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A host cell membrane microdomain is a critical factor for organelle discharge by *Toxoplasma gondii*

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ABSTRACT

Host cell microdomains are involved in the attachment, entry, and replication of intracellular microbial pathogens. Entry into the host cell of *Toxoplasma gondii* and the subsequent survival of this protozoan parasite are tightly coupled with the proteins secreted from organelle called rhoptry. The rhoptry proteins are rapidly discharged into clusters of vesicles, called evacuoles, which are then delivered to parasitophorous vacuoles (PVs) or nucleus. In this study, we examined the roles of two host cell microdomain components, cholesterol and glycosylphosphatidylinositol (GPI), in evacuole formation. The acute depletion of cholesterol from the host cell plasma membrane blocked evacuole formation but not invasion. Whereas the lack of host cell GPI also altered evacuole formation but not invasion, instead inducing excess evacuole formation. The latter effect was not influenced by the evacuole-inhibiting effects of host cell cholesterol depletion, indicating the independent roles of host GPI and cholesterol in evacuole formation. In addition, the excess formation of evacuoles resulted in the enhanced recruitment of host mitochondria and endoplasmic reticulum to PVs, which in turn stimulated the growth of the parasite.

1. Introduction

Toxoplasma gondii is a widespread and obligate intracellular parasitic protist. It can infect virtually all types of nucleated mammalian and avian cells. As an opportunistic human pathogen, this parasite causes diseases in immunocompromised individuals and in neonates following congenital infection. *T. gondii* belongs to the phylum Apicomplexa, which also includes *Plasmodium*, the causative agent of malaria, and *Cryptosporidium*, responsible for intestinal cryptosporidiosis. One of the features shared by apicomplexan parasites is the presence of an apical complex, which consists of specialized secretory organelles, micronemes and rhoptries. Both of them are involved in host cell invasion [1]. Apicomplexan parasites can infect different types of host cells, but these parasites share a conserved mode of host cell invasion. The sequential secretion of proteins from micronemes and rhoptries enables parasite motility, promotes close attachment of the parasite to the target cell, and leads to its active penetration [2]. Invasion by *T. gondii* is thought to be accompanied by two membrane maturation steps. Following attachment, rhoptry proteins (ROPs) are initially discharged into the host cytoplasm as multivesicular structures referred to as evacuoles (eVs), at least some of which fuse with the parasitophorous vacuole (PV) membrane, which is derived and then further modified from the host cell plasma membrane [3–6]. During invasion, a type of tight junctions, called moving junctions (MJs), form at the circular point of contact between the parasite surface and the host plasma membrane. Because invasion is a rapid process, completed within 15–30 s [7], eV formation is typically examined using cytochalasin D (CytD), which arrests gliding motility and active penetration. CytD-treated parasites still have a high binding capacity for host cells and are able to form MJs. However, while this approach allows detailed morphological characterization of eVs, their molecular composition, including the source of the eV membrane, has yet to be determined.

Microdomains (or lipid rafts) are tightly packed membrane domains rich in cholesterol and sphingolipids [8,9]. Host cell cholesterol is essential for the invasion of *T. gondii* [10] and is found at the PV membrane (PVM) shortly thereafter [11]. Interestingly, however, during the invasion of *T. gondii* [12,13] or *Plasmodium falciparum* [14], the majority of type I transmembrane proteins are excluded, while many other proteins

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within microdomains, such as GPI-anchored proteins, gain access to PVs harboring these parasites. This selective sieving process, called molecular sorting, seems to be controlled by MJs, but the precise mechanism involved is poorly understood. While exclusion of host transmembrane proteins may serve to avoid PVM fusion with host lysosomes [15,16], the significance of the inclusion of microdomain-associated molecules such as GPI-anchored proteins into these structures is unclear. It also remains to be determined whether the sorting of host cell membrane proteins that occurs during the invasion process also takes place during eV formation.

T. gondii extensively associates with host mitochondria and endoplasmic reticulum (ER) at the PVM, concomitant with and continuing after invasion. This phenomenon is observed in vitro and in cells from infected animals [17,18]. Both host cell structures may serve as a source of lipids for *T. gondii* proliferation or PV enlargement [19–21]. Because some ROPs quickly associate with the nascent PVM after their secretion, they are thought to play a role in the association between host organelles and the PVM [19]. ROP2, in particular, has been proposed as the physical link that tethers host mitochondria to the PVM [22]. However, it was recently shown that the knockout of all ROP2 family proteins (ROP2A, ROP2B, and ROP8) does not prevent parasite recruitment of host mitochondria [23]. Mitochondrial association factor 1 (MAF1) was recently identified as one of the mediator of host-mitochondrial association of *T. gondii* [24].

Here, we show that host plasma membrane components are incorporated into eVs and that the uptake of host cell components during eV formation is selective. In addition, we provide evidence that two microdomain components of the host plasma membrane, GPI and cholesterol, play important roles in the formation of eVs but not PVs. Specifically, whereas GPI deficiency causes an increase of eV size, cholesterol depletion results a decrease of eV number. Finally, we show that both *T. gondii* proliferation and the recruitment of mitochondria and ER to PVs are increased in GPI-deficient mammalian cells, probably because of the excess injection of ROPs. The proposed regulatory mechanism may allow *T. gondii* to flexibly adapt to host microdomain dynamics, which may in turn contribute to the extremely broad host range of this parasite.

2. Materials and methods

2.1. Reagents and antibodies

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise. CytD and calcium ionophore A23187 were dissolved in dimethylsulfoxide (DMSO) at 1 mM. Filipin III was dissolved in DMSO at 10 mg/ml and stored at −80 °C. Fluorescently conjugated secondary antibodies were obtained from Life technologies (Carlsbad, CA, USA).

2.2. Cell culture

Tachyzoites of the RH strain of *T. gondii* or genetically engineered mutant strains were maintained by serial passage in HFF grown in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) containing 10 mM HEPES, 10% fetal bovine serum (FBS; Bovogen Biologicals, Victoria, Australia), 2 mM L-glutamine, and 10 μg gentamicin/ml at 37 °C in an atmosphere of 5% CO_2, as described previously [28]. *T. gondii* expressing GFP-tagged ROP16 was a gift from Dr. M. Yamamoto (Osaka University, Japan). Wild-type (WT), GPI-deficient mutants (GPAA1 and M2S2), the complemented GPAA1 clone (cCHO), and GPI-anchored green fluorescent protein (GFP-GPI) of Chinese hamster ovary (CHO) cells were kindly provided from Dr. T. Kinoshita (Osaka University, Japan). The CHOs were grown as monolayers in Ham's F-12medium (Wako) containing 10% FBS.

2.3. PV and eV assays

Parasites were collected by scraping the cell monolayer and released from the host cells by passage through a 21-gauge needle. Extracellular parasites were filtered onto a polycarbonate membrane filter (3.0-μm pore size; Millipore, Bedford, MA, USA) and washed in Hanks' balanced salt solution (Wako) containing 0.1 mM EGTA and 10 mM HEPES [28]. Host cells were cultured on 12-mm glass coverslips (Matsunami glass, Osaka, Japan) for 48 h before the experiment. To promote the formation of eVs, the monolayers were challenged with *T. gondii* pretreated with 1 μM CytD for 10 min at room temperature, at a concentration of 10^8 parasites/ml in DMEM containing 10% FBS. Following challenge for 60 min at 37 °C in the presence of 1 μM CytD, the monolayers were washed in phosphate-buffered saline (PBS) pH 7.4 three times prior to fixation in 4% formaldehyde (FA), dissolved in PBS. For PV assays in Fig. 1, GFP-GPI or CHO monolayers on coverslips were challenged with 10^7 parasites/ml in DMEM containing 10% FBS for 60 min at 37 °C. Infected monolayers were washed with PBS and then fixed in 4% FA.

2.4. Indirect immunofluorescence assay and confocal microscopy

For immunofluorescence microscopy, the infected monolayers were fixed in 4% FA for 20 min at room temperature, and permeabilized by incubation at room temperature for 20 min in PBS containing 0.5% Triton X-100. The permeabilized monolayers were then rinsed three times with PBS, blocked twice in PBS containing 5% normal goat serum and 5% FBS for 10 min at room temperature, and rinsed three more times with PBS containing 1% FBS. Parasites were stained with mouse monoclonal antibody against the surface antigen of *T. gondii*, anti-SAG1 (DG52) [28]. The parasite proteins ROP1 and ROP2 were detected using rabbit or mouse anti-ROP1 and mouse anti-ROP2, respectively. Anti-SAG1, anti-ROP1, and anti-ROP2 were provided by Dr. L.D. Sibley (Washington University School of Medicine, St. Louis, MO, USA). The host cell proteins Src and Cav-1 were detected using anti-Src (36D10) rabbit monoclonal antibody (Cell Signaling Technology, Boston, MA, USA) and anti-Cav-1 rabbit polyclonal antibody (Cell Signaling Technology), respectively, with permeabilization by incubation in PBS containing 0.3% Triton X-100 and staining by overnight incubation with the primary antibodies, according to the manufacturer's instructions. The stained monolayers were then rinsed in PBS containing 1% FBS and incubated with Alexa Fluor (405, 488 or 594) conjugated goat anti-mouse or goat anti-rabbit antibodies diluted into PBS containing 1% FBS. The coverslips were rinsed in PBS and then in DW, mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA), and examined using a confocal laser scanning microscope (Zeiss LSM510 or 780, Carl Zeiss, Oberkochen, Germany).

2.5. Invasion assay

Cells were cultured on 12-mm glass coverslips for 48 h before the experiment, challenged with GFP-expressing *T. gondii* (GFP-Toxo) [30] at a concentration of 2×10^6 parasites/ml for 60 min at 37 °C, washed by PBS three times, and fixed in 4% FA. Extracellular parasites were labeled with anti-SAG1 followed by Alexa 594 staining, without permeabilization. LSM 510 was used to count the number of parasites and total of 300 parasites per coverslip were examined. The ratio of intercellular parasites (green-staining) to total 300 parasites (green plus yellow staining) is defined as invasion efficiency [28].

2.6. Depletion and repletion of host cell cholesterol

Cell monolayers grown on 12-mm glass coverslips were incubated with 10 or 15 mM MβCD for 30 min at 37 °C and then challenged