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論文題目：Functional characterization of dense granule protein 9 in *Toxoplasma gondii*
（トキソプラズマの GRA9 分子の機能解析）

要旨

The protozoan parasite *Toxoplasma gondii* is an obligate intracellular pathogen belonging to the phylum Apicomplexa and virtually infect any kind of warm-blooded animal, including humans. Approximately 25-30% of the world's population is infected with *T. gondii*. *T. gondii* is divided into three lineages: types I, II and III in North America and Europe according to its virulence in laboratory mouse strains. Type I strains are virulent, type II exhibits reduced virulence and type III is avirulent. *T. gondii* is acquired orally by ingestion of raw or undercooked meat containing cysts, or after ingestion of fresh water or vegetables spoiled with oocysts. Toxoplasmosis is an important worldwide zoonosis. However, at present, no safe and effective vaccine exists for prevention against toxoplasmosis. Thus, identification of the growth and virulence factors of this parasite is important for the development of control strategies.

Similar to other apicomplexan parasites, *T. gondii* possesses main three morphological distinct secretory organelles, micronemes, rhoptries, and dense granules. The contents of these organelles are coordinately secreted during the invasion process of the parasite, ensuring proper recognition, attachment, and entry to the host cell, as well as survival and further development in the parasitophorous vacuole (PV). Dense granule proteins (GRA) are parasitic molecules secreted to the PV during host invasion. More than 20 GRAs have been identified in *T. gondii*. Among these proteins, GRA1, 16 and 24 can

be detected within the vacuolar space; GRA2, 4, 6, 9 and 12 have been located at the intravacuolar membranous nanotubular network; GRA3, 5, 7, 8, 10, 15 and 22 localize to the PV membranes; and GRA16 and GRA24 target the host cell nucleus. Furthermore, GRA proteins are believed to play important roles in the lytic cycle of *T. gondii*. GRA2, 4 and 6 interact in a macromolecular complex to stabilize the network membrane GRA10 plays a significant role in the growth and propagation of intracellular *T. gondii*. Recently, a novel dense granule protein, GRA41, has been shown to regulate timing of egress which relates with calcium fluxes.

The main purpose of this study was to identify new factors that are important for parasite growth and virulence. An in-depth understanding of a protein's molecular character is useful for evaluating the function of the protein. Furthermore, it is a popular method to evaluate the protein's function by knocking out the gene. Dense granule proteins play major structural functions within the PV and the cyst of *T. gondii*. Moreover, their particular location within the PV allows them to be involved in various interactions between parasites and the host cells. GRA9 protein has been identified in *T. gondii*, although its role in the lytic cycle remains unclear.

In chapter 1, a detailed characterization of the *T. gondii* GRA9 was provided. Phylogenetic analysis was performed with GRA9 protein from different *T. gondii* strains and other species indicating GRA9 to be relatively conserved in the different strains of *T. gondii*. Sequence analysis showed that the nucleotide sequences of GRA9 in the type I RH and type II PLK strains were both 957 bp, which were consistent with the predicted sequences of type I (TGGT1_251540) and type II (TGME49_251540), respectively. Furthermore, alignment of predicted amino acid sequence showed that RH GRA9 contains one amino acid substitution compared with that of PLK GRA9. In immunoblot analysis, the recombinant GRA9 was reactive with the sera from mice infected with *T. gondii*,

indicating the immunogenicity of this protein. Furthermore, the polyclonal antisera raised against *T. gondii* recombinant GRA9 also recognized specific protein bands in the lysates of RH and PLK tachyzoites. It is notable that the expression level of GRA9 is significantly higher in the PLK strain than in the type I RH strain. This might suggest different roles of GRA9 in RH and PLK strains. All these information are useful for further elucidating the function of GRA9 protein in *T. gondii*.

In chapter 2, derivatives of *T. gondii* RH and PLK strains with a null mutation in GRA9 were generated using CRISPR/Cas9 system. The phenotypes of GRA9 in wild types, knockout and complemented strains were analyzed *in vitro* and *in vivo* using Vero cells and BALB/c mice, respectively. The phenotype analysis revealed that knockout of GRA9 in PLK parasites inhibited plaque formation and egress from PV. Both the plaque formation and egress ability of PLKΔGRA9 strain were restored by complementation with a synonymous allele of PLK strain GRA9. Mouse experiments demonstrated that loss of GRA9 in PLK strain significantly reduced the pathogenicity of *T. gondii*. However, there was no phenotypic differences between RH and RHΔGRA9 strains except the defect in host cell invasion. Overall, *T. gondii* GRA9 knockout only influenced the growth and virulence of PLK strain. These results indicate that GRA9 may be involved in parasite egress and virulence in mice in a strain-specific manner.

Overall, in the current study, a detailed molecular characterization of GRA9 was provided and its function in type I RH and type II PLK strains *in vitro* and *in vivo* was characterized. These results provide useful information for further understanding the mechanisms of how GRA9 is involved in the lytic cycle and virulence of *T. gondii*.