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A HT/PEXEL motif in *Toxoplasma* dense granule proteins is a signal for protein cleavage but not export into the host cell

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Abstract

Apicomplexan parasites, such as *Toxoplasma gondii* and *Plasmodium*, secrete proteins for attachment, invasion and modulation of their host cells. The host targeting (HT), also known as the *Plasmodium* export element (PEXEL), directs *Plasmodium* proteins into erythrocytes to remodel the host cell and establish infection. Bioinformatic analysis of *Toxoplasma* revealed a HT/PEXEL-like motif at the N-terminal of several hypothetical unknown and dense granule proteins. Hemagglutinin (HA)-tagged versions of these uncharacterized proteins show co-localization with dense granule proteins found on the parasitophorous vacuole membrane (PVM). In contrast to *Plasmodium,* these *Toxoplasma* HT/PEXEL containing proteins are not exported into the host cell. Site directed mutagenesis of the *Toxoplasma* HT/PEXEL motif, RxLxD/E, shows that the arginine and leucine residues are permissible for protein cleavage. Mutations within the HT/PEXEL motif that prevent protein cleavage still allow for targeting to the PV but the proteins have a reduced association with the PVM. Addition of a Myc tag before and after the cleavage site shows that processed HT/PEXEL protein has increased PVM association. These findings suggest that while *Toxoplasma* and *Plasmodium* share similar HT/PEXEL motifs, *Toxoplasma* HT/PEXEL containing proteins interact with but do not cross the PVM.

Keywords

Toxoplasma gondii; dense granule proteins; HT/PEXEL motif; protein processing; trafficking; secretion

Introduction

Plasmodium spp. and *Toxoplasma gondii* are obligate intracellular parasites of the phylum Apicomplexa that survive in a specialized membranous organelle known as the parasitophorous vacuole (PV). To invade and establish the PV, *T. gondii* sequentially discharges proteins from three secretory organelles – the micronemes, rhoptries (ROP) and dense granules (1, 2). The micronemes contain several adhesion proteins that allow the attachment of parasites to host cells (3). The rhoptries possess a series of virulence factors that differ between the three clonal linages of *T. gondii* (4). Rhoptry proteins are also known to interact with microneme proteins to form a “moving junction” that migrates down the parasite (5, 6). The dense granules secrete their contents (GRA proteins) to distinct

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subcompartments of the PV where they contribute significantly to the biogenesis and modification of this compartment at the interface with the host cell (7, 8). GRA proteins are important in maintaining the structures of the PV and potential nutrient acquisition (9). GRA15 is sufficient for host nuclear factor kappa B activation (10) and GRA1, GRA4 and GRA7 are effective antigens used in DNA vaccines (11, 12). These findings demonstrate that secretory proteins play a significant role in host cell modulation and immune responses.

Several targeting motifs to *T. gondii* secretory organelles have been characterized. The pro-domains of microneme protein SUB1 and rhoptry protein ROP4 are sufficient to target a reporter protein to the micronemes or rhoptries, respectively (13-15). Additional signals for microneme targeting include two tyrosine-based motifs, SYHYY and EIEYE, at the C-terminus of MIC2 (16). Targeting of the ROP2 family relies on both YXXφ and LL motifs within their cytoplasmic domains (13, 17). For dense granule proteins, it was thought that the signal peptide is necessary and sufficient for localization to dense granules (18); however, transmembrane-bearing GRA5 does not depend on the signal peptide traffic to the dense granule (7). GRA5 is secreted as a soluble protein into the PV, and becomes stably associated with the PVM (19). The N-terminal ectodomain of GRA5 and GRA6 mediate their dense granule targeting (7, 20), suggesting that a sorting element is found within N-termini of certain GRA proteins.

P. falciparum contains a sorting signal sequence (RxLxE/Q/D) at the N-termini of proteins exported into the host cell (21, 22). This sorting signal, called the host targeting (HT) or *Plasmodium* export element (PEXEL) motif, facilitates export of proteins to the host cytosol, where they remodel the host cytoskeleton to promote parasite survival (21, 23, 24). A similar RxLR-dEER motif was also discovered in secreted proteins of the Irish potato famine pathogen *Phytophthora infestans* (25). Analyzing the *T. gondii* genome with a clustering algorithm, we discovered three distinct polymorphic families that contain a HT/PEXEL-like motif near their N-terminus. One of these families is the previously characterized nucleoside triphosphate hydrolase (NTPase) family of GRA proteins (26). The other two families contain uncharacterized proteins annotated as hypothetical unknown. Our studies show that these hypothetical unknowns are new GRA proteins that localize to the PVM and PV. The *T. gondii* HT/PEXEL motif serves as a cleavage signal and may contributes to protein-protein associations. *T. gondii* HT/PEXEL containing proteins are not directed into the host cytosol, which indicates a different trafficking mechanism between *Toxoplasma* and *Plasmodium*.

Results

Discovery of a HT/PEXEL-like motif in new secretory protein families

The *T. gondii* genome was analyzed to identify protein families that: (i) contain an ER-type signal sequence, (ii) are highly polymorphic as an indication that they have evolved in response to antigenic pressure or functional diversification, and (iii) contain a HT/PEXEL motif because this sequence is important for host targeting in the malaria parasite (reviewed in (24)). Here we characterize three protein families from this search (Table 1). One of the protein families (Family 1, Table 1) has been reported as NTPases, which are GRA proteins. There is no evidence that *T. gondii* NTPases are exported into the host (26).

The other two protein families (Families 2 and 3 in Table 1) were uncharacterized and potentially secreted from the parasite into the host. From the bioinformatics search, both new families contain three members each; TGME49_087740, TGME49_000010 and TGME49_045430 for Family 2 and TGME49_041190, TGME49_041300, and TGME49_041610 for Family 3. Further genome analysis showed that Family 3 contains a

fourth member, TGME49_041305, which was not found in the bioinformatics search because the annotation is missing the first exon and thus the signal peptide (Figure S1).

HT/PEXEL motif does not export proteins to the host cell in cysts

To examine the localization of these new HT/PEXEL-containing proteins in *T. gondii*, C-terminal hemagglutinin (HA)-tagged versions of selected genes (TGME49_087740, TGME49_000010, and TGME49_041610) were generated to be expressed from the α-tubulin promoter. Cells infected with parasites expressing each HA-tagged protein were stained with anti-HA and anti-surface antigen 1 (SAG1) antibodies. While TGME49_087740 and TGME49_000010 were primarily localized on the PVM and TGME49_041610 was localized to the PV space as well as the PVM (Figure 1), none of the anti-HA staining appeared within the host cell. To increase the signal from the HA-tagged proteins, we examined host cells infected with multiple vacuoles and grown for extended time, but both of these conditions also did not show HA-tagged protein within the host cell (Figure S2). These data show that HT/PEXEL-containing *T. gondii* proteins are secreted and targeted to the PV and PVM, but not the host cytosol.

Within warm-blooded animals, *T. gondii* disseminates as a rapidly replicating form called a tachyzoite and establishes a chronic life-long infection as an encysted form called a bradyzoite. To examine if the *T. gondii* HT/PEXEL motif was mediating GRA protein translocation into the host cell during chronic infection, parasites expressing HA versions of TGME49_087740 and TGME49_000010 were induced to switch into bradyzoites in tissue culture and the localization was examined by immunofluorescence assay (IFA). Neither protein co-localized with either Dolichos biflorus agglutinin (DBA) a lectin that binds the bradyzoite cyst wall, or bradyzoite antigen 1 (BAG1), the bradyzoite-specific heat shock protein located within the parasite cytoplasm (Figure 2). These results suggest that HT/PEXEL-containing *T. gondii* proteins are secreted and targeted to the PV, but not translocated to host cytosol in both the tachyzoite and bradyzoite stages.

HT/PEXEL-like motif proteins co-localize with GRA proteins

Because the localization of the new HT/PEXEL-containing proteins was reminiscent of dense granule proteins, we co-stained TGME49_087740, TGME49_000010 and TGME49_041610 with GRA7. In extracellular parasites, TGME49_087740, TGME49_000010 and TGME49_041610 largely co-localized with GRA7 (Figure 3). In addition, within intracellular parasites, HA staining of TGME49_087740 and TGME49_000010 overlapped completely with GRA7 staining on the PVM. Due to their dense granule locations, we gave these HT/PEXEL containing proteins numbers in the GRA series. For Family 2, TGME49_087740 was named GRA19 and TGME49_000010 was named GRA20 (Table 1). GRA19 is only found in type II strains and type I strains lack GRA20. For family 3, TGME49_041610 was named GRA21.

GRA19, GRA20 and GRA21 are processed

To begin to analyze the processing of these new dense granule proteins, we examined the size of C-terminal HA-tagged versions of GRA19, GRA20 and GRA21 by immunoblot. All three proteins showed a higher molecular weight band close to the size predicted by the ToxoDB annotation. The higher molecular weight band sometimes appeared as a doublet, likely due to incomplete processing of the ER-type signal sequence. Each of the three proteins also showed a less abundant reactive band approximately 10 kDa smaller (Figure 4A). Analysis of tachyzoite cDNA showed that the open reading frame (ORF) of GRA19 matched the TGME49_087740 annotation and no alternatively spliced forms were present (data not shown). Transient transfection of GRA19HA and GRA20HA expression constructs

in the RH type I strain showed that two bands could also be detected in type I strains and they migrated at the same molecular weights as the bands from type II strains (Figure 4B).

46 kDa isoform of GRA19 is not due to phosphorylation

PVM-localized GRA7 proteins are phosphorylated in infected host cells, which results in two forms of GRA7 detected on immunoblots (27). To determine if the two forms of GRA19 resulted from protein phosphorylation, parasite lysates were treated with or without alkaline phosphatase prior to immunoblot analysis. In type II strains, multiple phosphorylated forms of GRA7 can be seen (Figure 4C) as opposed to the single isoform seen in type I strains (27). While the increased mobility of GRA7 was due to phosphorylation, the mobility of 46 kDa isoform of GRA19 was not affected by alkaline phosphatase treatment (Figure 4C). This result led us to hypothesize that the lower molecular weight band was a processed form of GRA19.

The HT/PEXEL motif is a signal for cleavage of GRA19

After removal of the signal peptide, microneme and rhoptry proteins undergo additional proteolytic processing to remove N-terminal pro-peptides. While this N-terminal processing has not yet been described for GRA proteins, we hypothesized that GRA19 is proteolytically processed after removal of the signal peptide because both GRA19 isoforms were seen with a C-terminal HA-tag. In addition, both intracellular and extracellular parasites expressed two forms of GRA19, suggesting that the processing occurs within the parasite and that host cells are not required for the protein processing (Figure S3). In *Plasmodium*, the HT/PEXEL motif is recognized by an aspartic protease plasmepsin V and cleaved in the ER (28, 29). To determine if the *T. gondii* HT/PEXEL motif was also a signal for protein processing, we generated single alanine substitutions in the GRA19 PEXEL motif at R (position 1), L (position 3), and D (position 5). Similar to the results seen for point mutations in the *Plasmodium* HT/PEXEL motif (30), *T. gondii* expressing GRA19 with R to A or L to A substitutions express only the large 46 kDa form of GRA19, but the D to A substitution in the 5th position did not affect cleavage (Figure 4D). These results suggest that R and L, but not D are part of the recognition sites for protein cleavage.

To further characterize the cleavage event, amino acids 1-99 of GRA19, including wild type or RA mutant HT/PEXEL motif were subcloned onto the N-terminus of GFP to create a GRA19 ^{NT}-GFP or GRA19RA ^{NT}-GFP chimera. GFP fused with the wild type HT/PEXEL motif at its N-terminus expressed two isoforms (Figure 4E). In contrast, when the R to A mutation was introduced, the small form of the GFP fusion was no longer detected. The small isoform of GFP fusion was observed at approximately 26 kDa, close to the predicted size of GFP, suggesting that the proteolytic cleavage occurred within the motif.

The HT/PEXEL motif is necessary but not sufficient for PVM localization

As GRA19 and GRA20 are proteolytically processed and have a propensity to associate with the PVM (Figures 1, 3 and 4), we hypothesized that the *T. gondii* HT/PEXEL motif contributes to the PVM association. To test this hypothesis, GRA19 with wild type or mutated HT/PEXEL motifs (RA, LA or DA) was localized by IFA. The vacuoles were considered positive for PVM localization when the GRA19 staining was continuous around at least 50% of the PVM, whereas staining within PV was scored as PVM negative localization. For GRA19 with wild type HT/PEXEL motif, the majority of the vacuoles had continuous staining around at least 50% of the PVM and thus were scored as PVM positive (Figure 5A). In contrast, site-directed mutagenesis of R, L or D amino acids within the HT/PEXEL motif significantly reduced PVM localization (Figure 5B). These results suggest that processing of the HT/PEXEL motif contributes to the PVM localization of GRA19. They also suggest that the negative charge at position 5 of the HT/PEXEL is not important

for proteolytic processing (Figure 4D) but instead is involved in interactions with the PVM. Site directed mutagenesis of the *Plasmodium* HT/PEXEL motif also showed that while the 5th position was not important for proteolytic processing, it was essential for export into the erythrocyte (30).

Processed form of GRA19 associates with the PVM, likely through protein-protein interactions

To determine if HT/PEXEL processing contributes to PVM association, we placed a Myc tag before or after the HT/PEXEL signal of GRA19. We speculated that a Myc epitope placed after the signal peptides but before the HT/PEXEL-motif (Myc^{21}GRA19-HA) would allow us to specifically localize the long form of GRA19. Likewise, a Myc inserted after residue 99 of GRA19 (Myc^{100}GRA19-HA) will tag both the short and long forms of GRA19 (diagramed in Figure 6A). As expected, immunoblot analyses of parasites expressing Myc^{21}GRA19-HA only detected the long form of GRA19 when using an anti-Myc antibody (Figure 6B). When the blot was probed with an anti-HA antibody, both forms of Myc^{21}GRA19-HA were recognized, suggesting that the Myc tag did not affect the cleavage event. Both anti-Myc and anti-HA antibodies detected short and long forms of Myc^{100}GRA19-HA, and the short form has a slightly higher molecular weight due to the presence of the Myc tag (Figure 6B).

Localization of Myc^{21}GRA19-HA using an anti-Myc antibody revealed that the long form of GRA19 localized largely to the dense granules with little staining in the PV (Figure 6C), whereas staining with an anti-HA antibody localized Myc^{21}GRA19-HA to the dense granules, PV and PVM. Labeling of Myc^{100}GRA19-HA with both anti-Myc and anti-HA antibodies localized GRA19 to the dense granules, PV and PVM. These data suggest that only the fully processed short form of GRA19 associates with the PVM.

To determine the nature of the membrane association of the processed form of GRA19, intracellular parasites expressing GRA19-HA were subjected to Triton X-114 extraction. We found that the majority of the processed form of GRA19 partitioned in the aqueous phase along with the soluble protein, Rop1 (Figure 6D). In contrast, the membrane bound surface protein SAG1 was detected solely in the TX-114 detergent phase. A minor 38kDa band was detected by anti-HA antibody in the detergent phase. This minor band likely resulted from protein processing at the second HT/PEXEL-like motif (RGLPL) at residue 68 of GRA19. Taken together, these results suggest that the processed form of GRA19 associates with the PVM possibly through protein-protein interactions.

Other GRA proteins contain HT/PEXEL motifs

As all three families from Table 1 were dense granule proteins and we saw that the R, L, and D residues were important for the proper cleavage and localization of GRA19, we investigated whether other dense granule proteins contained a HT/PEXEL motif. We performed a Multiple Expectation Maximization for Motif Elicitation (MEME) using the sequences from all known dense granule proteins and the two new protein families. The motif was refined by manual curation, by searching for sequences that resembled the pattern RxLxD/E within 140 residues after the predicted methionine. This analysis showed while most GRA proteins contain a HT/PEXEL-like motif (Table 2), in addition to the new members listed on Table 1, only GRA3, GRA5 and GRA15 contained the exact consensus RxLxD/E. There is a propensity for predicted HT/PEXEL motif to match the consensus sequence in GRA proteins that are known to interact with the PVM (Table 2).

Discussion

Apicomplexan parasites, including *T. gondii* and *Plasmodium* spp., secrete hundreds of proteins during invasion and the establishment of the PV. These invasion factors lead to key signaling events for the establishment of the infection. The *Plasmodium* secretome has been predicted and the HT/PEXEL motif is one of the signals for protein export (reviewed in (24)). However, up to date, only the plant pathogen *Phytophthora* is known to export proteins with a motif similar to HT/PEXEL (25). Using bioinformatics and manual curation, we discovered three families of HT/PEXEL-containing proteins in *T. gondii*. The family members examined so far localized to the dense granules and further analysis showed that the *T. gondii* HT/PEXEL motif is found in several GRA proteins that associate with the PVM.

Similar to most dense granule proteins, these newly found GRA proteins (GRA19, GRA20 and GRA21) do not have any homology with proteins of known function. They also do not contain predicted transmembrane domains. GRA19 is only found in type II strains and has been mapped to a 3.4-Mb region of chromosome X (31). Family members GRA20 are located on chromosome XII for type II and III strains, but type I strains do not contain GRA20. For the GRA21 family, all four members are in a tandem array on chromosome VI in all three strains. While it appears that type III strains only contain three GRA21 family members, analysis of the expressed sequence tags from type III strains shows a fourth GRA21 family member transcribed in the same direction in between TGVEG_089940 and TGVEG_089920. The high degree of sequence similarity within these families indicates gene duplication and may complicate functional analysis of individual members.

Results from figures 5 and 6 suggest that the cleaved GRA proteins traffic from dense granules to preferentially associate with the PVM. Site directed mutagenesis of the HT/PEXEL motif showed that the R in position 1 and the L in position 3 are important for proteolytic processing and PVM association of GRA19 (Figures 4 and 5). The unprocessed form of GRA19 appears to be primarily localized to the dense granules, whereas when both forms of GRA19 were tagged, PVM localization was visualized (Figure 6C), suggesting that protein processing contributes to PVM association. However, when the D residue was replaced by A, the protein processing was not affected but the PVM localization was reduced. It is likely that the PVM association requires this negatively charged residue. Positively charged domains are thought to interact with the strong negative curvature of PVM (32), thus this negatively charged amino acid is likely to be required for interacting with a protein and not the membrane directly. In addition, the processed form of GRA19 is mostly hydrophilic in TX-114 aqueous phase, indicating that GRA19 may associate with the PVM through protein-protein interactions instead of a direct interaction (Figure 6D). Future examination of the GRA19 will gain insight into the mechanism of its PVM localization.

While there is a propensity for *T. gondii* HT/PEXEL containing proteins to associate with the PVM, the exact biological role of this motif is unclear. In *Plasmodium*, the HT/PEXEL motif is necessary for binding the PI(3)P in the parasite ER to facilitate export to the host cell (33). In addition, a secondary PI(3)P independent signal downstream of the *Plasmodium* HT/PEXEL export signal, which can work in conjunction with the HT/PEXEL signal has also been identified (34). As in *Plasmodium*, PI(3)P in *T. gondii* has been localized at the apicoplast (33-35). However the presence and function of PI(3)P in the *T. gondii* ER lumen has not been investigated. Unlike *Plasmodium*, the *T. gondii* HT/PEXEL motif is not a recognition site for protein transport across the PVM into host cell. This lack of host cell export may be due to the divergence of the protein complex known as PTEX (*Plasmodium* translocon of exported proteins (24, 36) in *T. gondii.* The PTEX complex contains five proteins in *Plasmodium*, with only the thioredoxin being potentially represented in the *T.*

gondii genome (TGME49_093870). The PTEX complex is proposed to form a pore in the PVM to recognize, unfold, translocate and then recruit host cell HSP70 to re-fold the exported proteins (24, 36). Because most members of the PTEX complex are absent from *T. gondii*, it is not likely to transport HT/PEXEL containing proteins across the PVM.

To our knowledge, this is the first report of a GRA protein being proteolytically processed. Our data suggest that the proteolytic processing of the *T. gondii* HT/PEXEL may be similar to *Plasmodium,* but the identity of the *T. gondii* protease has yet to be established. In *Plasmodium*, the HT/PEXEL motif is recognized by an aspartic protease, plasmepsin V in the ER and processed at the conserved L with acetylation of the new N terminus (28, 29). In *T. gondii*, there are seven aspartic proteases, of which TgASP5 appears to be the ortholog of plasmepsin V (37). However, TgASP5 is localized to the Golgi (37), so whether it is responsible for cleavage of the *T. gondii* HT/PEXEL motif will be of interest in future studies. Furthermore, the N-terminal domain of GRA5 and GRA6 is essential for both sorting and post-secretory subcellular localization (7, 20). While MEME analyses revealed HT/PEXEL motifs in both of these GRA proteins, only GRA5 associates with the PVM and contains the consensus RxLxE/D.

As the microneme and rhoptry organelles also secrete their contents from the parasite, we examined all known microneme and rhoptry proteins for a HT/PEXEL motif by manual curation. While several microneme and rhoptry proteins contained a near consensus HT/PEXEL motif, only two microneme and seven rhoptry proteins contained the exact HT/PEXEL consensus RxLxD/E within the first 140 amino acids (Table 3). Other proteins that contain signal peptides such as the SAG1 related surface antigens (SRS) and SAG1 unrelated surface antigens (SUSA) do not contain a consensus RxLxD/E motif. Future mutagenesis studies that define the amino acids necessary for the cleavage and trafficking may broaden the list of *T. gondii* proteins containing a HT/PEXEL motif. Most ROP proteins contain the consensus SφXE/D (where φ is a hydrophobic amino acid), which has been shown to be a processing site for ROP1, ROP13, TgSUB2 and TLN1 (38-41). While ROP24 does not contain a consensus SφXE/D cleavage domain, it does have a consensus HT/PEXEL domain. Likewise, a long exposure of the cleavage products of ROP13 shows a lower molecular weight band that corresponds to the size ROP13 cleaved within the HT/PEXEL motif (41). The biological significance of cleavage of the HT/PEXEL, especially during stress conditions such as animal infection, will be an interesting avenue for future investigations.

Materials and Methods

Screen for candidate secreted proteins containing a HT/PEXEL motif

To identify candidates for secreted proteins in *T. gondii*, we *in silico* screened the predicted coding sequences in the *T. gondii* genome for polymorphic families enriched in sequences with a signal sequence and a HT/PEXEL motif. Polymorphic gene families were identified using TRIBE-MCL, N-terminal ER-like signal sequences (SS) were selected using SignalP 2.0, and HT/PEXEL motifs were recognized by manual curation (42). Briefly, the 7,793 predicted ORFs in the *T. gondii* genome (http://toxodb.org, 09/2006) were analyzed using TRIBE-MCL (43). The program was run with the following parameters: 1) e-value cutoff of 1 and the filter on to generate an all-against-all sequence similarity matrix; 2) e-value cutoff of 1e-4 and heavyweight setting for the Markov cluster (MCL) algorithm that generates the Markov matrix; 3) an inflation value of 2.8 and scheme _5 to group the sequences into protein clusters. The MCL output identified 82 families with 3 or more members. These families were further analyzed to select only those where three or more members contained a SS, yielding 23 families. Presence of an SS was established using a maxS cutoff of 0.82 in the SignalP 2.0 program (44).

We then searched these 23 families of candidate secreted proteins with manual curation for the presence of a HT/PEXEL motif, which lead to the identification of the three families listed in Table 1. Three members of family 1 and all members of families 2 and 3 have the motif Rx(LV)x(ED) within 110 residues of the signal sequence cleavage site. After we found that members of all three families localized to the dense granules, we combined MEME analysis and manual curation to identify motifs shared between all known dense granule proteins and the two new protein families (45). The MEME was performed on the 140 residues after the predicted methionine. Manual curation was used to identify sequences that resemble the HT/PEXEL motif in known dense granule, microneme, rhoptry, SRS and SUSA proteins.

Cell culture and parasite strains

The *T. gondii* type II strain PruΔ*HPT* (Pru with a deletion in the hypoxanthine-xanthine guanine phosphoribosyltransferase) was used for all experiments. Parasites were maintained as tachyzoites by serial passage on monolayers of human foreskin fibroblasts (HFF). Tachyzoites were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Gibco) and 2 mM L-glutamine at 37°C with 5% CO_2. Bradyzoites were grown in RPMI-1640 supplemented with 1% fetal bovine serum and 1% penicillin-streptomycin, buffered with 50 mM HEPES to pH 8 at 37°C with ambient CO_2 for 5 days.

Plasmid construction and transfection

To insert a HA tag, the ORF of GRA19, GRA20 and GRA21 were amplified using primers to generate an Nsi site at the initiator methionine and a C-terminal HA tag followed by an engineered PacI site at the translational stop codon. Primers are listed in Table S1. Primers 19HAF and 19HAR were used for GRA19, 20HAF and 20HAR were used for GRA20, and 21HAF and 21HAR were used for GRA21. The PCR products were cloned into pCR2.1 (Invitrogen) according to the manufacturer's protocols. The GRA genes were digested with NsiI and PacI and subcloned into pBC SK+DHFR, which is derived from pT/230 (46) and expresses genes from the *T. gondii* α-tubulin promoter. To obtain RA or DA site directed mutagenesis construct, sequences were amplified from Topo-GRA19 using primer sets RAF and RAR or DAF and DAR according to the manufacturer's protocol (Finnzymes, NEB). The LA mutation construct was generated by SOE-PCR using 19HAF and LAR for the first fragment and LAF and 19HAR for the second fragment. To generate GRA19^{NT}-GFP and GRA19RA^{NT}-GFP constructs, the GFP sequence was amplified using TgGFP-AatII-F and TgGFP-PacI-R and inserted in frame after 297nt of GRA19, where an AatII was created. The Myc sequence was inserted into the N terminal region or after 297 nt of GRA19 by SOE-PCR using primers listed. The resulting plasmids were subcloned into pBC SK+DHFR digested with NsiI and PacI. Twenty-five milligrams of each linearized construct was electroporated with 1×10^7 PruΔHPT parasites and stable transformants were selected as resistant to 1 μM pyrimethamine (47). For transient transfection, twenty-five milligrams of linearized GRA19HA or GRA20HA construct was electroporated with 1×10^7 RH parasites and parasites were collected 25 h after electroporation prior to western blot analysis.

Immunofluorescence assays

For intracellular *T. gondii*, parasite infected HFF cells on coverslips were fixed in methanol for 5 min or 3% formaldehyde for 20 min. For extracellular *T. gondii*, recently lysed parasites in media were applied to coverslips and allowed to attach for 30 min before fixing. For protein localization in bradyzoites, HFF cells on coverslips were inoculated with $2\text{-}4 \times 10^4$ tachyzoites for 3 h, then shifted to bradyzoite differentiation condition for five days. The parasites were fixed with 3% formaldehyde. After fixing, all coverslips were permeabilized and blocked in 0.2% TX-100/3% FBS/PBS at 4°C.

Primary antibodies for the epitope tags were mouse anti-HA (1:500, MMS-101P, Covance Innovative Antibodies, Princeton, NJ) or mouse anti-Myc-Tag Antibody (1:300, #2272, Cell Signaling Technology, Inc). For co-localization, we used SAG1, GRA4, GRA7 or BAG1 antibodies, or Biotinylated-DBA (Vector Labs). Secondary antibodies were Alexa Fluor 488-conjugated donkey anti-mouse and Alexa Fluor 633-conjugated goat anti-rabbit or streptavidin (Invitrogen). Coverslips were mounted onto slides by using VectaShield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Labs). Samples were examined using a motorized Zeiss Axioplan III instrument equipped with a rear-mounted excitation filter wheel, a triple-pass emission cube and a Hamamatsu Orca-AG charge-coupled-device (CCD) camera operated by OpenLabs 4.0 software (Improvision, Lexington, MA). Serial image stacks (0.2 μm Z-increment) were collected with a 100× PlanApo oil immersion lens and fluorescence images were deconvolved by using the Volocity software (Perkin-Elmer). PVM staining was scored as associated if all or at least half of PVM was observed within vacuoles containing at least 16 parasites. Two different clones were included and total four coverslips were counted with at least 50 vacuoles each coverslip. The statistics was evaluated by ANOVA.

Western blot analysis

Fresh lysed or intracellular tachyzoites were collected by centrifugation (450 × g for 10 min). Parasite lysates were separated on 10% polyacrylamide gels, transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Buckinghamshire, United Kingdom) and blocked in 5% skim milk in PBS/0.1% tween 20. Membranes were incubated with a primary antibody (anti-HA, 1:5000; anti-GFP, 1:5000; anti-Myc, 1:5000; anti-β-tubulin 1:5000) followed by incubation with a secondary antibody (donkey anti-mouse or donkey anti-rabbit) conjugated to horseradish peroxidase (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection was performed with the Amersham ECL plus or prime Western blotting system for chemiluminescence according to manufacturer's protocol (GE Healthcare, Buckinghamshire, United Kingdom). For GRA protein phosphorylation, parasite lysates were treated with or without alkaline phosphatase (New England Biolabs) for 90 min prior to immunoblot analysis.

Triton X-114 phase partitioning

To access whether GRA19 separates with membrane or soluble fractions, intracellular parasites were subjected to Triton X-114 phase partitioning. Cells were pelleted and lysed in 1% Triton X-114. Following a low speed spin at 12,000 rpm to remove insoluble material, the lysates were partitioned as previous described (48).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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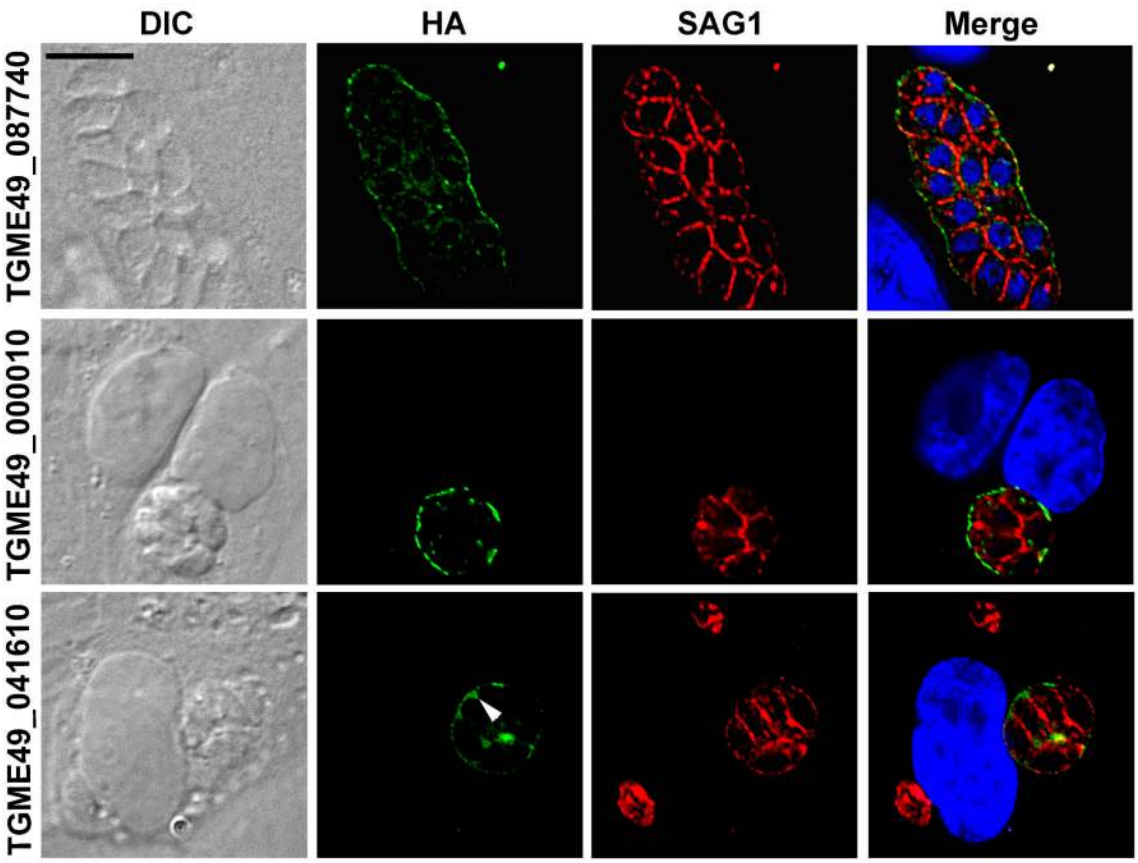

Figure 1. HT/PEXEL motif-containing proteins do not co-localize with a surface marker SAG1

HFF cells were infected with tachyzoites expressing HA-tagged TgME49_087740, TgME49_000010, or TgME49_041610 for 24 h and were fixed in 3% formaldehyde. HA tag was recognized by anti-HA antibody (green), SAG1 was recognized by anti-SAG1 antibody (red) and nuclei were stained with DAPI (blue). DIC, differential interference contrast. Scale bar: 10 microns. The arrow indicates protein in the PV space in between parasites for TgME49_041610.

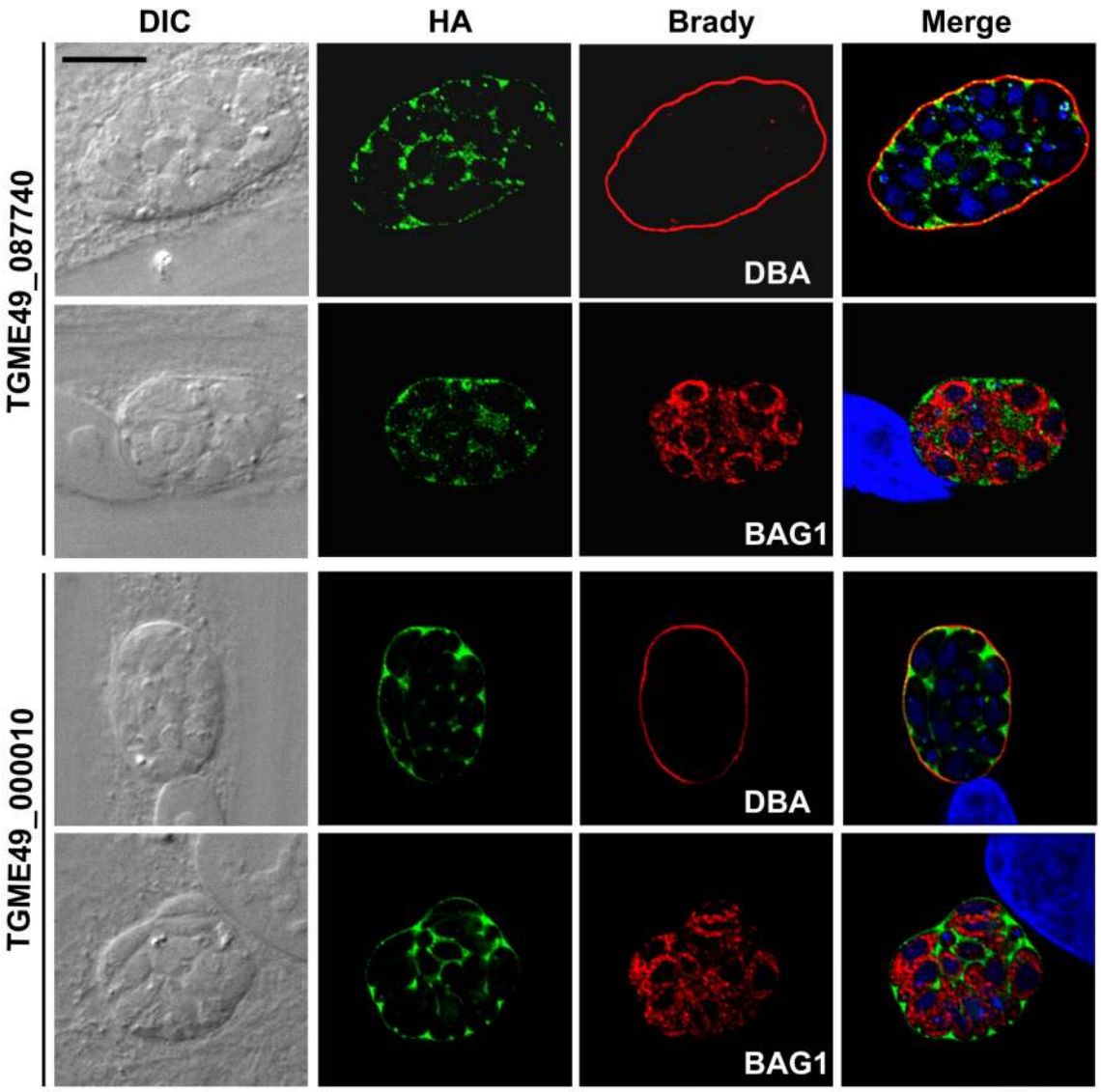

Figure 2. HT/PEXEL motif-containing proteins do not co-localize with bradyzoite markers DBA and BAG1

Parasites expressing HA-tagged TgME49_087740 or TgME49_000010 were grown in bradyzoite inducing conditions for 5 d. Parasites were fixed in 3% formaldehyde and stained with mouse anti-HA antibody (green), and either DBA or a rabbit polyclonal anti-BAG1 antibody (red). Nuclei were stained with DAPI (blue). DIC, differential interference contrast. Scale bar: 10 microns.

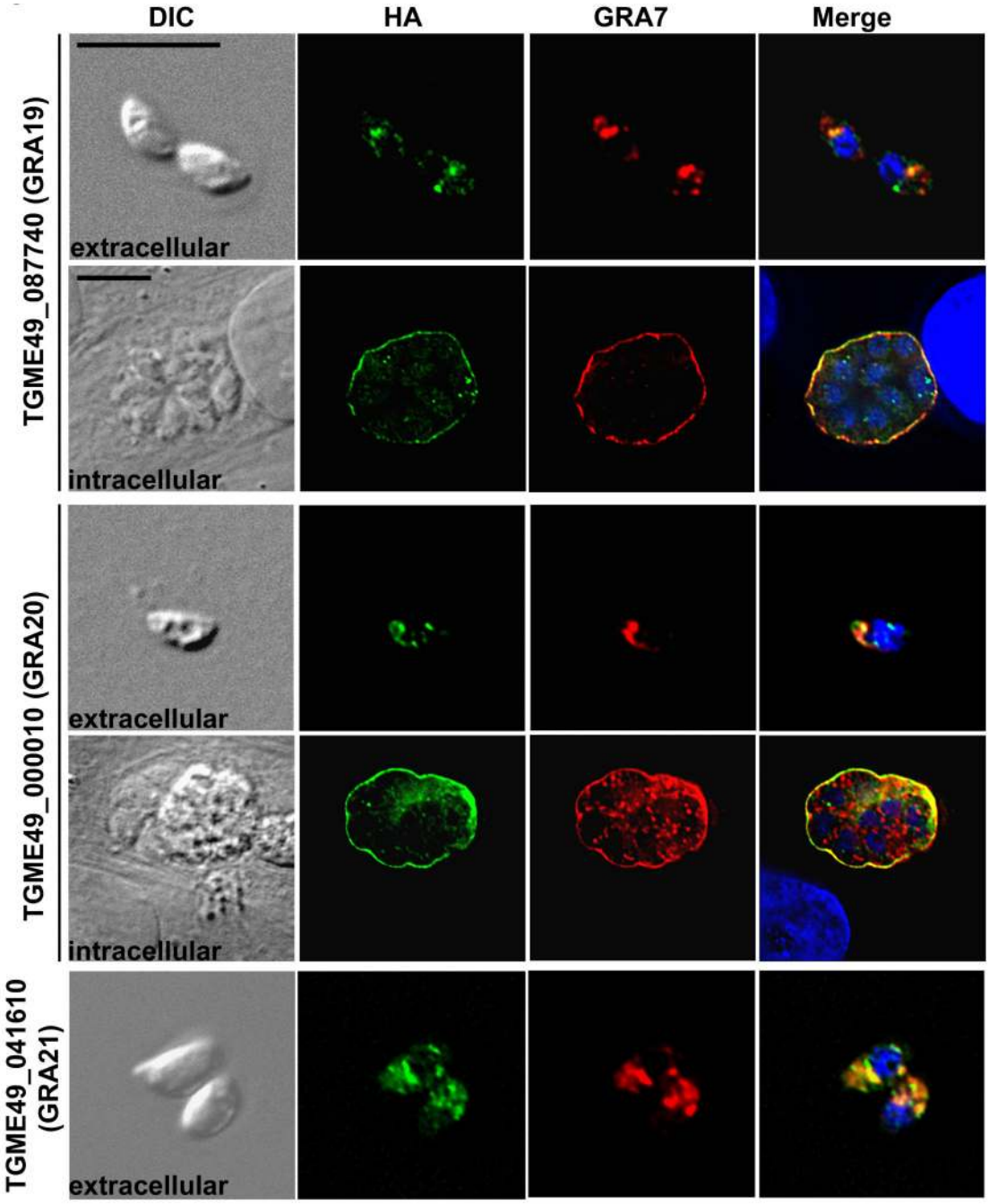

Figure 3. Co-localization of HT/PEXEL motif-containing and dense granule protein

For extracellular panels, recently lysed parasites were allowed to attach to coverslips for 30 min before fixing. For intracellular panels, HFF cells were infected with tachyzoites expressing HA-tagged GRA19 and GRA20 for 24 h. Parasites were fixed in 3% formaldehyde and stained with mouse anti-HA antibody (green), and rabbit polyclonal GRA7 antibody (red). Nuclei were stained with DAPI (blue). DIC, differential interference contrast. Scale bar: 10 microns.

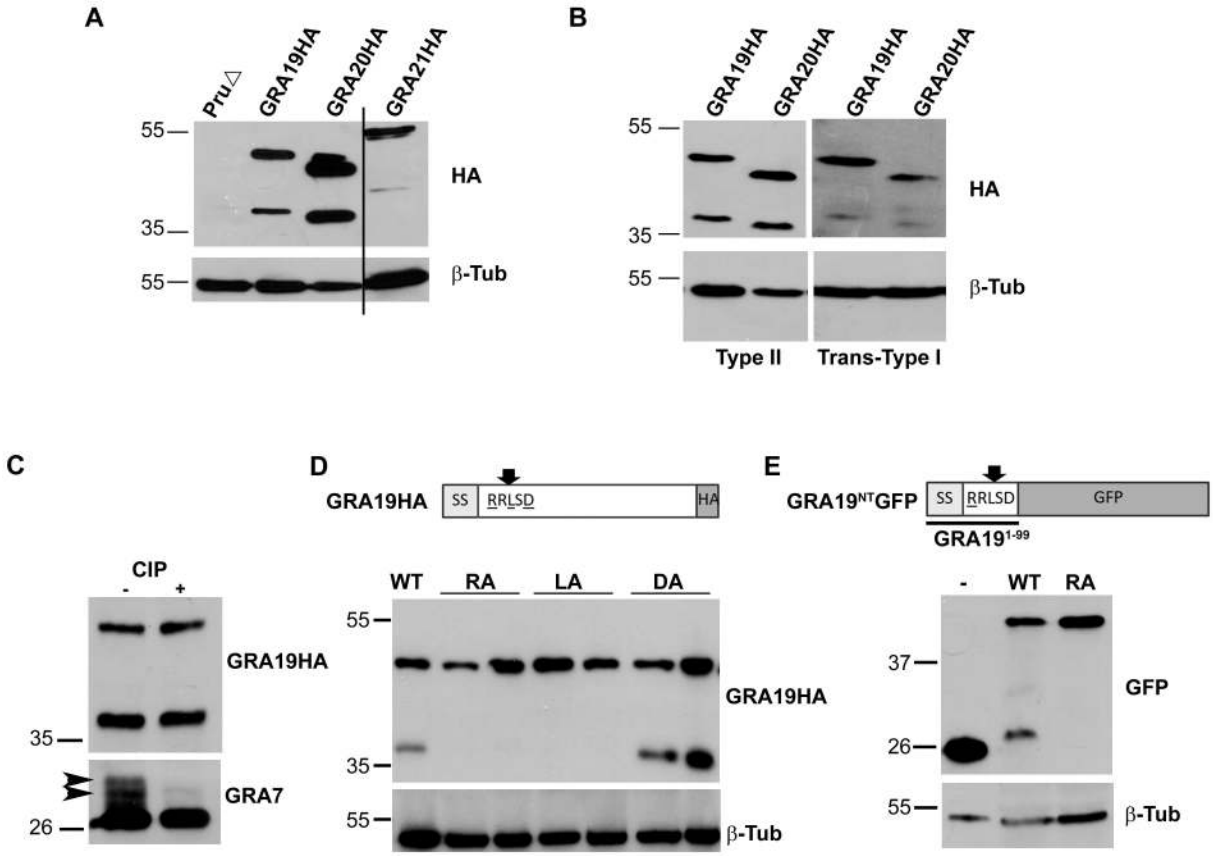

Figure 4. Amino acids R and L in the HT/PEXEL motif are recognition sites for protein processing

A) Lysates from PruΔ and parasites expressing GRA19HA, GRA20HA or GRA21HA were analyzed by immunoblot. The blots were probed with a mouse anti HA antibody and then re-probed with a rabbit anti β-tubulin as a loading control. B) Type I RH strains were transiently transfected with GRA19HA and GRA20HA constructs (Trans-Type I). Lysates were collected and analyzed on the same polyacrylamide gel as lysates from Type II parasites expressing GRA19HA and GRA20HA. The blots were probed with a mouse anti HA antibody and then re-probed with a rabbit anti β-tubulin as a loading control. C) Parasite lysates were incubated without (−) and with (+) calf intestinal phosphatase (CIP) for 90 min to determine if the modified forms of GRA19 were due to phosphorylation. Western blots were incubated with a mouse anti-HA to visualize GRA19HA. The blot was stripped and re-probed with rabbit anti-GRA7. Phosphorylated GRA7 forms were indicated by arrowheads. D) Diagram of GRA19HA expression construct shows the signal sequence (SS), potential secondary cleavage site marked with an arrow, the HT/PEXEL motif with the mutated amino acids underlined, and the C-terminal HA tag. For the immunoblot, GRA19HA with wild type (WT, first lane) or mutant HT/PEXEL motifs was visualized using anti-HA antibodies. The first lane of the blot is WT HT/PEXEL, next two lanes are an R to A mutant (RA), the next two lanes are an L to A mutant (LA) and the final two lanes are a D to A mutant (DA). To ensure that the GRA19 processing differences were due to the mutation and not insertion site within the genome, we examined at least two independent clones for each mutant. As a loading control, the blot was stripped and re-probed with anti-β-tubulin. E) Diagram of GRA19^{NT}GFP fusion construct shows the signal sequence (SS), potential secondary cleavage site marked with an arrow, the HT/PEXEL motif with the mutated R underlined and GFP coding region. Lysates from parasites expressing GFP alone (−), or

GFP fusions with the wild type (WT) or mutant HT/PEXEL motif (RA) were analyzed by western immunoblot using an anti-GFP antibody. The blot was stripped and re-probed with anti-β-tubulin as a loading control.

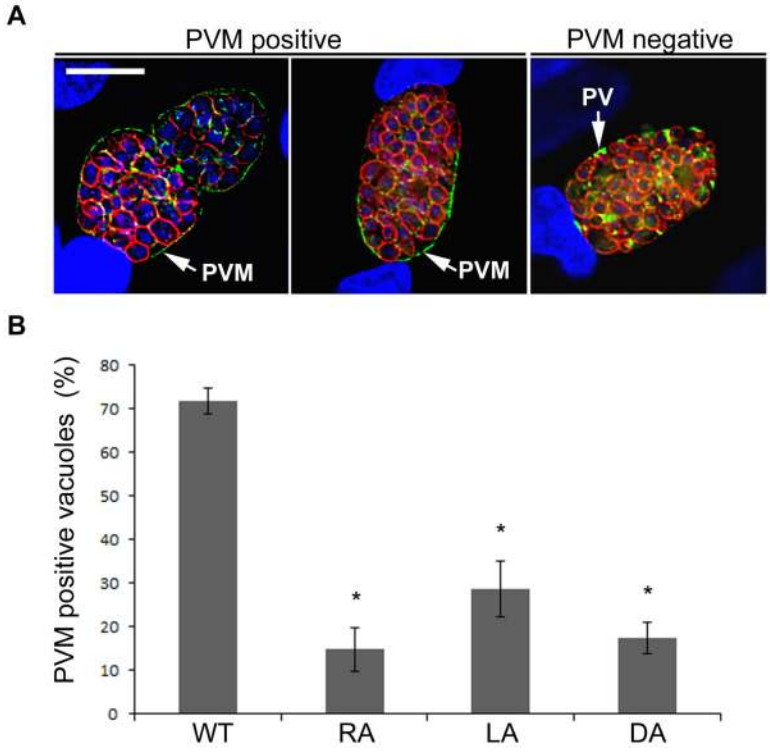

Figure 5. Mutant HT/PEXEL motif reduces PVM localization of GRA19

A) IFA images of HFF cells infected with parasites expressing GRA19HA-tagged for 24 h and fixed in 3% formaldehyde. HA tag was recognized by anti-HA antibody (green), SAG1 was recognized by anti-SAG1 antibody (red) and nuclei were stained with DAPI (blue). In the first two panels, GRA19 was considered "PVM positive" because at least 50% of the PVM had continuous GRA19 staining. The last panel is a representative of "PVM negative" with little GRA19 on the PVM. White arrows indicate PVM or PV localized GRA19 protein. B) Graph of the percentage of vacuoles positive for GRA19 on the PVM (Vacuoles PVM positive (%)) with either wild type (WT) or mutant HT/PEXEL motif. Single amino acid substitutions are R to A (RA), L to A (LA) and D to A (DA). The staining patterns were counted for at least 50 vacuoles with 16+ parasites each vacuole and data analyzed by ANOVA. (* $P<0.0001$, $n=4$) Scale bar: 10 microns.

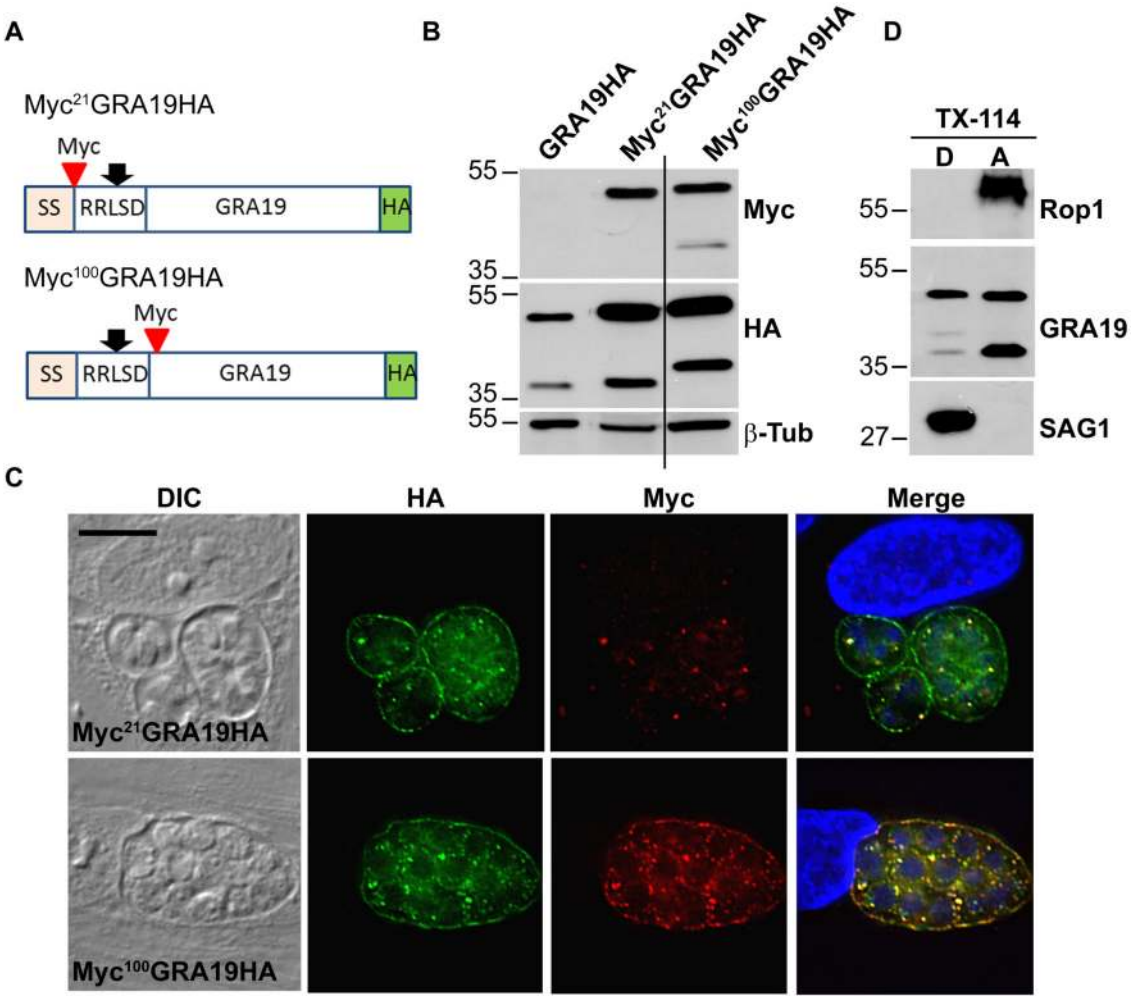

Figure 6. Processing of GRA19 contributes to PVM association

A) Schematic representation of the Myc-tag expression constructs Myc^{21}GRA19HA and Myc^{100}GRA19HA. The ER-type the signal sequence is labeled with SS, the Myc tag insertion site is labeled with a red triangle, potential secondary cleavage site marked with an black arrow, and the HA tag is labeled at the C-terminus. B) Immunoblot of lysates from parasites expressing Myc^{21}GRA19HA and Myc^{100}GRA19HA analyzed with an anti-Myc or anti-HA antibody. The blot was re-probed for anti-β-tubulin as a loading control. C) IFA images of HFF cells infected with parasites expressing Myc^{21}GRA19HA or Myc^{100}GRA19HA and stained with anti-HA antibody (green), anti-Myc antibody (red) and the nuclei with DAPI (blue). DIC, differential interference contrast. D) TX-114 phase partitioning of intracellular parasites expressing GRA19HA analyzed with anti-HA antibody. The soluble marker Rop1 is found in the aqueous phase (A), whereas the GPI-anchored SAG1 is detected in the detergent (membrane) phase (D). Scale bar: 10 microns.

Table 1

Families of HT/PEXEL motif-containing *T. gondii* proteins.

Family #	ToxoDB ID #	Gene Annotation
1	TgME49_025290	Nucleoside-triphosphatase, putative
	TgME49_077240	Nucleoside-triphosphatase I
	TgME49_077270	Nucleoside-triphosphatase II, putative
	TgME49_077720	Nucleoside-triphosphatase, putative
2	TgME49_087740	Hypothetical protein (renamed GRA19)
	TgME49_045430	Hypothetical protein
	TgME49_000010	Hypothetical protein (renamed GRA20)
3	TgME49_041190	Hypothetical protein
	TgME49_041300	Hypothetical protein
	TGME49_041305	Hypothetical protein
	TgME49_041610	Hypothetical protein (renamed GRA21)

Table 2

Identification of HT/PEXEL motif in N-termini of *T. gondii* likely GRA proteins.

Name	Signal peptide *(16)	Distance from start codon	PEXEL-like Motif	Characteristics/ localization (9, 49)
GRA1	Yes	118	RALNK$	Soluble in PV
GRA2	Yes	NA	NI	PVN
GRA3 #	Yes	110	**RELYD**	PVM, PVN, PVE
GRA4	Yes	38	REDKE	PVN
GRA5	Yes	58	**RSLFE**	PVM, PVE
GRA6	Yes	NA	NI	PVN
GRA7	Yes	85, 106, 134	REPLE, RSDAE, RSFKD	PVN, PVM, PVE
GRA8	Yes	NA	NI	PVM, PVE
GRA9	Yes	55, 97, 124	RREPE, RRLMG, RALQP	PVN
GRA10	NI	7, 121, 129	RLSSE, RKLSS, RLPPE	PVM
GRA11	Yes	113	RLLLP	NI
GRA12	NI	NA	NI	PVN
GRA14	Yes	89, 90, 124	RRLPL, RLPLD, RSLQS	PVM, PVN
GRA15	Yes	53, 77	RWLGY, **RDLSD**	PVM, PVN, PVE
NTPase (TGME49_025290)	NI	67, 118, 130	RDLRK, RFLGN, **RQLLE**	PVN, (PVM)
NTPase (TGME49_077720)	Yes	51, 71	RDLDS, RRSYE	PVN, (PVM)
NTPase I (TGME49_077240)	Yes	53, 113, 136	RNLET, RVVLE, RLLFQ	PVN, (PVM)
NTPase II (TGME49_077270)	Yes	53, 101, 113, 136	RNLET, RLIRE, RVVLE, RLLFQ	PVN, (PVM)
GRA19	Yes	68, 86	RGLPL, **RRLSD**	PVM, PV
TgME49_045430	Yes	68, 86	RGLPL, **RRLSD**	NI
GRA20	Yes	72, 90	RGLAL, **RRLSD**	PVM, PV
TgME49_041190	Yes	68, 83, 138	**RRLAE, RELVD,** RQKFE	NI
TgME49_041300	Yes	68, 83	**RWLAE, RELLD**	NI
TGME49_041305	Yes	68, 83	**RWLAE, RELLD**	NI
GRA21	Yes	68, 83, 139	**RRLAE, RELVD,** RDAFE	PV, PVM
Cyclophilin-18	Yes	38, 69, 95	RIILE, RIIPD, RRFDD	PV
TgPI-1	Yes	34, 89, 118	RNLEL, RPPKD, RELRL	PV
TgPI-2	Yes	NA	NI	Soluble in PV
Tg14-3-3	NI	17, 24, 111	RPLLV, RPARD, RNLLS	PV

HT/PEXEL motifs with exact consensus sequence are in bold; NI, not identified, refers to either the initiator methionine not being defined, the lack of a HT/PEXEL motif with the first 140 amino acids, or the exact localization not being determined; NA, not applicable; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; PVN, parasitophorous vacuole network; PVE, parasitophorous vacuole extensions;

*Signal peptides were predicted using SignaIP software;

#second in frame methionine used as it has a signal peptide predicted by SignaIP software.

Table 3

Identification of a consensus HT/PEXEL motif in N-termini of *T. gondii* microneme and rhoptry proteins.

Name	Signal peptide*(16)	Distance from start codon	PEXEL-like Motif	Characteristics/ localization (9, 49)
MIC10	NI	124	RGLRE	Microneme
ROM1	NI	5	RTLAD	Microneme
ROP4	NI	114	RPLAD	Rhoptry (PVM)
ROP13	Yes	138	RRLLD	Rhoptry
ROP24	Yes	38	RPLPE	Rhoptry
RAB11	Yes	105	RWLKE	Rhoptry
RON2L2	NI	8, 44	RQLPE, RTLND	Rhoptry
RON4L1	NI	107	RPLKD	Rhoptry
Toxolysin (TLN1)	Yes	35	RVLFD	Rhoptry

*Signal peptides were predicted using SignaIP software; NI, not identified, refers to the initiator methionine not being defined; PVM, parasitophorous vacuole membrane