

GRA Proteins of *Toxoplasma gondii*: Maintenance of Host-Parasite Interactions across the Parasitophorous Vacuolar Membrane

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Abstract: The dense granule of *Toxoplasma gondii* is a secretory vesicular organelle of which the proteins participate in the modification of the parasitophorous vacuole (PV) and PV membrane for the maintenance of intracellular parasitism in almost all nucleated host cells. In this review, the archives on the research of GRA proteins are reviewed on the foci of finding GRA proteins, characterizing molecular aspects, usefulness in diagnostic antigen, and vaccine trials in addition to some functions in host-parasite interactions.

Key words: *Toxoplasma gondii*, dense granule, GRA protein, PVM, host-parasite interaction

INTRODUCTION

Three secretory organelles are present in the cytoplasm of *Toxoplasma gondii*: microneme, rhoptry, and dense granule, which are known to function in the entry of the parasite and maintain intracellular host-parasite relationships, as unique to the parasites of the phylum Apicomplexa. The contents of the 3 secretory organelles have been released sequentially according to a cascade mode [1,2]. The micronemal proteins are released first, upon contact with host cells, and they are thought to function for the host cell recognition and attachment [3]. The contents of the rhoptries are released next, and they may function in the formation of the parasitophorous vacuole (PV) [4] with the aids of rhoptry neck proteins (RON) [5,6]. The dense granular proteins are exocytosed both during and after invasion into the PV. The exocytosed dense granular proteins either remain soluble in the lumen of the PV or they become associated with the PV membrane (PVM) or the tubulovesicular network (TVN) of membranous structure within the PV [7]. The dense granule proteins are thought to modify the environment within the PV, thereby functioning for intracellular survival and replication.

In the dense granule, there are 12 GRA proteins (GRA1-GRA14, but GRA11 and 13 are phantom-like) that have been previously identified in *T. gondii* tachyzoites [8-12] without sequence

homology to each other in addition to 2 isoforms of nucleotide triphosphate hydrolase (NTPase I and II) [13] and 2 protease inhibitors (TgPI 1 and 2) [14,15]. All the GRA proteins are identified as excretory/secretory antigen (ESP). They all contain signal sequences of 25-30 amino acids, except for GRA3 and GRA6, and this would target to the secretory pathway of the parasite. There are 22 and 23 amino acids in GRA3 and GRA6, respectively, upstream of the hydrophobic sequence, and these would function as stop-transfer sequences [16] rather than as long signal sequences including these sequences. Many GRA proteins also contain putative transmembrane domains (GRA3, GRA4, GRA5, GRA6, GRA7, GRA8, GRA10, GRA12, and GRA14). GRA7 and GRA10 have 2 putative transmembrane domains in between the fibronectin attachment motif RGD, especially. GRA1, GRA2, and GRA9 lack the transmembrane domain. GRA3 has been known to lack a transmembrane domain, but it associates with the PVM in an oligomeric form in GenBank U13771 [17]. The amino acid sequence deduced on the revised GRA3 (GRA3r) of GenBank AF414079 [18] has 2 potential transmembrane domains near the C-terminal.

Secreting into the PV, only GRA1, TgPIs, and NTPases remain primarily in the lumen of the vacuole [15,19]. Most GRA proteins are detected in close association with the membranous system of this compartment. Hence, a fraction of the GRA1, GRA3, GRA7, and NTPases pools, as well as GRA2, GRA4, GRA6, GRA9, GRA12, and GRA14 are detected more specifically associated with the vacuolar network membranes. In these membranes, GRA2, GRA4 and GRA6 participate to the formation of

• Received 8 September 2009, revised 28 September 2009, accepted 28 September 2009.

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a multimeric protein complex [20]. In contrast, GRA3, GRA5, GRA7, GRA8 and GRA10 are preferentially detected as PVM-associated proteins [10].

GRA1

GRA1 has been identified in tachyzoites as a polypeptide of 24 kDa that is an excreted-secreted antigen (ESA) and is cross-reactive with bradyzoites [21]. It is located in the dense granules of both tachyzoite and bradyzoite forms and showed that it is secreted within PV. Moreover $^{45}\text{Ca}^{2+}$ labeling as well as regional homologies indicate that this protein has Ca^{2+} -binding properties, suggesting its physiological importance in host cell invasion. Following host cell invasion, GRA1 was secreted into the lumen of the PV as a soluble protein that subsequently became peripherally associated with the membranous tubular network [19]. GRA1 was used as a marker of dense granule for the sequential secretion of 3 secretory organelles of *T. gondii* [22].

For the determination of B-cell epitope in GRA1, a library of cDNA fragments from *T. gondii* tachyzoites was displayed as fusion proteins to the amino-terminus of lambda bacteriophage capsid protein D [23], which revealed the existence of an immunodominant epitope (epi-24 peptide). The GRA1 DNA vaccine elicited CD8⁺ T-cells that were shown to have cytolytic activity against parasite-infected target cells and a GRA1-transfected cell line [24]. C3H mice immunized with the GRA1 DNA vaccine showed 75-100% protection, while 0-25% of mice immunized with the empty control vector survived challenge with *T. gondii* cysts.

Yeast two hybrid analysis with GRA1 as bait to the prey of HeLa cDNA library [25] results in the interaction with gene products of Mof4 family associated protein 1, coenzyme A synthase, laminin β 3, ribosomal protein L10a, NAD(P)H dehydrogenase, quinone 2, cofactor required for Sp1 transcriptional activation, subunit 2, and leukotriene B4 receptor 2.

GRA2

GRA2 was first found as a dense material trapped between parasite and vacuole membranes before either the vacuolar network or the vacuole membrane in immunofluorescence assay and immunoelectron microscopy at different stages after infection [26]. A monoclonal antibody (TG17.179) recognizes an ESA of 28.5 kDa named GRA 2, which is stored in the dense granules and secreted into the PV after host cell invasion. Screening

of an expression cDNA library with the mAb led to the isolation of the longest one being 1,030 bp [27], which consists of an 185 amino acid polypeptide (19.8 kDa) including a 23 amino acid signal sequence. The presence of many serine and threonine residues may indicate an O-glycosylation [28]. The predicted mature polypeptide shows an internal helical domain with 2 amphipathic α -helices that might be involved in the association of GRA2 with the membranous network within the PV. Following host cell invasion, GRA2 was secreted within multi-lamellar vesicles released from a specialized posterior invagination of the parasite [19]. The multi-lamellar vesicles assemble to form the intravacuolar network, which contains an integral membrane form of GRA2. The molecular basis of targeting to a network of membranous tubules that connect with the vacuolar membrane is dependent on the 2 amphipathic α -helices [29]. Cross-linking studies established that GRA4 and GRA6 specifically interact with GRA2 to form a multimeric complex that is stably associated with the intravacuolar network [20], which is based on both protein-protein and hydrophobic interactions, may participate in nutrient or protein transport within the vacuole.

T-cell blot analysis using SDS-PAGE-fractionated parasite extracts identifies the parasite Ag (s) involved in the maintenance of T-cell mediated long term immunity, 6 of 25 clones recognized *T. gondii* fractions in the 24- to 28-kDa range and proliferated to purified GRA2, 5 of 25 clones [30]. CD4⁺ T-cells specific for GRA2 may be involved in the maintenance of long term immunity to *T. gondii* in healthy chronically infected individuals. Passive immunization of mice with GRA2 mAb following challenge with a lethal dose of tachyzoites significantly increased survival compared with results for mice treated with control ascites [31].

GRA3

GRA3 is a 30 kDa protein located inside the dense granules of *T. gondii*, which is exocytosed after invasion into the PV to become associated with the PVM and extensions of the PVM that protrude into the cytoplasm [17]. PVM insertion of GRA3 is the first observed phenomena in *T. gondii* or related parasites of a protein which inserts into the vacuole membrane for some purpose other than to lyse that membrane. A partial cDNA encoding GRA3 was isolated from a *T. gondii* expression library using polyclonal and monoclonal antibodies to the mature GRA3 protein of tachyzoites. The cDNA of GRA3 encodes 2 methionines at the N-terminus followed by an open reading frame with a

hydrophobic region of 22 amino acids flanked by charged residues consistent with a signal sequence [32]. The endogenous dense granule marker GRA3 is secreted constitutively in a calcium-independent fashion using *T. gondii* NSF/SNAP/SNARE/Rab machinery that can interact functionally with their mammalian homologues [33]. Recently, previously published sequence for GRA3 is actually an artificial chimera of 2 proteins of molecular weight 65 kDa, shares the C-terminus with published GRA3 and possesses no significant sequence similarity with any protein thus far deposited in Genbank [34]. The corrected GRA3 has an N-terminal secretory signal sequence and a transmembrane domain consistent with its insertion into the PVM. GRA3 possesses a dilysine 'KKXX' endoplasmic reticulum (ER) retrieval motif that rationalizes its association with PVM and possibly the host cell ER.

Immunodominant regions encoded by GRA3 (and MIC3) genes on the human B-cell response against *T. gondii* infection is identified in a panel of recombinant phage clones carrying B-cell epitopes [35].

Yeast two hybrid analysis with GRA3 as bait to the prey of HeLa cDNA library [25] results in the interaction of host gene products of calcium modulating ligand (CAMLG), paired box gene 6, ribosomal protein S18, and FUN14 domain containing 1. Of these, CAMLG is an integral membrane protein which appears to be a new participant in the calcium signal transduction pathway [36], which functions similarly to cyclosporin A as binding to cyclophilin B and acting downstream of the TCR and upstream of calcineurin by causing an influx of calcium [37]. Modulation of intracellular calcium concentration with GRA3-CAMLG interaction leads to the inhibition of host cell apoptosis [38] for the longterm residence of invading intracellular parasites. In addition to this cellular physiological function, binding of GRA3 (and GRA5 and GRA6) to CAMLG in the intracellular integral membrane of ER is suggested to be the ligand of ER anchorage to PVM. Actually, fluorescences from GRA3 and CALMG are added in PVM [39] to be suggested a receptor-ligand function of the 2 proteins of which the binding mode of N-terminal hydrophobic interaction and insertion of ER-retrieval motif into ER membrane.

GRA4

GRA4 has been identified by the screening of clones from a *T. gondii* expression library with the immune serum from a *T. gondii*-infected rabbit and further screening using milk and in-

testinal secretions from mice orally infected with *T. gondii* cysts [40]. The deduced amino acid sequence contains a putative N-terminal signal sequence of 20 amino acids but no apparent glycolipid anchor sequence and a proline rich (12%) product with an internal hydrophobic region of 19 amino acids and a potential site of N-glycosylation. GRA4 was distributed throughout the lumen of the PV and only later became associated with the mature network (PVN) that is found dispersed throughout the vacuole [20]. The association of GRA4 with the network membranes is mediated by strong protein-protein interactions with GRA6 that has been predominantly influenced by hydrophobic interactions, and a phosphorylated form of this protein present within the vacuole showed increased association with the network membranes. Cross-linked GRA4 and GRA6 specifically interact with GRA2 to form a multimeric complex that is stably associated with the intravacuolar network, which may participate in nutrient or protein transport within the vacuole.

The 40 kDa GRA4 reacts strongly with milk IgA, weakly with intestinal IgA, and also with mucosal IgA. GRA4 stimulates primed mucosal T-lymphocytes from CBA/J and BALB/c mice whereas no proliferation with C57BL/6 T cells [41]. Peptide of 229-242 amino acids from GRA4 only induces detectable proliferation of primed-CBA/J T lymphocytes. This is further confirmed by T-cell blot analysis of 2-dimensionally separated *T. gondii* lysate [42]. GRA4 elicits both mucosal and systemic immune responses following oral infection of mice with cysts [43]. Protein C (amino acids 297-345) is particularly well recognized by serum IgG antibodies, milk IgA antibodies and intestinal IgA antibodies from *T. gondii* infected mice, by serum IgG antibodies from infected humans and sheep. A major B epitope is localized within the last 11 C-terminal residues of GRA4. A second epitope, recognized with lower frequency, is mapped within the region 318-334.

GRA4 has been attracting many researchers to find candidate for vaccine against this parasite. Whole coding sequence of GRA4 has been tried as a DNA vaccine, which results in a 62% survival of susceptible C57BL/6 infected mice [44]. Vaccine efficacy of recombinant GRA4 (rGRA4) and ROP2 (rROP2) proteins and a mix of both combined with alum is evaluated in C57BL/6 and C3H mice [45]. Challenge of rGRA4- or rGRA4-rROP2-vaccinated mice from both strains with ME49 cysts resulted in fewer brain cysts than the controls, whereas vaccination with rROP2 alone only conferred protection to C3H mice. These results suggest that GRA4 can be a good candidate for a multiantigen anti-*T. gondii* vaccine based on the use of alum as an adjuvant. A mul-

tiantigenic vaccine using SAG1 and GRA4 selected on the basis of previous immunological and immunization studies protects well against the infection in mouse [46]. Mortality of susceptible C57BL/6 mice reduced upon oral challenge with cysts of the 76K type II strain by 62% survival and the protection was further increased by co-inoculation with a plasmid encoding the GM-CSF by 87% survival. This DNA cocktail provided significant protection of less susceptible outbred Swiss OF1 mice against the development of cerebral cysts. These are due to the development of a specific humoral and cellular Th1 response to native *T. gondii* SAG1 and GRA4 antigens.

Yeast two hybrid analysis with GRA4 as bait to the prey of HeLa cDNA library [25] results in the interaction of gene products of cofilin 1 (CFL1), pyruvate kinase, tRNA splicing endonuclease 34 homolog, translocase of inner mitochondrial membrane 50 homolog (TIMM50), tumor susceptibility gene 101, nicotinate phosphoribosyltransferase domain containing 1, presenilin enhancer 2 homolog, WD repeat domain 68, RNA binding motif protein 9, thymidine kinase 1, MHC class I antigen (HLA-A*0201 allele, AY365426), cortactin (BC033889), and translocase of inner mitochondrial membrane 13 homolog (TIMM13). Among these, cofilin is a widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin in a pH-dependent manner [47,48]. It is involved in the translocation of actin-cofilin complex from cytoplasm to nucleus [49]. GRA4-cofilin interaction may exert to maintain intravacuolar network. After induction of apoptosis, cofilin is translocated to mitochondria before release of cytochrome c. Reduction of cofilin with siRNA resulted in inhibition of both cytochrome c release and apoptosis [50]. Of course GRA4 localizes within the PV, its intervening action with cytoplasmic cofilin to TIMM50 [51] and TIMM13 [52,53] from approaching mitochondria to PVM relates with anti-apoptotic function of these complex.

GRA5

GRA5, the P21 antigen of *T. gondii*, has been described as a dense granule component, secreted in the PV during host cell invasion [54]. The gene encoding GRA 5 is 834 bp long and does not contain any intron and the deduced amino acid sequence of an open reading frame encodes 133 amino acids of which a N-terminal hydrophobic region possesses the characteristics of a signal peptide of 25 amino acids and a second hydrophobic domain bordered by 2 hydrophilic regions strongly suggests a

transmembrane region. This molecular structure is supported by ultrastructural studies showing the association of the P21 antigen with the PVM. GRA5 is present both in a soluble form and in hydrophobic aggregates [55] in the targeted PVM. GRA5 is secreted as a soluble form into the PV after which becomes stably associated with the PVM as a transmembrane protein with its N-terminal domain extending into the cytoplasm and its C-terminus in the vacuole lumen.

Yeast two hybrid analysis with GRA5 as bait to the prey of HeLa cDNA library [25] results in the interaction of gene products of calcium modulating ligand (CALMG, NM_001745) and small glutamine-rich tetratricopeptide repeat (TRP)-containing alpha (SGTA, NM_003021). GRA5 binds to CALMG to modulate intracellular calcium concentration as GRA3 and GRA6, which leads to inhibition of apoptosis [38] for the long-term residence of the intracellular parasites. Structurally GRA5 binds to CALMG in the intracellular integral membrane of ER with coordination of GRA3 [39], which suggests to be the ligand of ER anchorage to PVM. And GRA5 also binds to SGTA, which is expressed ubiquitously as a housekeeping function interacting with 70 kDa heat shock protein and HSP90b [56,57].

GRA6

GRA6 has been characterized as a 32 kDa protein which localized in the dense granules of tachyzoites and in the PV closely associated to the network [58]. A cDNA of 1,600 bp encoding GRA6 potentially encodes a 230-amino-acid polypeptide, of which the deduced polypeptide contains 2 hydrophobic regions with the characteristics of transmembrane domains. The N-terminal domain does not fit the classical feature of a signal peptide. The central hydrophobic domain is flanked by 2 hydrophilic domains which contain 4 blocks of amino acids homologous to the GRA5 protein. The C-terminal hydrophilic region comprises 24% of glycine residues, which may indicate a structural role for GRA6 in the network. Following release into the PV, GRA6 was rapidly translocated to the posterior end of the parasite where, like previously reported for GRA2, it bound to a cluster of multi-lamellar vesicles that give rise to the network [20]. GRA6 gene is utilized as typing markers of sequence polymorphisms in the dense granule antigen [59]. Sequence alignment identified nucleotide polymorphisms at 24 positions out of 690 bp, which correlated with murine-virulence. Types I, II, and III could be distinguished from each other on the basis of 3, 10, and 6 variable positions, respectively. Two deletions of 15 bp

and 3 bp existed in the avirulent (type II) strains. With an exception, all polymorphic positions resulted in amino acid substitutions, and the 2 gaps of 15 bp and 3 bp caused the deletion of 6 amino acids in type II strains. Intra-specific polymorphisms were also found in the virulent group. The large variety of amino acid changes supports the view that the GRA6 protein plays an important role in the antigenicity and pathogenicity of *T. gondii*. And GRA6 stabilises tubular network with the aids of GRA2 after invasion [7]. The induction of nanotubules by the parasite protein GRA2 may be a conserved feature of amphipathic α -helical regions, which have also been implicated in the organization of Golgi nanotubules and endocytic vesicles in mammalian cells.

Yeast two hybrid analysis with GRA6 as bait to the prey of HeLa cDNA library [25] results in the interaction of gene products of calcium modulating ligand (CAMLG), spectrin repeat containing nuclear envelope 2, ATP synthase, H⁺ transporting, mitochondrial F1 complex, α subunit 1 (ATP5A1), and proteasome subunit α type 4 (PSMA4). GRA6 binds to CAMLG also to modulate intracellular calcium concentration as GRA3 and GRA5. GRA6 is secreted to PV to coordinate structurally to bind to CAMLG in the intracellular integral membrane of ER with GRA3 and GRA5 in PVM, which is suggested to be the ligand of ER anchorage to PVM. ATP5A1 is a subunit of mitochondrial ATP synthase which catalyzes ATP synthesis [60]. GRA6 binds to proteasome subunit, α type, 4 (PSMA4). Proteasomes, multicatalytic proteinase complexes, are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway [61,62]. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides.

GRA7

GRA7 was found by immunoscreening of an expression library constructed with *T. gondii* tachyzoite mRNA with sera from toxoplasmosis-positive humans [63], which contains a putative signal sequence and 2 hydrophobic regions in the C-terminal, the last of which has the characteristics of a membrane-spanning domain. After host cell invasion, the protein is secreted into the vacuolar network, the PVM, and into extensions protruding in the cytoplasm. A single mRNA transcript of 1.3 kb was detected by Northern blot [64], and the deduced 236 amino acid protein contains a putative N-terminal signal peptide, 1 site of potential N-linked glycosylation, and, close to the C-termi-

nus, a further hydrophobic, putative transmembrane domain. The p29 accumulates within the PV and PVM in tachyzoite infected cells whereas in bradyzoite-infected cells, p29 is present within the host cell cytoplasm [65]. Properties of GRA7 that are pertinent to its membrane targeting and to GRA7-directed immune resistance were studied in detail [66] that GRA7 is exclusively membrane-associated in both parasites and infected host cells with the hydrophobic stretch from amino acid 181-202 providing a possible membrane anchor.

Yeast two hybrid analysis with GRA7 as bait to the prey of HeLa cDNA library [25] results in genes of poly (rC) binding protein 1 (PCBP1) and thymosin beta 10 (TMSB10). PCBP1 along with PCBP2 and hnRNPK corresponds to the major cellular poly (rC)-binding proteins [67]. It contains 3 K-homologous (KH) domains which may be involved in RNA binding [68]. PCBP1 is also suggested to play a part in formation of a sequence-specific α -globin mRNP complex which is associated with α -globin mRNA stability [69].

GRA8, GRA9, GRA10, GRA12, and GRA14

GRA8 was found to be a proline-rich (24%) 38 kDa protein which is released into PV during or shortly after invasion and associates with the periphery of the vacuole [70]. The deduced amino acid sequence of GRA8 consists of a polypeptide of 267 amino acids, with an amino terminal signal peptide, 3 degenerate proline-rich repeats in the central region and a potential transmembrane domain near the carboxy terminus. GRA9 was found as a 41 kDa protein of 318 amino acids [71], which associates with the network of tubular membranes connected to the PVM. Like the other GRA proteins, GRA9 is secreted into the vacuole from the anterior end of the parasite.

GRA10 was found as a 36 kDa major proteins in the excretory/secretory proteins from *T. gondii* before the parasite's entry into host cells, and they are released into the PV during or shortly after invasion to be associated with the PVM [10]. The cDNA sequence encoding 364 amino acids of which the deduced amino acid sequence consists of a polypeptide of amino terminal signal sequence and 2 potential transmembrane domains in the middle of sequence not near the carboxy terminus. GRA10 has a RGD motif between the 2 potential transmembrane domains.

GRA12 is secreted into the PV from the anterior pole of the parasite soon after the beginning of invasion, transits to the posterior invaginated pocket of the parasite where a membranous tubulovesicular network is first assembled, and finally resides

throughout the vacuolar space, associated with the mature membranous nanotubular network similarly to both GRA2 and GRA6 [12]. And GRA14 is targeted to membranous structures within the vacuole known as the intravacuolar network and to the vacuolar membrane surrounding the parasite [11]. It has an unexpected topology in the PVM with its C terminus facing the host cytoplasm and its N terminus facing the vacuolar lumen.

Yeast two hybrid analysis with GRA8 as bait to the prey of HeLa cDNA library [25] results in genes of thymidine kinase 1, RNA binding motif protein 9, nucleotide binding protein 2, hydroxyacyl-coenzyme A dehydrogenase type II, actinin alpha 1, ATP synthase, H⁺ transporting mitochondrial F0 complex subunit C1, ribosomal protein SA, phosphoglycerate dehydrogenase, ribosomal protein L10, nitrilase family member 2, cadherin-like 24, eukaryotic translation initiation factor 3 subunit 2 β , pyruvate kinase, and cytochrome b5 reductase 3. Yeast two hybrid analysis with GRA9 as bait to the prey of HeLa cDNA library results in genes of filamin B β (actin binding protein 278), metallothionein 2A, and processing of precursor 7 ribonuclease P subunit. GRA10 secreted into PVM interacts with 7 genes of HLA-B associated transcript 8, signal transducer and activator of transcription 6 (STAT6), HSPC009 protein, TATA box binding protein (TBP)-associated factor (TAF1B), solute carrier family 10 (sodium/bile acid cotransporter family) member 3, RNA binding protein 1 (RANBP1), and NADH dehydrogenase subunit 1 of HeLa cells. Among these, STAT6 is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein plays a central role in exerting IL-4 mediated biological responses. It is found to induce the expression of BCL2L1/BCL-X(L), which is responsible for the anti-apoptotic activity of IL-4 [72]. It functions in the differentiation of T helper 2 (Th2) cells, expression of cell surface markers, and class switch of immunoglobulins [73]. GRA10 binds to TAF1B. Initiation of transcription by RNA polymerase I requires the formation of a complex composed of the TATA-binding protein (TBP) and 3 TBP-associated factors (TAFs) specific for RNA polymerase I. This complex, known as SL1, binds to the core promoter of ribosomal RNA genes to position the polymerase properly and acts as a channel for regulatory signals [74, 75]. And Ran/TC4-binding protein, RanBP1, interacts specifically with GTP-charged RAN. RANBP1 binds to RAN complexed with GTP but not GDP [76]. RANBP1 does not activate GTPase

activity of RAN but does markedly increase GTP hydrolysis by the RanGTPase-activating protein (RanGAP1) [77]. RANBP1 acts as a negative regulator of RCC1 by inhibiting RCC1-stimulated guanine nucleotide release from RAN [78].

FUTURE PERSPECTIVES

Despite many advances in the research of GRA proteins of *T. gondii* and the suggestion of their major role in the intracellular parasitism of the parasite across the PVM, many points of view still remain in question. What is the specific signal that targets the GRA proteins to the dense granules and that triggers secretion into the PV? What is the underlying mechanism that which GRA protein secretes into PV to organize intravacuolar network or PVM to face the host cellular components? Most importantly, what is the exact role of each GRA protein in the intracellular parasitism? With more updated molecular biological tools such as transfection skills, yeast two hybrid technique and information obtained by microarray of host cells before and after infection will help us to decipher the role of GRA proteins in the parasitism.

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