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Identification and purification of actin from the subpellicular network of *Toxoplasma gondii* tachyzoites

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Abstract

Toxoplasma gondii infects cells through dynamic events dependent on actin. Although the presence of cortical actin has been widely suggested, visualisation and localisation of actin filaments has not been reported. The subpellicular cytoskeleton network is a recently described structure possibly involved in the dynamic events. Using non-ionic detergent extractions, the cortical cytoskeleton network was enriched and used for the isolation and identification of actin. Actin was detected by Western blots in extracts of cytoskeleton networks, and it was localised by gold staining in the network and in both the apical end and the posterior polar ring. Actin was isolated from subpellicular cytoskeleton extracts by binding to DNase I, and it polymerised in vitro as filaments that were gold-decorated by a monoclonal anti-actin antibody. Filaments bound the subfragment 1 of heavy meromyosin, although with atypical arrangements in comparison with the arrowheads observed in muscle actin filaments. Treatment with cytochalasin D and colchicine altered the structural organisation of the subpellicular network indicating the participation of actin filaments and microtubules in the maintenance of its structure. Actin filaments and microtubules, in the subpellicular network, participate reciprocally in the maintaining of the parasite's shape and the gliding motility.

Keywords: Actin; Apicomplexans; Cytoskeleton; Colchine; Cytochalasin D; Motility; Subpellicular network; *Toxoplasma gondii*

1. Introduction

Toxoplasma gondii is a highly motile obligate intracellular protozoan that invades cells through an active process dependent on actin-myosin interactions (Ryning and Remington, 1978; Schwartzman and Pfefferkorn, 1983; Hiroshi et al., 1995; Dobrowolski and Sibley, 1996; Dobrowolski et al., 1997a,b). *Toxoplasma*, like other apicomplexan parasites, lacks locomotion organelles, however, it displays highly dynamic twirling–gliding movements over the substratum, without changing its cell shape (King, 1988; Mondragon et al., 1994; Mondragon and Frixione, 1996; Hakansson et al., 1999). Its cytoskeleton is constituted by a cage of 22 helical subpellicular microtubules organised from the polar ring and associated with the inner membrane complex (Dubremetz and Torpier, 1978; Russell and Sinden, 1981; Russell and Burns, 1984; Nichols and Chiappino, 1987). The conoid, a retractile apical organelle that is projected against the host cell plasma membrane during the active invasion (Chiappino et al., 1984; Werk, 1985), consists of spiral fibrous subunits (De Souza, 1974; Nichols and Chiappino, 1987; Morrissette et al., 1997) containing α tubulin (Hu et al., 2002). Tachyzoite motility and conoid extrusion are inhibited by cytochalasin D (CD), an actin filament disrupting drug as well as by butanedione monoxime (BME), a light chain ATPase inhibitor, suggesting the involvement of actin–myosin interactions (Dobrowolski and Sibley, 1996;

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Mondragon and Frixione, 1996; Hakansson et al., 1999; Herm-Gotz et al., 2002). Although *Toxoplasma* is a highly dynamic parasite, the presence of actin filaments has been debated because of the difficulties detecting them with fluorescent fallacidin or phalloidin or with conventional ultrastructural EM methods (Cintra and De Souza, 1985; Bannister and Mitchell, 1995; Dobrowolski et al., 1997a,b; Poupel et al., 2000). In contrast, by immuno-gold and immunofluorescence methods, the actin molecule has been found at the conoid and associated with the plasma membrane (Cintra and De Souza, 1985; Endo et al., 1988; Yasuda et al., 1988; Allen et al., 1997; Dobrowolski et al., 1997a,b). Jasplakinolide, a membrane-permeable actin-polymerising and filament-stabilising drug (Bubb et al., 1994), induces the polymerisation of actin filaments at the apical end without the induction of the conoid extrusion and with a reversible inhibition of the cell invasion suggesting a role for the actin polymerisation–depolymerisation dynamics as a requisite for motility and invasion (Shaw and Tilney, 1999). Biochemical evidence indicates that actin is mainly found in tachyzoites as the globular monomer (G-actin) (Dobrowolski et al., 1997b; Poupel and Tardieux, 1999), because of the participation of two actin monomer-sequestering proteins, ADF (Allen et al., 1997) and toxofilin, which also caps actin filaments to induce their disassembly (Poupel et al., 2000). Detection of myosin molecule TgMyoA beneath the plasma membrane suggests its participation to provide the dynamic force for the parasite motility (Herm-Gotz et al., 2002). Recently, a complex of proteins that includes the actin binding protein aldolase was described to mediate the molecular interactions between the C domains of *Plasmodium* TRAP and *Toxoplasma* MIC2 and the actin cytoskeleton (Jewett and Sibley, 2003).

Through extraction treatments, a cortical cytoskeleton composed of a subpellicular network associated with the subpellicular microtubules was reported, which was distributed along the tachyzoites (Mann and Beckers, 2001; Oliveira-Lima et al., 2001). TgIMC1 and TgIMC2, two proteins from the subpellicular network, are associated with the cytoplasmic face of the inner membrane complex between the subpellicular microtubules (Mann and Beckers, 2001). The subpellicular network assembles during the early stages of the development of daughter parasites, as a structure that gradually increases its stability associated with a proteolytic removal of the carboxy-terminus of TgIMC1 (Mann et al., 2002). High resolution SEM of detergent extracted tachyzoites revealed a net of actin-like filaments. The biochemical nature of this network had not been determined (Schatten et al., 2003).

In the present study, actin was identified and isolated from the subpellicular network cytoskeleton. A function for actin in the cytoskeleton network was determined by the exposure to drugs that alter the cytoskeleton integrity. Location of actin at the subcortical level associated with the subpellicular network suggests its participation in the dynamic behaviour of the parasite during gliding motility and cell invasion.

2. Materials and methods

All reagents and protease inhibitors were purchased from SIGMA, Chemical Co (St Louis, MO), unless otherwise indicated.

2.1. Animals

Balb/c mice used for parasite infections were maintained in an animal facility with regulated environment conditions of temperature, humidity and filtered air. Management was performed according to the country official norm NOM-062-ZOO-1999 for the production, care and use of laboratory animals (Mexico).

2.2. Maintenance and purification of T. gondii tachyzoites

RH strain tachyzoites were maintained by i.p. passages in female Balb/c mice (Mondragon et al., 1994). After cervical dislocation, parasites were recovered from i.p. exudates after a peritoneal washing with PBS (138 mM NaCl, 1.1 mM K_2PO_4, 0.1 mM Na_2HPO_4 and 2.7 mM KCl, pH 7.2) and purified by filtration through 5 μm pore polycarbonate membranes (Millipore Co, Bedford, MA).

2.3. Preparation of subpellicular networks for structural characterisation

Tachyzoites (1×10^3/ml) were settled on 200 mesh nickel grids covered with formvar film (Polysciences, Warrington, PA) for 5 min. Parasites were rapidly washed with PHEM (10 mM HEPES, 10 mM EGTA, 1 mM $MgCl_2$), 50 μg/ml *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 μg/ml *N*α-p-Tosyl-L-lysine choromethyl ketone (TLCK) and 17.4 μg/ml phenylmethylsulfonyl fluoride (PMSF) and incubated with 0.1% Triton X-100 in PHEM for 5 min, and then rinsed twice with PHEM to provide the subpellicular networks (modified from Hartwig, 1992). For structural analysis, networks were negatively stained with 1% uranyl acetate (Polysciences, Warrington, PA) and viewed through a JEOL 2000EX TEM at 80 keV (JEOL LTD, Japan). Other samples were dehydrated in increasing concentrations of ethanol, critical point dried in a CO_2 atmosphere in a Samdry®-780A apparatus (Tousimis Research Co, USA) and coated by the evaporation of Platinum–carbon (Pt/C) rods at 45° in a BAF 400T freeze fracture system (Balzers, Austria) and then observed in the TEM.

2.4. Actin immuno-gold staining

Subpellicular networks on the grids were blocked with 0.1% BSA in PHEM for 2 min and incubated overnight with