

***Trypanosoma cruzi* trypomastigotes induce cytoskeleton modifications during HeLa cell invasion**

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It has been recently shown that Trypanosoma cruzi trypomastigotes subvert a constitutive membrane repair mechanism to invade HeLa cells. Using a membrane extraction protocol and high-resolution microscopy, the HeLa cytoskeleton and T. cruzi parasites were imaged during the invasion process after 15 min and 45 min. Parasites were initially found under cells and were later observed in the cytoplasm. At later stages, parasite-driven protrusions with parallel filaments were observed, with trypomastigotes at their tips. We conclude that T. cruzi trypomastigotes induce deformations of the cortical actin cytoskeleton shortly after invasion, leading to the formation of pseudopod-like structures.

Key words: *Trypanosoma cruzi* - cell invasion - cytoskeleton - membrane protrusion

Trypomastigote forms of *Trypanosoma cruzi*, which is the causative agent of Chagas disease, that are derived from infected cells are the parasite form responsible for spreading mammalian infections. This process occurs in the acute phase of the disease or during reactivation following immunosuppression (Schofield et al. 2006, Alves & Mortara 2009). *T. cruzi* triggers several host cell receptors and signalling pathways to invade cells (Burleigh & Woolsey 2002, Yoshida & Cortez 2008). Recently, it was shown that the rapid compensatory endocytosis that follows membrane repair is one such mechanism that is exploited by trypomastigotes (Fernandes et al. 2011). Mechanical pressure exerted by parasites that are attached to or underneath host cells could be a source of plasma membrane wounding, which is required to induce an endocytic response. Another intriguing aspect that accompanies the invasion of HeLa and other cell types is the formation of cellular protrusions (Fernandes et al. 2011), or pseudopodia (Schenkman & Mortara 1992), which are associated with the early stages of invasion. Here, we used cytoskeletal preparations and electron microscopy to follow parasites that were either initiating contact with HeLa cells or were recently internalised. We show that the previously described trypomastigotes protruding from host cells are associated with actin filaments, indicating that this structure is derived from cortical cytoskeleton that is deformed by the parasite's active motility.

MATERIALS AND METHODS

HeLa cells were grown on glass coverslips and allowed to interact with Y strain trypomastigotes as described previously at a 100-200:1 parasite:cell ratio. After 15 min, when parasites are mostly initiating contact, or 45 min, when parasites have already invaded cells, the cells were washed with 0.1 M phosphate-buffered saline (37°C) five times to remove unattached parasites and treated with a membrane extraction solution containing 1% Triton X-100, 100 mM PIPES (pH 7.2), 4% sucrose, 1 mM MgCl₂, 10 μM taxol (Invitrogen) and 10 μM phalloidin (Sigma) [to stabilise microtubules and microfilaments, respectively (Sant'anna et al. 2005)] for 10 min with gentle rocking at room temperature. Then the samples were washed twice for 10 min in the same solution without detergent and fixed with 2% glutaraldehyde, 4% paraformaldehyde [Electron Microscopy Sciences (EMS), Hatfield, PA, USA], 140 mM NaCl, 2 mM CaCl₂ and 1 mM MgCl₂ in 0.1 M HEPES (pH 7.2) for 30 min. Samples were post-fixed with 1% OsO₄ (EMS) and 1% tannic acid (EMS) for 1 h each, dehydrated with ethanol and critical point-dried from CO₂ (CPD 020 Balzers-Tec). The slides were coated with a thin carbon layer (8 nm) (BAF 300, Balzers) and the glass coverslips were detached from the cell layer by immersion in 20% hydrofluoric acid (Sigma). Small pieces of the cell layer were transferred to distilled water for 10 min and then collected onto copper grids (300 mesh). Specimens were examined on a JEOL 1010 transmission electron microscopy (TEM) (Tokyo, Japan) at 80 kV and photographed with an AMT-Hamamatsu system. The images are presented in an inverted grey scale to enhance the contrast and facilitate visualisation.

RESULTS

The membrane extraction treatment with cytoskeleton-stabilising conditions revealed the interactions between parasites and HeLa cell cytoskeletons at a high

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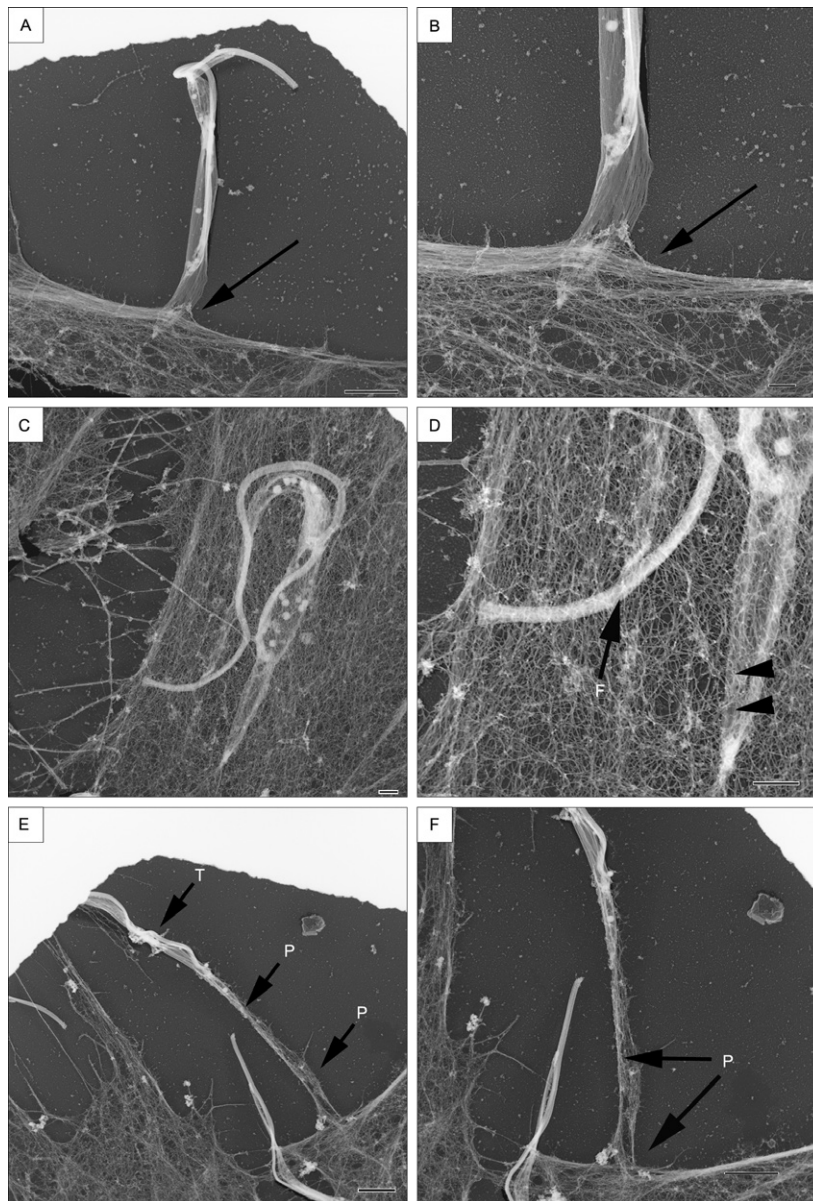
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resolution. Although TEM images are two-dimensional projections, the procedure used generates a three-dimensional perspective where the cytoskeletal fibres, especially thin actin filaments, could be clearly distinguished by their thickness (5-7 nm) and distribution. Trypomastigotes that were fixed 15 min after interacting with HeLa cells were observed in their initial invading profiles, where the posterior end of the parasite penetrates under the cell cytoplasm, resulting in an undulated cell cortex border filled with parallel-arranged actin filaments (Fig-

ure A, B). At more advanced periods of interaction with the host cells (45 min), when trypomastigotes are seen actively moving inside the host cytoplasm (Dvorak & Hyde 1973, Fernandes et al. 2011), the parasites fully invaded the cell cytoplasm and were surrounded by cytoskeletal filaments (Figure C, D). At later time periods (protruding), trypomastigotes were visualised at the tips in thick, column-like projections originating from the host cells that are rich in 5-7-nm filaments, which is compatible with the characteristics of actin filaments (Figure E, F).



Transmission electron microscopy of extracted membrane samples shows different steps of parasitic invasion into HeLa cell. A: overview of a *Trypanosoma cruzi* tryptomastigote (Y strain) with its posterior segment invading a host cell at 15 min of interaction (Bar = 2 nm); B: detail of A showing the cortical cytoskeleton (mostly microfilaments) with an undulated cell border caused by the parasite penetration under the host cell (Bar = 500 nm). Arrows in A and B indicate the site of cortical filament undulation; C: at 45 min, trypomastigotes were fully observed within the host cells with filaments running above and under the parasite (Bar = 500 nm); D: flagellum (F) and cell body are accommodated within the actin meshwork. Arrow also indicates cytoplasmic filaments over the flagellum and arrowheads, filaments underneath the parasite body (Bar = 500 nm). Host cells change their cytoskeleton in response to the parasite moving within the cytoplasm: E: *T. cruzi* tryptomastigote (T) protrude from HeLa cells and generates a microfilament-rich column-like projection (P) (Bar = 2 nm); F: detail of the HeLa cell actin protrusion (P) (Bar = 2 nm).

DISCUSSION

Our results reinforce the idea that *T. cruzi* trypomastigotes may be found underneath host cells during the initial stages of contact with HeLa cells that are attached to coverslips. Considering that the parasites are extremely motile (Hill 2003), focal adhesions may be regarded as potential sites of membrane wounding. The active parasite movement is likely to tear regions of the plasma membrane that are tightly attached to the substrate and therefore promote repair and the consequent endocytic process that aids in parasite invasion (Fernandes et al. 2011). Moreover, once they are inside cells, trypomastigotes move freely and actively in the cytoplasm, covered by the parasitophorous vacuole (PV) membrane and likely supported by cytoskeletal filaments. This intracellular movement of recently internalised *T. cruzi* trypomastigotes has been described in detail (Dvorak & Hyde 1973) and protruding events could also be seen in this study after approximately 30 min of invasion.

Using TEM, we were able to image parasites that were previously observed by field-emission scanning electron microscopy at the edges of cellular protrusions (Fernandes et al. 2011). Similar protrusions, called pseudopodia, are believed to be a result of the invasion process (Schenkman & Mortara 1992, Barbosa & Meirelles 1995). Interestingly, these protrusions were not sensitive to actin filament-disrupting agents and actin-rich cups were observed surrounding what was interpreted as an invading parasite, even in the presence of cytochalasin D (Schenkman & Mortara 1992). In addition, Schenkman and Mortara (1992) observed double-layered projections that may correspond to the protrusion phenomenon described by Fernandes et al. (2011), where parasites that are surrounded by the PV membrane protrude from the host cell due to their motility, stretch the plasma membrane and appear to be enveloped in both PV and host cell plasma membrane.

The observations by Fernandes et al. (2011) have been confirmed by our cytoskeletal images, which shed light on the initial interpretation of actin-rich and cytochalasin-insensitive pseudopodia around invading parasites (Schenkman & Mortara 1992). The formation of such actin-rich projections in the presence of cytochalasin D (Schenkman & Mortara 1992) supports the idea that they are formed by the active movement of the intracellular trypomastigotes toward the cell periphery, pushing away the host cell membrane and passively dragging the cortical meshwork of actin filaments. Altogether, these results provide an alternative explanation for this apparent paradox and propose that highly motile, recently internalised parasites can protrude from host cells and

appear to be enveloped by the host cell cortical actin in structures that resemble pseudopodia. However, because this stretching of the plasma membrane is driven by the parasite, these membrane extensions are not independent of *de novo* actin polymerisation.

Host cell invasion by *T. cruzi* trypomastigotes remains a highly a complex phenomenon that is likely initiated by the binding of several ligands to their receptors (Schofield et al. 2006, Alves & Mortara 2009), followed by the triggering of the repair pathway, which involves injury and rapid membrane internalisation by the endocytic machinery (Fernandes et al. 2011). Once inside the cell, trypomastigotes that are covered by the parasitophorous membrane actively move in the cytoplasm, providing the driving force that generates the protrusions that were described previously (Fernandes et al. 2011) and further characterised in this paper.

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