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Rhoptries: an arsenal of secreted virulence factors

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Abstract

Apicomplexan parasites use actin-based motility coupled with regulated protein secretion from apical organelles to actively invade host cells. Critical in this process are rhoptries, club-shaped secretory organelles that discharge their contents during parasite invasion into host cells. A proteomic analysis of the rhoptries in *Toxoplasma gondii* demonstrated that this organelle contains a number of novel rhoptry proteins (ROP) including serine-threonine kinases and protein phosphatases. A subset of ROP proteins have been shown to target to the moving junction, which plays a key role in invasion and parasitophorous vacuole formation. Other ROP proteins have various destinations in the host cell including the host cell nucleus and the parasitophorous vacuole, likely reflecting their distinct targets and roles. Forward genetic analysis recently revealed that secretory ROP kinases dramatically influence host gene expression and are the major parasite virulence factors. Thus, ROP proteins are functionally analogous (although not homologous) to effectors released by type III and IV secretion systems, which are factors that play an important role in bacterial virulence. Deciphering the role of ROP effectors may allow specific disruption of these factors, thus offering new options for preventing disease.

Introduction

Apicomplexan parasites actively penetrate a wide range of cell types, powering their way into the cell with a novel form of actin-based motility [1]. Invagination of the host cell plasma membrane during parasite entry occurs independently of changes in the host cell cytoskeleton or signaling and is not due to host-mediated internalization [2]. During invasion, the tight apposition of the host and parasite membranes forms a visible constriction known as the moving junction, which selectively restricts access of host proteins to the forming vacuole [3]. Most host cell membrane proteins are prevented from entering the forming vacuole by exclusion of transmembrane proteins with cytoplasmic tails and by a process that depends on protein partitioning in the lipid environment of the membrane [4]. This remarkable feat of invasion and vacuole remodeling takes a mere 15–20 seconds to complete, almost an order of magnitude faster than phagocytosis [2]. With motility as the driving force, invasion is mediated by distinct waves of regulated protein secretion from apical organelles, which are designed to release adhesins for attachment and deliver proteins to the host cell or parasite-containing vacuole [5,6].

Rhoptries: an apical secretory organelle involved in invasion

The waves of apical protein secretion begin with the release of microneme proteins, which are involved in attachment of extracellular parasites to the host membrane [6]. A second wave of secretion occurs from apical club-shaped organelles dubbed rhoptries [6] (Figure 1A). Rhoptry proteins (ROP) were first identified based on monoclonal antibodies generated to cell fractions enriched in this organelle [7]. Rhoptry proteins are trafficked from the ER through the Golgi by a conserved pathway [8] prior to being packaged into the apically located secretory organelles. In addition to a classic eukaryotic signal peptide for entrance into the secretory pathway, many rhoptry proteins also contain an additional “pro-domain” that is processed from the mature protein en route to the organelle [12]. For the soluble protein ROP1, the pro-domain has been shown to be necessary and sufficient for rhoptry targeting, although pro-domain cleavage is not necessary for rhoptry targeting or secretion during invasion [9–11]. The pro-domain may also play a role in maintaining rhoptry proteins as zymogens until they reach the rhoptries. Pro-domain cleavage appears to be mediated by the subtilisin protease TgSUB2, although processing roles for other rhoptry proteases cannot be excluded [12]. The processing site in ROP1 and TgSUB2 has been shown to occur at a cleavage site (P1–P4, SΦXE, Φ = hydrophobic) that is unusual for subtilisin proteases [12,13]. If processing turns out to be important for function, this unique pro-domain cleavage event may enable the design of protease inhibitors as therapies that specifically target the parasite.

Rhoptry secretion is initiated very rapidly after intimate contact and is completed within 2 min of invasion [6], implying that the contents of these organelles are vital to formation of the parasite-containing vacuole. Intriguingly, rhoptry protein secretion is still triggered by intimate contact when parasite invasion is blocked by cytochalasin D [14]. Tracking secretory proteins released under a cytochalasin D block revealed that they coalesce into vesicular structures (termed “evacuoles” since they lack a parasite) that resist endocytic fusion and recruit host mitochondria and ER, much like the mature parasite-containing vacuole [14]. Evacuoles are also seen during normal invasion and may be formed from the initial burst of secretion from the rhoptries that occurs in the initial stages of invasion [14] (Figure 1B). During this initial burst, the host plasma membrane is transiently disrupted potentially allowing access to the cytosol before the envelope reseals to envelope the parasite [15,16]. Based largely on these studies, it was proposed that: “secretion of rhoptries at the time of invasion served as a means to deliver effector proteins into the host cell” [14]. While this early hypothesis ultimately proved correct, progress was delayed by the complication that most known ROP proteins at the time had poor conservation and hence, it was difficult to predict their potential functions.

Rhoptry proteomics

Improved methods for obtaining highly purified rhoptries allowed for a more comprehensive analysis of the contents of the organelle via a proteomic approach [17•]. In sum, 38 novel proteins were identified and the purity of the fraction was verified by the production of antibodies in which 12/13 proteins were demonstrated to localize to the rhoptries [17•]. The most abundant proteins in the rhoptries are a family of related proteins, the ROP2 family (including ROP4, ROP7, and ROP8), which contain a conserved serine/threonine (S/T) kinase domain, although most members lack key residues predicted to be necessary for kinase activity [18]. The founding member of the family, ROP2, has been shown to traffic to the cytoplasmic face of the PVM where it is believed to mediate association of host cell mitochondria and ER [19,20] (Figure 1A). Although initially thought to adopt a transmembrane topology in the parasite-containing vacuole [19], this model has recently been questioned based on studies of the related protein ROP5, which also contains a conserved S/T kinase domain [21]. At the core of this domain is a hydrophobic alpha helix, which would be unable to extend across the membrane if the protein folds in a similar manner to well-studied S/T kinases [18]. While

additional members of the ROP kinase family were recognized by the proteomic approach, their significance was not appreciated until recent genetic studies implicated them as virulence determinants (see below). In addition to kinases, the proteome revealed protein phosphatases, insulinase-like and serine proteases, and the actin-binding protein Toxofilin [22], raising the possibility that they perform important functions after being delivered to the host cell cytosol. The rhoptry proteome also contains the small GTPase Rab11 which is likely involved in vesicle based trafficking to the organelle.

Perhaps the most intriguing find was a group of proteins with orthologues in *Plasmodium* (but not outside the Apicomplexa), which represented the first rhoptry proteins in common between these apicomplexans. Surprisingly, antibody localization showed that four of five of these proteins localized specifically to the duct-like neck portion of the rhoptries, leading to their designation as RON (Rhoptry Neck) proteins (Figure 1A). The fact that all of the RON proteins identified have homologues in *Plasmodium* indicates a conserved role throughout the Apicomplexa. In contrast, nearly all of the proteins localizing to the bulb portion of the organelle are unique to either *Toxoplasma* or *Plasmodium*, indicating these proteins are highly adapted to the niche in which these cells invade (e.g. nucleated cells for *Toxoplasma* and erythrocytes/hepatocytes for *Plasmodium*). Furthermore, all of the bulb-localized proteins examined thus far are secreted into the host cell during invasion. A striking example of secretion into the host cell came with the discovery of a protein phosphatase 2C (PP2C-hn) which is secreted from the rhoptries and targeted to the host nucleus during invasion [23] (Figure 1D,E). PP2C-hn contains a nuclear localization sequence, indicating that delivery to the host cytosol is followed by uptake via the host nuclear import machinery. Despite its interesting localization, the precise function of PP2C-hn has thus far been elusive.

Defining the moving junction

The importance of the RON proteins became immediately clear once they were examined in parasites that were arrested during host cell invasion [24•,25•]. Instead of being secreted into vacuoles and the nascent vacuole like the ROP proteins, the RON4 protein was shown to localize to the moving junction (Figure 1A). The proteins forming this point of contact at the host plasma membrane likely serve as an anchor, allowing the actin-myosin motor to “pull” the parasite into the nascent vacuole, although the mechanism of how these proteins are connected to the actin-myosin motor has not yet been determined. In addition, the moving junction appears to function as a molecular sieve, which is likely critical for establishing the non-fusogenic vacuole in which the parasite resides [26]. The selective exclusion of transmembrane proteins anchored in the cytoskeleton suggests that filtering takes place on the cytoplasmic face of the plasma membrane. Alternatively, many proteins appear to gain access to the vacuole by virtue of partitioning into lipid rafts [3,4]. This raises the intriguing possibility that the moving junction is a mechanism to order lipids within the bilayer and thus influence the protein composition of the vacuolar membrane.

Defining the topology of RON complexes and identifying other key players, including possible host cell proteins is crucial to defining the function of the moving junction. Three RON proteins (RON2, RON4, Twinscan4705 [RON5]) exist as a preformed complex within the rhoptry necks prior to release into the moving junction [25•]. Upon release, the RON2/4/5 complex associates with the micronemal protein AMA1, which is secreted from micronemes onto the plasma membrane where it is anchored by a transmembrane domain. Whether or not additional parasite or host proteins are involved and how this complex attaches to the host membrane is unknown. RON4 and RON5 appear to lack transmembrane domains or other obvious sequences that might enable membrane association of the complex. RON2 contains at least two predicted transmembrane domains and thus it may act as a bridge by inserting into the host plasma membrane [17••]. If so, it is possible that host proteins are not necessary for formation of the

moving junction and this would help to explain how *Toxoplasma* can invade any nucleated cell. This scenario shares intriguing similarities with the TIR proteins from enteropathogenic *E. coli*, in which the bacterium inserts its own receptors into the host cell for attachment [27]. Alternatively, the RON2/4/5/AMA1 protein complex could interact with host proteins at the moving junction, but such host receptors would need to be nearly universal to account for the broad host cell range infected by this parasite.

Pin-pointing effectors of virulence

Toxoplasma is equipped with genome sequences for three major lineages (<http://ToxoDB.org>) and excellent methods for reverse [28] and forward genetics [29]. Several recent studies have taken advantage of these tools and the highly clonal population structure of *Toxoplasma* [30], to map genes in the parasite that control important phenotypic differences between parasite lineages. These studies have led to the important discovery that ROP proteins play vital roles in modulating host cell signaling and parasite virulence.

One of the most dramatic phenotypes between *Toxoplasma* lineages is the difference in their acute virulence in mice: type I strains are uniformly lethal in all strains of mice, while types II and III are relatively nonvirulent, at least in outbred mice [30]. Taking advantage of this difference, the genetic basis for virulence in the type I lineage was mapped by analysis of progeny from a genetic cross between types I and III. Remarkably, these studies identified a single gene, designated ROP18, as the major determinant of virulence in the type I lineage [31••] (Figure 2A). The functional role of ROP18 was demonstrated by reverse genetics, taking advantage of the fact that type III strains naturally express extremely low levels of this gene [31••]. Transgenic parasites of the type III genotype expressing the type I allele of ROP18 showed an increased virulence of ~4 logs [31••]. ROP18 is one of the few members of the ROP2 clade that contains conserved residues required for S/T kinase activity [18]. Activity was subsequently demonstrated using *in vitro* expressed protein [32•], and the critical importance of the kinase activity was shown by point mutational analysis in the parasite [31••]. Insight into the biological function of ROP18 came from studies demonstrating it was secreted into the host cell during invasion, occupied vacuoles, and was ultimately trafficked to the parasite containing vacuole [31••,32•] (Figure 1C). The increased virulence of parasites expressing the type I allele of ROP18 is associated with enhanced replication of the parasite (Figure 2A), although other genetic loci also control differences in growth [31••,32•]. Type II and III strains also differ in virulence, but owing to their lower pathogenicity these phenotypes can only be appreciated in inbred mouse lines. A comparison of genes required for pathogenesis of type II vs. III strains by a similar genetic mapping strategy revealed that ROP18, as well as at least 4 other loci, also contribute to pathogenesis in these genetic backgrounds [33•].

Global analysis of how *Toxoplasma* alters host cell transcription led to identification of another secreted rhoptry protein, ROP16, that is targeted to the host cell nucleus [34••] (Figure 2B). A comparison of strain-specific differences in the induction of host cell transcriptional profiles detected by microarray was used to identify pathways that were differentially affected by parasite genes. This approach took advantage of genetic crosses between type II and III strains to map specific genomic regions in the parasite that mediate changes in host cell transcription ultimately leading to the identification of ROP16 as the effector [34••]. ROP16 is rapidly injected into host cells during invasion and it makes its way to the host cell nucleus owing to the presence of a nuclear localization sequence, although this element may not be crucial to its function. ROP16 is predicted to be an active S/T kinase and while its direct targets have not been identified, it acts to sustain the phosphorylation of STAT3, a negative regulator of Th1 immune responses. ROP16 is highly polymorphic and genetic analysis reveals that the allele shared by genotypes I and III is effective in mediating sustained phosphorylation of STAT3, while the allele found in type II does not [34••]. Activation of STAT3/6 may explain the much

lower levels of IL-12 that are induced by type I or III versus type II strains following infection of macrophages [34,35]. By modulating host gene expression in this manner, type I and type III strains may be able to avoid detection by the immune system while type II strains seem adapted to trigger early responses, which results in greater immune mediated pathology in mucosal and CNS models of infection in the murine host.

Conclusions and future directions

Recent studies have demonstrated that the rhoptries are central to host cell invasion and establishment of the intracellular vacuole. The remarkable number of targets of rhoptry proteins including the moving junction, vacuole, vacuolar membrane and host cell compartments highlight the versatility of the rhoptries in regulating different aspects of intracellular survival. Perhaps most exciting is the direct injection of polymorphic kinases into the host cell which revealed a novel mechanism for parasite alteration of host cell gene expression and enhanced growth leading to virulence. This process is mechanistically distinct, yet analogous to the secretion of bacterial effectors into host cells. While a great deal is known about specific targets and pathways influenced by the latter, very little is understood about the potential downstream alterations in the host cell due to ROP effectors. The molecular mechanism whereby ROP16 and ROP18 exert their influence remain uncertain, but are likely to involve phosphorylation of host substrates (Figure 2). Additionally, the S/T family of ROP kinases contains 7 other members, albeit many of them degenerate, that are also likely to make their way into the host cell. Given the excellent genetic and cell biological tools available in *Toxoplasma*, it is likely additional insights into the role of ROP effectors will be rapidly forthcoming.

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Figure 1.

Secretion of rhoptry proteins during host cell invasion. **A)** Diagram showing rhoptry secretion during invasion. The rhoptry neck proteins (RON2/4/5, red) are released into the moving junction where they likely aid in invasion and the filtering of host transmembrane proteins from the nascent vacuole. The rhoptry body proteins (ROPs, green) are initially released into the host cell where they can then be targeted to the host nucleus or to the cytoplasmic face of the vacuole. ROPs are also released into the vacuole during invasion. **B)** Immuno-EM showing ROP1 (immunogold label) released into the cytosol during invasion (used with permission [14]). Tg = parasite; R, rhoptry; PV, parasite-containing vacuole, arrows indicate the limit of the vacuole membrane, * denote satellite vesicles that also contain ROP1 (i.e. evacuoles). Scale bar = 250 nm. **C)** Immunofluorescence staining of ROP18 (green) and ROP1 (red) surrounding nascent parasite-containing vacuoles and in evacuoles within the host cell cytosol following invasion (used with permission [31••]). **D, E)** Targeting of the ROP phosphatase PP2C-hn to the host nucleus following secretion (phase contrast and immunofluorescence).

Figure 2.

Summary of two major ROP effectors in *Toxoplasma*. **A)** ROP18 is secreted into the host cell at the time of invasion and is subsequently targeted to the parasite-containing vacuole.

Expression of the type I allele confers increased growth and virulence [31••,32•]. **B)** ROP16 is released into the host cell but traffics to the host cell nucleus. The allele shared by types I and III induce prolonged phosphorylation of STAT3, a negative regulator of Th1 immunity [34••]. Both proteins have an N-terminal signal peptide and conserved S/T kinase domain.