Host Cell Invasion by *Toxoplasma gondii* Is Temporally Regulated by the Host Microtubule Cytoskeleton†‡

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*Toxoplasma gondii* is an obligate intracellular protozoan parasite that invades and replicates within most nucleated cells of warm-blooded animals. The basis for this wide host cell tropism is unknown but could be because parasites invade host cells using distinct pathways and/or repertoires of host factors. Using synchronized parasite invasion assays, we found that host microtubule disruption significantly reduces parasite invasion into host cells early after stimulating parasite invasion but not at later time points. Host microtubules are specifically associated with the moving junction, which is the site of contact between the host cell and the invading parasite. Host microtubules are specifically associated with the moving junction of those parasites invading early after stimulating invasion but not with those invading later. Disruption of host microtubules has no effect on parasite contact, attachment, motility, or rate of penetration. Rather, host microtubules hasten the time before parasites commence invasion. This effect on parasite invasion is distinct from the role that host microtubules play in bacterial and viral infections, where they function to traffic the pathogen or pathogen-derived material from the host cell's periphery to its interior. These data indicate that the host microtubule cytoskeleton is a structure used by *Toxoplasma* to rapidly infect its host cell and highlight a novel function for host microtubules in microbial pathogenesis.

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that is capable of causing disease in fetuses and immunocompromised individuals (23). The parasite infects a wide range of nucleated cells of most warm-blooded animals. The mechanisms underlying this wide tropism are not known but could be due to either the parasite infecting cells using a ubiquitously expressed host receptor and associated machinery, inserting its own receptor into the host cell's plasma membrane, or using multiple host cell receptors/machinery (5).

*Toxoplasma* invasion is a multistep, complex process consisting of parasite contact to host cells, intimate attachment, parasite motility, and then penetration (5). Host cell contact is a loose, low-affinity interaction that is mediated by parasite surface antigens. An unknown signal then triggers the release of proteins from a specialized secretory organelle called micronemes whose contents include proteins that function as adhesins. This is then followed by parasite gliding motility on the host cell surface. At some point, proteins from a second secretory organelle, named rhoptries, are exocytosed. Among these rhoptry proteins, several (RON2, RON4, RON5, and RON8) are part of a preformed complex that binds the previously secreted AMA1 microneme protein (1, 2, 20, 33). Together, these proteins form the moving junction complex, which defines the parasite entry site on the host cell plasma membrane. Parasite penetration occurs by the parasite propelling itself forward, via acto-myosin-dependent motility, into the host plasma membrane (35). This causes an invagination of the plasma membrane resulting in the formation of the parasitophorous vacuole (PV), which is the compartment that the parasite resides in throughout its time in the host cell. However, host plasma membrane-associated proteins are selectively incorporated into the developing PV such that glycosylphosphatidylinositol (GPI)-linked proteins are included, while single-pass transmembrane proteins are excluded (7, 24).

In contrast to parasite molecules that function during invasion, few host cell components involved in this process are known. A notable exception is the finding that host Arp2/3-dependent actin polymerization promotes *Toxoplasma* invasion (11). Nevertheless, how actin or other host molecules function during invasion remains to be determined. The host microtubule cytoskeleton has been widely studied for its role during receptor-mediated endocytosis, as well as in bacterial and viral infections, where microtubules act to facilitate cargo transport from the host cell periphery to the interior (8, 15, 27, 29, 40). Consistent with this role in cargo transport, host microtubules also promote trafficking of rhoptry proteins secreted into the host cell (12). However, whether this host cell structure functions during parasite invasion *per se* is unknown.

Here, we tested the hypothesis that host microtubules are used by *Toxoplasma* tachyzoites to penetrate into its host cell. Using synchronized parasite invasion assays, we find that disruption of host microtubules significantly reduces parasite invasion into host cells early after stimulating parasite invasion but not at later time points. Host microtubules are localized to the moving junction but, unlike their previously described role in pathogen invasion, host microtubules...
promote tachyzoite invasion by hastening the time that parasites initiate invasion.

**MATERIALS AND METHODS**

**Cell lines and parasites.** Human foreskin fibroblasts (HFFs), HeLa, Vero, and CHO cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). LLC-PK1 cells stably expressing green fluorescent protein (GFP)-tubulin were from Patricia Wadsworth (University of Massachusetts). CHO cells stably expressing N-terminal hemagglutinin (HA)-tagged CD59 were previously described (19). CD59 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Toxoplasma gondii strain TgSAG1 was used for all experiments. Transiently transfected, stably transformed, or recombinant parasites were used in all experiments.

**Toxin secretion.** CHO cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). CHO cells stably expressing N-terminal hemagglutinin (HA)-tagged CD59 were previously described (19). Transiently transfected, stably transformed, or recombinant parasites were used in all experiments.

**Tomato red fluorescent protein (TRFP).** Parasites were released from their host cells by passing them three times through a 27-gauge syringe needle. High potassium-based buffer, which permits attachment but blocks parasite penetration, was then used to wash parasites in high potassium buffer and then switching to a medium with a low-potassium-containing buffer (e.g., DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine, and antibiotics. Host cultures were maintained in DMEM plus 10% FBS. Parasites and cells were fixed and then blocked with 3% bovine serum albumin in 0.1% Triton X-100, permeabilized with 0.1% Triton X-100, and then stained with rat anti-HA (Roche Indianapolis, IN) and Alexa Fluor-488 goat anti-rabbit (Invitrogen). Images were analyzed by confocal microscopy as described above.

**Video microscopy.** A 35-mm² glass-bottom dish (World Precision Instruments, Sarasota, FL) containing either mock- or nocardozole-treated HFFs plated on 12-mm² coverslips that were fixed with 0.1% glutaraldehyde–4% formaldehyde for 15 min at room temperature and extensively washed with PBS. GFP + parasites (107) were added, followed by incubation for 1 h at 37°C. Parasites and cells were fixed and then blocked with 3% bovine serum albumin (BSA), and parasite plasma membrane deposits left behind by the gliding parasite were detected with rabbit anti-SAG1 as previously described (10).

**Confocal microscopy.** GFP-tubulin cells were infected with TRFP + parasites using the potassium-based synchronized invasion assay. At the indicated time points, cells were fixed with glutaraldehyde-formaldehyde and blocked with 3% BSA in PBS. Nonpermeabilized cells were stained with rabbit anti-SAG1 and Alexa Fluor-647 goat anti-rabbit (Invitrogen). Coverslips were mounted on glass slides and serial x-z-stack images (0.25-µm steps/section) were collected with a Leica SP2 MP confocal microscope. Images were deconvoluted and three-dimensional (3D) reconstructions developed using the Velocity 3D imaging software (Perkin-Elmer Waltham, MA). To properly assign colocalization with the moving junction, 3D reconstructions were necessary for all image analysis except for RON4, as discussed below. f-actin was detected in 3% paraformaldehyde-fixed HFFs with Alexa Fluor 568-conjugated phalloidin (Invitrogen). Microtubules were scored as localized to the parasite moving junction if one or more microtubules spanned across the width of the moving junction. If microtubules were not associated with the moving junction but spanned across the width (but not the length) of an invading parasite a score of “distal to moving junction” was assigned. Images were independently analyzed by two individuals in a nonblinded fashion, and their counting was found to significantly correlate with each other and thus was used to generate averages and standard deviations. Conventional confocal microscopy with the rhodamine chelator TRITC was used to visualize parasites when it was not possible because glutathione depletes actin filaments (21), and microtubules are poorly preserved in cells fixed only with formaldehyde. To detect HA-tagged CD59, cells were formaldehyde fixed, stained with rabbit anti-SAG1 Alexa Fluor-594 chicken anti-rabbit (Invitrogen), permeabilized with 0.1% Triton X-100, and then stained with rat anti-HA (Roche Indianapolis, IN), and Alexa Fluor-488 goat anti-rabbit (Invitrogen). Images were analyzed by confocal microscopy as described above.

**RESULTS**

Disruption of the host microtubule cytoskeleton transiently decreases Toxoplasma invasion. Toxoplasma gondii invasion is a multistep, complex process in which parasites adhere to and then penetrate into cells (5). These two steps can be dissociated by adding parasites to cells in a high-potassium-containing buffer, which permits attachment but blocks parasite penetration (16). Penetration can then be stimulated by replacing the medium with a low-potassium-containing buffer (e.g., DMEM) that is permissive for invasion. By allowing parasites to adhere to host cells in the high-potassium buffer and then switching to low-potassium buffer, parasite invasion can be synchronized. Using this synchronized invasion assay, we measured Toxoplasma invasion following detailed time points. Thus, freshly egressed GFP-expressing tachyzoites were added to HFFs in high potassium buffer and after 20 min parasite invasion was stimulated by adding invasion buffer. At each time point, the
cells were fixed and stained with antisera to the *Toxoplasma* surface protein SAG1 without permeabilization to discriminate between intracellular and extracellular parasites. The data indicated that parasite invasion began within 1 min of adding invasion medium and rapidly increased during the first 4 min, at which time 70% of the total number of parasites that would invade in the first hour had invaded. Over the next 56 min, parasite invasion continued but at a significantly reduced rate (Fig. 1A).

We next used a temperature-based synchronized parasite invasion as a second method to examine invasion kinetics. Parasites were added to host cells at room temperature, which is a temperature nonpermissive for invasion, and then immediately centrifuged at 250 × g for 3 min at room temperature. Invasion was initiated by transferring the cells to a 37°C heat block, after which the cells were fixed at specific times, and invasion was quantified by differential SAG1 staining. Relative to potassium-synchronized parasites, temperature-synchronized parasites invaded more slowly requiring 10 min before 70% of the parasites invaded (Fig. 1B).

We next tested how loss of host microtubules impacted *Toxoplasma* invasion by comparing invasion in the absence or presence of the microtubule depolymerizing agent, nocodazole. Mock- and nocodazole-treated host cells were infected with parasites using the potassium synchronization assay. The cells were fixed 1, 2, 5, and 60 min later, and invasion was quantified by differential SAG1 staining. The data indicated that in nocodazole-treated host cells, parasite invasion was reduced by more than 70% 1 min after adding invasion buffer and by ca. 50% after 2 min (Fig. 2A). By 5 min, no significant differences between mock- and drug-treated cells were observed ($P = 0.58$). Similarly, early but not late parasite invasion was significantly reduced when host cells were treated with demecolcine, which also destabilizes microtubules through a mechanism distinct from nocodazole, or taxol, which prevents microtubule depolymerization (Fig. 2B). The effect of these drugs on parasite invasion was not cell type specific since early, but not late, invasion of Vero and CHO cells was also reduced by nocodazole (see Fig. S2 in the supplemental material). Finally, nocodazole had a comparable effect on parasite invasion when invasion was synchronized using the temperature-shift assay (Fig. 2C).

Having established that host cell invasion was similarly affected by nocodazole with both synchronization methods, we chose to perform the rest of our assays with the potassium shift method since it more efficiently synchronizes invasion, which is a conclusion reached by others (16). This is most likely because parasites reach equilibrium more quickly and uniformly after the buffer switch than by shifting a room temperature plate of cells to a 37°C heat block. In addition, after parasites are pelleted onto host cells at room temperature they must first become intimately associated with the host cell since microneme secretion is negligible at room temperature (6).

Nocodazole is unlikely to target the parasite’s microtubule cytoskeleton since *Toxoplasma* tubulin is only sensitive to nocodazole at concentrations 1,000-fold higher than those used here (26). In addition, the parasite’s subpellicular microtubules are not dynamic during invasion and only disassemble as parasites progress through the cell cycle and replicate (32). For this reason, *Toxoplasma* invasion is unaffected even when extracellular tachyzoites are incubated with microtubule inhibitors that can specifically target the parasite’s, but not the host’s, microtubule cytoskeleton (32). To further ensure that nocodazole was not impacting the parasites in an unforeseen and irreversible manner, extracellular parasites were pretreated with nocodazole, washed, and then evaluated in the invasion assay using untreated host cells. As expected, pretreating parasites with nocodazole had no detectable effect on invasion (Fig. 2D). Although, we cannot rule out nocodazole having a reversible affect on the parasite, our data together with the above-mentioned studies indicate that the effects of nocodazole and the other microtubule inhibitors on parasite invasion are a consequence of the drugs affecting the host microtubule cytoskeleton.

**Host cell microtubules localize to the moving junction of early invading parasites.** We next assessed host microtubule localization and organization during parasite invasion by infecting GFP-tubulin expressing host cells with rFP' parasites using the synchronized invasion assay. At 2 min after the addition of invasion buffer, the cells were fixed and stained with anti-SAG1 antisera without permeabilizing the cells. Confocal z-stacks underwent 3D reconstruction to accurately determine...
Host microtubules hasten parasite penetration. Parasite invasion consists of at least four stages: host cell contact, intimate attachment, gliding motility, and penetration (5). To assess whether disruption of host microtubules affected the ability for parasites to engage in loose, low-affinity contact with the host cell, parasites were added to mock- or nocodazole-treated cells in high potassium buffer and then switched to invasion buffer for 2 or 60 min, at which time the cells were fixed without washing so as to not remove the loosely attached parasites. No differences were observed in the numbers of parasites remain-

![Graph](image)

**FIG. 2.** Host microtubules are important for *Toxoplasma* Invasion. (A) HFFs were treated with dimethyl sulfoxide (DMSO) or 1 μM nocodazole for 3 h at 37°C. GFP<sup>+</sup> parasites were then added to the cells in vehicle- or drug-containing high-K<sup>+</sup> buffer and incubated for 20 min. K<sup>+</sup> buffer was removed and replaced with DMSO- or nocodazole-containing invasion buffer. At the indicated times, cells were fixed, and the numbers of intracellular and extracellular parasites were determined by differential SAG1 staining. The averages and standard deviations of three independent experiments counting a minimum of 200 randomly selected parasites for each sample are shown. *, P < 0.05; **, P < 0.005 (Student t test). (B) HFFs were mock treated or treated with a 1 μM concentration of the indicated drug for 3 h at 37°C and then infected with GFP<sup>+</sup> parasites using the potassium synchronization method. At the indicated times, the cells were fixed and stained with anti-SAG1 to discriminate between extracellular and intracellular parasites. The averages and standard deviations of three independent experiments counting a minimum of 200 randomly selected parasites for each sample are shown (*, P < 0.05; **, P < 0.005 [Student t test]). (C) DMSO- or nocodazole-treated HFFs were infected with GFP<sup>+</sup> parasites using the temperature-shift synchronization method. At the indicated times, cells were fixed and stained with anti-SAG1 to discriminate between extracellular and intracellular parasites. The averages and standard deviations of three independent experiments counting a minimum of 200 randomly selected parasites for each sample are shown (***, P < 0.005 [Student t test]). (D) “Treated HFFs” are host cells pretreated with 1 μM nocodazole or DMSO for 3 h at 37°C and then infected with GFP<sup>+</sup> parasites. “Treated parasites” are extracellular GFP<sup>+</sup> parasites treated with 1 μM nocodazole or DMSO in DMEM for 1 h at 37°C and then washed. The washed parasites were added to host cells in high-K<sup>+</sup> buffer, and then invasion buffer was added. Two minutes later, the cells were fixed, and the numbers of invaded parasites were determined by SAG1 differential staining. The averages and standard deviations of three independent experiments counting a minimum of 200 randomly selected parasites for each sample are shown (*, P < 0.05 [Student t test]).
ing associated with the mock- or nocodazole-treated host cells (Fig. 6A). To next test whether nocodazole affects intimate attachment, the assay described above was repeated, but loosely associated parasites were removed by rigorous washing before the host cells were fixed. Nocodazole had no significant impact at either time point on the parasites remaining under these conditions (Fig. 6B). Numbers of intimately attached parasites remaining after 2 min increased at the 60-min time point, which is consistent with the finding that only a certain percentage of contact stage parasites will rapidly release their micronemes, while others will take longer to become intimately attached (17). Taken together with the finding that nocodazole reduced early but not late invasion, these data demonstrate that nocodazole has no detectable impact on parasite contact or intimate attachment. Coupled with the fact that nocodazole did not appear to have severe effects on host cell morphology under these conditions (see Fig. S1 in the supplemental material), these data also indicate that differences in parasite invasion are not due to the drug altering host plasma membrane surface area.

Gliding motility is a specialized mode of parasite motility on the host cell surface that precedes host cell penetration. A common readout for gliding motility is detection of parasite-derived surface depositions that appear as long, circular trails on serum-coated glass coverslips (10, 13). To examine whether disrupting host microtubules affected gliding motility, GFP parasites were incubated at 37°C with formaldehyde-fixed host cells that were either mock or nocodazole treated. After 60 min, the cells were fixed and stained to detect SAG1 deposited by motile parasites. No dramatic differences in SAG1 deposition were observed (Fig. 6C). It should be noted, however, that SAG1 deposition on host cells was not as widespread as on serum-coated coverslips. In addition, these depositions appeared as periodic deposits rather than the long continuous ones left on glass surfaces. The basis for these differences is unclear, but their irregular shape and size precluded us from being able to develop quantifiable criteria (e.g., trail length, intensity, etc.).

As Toxoplasma penetrates into its host cell, the parasite selectively incorporates host plasma membrane GPI-linked proteins into the developing PV, while excluding single-pass transmembrane proteins (7, 24). To test whether disrupting
host microtubules affected plasma membrane partitioning during penetration, cells stably expressing GPI or transmembrane versions of HA-tagged CD59, which is a naturally occurring GPI-linked protein (28, 31, 34), were mock or nocodazole treated and then infected with trFP⁺ parasites for 2 min using the synchronized invasion assay. PV association of either ver-

FIG. 4. Host microtubules associate with the moving junction of early, but not late, invading parasites. GFP-tubulin cells were infected with trFP⁺ parasites for 2 or 4 min and then fixed and analyzed by confocal microscopy. 3D reconstructions were used to determine how often and where (relative to the moving junction) host microtubules were associated with invading parasites. Means and standard deviations from three independent experiments counting 15 randomly selected invading parasites for each sample are shown (*, P < 0.005 [Student t test]).

FIG. 5. Host microtubules and RON4 colocalization at the moving junction. GFP-tubulin cells were infected with trFP⁺ parasites for 2 min, methanol fixed, and then stained to detect RON4 and SAG1. The white arrows are pointing to the moving junction identified as a ring of RON4 protein at the parasite constriction (scale bar, 5 μm). The RON4 staining in front of the moving junction is apical nonexocytosed protein (1).

FIG. 6. Disruption of host microtubules has no apparent affect on parasite contact, intimate attachment, or gliding motility. (A) GFP⁺ parasites in high-K⁺ buffer were added to mock-treated (■) or nocodazole-treated (□) HFFs and switched to invasion medium for the indicated times. The medium was then removed, and the cells were fixed without washing. The numbers of cell-associated GFP⁺ parasites and host nuclei were counted. Means and standard deviations from three independent assays counting 10 random fields for each sample are shown. (B) GFP⁺ parasites in high-K⁺ buffer were added to mock-treated (■) or nocodazole-treated (□) HFFs. The medium was removed, after which the cells were washed three times with ice-cold PBS and then fixed. The numbers of cell-associated GFP⁺ parasites and host nuclei were counted. The means and standard deviations from three independent experiments counting 10 random fields for each sample are shown. (C) GFP⁺ parasites were added to formaldehyde-fixed and washed mock- or nocodazole-treated cells for 60 min at 37°C. The cells were fixed and stained with anti-SAG1. Representative images from three independent experiments are shown.
sion of epitope-tagged CD59 was defined by using the criteria developed by Mordue et al. (24): a rim of staining enveloping the internalized region of the parasite. The incorporation of HA-CD59GPI into the developing PV was not significantly altered in nocodazole-treated host cells (Fig. 7A; CD59 GPI). Using HA-CD59, we did note a slight but not statistically significant increase in HA-CD59 associated with parasites invading nocodazole-treated cells \( (P = 0.078 \text{ [Student } t\text{-test]}) \). Thus, nocodazole did not significantly impact exclusion of HA-CD59 from the forming PV (Fig. 7B; CD59).

Confocal fluorescence video microscopy imaging of tRFP parasite invasion of GFP-tubulin cells was next used to measure rates of parasite invasion. To ensure that rates were properly determined, only parasites for which we could definitively identify the moving junction (defined as a constriction) starting at the anterior end and ending at the posterior end were included in the analysis. In mock-treated host cells, parasite invasion was completed within \( 23.1 \pm 6.0 \text{ s} \) \( (n = 20) \) (Fig. 8A). Similarly, penetration into nocodazole-treated cells was completed in \( 24.7 \pm 10.3 \text{ s} \) \( (n = 29) \), which was not significantly different than mock-treated cells \( (P > 0.79 \text{ Student } t\text{-test}) \). These data are also consistent with previous studies reporting that tachyzoites require 25 to 30 s to fully penetrate into the host cell (25, 35). Finally, these data further support our conclusion regarding gliding motility from the SAG1 deposition assays (Fig. 6C) since the parasite’s acto-myosin machinery power both gliding motility and host cell penetration (5, 13).

Collectively, our data revealed that parasite attachment, motility, and penetration rate were independent of host microtubules. These data led us to hypothesize that host microtubules shortened the time before parasites initiated invasion. To test this hypothesis, we used fluorescence video microscopy to determine how long after adding invasion media the moving junction could first be detected at the anterior end of the parasite. Since it took approximately 45 s after the addition of invasion buffer to select a field and initiate imaging, we expected that some parasites would have already completely invaded its host cell. We identified invaded parasites by two criteria: (i) the parasites remained stationary, and (ii) the parasites displaced host microtubules but were not surrounded by them, which is consistent with other work showing that host microtubules associate with the PV at least 60 min postinvasion (37).

In the absence of nocodazole, ca. 65% of parasites completed invasion before imaging could be started (Fig. 8B). Parasites that invaded after initiating invasion did so after ~147 s. In contrast, significantly fewer parasites penetrated nocodazole-treated cells before imaging began and those parasites that had invade began after ~245 s, which was significantly later than mock-treated cells. Together, these data demonstrate that host microtubules act to shorten the time before parasites initiate host cell invasion.

**DISCUSSION**

Host cell invasion, a critical step in *Toxoplasma*’s lytic life cycle, is composed of a series of finely orchestrated events involving attachment, gliding motility, moving junction formation, and penetration. The host cell factors involved in each of these stages are unknown. Here, we demonstrated that *Toxoplasma* invasion is temporally dependent on host microtubules. In contrast to the well-established function of microtubules during endocytosis, as well as during bacterial or viral host cell invasion (8, 15, 27, 29, 40), host microtubules affected *Toxoplasma* invasion by hastening the time that parasites initiated host cell invasion. These data are significant in that they define a host cell structure that functions during *Toxoplasma* invasion. In addition, they are, to our knowledge, the first demonstration that host microtubules define when an intracellular pathogen initiates host cell penetration.
Several critical cellular processes are dependent on the microtubule cytoskeleton, including cytokinesis, cell shape, and membrane trafficking. Since our assays were done both with quiescent fibroblasts and replicating cells (Vero, CHO, and LLCPK-1), it is unlikely that cell cycle regulation is associated with how host microtubules promote early parasite infection. In addition, dramatic changes in cell shape, which could affect the available surface area for parasite attachment, do not appear to underlie the effect of nocodazole on parasite invasion for two reasons. First, under the conditions in which we performed our assays, we did not note significant changes in cell shape or morphology. Second, the drug had no significant effect on numbers of parasites in contact with host cells. Moreover, we could not detect differences in numbers of intimately attached parasites, indicating that host microtubules have no apparent effect on micronemal adhesin secretion or for host plasma membrane expression of their receptors. Finally, the lack of an effect of nocodazole on the rate of parasite penetration indicates that host microtubule-based trafficking does not help the parasites to propel themselves into the host cell.

Host microtubule hastening of tachyzoite penetration could be a result of three different but not necessarily mutually exclusive mechanisms. First, host microtubules may organize a host plasma membrane-based protrusion that acts as a fulcrum for gliding parasites to use as a pivot to begin to penetrate into the host cell. The concentration of host microtubules on one side of the moving junction suggests that once a parasite encounters such a structure the parasite would begin to invade while the structure remained stable at the site of parasite invasion. Second, host microtubules may provide support to help withstand the compressive force induced by a parasite in contact with the host cell. Such a hypothesis is consistent with the tensegrity model of cellular responses to mechanical force. In this model, the cells are prestressed structures that use microtubules to provide resistance to compression (14). As examples, microtubules are required to help cells respond to mechanical stresses such as artificial stimuli (e.g., glass pipette-mediated plasma membrane deformation) or physiological stimuli (e.g., cardiomyocyte contractility or endothelial cell responses to thrombin) (3, 14, 38).

A third possibility is that host microtubules may aid in promoting the efficient formation of a functional moving junction. The moving junction complex is composed of the micronemal protein AMA1 and the preformed rhoptry-derived RON complex that only bind one another after they are secreted (1, 20). The RON complex is composed of several proteins that include RON2, which is associated with the host plasma membrane most likely as a multipass transmembrane protein, and RON4, RON5, and RON6 that are exposed to the host cytosol (2, 33). One current model of rhoptry secretion is that rhoptry proteins are released into the host cell within membranous whorled-structures (12). While trafficking of some rhoptry proteins to the interior of the cell is host microtubule dependent (12), it is unknown what would direct the RON complex to the host plasma membrane. Thus, we propose a model where the RON complex is secreted into the membranous whorls, associates with host microtubules either directly via one of its host cytoplasm-exposed proteins or indirectly by binding a microtubule-binding protein and uses these host microtubules to traffic to the host plasma membrane where it can bind AMA1 and form the moving junction. When host microtubules are disrupted, RON complex delivery to the plasma membrane would be less efficient and thus cause a lag in the time before parasites could begin to invade.

Association of host microtubules with the moving junction was defined as a specific process since host microtubules were associated significantly more often with early invading parasites than late invading ones. In addition, we and others (9) failed to detect host f-actin at the moving junction of a majority of invading parasites. Finally, host microtubules were asymmetrically organized at the moving junction instead of evenly dispersed around it. Thus, we do not believe that microtubule association with the moving junction was simply due to the enlarging PV pushing host cytoplasmic contents aside. Whether asymmetric organization is functionally significant is unclear, but this observation is consistent with all three of our hypotheses. For example, the microtubule-based plasma membrane protrusion used by the parasite as a pivot may remain intact as a parasite
continues to invade. Likewise, the microtubules may need to remain intact to help the cell withstand the compressive forces induced as a parasite continues to invade. Finally, host microtubules may not disperse after aiding in helping to sort rhoptry proteins.

Our data showed that nocodazole had a dramatic affect on early parasite invasion, which was overcome at later time points. However, it is unclear whether these data indicate that Toxoplasma gains entry to its host cell using distinct sets of host factors/pathways (at least one of which is rapid and microtubule dependent). Other pathogens with wide host cell tropisms also utilize multiple host cell pathways to gain entry. For example, Trypanosoma cruzi can infect host cells by either recruiting host lysosomes or by activating host phosphoinositide 3-kinase (36, 39). It is also possible that host microtubules are utilized as a parasite continues to invade. Finally, host microtubules remain intact to help the cell withstand the compressive forces induced as a parasite continues to invade. Likewise, the microtubules may need to remain intact to help the cell withstand the compressive forces induced as a parasite continues to invade. Finally, host microtubules may not disperse after aiding in helping to sort rhoptry proteins.

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