme–rhoptry complex. Thus, p104 appears to be a suitable marker to study the development of micronemes and rhoptries in *T. parva*. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Theileria parva*; Microneme/rhoptry biogenesis; Signal–anchor sequence

Abbreviations: AP, acceptor peptide; ER, endoplasmic reticulum; p104, *Theileria parva* 104 kDa microneme–rhoptry protein; p104t, *Theileria parva* 104 kDa microneme–rhoptry protein truncated after residue 301; RM, rough microsomes; SRP, signal recognition particle.

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1. Introduction

The phylum Apicomplexa encompasses many obligate intracellular parasites with mostly complex life cycles covering a large spectrum of host organisms. The characteristic apical complex, a set of organelles located at the anterior end of invasive stages, has given name to this taxon. The apical complex typically consists of several rhoptries, dense granules and micronemes. These organelles appear to differ not only in their ultrastructure but also in their function and protein composition. In general their contents can be discharged and they are implicated in entry into and remodelling of the host cell, as well as in release from the host cell (reviewed in [1,2]).

The biogenesis of rhoptries and micronemes is still poorly understood. The common view is that they are secretory organelles which acquire their contents from the endoplasmic reticulum (ER), possibly via a Golgi complex-like compartment. Where in the cell rhoptry and microneme specific proteins are diverted from the classical secretory pathway and which structural features of these proteins are involved in their sorting is entirely unclear.

Theileria parva is the causative agent of East Coast Fever, a cattle disease of economic importance in endemic areas of subsaharan Africa [3]. In this organism a single apical complex specific protein has been identified, the 104 kDa microneme–rhoptry protein (p104). p104 is localized to the microneme–rhoptry complex of the sporozoite, the stage invading bovine lymphocytes. The gene for it encodes a 924 residue polypeptide, which appears to be processed to smaller proteins sized 90, 85, and 35 kDa. The primary product contains two hydrophobic segments, one at the N-terminus, consisting of residues 3–19, and one at the C-terminus, consisting of residues 905–924 [4].

Here, we report on the biogenesis of p104 in a cell-free expression system, where it becomes integrated into ER membranes and N-glycosylated. Transport is mediated via a non cleaved N-terminal signal–anchor sequence and appears to occur co-translationally. We suggest p104 to be an ideal marker molecule for studying biogenesis of rhoptries and micronemes in *T. parva*.

2. Materials and methods

2.1. Plasmid construction

Plasmid pUC19-p104, containing a cDNA for p104 mRNA in vector pUC19, was obtained from Dr K.P. Iams, ILRI, Nairobi. A cDNA fragment containing the entire coding region was excised with *Dra*I from plasmid pUC19-p104 and ligated into the *Sma*I site of plasmid Bluescript SKII (Stratagene, La Jolla, CA). After bacterial transformation a clone containing the p104 insert under control of the T7 promoter was identified by restriction analysis (pSK-p104T7) and used for in vitro transcription.

2.2. In vitro transcription and translation

Plasmid pSK-p104T7 was linearized with *Not*I and transcribed in vitro with T7 RNA polymerase as described [5]. For some experiments pSK-p104T7 was cut with *Hind*III to generate a 3' end truncated transcript, encoding amino acids 1–301 of p104 (p104t). Aliquots of transcription reaction were translated in wheat germ lysate in the presence of L-[³⁵S]methionine and either in the absence or presence of dog pancreas rough microsomes (RM) as described [6]. In some experiments glycosylation at asparagine residues was blocked by addition of acceptor peptide (AP) benzoyl-Asn-Leu-Thr-N-maleimide to a final concentration of 30 mM [7]. SRP-stripped microsomes were prepared by high salt extraction essentially as described [8].

2.3. Post-translational assays

Proteinase K treatment of translation mixtures was performed as described [9]. Carbonate extraction of membranes was modified [10]: 50 µl of translation mix were layered over a 200 µl cushion of 0.25 M sucrose, 0.5 M KCl, 5 mM DTT, 3 mM MgCl₂, 50 mM HEPES KOH pH 7.5 and spun in a Beckman TLA 100.3 rotor (50 000 rpm, 15 min, 4°C). The resulting supernatant was removed and the pellet was resuspended in 50 µl 0.1 M Na₂CO₃ pH 11, incubated for 15 min on ice, layered over a cushion of 0.25 M sucrose, 0.1 M Na₂CO₃ pH

11, and spun as above. Supernatant and pellet fractions were recovered. SDS-polyacrylamide gel electrophoresis of reduced samples and fluorography were essentially as described [5].

3. Results and discussion

3.1. p104 is translocated across and processed by endoplasmic reticulum-derived membranes

We employed a wheat germ lysate *in vitro* translation system supplemented with dog pancreas RM, where entry of proteins into the ER and modification by signal peptide cleavage and glycan addition to asparagine residues can be reconstituted. We transcribed and translated p104 *in vitro* and obtained a protein of apparent molecular mass of about 110 kDa (Fig. 1, lane 1). In the presence of RM one higher molecular mass species was synthesized (lane 2). Since p104 contains five potential *N*-glycosylation sites (positions 42, 83, 200, 331, 401), we hypothesized that p104 was both membrane translocated and processed by *N*-glycan addition in the ER lumen. In the *in vitro* expression system addition of one *N*-linked glycan results in an increase of apparent mass by about 2.5 kDa. Thus detection of signal peptide cleavage could be obscured by concomitant glycan addition. Therefore we used inhibition of *N*-glycosylation by competition with acceptor peptide (AP) both to test for signal peptide cleavage in the absence of glycosylation and to confirm the expected glycosylation [7]. When RM and AP were present during translation, the processed form(s) were not detected (lane 5). However, the product was completely resistant to exogenously added proteinase K (lane 4) unless detergent was added (lane 3). The translation products smaller than 110 kDa are most likely due to premature termination.

These results demonstrate that p104 was translocated across the membrane of the ER and *N*-glycosylated. However, it is unclear whether p104 is glycosylated *in vivo* and whether *T. parva* performs protein *N*-glycosylation at all. For the apicomplexans *Cryptosporidium parvum* [11], *Tox-*

oplasma gondii [12], and *Plasmodium falciparum* [13] *N*-glycosylation has been reported.

3.2. The *N*-terminus of p104 carries a non-cleaved signal sequence for translocation

Preventing *N*-glycosylation we could not detect appearance of a slightly smaller sized form of p104, the expected product of signal peptide cleavage (Fig. 1, lane 5). However, the putative cleavage would remove some 20–30 residues from a total of 924 and cleaved and uncleaved p104 may well not be separated sufficiently on the gel. Therefore we used a C-terminal truncation of p104 (p104t) consisting of residues 1–301 of predicted molecular mass of 33 915. The removal of the putative signal peptide from this mutant would be clearly detectable as an increase of mobility in our gel analysis system [9]. p104t

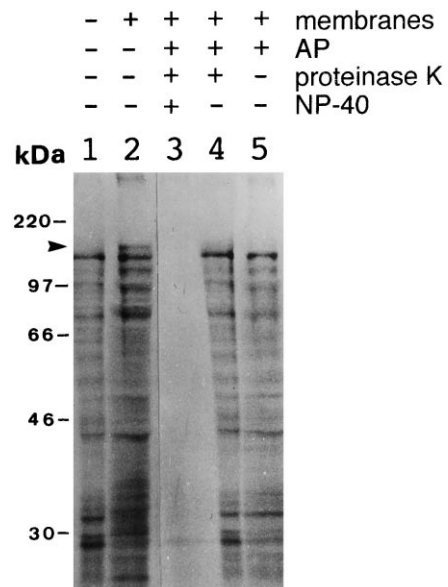


Fig. 1. p104 is translocated across and *N*-glycosylated by membranes. p104 was translated *in vitro* in the absence (lane 1) or presence of membranes (lanes 2–5) with (lanes 3–5) or without AP (lanes 1,2) for *N*-glycosylation as indicated. After translation in the presence of membranes and AP aliquots of the assay were treated with proteinase K either in the absence (lane 4) or presence (lane 3) of detergent NP-40 as indicated. Samples were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The mobilities of standard proteins are indicated and glycosylated p104 is marked by an arrowhead.

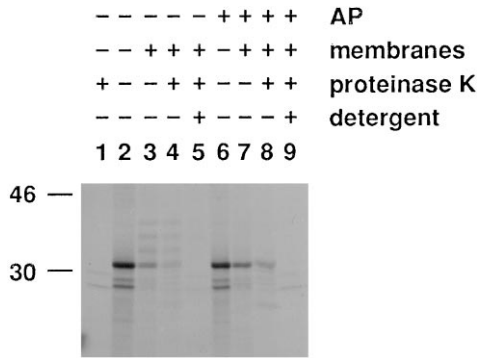


Fig. 2. The N-terminus of p104 carries a non-cleaved signal peptide. p104 truncated after residue 301 was translated in vitro either in the absence or presence of membranes with or without acceptor peptide (AP) as indicated. After translation in the presence of membranes and AP aliquots of the assay were treated with proteinase K either in the absence or presence of detergent NP-40 as indicated. Samples were analyzed as in Fig. 1. The mobilities of standard proteins are indicated as kDa values.

contains three of the five potential *N*-glycosylation sites of p104. As shown in Fig. 2, p104t was translated in vitro in the absence (lanes 1,2,6) or presence of RM (lanes 3–5,7–9), either without (lanes 1–5) or with AP present (lanes 6–9). In the absence of RM a 31 kDa translation product was expressed (lanes 2,6), which was sensitive to treatment with proteinase K (lane 1). In the presence of RM (lane 3) three additional products sized 33, 36 and 39 kDa were synthesized. These three additional products were not generated when AP was present during translation (lane 7) and were completely resistant to treatment with proteinase K (lane 4) unless detergent was added (lane 5), indicating that all three potential *N*-glycosylation sites present were used and that the N-terminal fragment of p104 was entirely translocated across the membrane. Translation in the presence of RM and AP yielded the 31 kDa product as a protease resistant protein (lane 8), which was digested upon addition of detergent (lane 9), demonstrating that AP did not inhibit membrane translocation.

A slightly smaller sized, protease protected product, which would be expected upon cleavage of the signal peptide, could neither be detected in the absence (lanes 3,4) nor in the presence of AP

(lanes 7,8). Thus, we conclude that the hydrophobic sequence at the N-terminus of p104 functions indeed as the core of a signal sequence for targeting to and translocation across the ER membrane and that the p104 signal sequence belongs to the minor class of non-cleaved ER signal sequences. Our experimental data are in agreement with statistical analysis of characterized signal sequences, which shows that the p104 signal lacks a consensus sequence for cleavage by signal peptidase [14]. Previously it has been shown that *T. parva* exhibits a similar signal peptidase activity as in the cell-free system [5]. Therefore, we assume that the p104 signal sequence is not cleaved in vivo.

Non-cleaved signal sequences can have two principal fates. They can either leave the ER membrane or they can anchor the polypeptide in the membrane as so-called signal–anchor sequences. If the p104 signal sequence functions as membrane anchor, only a few amino acids would be accessible to exogenously added protease. Our analysis shown in Fig. 2 can not distinguish between the two possibilities.

3.3. p104 is membrane anchored by its N-terminal signal sequence

To test whether the p104 signal has a signal–anchor function we applied carbonate extraction analysis, which allows separation of integral membrane proteins from soluble and peripheral membrane proteins. We analysed full-length p104 (Fig. 3, panel A), p104t (panel B), a cytosolic *T. parva* heat shock protein [15] (panel C), the transmembrane glycoprotein invariant chain, carrying a non-cleaved signal–anchor [16] (panel D), and the secretory protein lysozyme, carrying a cleaved signal sequence [17] (panel E) in a single experiment. The proteins were translated in vitro in the presence of RM (lane 1 in all panels). After translation an aliquot of each assay was pelleted through a sucrose cushion containing 0.5 M KCl. The resultant supernatants, containing non translocated proteins, were analysed in lanes 2. The membrane pellets were extracted with 0.1 M Na₂CO₃ pH 11.0 and pelleted through an alkaline sucrose cushion. The resultant supernatants, con-

taining luminal, completely translocated as well as peripheral proteins, and the pellets, containing proteins anchored via a membrane integrated peptide segment, were analysed in lanes 3 and 4, respectively.

Membrane-processed full-length p104 (g) was found in the carbonate extracted membrane pellet but not in the supernatants, suggesting that p104 is membrane integrated (panel A, lane 4). However, integration of p104 could occur via either of its two hydrophobic segments or by both of them. p104t exhibited a very similar distribution. The three glycosylated forms (g) were found in the carbonate extracted pellet exclusively, demonstrating that the N-terminus of p104 is membrane anchored (panel B, lane 4). Analysis of character-

ized control proteins gave the expected results, e. g. the cytosolic protein was found in the salt wash supernatant (panel C, lane 2), the glycosylated forms (g) of the transmembrane protein invariant chain were almost exclusively found in the carbonate extracted membrane pellet (panel D, lane 4) and the membrane processed form (p) of the secretory protein lysozyme was almost exclusively found in the carbonate extract (panel E, lane 3). Minute amounts of membrane processed transmembrane protein in the salt wash and carbonate extract supernatant fractions (panel D, lanes 2 and 3) are most likely due to carry over, and the presence of some processed secretory protein in the salt wash (panel E, lane 2) could be due to leakiness of microsomes. Since the controls

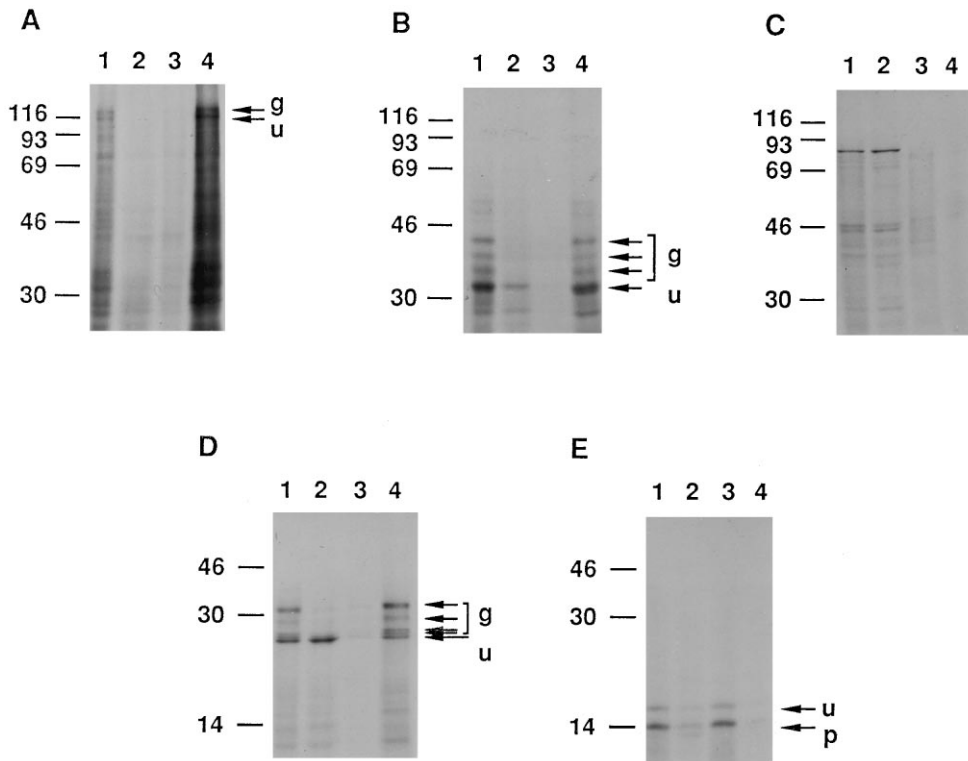


Fig. 3. p104 contains a N-terminal signal–anchor sequence. Full-length p104 (panel A), p104t (panel B), cytosolic heat shock protein 90 (panel C), transmembrane glycoprotein I γ carrying a signal–anchor sequence (panel D), and secretory protein lysozyme carrying a cleaved signal peptide (panel E) were translated *in vitro* in the presence of membranes (lane 1 in all panels). Aliquots of the assays were treated with high salt buffer, the membranes pelleted and extracted with carbonate buffer pH 11. Samples were analyzed as in Fig. 1. Lanes 2 represent proteins in the high salt wash, lanes 3 represent the soluble fraction after carbonate extraction, and lanes 4 the membrane pellet fraction. The mobilities of standard proteins are indicated as kDa values. u marks unprocessed; g, glycosylated; and p, proteolytically processed forms.

demonstrated that the carbonate extraction was working properly, we concluded that the N-terminus of p104 functions as a signal–anchor sequence.

Signal–anchors sequences compared to cleaved signal sequences have generally longer hydrophobic segments allowing stable membrane integration [18]. It is unclear whether the second hydrophobic segment of p104, consisting of 20 uncharged residues at the very C-terminus, functions as a second membrane anchor. Nonetheless p104 behaves as an integral membrane protein with virtually no cytoplasmic segment.

3.4. Translocation of p104 is dependent on SRP

Translocation across the ER membrane can occur either during or after translation. Co-translational translocation is characterized by recognition of the signal sequence on the nascent polypeptide chain by SRP, which mediates targeting of the nascent chain-ribosome-SRP complex to the ER membrane. In contrast post-translationally translocated polypeptides are transported to the ER independently of SRP. Post-translational translocation is exceptional and best studied in the lower eucaryote *Saccharomyces cerevisiae* where it appears to play a larger role [19,20].

To verify co- or post-translational translocation of p104 we tested whether p104 translocation was dependent on SRP (Fig. 4). p104 was translated in vitro without RM (lane 1), and with stripped RM in the absence (lane 2) or presence (lane 3) of SRP. With stripped RM only, p104 is not processed (lane 2). When stripped RM and SRP are added, p104 is processed (lane 3), demonstrating that the stripped membranes are still translocation competent. Thus, translocation of p104 was clearly dependent on SRP and we conclude that p104 is translocated co-translationally across the ER membrane.

3.5. Implications of our studies

We have shown here that in an in vitro expression system *T. parva* 104 kDa microneme–rhoptry protein is integrated into the ER membrane in

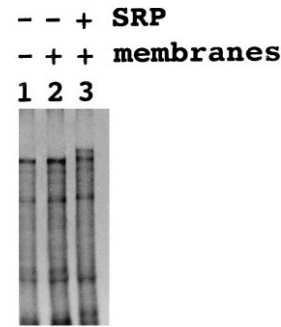


Fig. 4. Membrane processing of p104 is dependent on signal recognition particle (SRP). p104 was translated in vitro either in the absence or presence of membranes stripped from SRP, with or without SRP added as indicated. Samples were analyzed as in Fig. 1.

a SRP-dependent manner via an uncleaved N-terminal signal sequence. p104 has virtually no cytoplasmic portion and thus it represents a rather unusual type of membrane protein, structurally resembling Influenza virus neuraminidase [21]. According to our knowledge p104 is the first apical complex organelle protein described to have a signal–anchor sequence. For other proteins routed to rhoptries and micronemes cleavage of their N-terminal signal peptides for entry into the ER has been documented, e.g. for *Plasmodium falciparum* RAP-1 [22], *Eimeria tenella* Etmic-2 [23], and pSM/1.6 antigen of *Sarcocystis muris* [24].

Interestingly, p104 seems to share structural features with the circumsporozoite protein and the sporozoite surface protein 2 of *Plasmodium yoelii*. Both contain a hydrophobic stretch at the N-terminus postulated to be a signal peptide, and another one at the C-terminus, postulated to be a membrane anchor [25,26]. For both proteins the putative signal peptide has a sufficiently long hydrophobic core region to anchor the protein in the membrane and there is no bona fide cleavage site for signal peptidase [27]. Thus both proteins might have a signal–anchor like p104. Furthermore they have regions homologous to a segment of p104, consisting of residues 557–588. The circumsporozoite protein has one stretch and the sporozoite surface protein 2 has two stretches with 41–44% identity with the p104 region. Both

proteins are transported to the cell surface, most likely after passing through the ER. Immunoelectron microscopical analysis suggested that both proteins are partially located in micronemes in *P. yoelii* sporozoites [28].

Features of signal–anchor regions are determinants of the topology of integral membrane proteins. Hydrophobic protein segments shorter than 19 residues and a positive net charge on the N-terminal side favour translocation of the polypeptide segment C-terminal of the anchor with the N-terminal region staying in the cytoplasm [29,30]. The hydrophobic stretch of p104 consists of 17 residues (no. 3–19) and charged residues in the adjacent regions are lysine at position 2 and aspartic acid at positions 20 and 34 [4]. Thus the observed topology of p104 agrees with existing models for signal–anchor function in eucaryotic membrane protein biogenesis.

It has been suggested that p104 is further processed to several smaller antigens, sized 90, 85, and 35 kDa, in the sporozoite stage [4]. Most likely the 90 and 85 kDa antigens are derived by C-terminal truncation and both would still be membrane integrated by the N-terminal signal–anchor.

We have previously shown that in the *T. parva* schizont stage the secretory pathway operates and obtained strong evidence that a signal sequence for ER translocation is functional and processed by cleavage [5]. Taken together the general mechanisms of protein targeting to and integration into the ER membrane appear to be functional in *T. parva*. Based on our findings we hypothesize that in vivo p104 is co-translationally integrated into the membrane of the ER and further routed to the microneme–rhoptry complex. In line with this model are data from ultrastructural analysis of merogony in *T. parva*, which led to the proposal that rhoptries arise from smooth ER [31]. p104 could serve as a marker to follow up the biogenesis of rhoptries and micronemes in *T. parva*. The former are present in sporozoites and merozoites and the latter in sporozoites only [32,31]. Besides being the only known *T. parva* microneme/rhoptry protein, the membrane anchoring of p104 and its potential for C-terminal proteolytic processing let it appear attractive as a tool for analysis of intracellular transport pathways.

In the eucaryotic secretory pathway proteins can either be routed by sorting signals, i.e. structural features determining transport to or retention in a certain compartment, or in the absence of such features by default to the cell surface. It appears reasonable to assume that sorting signals for rhoptries and micronemes exist. None are known so far. For p104 its membrane topology argues against any sorting mechanism dependent on a cytoplasmic portion. However, it is conceivable that p104 might use a cytoplasm located sorting machinery indirectly by associating with another protein. For *T. parva* the analysis of signals for intracellular protein sorting using expression of mutant proteins is currently not possible. However, this methodology is available for *Plasmodium* sp. and *Toxoplasma gondii* [33,34]. If p104 was transported to apical complex compartments in these organisms, it would be possible to characterize sorting signals of p104 in those species.

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