Review

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Toxoplasma secretory granules: one population or more?

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In *Toxoplasma gondii*, dense granules are known as the storage secretory organelles of the so-called GRA proteins (for dense granule proteins), which are destined to the parasitophorous vacuole (PV) and the PV-derived cyst wall. Recently, newly annotated GRA proteins targeted to the host cell nucleus have enlarged this view. Here we provide an update on the latest developments on the *Toxoplasma* secreted proteins, which to date have been mainly studied at both the tachyzoite and bradyzoite stages, and we point out that recent discoveries could open the issue of a possible, yet uncharacterized, distinct secretory pathway in *Toxoplasma*.

The *T. gondii* life cycle

T. gondii is an apicomplexan parasite that can virtually infect any kind of warm-blooded animal, including human beings. The parasite is acquired orally, after ingestion of raw or undercooked meat containing cysts, or after ingestion of fresh water or vegetables spoiled with oocysts. In its intermediate hosts, *Toxoplasma* multiplies in an asexual manner. Once liberated from the resistant cyst or oocyst wall in the duodenum, the bradyzoites or the sporozoites, respectively, differentiate into tachyzoites that disseminate into the entire organism. Within immune-privileged organs (i.e., muscle, heart, brain, retina, and testicles), tachyzoites that had been dividing by endodyogeny within intracellular PVs differentiate into bradyzoites while the PVs transform into intracellular cysts that may remain dormant for years as long as the immune pressure remains stable, ensuring transmission of the parasite by carnivorism (for reviews, see [1,2]).

In the small intestine of young felids, *Toxoplasma* behaves as a typical coccidian parasite, expanding by asexual endopolygeny to form merozoites. Eventually these merozoites differentiate into gametes. Fertilization in felid-definitive hosts leads to the production of millions of oocysts that are released into the environment with cat feces. The cells contained in the oocysts must then undergo a last step of division to generate infectious sporozoites. These mature oocysts, which are resistant to all normal laboratory disinfectants, are likely responsible for efficient spreading of the parasite to all non-carnivorous animals (for reviews, see [1,2]).

The host–parasite interactions that govern the sexual cycle of *Toxoplasma* in felids are likely highly specific and due to the refractoriness of the coccidian stages to cell culture, cell biology studies on this fascinating cycle are still limited. The recent determination of global gene expression of merozoites and oocysts harvested from cat intestines revealed dramatic differences from that of the tachyzoite and bradyzoite stages. In particular, most genes encoding secreted proteins, including those secreted by the dense granules, were shown to be downregulated [3–5]. This review will thus focus mainly on the tachyzoite and bradyzoite stages, which are amenable to cell culture and reverse genetics.

Dense granule subpopulations or novel secretory organelles in *Toxoplasma*?

The success of *T. gondii* as an intracellular parasite in intermediate hosts and in tissue culture relies on sequential secretion from Apicomplexa-specialized secretory organelles and mobilization of particular elements of its cytoskeleton to invade host cells and form a PV, inside which it multiplies [6]. Three types of morphologically distinct secretory organelles have been described in *T. gondii*. Among these, the Apicomplexa-conserved micronemes and rhoptries, which are localized at the apical part of the invasive stages, are involved in parasite attachment to the host cell and the early steps of PV formation, respectively [7]. In *T. gondii*, a third type of dense core secretory organelles, named the dense granules, has been characterized. The secretion of their contents, the GRA proteins, was shown to occur consecutively to the exocytosis from micronemes and rhoptries, at the end of the invasion process, once the PV has formed [8]. The post-secretory localization of the GRA proteins in both the PV and the cyst wall, combined with results obtained from the phenotypic analysis of parasites knocked-out for the expression of several *GRA* genes, have led to the understanding that GRA proteins are important for the maturation of the PV into a metabolically active compartment and to its subsequent transformation into a cyst [6,9]. Recent studies have challenged this view and have reported the specific targeting of some so-called dense granule proteins in the host cell nucleus, where they reprogram host gene expression [10]. Other 'GRA'-like proteins have been recently annotated in the ToxoDB database (version 12.0 released September 10, 2014: http://toxodb.org/toxo/). Here, we

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provide an update on the increasing number of newly characterized GRA proteins at both the tachyzoite and bradyzoite stages by summarizing first the discovery of the GRA proteins and reviewing the common properties of validated GRA proteins. We then discuss the possibility that some of these proteins actually do not originate from the conventional dense granules but from another type of *Toxoplasma* secretory organelle that is not yet characterized, leading us to propose new features to convey distinctions between validated GRA proteins and less well characterized proteins.

Defining the dense granules

Since the 1960s, as soon as transmission electron microscopy (EM) offered sufficient resolution to observe the internal organelles of *Toxoplasma*, dense granules have been so called because of their morphology: they were observed as one single population of microspheres of approximately 200 nm in diameter, enclosed in a unit membrane, and evenly dispersed on each side of the parasite nucleus. Most importantly, their dark appearance under the electron beam (Figure 1A) suggested that they could correspond to a protein storage compartment. Dense granules, which vary in number depending on the parasite stage, are most prevalent at the tachyzoite stage in *Toxoplasma* [11].

Discovery and nomenclature of the GRA proteins

The development of monoclonal antibodies specific to *Toxoplasma* proteins secreted *in vitro* after the use of an artificial secretion inducer (heat-inactivated serum) led to the cloning of a unique gene encoding a protein that was localized by immuno-EM within the dense granules of both the tachyzoite and the bradyzoite stages of the coccidian parasite *T. gondii* [12,13]. Until that time, the *Toxoplasma* proteins had been referred to as their apparent molecular weight on SDS-PAGE, for example P30 being the 30-kDa major surface protein of the tachyzoite stage. However, with the growing number of characterized proteins, Sibley and collaborators proposed in 1991 the use of a common nomenclature for *Toxoplasma* genes and proteins [14]. This nomenclature was inspired by that of yeast and allowed for the dubbing of any characterized protein based on its enzymatic activity or its subcellular localization. Accordingly, the first characterized GRA protein that was localized in the dense granules of both tachyzoites and bradyzoites, for which no function could be proposed, was referred to as GRA1 [13].

The main features of the GRA proteins

Today, the full proteome of dense granules still remains unknown, and awaits the purification of these organelles, which has not yet been reported for *Toxoplasma* or for any other Apicomplexa. Nevertheless, during the last two decades, 16 *GRA* genes (*GRA1*, *GRA2*, *GRA3*, *GRA4*, *GRA5*, *GRA6*, *GRA7*, *GRA8*, *GRA9*, *GRA12*, *GRA14*, *GRA19*, *GRA20*, *GRA21*, *GRA23*, *GRA25*, here referred to as the 'canonical' *GRA* genes) and their encoded proteins have been characterized (Figure 2) [15,16]. Despite the fact that these proteins failed to share homology between themselves or with proteins of known functions, they were defined as the family of GRA proteins based purely on their unique colocalization within the dense granules: their localization was determined by immunofluorescence after using 0.1% of triton X-100, a non-ionic polyoxyethylene

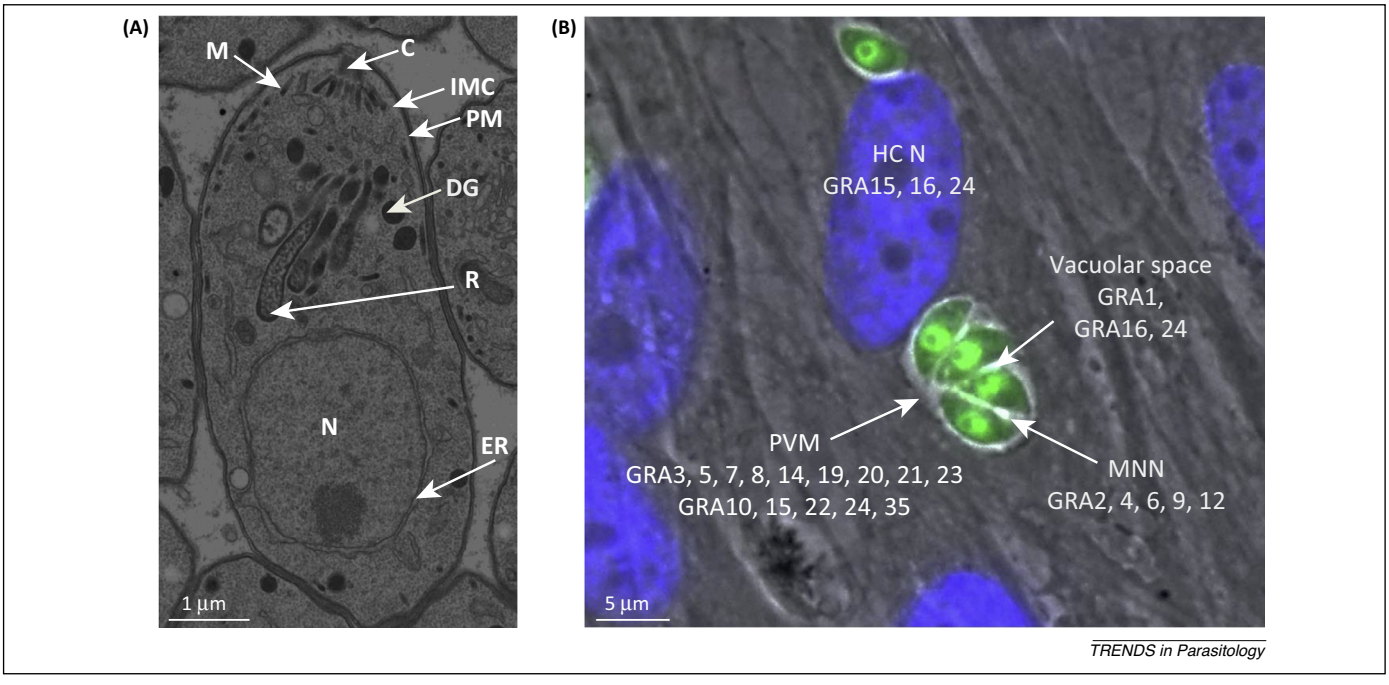

Figure 1. **(A)** The main intracellular organelles of *Toxoplasma gondii* as they appear on a longitudinal section of a tachyzoite observed by transmission electron microscopy (EM). Abbreviations: C, conoid; DG, dense granule; ER: endoplasmic reticulum; IMC, inner membrane complex; M, microneme; N, nucleus; PM, plasma membrane; R, rhoptry. **(B)** A schematic representation of the final localizations of the dense granule (GRA) proteins in infected cells. Human foreskin fibroblasts (HFFs) infected overnight with RH tachyzoites expressing green fluorescent protein [63] and fixed with 4% paraformaldehyde for 20 minutes. Host cell and parasite nuclei were labelled with Hoechst reagent No. 33342. Once secreted from the dense granules, GRA1, GRA16, and GRA24 can be detected within the vacuolar space; GRA2, 4, 6, 9, and 12 associate preferentially with the intravacuolar membranous nanotubular network (MNN); GRA3, 5, 7, 8, 14, 19, 20, 21, 23 and GRA10, 15, 22, 24, 25 associate with the parasitophorous vacuole membrane (PVM). GRA15, 16, and 24 are also targeted to the host cell nucleus (HC N).