Host Small GTPases in Apicomplexan Parasite Infection

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The Apicomplexa are obligate intracellular parasites responsible for several important human diseases. These protozoan organisms have evolved several strategies to modify the host cell environment to create a favorable niche for their survival. The host cytoskeleton is widely manipulated during all phases of apicomplexan intracellular infection. Moreover, the localization and organization of host organelles are altered in order to scavenge nutrients from the host. Small GTPases are a class of proteins widely involved in intracellular pathways governing different processes, from cytoskeletal and organelle organization to gene transcription and intracellular trafficking. These proteins are already known to be involved in infection by several intracellular pathogens, including viruses, bacteria and protozoan parasites.

Keywords: Apicomplexa ; GTPases ; host–parasite interactions ; host-targeted therapies

1. Roles of the Host Small GTPases in Intracellular Pathogen Infection

Small GTPases are a superfamily of small proteins of 21–30 kDa involved in signal transduction and characterized by a common “G domain”, which binds the nucleotide guanosine-triphosphate (GTP) and hydrolyzes it to guanosine-diphosphate (GDP). GTPases act like a switch, alternating between an active GTP-bound conformation, in which they bind downstream effectors, and a GDP-bound conformation, in which they are inactive. In the latter conformation, guanosine-diphosphate (GDP) will tend to dissociate from the GTPase, thus allowing a new GTP molecule to bind [1]. Interchange between GTP and GDP is regulated by different classes of GTPase interactors. Guanine nucleotide exchange factors (GEFs) promote GDP release and GTP loading, thus activating the signal. Each GTPase can interact with different GEFs, thus integrating signals from various sources. GTPase activating proteins (GAPs) bind to GTPases in their active conformation, improving their catalytic activity and allowing the protein to quickly return to its inactive state, thus shortening signal duration. Guanine-nucleotide-dissociation inhibitors (GDIs) maintain the GTPase in an off-state, preventing GDP dissociation, thus inhibiting the GTPase activity [2][3].

Small GTPases are divided into five families based on their sequence and structure: Rho, Rab, Ras, Arf and Ran families [4]. These proteins are exploited by all classes of intracellular pathogens both to invade the host cell and to develop and grow once inside the host. Bacteria have evolved different strategies to modulate the host small GTPases. The first consists of producing effectors that mimic GTPase modulators, such as GEFs, GAPs and GDIs. The best documented example of this strategy is the bacterium *Salmonella enterica*, a pathogen that has evolved a unique ability to up- and downregulate the activity of the host small GTPases Cdc42 and Rac1, two actin cytoskeleton key regulators. Upon invasion, *Salmonella* injects, through a needle-like appendage, two bacterially encoded GEFs, SopE and SopE2, inside the host cell. These proteins activate Cdc42 and Rac1, initiating a signal transduction cascade in the host cell that induces actin cytoskeletal rearrangements and membrane ruffling, aimed at facilitating bacterial engulfment and internalization [5]. The host cell morphological changes associated with bacteria uptake are rapidly reversed by *Salmonella* to ensure a minimally disrupted environment to live in. Once inside the host cell, these bacteria produce the secreted effector protein (SptP), a protein that antagonizes Cdc42 and Rac1 by mimicking a GAP and facilitates host cell actin cytoskeleton recovery [6]. SptP is the smallest GAP known so far and shows significant differences in its structure if compared to eukaryotic GAPs. Nevertheless, by analyzing the crystal structure of a SptP–Rac1 complex, it was shown that these differences are not exposed to the protein surface and SptP revealed an unusual GAP architecture that extensively mimics host functional homologs [7]. SptP constitutes an important example of convergent evolution between pathogen and host. Similar strategies have also been reported for many other bacteria that express factors mimicking all classes of GTPase modulators [8].

Another way widely used by intracellular pathogens to hijack the host small GTPases is by modifying them with post-translational modifications, such as ADP-ribosylation, glucosylation, deamidation and transglutamination, resulting either

in their inactivation or activation. For instance, the bacterium *Clostridium botulinum* delivers into the host cell an exoenzyme called C3 that ADP-ribosylates the host small GTPase RhoA, inactivating its signaling pathway [9].

Another example of bacterial toxins modulating host small GTPases through post-translational modifications is the toxin cytotoxic necrotizing factor (CNF) from *Escherichia coli* and *Yersinia pseudotuberculosis*. CNF deaminates the host GTPases Rac1, RhoA and Cdc42, keeping them in an active state, thus stimulating actin polymerization and favoring bacterial entry [10].

Post-translational modifications can also modulate degradation of the host small GTPases through the ubiquitin–proteasome system. This is the case of a toxin called protein adenylyltransferase (VopS), expressed by *Vibrio parahaemolyticus*, that AMPylates Rac1 and RhoA, hindering their interaction with the E3 ubiquitin ligase, thus reducing their degradation. The consequent activation of Rac1 and RhoA signaling pathways inhibits several factors involved in the host response to bacterial infection [11]. On the contrary, the bacterium *Legionella pneumophila* secretes enzymes that ubiquitinate the host small GTPases Rab33b and Rab1, increasing their degradation in order to manipulate host vesicle trafficking to form a protective vacuole in which the bacterium will spend its intracellular life [12].

All classes of small GTPases were also shown to be involved in infection by viruses, at different stages of the viral life cycle, from cell entry [13][14][15][16][17][18], to replication [19][20][21], to late assembly/release of enveloped viruses [18].

Small GTPases are also involved in infection by some intracellular parasites. The three GTPases Rac1, Cdc42 and Arf6 were shown to be involved in host cell invasion by the flagellate parasite *Trypanosoma cruzi* [22][23], the etiological agent of Chagas disease. During *T. cruzi* invasion, these GTPases modulate the actin cytoskeleton at the site of parasite entry, favoring parasite penetration [22][23][24]. A similar mechanism was found also during invasion by the intracellular parasite *Leishmania donovani* that infects macrophages. Rac1 and Arf6 are activated during *L. donovani* entrance and mediate its phagocytosis by acting on actin modulation and on membrane recycling at the site of entry [25]. After invasion, Rac1 localizes to the phagosome where *L. donovani* resides and here, together with Cdc42, participates in the formation of an F-actin shell around the phagosome that arrests phagosomal maturation [26].

An overview on how apicomplexan parasites exploit the host small GTPases to their advantage will be presented.

2. Host Small GTPases in Apicomplexan Infection: The Roles of Rho GTPases

The Rho family comprises about 25 different members, involved in several functions, such as cytoskeleton modulation, cell polarity and cell cycle progression. They act as molecular switches in several pathways that link plasma membrane receptors to cytoskeletal reorganization. Many Rho GTPases can undergo prenylation on their C-terminal domain. This post-translational modification increases the protein hydrophobicity and promotes its subcellular localization to the plasma membrane [27]. Among Rho GTPases, the most studied are RhoA, Rac1 and Cdc42, due to their involvement in tumor progression and metastasis [28].

Because of their role in actin cytoskeleton modulation, GTPases belonging to the Rho family are involved in infection by many intracellular pathogens [7][15][16][17][25][26][29][30][31][32][33][34][35][36][37][38], including several apicomplexan parasites, such as *Plasmodia* [39][40][41][42], *T. gondii* [43][44], *C. parvum* [45][46] and *Theileria annulata* [47]. Moreover, several commercially available inhibitors of Rho GTPases have shown in vitro efficacy against intracellular pathogens, including bacteria [48][49], viruses [50][51] and protozoan parasites [52][53].

The Rho GTPase Rac1 was shown to play an important role in infection by both *T. gondii* and *P. falciparum*. *T. gondii* recruits Rac1 to its PV membrane (PVM) and transfection of a Rac1 dominant-negative mutant (Rac1-N17) impairs the GTPase recruitment, indicating that its activation is required for mobilization to the PVM. The amount of active Rac1 was higher in infected cells compared to the uninfected control, suggesting that the parasite induces Rac1 activation. Moreover, both transfection of a Rac1 dominant-negative form and down-regulation of the endogenous protein by RNA interference led to a significant reduction in parasite invasion rates [43], showing that the GTPase is required for efficient invasion.

Because of its role in many types of cancer, Rac1 has been widely studied and several chemical inhibitors of the GTPase are commercially available and were shown to reduce invasion rates of many intracellular pathogens [16][31][33][35][54]. Several Rac1 inhibitory compounds were tested, targeting either interaction with GEFs or nucleotide exchange activity, on *P. falciparum* in vitro cultures and showed that twelve inhibitors have antimalarial activity. Among them, three showed a

half inhibitory concentration below 1 µM. The most efficient inhibitors were also tested for their cytotoxicity, giving good results on human microvascular endothelial cells (HMEC-1) [40].

The small GTPase Cdc42 was shown instead to be involved in *C. parvum* infection. *C. parvum* primarily infects intestinal epithelia but can also infect other types of epithelia [55]. When ingested, *C. parvum* oocysts excyst in the gastrointestinal tract and release infective sporozoites. The parasites then attach to the apical membrane of the host epithelial cells, inducing the formation of membrane protrusions that encapsulate the sporozoite and form an intracellular but extracytoplasmic parasitophorous vacuole (PV) [56].

3. Host Small GTPases in Apicomplexan Infections: The Roles of Rab GTPases

The Rab family is the largest group of small GTPases, with more than 60 members localized to different intracellular membranes. They are characterized by a reversible association with membranes through geranylgeranyl groups that are attached to their C-terminal end. Rab GTPases are involved in the modulation of vesicular trafficking and protein transport. Each intracellular membrane is characterized by specific Rab proteins [4][57].

Apicomplexan parasites manipulate Rab GTPases by promoting or antagonizing their function, in order to modulate cellular trafficking pathways, move host vesicles near to or inside their PVM, scavenge nutrients from host vesicles and control host phagocytic activity [58].

For instance, *T. gondii* preferentially internalizes vesicles involved in Golgi trafficking, in order to scavenge sphingolipids and other nutrients from the host, which the parasite is auxotrophic for [59]. To facilitate this, some *T. gondii* strains relocate the host Golgi near the PV and fragment it in ministacks [60].

In particular these parasites are able to selectively recognize and internalize to their PV vesicles bound to specific Rab GTPases [59]. Among them, Rab14 and Rab43, two Golgi GTPases, are a clear example of Rab manipulation for parasite needs. When dominant negative Rab14 and Rab43 mutants were expressed in the host cells, sphingolipids showed an aberrant localization in large foci on and within the PVM. Moreover, the sphingolipid amount in the PV lumen was significantly reduced compared to the control, indicating that Rab14 and Rab43 activity is required for an efficient uptake of host sphingolipids [60]. It was also shown that the GTP-bound form of Rab14 is preferentially internalized to the PV, compared to the GDP-bound form, confirming that GTPase activation is required for vesicle delivery to the PV lumen [59]. This mechanism has already been shown for other Rabs, such as Rab1, suggesting this may be a common feature in Rab vesicle internalization [59].

Moreover, *P. berghei* exploits host Rab GTPases to acquire nutrients from the host hepatocyte. Like *T. gondii*, *P. berghei* fragments the host cell Golgi, in order to increase the surface interaction between this organelle and the PVM. It was shown that transfection of a dominant-negative mutant of Rab11a, a GTPase that regulates endosome recycling in the trans-Golgi, prevents the Golgi fragmentation exerted by the parasite and significantly reduces both the number of parasites reaching maturation and parasite size [61].

Rab GTPases are also key regulators of vesicular trafficking in the endocytic pathways in macrophages [62]. Some apicomplexan parasites can interfere with these proteins in order to subvert the host immune response [58].

For instance, *P. berghei* parasites modulate Rab gene transcription in macrophages to their own advantage. It was shown that when macrophages were incubated with *P. berghei*-infected erythrocytes, the expression levels of nine different Rabs regulating phagocytosis increased. In particular, Rab14 plays a protective role for the parasite, since its silencing causes a two-fold increase in phagocytosed parasites, while its overexpression causes a significant decrease [63]. In macrophages, Rab14 is involved in the trafficking to the plasma membrane of CD36, a receptor involved in *P. berghei* phagocytosis. As a matter of fact, it was shown that its silencing leads to an increase in CD36 surface expression due to a reduction in its internalization [64]. It was proposed that the primary uptake of *P. berghei*-infected erythrocytes increases the levels of Rab14, leading to a reduction in CD36 receptor exposed on the macrophage surface, thus inhibiting subsequent phagocytic activity and decreasing parasite clearance [63][64].

4. Host Small GTPases in Apicomplexan Infection: The Roles of Ras, Arf and Ran GTPases

The Ras family includes GTPases with a high degree of sequence identity and function redundancy that are important regulators of signaling pathways linking extracellular signals to gene transcription, cell proliferation, differentiation and

morphology. The Ras genes were the first small GTPase genes to be identified and are the most common oncogenes in human cancer [65].

T. gondii parasites infect a variety of human cells, including dendritic cells (DCs), antigen-presenting cells that trigger adaptive immune response. Infected DCs show a hypermigratory phenotype characterized by amoeboid motility that guarantees parasite dissemination inside the host organism. In order to promote hypermotility, *T. gondii* induces gamma-aminobutyric acid (GABA) secretion and activation of its ionotropic receptor, leading to calcium influx. The increase in Ca^{2+} concentration induces the activation of calmodulin (CaM) and CaM kinase II (CaMkII), which in turn activates the GTPase Ras. Moreover, the MET receptor tyrosine kinase is activated upon *T. gondii* infection and its signaling also activates Ras, which is thus a nodal factor coordinating signals from both GABA and MET receptors. Ras activation leads to extracellular signal-regulated kinase 1/2 (Erk1/2) phosphorylation, which in turn phosphorylates proteins in the nucleus and in the cytoplasm that maintain hypermotility [66].

The ADP-ribosylation factor family (Arf) is composed of six proteins with overlapping functions, characterized by an N-terminal amphipathic domain of 14 amino acids that is myristoylated. When the Arf GTPase is bound to GDP, the N-terminal domain faces towards the catalytic site and is autoinhibitory. When the protein is bound by a GEF, the myristoylated domain swings out of the protein and ensures its association to membranes, allowing GTP binding and consequent GTPase activation.

Arf GTPases mainly regulate organelle structure, membrane biosynthesis, trafficking and interaction with the actin cytoskeleton, lipid transport in the Golgi network and recruitment of proteins that regulate cargo sorting into vesicles. Arfs localize to membranes throughout the cell, including the plasma membrane and the membranes of the secretory, endosomal and lysosomal pathways [67][68].

Arf6, a GTPase involved in membrane trafficking during endocytosis [69], was shown to be involved in *T. gondii* invasion of the host cell. The GTPase is recruited to the PV of invading parasites, and when its activity is inhibited by transfecting the host cell with a dominant-negative form of the GTPase, or when the endogenous Arf6 is inactivated by RNA interference, parasite invasion rates decrease. This indicates that Arf6 is important for *T. gondii* invasion of the host cell, possibly playing a role in membrane invagination and PV formation. In this process, Arf6 is triggered by phosphatidylinositol 3-kinase (PI3-kinase), a kinase that is activated upon invasion. In turn, Arf6 activates phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase) that is responsible for generating phosphatidylinositol 4,5-biphosphate (PIP2), which surrounds the invading parasite and is known to regulate actin cytoskeleton dynamics [70].

The Ran family includes only one member, the protein Ran, characterized by an acidic tail at its C-terminus and by the absence of a membrane-binding motif, present in all the other small GTPases. Ran, unlike the other GTPases that are either cytoplasmic or associated with membranes, localizes both in the nucleus and the cytoplasm. Ran is involved in the regulation of several cellular functions, such as cell cycle progression through the regulation of the cell spindle apparatus and nuclear envelope formation during mitosis, cytoplasm–nucleoplasm transport and RNA nuclear export, and synthesis and processing during interphase [4][71].

The only apicomplexan parasite that was shown to manipulate Ran is *T. annulata*. This is not surprising, since *Theileria* parasites deeply alter the host cell cycle, differentiation and motility to favor their spread in the host organism [72]. They do so both by modulating the host gene transcription and by manipulating its signaling pathways.

These parasites, unlike other apicomplexan, are free in the host cytoplasm and are not surrounded by a PV. In infected cells, the parasite forms the so-called annulate lamellae (AL), a membranous structure localized in close proximity to the parasite, containing pores similar to the nuclear pore complexes (NPCs) [73]. It was recently demonstrated that the components of the AL, including Ran, are in fact derived from the host NPCs. The role of Ran may be linked to the delivery of soluble parasite factors to the host nucleus, in order to subvert gene transcription [74]. Interestingly, two proteins that regulate Ran, the Ran GTPase-activating protein 1 (RanGAP1) and the Ran-binding protein 2 (RanBP2), which are both part of the NPCs, were found to associate with *Theileria* parasites, thus indicating some sort of regulation of host Ran GTPase in proximity to the parasite surface [75].

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