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The Hsp90 co-chaperone p23 of *Toxoplasma gondii*: Identification, functional analysis and dynamic interactome determination

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Abstract

Toxoplasma gondii is among the most successful parasites, with nearly half of the human population chronically infected. Recently a link between the *T. gondii* Hsp90 chaperone machinery and parasite development was observed. Here, the *T. gondii* Hsp90 co-chaperones p23 and Hip were identified mining the *Toxoplasma*- database (www.toxodb.org). Their identity was confirmed by domain structure and blast analysis. Additionally, analysis of the secondary structure and studies on the chaperone function of the purified protein verified the p23 identity. Studies of co-immunoprecipitation (co-IP) identified two different types of complexes, one comprising at least Hip-Hsp70-Hsp90 and another containing at least p23-Hsp90. Indirect immunofluorescence assays showed that Hip is localized in the cytoplasm in tachyzoites and as well in bradyzoites. For p23 in contrast, a solely cytoplasmic localization was only observed in the tachyzoite stage whereas nuclear and cytosolic distribution and colocalization with Hsp90 was observed in bradyzoites. These results indicate that the *T. gondii* Hsp90-heterocomplex cycle is similar to the one proposed for higher eukaryotes, further highlighting the implication of the Hsp90/p23 in parasite development. Furthermore, co-IP experiments of tachyzoite/bradyzoite lysates with anti-p23 antiserum and identification of the complexed proteins together with the use of the curated interaction data available from different source (orthologs and *Plasmodium* databases) allowed us to construct an interaction network (interactome) covering the dynamics of the Hsp90 chaperone machinery.

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Nucleotide and protein sequences reported in this paper are available in the GenBank™ databases under the accession N° DQ680015 (Hip) and DQ680014 (p23).

Keywords

Toxoplasma gondii; development; Hsp90 heterocomplex; p23; protein prediction interaction; bioinformatic

1. Introduction

Toxoplasma gondii is a ubiquitous, obligate, intracellular protozoan parasite that infects a large number of warm-blooded animals. In humans, *T. gondii* follows an asexual replication cycle, characterized by two stages: rapidly growing 'tachyzoites' and latent 'bradyzoite' tissue cysts. Tachyzoites are responsible for acute disease and congenital neurological birth defects, while the slowly dividing bradyzoite form may remain latent within the tissues for many years, representing a potential threat to immune-compromised patients. Both developmental stages are essential for disease and parasite propagation.

Stress has been shown to induce bradyzoite formation and heat shock proteins (Hsp) are likely to play an important role during stage conversion [1]. The expression of the heat shock proteins Hsp60, 70 and 90 is increased during conversion from tachyzoites to bradyzoites [1,2]. In this context, Radke et al., [3] performed serial analysis of gene expression (SAGE) to define the *T. gondii* transcriptome of the intermediate-host life cycle that leads to the formation of the bradyzoite/tissue cyst. In their study, an increase in Hsp90 mRNA occurs within the first 24 h of bradyzoite development, suggesting that Hsp90 mRNA may be an early bradyzoite marker. In this context, we recently showed that subcellular localization of the *T. gondii* Hsp90 is also developmentally regulated [2]. Furthermore, geldanamycin, a benzoquinone ansamycin antibiotic capable of binding and disrupting the function of Hsp90, blocked the conversion from tachyzoite to bradyzoite and vice versa, suggesting an important role of this protein in the regulation of stage inter-conversion [2]. Due to lack of drugs capable of eliminating tissue cysts, up to now there is no effective treatment for chronic toxoplasmosis. Thus, the *T. gondii* Hsp90 emerges as an interesting target for drug development also because of its appearing pleiotropic role, including invasion and replication [2,4].

Hsp90 does not act as a regular chaperone in the folding of non-native proteins. Instead, it binds to substrate proteins (client proteins) that are in a near-native state, at an advanced stage of folding [5]. In addition to protein folding activity, Hsp90 has an alternative function associated with the assembly of multi-protein complexes and their turnover. In the cell, Hsp90 is chaperoning more than 100 'client proteins', most of them involved in signal transduction, regulation of the cell cycle or regulation of transcription and thereby influencing development and evolution [6]. In higher eukaryotes, Hsp90 is regulated by further proteins, so called co-chaperones, which participate in dynamic multi-chaperone complexes [6,7]. Co-chaperones can regulate the ATP-hydrolysis of Hsp90, influence its affinity for client proteins [8], target it to its client protein [9,10] or to a particular subcellular compartment [9–11]. In studies based on glucocorticoid receptor (GR) maturation co-chaperones were determined to be involved in achieving efficient Hsp90-heterocomplex assembly: Hsp90, Hsp70, Hsp organizing protein (Hop), p23, an Hsp90-binding co-chaperone and Hsp40 [6]. Another co-chaperone, the Hsp70 interacting protein (Hip), has also been purified by co-immunoprecipitation (co-IP) [12]. Mechanistically, Hip was detected in early Hsp90-heterocomplex (formed by Hsp40-Hip-Hsp70-client protein-Hop-Hsp90). By contrast, p23 enters at late stage of the cycle, leading to complete inhibition of the ATPase activity and increasing the apparent affinity of Hsp90 for ATP [8,13–15].

Despite the observation that the Hsp70/Hsp90 cycle may be involved in apicomplexan parasites propagation, only *Plasmodium* Hip and p23 co-chaperones have been identified and preliminary characterized so far [16,17]. Here, we set out to elucidate the role of Hsp90-heterocomplex during *T. gondii* differentiation. We studied Hip and p23 interactions in *T. gondii* and assessed subcellular localization of Hip and p23 during tachyzoite-bradyzoite conversion. Additionally, basic structural and functional characteristics of p23 were determined to further confirm the identity of this Hsp90 co-chaperone. Finally, putative interactors of p23 and Hsp90 during tachyzoite and bradyzoite stages were identified by mass spectrometry analysis following co-IP.

2. Materials and Methods

2.1 *In silico* analysis

In order to identify proteins of *T. gondii* Hsp70/Hsp90 machinery we searched Toxodb (www.toxodb.org) for Hsp90 and its putative binding proteins on the basis of the respective domains: Hsp90, DNAJ, TPR, P23 and Aha. To identify Hip and p23 proteins, the *Plasmodium berghei* Hip, p23, *Rattus norvegicus* Hip and human p23 (AN: **Q08168**, **XP_680236**, **P50503** and **L24804**, respectively) sequences were used to blast (www.toxodb.org blastp). Several amino acids and their respective DNA were retrieved from ToxoDB web page. Identity was further analyzed by Blast programs (www.ncbi.nlm.nih.gov.ar). β -sheet was deduced by using the <http://protevo.eb.tuebingen.mpg.de/toolkit>. Mitochondria and chloroplast transit peptides were analyzed by Predotar software at <http://urgi.versailles.inra.fr/predotar/predotar.html>

2.2 cDNA cloning, sequencing and sequence analysis

Putative Hip and p23 open reading frames (ORF) were amplified from cDNA. Total RNA was extracted using TRIzol® Reagent (Invitrogen™ Life Technologies) according to the manufacturer's instructions. Two micrograms of total RNA from tachyzoites was reverse transcribed to cDNA with 200 U of M-MLV reverse transcriptase (Invitrogen Life Technologies) and oligo(dT)12–18. For PCR the following primers were used: Hip: sense 5'-GGATCCATGGCGGCGCTCAGCCCTCA and antisense 5'-GTCTGACTTACATAGGAGAGCCAGTTG; p23: sense 5'-CTGCAGATGGCGGACGCACAAGTTCA and antisense 5'-GTCTGACTCAAGCCTGCGCAGCTTCTG. *Bam*HI and *Kpn*I sites were included in the sense and antisense primers respectively (underlined sequence). These fragments were cloned in pGEM T easy vector (Promega). The 5' Untranslated region (UTR) of both *p23* and *hip* and 3'UTR of *p23* mRNA were sequenced by reverse transcriptase-PCR using new primers: for *p23* (sense (5'-CCGTCGCGACTTTTTTTGCGA) and an antisense (5'-AACACGACGCCCGTCAGCTCC). For *hip* 5'UTR sequence a new sense primer was designed (5'-GGACGTGTTTAATCCATTCT).

2.3 Expression and Purification of recombinant *T. gondii* Hsp70/Hsp90 co-chaperones

The open reading frames of *T. gondii* *hip* and *p23* genes were sub-cloned into the prokaryotic expression vector pRSET A (Invitrogen Life Technologies) downstream and in frame with a sequence that encodes an N-terminal 6xHis-tag. *Bam*HI and *Kpn*I restriction sites were engineered into the 5' and 3' primers respectively. *Escherichia coli* BL21 (DE3) were freshly transformed with the expression plasmids. Cultures were grown to OD₆₀₀ = 0.6 before protein expression induction by the addition of isopropyl thio- β -D-galactoside (2 mM). After overnight incubation at 30°C, the cells were harvested and purified in soluble form using a commercial HisTrap 5ml column (GE Healthcare) according to the manufacturer's instructions.

2.4 Circular dichroism spectroscopy

The content of structural elements was determined by Far-UV-CD spectroscopy, using a Jasco J-715 spectropolarimeter (Jasco, Großstadt, Germany). The measurements were carried out in quartz cuvettes with 0.02 cm pathlength at a protein concentration of 0.3 mg/ml. Far UV-spectra were recorded in 50 mM Tris/HCl, 2 mM EDTA, pH 8.0 at 20°C; 16 spectra were accumulated and all spectra were buffer-corrected. Structural elements were calculated using the CD spectra deconvolution software CDNN [18 R. and Jaenicke, R. (1992) Protein Eng. 5, 191–195)].

2.5 Chaperone activity

Chaperone activity of *T. gondii* p23 was assayed as described for human p23 [19]. To examine the influence of *T. gondii* p23 on the thermal aggregation of citrate synthase (CS), a solution of 30 µM CS (monomer) was diluted 1:30 in 40 mM HEPES/KOH pH 7.5, equilibrated at 44°C, in the presence and in the absence of p23. Aggregation kinetics was measured in a Cary 50 spectrophotometer (Varian, Darmstadt, Germany) following the change in light scattering at 360 nm.

To determine the influences of p23 on the thermal inactivation of CS, CS was incubated in the absence or presence of *T. gondii* p23 in 40 mM Hepes/KOH, 0.5 mM DTT, pH 7.5 at 45°C. CS activity was assayed taking aliquots at time points indicated in 50 mM Tris/HCl, 2 mM EDTA, pH 8.0 at 25°C as described previously [19,20]

2.6 Production of antibodies

C3H mice were immunized with 10 µg of Hip and p23 recombinant proteins in complete Freund's adjuvant (Sigma-Aldrich™ de Argentina S.A) and boosted every two weeks with three successive injections with identical doses of the respective recombinant protein in incomplete Freund's adjuvant (Sigma-Aldrich™ de Argentina S.A.).

2.7 Parasite *in vitro* differentiation and manipulation

Parasite differentiation and co-localization analysis were further described elsewhere [2]. Briefly, tachyzoites of avirulent PK strain were grown *in vitro* in human foreskin fibroblasts (HFF) and induced to differentiate into bradyzoites for 4 days in low CO₂ concentration (0.03%) and pH 8.1. Bradyzoite induction was assessed and followed by murine mAb α p34 T82C2 (specific marker of bradyzoite P34 surface antigen) a gift from J. F. Dubremetz, Université de Montpellier II, France or by rabbit polyclonal anti-BAG1 (specific marker of cytoplasmic BAG1 small heat shock protein) obtained in our laboratory [21]. Antibodies specific to the tachyzoite surface protein SAG1 (murine mAb α p30 T4IE5) a gift from J. F. Dubremetz, (Université de Montpellier II, France), to the cytoplasmic/nuclear Hsp90 (rabbit polyclonal anti-Hsp90, C-terminal region) [2,22]. Following incubation with primary antibodies, cells were washed three times with PBS, and then incubated with the corresponding secondary antibodies (1:4000). Alexa fluor goat anti-rabbit 350 or Alexa fluor goat anti-mouse 488 (Invitrogen Argentina Ltda.). Cover slips were washed three times and mounted in Fluoromont G (Southern Biotechnology Associates) and viewed using a Zeiss Axiovert 35 inverted microscope equipped with a 100 W Hg-vapor lamp and epifluorescence filter sets or a Nikon Model Eclipse E600 (magnification 100X, numerical aperture 1,40 at 24°C). Propidium iodide (PI, Sigma-Aldrich™ Argentina S.A) used to stain the nucleus. Fluorescences were recorded separately, given a colour (red, green or blue) and the images were analyzed by Image-Pro Plus version 5.1.0.20 and merged using Adobe Photoshop.

2.8 Immunoblot and co-immunoprecipitation (co-IP) analysis

In order to carry out immunoblot studies in both tachyzoites and induced bradyzoites, medium was removed and the cells were washed once with PBS, the monolayer was scraped and passed five times through a 27-gauge needle, bradyzoites were also passed once through a 30-gauge needle to release parasites from the host cells. The parasites were then centrifuged at 1,800 rpm for 10 min at room temperature and resuspended in sterile PBS and counted in a Neubauer hemocytometer chamber. Both stages of parasites were purified from the host cell material by passage through a 3 mm-pore size filter (Amersham Hybond, GE Healthcare Argentina S.A.).

In order to perform Western blot, proteins from parasites and human foreskin fibroblasts (HFF) were extracted in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed and transferred on nitrocellulose. Non-specific binding sites were blocked with 5% non-fat-dried milk in PBS containing 0.05% Tween-20 (PBS-T) and the membranes were then incubated (1 h, room temperature) with polyclonal antibodies diluted 1:500. The membranes were washed with PBS-T prior to incubation with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibodies, diluted 1:5,000 (Santa Cruz Biotechnology). Immunoreactive protein bands were visualized by NBT-BCIP (Sigma-Aldrich™ Argentina S.A.).

To perform co-IP studies, tachyzoites and *in vitro* induced bradyzoites of *T. gondii* PK (Me49) were incubated 2 h at 4°C in lyses buffer (0.1% [v/v] NP40, 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 4 mM EDTA, sodium molybdate 20mM, Sigma inhibitors cocktail [1 mM PMSF, 104 mM 4-{2-aminoethyl}benzenesulfonyl fluoride, 80 mM aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64]) and pelleted 20 min at 12,000 g at 4°C. In parallel, the antibody (1:500 to 1,000) was incubated with protein A/G-Sepharose (Sigma-Aldrich™ de Argentina S.A) in lysis buffer for 2 h at 4 °C. Supernatants of parasite lysates were then incubated in lysis buffer plus antibody (1:500 to 1,000) and protein A/G-Sepharose for 2 h at 4°C in gentle rocking. After the resin was washed four times with lysis buffer and 2 times with wash buffer (0.5% [v/v] NP40, 50 mM Tris-HCl pH=8.3, NaCl 1M), the antigen was eluted by boiling in sample buffer and separated by SDS-PAGE.

2.9 Mass spectrometry analysis

Tachyzoite and bradyzoite proteins were pulled down with the anti-p23 and -Hsp90 antibodies, separated on a 10% polyacrylamide gel and stained with Coomassie Blue R-250 (Fig. S1). Negative controls with pre-immune sera did not result in any visible protein bands. Bands of interest were excised (Fig. S1), and de-stained with three progressive washes of 25 mM NH₄HCO₃ in 50% acetonitrile, and then followed by reduction with 200 µl of 10 mM dithiothreitol (DTT) in 100 mM NH₄HCO₃ for 1 h at 56 °C and alkylation with 55 mM iodoacetamide in 200 µl of 100 mM NH₄HCO₃ in the dark for 30 min. The hydrated gel slice was covered with 30 µl of trypsin solution (10 ng/µl) and incubated overnight at 37°C. A small amount of 10% acetic acid was then added to the tube to stop the digestion. The sample was then centrifuged at 3,000 r.p.m., and the supernatant was saved for analysis by electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC-MS). A fused-silica microcapillary LC column (12 cm x 75 µm id) packed with C18 reversed phase resin (Magic C18AQ, 5-µm particle size, 20-nm pore size, Michrom Bioresources, Auburn, CA) was used with nanospray ESI. The nanospray-ESI was fitted onto a linear quadrupole ion trap (LTQ) mass spectrometer (Thermo Electron, San Jose, CA) that was operated in a collisional-induced dissociation mode to obtain both MS and MS/MS spectra. Samples of tryptic peptides were loaded onto the microcapillary column and separated by applying a gradient from 5–80% acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min for 55 min.

Mass spectrometry data were acquired in data-dependent acquisition mode, in which a full MS scan was followed by 10 MS/MS spectra of the most abundant ions.

After an LC-MS run was completed and spectra obtained, the spectra were searched against the ToxoDB 4.1 protein database (www.toxodb.org) using SEQUEST (Bioworks software package, version 3.3, Thermo Electron,). The search parameters permitted ± 1.0 Da peptide MS tolerance, and ± 1.0 Da MS/MS tolerance. Oxidation of methionine and carboxymethylation of cysteines were allowed as variable modifications. Up to two missed tryptic cleavages of peptides were considered. The cutoff for SEQUEST assignment was a cross-correlation score (Xcorr) greater than 1.9, 2.5, and 3.8 for peptide charge states of 1, 2, and 3, respectively; and a delta-correlation score (ΔC_n) >0.1 .

2.10 Interactome map

After collecting data from proteomics experiment, a network of Hsp90 and p23 interactors was constructed using network visualization and the modeling tool of Cytoscape (<http://www.cytoscape.org>) [23]. A putative name was assigned to each interactor according to ToxoDB 4.1 and Uniprot databases (<http://beta.uniprot.org/>). Subcellular localization information of proteins was obtained from ToxoDB 4.1, or predicted according to gene ontology data of orthologs of the putative *Toxoplasma* proteins (obtained from Uniprot and ToxoDB). With this information and using Cerebral (a Cytoscape plugin) [24], a network basing on intracellular localization was arranged. All interactions were compared mining Hsp90 and p23 interactomes present in public protein-protein interaction (PPI) databases like POINT (<http://point.bioinformatics.tw>) and the specific database of protein interaction network for *Plasmodium falciparum* (<http://apidb.org/apidb/showQuestion.do?questionFullName=GeneQuestions.GenesByFunctionalInteraction>), derived from yeast two-hybrid studies [25,26]. Based on the Cytoscape generated diagrams, Hsp90 and p23 interactomes were schematized in a tachyzoite and bradyzoite draw.

3. Results

3.1 Identification of novel Hsp90 genes and Hsp90-cochaperones of *T. gondii*

In order to determine the number of *Toxoplasma* Hsp90 proteins, a database search in the *Toxoplasma* genome project (www.toxodb.org) was performed. Four putative proteins with Hsp90 signature were found: the previously described Hsp90 (Accession [AY344115](#)) detected as extracellular, cytosolic and nuclear [2,4], the Hsp90-protein described by Saksouk *et al.* [27] which co-immunoprecipitate (co-IP) with parasite histones (Accession [AY53805](#)), and the not previously described genes 83.m01184 and 583.m05357. *In silico* analysis [AY53805](#) revealed a signal peptide (possible cleavage site between amino acids 24 and 25), an endoplasmic reticulum retention motif (HDEL) in the C-terminus (66.7 % of endoplasmic reticulum prediction value). Therefore, it could be assumed that this protein is a *T. gondii* GRP94. The other two potential new proteins with Hsp90 signature ("gene IDs" 83.m01184 and 583.m05357) included mitochondrial and mitochondrial/chloroplast transit peptides in the N-terminal region of their sequences and lacks the C-terminal region of Hsp90, which contains the highly conserved MEEVD motif that terminates Hsp90 and is the binding site for the Hop co-chaperone during the Hsp90-heterocomplex cycle [28]. The gene ID 83.m01184 also showed homology (identity of 42%) to Hsp75/TNF alpha receptor (TRAP1), whereas the identity with Hsp90 ([AY344115](#)) is lesser than 28%.

T. gondii cytoplasmic Hsp70 and immunophilin FBCP were already described, and putatives Hsp40, Hip, Hop, Aha1, p23 and PP5 Hsp90-co-chaperones were also identified after searching the ToxoDB database (Table 1).

3.2 Cloning and sequence characterization of *T. gondii* Hip as early marker for the Hsp90-heterocomplex cycle

In higher eukaryotes, Hip was shown to be associated to Hsp70 early in the Heterocomplex-Hsp90 [9], becoming a stage marker for the Hsp90-heterocomplex cycle. The cDNA for gene 44.m00052 present an ORF of 1,278-bp which encodes for a protein of 425 amino acids with a theoretical mass of 45.1-kDa. Hip proteins comprise a series of consecutive modules [12]. Comparison to the *R. norvegicus*, *P. berghei* and *T. gondii* Hip proteins showed high overall similarity (Fig. 1A). *T. gondii* Hip showed a highly conserved structure (Fig. 1B), revealing the first 15 residues necessary for homo-oligomerization, a negatively charged residues region preceding the TPR domain and the Sti1 domain containing two “DPEV”-motifs (Fig. 1B). In the “GGMP” rich region, two “GGMP”, two “GGFP” and several “GGXX” repeats are present (Fig. 1A).

The TPR motif is a 34-amino acid sequence occurring three times in the protein in slight variation. It forms α -helices (A and B) that fold back on each other resulting in a compactly folded domain [29]. There is a consensus pattern of hydrophobic and hydrophilic residues (b and l in Fig. 1C), positions 8, 20 and 27 being small hydrophobic residues. Position 32 is frequently a proline [30]. Analysis of the three TgHip TPR motifs showed that they contain nearly all of the conserved residues and predicted two α -helices (Fig. 1C).

3.3 Cloning and structural characterization of *T. gondii* p23

Searching *Toxoplasma* sequence databases with *Plasmodium* and human p23 (AN: **XP_680236** and **L24804** respectively) resulted in the identification of three putative p23-containing proteins: 645.m00320 (TGME49_121520: annotated as p23 co-chaperone), 541.m01195 (TGME49_105820: annotated as Calcyclin binding protein) and 44.m02666 (TGME49_031590: annotated as SGS domain-containing protein). The two latter have one additional SGS domain, a feature of SGT1 and calcyclin binding proteins. The ID 645.m00320 sequence presented the highest identity values when compared with *P. berghei* and human p23 (38% and 32% respectively), meanwhile 541.m01195 and 44.m02666 presented identity values ranging between 23% to non-significant identity). Based on these data it represented the most likely candidate to be the *T. gondii* p23 related to Hsp90-heterocomplex [17]. Therefore, the respective cDNA was cloned and sequenced. It showed an ORF of 681-bp encoding a 226-amino acid protein (Fig. 2A) with a theoretical mass of 24.8-kDa. The amino acid sequence and its similarity with *P. berghei* p23 and Human p23 proteins are shown in Figure 2 (A). As expected, p23 is composed by an N-terminal anti-parallel β -sandwich domain and a C-terminal unstructured acidic region [31]. Yeast p23-like homologues presented also a “GGM/A” situated within the C-terminal region [32,33]. *T. gondii* p23 presented a characteristic p23 domain which showed the 8 putative β -sheets and presented a C-terminal region rich in acidic residues (Fig. 2A). It also contained a “GGM” rich region. By evolutionary tracing (ET), Zhu and Tygat [34] identified conserved residues along the p23 sequences (8W, 79K, 86W, 89L, 103F/W and 106W in human p23), which are also present in *T. gondii* p23 (asterisks in Fig. 2A).

With the aim to analyze the protein properties of *T. gondii* p23 we cloned the gene, expressed the respective protein in *E. coli*, and purified it to homogeneity. In order to investigate the secondary structure of *T. gondii* p23 experimentally, we analyzed the protein by far UV CD spectroscopy. The spectrum revealed a minimum between 204 and 208 nm indicative of a protein containing mainly β -sheets and a significant content of random coil (Fig. 2B). Using the deconvolution software CDNN, a content of ~ 46 % β -sheet, 17 % β -turn, 30 % random coil and only ~ 6 % α -helix was calculated. These data are in agreement with the gene-based predictions of a β -sandwich structure and structural data on human and yeast p23 [31,35,36].

Despite of its modulating interaction with Hsp90, a functional key characteristic of p23 is its ability to act as a molecular chaperone itself. To test whether *T. gondii* p23 exhibits chaperone activity, as previously shown for its human or yeast homologues, [31,37] we performed thermal unfolding assays using citrate synthase (CS) as a model substrate. CS gets inactivated and aggregates irreversibly at temperatures above 40°C [19]. As shown in figure 3A, the spontaneous aggregation of CS, as monitored by light scattering at 44°C, reached a maximum value after approximately 40 min. Increasing concentrations of *T. gondii* p23 led to a concentration-dependent inhibition of CS aggregation. Interestingly, molar ratios of 2:1 p23:CS (monomers) already resulted in half maximum and ratios of 4:1 p23:CS in total suppression of the aggregation of CS. In comparison to human or yeast p23, which show half maximal aggregation suppression at ratios of ~5:1 and 10:1 respectively, this indicates a significantly higher (~2–5 fold) chaperone activity for the *T. gondii* protein [31,37].

A second interesting functional feature of previously described p23 proteins is their ability to slow down the inactivation of model substrates. In contrast to aggregation, inactivation is an early step on the unfolding pathway of substrates like CS. To investigate if *T. gondii* p23 is also able to bind early unfolding intermediates of CS, we analyzed the influence of the *T. gondii* p23 on CS inactivation. Similar to human and yeast p23, the *T. gondii* protein was able to slow down the inactivation reaction significantly in a concentration-dependent manner (Fig. 3B). Interestingly, already sub-stoichiometric amounts of p23 influenced CS inactivation. The maximum effect could be seen at molar ratios of 4:1 p23:CS (monomers). This observation implicates a transient binding behavior of p23 to CS.

Taken together, compared to human and yeast p23, *T. gondii* p23 shows highly similar structural properties but overall seems to be an even stronger chaperone *in vitro*.

3.4 Hip and p23 are integrated in different Hsp90 complexes

To investigate if *T. gondii* p23 interacts with Hsp90 and is integrated in similar interaction schemes than human or yeast p23 we first generated anti-p23 and as well anti-Hip antibodies to be able to address their protein-protein interactions by Western blot, co-immunoprecipitation (Co-IP) and immune-fluorescence analysis (IFA). Western-blot analysis showed a single band of 46-kDa which corresponds to the predicted protein size in tachyzoite lysate and coinciding with the one recognized by anti-His antibody in extracts of purified recombinant Hip (Fig. 4). Similarly, anti-*T. gondii* p23 antibody reacted with a single band of 25-kDa on tachyzoite lysate, also matching with the one recognized by anti-His antibody in extracts of purified recombinant Tgp23 (Fig. 4).

In order to demonstrate that the Hsp90-heterocomplex maturation pathway occurring in *T. gondii* is similar to that observed in higher eukaryotes, co-IP assays were performed using anti-Hsp90, anti-Hip and anti-p23 antibodies and analyzed by Western blot with all of them. A commercial antibody against Hsp70 was included to detect this protein in the analyzed complexes. Pull down of Hsp90, Hip and p23 proteins (Fig. 5) revealed that Hip is associated to Hsp70 and Hsp90 but not with p23, whereas p23 interacted with Hsp90 but not with Hip or Hsp70 (Fig. 5).

3.5 p23, but not Hip, translocates to the nucleus during bradyzoite development

Recently we have reported that cytoplasmic Hsp90 translocates to the nucleus when *T. gondii* differentiates to bradyzoite [2]. In order to investigate if some of the Hsp90 co-chaperones behave similarly, IFAs on intracellular tachyzoite and bradyzoites were carried out (Fig. 6). Intracellular, non-virulent *T. gondii* PK cells were grown in tachyzoites and bradyzoites conditions (37°C, 5% CO₂ and 37°C, 0.03% CO₂, pH 8.1, respectively). Hip

showed a cytoplasmic localization in tachyzoite and bradyzoite stages (Fig. 6), without any co-localization with propidium iodide stained nuclei. Co-localization of Hip with cytoplasmic Hsp90, revealed by the overlaid fluorescence signal, confirmed the potential interaction between both proteins (Fig. 6).

For p23, a cytoplasmic localization was observed in tachyzoite samples whereas in bradyzoites both, a cytosolic and nuclear distribution was observed. Furthermore, co-localizing with the cytoplasmic/nuclear Hsp90 was indicated by the overlaid fluorescence (Fig. 6).

3.6 Interactome analysis of *T. gondii* p23

The protein composition of the p23 interactome during bradyzoite development was determined from a differentiated PK strain by immunoprecipitation and mass spectrometry analysis. In order to perform a comparative analysis, tachyzoite lysates of PK strain were also studied. Immunoprecipitation with p23 antibody was more efficient than with Hsp90 antibody (data not shown). Preimmune serum sample gave a clean immunoprecipitation-pattern (Fig. S1). In addition, the use of an anti-*T. gondii* Hsp20 antibody as independent control, did not pull down any protein (data not shown and [38]). Overall, 43 parasite proteins that co-IP with anti-p23 antibody either from tachyzoite or bradyzoite lysates were identified (Table 2, S1 and S2). Almost all of them are involved in ATP production/glycolysis (1–7) and translation (8–13) or are chaperones and other proteins with protein binding properties (24–33). Additionally, some further proteins from rhoptry, microneme, dense granules and histones were pulled down (Table 2, Table S3).

A protein-protein interaction (PPI) model of the *T. gondii* Hsp90/p23 proteins was created by combining our data sets obtained from *T. gondii* p23 IP-mass spectrometry analysis (Table S3), *T. gondii* Hsp90 IP-mass spectrometry (Table S3) and co-IP assays/microscopy co-localization with their subcellular compartmentalization and temporal synchronicity [tachyzoite or bradyzoite stages] (Fig. 7 and Cytoscape file S1). This PPI map was compared with Hsp90/p23 interactomes generated from: 1) the web-based resource POINT (<http://point.bioinformatics.tw/Welcome.do>) which integrates data from several protein prediction interactors (PPI) repositories (IntAct, Mint, DIP, BIND, BioGRID, etc.) into interolog maps, which are based on the assumption that a pair of interacting proteins (interologs) in one species predicts an interaction of orthologous proteins in another species, and 2) from the PPI database for *P. falciparum*, derived from yeast two-hybrid studies [25,26]. Interactome attributes and interactors data are shown in Table S4. Among all the p23 interactors, 15 were also detected in PPI databases (Table 2). Taking together 23 interactors in tachyzoites and bradyzoites and Hsp90 interactors in tachyzoites (Fig. 5 and Table S3) we built an Hsp90/p23 interactome network (Fig. 7). In this network 77% are proteins conserved in other organisms, 65% present as far as mammals and another 12% conserved in addition in *P. falciparum* (Table S3). From this pool of evolutionary conserved interactors, 43% and 35% of the interactions found in this study were also found in the interolog PPI map and *P. falciparum* PPI databases, respectively (Fig. 7 and Table S3). The interactome maps also indicated a wide range of subcellular localizations and functions of putative Hsp90/p23 targets in both parasite stages (summarized in Fig. 7, Table S3 and Cytoscape file S1).

4. Discussion

Recently we described the importance of *T. gondii* Hsp90 in the two asexual stages of interconversion (tachyzoite-bradyzoite) of the parasite [2]. Based on *in silico* analysis, the previously described Hsp90 [2] presented all the requisites to be a candidate to assemble into the Hsp90/70-heterocomplex in *T. gondii*, a prediction supported here by experiments

that show that Hsp90 interacts with the respective co-chaperones Hip and p23. *T. gondii* Hip and p23 showed the typical hallmarks of proteins from these families. *T. gondii* p23 sequence and determined secondary structure was comparable to the sequence and structure of human or yeast p23 [32,33], indicating conservation of the basic IgG like, globular, β sandwich fold throughout the protein family [35,36]. Moreover the *T. gondii* p23 chaperone activity showed to be stronger than its human and yeast counterparts, which could be important for the parasite during bradyzoite development usually triggered by stressful environments [39]. Both *T. gondii* Hip and p23 interacts with Hsp90, but not directly with each other. In higher eukaryotes Hip is a marker of the early Hsp90-heterocomplex, whereas p23 binds to Hsp90 dimers at a later stage [8,13,14]. In comparison, a similar Hsp90-heterocomplex-cycle pathway is occurring in *T. gondii*.

Additionally, we showed that p23 translocates to parasite nucleus in the bradyzoite stage as it was observed for parasite Hsp90 [2]. Recently Picard *et al* [40] demonstrated by FRAP analysis that Hsp90 and p23 are always co-localizing, which is consistent with the notion that the bulk of p23 is engaged in a complex pattern of Hsp90-mediated interactions. Probably in *T. gondii* p23 and Hsp90 could be working as a complex rather than in separated ways. Several studies showed that in mammals and yeast, Hsp90 and its co-chaperone p23 are selectively recruited to gene expression regulatory complexes and that they play a role in telomere maintenance, suggesting extensive functions for these molecular chaperones in gene regulation and formation and maintenance of a particular state of the chromatin [41–44]. Direct interaction between Hsp90 and core histones or chromatin remodeling complexes has been suggested [45–47]. p23 pulled down with H2AZ and H4 in tachyzoite and bradyzoite stages respectively. Recently, *Toxoplasma* H2AZ showed to be associated to active chromatin whereas the variant H2AX and H4K20me1 (H4 mono-methylated in lysine 1) are enriched at silent promoters and its expression is increased during bradyzoite differentiation [48]. There might be a connection between Hsp90-heterocomplex and chromatin regulation in *Toxoplasma* as well.

Co-IP experiments identified several proteins that could be assisted by p23 during bradyzoite development. Some of these proteins could be contaminants (e.g. SAG1, a tachyzoite stage specific protein that appear in bradyzoite pulled down sample); therefore their interaction with p23 or p23/Hsp90 complex should be confirmed. The stage specific interaction also should be confirmed by additional experiments. On the other hand, some p23 interactors were not detected because of analyzing only coomassie stainable bands. It is interesting to note that although there is no antecedent of p23-IP analysis in other organisms, 15 out of the 43 proteins that pulled down in the IP performed with anti-p23 of *T. gondii* were also identified as p23-interacting proteins in other cell systems or interactome networks [49,50]. Since at least 8 proteins are specific of *T. gondii* (micronemes, rhoptry, dense granules and hypothetical proteins) the coincidence degree between our co-IP pool of conserved proteins and PPI databases (near 43%) suggest that our pull down experiment was highly specific. The complete set of Hsp90/p23 interactors that we found suggests that the Hsp90/p23 machine keeps its evolutionary conserved functions with slight specializations for the parasite biology [49,51].

Most of the putative p23-interacting proteins are involved in glycolysis, ATP production, protein synthesis, protein assistance (chaperones and foldases), some kinases and phosphatases (Table 2 and Table S2). Noteworthy, during bradyzoite development the parasite mitochondria is down-regulated [52,53], and accumulate amylopectin granules [54], indicating that in bradyzoites the predominant pathway to obtain energy is glycolysis. Moreover, it was demonstrated that pyruvate kinase and lactate dehydrogenase, presented a markedly higher activity in bradyzoites than in tachyzoites, and over-expression of *T. gondii* lactate dehydrogenase enzymes LDH1 and LDH2 enhances parasite differentiation under

alkaline conditions [55], suggesting that lactate production is particularly important in this developmental form [54]. Recently a deoxyribose phosphate aldolase-like gene expressed in bradyzoite stage was identified [56]. Despite almost all of the enzymes involved in glycolysis are expressed in both parasite stages, some of them were found to be overexpressed in bradyzoite stage in *Neospora caninum*, a closely related parasite of *T. gondii* [57]. In this context, p23 could be assisting parasite glycolytic enzymes during bradyzoite conversion to ensure enough ATP generation in the new parasite scenario. Similarly, p23 could be assisting the translational machinery to generate new proteins during early bradyzoite transformation.

The interactome map generated could be an important tool for future studies in *T. gondii* as well as other cell systems. We could identify novel potential interactors, some of them specific proteins of *T. gondii* (Mic3, Rop4, Rop5, Rop9, Gra5, Gra7). Recently Gra5, but not other dense granule proteins, was shown to interact with Hsp90 at least in tachyzoite stage [58]. *N. caninum* Rop9 and Gra9 showed to be over expressed in bradyzoite stage [57]. Nevertheless, it should be noted that all of them could be in complexes throughout the secretory pathway, suggesting that Hsp90/p23 complex could be assisting the stabilization of these complexes.

The predicted pleiotropic role of parasite Hsp90 and in addition p23, is evidenced by the intracellular localization of its targets from the nucleus and apicoplast to the secretory organelles dense granules, rothtries and micronemes and by the wide range of annotated and predicted function of their interactors. Several cell cycle kinases, phosphatases and transcription factors are client proteins of the Hsp90/p23 complex [6]. Here, we identified some proteins of this type but their role during parasite differentiation remains currently uncertain. The inability to obtain classical cell cycle kinases or transcription factor-like proteins could be due to the fact that they are low abundance proteins.

Taken together, we showed that the Hsp90-hetercomplex cycle is present in *T. gondii*. Additionally we showed that *T. gondii* co-chaperone p23 has some differences in sequence structure and activity in comparison with human p23 that could be important for parasite biology. Finally, the p23 interactome network, presented here, will allow making predictions and hypothesis about the dynamics of the Hsp90/p23 chaperone machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of *T. gondii* Hip co-chaperone

A. Sequence alignment generated by clustal W (Bioedit program). Letters in black indicate identical residues; letters in grey indicate conserved residues. Gaps are indicated by dashes and were introduced to improve the alignment. TgHip, *T. gondii* Hip (Accession number [AN]: **DQ680015**), PbHip, *P. berghei* Hip (AN: Q08168), RnHip, *R. norvegicus* Hip (AN: P50503). TgHip and RnHip sequences showed 36% of identity and 55% of positives, whereas with PbHip identity was of 45% and positives were 58% (Blast2 program at www.ncbi.nlm.nih.gov/Blast). **B.** Schematic representation of *T. gondii* and *R. norvegicus* Hip proteins with the putative protein motifs. Numbers denote the amino acid positions of motifs of the protein. **C.** Sequence alignment of the *T. gondii* and *R. norvegicus*'s tetratricopeptide units from the TPR domains of Hip proteins. Consensus primary structure of the TPR domains: "b": hydrophobic aminoacids, "l": hydrophilic aminoacids. Above, the schematic representation of α helices determined by prediction of secondary structure is indicated. Conserved amino acids are marked in black. Stars indicate the most important positions in this structure (residues 4, 8, 11, 20, 24 and 27). The amino acid in position 32 is normally a hydrophobic residue (e.g. proline).

Figure 2. Characterization of *T. gondii* p23

A. Sequence alignment was generated by clustal W (Bioedit program). Letters in black indicate identical residues; letters in grey indicate conserved residues. Gaps are indicated by dashes and were introduced to improve the alignment. Tgp23, *T. gondii* p23 (Accession number [AN]: **DQ680014**), Pbp23, *P. berghei* p23 (AN: XP_680236), Hup23, human p23 (AN: L24804). TgHip and Pbp23 sequences showed 40% of identity and 57% of positives, whereas with Hup23 it was of 28% of identity and 45% of positives (Blast2 program at www.ncbi.nlm.nih.gov/Blast). Domains, β -sheet regions and conserved residues are indicated in figure. β -sheet was deduced by using the <http://protevo.eb.tuebingen.mpg.de/toolkit>. **B.** *T. gondii* p23 shows the characteristic, (β -sheet rich secondary structure. The far-UV-CD spectrum of the native *T. gondii* p23 was recorded at a protein concentration of 0.3 mg/ml in 50 mM Tris/HCl, pH 8.0 at 20°C. *Inset:* Calculated percentage of secondary structure elements.

Figure 3. Determination of *T. gondii* p23 chaperone activity

A: Influence of p23 on the thermal aggregation of CS. CS (final concentration: 1.0 μM) was diluted into a thermostated solution (43°C) containing 1.0 μM (□), 2.0 μM (◇), 4.0 μM (▽) or 8.0 μM (Δ) p23. Circles (○) represent the spontaneous aggregation of CS at 43°C. The kinetics of aggregation was determined by measuring the light scattering of the samples at 400 nm. **B:** Influence of p23 on the thermal inactivation of CS. Inactivation of CS (0.15 μM) at 43°C in the absence (○) and in the presence of increasing concentrations of p23; 0.075 μM (+), 0.15 μM (□), 0.3 μM (◇) or 0.6 μM (▽).

Figure 4. Detection of native Hip and p23 proteins

Western-blot with murine anti-*T. gondii* Hip (α -TgHip), murine anti-*T. gondii* p23 (α -Tgp23) and murine anti-Histidines (α -His) antibodies. Tg: *T. gondii* PK strain lysate. Protein extract from uninfected HFF cells were also assayed. Preimmune serum samples did not show reactivity (data not shown). Migration of size marker is indicated (in kilo Daltons [kDa]).

Figure 5. Co-IP analysis of Hsp90, Hip and p23 proteins

Toxoplasma gondii PK strain lysates (Tg) were used for immunoprecipitation (IP) using either preimmune (Pre) or specific α Hsp90, α Hip and α p23 antisera (vertical columns). Pulled down materials were analyzed by western blot with specific sera antibodies and a commercial anti-Hsp70 antibody (horizontal columns).

Figure 6. Indirect immune-fluorescence assay (IFA) and subcellular localization of Hip and p23 in intracellular PK tachyzoites (Tz) and bradyzoites (Bz)

PK Tz were grown *in vitro* in human foreskin fibroblasts (HFF) and induced to Bz.[2]. To induce Bz formation in PK strain, parasites were grown 4 days at pH 8.1 and low CO₂). IFA was performed on fixed intracellular parasites with antibodies against *T. gondii* Hip (murine serum), -p23 (murine serum) and -Hsp90 (rabbit serum) antibodies [2]. Propidium iodide (PI) staining reveals the location of the nucleus (red color). Mouse anti-P30 (α -SAG1) or anti-P34 serum (α -p34, mAb T82C2)[59] were used as marker of Tz and Bz respectively (data not shown). Rabbit anti-BAG1 polyclonal serum was used as cytoplasmic bradyzoite marker. The efficiency of *in vitro* conversion to Bz was 82–88%. White arrows heads are signaling the localization of the nucleus in both panels.

Figure 7. Differential interactome of Hsp90/p23 complex in tachyzoites and bradyzoites
 Interactors are visualized in a scheme of *T. gondii* tachyzoite and bradyzoite. Combined interactome of Hsp90 and p23 proteins was built using the Cytoscape platform based on the data obtained from proteomic analysis and verified by: co-IP assays from the present study (grey interactors), mining Hsp90 and p23 interactomes present in the public PPI database POINT (<http://point.bioinformatics.tw>) and the specific database for *P. falciparum* (<http://apidb.org/apidb/showQuestion.do?questionFullName=GeneQuestions>) (rectangle and dashed contour, respectively). Cellular localization is inferred from Gene Ontology data. ACT, actin; ADT, ADP/ADT carrier; BCKDH, 2-oxoisovalerate dehydrogenase; CLH, clathrin heavy chain; CRMP, Cysteine repeat modular protein; EF1-A, elongation factor 1

alpha; EF-2, elongation factor 2; ENO2, enolase 2; FBP/aldo, Fructose 1,6 biphosphatase aldolase; GAPDH, glyceraldehyde-3-phosphate dehydorgenase; LDH, lactate dehydrogenase 1, MyoA, myosin A; NTP1, nucleoside triphosphatase I; PDIA, protein disulfide isomerase; PKM, pyruvate kinase I; Trp and Asn-tRNAs, tryptophanyl and asparaginyln tRNA synthase.

Table 1Identification of *T. gondii* Hsp90 and Hsp90-heterocomplex related proteins

Gene ID Release 5	Gene ID Release 4	Domains [*] /signal sequences	Chromosome	Protein identification ⁺ (GenBank)
TGME49_088380 TGGT1_035020 TGVEG_082150	80.m00001	Histidine kinase-like ATPase, Hsp90, HtpG, C-terminal EEVD	IX	Hsp90 (AN: AY344115)
TGME49_044560 TGGT1_045380 TGVEG_104350	49.m00060	Histidine kinase-like ATPase, Hsp90, HtpG, C-terminal KDEL	VI	GRP94 <i>putative</i> (AN: AAY53805)
TGME49_092920 TGGT1_073160 TGVEG_014980	83.m01184	Histidine kinase-like ATPase, Hsp90, HtpG N-terminal mitochondria/chloroplast transit peptide	Ia	Hsp75 or TRAP1 <i>putative</i>
TGME49_110430 TGGT1_086650 TGVEG_094510	583.m05357	Histidine kinase-like ATPase, Hsp90, HtpG, N-terminal mitochondria/chloroplast transit peptide	XI	Hsp90Chloroplast <i>putative</i>
TGME49_073760 TGGT1_112840 TGVEG_021100	59.m00003	Hsp70, DnaK, actin-like ATPase, C-terminal EEVD	VIII	Hsp70 (AN: AAC72002)
TGME49_111240 TGGT1_087560 TGVEG_095340	583.m05418	1 DnaJ, G-Rich region, DnaJ_CXXCXGXXG repeats/Zinc finger CR-type profile, 1 DnaJ_C, HPD sequence	XI	TgYdJ1 <i>putative</i>
TGME49_032660 TGGT1_114860 TGVEG_022980	44.m00052	1 TPR, 1 STH1, C-terminal DP repeat	VIII	Hip <i>putative</i>
TGME49_052220 TGGT1_001140 TGVEG_055940	52.m00012	3 TPR, C-terminal DP repeat	III	Hop <i>putative</i>
TGME49_121520 TGGT1_064560 TGVEG_059010	645.m00320	1 P23/CS	Ib	p23 <i>putative</i>
TGME49_112200 TGGT1_088450 TGVEG_096300	583.m05469	1 TPR, 1 Protein phosphatase 2A homologue/ Calcineurin- like phosphoesterase	XI	Immunophilin PP5-like <i>putative</i>
TGME49_083850 TGGT1_035880 TGVEG_032190	76.m00007	1 FKBP_C, 1 TPR, 1 cyclophilin	V	Immunophilin FBCP (AN: AAX51680)
TGME49_010800 TGGT1_124070 TGVEG_008760	27.m00826	Aha1_N, Activator of Hsp90 ATPase	IV	Aha 1 <i>putative</i>

Release 4*
NCBI Conserved Domain Search⁺
based on domains, signal sequences and/or blastp identityTGME49, *T. gondii* strain Me49, TGGT1, *T. gondii* strain GT1 and TGVEG, *T. gondii* strain VEG

Table 2

Proteomic analysis of proteins pulled down with anti-P23 antibody in tachyzoite and bradyzoite stages

Function	Protein name	stage	Gene ID Release 5	Gene ID Release 4	Peptide freq (Tz/Bz)	Interactome analysis (db) *
Glycolysis/ ATP generation	1 Triose phosphate isomerase I	Bz	TGME49_025930	42.m00050	3	
	2 Glyceraldehyde-3-phosphate dehydrogenase I	Bz	TGME49_089690	80.m00003	10	
	3 Phosphoglycerate kinase I	Bz	TGME49_118230	641.m00193	7	
	4 Enolase 2 (ENO2)	Tz,Bz	TGME49_068850	59.m03410	4/8	yes
	5 Pyruvate kinase I	Bz	TGME49_056760	55.m00007	5	yes
	6 Lactate dehydrogenase I (LDH1)	Tz,Bz	TGME49_032350	44.m00006	9/13	yes
	7 Fructose-1,6-bisphosphate aldolase	Tz,Bz	TGME49_036040	46.m00002	6/9	yes
Translation machinery/ Protein metabolism	8 Elongation factor 1alpha	Tz,Bz	TGME49_086420	76.m00016	14/14	
	9 Elongation factor 2	Tz,Bz	TGME49_005470	20.m03912	5/4	yes
	10 Asparaginyl-tRNA synthase	Bz	TGME49_070510	59.m03518	2	yes
	11 Tryptophanyl tRNA Synthase	Bz	TGME49_088360	80.m00063	4	
	12 nascent polypeptide-associated complex	Tz	TGME49_005560	20.m03918	3	
	13 Cytosol aminopeptidase	Bz	TGME49_090670	80.m00088	4	
Kinases/ Phosphorylases/ Phosphatases	14 Rhopty protein 4 (protein kinase Rop4))	Bz	TGME49_095110	83.m02145	9	
	15 Rhopty protein 5 (protein kinase Rop5)	Bz	TGME49_108080	551.m00238	13	
	16 Uridine Phosphorylase	Bz	TGME49_110640	583.m00630	3	
	17 Nucleoside triphosphatase I	Tz,Bz	TGME49_077240	65.m00001	5/7	
	18 Ser-Thr phosphatase 2C	Tz,Bz	TGME49_031850	44.m00037	1/7	yes
Cytoskeleton/motility/ Traffic	19 Actin	Tz,Bz	TGME49_009030	25.m00007	3/15	yes
	20 Myosin A	Tz	TGME49_035470	46.m00001	21	yes
	21 Clathrin heavy chain	Tz	TGME49_090950	80.m02298	2	

Function	Protein name	stage	Gene ID Release 5	Gene ID Release 4	Peptide freq (Tz/Bz)	Interactome analysis (db) *
Redox homeostasis	Thioredoxin	Bz	TGME49_009950	25.m00203	4	
	Protein disulfide isomerase	Tz,Bz	TGME49_011680	27.m00003	17/21	
Chaperones/ protein binding	Hsp101/ClpA	Tz	TGME49_057990	55.m04729	5	
	Grp94	Tz,Bz	TGME49_044560	18/4		
	Hsp90	Bz	TGME49_044560	80.m00001	7	yes
	Hsp70	Tz,Bz	TGME49_073760	59.m00003	11/8	yes
	Organellar Hsp70	Bz	TGME49_051780	50.m00085	2	yes
	Hsp70/BiP	Tz,Bz	TGME49_111720	583.m00009	2/24	yes
	Hsp60	Bz	TGME49_047550	50.m00006	5	
	Hsp20	Tz,Bz	TGME49_032940	44.m02755	224/69	
	14.3.3	Bz	TGME49_063090	55.m00015	5	yes
	Hypotetical protein, WD repeat containing protein	Tz	TGME49_056860	55.m04655	1	
Others	Surface antigen SAG1	Bz	TGME49_033460	44.m00009	9	
	B-antigen	Tz,Bz	TGME49_062470	55.m04993	2	
	Dense granule 7 (Gra7)	Bz	TGME49_003310	20.m00005	11	
	Dense granule 5 (Gra5)	Tz	TGME49_086450	76.m00004	2	
	Rhoptry protein 9 (Rop9)	Bz	TGME49_043730	49.m00048	7	
	Microneme protein 2 (Mic2)	Tz,Bz	TGME49_001780	20.m00002	2/3	
	Histone 4 (H4)	Bz	TGME49_044560	49.m03134	4	
	Histone 2A variant Z (H2AZ)	Tz	TGME49_100200	145.m00002	4	yes
	Hypotetical protein	Bz	TGME49_034380	46.m01601	4	
	Hypotetical protein	Bz	TGME49_063850	55.m08199	2	

1–43, list order number of identified proteins in toxodb

* Plasmodium Y2H and orthologs data bases

Tz: tachyzoite

Bz:bradyzoite