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Functional domains of the *Toxoplasma* GRA2 protein in the formation of the membranous nanotubular network of the parasitophorous vacuole

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Abstract

Amphipathic α-helices have been proposed as the general means used by soluble proteins to induce membrane tubulation. Previous studies had shown that the GRA2 dense granule protein of *Toxoplasma gondii* would be a crucial protein for the formation of the intravacuolar membranous nanotubular network (MNN) and that one of the functions of the MNN is to organise the parasites within the parasitophorous vacuole. GRA2 is a small protein (185 amino acids), predicted to contain three amphipathic α-helices (α1: 70–92; α2: 95–110 and α3: 119–139) when using the standard programs of secondary structure prediction. To investigate the respective contribution of each α-helix in the GRA2 functions, we used Δ*GRA2-HXGPRT* knock-out complementation: eight truncated forms of GRA2 were expressed in the deleted recipient and the phenotypes of these mutants were analysed. This study showed that: (i) α3, when associated with the N-terminal region (NT) and the C-terminal region (CT), is sufficient to target the protein to the parasite posterior end and to induce formation of membranous vesicles within the vacuole. However, when associated only with CT, α3 is not sufficient to provide the hydrophobicity required for membrane association; (ii) the α1α2 region is alone not sufficient to induce membrane tubulation within the PV; and (iii) only one mutant, NT-α1α2α3, restores most of the biochemical and functional properties of GRA2, including traffic to the dense granules, secretion into the vacuole, association with vacuolar membranes, induction of the MNN formation and organisation of the parasites within the vacuole.

Keywords: *Toxoplasma gondii*; Protozoa; Parasitophorous vacuole; Tubular membranes; Membrane association; Amphipathic α-helix

1. Introduction

In eukaryotic cells, formation of tubular membranes is of general interest because it is the starting mechanism which drives formation of all the sub-cellular compartments involved in protein synthesis, modification, selection and trafficking. Despite an increasing number of studies investigating this mechanism, it is not yet fully understood. Specific composition in lipids or asymmetry in the lipid composition between membrane leaflets have been shown to be sufficient triggers to induce the initial membrane curvature responsible for the formation of

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membranous tubules (Matsuo et al., 2004; Sonnino et al., 2007). However, proteins also contribute to the formation of membranous tubules by applying pulling or bending forces on the membrane, thus reducing local tensions induced by lipid asymmetry (Zimmerberg and Kozlov, 2006). Amongst the proteins which can induce changes in membrane curvature and/or act on the membrane to change its properties, are the amphitropic proteins which contain amphipathic α-helices (Cornell and Taneva, 2006). Lee et al. (2005) thus hypothesized that insertion of amphipathic α-helix(ces) into a membrane bilayer could be the general mechanism used by cytosolic soluble proteins to initiate membrane curvature.

The intracellular apicomplexan parasite *Toxoplasma gondii* resides within its host cell, in a compartment named the parasitophorous vacuole (PV). The PV is formed during active parasite invasion from both the host cell plasma membrane and parasite secretory products (Håkansson et al., 2001; Charron and Sibley, 2002, 2004). This non-fusogenic compartment, devoid of most of the host cell proteins (Mordue et al., 1999), allows free diffusion of molecules up to 1300 Da (Schwab et al., 1994). One of the most intriguing features of the *Toxoplasma* PV is the presence of a network of membranous nanotubes, here referred as the membranous nanotubular network (MNN). It is assembled 10–20 min post-invasion at the posterior end of the parasite. The membranous nanotubes of 40–60 nm in diameter are decorated with intramembrane particles (Lemgruber et al., 2007), extend up to 1 μm in length within the vacuolar space, between the parasites, and connect with the PV membrane (Sibley et al., 1995; Magno et al., 2005). The function of the MNN is not yet clear; current hypotheses involve its participation in parasite intracellular development by increasing the surface of exchange between parasites and the host cell (Coppens et al., 2006; for a review, Mercier et al., 2005). Alternatively, a structural role in maintaining an ordered arrangement of daughter parasites during parasite division within the PV is proposed (Magno et al., 2005; R. Mondragon-Flores, personal communication).

Shortly after invasion, the parasite secretes numerous proteins from Apicomplexa-specific electron dense vesicles named the dense granules (reviewed by Mercier et al., 2005, 2007). Most of the dense granule proteins, including a subset named the GRA proteins (Sibley et al., 1991), do not share any obvious homology with proteins of known function; thus, their high level of expression and timing of release into the PV led to the hypothesis that they would play an important role in maturation of the newly formed PV (Dubremetz et al., 1993). Amongst the nine GRA proteins described so far, GRA2, GRA4, GRA6, GRA9 and to a lesser extent, GRA3, GRA5 and GRA7, become associated with the MNN following secretion into the PV (for reviews see Mercier et al., 2005, 2007). One protein, GRA2, is particularly important for the MNN formation. Upon publication of the *GRA2* sequence, GRA2 was predicted to contain a N-terminal signal peptide of 23 amino acids, two amphipathic α-helices, comprising 18 (region 69–87) and 17 (region 99–116) amino acids in frame with two hydrophilic domains of 46 and 69 amino acids in the N- and C-terminus, respectively (Mercier et al., 1993). Subsequent studies performed on an HA9 tagged form of GRA2 expressed in the wild-type parasite showed that both the aforementioned amphipathic α-helices are essential for stable association of the protein with the vacuolar network membranes (Mercier et al., 1998a). Furthermore, analysis of mutant parasites with their unique *GRA2* gene deleted showed that GRA2 organises the vacuolar components into elongated membranous tubules forming a network (Mercier et al., 2002; R. Mondragon-Flores, personal communication).

The increasing number of methods available to predict the secondary structure of proteins led us to again analyse the GRA2 protein sequence. The existence of a third amphipathic α-helix was predicted, placing α1 in the 70–92 amino acid region, α2 in the 95–110 region and α3 in the 119–139 region. This work was thus undertaken to investigate the contribution of each amphipathic α-helix in the MNN formation and in parasite organisation by carrying out targeted deletions within the GRA2 coding sequence. The Δ*GRA2* mutant was complemented with eight truncated forms of GRA2 and traffic, membrane association and function within the PV of mutant GRA2 proteins were explored.

2. Materials and methods

2.1. GRA2 predicted secondary structure

The GRA2 protein sequence (185 amino acids) was analysed using the secondary structure prediction methods available at the Expert Protein Analysis System proteomics server (ExPASy server) (http://kr.expasy.org/), i.e. jpred (http://www.compbio.dundee.ac.uk/~www-jpred/results/jp_HR6pDYj/jp_HR6pDYj.results.html), nnpredict (http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html), porter (http://distill.ucd.ie/porter/), predict protein (http://cubic.bioc.columbia.edu/predictprotein/submit _def.html), prof (http://www.aber.ac.uk/cgi-bin/user/~phiwww/get_prof_info), psa (http://bmerc-www.bu.edu/psa/request.htm), psipred (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html), sopma (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), GOR (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page=npsa_gor4.html), HNN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html), or using the Amphipaseek In Plane-Membrane Anchors prediction (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_amphipaseek.html) available at Pôle BioInformatique Lyonnais Gerland (http://pbil.ibcp.fr/htm/index.php). The amphipathic α-helices were represented using Protean from the DNASTAR software (Lasergene, Madison, WI) or Pepwheel available at EMBOSS (http://bioweb.pasteur.fr/seqanal/EMBOSS/).