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Ribosomal protein P2 localizes to the parasite zoite-surface and is a target for invasion inhibitory antibodies in *Toxoplasma gondii* and *Plasmodium falciparum*

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ABSTRACT

In the malarial parasite *Plasmodium falciparum*, the conserved ribosomal stalk protein P2 (PfP2) exhibits extra-ribosomal stage-specific oligomerization and trafficking to the host red cell membrane. Antibodies directed against PfP2 arrested cell division. We sought to examine whether P2 from a closely related Apicomplexan parasite, *Toxoplasma gondii*, exhibits similar properties in terms of its oligomeric status as well as such unique host-cell localization. Circular dichroism spectroscopy of recombinant P2 from *T. gondii* (TgP2) showed a structure similar to that of PfP2, but unlike PfP2, which forms SDS- and DTT-resistant oligomers, TgP2 exhibited only a weak SDS-resistant dimerization. Also, unlike PfP2 localization to the infected erythrocyte surface, TgP2 did not localize to the host membrane in *T. gondii* infected human foreskin fibroblast cells. However, P2 protein was detected on the free tachyzoite surface, corroborated by localization of epitope-tagged P2 transfected in *T. gondii*. The presence of P2 on the surface of *P. falciparum* merozoites was also observed, and specific antibodies raised against the P2 protein blocked both *T. gondii* and *P. falciparum* zoite invasion of the host cells. Thus, although certain moonlighting functions of the acidic ribosomal protein P2 are different amongst *P. falciparum* and *T. gondii*, the P2 protein localizes to the surface of the invasive zoite form, and appears to constitute a potential target for host cell invasion inhibition in both the Apicomplexan infections.

1. Introduction

The stalk of the large ribosomal subunit plays an essential role in translation elongation in all organisms. The stalk is formed by a set of small acidic protein dimers that bind to a larger core protein, which in turn interacts with the highly conserved GTPase associated region of the large rRNA that binds the entire complex to the ribosome [1]. In eukaryotes, the P-proteins form this lateral stalk complex of the large ribosomal subunit, comprising a 32 to 35 kDa P0 protein at the core, to which bind two heterodimers of acidic ribosomal proteins P1 and P2 (about 12–14 kDa), ultimately forming the stalk P0-(P1/P2)2 complex [2]. Some eukaryotic species possess more than one form of P1 and P2 proteins [3], and a third family of acidic P proteins, P3, has been described in plants [4]. Studies in yeast have demonstrated that while the core P0 protein is indispensable for cell survival and cannot be deleted, P1/P2 deletions are viable under rich growth conditions, indicating a dispensable role for P1/P2 in the house-keeping protein synthesis [5].

What then may be the alternate roles of these extremely conserved P1/P2 proteins across eukaryotic organisms? In yeast, P1/P2 protein deletions exhibit stress sensitivity [6]. There have been associations of ribosomal P2 protein expression with cancer, but those have been subscribed to altered cellular protein synthesis [7]. P1/P2 heterodimers are also reported to be the only ribosomal proteins that exist in the cytosol, free of ribosomes, with shuttling of heterodimers occurring between the ribosome and the cytosolic pool [1,8]. Heterogeneity of P-proteins in ribosomal composition has been reported, and ribosomes deficient in P1/P2 proteins have been observed in the stationary phase of growth [9]. The cytoplasmic pool of P1/P2 proteins is large, and experimental evidence indicates that the free stalk proteins participate in intracellular stimulation of some ribosome-inactivating proteins (RIP) [10]. Thus, additional extraribosomal functions of these proteins are not unexpected. However, other than growth and ribosome related functions, P2 protein has been documented to play roles at the cell surface.

P1 and P2 proteins are reported to be stably associated with the cell wall component of *Saccharomyces cerevisiae* under a variety of growth conditions [11]. In *Neisseria gonorrhoeae*, the functional orthologue of P2 (L12), is shown to be surface exposed, and has been implicated in cell invasion [12]. In *Plasmodium* species the P2 protein has been demonstrated to translocate to the infected host red blood cell surface at the onset of cell division, and anti-PfP2 monoclonal antibodies arrest parasite cell division [13]. This translocation to the cell surface was accompanied by extensive SDS-resistant oligomerization, and P2 at the

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infected erythrocyte surface was present exclusively as an SDS-resistant homo-tetramer [13,14].

Toxoplasma gondii, another Apicomplexan parasite closely related to *Plasmodium*, is an obligate intracellular pathogen of humans and most other warm-blooded vertebrates. Toxoplasma invasion into human cells appears to have mechanisms similar to that of Plasmodium [15]. Considering the high degree of similarity of *T. gondii* P2 protein to *Plasmodium* P2 protein, we examined the properties of P2 protein of *T. gondii*. Here we report that unlike *Plasmodium* P2, *T. gondii* P2 protein does not translocate to host cell surface, nor does it undergo oligomerization. However, we observed a novel extraribosomal localization of TgP2 to the free invasive tachyzoite surface. We also observed the presence of *P. falciparum* P2 protein on the merozoite surface, and demonstrated that both *Plasmodium* and *Toxoplasma* zoite-surface P2 proteins were targets of invasion inhibitory antibodies.

2. Materials and methods

2.1. Parasites and cell culture

T. gondii RH strain tachyzoites were maintained by serial passage in human foreskin fibroblasts (HFF) cultured in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Hyclone), 50 μg/ml penicillin–streptomycin (Invitrogen). Parasites were harvested by scraping and syringe passage serially using 20G and 23G needles (Becton-Dickinson).

P. falciparum 3D7 strain parasites were maintained in culture as described earlier [13]. Human blood was collected in K2 EDTA vacutainers (BD Biosciences) as an anticoagulant. After removing the leukocytes, the erythrocytes were washed and resuspended in complete RPMI (cRPMI; RPMI with 0.5% Albumax). Asexual stages of the *P. falciparum* 3D7 strain were cultured in vitro and maintained at 5% haematocrit in cRPMI at 37 °C in a humidified chamber containing 5% CO2. Synchronization of the erythrocytic stages of the parasite was carried out using 5% sorbitol and 60% Percoll [13].

2.2. Cloning of TgP2 for transfection and recombinant protein expression

The sequence for P2 was retrieved from the *T. gondii* database (toxodb.org) to design primers for amplification of the full-length gene from a Toxoplasma cDNA library (kind gift from Prof. David Roos). For generating an epitope tagged TgP2, the gene was amplified with the following primers; forward primer: ATGCCCATGGCAATGAAA TACGTCGC and reverse primer: ATGCCTTAAG*ACGCGTAGTCGGGGACGT CGTAGGGGT*AGTCGAAGAGCGAGAAGC, with the NcoI and AflII restriction sites underlined and the sequence to introduce the HA9 epitope (YPYDVPDYA) indicated in italics. The PCR product was digested with NcoI and AflII and ligated to the transfection vector pBS′-S1/HXGPRT/S as described earlier [16].

For recombinant protein expression, full length TgP2 was cloned into the BamHI and SalI sites of a pET28a(+) vector using the following primers—forward: AGGATCCATGGCAATGAAATACGTCGC reverse: ATGCGTCGACTTAGTCGAAGAGCGAGAAGC, with BamHI and SalI (New England Biolabs) sites underlined. Restriction digests were carried out as per the manufacturer's recommendations and ligations were carried out using T4 DNA ligase (New England Biolabs) at 16 °C for 16 h. All clones were confirmed by sequencing.

2.3. Recombinant protein expression and purification

The TgP2 cloned in pET28a was transformed in *Escherichia coli* BL21DE3 strain and protein expression was induced with 0.5 mM IPTG overnight. Recombinant protein was then purified using nickel-nitrilotriacetic acid beads (Qiagen). Recombinant PfP2 was purified as described earlier [14].

2.4. SDS-PAGE and immunoblotting

Concentration of recombinant protein was estimated using bicinchoninic acid (BCA) (Thermo Scientific Pierce) before separation by SDS-PAGE. 10 μg of recombinant protein samples was mixed with loading dye (50 mM Tris–Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) with or without reducing agent (10 mM DTT) as indicated and heated for 10 min at 95 °C before loading onto a 12% SDS-PAGE gel.

Harvested parasites (~10^6 tachyzoites) were mixed with SDS loading dye and resolved on a 15% SDS-PAGE gel. A fully confluent HFF T25 flask was infected with ~10^7 tachyzoites for 24 h after which uninvaded parasites were removed by aspirating medium. To this, and another uninfected flask of confluent HFFs, 200 μl of SDS-PAGE loading dye was added to lyse cells. Around 10 μl of this from each flask was heated at 95 °C and loaded per well onto a 15% SDS-PAGE for immunoblotting.

Proteins were transferred to methanol-activated polyvinylidenefluoride (PVDF) membrane (Millipore) using anode buffer (25 mM Tris–Cl, pH 10.4, 10% methanol) and the TransBlot Semi-Dry Transfer system (Bio-Rad). Membranes were blocked with 5% non-fat skimmed milk powder in 1X PBS containing 0.05% Tween-20 overnight and probed with specific antibodies. PfP2-specific mouse monoclonal antibody E2G12 and TgP2-specific rabbit antibodies were obtained from Bioklone (Chennai, India). Polyclonal mouse antibodies against PfP2 and TgP2 were raised in-house. Primary antibody dilution was made in 1X PBS containing 0.05% Tween-20 and incubated with the membrane for 1 h at room temperature, followed by washes and suitable HRP-coupled secondary antibody incubation before enhanced chemiluminiscence (ECL) detection. The blots were developed using the ECL Plus (GE Healthcare) substrate and detected using X-ray films. The following antibody dilutions were used: mouse anti-TgP2 polyclonal antibody—1:1000, rabbit anti-TgP2 antibody—1:1000.

2.5. Circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra of the protein at 25 °C were recorded on a JASCO-J810 spectropolarimeter (Jasco, Hachioji, Japan) using a 0.2 cm cell. Spectra for TgP2 and PfP2 were recorded. The protein concentration was 30 μM. The CD machine was pre-calibrated with iolar nitrogen for 1 h before starting. The samples at appropriate conditions were equilibrated at least 10–12 h before CD measurements. Each spectrum was an average of three scans (slit width of 1 nm). The data were plotted as molar ellipticity versus wavelength (from 200 to 250 nm).

2.6. Immunofluorescence

Harvested *T. gondii* tachyzoites were fixed in 4% paraformaldehyde (PFA) in PBS to be used for immunofluorescence. Parasites were allowed to settle onto the wells of 8-well chamber slides (Thermo Scientific), and the immunostaining was carried out under permeabilizing and non-permeabilized conditions. Where indicated, cells were permeabilized with 0.25% Triton-X 100 in PBS for 10 min. Blocking was carried out using 3% bovine serum albumin in PBS for 30 min before addition of primary antibody and incubation at room temperature for 1 h. This was followed by washes and staining with suitable secondary antibodies for 45 minutes at room temperature. Cells were then stained with DAPI and coverslips were mounted on the slides with Vectashield mountant.

For intracellular *T. gondii*, HFFs were grown in 8 well chamber slides and infected for 24 h with *T. gondii* before fixing in 4% PFA, permeabilizing for 10 min with 0.25% Triton-X 100 and blocking, followed by immunostaining as described above.

For *P. falciparum*, infected erythrocytes were harvested by centrifugation at 800 *g* for 10 min in a swing bucket rotor and fixed for 30 min in 4% PFA + 0.025% glutaraldehyde in PBS. Cells were permeabilized and blocked as above before immunostaining.