CASSIANO MARTIN BATISTA

PRODUÇÃO DE ANTICORPOS CONTRA TcHIP E CRUZIPAÍNA DE *TRYPANOSOMA CRUZI*, COM APLICAÇÃO NO ESTUDO DE ENDOCITOSE EM AMASTIGOTAS

Curitiba/PR
Fevereiro-2014

INSTITUTO CARLOS CHAGAS

Mestrado em Biociências e Biotecnologia

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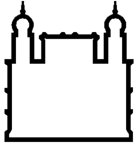
CASSIANO MARTIN BATISTA

Curitiba/PR

Fevereiro-2014

Lombada

DISSERTAÇAO M -ICC*	C.M. BATISTA	2014



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INSTITUTO CARLOS CHAGAS

Pós-Graduação em Biociências e Biotecnologia

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Dissertação apresentada ao Instituto Carlos Chagas/Fiocruz-PR como parte dos requisitos para obtenção do título de Mestre em Biociências e Biotecnologia

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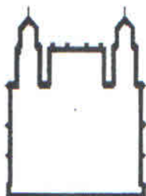

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Ata da Sessão Pública de exame de dissertação para obtenção do grau de **Mestre** em Biociências e Biotecnologia.

Aos 24 dias do mês de fevereiro de dois mil e quartoze, às quartoze horas, nas dependências do Instituto Carlos Chagas/Fiocruz Paraná, reuniu-se a Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Biociências e Biotecnologia, composta pelos Professores: Dr. Stênio Perdigão Fragoso, Dr. Bruno Dallagiovanna Muniz e Dra. Dorly de Freitas Buchi, com a finalidade de julgar a dissertação do(a) candidato(a) **Cassiano Martin Batista**, intitulada: **"Produção de anticorpos contra TcHIP e Cruzipaína de Trypanosoma Cruzi, com aplicação no estudo de endocitose em amastigotas"**, para obtenção do grau de Mestre em Biociências e Biotecnologia. O(a) candidato(a) teve até 45 (quarenta e cinco) minutos para a apresentação, e cada examinador teve um tempo máximo de arguição de 30 (trinta) minutos, seguido de 30 (trinta) minutos para resposta do(a) candidato(a) ou de 60 (sessenta) minutos quando houve diálogo na arguição. O desenvolvimento dos trabalhos seguiu o roteiro de sessão de defesa, estabelecido pela Coordenação do Programa, com abertura, condução e encerramento da sessão solene de defesa feito pelo Presidente **Dr. Stênio Perdigão Fragoso**. Após haver analisado o referido trabalho e arguido o(a) candidato(a), os membros da banca examinadora deliberaram pela "......aprovação......", habilitando-o(a) ao título de Mestre em Biociências e Biotecnologia, condicionada à implementação das correções sugeridas pelos membros da Banca Examinadora e ao cumprimento integral das exigências estabelecidas no Regimento Interno deste Programa de Pós-Graduação.

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Aos meus queridos avôs, David Martin e Benedicto Baptista, *in memoriam*.

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“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.”

Albert Einstein

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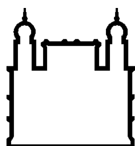

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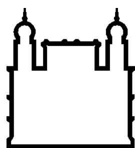
Produção de anticorpos contra TcHIP e cruzipaína de *Trypanosoma cruzi* , com aplicação no estudo de endocitose em amastigotas

RESUMO

DISSERTAÇÃO DE MESTRADO

Cassiano Martin Batista

A endocitose é um evento biológico já bem descrito em formas epimastigotas de *Trypanosoma cruzi*, onde macromoléculas são internalizadas através da bolsa flagelar e/ou do complexo citóstoma/citofaringe e direcionadas aos reservossomos. Reservossomos são grandes organelas localizadas na região posterior do parasito que estocam moléculas ingeridas e contém enzimas lisossomais, como por exemplo a cruzipaína. O objetivo desta dissertação foi produzir anticorpos policlonais ou monoclonais (mAbs) contra proteínas identificadas na proteômica de reservossomos (cruzipaína e TcHIP), e utilizá-las como marcadores de organelas da via endocítica e em estudos de endocitose em formas amastigotas de *T. cruzi*. Proteínas recombinantes foram expressas em *E. coli*, purificadas e inoculadas em camundongos Balb/c. A reatividade dos anti-soros foi confirmada por *western blot* contra extrato total de *T. cruzi* e contra proteína recombinante. Imunolocalização utilizando anticorpos policlonais anti-TcHIP mostrou que esta proteína está presente no complexo de Golgi em *T. cruzi*, o que foi confirmado por co-localização com TcRab7-GFP. Por outro lado, anticorpos policlonais obtidos contra cruzipaína recombinante demonstraram esta proteína no Golgi e em reservossomos de epimastigotas de *T. cruzi*. Para a produção de mAbs, esplenócitos de um camundongo imunizado com cruzipaína recombinante foram fusionados com células de mieloma da linhagem P3X63Ag8.653 (ATCC CRL-1580). Os hibridomas foram triados por ELISA, *western blot* e imunofluorescência indireta. O hibridoma mais estável foi selecionado para diluição limitante e um clone contra a cruzipaína recombinante (mAb CZP-315.D9) foi obtido, específico para reservossomos. Este anticorpo foi utilizado como ferramenta para investigar a co-localização de transferrina ingerida com a cruzipaína em formas amastigotas. Foi também possível demonstrar pela primeira vez por citometria de fluxo a atividade endocítica em formas amastigotas de *T. cruzi*.



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Production of antibodies against TcHIP and cruzipain of *Trypanosoma cruzi*, with application on study of endocytosis in amastigotes

ABSTRACT

MASTER DISSERTATION

Cassiano Martin Batista

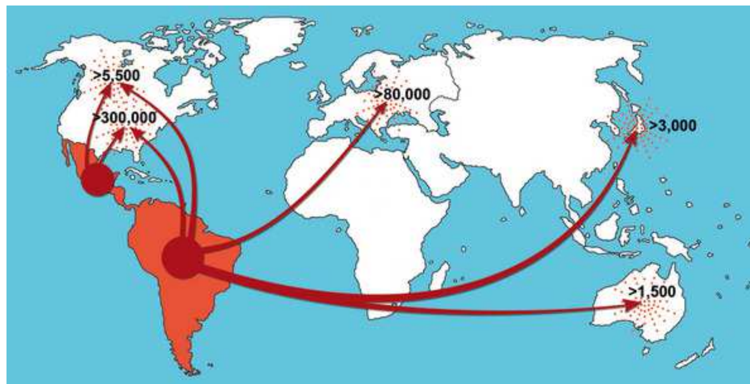
Endocytosis is a biological event well described in *Trypanosoma cruzi* epimastigotes, where macromolecules are ingested via the flagellar pocket and/or the cytostome/ cytofarynx complex and then targeted to reservosomes. Reservosomes are large organelles found at the posterior region of the cell body that store ingested molecules and contain lysosomal enzymes, such as cruzipain. Aim of this dissertation was to produce polyclonal or monoclonal antibodies (mAbs) against proteins identified in proteomic analyzes of reservosomes (cruzipain and TcHIP), and use these proteins both to label organelles of the endocytic pathway and to study endocytosis in *T. cruzi* amastigotes. Recombinant proteins were expressed in *E. coli*, purified and inoculated into Balb/c mice. Reactivity of the anti-sera was confirmed by western blot using total extracts of *T. cruzi* and recombinant proteins. Immuno-localization using polyclonal antibodies anti-TcHIP showed this protein in the Golgi apparatus of *T. cruzi*, which was confirmed by co-localization with GFP-TcRab7. Polyclonal antibodies against recombinant cruzipain recognized the protein in the Golgi and reservosomes of *T. cruzi* epimastigotes. For production of mAbs, splenocytes obtained from a mouse immunized with recombinant cruzipain were fused with the P3X63Ag8.653 myeloma cell line (ATCC CRL-1580). Hybridomas were then screened by ELISA, western blot and immunofluorescence. The most stable hybridoma was selected for limiting dilution and one clone against recombinant cruzipain (mAb CZP-315.D9) was obtained, specific for reservosomes. This antibody was then used as a tool to investigate in amastigotes the co-localization of uptaken transferrin with cruzipain. It was also possible to demonstrate for the first time by flow cytometry the endocytic activity in *T. cruzi* amastigotes.

1. INTRODUÇÃO

1.1. A DOENÇA DE CHAGAS

A doença de Chagas, também conhecida como tripanossomíase americana, afeta 7 a 8 milhões de pessoas no mundo, principalmente na América Latina (Coura e Viñas, 2010; Salomon, 2012). Além disso, cerca de 80 milhões de pessoas estão em áreas sob risco de infecção (Fig. 1.1) (Coura e Viñas, 2010). Esta enfermidade, causada pelo protozoário hemoflagelado *Trypanosoma cruzi*, foi descrita pelo médico e pesquisador brasileiro Carlos Chagas (1878-1934), que também descreveu o complexo ciclo de vida deste parasito (Chagas, 1909).

Figura 1.1. Distribuição mundial da doença de Chagas. O foco da doença se concentra nas Américas do Sul e Central e no México. No entanto, há casos disseminados por todo o mundo, principalmente pela migração de pessoas infectadas e pela falta de diagnóstico em países não endêmicos (Adaptado de Coura e Viñas, 2010).



A doença de Chagas apresenta um curso clínico lento e crônico. Alguns dias após a infecção ocorre a fase inicial aguda, caracterizada pela detecção do parasito por exame de sangue e sintomatologia variada e inespecífica. Após alguns meses a parasitemia diminui e o parasito permanece latente em tecidos, dando início à fase crônica assintomática, também chamada de indeterminada. Após anos de silenciamento, cerca de 30-40% dos pacientes podem desenvolver quadros clínicos típicos da doença, como cardiomiopatias e problemas digestórios e/ou neurológicos (Urbina, 2010).

Existem dois fármacos disponíveis para o tratamento da doença, o nirfutimox (cujo uso foi interrompido no Brasil devido aos elevados efeitos colaterais) e o benzonidazol. Entretanto, ambos apresentam baixa eficácia na fase crônica (fase na qual ocorre a maioria dos diagnósticos) e efeitos colaterais exacerbados, além de existirem cepas de parasitas resistentes a ambos os fármacos (Filardi e Brener, 1987; Murta e

Romanha, 1998; Urbina, 2010). Diante desta problemática, torna-se necessária a busca por terapias alternativas para a doença de Chagas, bem como investimento em linhas de pesquisa que visam elucidar eventos biológicos vitais no *T. cruzi*, tais como a endocitose de nutrientes, a fim de encontrar novos alvos terapêuticos.

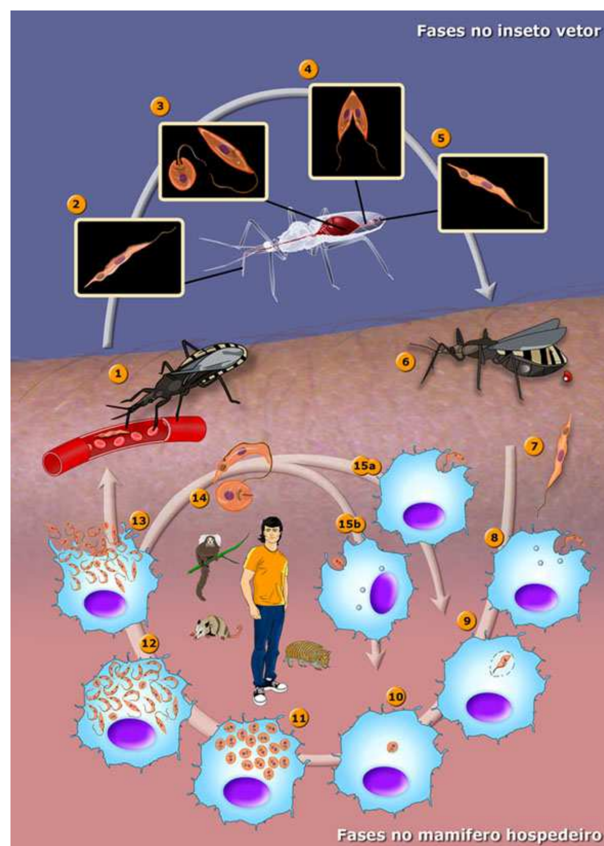
1.2. O *TRYPANOSOMA CRUZI*

O protozoário uniflagelado *T. cruzi* (Euglenozoa: Kinetoplastea) pertence à família Trypanosomatidae. Outros protozoários patogênicos pertencem a esta família, como o *T. brucei* (causador da doença do sono na África) e diversas espécies de *Leishmania* (que causam as leishmanioses). Os tripanossomatídeos possuem características morfológicas peculiares e específicas, sendo a principal delas a presença do cinetoplasto, uma região da mitocôndria onde se localiza um acúmulo de DNA (kDNA). Estes parasitos possuem apenas um flagelo, que se origina de uma bolsa flagelar que alterna sua localização (anterior ou posterior) de acordo com a forma evolutiva do parasito (De Souza, 2002).

A região anterior do corpo do *T. cruzi* (de onde o flagelo emerge) é de extremo interesse no estudo da biologia celular deste parasito, pois é neste local que se concentram os portais de endocitose (bolsa flagelar e complexo citóstoma/citofaringe) e exocitose (complexo de Golgi e bolsa flagelar), os quais representam processos vitais para a sobrevivência do parasito (descrito em mais detalhes no item 1.3.2).

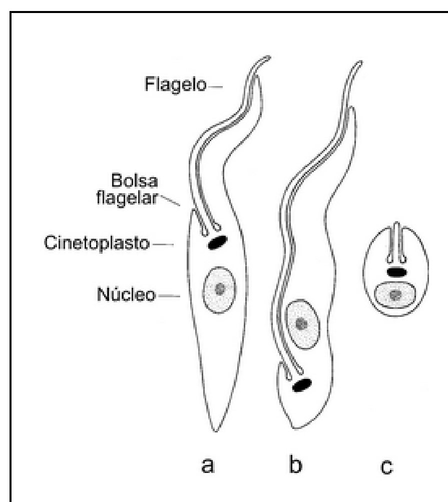
O *T. cruzi* possui um ciclo de vida complexo com alternância entre um hospedeiro invertebrado e um vertebrado, podendo infectar diferentes espécies de mamíferos, inclusive humanos (Fig. 1.2). No tubo digestório do hospedeiro invertebrado (insetos vetores da subfamília Triatominae), o parasito apresenta pelo menos duas formas evolutivas distintas: epimastigotas (replicativas, no intestino médio) e tripomastigotas metacíclicas (infectivas, na ampola retal). Já no hospedeiro vertebrado ocorrem principalmente as formas evolutivas amastigotas intracelulares (replicativas e infectivas) e tripomastigotas sanguíneas (infectivas) (De Souza, 1984). Além da transmissão vetorial, também pode ocorrer transmissão transfusional, congênita ou oral, esta última caracterizada pela ingestão de alimentos contaminados com o parasito, sendo essa via, atualmente, a principal responsável por surtos agudos da doença de Chagas no Brasil (Coura e Viñas, 2010; Steindel et al., 2008; Carter et al., 2012).

Figura 1.2. O ciclo de vida do *Trypanosoma cruzi*. Contaminação do inseto vetor (1) ocorre quando este pica um mamífero infectado e ingere formas tripomastigotas sanguíneas (2), que migram para a porção anterior do estômago e se diferenciam em epimastigotas e algumas formas esferomastigotas (3). Formas epimastigotas alcançam o intestino e se multiplicam por divisão binária (4), aderindo às membranas perimicrovilares secretadas pelas células intestinais. Epimastigotas podem alcançar o intestino posterior, onde iniciam a diferenciação em tripomastigotas metacíclicos (5), que aderem à cutícula que reveste o epitélio do reto e ampola retal do inseto. Contaminação do hospedeiro vertebrado ocorre quando o inseto infectado pica o mamífero. Neste processo o inseto geralmente defeca e urina (6), depositando assim sobre a pele ou mucosa do mamífero as formas tripomastigotas metacíclicas, que são infectivas (7), porém não replicativas. A invasão ocorre quando o hospedeiro se coça, gerando lesões da pele por onde o parasita pode penetrar, ou através das mucosas. Os tripomastigotas acessam a circulação sanguínea, aderindo e invadindo diferentes células nucleadas, tais como macrófagos (8), células musculares e epiteliais. Intracelularmente, os tripomastigotas se localizam em vacúolos parasitóforos (9). No interior do vacúolo os tripomastigotas se diferenciam em amastigotas e ocorre a lise da membrana do vacúolo (10). No citoplasma, as amastigotas se multiplicam por fissões binárias sucessivas (11) e em seguida se diferenciam em tripomastigotas, passando por uma forma de transição (12) antes da célula hospedeira ser rompida pelo excesso de parasitos (13). Lise da célula hospedeira pode ocorrer antes da total diferenciação de amastigotas em tripomastigotas, o que gera o aparecimento de diferentes formas no meio externo (14). No meio extracelular, os tripomastigotas (15a) e amastigotas (15b) podem infectar novas células (Fonte de acesso: Atlas didático, disponível em http:// labspace.open.ac.uk/ mod/ resource/view.php?id=459540&direct=1).



Nas diferentes formas evolutivas do *T. cruzi* o cinetoplasto (região de acúmulo do DNA mitocondrial e de onde se origina o flagelo) ocupa diferentes localizações intracelulares, o que determina a localização da bolsa flagelar (e consequentemente, do flagelo). Portanto, (a) formas epimastigotas possuem um flagelo anterior longo que emerge da bolsa flagelar e cinetoplasto localizados na região anterior ao núcleo, (b) formas tripomastigotas possuem um flagelo longo anterior emergindo da bolsa flagelar e cinetoplasto localizados na região posterior ao núcleo, e (c) formas amastigotas possuem flagelo curto emergindo da bolsa flagelar e o cinetoplasto localizados na região anterior ao núcleo (Fig. 1.3) (Revisto por De Souza, 2002).

Figura 1.3. Cinese do cinetoplasto em formas epimastigotas, tripomastigotas e amastigotas de *T. cruzi*. Em epimastigotas (a) e amastigotas (c), o cinetoplasto está localizado na região anterior do parasito. Nas formas tripomastigotas (b), o cinetoplasto está localizado na região posterior. Essa movimentação altera a localização da bolsa flagelar e, consequentemente, do flagelo (Fonte de acesso: http://dc205.4shared.com/doc/kCQWTzat/preview.html, com modificações).

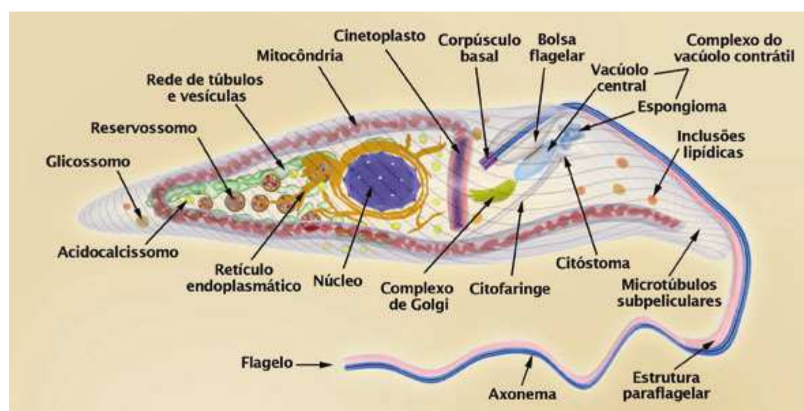


O *T. cruzi* possui organelas comuns a outras células eucarióticas, e outras específicas (Fig. 1.4). A mitocôndria única do parasito se estende por todo o corpo celular e concentra seu DNA em uma região especializada denominada cinetoplasto. O complexo de Golgi único está sempre localizado lateralmente ao cinetoplasto, na região da bolsa flagelar (revisto por De Souza, 2002). Como na maioria das células eucarióticas, o complexo de Golgi de *T. cruzi* possui uma região de entrada *Cis* (*Cis Golgi Network*) associada a uma cisterna do retículo endoplasmático, e uma região de saída *Trans* (*Trans Golgi Network*) rica em vesículas exocíticas (Figueiredo e Soares, 1995). Também associado à bolsa flagelar encontra-se um sistema osmo-regulador,

formado por um vacúolo contrátil alimentado por diversos túbulos coletores (Girard-Dias et al., 2012).

Os glicossomos são um tipo especializado de peroxissomos, onde as enzimas da via glicolítica do parasito se concentram (Michels e Opperdoes, 1991). Os acidocalcissomos são ricos em cálcio e fosfato e possuem pH ácido, estando presentes em maior quantidade em epimastigotas (Docampo e Moreno, 1999). Os reservossomos são organelas peculiares de epimastigotas, localizados na porção posterior da célula, concentrando a reserva de nutrientes ingeridos por endocitose (Soares e De Souza, 1988; Soares e De Souza 1991; Soares et al., 1992).

Figura 1.4. Esquema estrutural de epimastigotas de *T. cruzi*. O parasito possui organelas típicas de células eucariotas (núcleo, retículo endoplasmático, mitocôndria, complexo de Golgi), mas também organelas e estruturas especializadas (glicossomos, reservossomos, cinetoplasto e citóstoma). (Fonte de acesso: Atlas didático: http://labspace.open.ac.uk/mod/resource/ view.php?id=459544-).



1.3. A VIA ENDOCÍTICA

Em células eucarióticas a endocitose é um mecanismo básico para ingestão de macromoléculas, as quais são degradadas no sistema endossomal-lisossomal, gerando assim precursores para diversas vias metabólicas. Em geral, a formação de vesículas endocíticas ocorre por toda a extensão da superfície celular. Entretanto, em tripanossomatídeos a endocitose ocorre em um compartimento bem definido, a bolsa flagelar (revisto por De Souza et al., 2009). Surpreendentemente, as formas epimastigotas do *T. cruzi* possuem um segundo portal endocítico, o complexo citóstoma/citofaringe, também localizado na região anterior da célula (revisto por De Souza et al., 2009).

1.3.1. A via endocítica em células eucarióticas

Existem três mecanismos endocíticos básicos: fagocitose, macropinocitose e a endocitose propriamente dita. A fagocitose se caracteriza por emissão de expansões de membrana plasmática que envolvem a partícula a ser ingerida, sustentadas por filamentos de actina. Dentro da célula o material ingerido se localiza em vacúolos conhecidos como fagossomos, onde é degradado pela ação de enzimas lisossomais. A macropinocitose se caracteriza pela ingestão de pequenas quantidades de fluidos extracelulares através de microprojeções de membrana sustentadas por filamentos de actina. O material ingerido é então direcionado para o sistema endossomal-lisossomal. Já na endocitose propriamente dita há formação e brotamento de pequenas vesículas por invaginações da membrana plasmática, as quais contém as macromoléculas a serem ingeridas. O brotamento destas vesículas pode ser: (a) dependente ou independente de revestimento de clatrina ou caveolina, mas dependente de dinamina, ou (b) independente de revestimento de clatrina, caveolina e sem envolvimento da dinamina, mas com participação de flotilina-1 e flotilina-2 (revisto por Doherty e McMahon, 2009).

Após as vesículas endocíticas perderem seus revestimentos (caso estes estejam presentes) elas se fusionam com os endossomos iniciais (pH 6,5), sendo o conteúdo endocitado transferido para os endossomos tardios (pH 6.0), os quais contém enzimas lisossomais ainda inativas (oriundas do complexo de Golgi). Os endossomos tardios se fusionam com os lisossomos (pH 5,0), onde as enzimas lisossomais são ativadas. Os produtos da digestão são então utilizados em diversas vias metabólicas (revisto por Houtari e Helenius, 2011).

1.3.2. A via endocítica em tripanossomatídeos

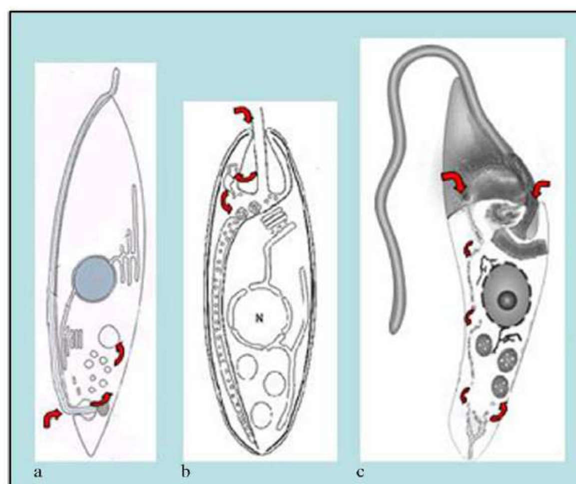
Em tripanossomatídeos ocorre apenas a endocitose propriamente dita, que ocorre apenas pela região da bolsa flagelar, já que a rigidez da membrana plasmática, causada pela presença de microtúbulos subpeliculares, impede a formação de vesículas ao longo do corpo celular (De Souza, 2002). Em tripanossomatídeos, a endocitose é bem estudada no *Trypanosoma brucei*. Neste parasito a endocitose é regulada de acordo com o estágio evolutivo da célula: a atividade endocítica é dez vezes maior em formas tripomastigotas sanguíneas, quando comparada com formas tripomastigotas procíclicas (Morgan et al., 2001). Neste parasito, as maquinarias endocíticas e secretoras estão localizadas na região posterior da célula (Fig. 1.5.a) e a captação de macromoléculas

externas está restrita à bolsa flagelar (Webster, 1989; Landfear e Ignatushchenko, 2001). A partir da bolsa flagelar, vesículas com revestimento de clatrina brotam carregando as macromoléculas ingeridas, que são entregues aos endossomos iniciais. Em *T. brucei* existem duas classes de vesículas com revestimento de clatrina: c*lathrin coated vesicles* de classes I e II (CCV-I e CCV-II, respectivamente) (Grunfelder et al., 2003). As CCV-I são grandes vesículas (135 nm), específicas de formas sanguíneas e ricas em macromoléculas ingeridas da superfície celular, originadas de vesículas com revestimento que brotaram da bolsa flagelar. Essas vesículas se fundem com cisternas endossomais. As CCV-II são pequenas vesículas (50-60 nm) que concentram traçadores de fase fluida, que seriam destinados à reciclagem (Engstler et al., 2004). Estas vesículas brotam em torno das cisternas endossomais e se fusionam com endossomos de reciclagem. Vesículas com revestimento de clatrina também são visualizadas brotando da rede *trans* do Golgi, provavelmente carregando enzimas lisossomais para a degradação das macromoléculas ingeridas contidas nos lisossomos. Os lisossomos de *T. brucei* estão localizados na região posterior, sendo organelas únicas ou um agrupamento de organelas (Webster, 1989; Alexander et al., 2002). Um marcador de lisossomos de *T. brucei* é a glicoproteína p67, que é estruturalmente similar às LAMPs (*lysosome associated membrane proteins* - marcadoras de lisossomos em mamíferos) (revisto por De Souza et al., 2009).

Em *Leishmania*, a endocitose já foi descrita tanto em formas promastigotas quanto em amastigotas (De Souza Leão et al., 1995; Waller e McConville, 2002). Formas promastigotas utilizam exclusivamente a bolsa flagelar para ingerir macromoléculas, que têm como destino o sistema endossomal-lisossomal (Fig. 1.5.b). Vesículas com revestimento de clatrina já foram observadas brotando da bolsa flagelar (Weise et al., 2000). A presença do gene codificante para esta proteína no genoma e a imunolocalização de clatrina concentrada na região da bolsa flagelar confirmam a expressão de clatrina nestes protozoários (Denny et al., 2005). Existem três organelas específicas da via endocítica em *Leishmania*: (a) corpos multivesiculares, adjacentes ao complexo de Golgi com 200-300 nm de diâmetro e com função indeterminada; (b) estruturas tubulares, localizadas lateralmente ao complexo de Golgi e à bolsa flagelar com 60 nm de diâmetro, que podem corresponder aos endossomos iniciais de promastigotas, pois constantemente estão ligadas a vesículas e acumulam traçadores endocíticos de fase fluida, e (c) túbulos multivesiculares, que ocupam as regiões anterior e posterior do parasito e contém pequenas vesículas de tamanho (100-200 nm de

diâmetro) e morfologia variáveis. Os túbulos multivesiculares acumulam traçadores endocíticos de fase fluida e são as últimas organelas da via (revisto por De Souza, 2009; Ghedin et al., 2001).

Figura 1.5. As vias endocíticas dos tripanossomatídeos. a) Formas tripomastigotas sanguíneas de *T. brucei*. A bolsa flagelar, por onde o parasito ingere macromoléculas, está localizada na região posterior, onde os outros compartimentos endocíticos também estão; b) Formas promastigotas procíclicas de *Leishmania*. A bolsa flagelar está localizada na região anterior e o destino final é a região posterior; c) formas epimastigotas de *T. cruzi*. Para a ingestão de nutrientes, o parasito conta também com o complexo citóstoma/citofaringe, localizado na região anterior. Independente da origem, macromoléculas ingeridas são direcionadas aos reservossomos, grandes vesículas localizadas na região posterior do parasito. As setas indicam os portais de endocitose (adaptado de De Souza et al., 2009).



Foi demonstrado que formas amastigotas de *Leishmania* podem ingerir macro-moléculas por processo mediado por clatrina através da bolsa flagelar (Denny et al., 2005). As macromoléculas ingeridas são degradadas nos megassomos (assim denominados por seu grande tamanho), descritos em *L. mexicana* (Alexander e Vickerman, 1975), *L. amazonensis* (Coombs et al., 1986) e em *L. chagasi* (Alberio et al., 2004), que correspondem ao destino final da via. Megassomos possuem características de lisossomos, tais como caráter ácido e presença de enzimas lisossomais. Uma dessas enzimas é uma cisteína proteinase, que é considerada um marcador de megassomos. Interessantemente, promastigotas possuem várias vesículas que acumulam esta cisteína proteinase, motivo pelo qual estas estruturas são consideradas precursoras dos megassomos de amastigotas (Ueda-Nakamura et al., 2001).

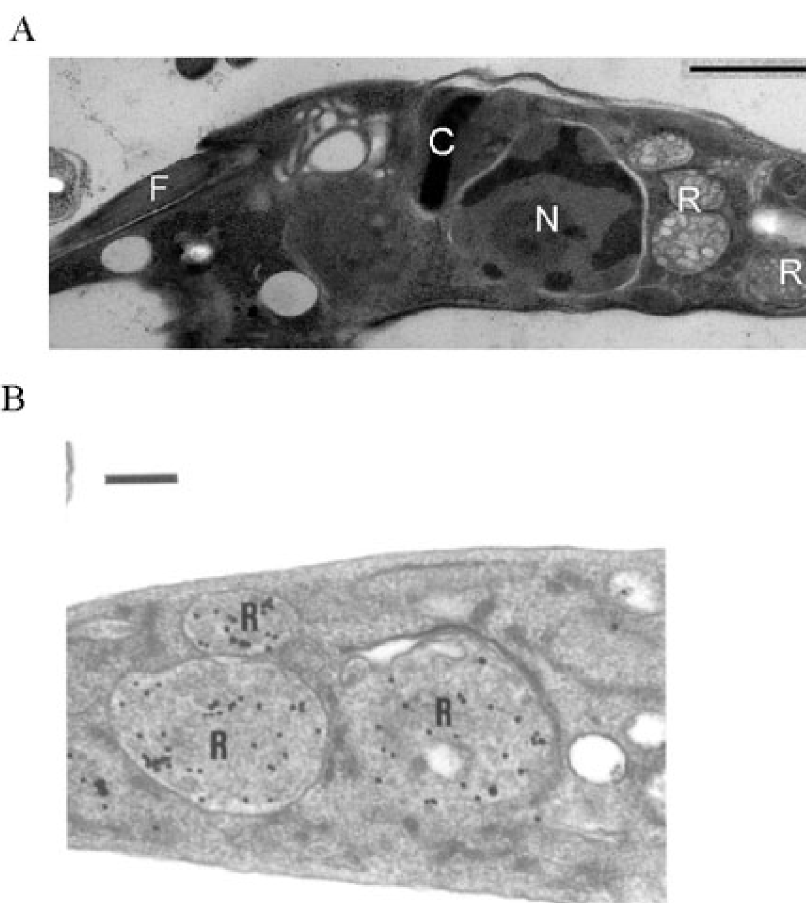
Em *T. cruzi* a endocitose está bem descrita apenas em formas epimastigotas. Não foi possível demonstrar a endocitose de proteína (transferrina acoplada a ouro coloidal) em formas tripomastigotas (Corrêa et al., 2002) e os estudos em amastigotas são inconclusivos (Soares e De Souza, 1991; Lima e Villalta, 1990). Em epimastigotas existem duas vias de endocitose de macromoléculas: a bolsa flagelar, como descrito em outros tripanossomatídeos, e o citóstoma, que é específico de formas epimastigotas e amastigotas de *T. cruzi* (Fig. 1.5.c). O citóstoma é uma invaginaçao da membrana plasmática na região anterior da célula revestida por microtúbulos especiais e que penetra na célula e atinge a região nuclear do parasito, formando o complexo citóstoma/citofaringe (Milder e Deane, 1969; Girard-Dias et al., 2012; revisto em De Souza et al., 2009). Uma análise quantitativa mostrou que 85% das macromoléculas ingeridas estão associadas ao citóstoma (Porto-Carreiro et al., 2000). Marcação na região da citofaringe após incubação com laranja de acridina (que possui afinidade por compartimentos ácidos) e a presença de uma H^+-ATPase (tipo P) indicam o caráter ácido desta estrutura (Porto-Carreiro et al., 2000; Vieira et al., 2005). Macromoléculas são rapidamente internalizadas em pequenas vesículas, que brotam a partir da citofaringe e/ou da bolsa flagelar.

Evidências de endocitose mediada por receptor (Figueiredo e Soares, 2000) e de brotamento de vesículas com revestimento, contendo material ingerido a partir da bolsa flagelar (Corrêa et al., 2008) são indicativos de presença de clatrina em *T. cruzi*. Vesículas com revestimento também foram observadas brotando da rede *trans* de Golgi (Sant'Anna et al., 2004). O uso de um anticorpo contra clatrina bovina resultou em marcações no complexo de Golgi e na região da bolsa flagelar (Corrêa et al., 2008), demonstrando a expressão de um homólogo de clatrina bovina em *T. cruzi*. Entretanto, tratamentos para inibir endocitose mediada por receptor não impediram a ingestão de transferrina, demonstrando a ocorrência simultânea de endocitose independente de receptor pelo parasito (Corrêa et al., 2008). Outro dado importante foi a retenção de transferrina no citostóstoma quando esta foi incubada com o parasito a 12ºC, indicando que o parasito não possui um endossomo inicial correspondente ao das células de mamíferos, já que nesta temperatura a carga fica retida nestes compartimentos (Figueiredo e Soares, 2000).

Macromoléculas ingeridas possuem um destino final peculiar em *T. cruzi*: os reservossomos (Figs. 1.5.c e 1.6) (Soares e De Souza, 1988, Soares e De Souza, 1991). Estas organelas são classificadas como compartimentos pré-lisossomais ou endossomos

tardios, devido ao pH ácido em torno de 6,0, mantido por um bomba de prótons ATPase tipo P (Vieira et al., 2005) e também devido à presença de enzimas lisossomais, tais como a principal cisteína proteinase de *T. cruzi*, a cruzipaína (Cazzulo et al., 1990a; Soares et al., 1992). A principal e mais conhecida função dessas organelas é a estocagem do material ingerido (Fig. 1.6), o qual co-localiza com a cruzipaína (Soares e De Souza, 1991; Soares et al., 1992).

Figura 1.6. Os reservossomos de *T. cruzi* são organelas de estoque de macromoléculas ingeridas. (A) Localização dos reservossomos (R) na região posterior ao núcleo (N) do parasito. Cinetoplasto (C); Flagelo (F) Barra= 0,5 µm. (Modificado de Girard-Dias et al., 2012). (B) Formas epimastigotas incubadas com transferrina acoplada a ouro coloidal e processadas para microscopia eletrônica de transmissão. O material ingerido é estocado nos reservossomos (R), grandes vesículas localizadas na região posterior da célula. Barra= 0,25 µm. (Adaptado de Soares et al., 1992).



A reserva energética dos reservosomos é consumida durante o processo de diferenciação *in vitro* de epimastigotas para tripomastigotas metacíclicos (metaciclogênese) (Figueiredo et al., 1994; Soares, 1999; Figueiredo et al., 2004). Os reservossomos também estocam lipídeos, formando inclusões lipídicas, além de

armazenar grandes quantidades de colesterol (Soares e De Souza, 1988; Sant'Anna et al., 2008a; Pereira et al., 2011).

Com a obtenção de uma fração purificada dos reservossomos (Cunha-e-Silva et al., 2002) foi possível realizar a proteômica do seu conteúdo (Sant'Anna et al., 2009). Além da presença de proteases típicas de endossomos tardios e lisossomos, tais como cruzipaína e serina carboxipeptidase, diversas outras proteínas foram encontradas, podendo indicar novas funções para os reservossomos (Sant'Anna et al., 2009). Sant'Anna e colaboradores também observaram que a cruzipaína e a serina carboxipeptidase, além de co-localizarem em formas epimastigotas, co-localizam também em formas amastigotas e tripomastigotas, em organelas classificadas como *lysosome*-*like* (Sant'Anna et al., 2008b).

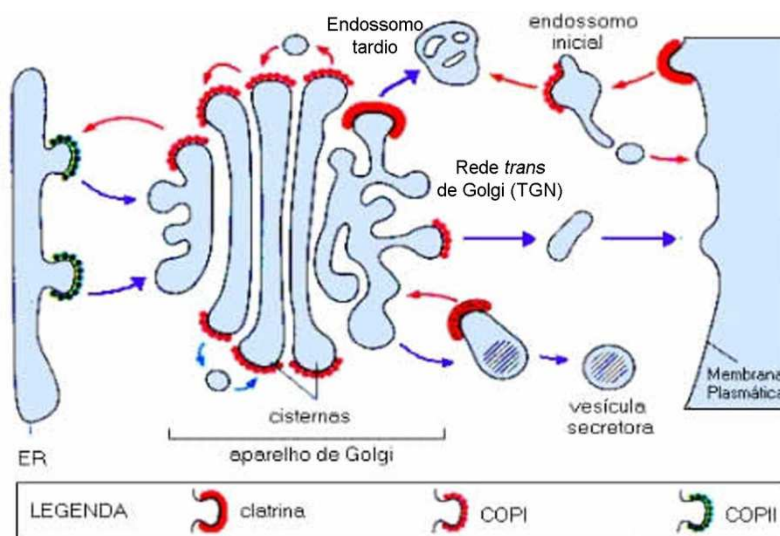
A endocitose de nutrientes em formas amastigotas intracelulares (encontradas no hospedeiro vertebrado) permanece não elucidada. Existem algumas evidências de que esse evento biológico ocorra nestas formas, pela: (a) presença de citóstoma (revisto por De Souza et al, 2009), (b) presença de uma organela lisossomo/reservossomo-*like* (Sant'Anna et al., 2008b), (c) presença de um receptor específico para transferrina humana (Lima e Villalta, 1990) e (d) diminuição significativa de sinal radioativo quando transferrina radioativa foi incubada com formas amastigotas a 4ºC e os parasitos foram tratados com ácido acético (sugerindo remoção da transferrina aderida à superficie das células), em relação ao grupo não tratado; mas quando transferrina radiotiva foi incubada a 37ºC e os parasitos foram tratados com ácido acético houve pouca mudança no sinal radioativo, sugerindo que nesta temperatura a transferrina foi internalizada (Lima e Villalta, 1990). Entretanto, os autores deste trabalho não estudaram o mecanismo de captação da transferrina, nem a localização final do material ingerido.

1.3.3. Transporte de enzimas lisossomais a partir da rede *trans* de Golgi aos lisossomos

Em células eucarióticas superiores as enzimas lisossomais geralmente são glicoproteínas produzidas no retículo endoplasmático e editadas no complexo de Golgi. O complexo de Golgi compreende uma complexa rede de cisternas e vesículas com três porções definidas: (a) as cisternas da face *cis*, que recebem as enzimas lisossomais oriundas do retículo endoplasmático, (b) as cisternas mediais, onde as enzimas recebem a maioria das modificações pós-traducionais, como glicosilações (em sua grande maioria, adição de manose-6-fosfato) e sulfatações e (c) as cisternas da face *trans*, de

onde vesículas com as enzimas lisossomais brotam e são direcionadas a uma complexa estrutura adjacente, denominada rede *trans* de Golgi (TGN: *trans Golgi network*). Existem duas vias principais pelas quais as enzimas lisossomais são direcionadas aos lisossomos: uma dependente de receptor de manose-6-fosfato e outra independente deste receptor. A partir da TGN, as enzimas lisossomais são transportadas em vesículas com revestimento de clatrina, com direcionamento ao sistema endossomal-lisossomal (Fig. 1.7), para assim exercer a sua função de digerir o material endocitado (revisto por Braulke e Bonifacino, 2009). Desta maneira, há uma integração entre as vias de exocitose e endocitose.

Figura 1.7. Direcionamento de enzimas lisossomais aos endossomos tardios. Enzimas lisossomais são glicoproteínas produzidas no retículo endoplasmático (ER), de onde brotam em vesículas revestidas por COP-II com direcionamento ao complexo de Golgi. Macromoléculas pertencentes ao retículo endoplasmático retornam em vesículas revestidas por COP-I. No complexo de Golgi as enzimas lisossomais sofrem modificações pós-traducionais, mais comumente glicosilações e sulfatações. Após estas modificações, as enzimas são transportadas em vesículas revestidas por clatrina para os endossomos tardios. Após a maturação dos endossomos tardios em lisossomos (não demonstrado na figura), as enzimas lisossomais passam a digerir o material ingerido (Fonte de acesso: http:// biologiacelulareestrutural.blogspot.com.br).



Pouco se sabe sobre o transporte de enzimas lisossomais em *T. cruzi* e o que se sabe é controverso. A principal cisteína proteinase de *T. cruzi*, a cruzipaína ou gp57/51, é uma glicoproteína rica em manose-6-fosfato que possui homologia com catepsina-L de mamíferos e papaína de plantas, que são enzimas lisossomais (revisto por Cazzulo, 1997). Além disso, por possuir papéis na nutrição, diferenciação e interação parasito-

célula hospedeira, a cruzipaína é considerada um fator de virulência e um alvo terapêutico para a doença de Chagas (revisto por Scharfstein, 2010).

Embora a cruzipaína seja rica em manose-6-fosfato, Cazzulo e colaboradores não identificaram nenhum receptor para manose-6-fosfato em *T. cruzi* (Cazzulo et al., 1990b), permanecendo não elucidada a via de direcionamento de cruzipaína e outras enzimas para o sistema endossomal do parasito. Acredita-se que esse mecanismo envolva uma via independente de receptor de manose-6-fosfato (Cazzulo et al., 1990b). Existem evidências de que esse transporte ocorra no parasito: (a) anticorpos produzidos contra a cruzipaína nativa reconhecem a enzima majoritariamente em duas localizações subcelulares em epimastigotas de *T. cruzi*, no Golgi (lateral ao cinetoplasto) e nos reservossomos (Murta et al., 1990; Souto-Padrón et al., 1990; Sant'Anna et al, 2008b), (b) como citado no item anterior, em reservossomos a cruzipaína co-localiza com transferrina ingerida (Soares et al., 1992) e possui a função de digestão do material endocitado. A presença de cruzipaína em organelas lisossomo/reservossomo-like de formas evolutivas encontradas no hospedeiro vertebrado (tripomastigotas e amastigotas) sugere a presença desta via em todas as formas evolutivas de *T. cruzi* (Sant'Anna et al., 2008b). Assim, a elucidação do mecanismo de transporte da cruzipaína do Golgi para os reservossomos é um interessante modelo de estudo na tentativa de bloquear esta via, tendo em vista o papel importante desta enzima e dos reservossomos na nutrição do parasito e na sua diferenciação (revisto por Soares, 1999; Figueiredo et al., 2004).

1.4. A TcHIP

A partir de uma fração purificada de reservossomos de formas epimastigotas de *T. cruzi* foi possível estabelecer a proteômica desta importante organela (Sant'Anna et al., 2009). Como esperado, a análise proteômica confirmou a presença de cruzipaína. Entretanto, foram também identificadas algumas proteínas sem provável função relacionada aos reservossomos, dentre elas a *Huntingtin Interacting Protein* (TcHIP) (Sant'Anna et al., 2009). A TcHIP é uma zDHHC palmitoil transferase putativa que possui homologia com a proteína HIP-14 de humanos, a qual está localizada no complexo de Golgi e tem como função realizar a palmitoilação (adição de ácido palmítico) da proteína Huntingtina, regulando a sua função e localização (Singaraja et al., 2002; Yanai et al., 2006). Outra proteína homóloga à TcHIP, a PfAnkDHHC, está descrita em *Plasmodium falciparum*, com localização subcelular também no complexo de Golgi (Seydel et al., 2005). Em ambos os estudos foram utilizados anticorpos para a

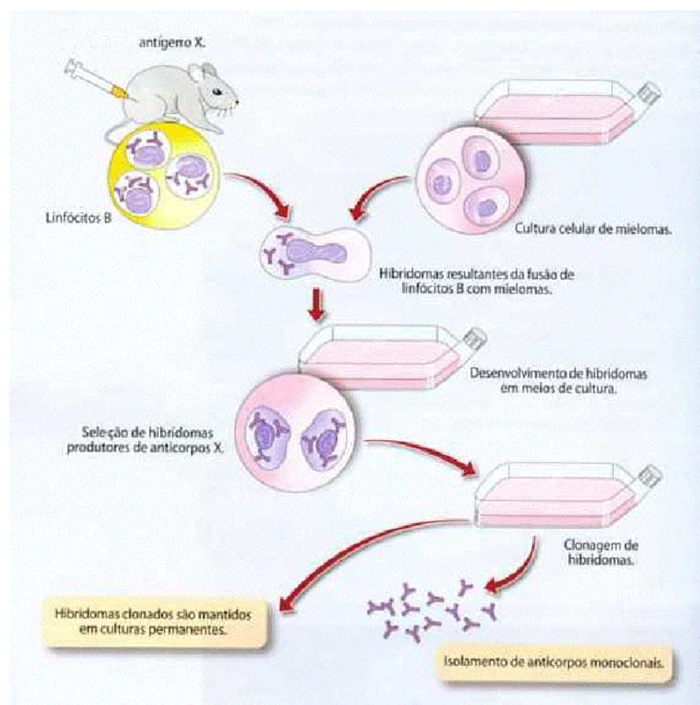
determinação da localização subcelular das respectivas palmitoil transferases. Entretanto, ainda não estão descritas na literatura zDHHC palmitoil transferases com localização no sistema endossomal-lisossomal.

1.5. Anticorpos Monoclonais

Anticorpos produzidos pelo sistema imune de eucariotos superiores podem ser utilizados como importantes ferramentas para a produção de marcadores celulares (Heine et al., 2011). Os anticorpos podem ser classificados como policlonais e monoclonais. Os anticorpos policlonais detectam vários epítopos de um antígeno, o que pode comprometer sua especificidade. Além disso, para a sua produção é necessário coletar o soro do animal imunizado, havendo a necessidade de repetir o processo quando o estoque do soro termina, dificultando a reprodutibilidade dos resultados e levando a questões éticas devido ao uso exacerbado de cobaias (Nelson et al., 2000).

Em contrapartida, os anticorpos monoclonais (mAbs), provenientes de um clone de uma linhagem de células B do baço de uma cobaia previamente imunizada, detectam apenas um epítopo do antígeno, garantindo maior especificidade quando utilizados como marcadores celulares. Para a sua produção é necessária a fusão de células B do baço com células de linhagem tumoral (mieloma), o que permite a manutenção *in vitro* das células híbridas por tempo indeterminado (Fig. 1.8). Além disso, essa técnica permite a produção de anticorpos em larga escala sem a sucessiva utilização de cobaias, pois a síntese ocorre em células cultivadas *in vitro,* reduzindo os impactos éticos (Nelson et al., 2000). Por essas vantagens, mAbs têm sido cada vez mais empregados (Lei et al., 2011; Yin et al., 2013; Doria-Rose et al., 2012).

Figura 1.8. Produção de anticorpos monoclonais (mAbs) *in vitro*. Primeiramente, a proteína de interesse (antígeno X) é inoculada em um animal de laboratório (rato, camundongo, coelho, etc.), levando à uma resposta imune. Em seguida, o baço do animal imunizado é retirado e os esplenócitos são fusionados com células de mieloma (de mesma origem do animal de laboratório), resultando em células híbridas (hibridomas) com crescimento inderteminado *in vitro*. Após a fusão é feita a seleção de hibridomas produtores de anticorpos estáveis contra a proteína de interesse. A estabilidade do hibridoma é testada por etapas de congelamento/descongelamento, com verificação da produção de anticorpos. Para obtenção de mAbs o hibridoma estável é submetido à clonagem, normalmente pela técnica de diluição limitante. (Fonte de acesso: http://clientes.netvisao.pt/ltomas/ A%20Biotecnologia.htm).



Não há dados na literatura que confirmem a presença da via endocítica em formas amastigotas de *T. cruzi*, apesar de haverem evidências (Lima e Villalta, 1990; Sant'Anna et al., 2008b; De Souza et al. 2009). Parte da dificuldade provém da falta de marcadores específicos de organelas que fazem parte da via endocítica, como por exemplo os reservossomos (Cunha-e-Silva et al., 2006). Assim, este trabalho possui como objetivo estudar a endocitose em formas amastigotas de *T. cruzi*, utilizando anticorpos policlonais ou monoclonais obtidos contra marcadores celulares de endocitose. Como marcadores foram selecionadas as proteínas cruzipaína e TcHIP, por estarem presentes na proteômica dos reservossomos (Sant'Anna et al., 2009).

2. OBJETIVOS

2.1. OBJETIVO GERAL

Produzir anticorpos policlonais ou monoclonais (mAbs) contra proteínas marcadoras da via endocítica, com a finalidade de analisar a endocitose de macromoléculas por formas amastigotas de *Trypanosoma cruzi*.

2.2. OBJETIVOS ESPECÍFICOS

- Obter anticorpos policlonais ou monoclonais contra cruzipaína e TcHIP de *T. cruzi*;

- Determinar a expressão e localização celular de cruzipaína e TcHIP nas diferentes formas evolutivas de *T. cruzi* (epimastigotas, tripomastigotas e amastigotas);

- Avaliar o processo de endocitose em amastigotas de *T. cruzi*, utilizando cruzipaína como marcador de organelas endocíticas.

3. ARTIGOS

Os resultados obtidos durante o desenvolvimento desta dissertação de mestrado foram compilados em três manuscritos, conforme descrito a seguir: um artigo completo referente à localização subcelular de TcHIP que foi publicado na revista Experimental Parasitology (Artigo 1). Um manuscrito descrevendo a produção de um anticorpo monoclonal contra cruzipaína, o qual está aceito para publicação na revista Biomed Research International (Artigo 2). Além disso, um manuscrito abordando resultados recentes sobre a presença de endocitose em formas amastigotas de *T. cruzi* que está submetido para a revista Parasites and Vectors (Artigo 3).

Artigo 1

Batista CM, Kalb L, Moreira CMN, Batista GT, Eger I, Soares MJ. Identification and subcellular localization of TcHIP, a putative Golgi zDHHC palmitoyl transferase of *Trypanosoma cruzi*. Exp Parasitol 2013;134: 52-60.

Artigo 2

Batista CM, Medeiros LC, Eger I, Soares MJ. mAb CZP-315.D9: an anti-recombinant cruzipain monoclonal antibody that specifically labels the reservosomes of *Trypanosoma cruzi* epimastigotes. Biomed Research International, 2014, doi:10.1155/2014/714749.

Artigo 3.

Batista CM, Kessler RL, Eger I, Soares MJ. Transferrin uptake by *Trypanosoma cruzi* intracellular and axenic amastigotes as analyzed by flow cytometry and immunofluorescence. Submmited manuscript.

4. RESULTADOS

4.1. ARTIGO 1

A TcHIP é uma zDHHC palmitoil transferase que foi detectada após análise proteômica dos reservossomos de *T. cruzi* (Sant'Anna et al., 2009). Entretanto, nenhum estudo confirmatório envolvendo a localização subcelular desta proteína foi realizado. Para investigar a expressão e localização de TcHIP em *T. cruzi*, anticorpos policlonais foram obtidos. Diferente do demonstrado pela análise proteômica, a localização subcelular de TcHIP mostrou-se no complexo de Golgi, nas diferentes formas evolutivas de *T. cruzi*. Dados da literatura mostram que outras zDHHC palmitoil transferases homólogas à TcHIP em *Homo sapiens* e *Plasmodium falciparum* também estão localizadas no complexo de Golgi (Singaraja et al., 2002; Seydel et al., 2005), corroborando os resultados de imunolocalização obtidos para TcHIP.

Estes resultados foram reunidos para a publicação no artigo "*Identification and subcellular localization of TcHIP, a putative Golgi zDHHC palmitoyl transferase of Trypanosoma cruzi*" na revista *Experimental Parasitology* (ELSEVIER), que possui fator de impacto 2,154.

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Identification and subcellular localization of TcHIP, a putative Golgi zDHHC palmitoyl transferase of *Trypanosoma cruzi*

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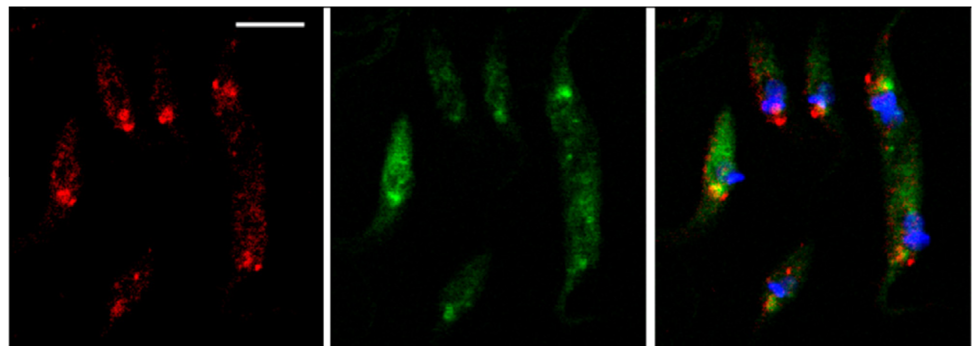
HIGHLIGHTS

- TcHIP is a putative zDHHC palmitoyl transferase of *T. cruzi.*
- TcHIP is expressed in all developmental forms of *Trypanosoma cruzi.*
- TcHIP colocalized with TcRab7, a Golgi marker of *T. cruzi.*

GRAPHICAL ABSTRACT

Confocal microscopy showing TcHIP immunolocalization at the Golgi complex of *T. cruzi* epimastigotes. Red: TcHIP; Green: TcRab7/GFP (Golgi marker); Merged image to the right: Blue–nucleus and kinetoplast; Yellow–TcHIP-TcRab7/GFP colocalization. Bar = 5 μm.



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ABSTRACT

Protein palmitoylation is a post-translational modification that contributes to determining protein localization and function. Palmitoylation has been described in trypanosomatid protozoa, but no zDHHC palmitoyl transferase has been identified in *Trypanosoma cruzi*, the etiological agent of Chagas disease in Latin America. In this study we identify and show the subcellular localization of TcHIP (Tc00.1047053508199.50), a putative *T. cruzi* zDHHC palmitoyl transferase. Analysis of the deduced protein sequence indicates that it contains ankyrin repeats (Ank and Ank2) and the zDHHC conserved domain, typical of zDHHC palmitoyl transferases. A TcHIP polyclonal antiserum obtained from mice immunized with the purified recombinant protein was used to study the presence and subcellular localization of the native enzyme. In western blots this antiserum recognized a protein of about 95 kDa, consistent with the predicted molecular mass of TcHIP (95.4 kDa), in whole extracts of *T. cruzi* epimastigotes, metacyclic trypomastigotes and intracellular amastigotes. Immunolocalization by confocal microscopy showed TcHIP labeling at the Golgi complex, co-localizing with the *T. cruzi* Golgi marker TcRab7-GFP. Transfectant *T. cruzi* epimastigotes containing a construct encoding TcHIP fused to proteins A and C (TcHIP/AC) were obtained. In western blotting experiments, the TcHIP polyclonal antiserum recognized both native and TcHIP/AC proteins in extracts of the transfectants. Confocal microscopy showed co-localization of native TcHIP with TcHIP/AC. These findings demonstrate the presence of a putative zDHHC

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palmitoyl transferase (TcHIP) containing ankyrin and zDHHC domains in different developmental forms of *T. cruzi*, and its association with the Golgi complex.

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1. Introduction

Palmitoylation is a type of acylation that involves addition of palmitic acid to a protein. This modification can regulate the protein's localization, function, membrane anchoring, lipid raft segmentation and trafficking (Corvi et al., 2011). It is catalyzed by two different families of palmitoyl transferases. One group present a cysteine-rich domain, containing a zinc molecule and the "asp-his-his-cys" motif (zDHHC), which is responsible for their function (Putilina et al., 1999). The other enzyme type is the membrane-bound *O*-acyltransferase (MBOAT), which is involved in palmitoylation of various signaling proteins (Corvi et al., 2011).

The human Huntingtin Interacting Protein 14 (HIP-14) is a zDHHC palmitoyl transferase that is associated with the Golgi complex (Singaraja et al., 2002) and regulates the trafficking and function of the neuronal protein huntingtin (Yanai et al., 2006). The enzyme PfAnkDHHC palmitoyl transferase (containing ankyrin and zDHHC domains) is found in the protozoan *Plasmodium falciparum* and, like its human homologue HIP-14, is also found at the Golgi complex (Seydel et al., 2005). Although protein palmitoylation has been described in several apicomplexan protozoa, such as *Eimeria* spp. (Donald and Liberator, 2002), *Toxoplasma* spp. (Gilk et al., 2009) and *P. falciparum* (Rees Channer et al., 2006; Russo et al., 2009), little is known about the presence and function of palmitoyl transferases in trypanosomatid protozoa.

Myristoylation is an acyl modification that increases the total hydrophobicity of the protein. Some proteins also require palmitoylation at their N-terminus after myristoylation, for stable membrane attachment and/or protein translocation to rafts/caveolae or intracellular liquid-ordered domains (Levental et al., 2010; McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001; Shahinian and Silvius, 1995; Webb et al., 2000). No such dually acylated protein have been reported in the trypanosomatids *Trypanosoma brucei, Trypanosoma cruzi* or *Leishmania major* (Emmer et al., 2011; Godsel and Engman, 1999; Tull et al., 2004; Tyler et al., 2009). In *T. brucei*, the etiological agent of African trypanosomiasis, a palmitoyl transferase containing the zDHHC domain is required for sorting of calflagin (a Ca^{2+}-binding protein) to the flagellar membrane (Emmer et al., 2009). The localization of calflagins depends on their acylation status: calflagins that are only myristoylated are targeted to the cell body membrane; if after myristoylation they are palmitoylated at their N-terminus, they are directed to the flagellar membrane (Emmer et al., 2011). The flagellar calcium-binding protein (FCaBP) of *T. cruzi* is another dually acylated protein (Godsel and Engman, 1999), dual acylation being necessary for protein targeting to both flagellum and lipid rafts at the flagellar membrane of this parasite (Tyler et al., 2009). Dual acylation is also essential for the export of phosphatidylinositol-phospholipase C (PI-PLC) to the outer surface of *T. cruzi* intracellular amastigotes (Furuya et al., 2000; Martins et al., 2010). Furthermore, the small myristoylated and palmitoylated SMP-1 protein is targeted to the flagellar membrane in *Leishmania major* (Tull et al., 2004). These various data indicate that protein acylation plays an important role in targeting proteins to the flagellar and other cell membranes in trypanosomatids.

No zDHHC palmitoyl transferase has previously been described in *T. cruzi*, the etiological agent of Chagas disease in Latin America. The aim of this work was to identify a palmitoyl transferase in this parasite and assess its expression level and subcellular localization. We report the presence of a zDHHC palmitoyl transferase (TcHIP), containing ankyrin and zDHHC domains, in various developmental forms of *T. cruzi*, associated with the Golgi complex.

2. Material and methods

2.1. Reagents

Alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibodies, mouse anti-histidine antibody, rabbit anti-protein A antibody, neomycin (G418), bromophenol blue, β-mercaptoethanol, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), Dulbecco's Modified Eagle's Medium (DMEM) and Freund adjuvant were purchased from Sigma Co. (St. Louis, MO, USA). Hoechst 33342, goat anti-mouse antibodies coupled to AlexaFluor-488 or AlexaFluor-594, Bench Mark prestained Protein Ladder and Bench Mark Protein Ladder were purchased from Life Technologies-Invitrogen Co. (Carlsbad, CA, USA). Coomassie Blue R-250 was purchased from Merck Co. (Darmstadt, Germany). Alu-Gel-S adjuvant was purchased from Serva Electrophoresis GmbH Co. (Heidelberg, Germany). Fetal calf serum (FCS) was purchased from Cultilab Ltda. (Campinas, SP, Brazil). Isopropylthio-β-galactoside (IPTG) was purchased from Anresco Laboratories Inc. (San Francisco, CA, USA).

2.2. Parasites

Cultured epimastigotes of *T. cruzi* clone Dm28c (Contreras et al., 1988) were maintained by weekly passages at 28 °C in liver infusion tryptose (LIT) medium (Camargo, 1964) supplemented with 10% heat inactivated FCS. For TcHIP cloning, DNA was extracted from three-day-old cultured epimastigotes by a phenol–chloroform method (Sambrook et al., 1989).

In vitro-derived *T. cruzi* metacyclic trypomastigotes were obtained by incubating epimastigotes in TAU3AAG medium, according to a previously described metacyclogenesis (epimastigote-to-trypomastigote differentiation) protocol (Contreras et al., 1985). After 72 h of cultivation in this medium, about 80% of the cells in the supernatant were in the trypomastigote form.

To obtain *T. cruzi* intracellular amastigotes, 5×10^4 Vero cells (ATCC CCL-81) were seeded on circular glass coverslips and maintained at 37 °C in DMEM supplemented with 5% FCS in a humidified 5% CO_2 atmosphere. After 24 h, the cells were infected with *in vitro*-derived metacyclic trypomastigotes (ratio: 10 parasites per host cell). After 4 h of interaction, the cell monolayers were washed with PBS to remove non-adherent parasites and then further incubated in the same conditions. Intracellular amastigotes were obtained three days post-infection. Intracellular amastigotes were visualized by confocal microscopy of infected Vero cells.

2.3. In silico analysis

The TritrypDB database was searched for a *T. cruzi* gene encoding an aminoacid sequence of a putative palmitoyl transferase and a gene with the i.d. Tc00.1047053508199.50 (TcHIP) was identified. The deduced aminoacid sequence of TcHIP was aligned with the Protein Blast algorithm (Blastp-NCBI, Bethesda, MD, USA). For domain analysis pFAM software (Sanger Institute, Cambridge, UK) was used. The ClustalW algorithm (EMBL, Heildeberg, Germany) was used for multiple aminoacid sequence alignments by using the randomly selected sequences of putative zDHHC

palmitoyl transferases in several organisms, with the NCBI accession numbers: XP_80987.1 (*T. cruzi*), XP_827117.1 (*T. brucei*), XP_001561741.1 (*Leishmania braziliensis*), XP_888588.1 (*L. major*), NP_648824.1 (*Drosophila melanogaster*), NP_056151.2 (*Homo sapiens*), NP_766142.2 (*Mus musculus*), NP_010550.1 (*Saccharomyces cerevisae*) and XP_001351109.1 (*Plasmodium falciparum*).

2.4. Cloning, expression and purification of TcHIP

A 900 bp segment at the 5′ end of the gene that encodes TcHIP (2610 bp) was used to design primers (Forward: 5′ATGCAGGTG-TTTGGCGCTCGGATG-3′ and Reverse: 5′TCAGCAACAACGAACGCA-GA-3′) with recombination sites (attBs) to enable entry into the Gateway cloning platform (Life Technologies-Invitrogen, USA). *Escherichia coli* strain BL21 (DE3) pLys was used for recombinant protein production (TcHIP+pDEST17 vector expressing histidine tag), performed by adding 1 mM IPTG to the cell culture. Production of the recombinant protein was analyzed by SDS–PAGE (Laemmli, 1970) stained with Coomassie Brilliant Blue R-250. The insoluble fraction of the recombinant protein was purified from the polyacrylamide gel by elution, and was analyzed by SDS–PAGE.

To confirm the presence of histidine-tagged recombinant protein, western blot (Towbin et al., 1979) was performed by using an anti-histidine antibody diluted to 1:3000 in blocking buffer (PBS containing 0.05% Tween and 5% non-fat dry milk). For competitive binding analysis, 50 μg of purified recombinant protein was incubated for one hour with the anti-histidine antibody (1:3000). The resulting immune-complex was then incubated with a nitrocellulose membrane containing the purified recombinant protein.

2.5. Protein extraction

Three-day-old culture epimastigotes (3×10^7 parasites ml^{-1}) and *in vitro*-derived metacyclic trypomastigotes (3.5×10^8 parasites $ml^{-1)}$ were resuspended in non-denaturing buffer-A (20 mM Tris–HCl pH 8.0; 300 mM NaCl; 1 mM PMSF). Isolated intracellular amastigotes (10^9 parasites ml^{-1}) and uninfected Vero cells (3×10^6 cells ml^{-1}) were resuspended in denaturing buffer-B (40 mM Tris–HCl pH 6.8; 1% SDS; 360 mM β-mercaptoethanol; 6% glycerol; 0.005% bromophenol blue). Parasites and Vero cells were lysed by eight vortex-ice bath cycles and the cell homogenates were then centrifuged at 9000*g* for 10 min. The protein content of the extract was determined by a micro-BCA (bicinchoninic acid) assay (Smith et al., 1985). All protein extracts were ressuspended in denaturating buffer B and 30 μg aliquots of protein were boiled for 5 min at 100 °C and applied to polyacrylamide gels.

2.6. Generation of T. cruzi transfectants

To generate transfectant epimastigotes expressing TcHIP fused to proteins A and C (TcHIP/AC) at its amino-terminus, primers were designed (Forward: 5′GGGGACAAGTTTGTACAAAAAAGCAGGCTT-CATGTCATCATCACCGTCATTGTTA-3′ and Reverse: 5′GGGGACCAC-TTTGTACAAGAAAGCTGGGTCCACGGCGTTCATCTTTCACCT-3′) to amplify the TcHIP gene (2610 bp) without the stop codon and give a fragment suitable for use with the Gateway cloning platform. PCR using minipreparations of plasmid vector containing the TcHIP gene fused to pTcGWPTP as a template was performed to confirm the appropriate insertion. The pTcGWPTP vector contains sequences encoding proteins A and C and is a modification of the pTcGWGFP vector, previously described by Batista et al. (2010). For annealing, the Forward primer corresponding to the part of pTcGWPTP vector encoding the N-terminus (5′-GGGCATGCA-TGGCAGGCCTTGCGCAC-3′) and the Reverse primer of the TcHIP gene were used. TcHIP/AC epimastigotes were transfected, selected and maintained as previously described (Batista et al., 2010). Western blot with an anti-protein-A antibody diluted to 1:20,000 was used to confirm the presence of protein-A in protein extracts (60 μg) of TcHIP/AC epimastigotes.

TcRab7 is a Golgi marker in *T. cruzi* (Araripe et al., 2004). Thus, a plasmid vector encoding TcRab7 fused to GFP (kindly provided by Michel Batista, Instituto Carlos Chagas/FIOCRUZ-Paraná) was used to obtain TcRab7-GFP transfectant *T. cruzi* epimastigotes, as previously described (Batista et al., 2010).

2.7. Polyclonal antiserum and Western blot

Three male albino Swiss mice (30–45 days) were intraperitoneally immunized with 50 μg of purified TcHIP recombinant protein plus complete Freund's adjuvant, followed by four doses of 20 μg TcHIP+Alu-Gel-S, one every two weeks. A polyclonal anti-TcHIP serum was then obtained by cardiac puncture. This experiment was conducted in strict accordance with the recommendations of the Guide for Animal Use of the FIOCRUZ Committee on Animal Experimentation (protocol number P-0434/07).

This polyclonal antiserum (diluted at 1:200 in blocking buffer) was used in western blotting experiments to study the expression of the native TcHIP in protein extracts from *T. cruzi* epimastigotes, metacyclic *in vitro*-derived trypomastigotes and intracellular amastigotes, as well as in Vero cells. ImageJ 1.45s software (National Institute of Mental Health-NIMH, Bethesda, Maryland, USA) was used for densitometry analysis to evaluate the protein expression levels. Values for TcHIP expression in the different *T. cruzi* developmental stages were normalized to the signal given by a murine anti-TcActin serum (diluted at 1:200 in blocking buffer). For competitive binding analysis, 50 μg of purified recombinant protein was incubated for one hour with the anti-TcHIP serum (1:200). The resulting immune-complex was then incubated with a nitrocellulose membrane containing an epimastigote protein extract.

2.8. Fluorescence microscopy assays

T. cruzi epimastigotes, *in vitro*-derived metacyclic trypomastigotes and intracellular amastigotes were washed twice in PBS, fixed for 10 min with 4% paraformaldehyde and incubated for one hour at 25 °C with the pre-immune or the polyclonal TcHIP antisera diluted at 1:150 in incubation buffer (PBS pH 7.4 containing 1% BSA). After three washes in PBS, the samples were incubated in the same conditions with a secondary goat anti-mouse antibody coupled to AlexaFluor 488 or 594 diluted at 1:600 in incubation buffer. The samples were washed three times with PBS, incubated for 5 min with 1.3 nM DNA marker Hoechst 33342, and studied under a Leica SP5 confocal laser microscope (Leica Microsystems, Wetzlar, Germany).

For competitive binding assays, 50 μg of purified TcHIP recombinant protein was incubated for 1 h with the TcHIP polyclonal antiserum diluted in incubation buffer to 1:150. The resulting immune-complex was then incubated with T. *cruzi* epimastigotes and the experiment proceeded as described above.

To study co-localization of native TcHIP in transfectant epimastigotes expressing TcHIP/AC, three-day-old transfectant epimastigotes were washed twice in PBS, fixed for 30 min with 4% paraformaldehyde and incubated for 1 h at 37 °C with the TcHIP polyclonal antiserum (1:150) or with the protein-A antibody (1:40,000). The samples were washed three times in PBS, and incubated in the same conditions with a secondary goat anti-mouse antibody coupled to AlexaFluor 488 (1:600) and a goat anti-rabbit antibody coupled to AlexaFluor 594 (1:600). A negative control was performed by incubating the protein-A antibody with

wild-type epimastigotes. The experiment then proceeded as described above.

To study co-localization of native TcHIP in transfectant epimastigotes expressing TcRab7/GFP, three-day-old cultured transfectant epimastigotes were washed twice in PBS, fixed for 30 min with 4% paraformaldehyde and incubated for one hour at 37 °C with the TcHIP polyclonal antiserum (1:150). The samples were washed three times in PBS, incubated in the same conditions with a secondary goat anti-mouse antibody coupled to AlexaFluor 594 (1:600). The experiment then proceeded as described above.

3. Results

3.1. Identification of the TcHIP gene

A gene with i.d. Tc00.1047053508199.50 was identified in the TritrypDB database. This gene is 2610 bp long, maps between nucleotide positions 166,176 and 168,785 of the *T. cruzi* chromosome 8 and encodes a putative Huntingtin Interacting Protein (HIP) of 869 amino acids (95.4 kDa). Amino acid alignments revealed that TcHIP is conserved in trypanosomatids (Table 1), with maximum identity of between 35% (with the sequence in *L. major*) to 40% (with the sequence in *T. congolense*). Furthermore, three TcHIP i.ds. were observed in *T. cruzi* and two in *T. brucei*, related to different haplotypes and strains of these parasites (Table 1).

3.2. TcHIP is an ankyrin and putative zDHHC palmitoyl transferase

Analysis of TcHIP gene revealed that the encoded protein contains ankyrin repeats (Ank and Ank-2) and the conserved zDHHC domain (Fig. 1A). As expected, the conserved motif "asp-his-his-cys" (zDHHC domain) was found between the amino acids 400–700 in all organisms (Fig. 1B), with, in a few cases the second histidine (H) replaced by tyrosine (Y; in *S. cerevisae, T. cruzi, T. brucei*) or phenylalanine (F; in *Leishmania*). Some conserved amino acids were also found in the region near the zDHHC motif, notably glycine and asparagine.

3.3. Cloning of TcHIP in E. coli and purification of the recombinant protein

A fragment of approximately 900 bp encoding TcHIP was amplified by PCR from genomic DNA of *T. cruzi* strain Dm28c and inserted into the vectors pDONR 221 and pDEST17. Both constructs were confirmed by PCR and nucleotide sequencing. Expression of the insoluble recombinant his-tagged TcHIP protein from pDEST17 in *E. coli* was evaluated by SDS–PAGE (Fig. 2A) and western blotting with anti-histidine antibodies (Fig. 2B). Total extracts of bacteria without the expression vector were used as negative controls (see Fig. 2A, lane 1 for the electrophoretic banding pattern) and gave no signal with the anti-histidine antibody (Fig. 2B, lane 1). Extracts of the uninduced bacteria with the expression vector showed basal expression of a protein with about 40 kDa (Fig. 2A, lane 2), recognized by the anti-histidine antibody (Fig. 2B, lane 2). After 3 h of induction with 1 mM IPTG, the protein banding at about 40 kDa was much more abundant (Fig. 2A, lane 3). This electrophoretic profile was consistent with the expected molecular mass of the TcHIP recombinant protein (33 kDa for the TcHIP sequence plus 6 kDa for the histidine tag) and the protein was recognized by the anti-histidine antibody (Fig. 2B, lane 3).

This 40 kDa polypeptide was purified by elution, analyzed by SDS–PAGE (Fig. 2A, lanes 4, 5 and 6) and the presence of the TcHIP recombinant protein was confirmed by western blotting with an anti-histidine antibody (Fig. 2B, lanes 4, 5 and 6). The bands at lower molecular mass (present in the elutions) were different from those for induced bacterial extracts, and were recognized by the anti-histidine antibody. A competitive western blot assay was performed to confirm the specificity of the anti-histidine antibody for the purified recombinant TcHIP protein: pre-incubation of purified recombinant TcHIP with the anti-histidine antibody decreased its binding to the recombinant protein bound to the nitrocellulose membrane (data not shown).

3.4. TcHIP expression in different T. cruzi developmental stages

The murine polyclonal anti-TcHIP serum recognized a protein of about 95 kDa in *T. cruzi* extracts (Fig. 2C, lanes 1, 2 and 3), consistent with the expected molecular mass of the native TcHIP (95.4 kDa). Normalizing the signals to that obtained with a murine polyclonal serum against TcActin (42 kDa) as a reference, indicated that TcHIP was similarly abundant in various *T. cruzi* developmental stages (Fig. 2C, 2D). However, native TcHIP expression was higher in five-day-old epimastigotes (Supplementary material S1). As expected, no reaction was observed after incubation of epimastigote protein extracts with the pre-immune antiserum (Fig. 2C, lane 4). Although a cross-reaction with Vero cell extracts was observed, the reacting protein band was slightly lower (Fig. 2C, lane 5). Pre-incubation of the TcHIP antiserum with purified recombinant TcHIP completely abolished recognition of native TcHIP in protein extracts of *T. cruzi* epimastigotes (data not shown), confirming the specificity of the TcHIP antiserum.

3.5. Generation of TcHIP/AC transfectant epimastigotes

The TcHIP gene was inserted into a pTcGWPTP vector such that proteins A/C would be fused to the N-terminus of the encoded protein. Analysis by PCR confirmed that the amplicon had the expected size (2607 bp of TcHIP gene, plus about 400 bp of the PTP

Table 1
Amino acid sequence comparison of *Trypanosoma cruzi* Huntingtin Interacting Protein (TcHIP), showing the maximal percentage of identical amino acids (max identity) and the percentage of related amino acids (similarity) in the compared sequences. Sequence length represents the number of aligned amino acids used for comparison.

Accession nr.	Description	Max identity (%)	Similarity (%)	Sequence length
XP 80987.1	HIP *T. cruzi*	100	100	869
EKG01838.1	HIP *T. cruzi*	97	98	871
XP 816878.1	HIP *T. cruzi*	96	98	869
EKF30315.1	HIP *T. cruzi marinkellei*	87	91	875
XP 827117.1	HIP *T. brucei*	39	54	880
CBH 12344.1	HIP *T. brucei*	39	54	882
CCC92865.1	HIP *T. congolense*	40	56	885
CCC20477.1	HIP *T. vivax*	39	55	812
XP 001561741.1	HIP *Leishmania braziliensis*	36	52	281
XP 888588.1	HIP *L. major*	35	53	280
NP 056151.2	Palmitoyl transferase ZDHHC17 *Homo sapiens*	30	47	122
NP 010550.1	Akr1p *S. cerevisae*	36	62	47

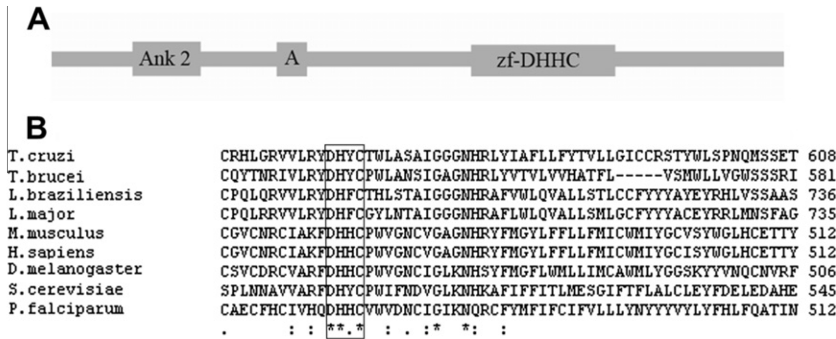

Fig. 1. *In silico* analysis of the deduced aminoacid sequence of TcHIP. (A) Domain analysis of the TcHIP aminoacid sequence with pFAM software. The Ank-2 repeat (aminoacids 102–182) and the Ank repeat ("A", aminoacids 270–302) are structural domains. The zDHHC domain (aminoacids 491–658) contains the motif in a cysteine-rich sequence. (B) Multiple aminoacid alignment, with the ClustalW algorithm, of randomly chosen putative zDHHC palmitoyl transferases encoded in the genome of various organisms shows conservation of the "asp-his-his-cys" (zDHHC domain, box) motif at aminoacids 400–700, and the presence of other conserved aminoacids such as proline (P), arginine (R) and glycine (G). (*): identical aminoacid residues in all sequences; (:): different, but highly conserved (very similar) aminoacids in the sequences; (.): different aminoacids that are somewhat similar in the sequences.

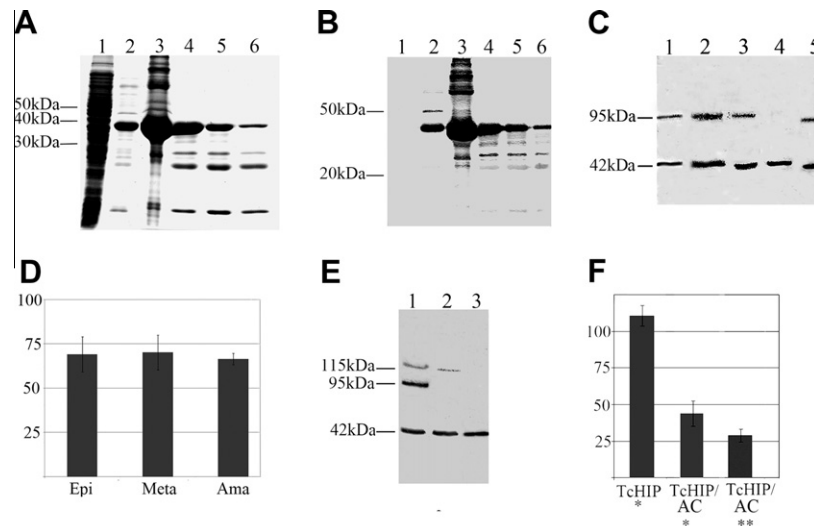

Fig. 2. Generation of TcHIP antiserum and TcHIP fusion transfectants. (A and B) Generation of recombinant TcHIP protein, 13% SDS–PAGE polyacrylamide gel (A) and western blot using an anti-histidine antibody (B). Lane 1 – control: whole extract of *Escherichia coli* without the pDEST expression vector; lane 2 – extract of uninduced *E. coli* containing the plasmid vector carrying the TcHIP insert; lane 3 – extract of *E. coli* induced with 1 mM IPTG; lanes 4, 5 and 6 – first, second and third elutions of the TcHIP protein band (about 40 kDa) from the polyacrilamide gel. (C) Native TcHIP expression in various *T. cruzi* developmental stages. Whole cell extracts were incubated with anti-TcActin and anti-TcHIP polyclonal antisera. Lane 1 – epimastigotes; lane 2 – *in vitro*-derived metacyclic trypomastigotes; lane 3 – intracellular amastigotes; lane 4 – control: epimastigotes incubated with anti-TcActin polyclonal antibody and a pre-immune antiserum; lane 5 – control: whole Vero cell extract incubated with TcActin and TcHIP polyclonal antibodies. (D) TcHIP integrated density ($n = 3$) as analyzed by ImageJ using a murine anti-TcActin polyclonal serum as a normalizer. (E) Analysis of TcHIP fused to proteins A/C by western blotting. Lane 1: protein extract of TcHIP/AC epimastigote transfectants incubated with TcHIP antiserum; lane 2: protein extract of TcHIP/AC epimastigote transfectants incubated with anti-protein-A; lane 3: control: whole epimastigote protein extract incubated with protein-A antibody. (F) Expression of native TcHIP and TcHIP/AC in epimastigotes as evaluated by ImageJ ($n = 3$) using a murine anti-TcActin polyclonal serum as a normalizer. (*): protein extract of TcHIP/AC epimastigotes incubated with TcHIP antiserum. (**): protein extract of TcHIP/AC epimastigotes incubated with anti-protein-A tag. The Bench Marker Protein Ladder was used for molecular mass reference.

gene, data not shown). Two other fragments were also amplified, corresponding to the plasmid DNA used as a template for the amplification. Epimastigotes transfected with the TcHIP/AC construct were selected, and western blot analysis with anti-protein-A antibody confirmed the presence of a protein-A fusion, at about 115 kDa, in the protein extracts (Fig. 2E). The TcHIP antiserum recognized two proteins at about 95 kDa and 115 kDa, in these extracts, corresponding to the native TcHIP and TcHIP/AC, respectively.

Incubation of the anti-protein-A tag antibody with wild-type epimastigote protein extract abolished its specific binding in western blots; the only band observed was the 42 kDa protein that corresponds to the TcActin normalizer. Densitometry analysis was used to compare the expression levels of native TcHIP and TcHIP/AC proteins. The TcHIP/AC band intensity was in all cases weaker, regardless of whether antibodies to TcHIP or to the protein A tag were used; this indicates that native TcHIP was highly expressed (Fig. 2F).

3.6. Immunolocalization of TcHIP

Immunolocalization of TcHIP in *T. cruzi* parasites by confocal microscopy revealed a strong positive spot at the anterior end of the cells, close to the kinetoplast and the flagellar pocket of epimastigotes and intracellular amastigotes (Fig. 3B, C, J, K). In many cases there was labeling lateral to the kinetoplast (Fig. 3C, K). In *in vitro*-derived metacyclic trypomastigotes, this single positive spot was located between the kinetoplast and the nucleus (Fig. 3F, G). This localization is compatible with the single Golgi

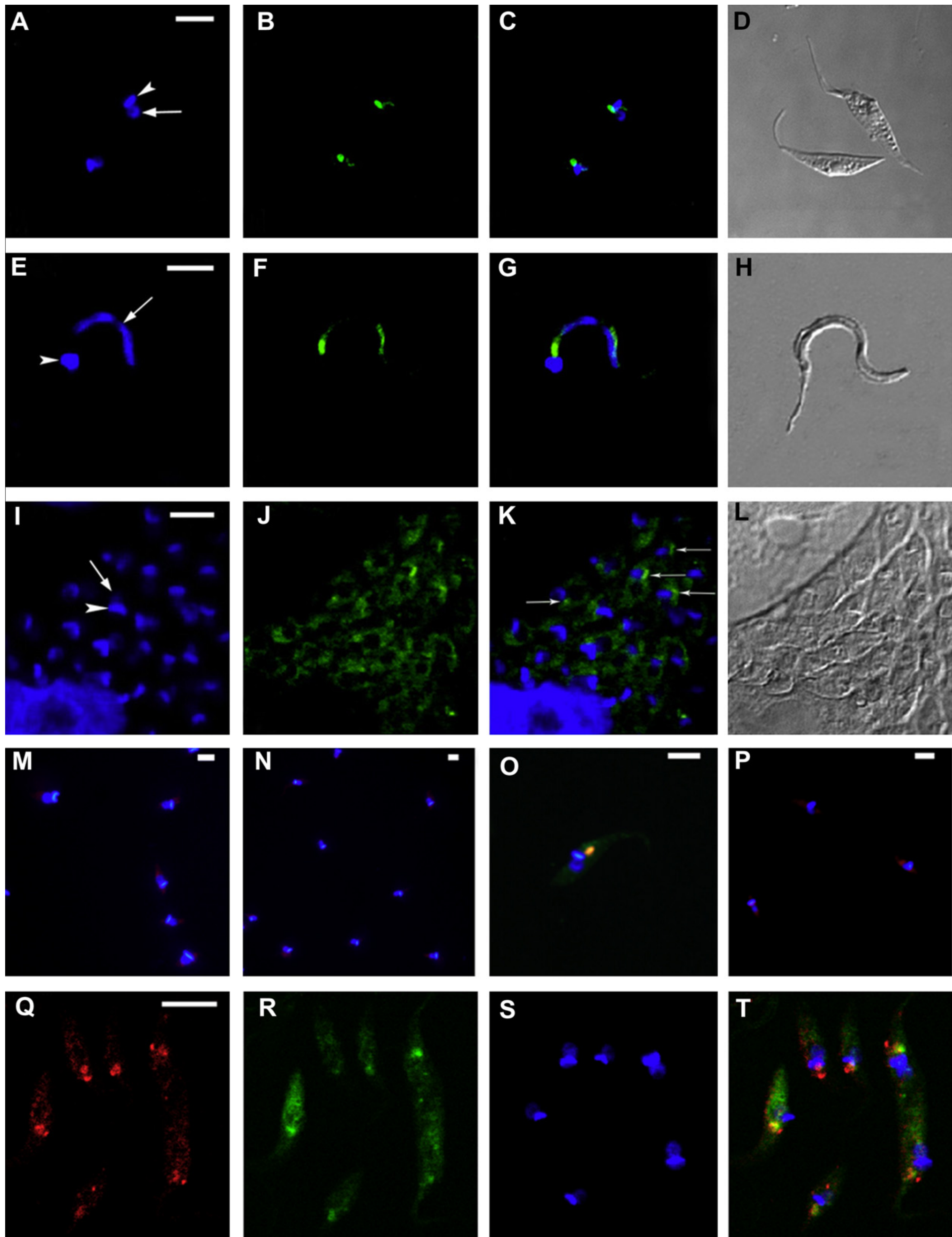

Fig. 3. Immunolocalization of TcHIP in *T. cruzi* by confocal laser microscopy, with co-localization with TcHIP/AC and TcRab7/GFP. (A–D) epimastigotes; (E–H) *in vitro* derived trypomastigotes; (I–L) intracellular amastigotes; (M) Control: competitive immunofluorescence with epimastigotes, using TcHIP antiserum pre-incubated with purified recombinant TcHIP; (N) Control: epimastigotes incubated with pre-immune antiserum; (O) TcHIP/AC transfectant epimastigotes; (P) Control: wild-type epimastigotes incubated with protein-A antibody; (Q–T) TcRab7/GFP transfectant epimastigotes. Panels A,E,I,M,S: Staining of nucleus (large arrow) and kinetoplast (arrowhead) with Hoechst 33342; Panels B,F,J,N: TcHIP labeling with AlexaFluor 488. The labeling appears as small dots close to the kinetoplast; Panels C,G,K,O: Merged images of Hoechst and AlexaFluor 488 staining; Panels D,H,L: DIC (interferencial contrast) image of the parasite body; Panel Q,M,N: TcHIP labeling with AlexaFluor 594; Panel O: native TcHIP staining with AlexaFluor 488 and TcHIP/AC staining with AlexaFluor 594; Panel P: protein-A labeling with AlexaFluor 594; Panel R: TcRab7/GFP staining; Panel T: merged images of Hoechst, AlexaFluor 594 and TcRab7/GFP stainings. The yellow color indicates co-localization of the markers. Bars = 5 μm.

complex of trypanosomatids, which is found close to kinetoplast, in the flagellar pocket region.

Competitive immunofluorescence assays were used to confirm the specificity of the TcHIP antiserum. As expected, pre-incubation of purified recombinant TcHIP with the TcHIP antiserum completely abolished the labeling described above (Fig. 3M). Epimastigote forms were also incubated with pre-immune serum and this resulted in weak diffuse labeling throughout the cytoplasm (Fig. 3N). These results confirmed those of competitive and pre-immune western blot assays, and demonstrate the specificity of the antibody.

Incubation of TcHIP/AC transfectant epimastigotes with anti-protein-A tag antibodies resulted in labeling in part of the population, and in all cases this labeling was close to the kinetoplast and flagellar pocket (Supplementary material S2). When the anti-protein-A tag antibody was incubated with wild-type epimastigotes, no cytoplasmic labeling was detected (Fig. 3P, negative control). Incubation of TcHIP/AC epimastigotes with both anti-protein-A tag and TcHIP antisera resulted in co-localization of the two labels (Fig. 3O), further confirming the specificity of the TcHIP antiserum.

Finally, transfected *T. cruzi* epimastigotes expressing the Golgi marker TcRab7-GFP were probed with the TcHIP polyclonal antiserum: the two labels were superimposed, confirming that the TcHIP was present in the Golgi apparatus (Fig. 3Q, R, T).

4. Discussion

Palmitoylation is an important post-translational protein modification, and can alter protein localization and function. Study of palmitoyl transferases, enzymes that catalyze this modification, should help in understanding the role of such modifications in the cell biology of parasites. Indeed, protein acylation has already been described in parasitic protozoa (Corvi et al., 2011), which consequently incorporate lipids, including palmitic acid, from their hosts into their own protein (Coppens et al., 2006; Quittnat et al., 2004).

In silico searches allowed us to find a putative zDHHC palmitoyl transferase (TcHIP) encoded in the genome of the trypanosomatid protozoan *T. cruzi*. Aminoacid alignments showed that TcHIP is conserved in trypanosomatids, suggesting that it may have similar functions in all these parasites. The TcHIP protein has ankyrin repeats (Ank and Ank-2) and the zDHHC domain. Ankyrin repeats consist of repetitive stretches of 33 residues of glutamine and lysine, as also described in signaling proteins. They mediate protein–protein interactions and are among the most common structural motifs in known proteins (Mosavi et al., 2004). Palmitoyl transferases also have cysteine-rich domains containing the "asp-his-his-cys" (DHHC) motif involved in adding palmitic acid to proteins usually anchored to Golgi complex membranes (Putilina et al., 1999). Most zDHHC palmitoyl tranferases also present ankyrin and plexin repeats, and these may be responsible for either substrate specificity or interaction with a possible regulator (Corvi et al., 2011).

Multiple aminoacid alignments showed substantial conservation of some residues in the zDHHC palmitoyl transferases in several eukaryotic organisms including *T. cruzi*; some of these conserved aminoacids are typical of the ankyrin repeats, such as alanine and lysine (Mosavi et al., 2004), and some are typical of the zDHHC domain, such as aspartate, histidine and cysteine, which correspond to the "asp-his-his-cys" motif (Putilina et al., 1999). Only a few substitutions of the second histidine were observed, replaced by tyrosine (DHYC) in *S. cerevisae, T. cruzi, T. brucei* and phenylalanine in *Leishmania*. Interestingly, it has been shown that the replacement of the second histidine with tyrosine in the zDHHC motif in *S. cerevisae* does not reduce the enzyme activity, as demonstrated by *in vivo* and *in vitro* experiments; the *S. cerevisae* zDHHC palmitoyl transferase (Akr1p) containing the DHYC motif is required for casein kinase Yck2p palmitoylation (Roth et al., 2002). Further work is required to assess the significance of these sequence variants for *T. cruzi* TcHIP expression and whether they are required for enzyme function. Multiple alignments also revealed both similar and somewhat similar aminoacids at numerous positions, contributing to the conservation of these domains in the various different organisms.zDHHC palmitoyl transferases are usually found at the endoplasmic reticulum, and Golgi and plasma membranes (Ohno et al., 2006). A recent report suggested that the Golgi complex may act as a hub for palmitoylation of peripheral proteins, and this may be the case for various substrates of zDHHC palmitoyl transferases (Michaelson et al., 2002). An example is huntingtin in human cells that is palmitoylated by the Golgi zDHHC palmitoyl transferase HIP-14 (homologue to TcHIP), which regulates its trafficking and function (Singaraja et al., 2002; Yanai et al., 2006). Furthermore, HIP14 co-localizes with coated vesicle markers, such as γ-adaptin (Golgi Adaptor Complex 1) and clathrin (Singaraja et al., 2002). Accordingly, it has been shown that PfAnkDHHC of *P. falciparum*, a zDHHC palmitoyl transferase orthologue of HIP-14 and TcHIP, is localized in the Golgi complex (Seydel et al., 2005).

To study the expression of native TcHIP, we obtained a portion of the gene, fused it to a sequence encoding a histidine tag, and expressed it in a prokaryotic vector. A 40 kDa polypeptide was purified by elution and confirmed to be recombinant TcHIP by western blotting with an anti-histidine antibody. Western blotting of the eluted protein gave additional protein bands with lower molecular mass recognized by anti-histidine antibodies, which were probably TcHIP degradation or proteolysis products.

The purified polypeptide was used to immunize mice to obtain an anti-TcHIP polyclonal serum. Western blotting experiments showed that the antiserum recognized a major polypeptide of about 95 kDa, consistent with the expected molecular mass of the predicted TcHIP protein, in different developmental stages of *T. cruzi*; this protein was expressed at similar levels in the different stages. However, the intensity of the TcHIP protein band was higher for extracts of five day-old epimastigotes than three day-old epimastigotes (Supplementary material S1). This may indicate differential expression of the protein during culture growth. Curiously, transcriptomic data for TcHIP for the *T. cruzi* life cycle available from the TritrypDB database (data not shown) are not consistent with our expression data. This may be related to post-transcriptional regulation processes, common in trypanosomatids and which depend on extracellular signals (De Gaudenzi et al., 2011; Araújo and Teixeira, 2011).

There was cross-reaction of the TcActin and TcHIP antisera with Vero cells extracts, but the protein bands recognized were of slightly lower molecular mass. This was presumably due to recognition of orthologous proteins with lower molecular mass, or a technical artifact.

We report TcHIP expression in different developmental stages of *T. cruzi*. Protein palmitoylation may be a crucial modification of membrane proteins involved in parasite-host cell interaction and invasion, modulating their functions. A recent report showed that 2-bromopalmitate (2BP), a palmitoylation inhibitor, alters gliding, host cell invasion and parasite morphology in the Apicomplexa protozoan *Toxoplasma gondii* (Alonso et al., 2012). Thus, it would be interesting to explore whether inhibition of protein palmitoylation in *T. cruzi* amastigotes and trypomastigotes (mammalian forms) could be exploited as a metabolic target.

To confirm TcHIP expression and localization in *T. cruzi* epimastigotes, transfectant epimastigotes containing TcHIP fused to proteins A/C (TcHIP/AC) were generated. Western blot confirmed the presence of proteins A/C in the protein extract of TcHIP/AC

epimastigotes. However, TcHIP/AC labeling was weaker than that of native TcHIP with both TcHIP antiserum and protein-A antibody, possibly due to expression of the fused protein in only part of the population (Supplementary material S2). Indeed, when 30 μg of TcHIP/AC epimastigote protein extract was used, intensity of the TcHIP/AC protein band was weak (data not shown). Therefore, we decided to use 60 μg of protein extract from TcHIP/AC transfectant epimastigotes in experiments.

Confocal microscopy and the polyclonal TcHIP antiserum were used for immunolocalization of the native TcHIP. In epimastigotes, *in vitro*-derived metacyclic trypomastigotes and intracellular amastigotes, labeling was found as a single spot close to the kinetoplast and flagellar pocket. Competitive immunofluorescence and co-localization of TcHIP in epimastigotes expressing TcHIP/AC confirmed the specificity of the antiserum for TcHIP. The cellular localization was compatible with the Golgi complex of trypanosomatids, which is always close to the proximal region of the flagellar pocket (near the kinetoplast), usually with the trans-side facing the flagellar pocket (Girard-Dias et al., 2012; Soares and De Souza, 1988). TcRab7 is a Golgi marker in *T. cruzi* (Araripe et al., 2004; Batista et al., 2010), and we found it to co-localize with TcHIP, confirming that TcHIP localizes at the Golgi complex. This result is in accordance with our *in silico* analysis, which demonstrated the presence of the ankyrin repeats and a zDHHC domain in TcHIP, a pattern typical of other homologous palmitoyl transferases (HIP-14, PfAnkDHHC) that are associated with the Golgi complex in other eukaryotic cell types (Singaraja et al., 2002; Seydel et al., 2005).

A palmitoyl proteome of *T. brucei* has recently been described by LC/MS-MS and diverse types of palmitoylated proteins were found, including proteases, antigens/surface proteins, membrane transporters and vesicle/trafficking proteins. One of these proteins is a cysteine protease precursor (Emmer et al., 2011). *T. cruzi* epimastigotes contain a major cysteine protease (cruzipain), which is a mannose-rich glycoprotein that accumulates in reservosomes (De Souza et al., 2009; Soares et al., 1992). Protein alignments showed that cruzipain (NCBI accession number: XM_813485.1) shares 59% identity with the *T. brucei* cysteine protease precursor (NCBI accession number: P14658). Reservosomes are the end compartment of the endocytic pathway, where ingested proteins accumulate (De Souza et al., 2009). *T. cruzi* lacks a mannose-6-phosphate receptor (Cazzulo et al., 1990), and it is still not known how cruzipain is routed from the Golgi complex to the reservosomes. Our study indicates that TcHIP is a putative palmitoyl transferase, associated with the Golgi complex of *T. cruzi*. Palmitic acid is attached to cysteine residues usually through a thioester bond (Linder and Deschenes, 2003). Cruzipain has an N-teminal cysteine (C) residue, and several other C residues scattered throughout the aminoacid sequence. Thus, it is tempting to speculate that palmitoylation may direct cruzipain (and/or other glycoproteins) from the Golgi to reservosomes.

Endosomal integral membrane proteins involved in vesicular trafficking, such as μ-adaptin (Golgi Adaptor Complex 1), Rab1, Rab7 and TcHIP, have been found in the subcellular proteomes of a purified reservosomal fraction (Sant'Anna et al., 2009), indicating dynamic trafficking of vesicles between the Golgi and reservosomes. Our immunolocalization analyzes did not reveal any TcHIP labeling in reservosomes. It has been shown that each epimastigote form contains only a few reservosomes, all at their posterior end (Soares and De Souza, 1988). It is possible that TcHIP would be enriched and detectable in a purified reservosomal fraction, but is present at non-detectable levels in reservosomes of intact cells processed for immunolocalization studies. It is also possible that the detection of TcHIP in this purified reservosomal fraction may have been due to contamination. Further studies are required to characterize the importance of TcHIP in the cell biology of *T. cruzi*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2013.01.023.

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4.2. ARTIGO 2

Dados de proteômica de reservossomos de *T. cruzi* mostraram a presença de cruzipaína nessa organela (Sant'Anna et al., 2009), sendo uma proteína amplamente aceita como marcador de reservossomos (Murta et al., 1990; Souto-Padrón et al., 1990; Soares et al., 1992). Como a cruzipaína é processada no Complexo de Golgi, ela pode ser localizada tanto no Golgi quanto nos reservosomos. Atualmente, nenhum anticorpo descrito na literatura reconhece exclusivamente a cruzipaína processada e endereçada aos reservossomos. Com o objetivo de produzir um anticorpo que reconhece exclusivamente a cruzipaina presente nos reservossomos para posteriores estudos de endocitose, neste trabalho foi produzido e caracterizado um anticorpo monoclonal (mAb CZP-315.D9) contra a cruzipaína recombinante de *T. cruzi* (TcCruzipaína).

Por ser o primeiro anticorpo monoclonal produzido contra a cruzipaína recombinante a reconhecer especificamente os reservossomos, os dados obtidos geraram um manuscrito que está publicado em um número especial (*Cell Biology of Pathogenic Protozoa and their Interaction with Host Cells*) da revista *Biomed Research International* (Hidawi corporation), com fator de impacto 2,88.

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Research Article

mAb CZP-315.D9: An Antirecombinant Cruzipain Monoclonal Antibody That Specifically Labels the Reservosomes of *Trypanosoma cruzi* Epimastigotes

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Reservosomes are large round vesicles located at the posterior end of epimastigote forms of the protozoan *Trypanosoma cruzi*, the etiological agent of Chagas disease. They are the specific end organelles of the endocytosis pathway of *T. cruzi*, and they play key roles in nutrient uptake and cell differentiation. These lysosome-like organelles accumulate ingested macromolecules and contain large amounts of a major cysteine proteinase (cruzipain or GP57/51 protein). Aim of this study was to produce a monoclonal antibody (mAb) against a recombinant *T. cruzi* cruzipain (TcCruzipain) that specifically labels the reservosomes. BALB/c mice were immunized with purified recombinant TcCruzipain to obtain the mAb. After fusion of isolated splenocytes with myeloma cells and screening, a mAb was obtained by limiting dilution and characterized by capture ELISA. We report here the production of a kappa-positive monoclonal IgG antibody (mAb CZP-315.D9) that recognizes recombinant TcCruzipain. This mAb binds preferentially to a protein with a molecular weight of about 50 kDa on western blots and specifically labels reservosomes by immunofluorescence and transmission electron microscopy. The monoclonal CZP-315.D9 constitutes a potentially powerful marker for use in studies on the function of reservosomes of *T. cruzi*.

1. Introduction

The kinetoplastid protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease, which affects about eight million people in the 18 countries in which it is endemic, mostly in Latin America [1, 2]. This parasite has a complex life cycle, with two developmental stages in the insect host (replicative epimastigotes and infective metacyclic trypomastigotes) and two stages in mammalian hosts (replicative intracellular amastigotes and infective bloodstream trypomastigotes). Macromolecule endocytosis plays an important role in this flagellate protozoan, allowing survival in the very different environments it colonizes. The endocytosis pathway has been elucidated mainly in epimastigote forms: molecules enter the cells via the flagellar pocket and cytostome, both located in the anterior region of the cell and accumulate in the reservosomes, the end compartments of the endocytosis pathway [3–6].

Reservosomes are large round vesicles located at the posterior end of *T. cruzi* epimastigotes [7]. The lack of molecular markers for cytoplasmic compartments in this parasite makes it difficult to clarify all the functions of reservosomes, which have characteristics typical of prelysosomes, lysosomes, and recycling compartments [8]. Subcellular localization [9] and proteomics [10] experiments have shown reservosomes to contain large amounts of a cysteine proteinase, known as cruzipain [11] or GP57/51 [12]. The native GP57/51 has been isolated from epimastigotes and used to generate a monoclonal antibody (mAb) [13]. Subcellular localization experiments demonstrated the presence of this protein in vesicles of the endosomal/lysosomal system and close to the flagellar pocket [12, 14]. At about the same time, the native

cysteine proteinase (cruzipain) was isolated and characterized [11, 15]. A monospecific rabbit polyclonal antibody against this protein labeled reservosomes, the membrane lining the cell body and flagellum, the inside of the flagellar pocket, and even the cytostome [16]. Thus, no antibody directed against cruzipain has yet been reported to label reservosomes specifically, despite the accumulation of the enzyme in this organelle.

We report here the characterization of a mouse monoclonal antibody (mAb CZP-315.D9) against recombinant *T. cruzi* cruzipain (TcCruzipain) that specifically recognizes reservosomes. This mAb has potential as a powerful molecular marker for studies on the function of this organelle.

2. Materials and Methods

2.1. Ethics Statement. Experiments involving animals were approved by the Ethics Committee of Fiocruz (Protocol P-47/12-3 with license number LW-15/13).

2.2. Reagents. Polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), l-*trans*-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane (E-64), alkaline phosphatase (AP)-conjugated goat anti-mouse or goat anti-rabbit antibodies, mouse anti-histidine antibody, rabbit anti-protein A antibody, bromophenol blue, β-mercaptoethanol, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), HT (hypoxanthine and thymidine) medium, HAT (hypoxanthine, aminopterin, and thymidine) medium, and Roswell Park Memorial Institute-1640 (RPMI-1640) medium were purchased from Sigma Co. (St. Louis, MO, USA). Transferrin-Alexa 633, horse-radish peroxidase (HRP-) goat anti-mouse IgG (H+L), Hoechst 33342, goat anti-mouse antibodies coupled to AlexaFluor-488 or AlexaFluor-594, goat anti-rabbit antibody coupled to AlexaFluor 594, Bench Mark prestained Protein Ladder, and Bench Mark Protein Ladder were purchased from Life Technologies-Invitrogen Co. (Carlsbad, CA, USA). Alu-Gel-S adjuvant was purchased from Serva Electrophoresis GmbH Co. (Heidelberg, Germany). Fetal calf serum (FCS) was purchased from Cultilab Ltda (Campinas, SP, Brazil). Isopropylthio-β-galactoside (IPTG) was purchased from Anresco Laboratories Inc. (San Francisco, CA, USA). SureBlue TMB Substrate was purchased from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, MD, USA). Bradford solution was purchased from BIO-RAD (Hercules, CA, USA).

2.3. Parasites. Cultured epimastigotes of *T. cruzi* clone Dm28c [17] were maintained at 28°C by weekly passages in liver infusion tryptose (LIT) medium [18] supplemented with 10% heat-inactivated fetal calf serum (FCS). For TcCruzipain cloning, DNA was isolated by phenol-chloroform extraction [19], from three-day-old cultures of epimastigotes.

2.4. Construction and Purification of Recombinant TcCruzipain Protein. The whole gene encoding *T. cruzi* cruzipain (TcCruzipain, 1404 bp, gene ID Tc00 .1047053507603.260) was used to design primers (Forward: 5′-ATGTCTGGCTGGGCTCGTGCGCTG-3′ and Reverse: 5′-TCAGAGGCGACGATGACGGCTGTGGGTA-3′) with recombination sites (attBs) for use on the Gateway cloning platform (Life Technologies-Invitrogen, USA). *Escherichia coli* strain C43+ was used for recombinant protein production (TcCruzipain + pDEST17 vector expressing a histidine tag), which was induced by incubating the cell culture for 7 h with 1 mM IPTG. The production of the recombinant protein (50 kDa TcCruzipain + 6 kDa histidine tag) was confirmed by western blotting with a probe directed against the histidine tag, and the recombinant protein was purified from the polyacrylamide gel by elution.

2.5. Construction of Recombinant Cruzipain Domains. The whole cruzipain gene was used for domain analysis by pFAM software (Sanger Institute, Cambridge, UK). Cruzipain has three protein domains: pre-pro (aminoacids 38–94), catalytic (aminoacids 123–335), and C-terminal extension (aminoacids 337–417). The nucleotide sequence encoding each protein domain was used to design specific primers, as follows: (a) pre-pro (nucleotides 1 to 368), Forward: 5′-ATG-TCTGGCTGGGCTCGTGCG-3′ and Reverse: 5′-CGCGCC-CAACTACCTCAACCTTCAC-3′; (b) catalytic (nucleotides 369 to 1005), Forward: 5′-CCCGCGGCAGTGGATTG-3′ and Reverse: 5′-CACCGCAGAGCTCGCCTCCTCC-3′; (c) C-terminal extension (nucleotides 1011 to 1404), Forward: 5′-GGTCCCGGTCCCACTCCTGAGCCA-3′ and Reverse: 5′-TCAGAGGCGGCGATGACGG-3′. Primers had recombination sites (attBs) for use on the Gateway cloning platform (Life Technologies-Invitrogen, USA). *Escherichia coli* strain C43+ was used for recombinant protein production (TcCruzipain protein domains + pDEST17 vector expressing a histidine tag), which was induced by incubating the cell culture for 4 h with 1 mM IPTG. Production of recombinant proteins was confirmed by western blot with a probe directed against the histidine tag.

2.6. Monoclonal Antibody Production. Three male BALB/c mice (30–45-days old) received four intraperitoneal doses of 20 μg TcCruzipain + Alu-Gel-S and a last intravenous (without Alu-Gel-S) injection, separated by intervals of one week. The animals were checked before immunization for antibody cross reactivity with protein extracts of *T. cruzi* epimastigotes (preimmune serum) by western blot assay.

The spleen of a TcCruzipain-reactive mouse was used in a cell fusion protocol [20]. Spleen cells were obtained by filtration, centrifugation, and washing and were fused with Ag8XP3653 myeloma cells (generously supplied by Dr. Carlos R. Zanetti, from Laboratório de Imunologia Aplicada, Universidade Federal de Santa Catarina, Brazil) in the presence of 50% polyethylene glycol (PEG). After fusion, the cells were resuspended at a density of 2.5×10^6 cells/mL in RPMI medium supplemented with 20% FCS and 100 μL of this suspension was added to each well of a 96-well plate. The cells were allowed to grow for 24 h at 37°C, under an atmosphere containing 5% CO_2, and 100 μL of HAT medium was then added to the cell culture. The medium was replaced every 48 h. Hybrid cells were selected

over a period of 14 days, and the medium was then replaced with HT medium for an additional four days. Hybrid cells were selected and propagated in RPMI medium containing 20% FCS. Positive hybridomas were selected by indirect ELISA, western blotting, and indirect immunofluorescence (see below).

The most stable hybridoma in cryosurvival assays [20] was cloned by limiting dilution. The SBA Clonotyping-HRP System (Southern Biotech, Birmingham, USA), based on capture ELISA, was used to identify mAb isotype, in accordance with the manufacturer's instructions. Positive hybridomas and clones were cryopreserved at the Laboratório de Biologia Celular (ICC/FIOCRUZ-PR).

2.7. ELISA. For indirect ELISA, recombinant TcCruzipain (0.15 μg/well) was adsorbed onto 96-well immunoplates (Nunc, Roskilde, Denmark) by incubation overnight at 4°C with sensitizing buffer (0.05 M sodium carbonate and sodium bicarbonate, pH 9.6). The plates were then blocked by incubation for 1 h with 5% nonfat milk powder in PBS supplemented with 0.01% Tween 20 (PBS-T). The hybridoma supernatants were added to the immunoplates and incubated for 1 h at 37°C. The plates were washed five times with PBS-T and incubated for 1 h at 37°C with HRP-conjugated goat anti-mouse IgG (1 : 4,000). The plates were then washed five times with PBS-T and immunoreactivity was visualized with the SureBlue TMB Substrate, with optical density (OD) being read at 450 nm in an EL800 ELISA reader (BioTek, Winooski, VT, USA). Only OD values higher than 0.300 were considered positive.

2.8. Western Blot. For expression analysis of native cruzipain on *T. cruzi* epimastigotes, total protein extracts of the parasites were prepared by resuspending PBS-washed parasites (10^9 cells/mL) in denaturing buffer A (40 mM Tris-HCl pH 6.8; 1% SDS; 360 mM β-mercaptoethanol). PMSF (1 mM) and E-64 (100 μM) were used as protease inhibitors. Protein content was determined in a Bradford assay [21]. The samples were resuspended in denaturing buffer B (40 mM Tris-HCl pH 6.8; 1% SDS; 360 mM β-mercaptoethanol; 6% glycerol; 0.005% bromophenol blue) and boiled at 100°C for 5 min. Protein extracts (15 μg protein/lane) were fractionated by SDS-PAGE in 10% polyacrylamide gels and the resulting bands were transferred onto nitrocellulose membranes (Hybond C, Amersham Biosciences, England), according to standard protocols [19, 22]. Following protein transfer, the membranes were blocked by incubation with 5% nonfat milk powder/0.05% Tween-20 in PBS. The membranes were then incubated for 1 h with blocking buffer containing preimmune serum (diluted 1 : 200), antirecombinant TcCruzipain polyclonal serum (diluted 1 : 500), antirecombinant TcCruzipain hybridoma (CZP-315) supernatant, or antirecombinant TcCruzipain monoclonal antibody (mAb CZP-315.D9, diluted 1 : 100). The membrane was washed three times in 0.05% Tween-20/PBS and then incubated for 1 h with AP-conjugated rabbit anti-mouse IgG (diluted 1 : 10,000). A polyclonal antirecombinant actin (TcActin; diluted at 1 : 200) mouse serum [23] was used for normalization. The membrane was then washed three times with 0.05% Tween-20/PBS and the reactive bands were visualized with BCIP-NBT solution, as described by the manufacturer.

To verify the specificity of mAb CZP-315.D9, whole protein extracts of *E. coli* (15 μg protein/lane) and purified recombinant TcCruzipain (2 μg protein/lane) were fractionated by SDS-PAGE in 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with either the antirecombinant TcCruzipain polyclonal serum (diluted 1 : 1000 in blocking buffer) or the mAb CZP-315.D9 (diluted 1 : 100 in blocking buffer). The membrane was washed three times in 0.05% Tween-20/PBS and then incubated for 1 h with AP-conjugated rabbit anti-mouse IgG (diluted 1 : 10,000). The membrane was then washed three times with 0.05% Tween-20/PBS and the reactive bands were visualized with BCIP-NBT solution, as described by the manufacturer.

For analysis of cruzipain domain labeling, protein extracts of *E. coli* vector (with cruzipain domains) were fractionated by SDS-PAGE (15 μg protein/lane) in 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with the antirecombinant TcCruzipain polyclonal serum (diluted 1 : 1000) or with the mAb CZP-315.D9 (diluted 1 : 100). The experiment then continued was described above.

2.9. Fluorescence Microscopy. *T. cruzi* epimastigotes were washed twice in PBS, fixed by incubation for 30 min with 4% paraformaldehyde, permeabilized by incubation for 5 min with PBS/0.5% Triton, and incubated for one hour at 37°C with preimmune serum diluted 1 : 150 in PBS pH 7.4 containing 1.5% BSA (incubation buffer), anti-TcCruzipain polyclonal serum diluted 1 : 500 in incubation buffer, anti-TcCruzipain hybridoma (CZP-315) supernatant, or antirecombinant TcCruzipain mAb (CZP-315.D9) diluted 1 : 40. Samples were washed three times with PBS and then incubated, in the same conditions, with goat anti-mouse secondary antibody coupled to AlexaFluor 488 or 594 diluted 1 : 600 in incubation buffer. The samples were washed three times with PBS, incubated for 5 min with 1.3 nM Hoechst 33342 (DNA marker), and examined under a Leica SP5 confocal laser microscope (Leica Microsystems, Wetzlar, Germany).

We further analyzed colocalization of native cruzipain in the Golgi apparatus in transfected epimastigotes expressing the *T. cruzi* Golgi marker TcHIP/AC [24]. Three-day-old culture transfected epimastigotes were washed twice in PBS, fixed for 30 min with 4% paraformaldehyde, and incubated for one hour at 37°C with anti-TcCruzipain mouse polyclonal serum (1 : 500) and rabbit anti-protein A antibody (1 : 40,000). The samples were washed three times in PBS and incubated, in the same conditions, with the secondary antibodies: goat anti-mouse antibody coupled to AlexaFluor 488 and goat anti-rabbit antibody coupled to AlexaFluor 594 (both diluted 1 : 600). Fluorescence microscopy was then carried out as described above.

2.10. Endocytosis Assay. *T. cruzi* epimastigotes were washed twice in PBS and then subjected to nutritional stress in PBS for 15 min at 25°C. They were then incubated for 30 min at 28°C with transferrin coupled to Alexa 633 (1 mg/mL) diluted 1 : 40. This period was long enough for the ingested

Table 1: Characterization of mAb CZP-315.D9 with the SBA Clonotyping-HRP System. Numbers are the optical density (OD) values read at 450 nm. Only OD values above 0.300 were considered positive. RPMI medium was used as a negative control.

	Ig (H+L)	IgM	IgA	IgG1	IgG2a	IgG2b	IgG3	Kappa	Lambda
Anti-TcCruzipain serum	1.207	0.852	0.585	1.24	0.816	0.996	0.19	1.125	0.33
Hybridoma 315	0.99	0.085	0.077	1.259	0.512	0.078	0.069	0.362	0.058
mAb CZP-315.D9	0.788	0.055	0.056	1.105	0.085	0.164	0.065	0.459	0.133
Medium (RPMI)	0.059	0.056	0.049	0.064	0.068	0.061	0.072	0.108	0.059

transferrin to accumulate in the reservosomes [3]. For the colocalization of transferrin with native cruzipain, the fed parasites were then fixed by incubation for 30 min with 4% paraformaldehyde, permeabilized by incubation for 5 min with 0.5% Triton in PBS, and incubated with the CZP-315.D9 mAb diluted 1 : 40 in incubation buffer. The samples were washed three times in PBS and then incubated, in the same conditions, with a goat anti-mouse secondary antibody coupled to AlexaFluor 488 (1 : 600) in incubation buffer. The samples were washed three times with PBS, incubated for 5 min with 1.3 nM Hoechst 33342, and examined under a Leica SP5 confocal laser microscope.

2.11. Transmission Electron Microscopy. Culture epimastigotes were collected by centrifugation, washed three times in phosphate buffer (pH 7.2), and fixed for 1 h at room temperature with 0.1% glutaraldehyde + 4% paraformaldehyde in 0.1 M phosphate buffer. The cells were then washed in phosphate buffer, dehydrated in graded ethanol series, and infiltrated overnight at low temperature (−20°C) with a 1 : 1 dilution of ethanol 100%: Lowicryl K4M or Lowicryl K4M MonoStep resin (EMS, Hatfield, PA, USA). After embedding for 6 h in pure resin, the samples were polymerized for 48 h at −20°C under UV light. Ultrathin sections (70 nm) were collected on nickel grids, incubated for 30 min with 50 mM ammonium chloride in PBS (pH 7.2), and then incubated for 1 h with mAb CZP-315.D9 diluted 1 : 20 in incubation buffer. After washing in this buffer, the grids were incubated for 1 h with a rabbit anti-mouse antibody coupled to 10 nm gold particles diluted at 1 : 20 in incubation buffer. After washing in buffer and distilled water, the grids were stained for 45 min with 5% uranyl acetate and for 5 min with lead citrate and observed in a JEOL 1200EXII transmission electron microscope operated at 80 kV.

3. Results

3.1. Production, Characterization, and Specificity of the Anti-TcCruzipain Monoclonal Antibody. The *T. cruzi* cruzipain gene was amplified, cloned (as confirmed by sequencing), and expressed in *E. coli*, producing a 56 kDa recombinant protein (50 kDa of TcCruzipain sequence + 6 kDa of his-tag) that was purified and used to immunize BALB/c mice. The mouse with the most responsive and specific anti-TcCruzipain serum (as determined by western blotting and subcellular localization by indirect immunofluorescence) was chosen for fusion of splenocytes with myeloma cells. Seven positive hybridomas were detected by indirect ELISA. The most stable hybridoma (CZP-315) was used to obtain clones by limiting dilution. An IgG1 isotype (OD value: 1.105) and kappa-positive (OD value: 0.459) monoclonal antibody (mAb CZP-315.D9) was obtained after selection by indirect ELISA, western blotting, and indirect immunofluorescence assays (Table 1).

A western blotting assay was performed to compare the reactivity of the anti-TcCruzipain polyclonal serum and the mAb CZP-315.D9 to *E. coli* protein extracts and to purified recombinant TcCruzipain. The anti-TcCruzipain serum recognized three protein bands between 80 and 110 kDa in *E. coli* (Figure 1(a), Ec lane 1) and several protein bands with the recombinant TcCruzipain (Figure 1(a), Czp lane 1), but with higher reactivity to a protein band between 50 and 60 kDa, compatible with TcCruzipain (50 kDa TcCruzipain + 6 kDa histidine tag). On the other hand, the mAb CZP-315.D9 recognized no protein bands in *E. coli* (Figure 1(a), Ec lane 2) but recognized the protein band between 50 and 60 kDa in the purified TcCruzipain fraction (Figure 1(a), Czp lane 2). Furthermore, both polyclonal and monoclonal antibodies recognized three protein bands below 50 kDa in the TcCruzipain fraction.

We further assessed the specificity of the CZP-315.D9 mAb against whole-epimastigote extracts by western blotting. Both anti-TcCruzipain polyclonal serum and CZP-315 hybridoma supernatant recognized two protein bands between 50 and 60 kDa, whereas the CZP-315.D9 mAb recognized mainly the protein band with about 50 kDa. The preimmune serum did not recognize any proteins. Actin (42 kDa), used for normalization, was detected with a polyclonal anti-TcActin mouse serum (Figure 1(b)).

Western blot assay was performed to determine which cruzipain domain (pre-pro domain, catalytic domain or C-terminal extension) was recognized by the polyclonal serum and by mAb CZP-315.D9. While the polyclonal serum recognized all protein domains and crossreacted with *E. coli* (protein bands below 50 kDa, Figure 1(c)), the mAb CZP-315.D9 did not, or weakly, recognize the pre-pro domain (Figure 1(c)).

3.2. Localization of Cruzipain in Reservosomes and Colocalization with Ingested Transferrin. T. cruzi epimastigotes were incubated with preimmune serum, anti-TcCruzipain polyclonal serum, CZP-315 hybridoma supernatant, or mAb CZP-315.D9. As expected, no labeling was observed with the preimmune serum (Figure 2(a)). The anti-TcCruzipain polyclonal serum labeled several round spots at the posterior end of the parasites (reservosomes) and a single spot at the anterior end of the cells, lateral to the kinetoplast (Figure 2(b)), corresponding to the Golgi complex (see below). The CZP-315

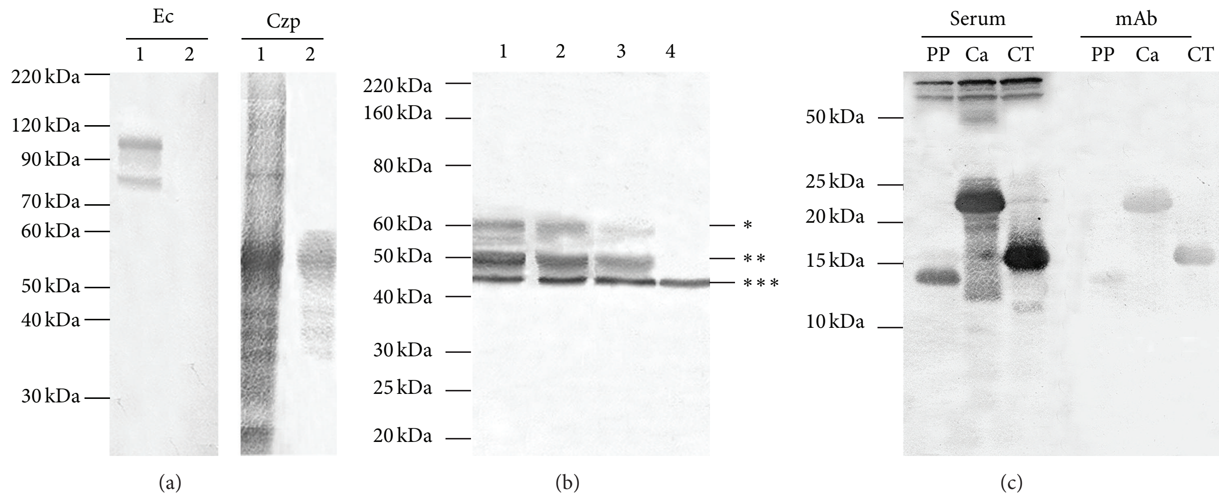

Figure 1: Western blot analysis of antibodies against TcCruzipain. (a) Protein extracts of *E. coli* (Ec) and purified recombinant TcCruzipain (Czp) incubated with anti-TcCruzipain polyclonal serum (lane 1) or CZP-315.D9 monoclonal antibody (lane 2). (b) Total protein extracts of *T. cruzi* epimastigotes incubated with anti-TcCruzipain serum (lane 1), CZP-315 hybridoma supernatant (lane 2), CZP-315.D9 monoclonal antibody (lane 3) or preimmune serum (lane 4). Actin was used for normalization. (c) Protein extracts of vector-bacteria containing recombinant cruzipain domains incubated with polyclonal antibodies (serum) or with mAb CZP-315.D9 (mAb) against TcCruzipain. PP: prepro domain; Ca: catalytic domain; CT: C-terminus domain. The Bench Mark Protein Ladder was used to determine molecular weights. *Immature cruzipain; **mature cruzipain; ***TcActin.

hybridoma supernatant and the mAb CZP-315.D9 recognized only the round spots (reservosomes) at the posterior end of the parasites (Figures 2(c) and 2(d)).

Incubation of TcHIP/AC transfectant epimastigotes with both anti-protein-A tag and anti-TcCruzipain polyclonal sera showed colocalization of the protein A tag and TcCruzipain at a single spot at the anterior end of the cells, lateral to the kinetoplast, corresponding to the single Golgi complex of the parasites (Figures 2(g) and 2(h)). No colocalization was observed at the posterior end of the parasites, which displayed only cruzipain labeling in several round structures (reservosomes).

An endocytosis assay was performed to validate the mAb CZP-315.D9. Alexa 633-conjugated transferrin was internalized and directed to the reservosomes, where it colocalized with cruzipain labeling (Figures 2(i)–2(l)).

We further assessed the immunolocalization of cruzipain by transmission electron microscopy (TEM). After incubating mAb CZP-315.D9 with ultrathin sections of epimastigote forms, gold labeling was found specifically in reservosomes (Figure 3). Weaker labeling was found in reservosomes from cells embedded with Lowicryl resin, which appeared electronlucent (Figures 3(a)–3(c)). More intense labeling was found in reservosomes from cells embedded with Lowicryl MonoStep resin (Figures 3(d) and 3(e)), which appeared more electrondense.

4. Discussion

Reservosomes are large round vesicles at the posterior end of *T. cruzi* epimastigote forms, in which the macromolecules taken up by the parasites accumulate [3]. Reservosomes are thus specific end organelles of the endocytosis pathway of this protozoan and can be used as exclusive markers/targets for these parasites. Proteomics analyses have shown that reservosomes contain several lysosomal enzymes [10], including a major cysteine proteinase known as cruzipain [11] or GP57/51 [12]. However, the antibodies against cruzipain currently available do not specifically target the reservosomes [11, 12]. We, therefore, aimed to produce a monoclonal antibody (mAb) against recombinant cruzipain (TcCruzipain) that specifically labeled reservosomes.

Indirect immunofluorescence assays to detect cruzipain in *T. cruzi* epimastigotes showed that (a) following incubation with a polyclonal serum against TcCruzipain, labeling was restricted to the reservosomes and in a single spot lateral to the kinetoplast and (b) following incubation with hybridoma supernatant and the mAb against TcCruzipain, labeling was restricted to the reservosomes. Immunolocalization of cruzipain by transmission electron microscopy showed gold labeling specifically in reservosomes. More intense labeling in electrondense reservosomes could be due to sample preservation in different resins (Lowicryl K4M and Lowicryl K4M MonoStep). Previous antibodies against cruzipain have labeled reservosomes, the membrane lining the cell body and flagellum, the inside of the flagellar pocket, and even the cytostome [9, 12, 14, 16]. Our monoclonal antibody, therefore, appears to be a suitable tool for the specific labeling of reservosomes.

TcHIP is a marker of the Golgi apparatus of *T. cruzi* [24]. Incubation of TcHIP/AC-transfected epimastigotes with both anti-protein-A tag and anti-TcCruzipain polyclonal sera revealed colocalization of protein A and TcCruzipain in a single spot at the anterior end of the cells, close to the kinetoplast, in a region corresponding to the Golgi complex. Cruzipain is a glycoprotein that is edited in the Golgi complex

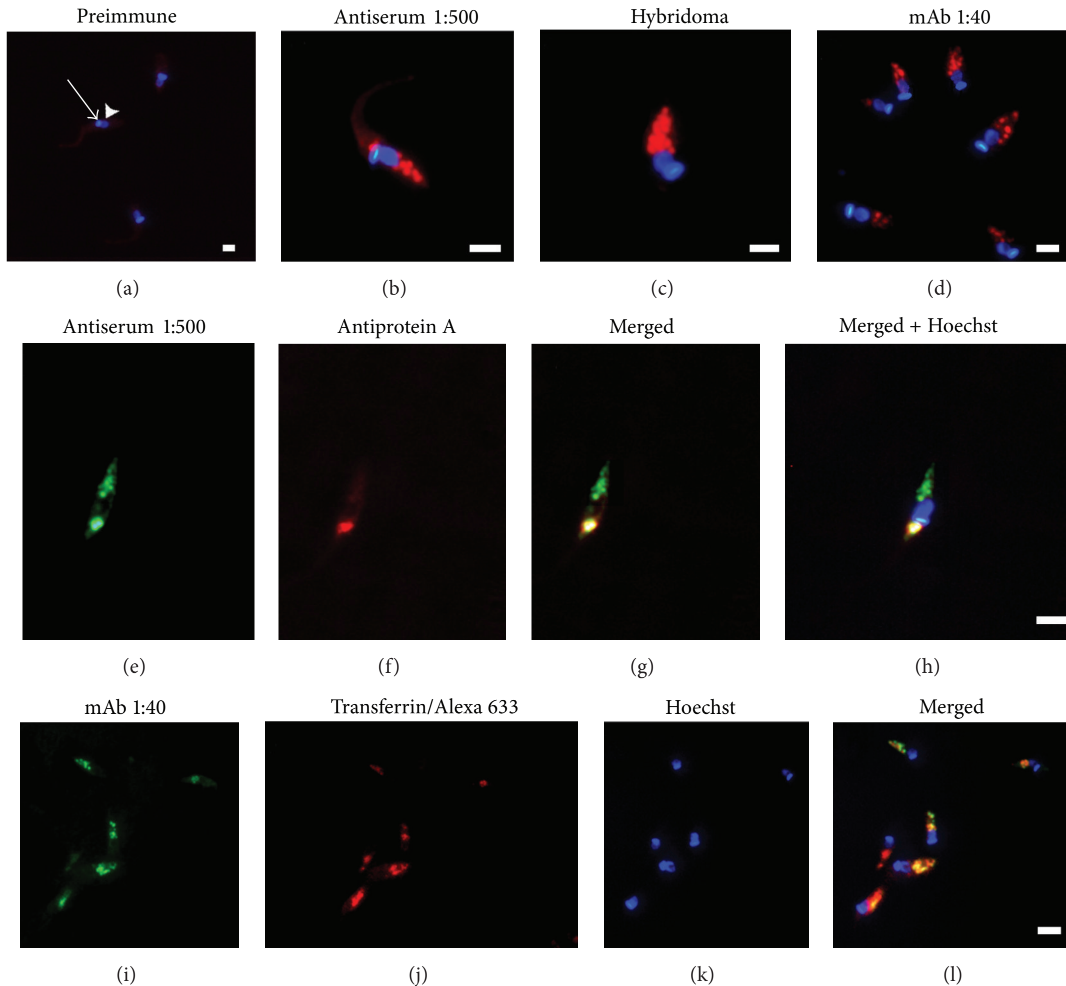

Figure 2: Immunolocalization of cruzipain and its colocalization with TcHIP/AC and ingested transferrin in *Trypanosoma cruzi* epimastigotes. The nucleus (arrowhead) and kinetoplast (arrow) are stained blue with Hoechst 33342. (a) Incubation with preimmune serum. (b) Incubation with anti-TcCruzipain serum. Note labeling of reservosomes and a single spot at the anterior end of the cell. (c) Incubation with CZP-315 hybridoma supernatant. Note that only reservosomes are labeled. (d) Incubation with CZP-315.D9 monoclonal antibody. Labeling is specific for reservosomes. ((e)–(h)) Colocalization of anti-TcCruzipain serum (green staining) with the Golgi marker TcHIP (red staining) in TcHIP/AC-transfected epimastigotes. ((i)-(l)) Endocytosis assay with transferrin coupled to Alexa 633 (red staining), and its colocalization with cruzipain (green staining) in epimastigotes, resulting in yellow staining. Bars = 5 μm.

and then directed to the endosomal/lysosomal system via the *trans*-Golgi network [25, 26]. Our polyclonal serum, therefore, also recognized immature cruzipain in transit through the Golgi complex, whereas the CZP-315 hybridoma and CZP-315.D9 mAb recognized the mature cruzipain in the reservosomes.

In western blot assays with whole extracts of *T. cruzi* epimastigote forms, both the anti-TcCruzipain serum and the CZP-315 hybridoma supernatant recognized two protein bands between 50 and 60 kDa, whereas the CZP-315.D9 mAb reacted strongly with a protein band at about 50 kDa. Cruzipain is produced as a 57 kDa protein, from which 6 kDa is cleaved to generate the mature cysteine protease, which thus has a molecular weight of 51 kDa (GP57/51) [12]. These data thus indicate that our CZP-315.D9 mAb recognizes mainly the mature enzyme in the reservosomes. Western blot assay to compare recognition of the anti-TcCruzipain polyclonal serum and the mAb CZP-315.D9 to purified recombinant TcCruzipain showed that the monoclonal recognized mainly a protein band between 50 and 60 kDa (50 kDa TcCruzipain + 6 kDa histidine tag), thus confirming the higher specificity of this mAb, as compared to a polyclonal antiserum. Both polyclonal and monoclonal antibodies recognized three protein bands below 50 kDa in a TcCruzipain fraction, probably due to proteolysis.

Cruzipain has three protein domains: pre-pro, catalytic, and C-terminal extension [27]. Our polyclonal serum recognized all protein domains by western blot analysis. On the other hand, mAb CZP-315.D9 recognized the catalytic domain and the C-terminal extension but did not, or weakly, recognize the pre-pro domain. This double binding can be dependent on conformational epitopes. No labeling with

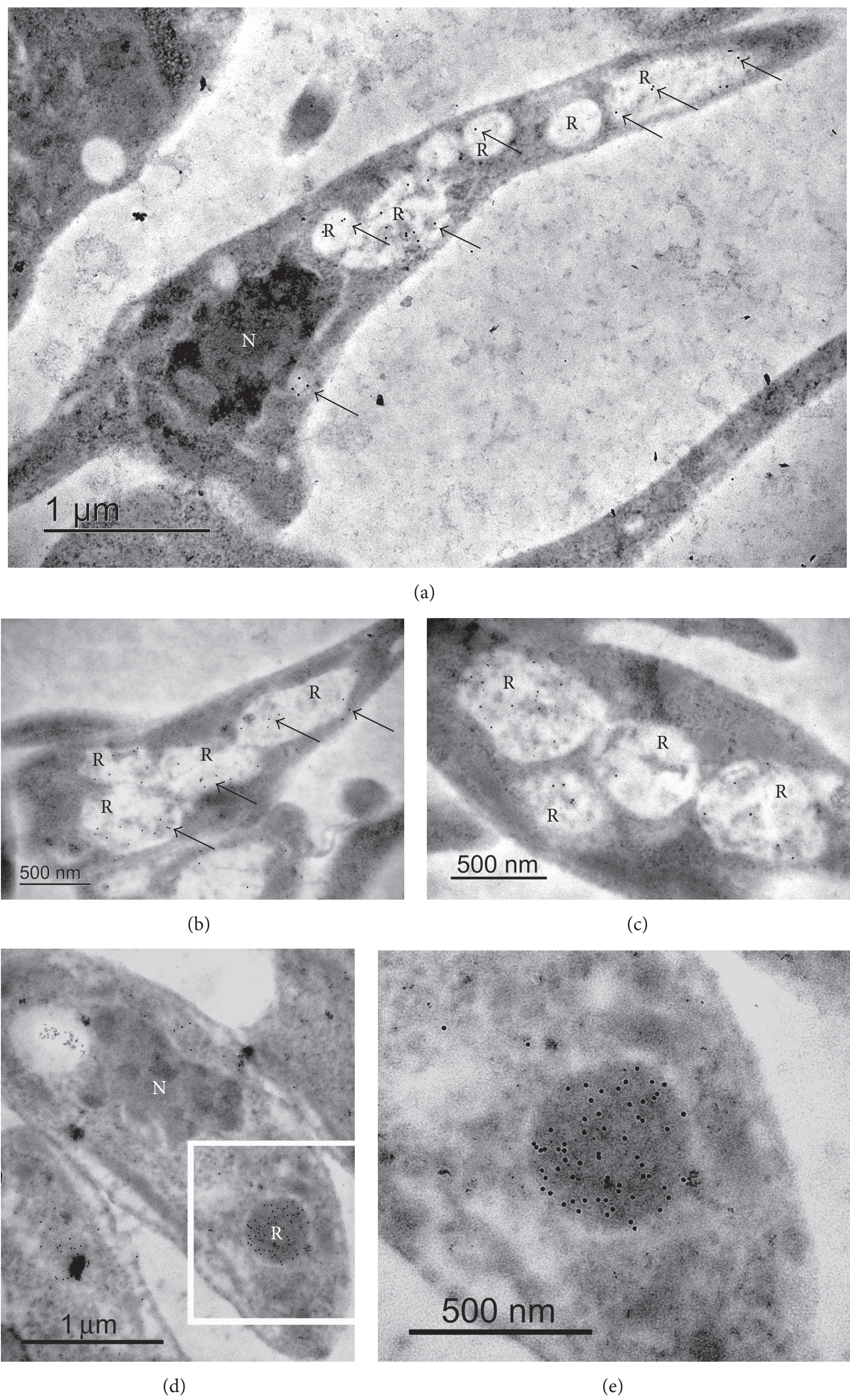

Figure 3: Immunolocalization of cruzipain in *Trypanosoma cruzi* epimastigotes by transmission electron microscopy. Ultrathin sections were incubated with the mAb CZP-315.D9, followed by a secondary antibody coupled to 10 nm gold particles. Note the specific gold labeling (arrows) in the reservosomes. Weaker labeling was found in cells embedded with Lowicryl K4M resin ((a)–(c)), while more intense labeling was found in the electrondense reservosomes of cells embedded with Lowicryl K4M MonoStep resin ((d)-(e)). (e) shows a high magnification of the area delimited in (d). N: nucleus; R: reservosome.

the pre-pro domain indicates why the mAb CZP-315.D9 does not recognize the immature cruzipain present in the Golgi complex.

An endocytosis assay was carried out with epimastigotes to validate the mAb CZP-315.D9. Transferrin ingested by the parasites was clearly colocalized with cruzipain labeling in the reservosomes. Thus, we demonstrate here, for the first time, the production of a specific mAb against reservosomal cruzipain. Monoclonal antibodies present several advantages over polyclonal sera, such as specificity, reproducibility, and ethical advantages [28]. The mAb produced in this study thus appears to be a potentially powerful molecular marker for studies on the function of this species-specific organelle, which plays an important role in the endocytosis of nutrients and cell differentiation (metacyclogenesis) in *T. cruzi* [29, 30].

5. Conclusions

We report here the production of a kappa-positive monoclonal IgG antibody (mAb CZP-315.D9) that recognizes recombinant *T. cruzi* cruzipain (TcCruzipain). This mAb binds mainly to a protein with a molecular weight of about 50 kDa on western blots and specifically labels reservosomes in *T. cruzi* epimastigotes by immunofluorescence and transmission electron microscopy. It thus constitutes a potentially powerful marker for use in studies on the function of these organelles.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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4.3. ARTIGO 3

Após a produção, caracterização e validação de um anticorpo monoclonal contra a cruzipaína (ARTIGO 2) a próxima etapa desta dissertação foi realizar ensaios de endocitose em formas amastigotas de *T. cruzi*. Os resultados obtidos, exibidos a seguir em forma de manuscrito submetido, demonstram a presença de atividade endocítica em amastigotas, porém menor do que em epimastigotas. Ensaios de endocitose de complexos transferrina-AlexaFluor analisados por citometria de fluxo em amastigotas intracelulares ou axênicos demonstraram a ocorrência de endocitose por estas formas evolutivas. O sinal de fluorescência foi maior em parasitos incubados com transferrina a 37°C em comparação aos parasitos incubados a 4°C, temperatura na qual a transferina não é internalizada em epimastigotas (Soares et al., 1992), independente de tratamento com ácido acético. Por microscopia de fluorescência, a transferrina ingerida co-localiza com a marcação de cruzipaína em amastigotas, indicando internalização e direcionamento da proteína para as estruturas reservossomos-*like* destas formas. Estes dados podem trazer novas perspectivas sobre o papel da endocitose como um alvo para o desenvolvimento de fármacos efetivos contra o estágio intracelular do parasito, predominante na fase crônica das infecções em humanos.

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ARTIGO SUBMETIDO

Transferrin uptake by *Trypanosoma cruzi* intracellular and axenic amastigotes as analyzed by flow cytometry and immunofluorescence

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Abstract

Background: *Trypanosoma cruzi* epimastigotes are able to uptake, internalize and store macromolecules in reservosomes, the end endocytic organelles in this protozoan parasite. However, few studies have focused on this crucial biological event in the more clinically relevant intracellular amastigote form, the proliferative stage found in the mammalian host. **Methods:** Transferrin uptake was analyzed by flow cytometry and fluorescence microscopy by incubating transferrin-AlexaFluor 633 at 4ºC or 37ºC for 30 min following treatment with or without acetic acid. **Results:** Here we present evidence of transferrin uptake in *T. cruzi* axenic and intracellular amastigotes. Ingested transferrin co-localized with cruzipain, a reservosome marker. **Conclusions:** Our data indicate that *T. cruzi* amastigotes have endocytic activity and that reservosome/lysosome-like organelles of amastigotes can store ingested extracellular macromolecules. These findings may open new perspectives for the development of chemotherapeutic targets against *T. cruzi.*

Keywords: cruzipain, endocytosis, intracellular amastigotes, lysosome-like organelles, transferrin, *Trypanosoma cruzi*

Background

Endocytosis is a biological event that is well characterized in epimastigotes (proliferative forms found in the insect vector) of the hemoflagellate *Trypanosoma cruzi* (Euglenozoa: Kinetoplastea), the etiological agent of Chagas disease [1-3]. In trypanosomatids, endocytosis of transferrin is a major source of iron ions, used in crucial processes including DNA replication, antioxidant defense, mitochondrial respiration and synthesis of the modified base J [4].

Transferrin is the major iron transporter protein of mammalian cells [5]. In *T. cruzi*, transferrin and other molecules are ingested via endocytic vesicles formed at the cytostome/cytopharynx complex and/or the flagellar pocket membrane, both located at the anterior cell end, and are directed to the reservosomes, where they co-localize with cruzipain, the major cysteine proteinase of *T. cruzi* [1,5,6,7]. However, the endocytic pathway is poorly known in the proliferative intracellular amastigote stage of *T. cruzi* (found in the mammalian host), the more clinically relevant form of this parasite [1,8].

It has been shown that *T. cruzi* amastigotes present receptors for human transferrin [9] and require iron for their growth in axenic conditions [10], in peritoneal macrophages *in vitro*, and in mice [11]. It has been suggested that amastigotes are able to internalize holo-transferrin (iron-loaded transferrin), as shown by the inability of acid treatment to dissociate amastigote bound holo-transferrin at 37ºC, as opposed to dissociation at 4ºC [9]. Ultrastructural analyses showed transferrin-gold complexes bound to the amastigote surface, but intracellular labeling was absent [1]. More recently, it was shown that intracellular amastigotes in cardiomyocytes can uptake transforming growth factor-β (TGF-β) from the host by an unknown receptor-mediated endocytosis mechanism in order to control their own intracellular life cycle [12], but the exact localization of the internalized TGF-β was not demonstrated.

All *T. cruzi* developmental forms present lysosome-related organelles, as shown by co-localization of serine carboxypeptidase and cruzipain in axenic epimastigotes, intracellular amastigotes and tissue culture derived amastigotes and trypomastigotes obtained from the culture supernatant [8]. It has been suggested that the lysosome-like organelles of intracellular amastigotes, contrary to the reservosomes of epimastigotes, are not related to storage of extracellular macromolecules uptaken by the parasite [8].

Our group has produced a monoclonal antibody (mAb CZP-315.D9) that reacts with recombinant TcCruzipain and specifically labels reservosomes of *T. cruzi* epimastigotes [13]. Thus, in the present work we have used this mAb to analyze by fluorescence microscopy the co-localization of ingested transferrin with cruzipain in lysosome-like organelles of *T. cruzi* axenic and intracellular amastigotes. Flow cytometry analysis of transferrin uptake also demonstrated the ability of *T. cruzi* amastigotes to internalize extracellular protein.

Methods

Reagents

Bovine serum albumin (BSA), Roswell Park Memorial Institute-1640 (RPMI-1640) medium and anti-transferrin goat IgG were purchased from Sigma Co. (St. Louis, MO, USA). Transferrin-Alexa 633, Hoechst 33342, goat anti-mouse antibody coupled to AlexaFluor-488 and rabbit anti-goat antibody coupled to AlexaFluor 594 were purchased from Life Technologies-Invitrogen Co. (Carlsbad, CA, USA). Fetal calf serum (FCS) was purchased from Cultilab Ltda (Campinas, SP, Brazil). Ssp4-2C2 monoclonal antibody was gently given by Dr. Norma Andrews (Department of Cell Biology and Molecular Genetics, University of Maryland, MD, USA)

Parasites

Culture epimastigote forms of *Trypanosoma cruzi* clone Dm28c [14] were maintained by weekly passages at 28°C in liver infusion tryptose (LIT) medium [15] supplemented with 10% heat inactivated fetal calf serum (FCS).

In vitro-derived *T. cruzi* metacyclic trypomastigotes were obtained by incubating epimastigotes in TAU3AAG medium, according to a previously described metacyclogenesis (epimastigote-to-trypomastigote differentiation) protocol [16]. After 72 h of cultivation in this medium, about 50% of the cells in the supernatant were under the trypomastigote form.

Vero cells (ATCC CCL-81) were maintained in 150 cm^2 cell culture flasks (Corning Incorporated, Corning, NY, USA) at 37°C in RPMI supplemented with 5% FBS in a humidified 5% CO_2 atmosphere. After 24 h the cells were infected with *in vitro*-derived metacyclic trypomastigotes (ratio: 10 parasites per host cell). After 4 h of interaction the host cells were washed with PBS to remove non-adherent parasites and then further incubated at the same conditions. Production of trypomastigotes then

peaked after four days of infection. Cell-derived trypomastigotes released into the supernatant were harvested by centrifugation at 3,000*g* for 10 min.

To release intracellular amastigotes, infected Vero cells were lysed 48 hours post-infection by cavitation, using 180 psi pressure for 5 min with a SPAN pump with nitrogen pressurized gas (Parr Instrument Company, Moline, IL, USA). Intact Vero cells were removed by centrifugation at low speed (10 min, 800*g*) and amastigotes were recovered from the supernatant.

Axenic amastigotes were obtained by *in vitro* amastigogenesis [17]. Briefly, cell derived trypomastigotes were collected from the supernatant of infected Vero cells after 4 days of infection, washed with PBS and incubated in high glucose DMEM medium at pH 5 and 37ºC in a humidified 5% CO_2 atmosphere. After 24 hours, almost 100% of the cells had an amastigote shape.

Endocytosis assay

Amastigotes (intracellular and axenic) and axenic epimastigotes (positive control) were submitted to a previously described endocytosis assay [1], using $2x10^6$ parasites. Briefly, after 15 min under stress with PBS at 25°C, the cells were incubated with transferrin-Alexa 633 (1 mg/mL) diluted at 1:20. Duplicates were incubated for 20-30 min at 37°C (amastigotes) or 28°C (epimastigotes). Alternatively amastigotes and epimastigotes were incubated at 4°C, as it is known that at 4°C transferrin binds to its receptor, but it is not internalized [6]. Negative controls were carried out in absence of transferrin.

Transferrin dissociation assay

A protocol was performed to dissociate membrane bound transferrin, as previously described [9], with modifications. Amastigotes were submitted to the endocytosis assay as described above and then incubated for 5s at 4ºC with 0.25M

acetic acid and 0.5M sodium chloride. The pH was then neutralized with 1M sodium acetate. Transferrin dissociation from the parasite surface was analyzed by flow cytometry as described below.

Flow cytometry

Following transferrin-Alexa633 internalization, analyses were carried out in a FACSCAria II equipment (Becton-Dickinson, San Jose, USA), with fluorescence detection with a 660/20 nm band-pass filter. A total of 20,000 events were acquired in the scatter regions (FSCxSSC), previously shown to correspond to parasite cells [18]. Data analysis was performed with FlowJo software (Treestar software). The normalized transferrin fluorescence median level was obtained from the ratio of fluorescence intensity between treated and non-treated cells.

To detect the amastigote-specific surface protein Ssp4 [19], *T. cruzi in vitro*-derived metacyclic trypomatigotes and amastigotes (both intracellular and axenic) were washed twice in PBS and fixed for 15 min with 4% paraformaldehyde. The cells were then incubated for 30 min at 28°C with an anti-Ssp4 antibody [19] diluted at 1:3200 in PBS pH 7.2. The samples were washed with PBS and then incubated for 30 min, in the same conditions, with a goat anti-mouse secondary antibody coupled to AlexaFluor 488 diluted at 1:1000 in PBS. The cells were washed with PBS and immediately analyzed by flow cytometry using a 530/30 nm band pass filter. Data analysis was performed with FlowJo software. In parallel, anti-Ssp4 stained cells were adhered to poly-L-lysine coated slides and observed under a Nikon E600 epifluorescence microscope (Nikon Instruments, Tokyo, Japan).

Fluorescence microscopy

T. cruzi amastigotes (axenic and intracellular) and epimastigotes were washed twice in PBS, fixed for 30 min with 4% paraformaldehyde, permeabilized for 5 min

with PBS/0.5% Triton and incubated for one hour at 37°C with an anti-recombinant TcCruzipain monoclonal antibody (CZP-315.D9 mAb) diluted at 1:40 in incubation buffer (PBS with 1.5% BSA). Samples were then washed three times with PBS and incubated, in the same conditions, with a goat anti-mouse secondary antibody coupled to AlexaFluor 488 diluted at 1:600 in incubation buffer. The cells were washed three times with PBS, incubated for 5 min with 1.3 nM Hoechst 33342 (DNA marker) and examined under a Nikon Eclipse E600 epifluorescence microscope.

For the co-localization of transferrin-Alexa633 with native cruzipain, parasites submitted to the endocytosis assay were fixed for 30 min with 4% paraformaldehyde, permeabilized for 5 min with 0.5% Triton in PBS and incubated with both the CZP-315.D9 mAb diluted at 1:40 in incubation buffer and an anti-transferrin antibody diluted at 1:150 in incubation buffer. The samples were washed three times in PBS and then incubated, in the same conditions, with a goat anti-mouse secondary antibody coupled to AlexaFluor 488 and rabbit anti-goat secondary antibody coupled to AlexaFluor 594 (to enhance the Alexa633 signal), both diluted at 1:600 in incubation buffer. The samples were washed three times with PBS, incubated for 5 min with 1.3 nM Hoechst 33342 and examined under a Nikon Eclipse E600 epifluorescence microscope.

Results and Discussion

Epimastigote forms of *T. cruzi* are able to uptake, internalize and store extracellular proteins in reservosomes, the end specific organelles of the endocytic pathway of these forms [1,3]. However, up to now the endocytic pathway has been poorly studied in the proliferative intracellular amastigote forms, which are prevalent in chronic chagasic patients and thus represent the more clinically relevant forms of *T.*

cruzi [20]. Therefore, studies on crucial biological events, such as endocytosis, could shed some light on specific pathways that could represent targets for the development of therapies against Chagas disease.

The few works that investigated transferrin uptake by intracellular amastigotes indicated that transferrin binds to the parasite cell surface via a receptor, but no internalization was observed [9,1]. However, amastigotes request iron for optimal growth in cell-free medium [9], peritoneal macrophages [10] and mice [11], probably obtaining iron from transferrin. Evidence for an endocytic pathway in amastigotes is the presence of lysosome-like organelles [8] with large electron-lucent rods [21].

In order to analyze endocytosis by *T. cruzi* amastigotes, we have developed a protocol to obtain intracellular amastigotes after Vero cell lysis by cavitation. To certify the amastigote stage, the expression profile of the *T. cruzi* amastigote marker Ssp4 [19] was compared in different *T. cruzi* developmental forms by flow cytometry (Fig. 1A). Only axenic and intracellular amastigotes showed intense Ssp4 expression (Fig. 1A). This result confirmed that parasites isolated from infected Vero cells corresponded to amastigotes.

Expression of Ssp4 and cruzipain in intracellular amastigotes was further evaluated by indirect imunofluorescence (Fig. 1B). Strong reaction for Ssp4 was observed at the cell surface (Fig. 1B, c) as previously described [19]. Subcellular localization of cruzipain was analyzed with a monoclonal antibody against recombinant TcCruzipain (Fig. 1B, e-h) and positive reaction occurred only at the post-nuclear region (Fig. 1B, g), compatible with the localization of lysosome-like organelles [8].

Intensity of the fluorescence signal was higher in epimastigotes incubated with transferrin-Alexa633 for 30 min at 28°C, as compared to controls without transferrin or incubated at 4°C (Fig. 2A). Fluorescence intensity decreased when parasites (from both

28°C or 4°C conditions) were incubated with acetic acid (Fig. 2A). However, the fluorescence signal from acetic acid treated parasites incubated at 28°C was higher than that of parasites incubated at 4°C, indicating transferrin internalization.

In axenic amastigotes a similar effect was observed, but the fluorescence signal in cells incubated at 37°C was slightly higher than that from cells incubated at 4°C (Fig. 2A). Fluorescence intensity decreased 56% when parasites where incubated with transferrin at 4°C and then treated with acetic acid. However, there was a minor decrease (3%) with parasites incubated at 37°C, indicating protein internalization.

Similar effect also occurred in intracellular amastigotes: the fluorescence signal in cells incubated at 37°C was higher than that from cells incubated at 4°C (Fig. 2A). After treatment with acetic acid fluorescence intensity decreased in both cases, but higher signal was observed in cells incubated at 37°C, indicating internalization of transferrin.

Normalized medians of the fluorescence peaks were graphically plotted (Fig. 2B) and showed that endocytic activity was higher in epimastigotes and lower in amastigotes, with axenic amastigotes showing only minimal activity (Fig. 2B). Our data on acetic acid treated amastigotes are consistent with those obtained by Lima and Villalta (1990), who suggested internalization of transferrin at 37°C, but they did not perform cell localization studies.

To verify the internalization of transferrin and its possible co-localization with cruzipain, parasites submitted to the endocytosis assay were processed for immunofluorescence by using specific antibodies to these proteins (Figs. 3 and 4). In epimastigotes incubated at 4°C, cruzipain was detected only at the reservosomes, located at the posterior cell region (Fig. 3A), while transferrin labeling was found only close to the nucleus, in a region compatible with the cytostome/cytopharynx bottom

[23,24]. In epimastigotes incubated at 28°C there was co-localization of transferrin with cruzipain at the reservosomes (Fig. 3B), as previously shown [6].

In axenic amastigotes incubated with transferrin at 4°C no co-localization with cruzipain was observed (Fig. 4A, B). In control cells and in cells treated with acetic acid, transferrin labeling was found disperse at the cell surface, with spots at the anterior and posterior region, while cruzipain was found at the posterior region of the parasites. On the other hand, in parasite incubated at 37°C intracellular co-localization occurred at the posterior cell region (Fig. 4C,D), although transferrin could be also found in single spots at the anterior cell region.

In intracellular amastigotes incubated at 4°C no co-localization occurred: transferrin labeling was dispersed at the cell surface, with discrete spots at the anterior cell region, while cruzipain was found at the posterior region with spots dispersed throughout the cell (Fig. 4F, G). In intracellular amastigotes incubated at 37°C, eventual co-localization of transferrin and cruzipain occurred in lysosome-like organelles at the posterior region (Fig. 4H, I). Transferrin was also found in discrete spots at the anterior region, compatible with the flagellar pocket and the cytostome [1], while cruzipain was located at the post-nuclear lysosome-like organelles [8]. Thus, our findings demonstrate transferrin internalization and targeting to lysosome-like organelles, despite a previous work [8] that suggested absence of relationship between extracellular macromolecules and lysosome-like organelles in intracellular amastigotes.

In summary, our data on the endocytic pathway of *T. cruzi* amastigotes may change the perspective for drug targeting against this intracellular stage of the parasite. Next step is the search for specific metabolic inhibitors that could impair the uptake of macromolecules as a tool to eliminate this intracellular stage.

Conclusions

Our data indicate that *T. cruzi* amastigotes have endocytic activity and that reservosome/lysosome-like organelles of amastigotes can store ingested extracellular macromolecules. These findings may open new perspectives for the development of chemotherapeutic targets against *T. cruzi.*

Competing interests

The authors declare that they have no competing interests.

Authors` Contributions

CMB carried out the endocytosis assays, acquired and analyzed the fluorescence microscopy data and draft the manuscript. RLK developed the cavitation protocol, acquired and analyzed the flow cytometry data and helped to draft the manuscript. IE conceived the study and participated in its design and coordination. MJS conceived the study, participated in its design and coordination and edited the final version of the manuscript. All authors had approved the final manuscript.

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Figure Legends

Figure 1. Validation of *T. cruzi* intracellular amastigotes obtained by cavitation. A) Flow cytometry analysis. Axenic and intracellular amastigotes, epimastigotes and *in vitro* derived trypomastigotes were incubated with a Ssp4 antibody (specific amastigote marker). Trypomastigotes and epimastigotes presented low fluorescence signal (autofluorescence), while axenic and intracellular amastigotes showed higher fluorescence intensity. **B)** Fluorescence microscopy analysis of intracellular amastigotes. a-d) Incubation with Ssp4 antibody. Note localization at the cell surface; e-h) Incubation with CZP-315.D9 monoclonal antibody. Labeling is specific in lysosome-like organelles. The nucleus (arrow) and kinetoplast (arrowhead) are stained blue with Hoechst 33342. Differential interferencial contrast (DIC) was used to visualize the parasite morphology. Bars = 5 µm.

Figure 2. Endocytosis assay by flow citometry of *T. cruzi* epimastigotes, axenic amastigotes and intracellular amastigotes. A) Incubation with transferrin at different temperatures. Negative control (black) was performed without transferrin incubation. N=20,000 events per duplicate. **B)** Normalized medians of the fluorescence peaks. N=2.

Figure 3. Endocytosis assay with *T. cruzi* epimastigotes, as analyzed by fluorescence microscopy. The cells were allowed to ingest transferrin and then incubated with an anti-transferrin antibody (red staining) and an anti-cruzipain (CZP, mAb CZP-315.D9) antibody (green staining). **A-D)** incubation at 4°C. No co-localization was observed. **E-H)** Incubation at 28°C. Note co-localization of transferrin and cruzipain, resulting in yellow staining in reservosomes. The nucleus (arrow) and

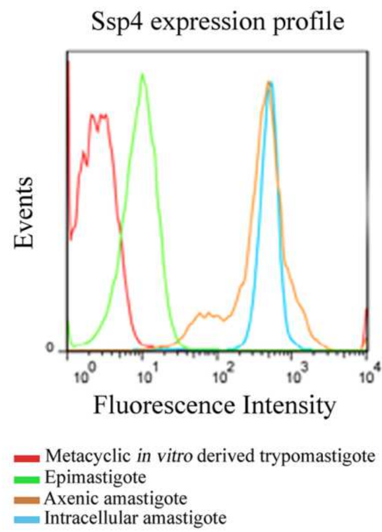
kinetoplast (arrowhead) are stained blue with Hoechst 33342. Differential interferencial contrast (DIC) was used to visualize the parasite morphology. Bars = 5 µm.

Figure 4. Endocytosis of transferrin by *T. cruzi* amastigotes, as analyzed by fluorescence microscopy. The cells allowed to ingest transferrin and then incubated with an anti-transferrin antibody (transf, red staining) and an anti-cruzipain (CZP, mAb CZP-315.D9) antibody (green staining). **A-B)** Axenic amastigotes incubated with transferrin at 4ºC. No co-localization was observed. **C-D)** Axenic amastigotes incubated with transferrin at 37ºC. Co-localization (yellow color) was usually observed at the posterior cell region. **F-G)** Intracellular amastigotes incubated with transferrin at 4ºC. No co-localization was observed. Note dispersed localization of cruzipain in these parasites. **H-I)** Intracellular amastigotes incubated with transferrin at 37ºC. Co-localization with cruzipain was observed at the posterior region of the cells. The nucleus (arrow) and kinetoplast (arrowhead) are stained blue with Hoechst 33342. Differential interferencial contrast (DIC) was used to visualize the parasite morphology. *Acetic acid treatment. Bars = 5 µm.

Figures:

Figure 1:

A



B

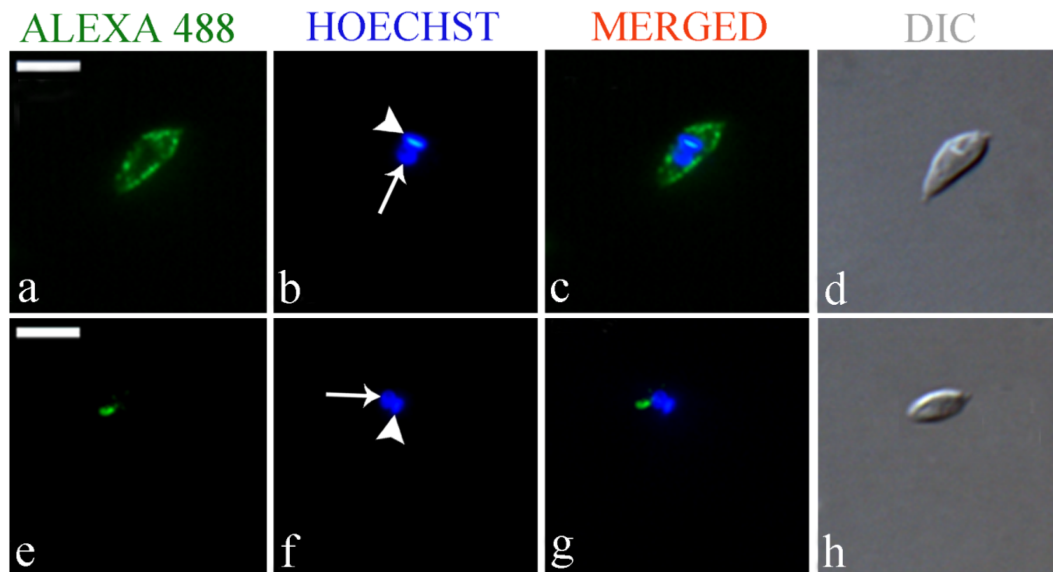


Figure 2:

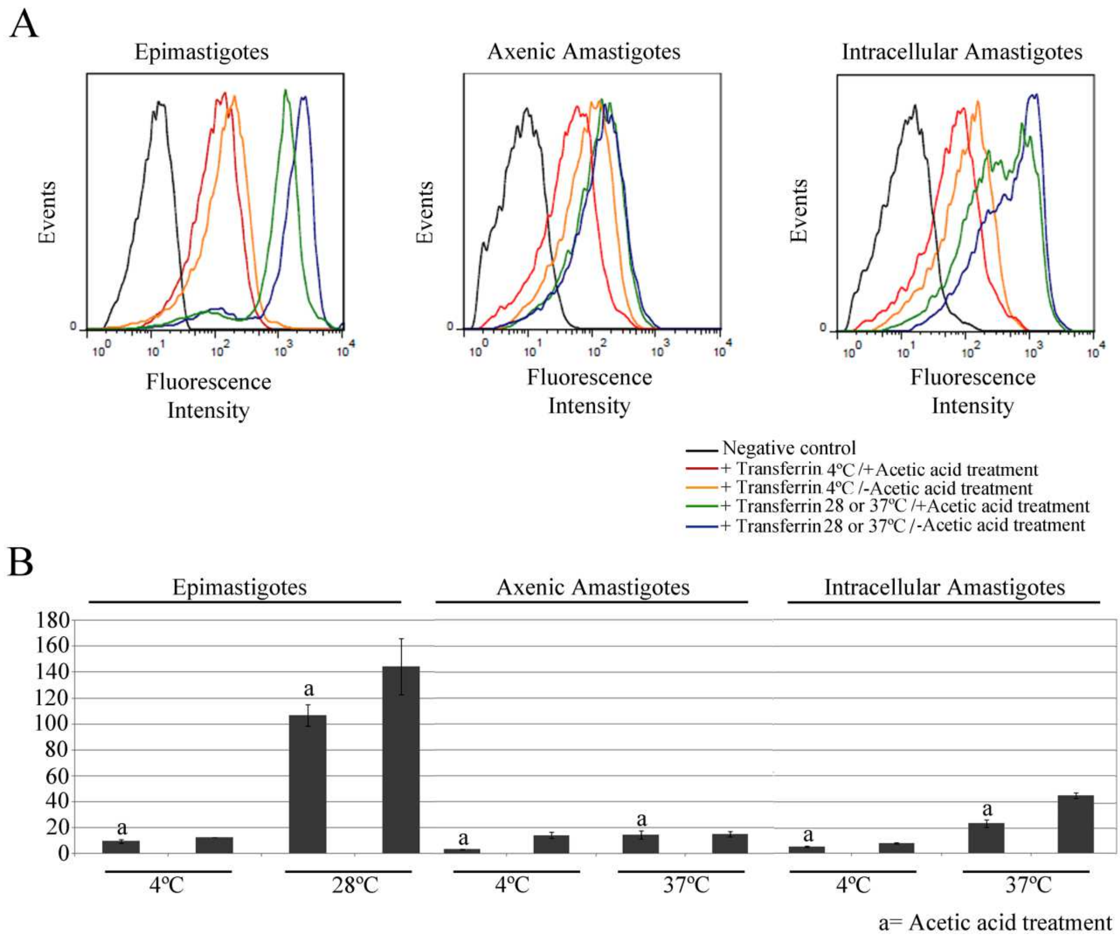


Figure 3:

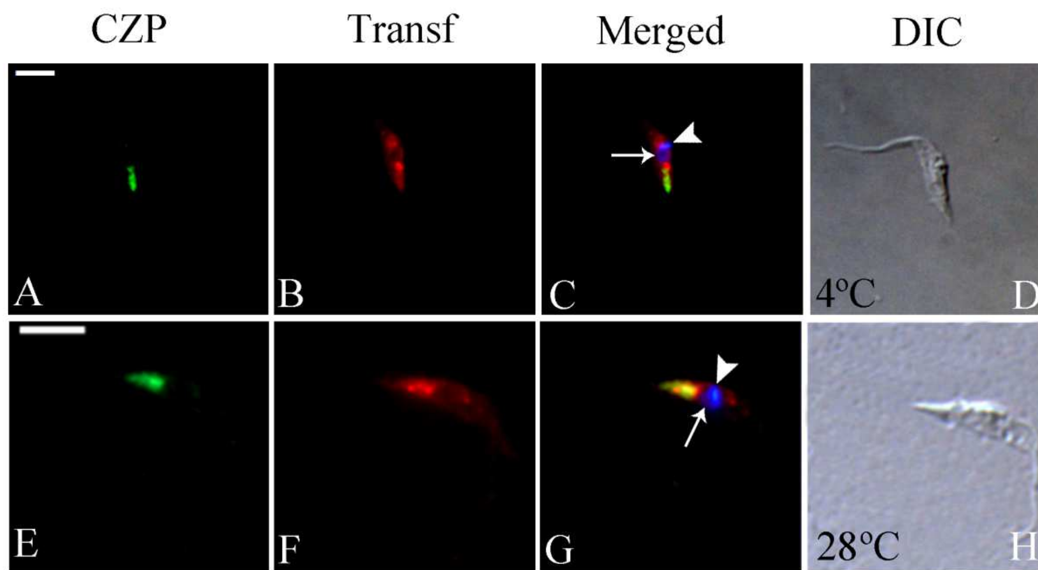
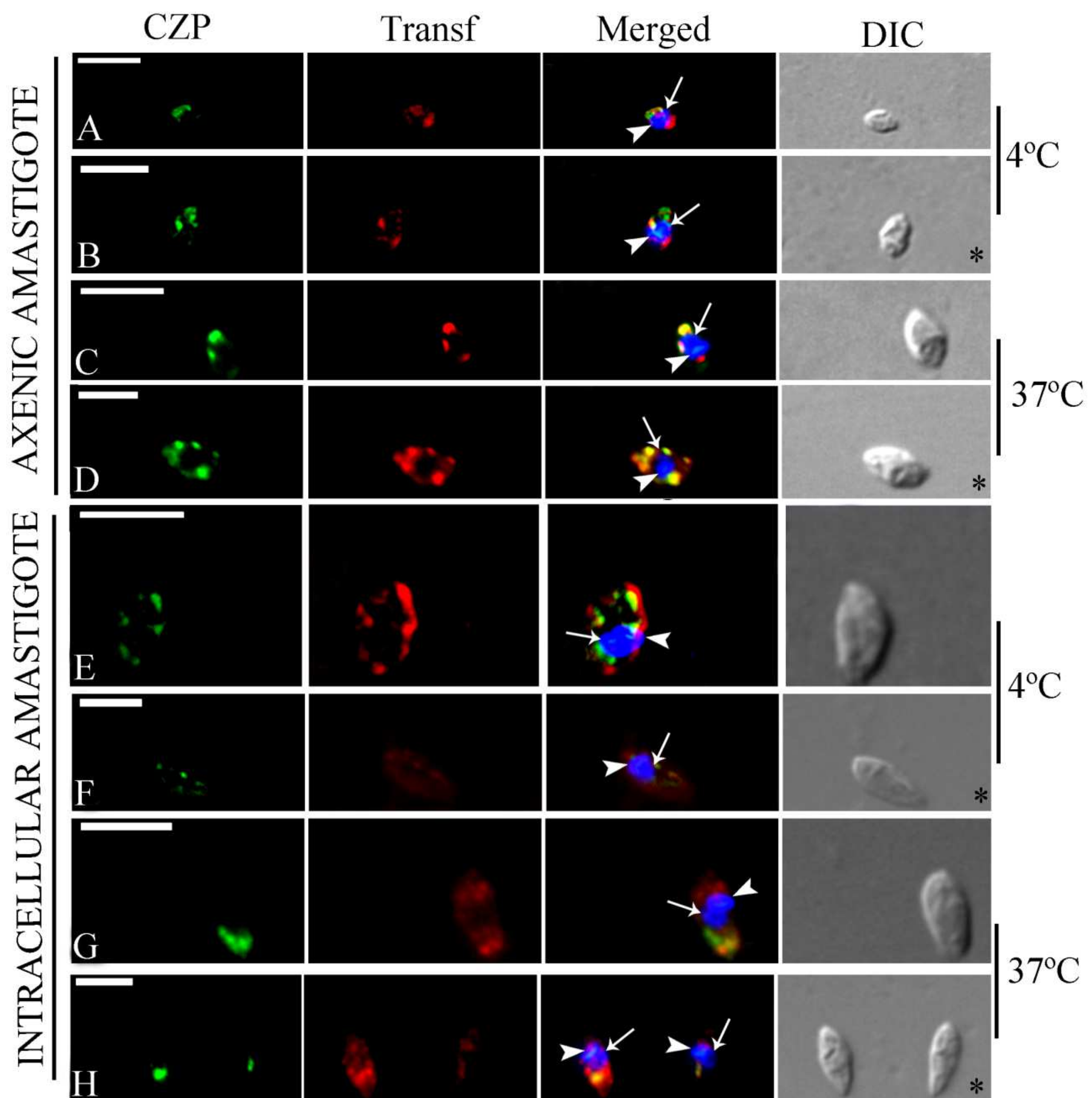


Figure 4:



5. DISCUSSÃO

A endocitose é um evento biológico já caracterizado em formas epimastigotas de *T. cruzi*: macromoléculas podem ser internalizadas a partir da bolsa flagelar ou do citóstoma, ambos localizados na região anterior do parasito. O destino final do material endocitado são os reservossomos, localizados na região posterior, que estocam o material ingerido, o qual é consumido durante o processo de diferenciação para tripomastigotas metacíclicos *in vitro* (Soares e De Sousa, 1991; Figueiredo et al., 2004). Outras características dos reservossomos são o pH 6,0, característico de organelas pré-lisossomais (endossomos tardios) e a presença de enzimas lisossomais, tais como a cruzipaína (ou gp57/51) (Cazzulo et al., 1990a; Murta et al., 1990; Souto-Padrón et al, 1990; Soares et al., 1992).

Entretanto, até o momento não estava bem definido o processo de endocitose em formas amastigotas e tripomastigotas, presentes no hospedeiro vertebrado. Não há evidencias de endocitose em formas tripomastigotas (Corrêa et al., 2002). Já em formas amastigotas intracelulares, existem evidências de endocitose (Lima e Villalta., 1990; Sant' Anna et al., 2008b). Por serem as formas mais prevalentes em pacientes crônicos chagásicos, os amastigotas intracelulares são os mais relevantes clinicamente (Coura e Borges Pereira, 2011). Neste sentido, o objetivo desta dissertação foi produzir anticorpos contra marcadores de reservossomos para estudar a endocitose em formas amastigotas de *T. cruzi*. Primeiramente, foram selecionadas a TcHIP e a cruzipaína, enzimas descritas após estudos de proteômica dos reservossomos (Sant'Anna *et al*, 2009).

A TcHIP é uma zDHHC palmitoil transferase putativa, uma vez que contem os domínios ankirina e zDHHC típicos destas enzimas (Putilina et al., 1999; Mosavi et al., 2006). zDHHC palmitoil transferases são enzimas que contém o motivo aspartato-histidina-histidina-cisteína (DHHC) e geralmente se localizam no complexo de Golgi. Estas enzimas realizam a palmitoilação, uma modificação pós-traducional que facilita a inserção de proteínas em membranas e que regula principalmente a hidrofobicidade, a localização e a função das proteínas palmitoiladas (revisto em Greaves e Chamberlain, 2011). A palmitoilação está descrita em protozoários parasitas, tais como os apicomplexas e os tripanossomatídeos (revisto em Corvi et al., 2011). Em *Trypanosoma brucei* foi realizado um estudo global sobre a palmitoilação (Emmer et al., 2011). Neste estudo, foram identificadas doze enzimas com o domínio DHHC. Após silenciamento

individual de cada uma dessas enzimas por RNA de interferência (RNAi) os autores observaram que o parasito consegue proliferar normalmente, indicando redundância enzimática. Porém, parasitos tratados com 2-bromopalmitato (2-BP), um inibidor de palmitoilação, não conseguem proliferar, indicando que a palmitoilação é um processo essencial para o desenvolvimento de *T. brucei* (Emmer et al., 2011). Entretanto, em *T. cruzi*, nenhuma palmitoil transferase foi ainda identificada e caracterizada, apesar da palmitoilação poder representar uma importante modificação pós-traducional neste parasito.

Utilizando anticorpos policlonais contra uma zDHHC palmitoil transferase putativa de *T. cruzi* (TcHIP) recombinante purificada foi possível observar marcação na região anterior das células, lateral ao cinetoplasto, em formas epimastigotas e amastigotas de *T. cruzi* (ARTIGO 1). Em formas tripomastigotas, a marcação foi entre o núcleo e o cinestoplasto. Estas marcações são compatíveis com a localização do complexo de Golgi de *T. cruzi*, o que foi confirmado por co-localização com TcRab7-GFP, uma proteína já descrita como marcadora de complexo de Golgi deste parasito (Araripe et al., 2004; Batista et al., 2010). Proteínas homólogas à TcHIP também se localizam no complexo de Golgi (Singaraja et al., 2002; Seydel et al., 2005; Frénal et al., 2013), sendo que essa organela, em mamíferos, parece ser um centro de palmitoilação de proteínas periféricas de membrana (revisto em Aicart-Ramos et al., 2011). Dessa forma, os dados da literatura somados aos resultados de imunolocalização de TcHIP em *T. cruzi* convergem para confirmar a presença dessa proteína majoritariamente no complexo de Golgi deste protozoário.

Na proteômica dos reservossomos foram identificadas outras proteínas de complexo de Golgi envolvidas em tráfego de vesículas, tais como a subunidade μ do complexo adaptador AP-1 e as Rabs 1 e 7 (Sant'Anna et al., 2009), o que pode indicar: (a) dinâmica de vesículas entre o Golgi e os reservossomos, ou (b) contaminação da fração de reservossomos com proteínas de Golgi. Em nossos resultados não foi possível detectar a presença de TcHIP nos reservosomos por microscopia de fluorescência. É possível que Sant' Anna e colaboradores (2009) tenham detectado a TcHIP em uma fração enriquecida de reservossomos por terem utilizado uma técnica muito mais sensível, como a espectrometria de massas. Pode ser que a menor sensibilidade da imunofluorescência não tenha permitido a visualização de uma fraca marcação decorrente de pequena quantidade de TcHIP possivelmente presente nos reservossomos.

Como não foi identificado em *T. cruzi* um receptor para manose-6-fosfato, ainda não se sabe como as enzimas lisosomais são direcionadas do Golgi aos reservossomos (Cazzulo et al., 1990b). Recentemente foi descrita a proteômica de uma fração enriquecida de proteínas palmitoiladas em *T. brucei* (Emmer et al., 2011). Dentre as várias proteínas identificadas, uma delas era uma cisteína proteinase precursora, com 59% de identidade com a cruzipaína. A palmitoilação ocorre em resíduos de cisteína das proteínas alvo (Linder e Deschenes, 2003). A cruzipaína possui resíduos de cisteína em toda a sua sequência polipeptídica. Portanto, uma hipótese é que palmitoilação pode direcionar a cruzipaína e outras enzimas do Golgi para os reservossomos. Mais estudos serão necessários para testar esta hipótese.

Uma vez que TcHIP não apresentou localização subcelular nos reservossomos, em nossa dissertação priorizamos a produção de anticorpos monoclonais contra a cruzipaína (ARTIGO 2). A cruzipaína ou gp57/51 é a principal cisteína proteinase de *T. cruzi*, possuindo papel fundamental na nutrição e na interação parasito-célula hospedeira, sendo considerada também um fator de virulência (revisto por Scharfstein, 2010) e um marcador de reservossomos. No entanto, anticorpos gerados contra cruzipaína não reconhecem apenas este compartimento celular em epimastigotas de *T. cruzi* (Murta et al., 1990; Souto-Padrón et al., 1990). Durante nossa produção de anticorpos monoclonais foram realizadas várias triagens por ELISA, *western blot* e imunofluorescência (Nelson et al., 2000) para selecionar hibridomas produtores de anticorpos contra a cruzipaína. O objetivo foi selecionar um hibridoma que secretasse anticorpos que reconhecessem exclusivamente a cruzipaína localizada nos reservossomos.

O soro policlonal do camundongo imunizado com cruzipaína recombinante de *T. cruzi* reconheceu a cruzipaína nativa tanto em reservossomos quanto no complexo de Golgi em epimastigotas selvagens e mutantes expressando TcHIP fusionada a proteina/AC. Entretanto, após fusão de células do baço, triagens e teste de congelamento/descongelamento, um hibridoma secretor de anticorpos específicos para reservossomos foi selecionado (hibridoma CZP-315) e em seguida um clone foi obtido. Este clone (mAb CZP-315.D9) foi caracterizado por ELISA de captura, sendo os anticorpos monolonais do tipo IgG-1 e kappa positivo. Contrariamente ao soro do animal imunizado, o anticorpo monoclonal reconheceu especificamente e exclusivamente a cruzipaína presente nos reservossomos, tanto por microscopia de fluorescência quanto por eletrônica de transmissão.

Para validar o mAb CZP-315.D9, foi realizado um ensaio de endocitose. Formas epimastigotas de *T. cruzi* foram incubadas com transferrina-Alexa 633 por 30 min a 28ºC, condições adequadas para o material ser internalizado e direcionado aos reservossomos (Soares e De Souza, 1991). Marcação para cruzipaína (mAb CZP-315.D9) e transferrina ingerida co-localizou nos reservossomos, como já havia sido descrito na literatura (Soares *et al*., 1992). Portanto, o mAb CZP-315.D9, produzido no presente trabalho (ARTIGO 2), pode ser considerado um marcador específico de reservossomos, constituindo uma importante ferramenta para estudos que buscam elucidar a função desta organela e o destino de moléculas endocitadas.

Para ensaios de endocitose em amastigotas de *T. cruzi* (ARTIGO 3) foi desenvolvido um protocolo para recuperação de amastigotas intracelulares por cavitação. Amastigotas axênicos obtidos por amastigogênese secundária (Hernandes et al., 2010) também foram utilizados nos ensaios. Análises por citometria e imunofluorescência indireta utilizando um anticorpo monoclonal contra Ssp4, um marcador de amastigotas (Andrews et al., 1987), confirmaram o estágio evolutivo dos parasitos utilizados nos experimentos.

Ensaios de endocitose com os amastigotas recuperados e também com amastigotas axênicos (obtidos da diferenciação *in vitro* de tripomastigotas de cultura em amastigotas) foram feitos incubando-se os parasitos com transferrina-Alexa 633 a 4ºC (adesão da proteína a receptores de superfície, mas não internalização) ou a 37ºC (adesão e internalização), seguindo-se análise por citometria de fluxo. Epimastigotas foram utilizados como controle positivo (incubação a 28ºC). Incubação de amastigotas a 4ºC resultou em transferrina não internalizada, como já demonstrado também para epimastigotas (Soares et al., 1992; Figueiredo e Soares, 2000). Por outro lado, a citometria de fluxo demonstrou intensidade de fluorescência maior em epimastigotas e amastigotas incubados a 28ºC e a 37 ºC, respectivamente. Em amastigotas axênicos, o deslocamento do sinal de fluorescência foi menor, indicando menor atividade endocítica. Para confirmar estes dados foi realizado um ensaio de endocitose nas mesmas condições anteriores, seguido de tratamento com ácido acético, de acordo com Lima e Villalta (1990), e análise por citometria de fluxo. Após o tratamento a intensidade de fluorescência diminuiu em todas as variáveis. Entretanto, nos parasitos que foram incubados com transferrina a 28 ou 37ºC a intensidade de fluorescência continuou maior do que nos que foram incubados à 4ºC, indicando a internalização.

Para estudar a possível internalização da transferrina e co-localização com cruzipaína, amastigotas foram incubados com o anticorpo monoclonal CZP-315.D9 e com anticorpo contra transferrina e então analizados por microscopia de fluorescência. Como resultado, houve co-localização de transferrina com cruzipaína nas estruturas lisosossomo/reservossomo-*like* dos amastigotas incubados a 37ºC, mesmo após tratamento com ácido acético, sugerindo fortemente a internalização da transferrina e direcionamento para estas organelas. Estes dados demonstram pela primeira vez a atividade endocítica em formas amastigotas e complementam dados anteriores, os quais não relacionavam os lisossomos-*like* dos amastigotas com estoque de macromoléculas ingeridas (Sant`Anna et al., 2008b).

Estes dados podem trazer novas perspectivas sobre o papel da endocitose como um alvo para o desenvolvimento de fármacos efetivos contra o estágio intracelular do parasito, predominante na fase crônica das infecções em humanos. Portanto, a pesquisa de inibidores metabólicos que possam impedir a captação de macromoléculas, com consequente eliminação dos parasitos intracelulares, pode ser uma nova abordagem para o desenvolvimento de um possível tratamento eficaz contra a doença de Chagas.

6. CONCLUSÕES

1. Estudos de imunolocalização mostraram a expressão e localização de uma zDHHC palmitoil transferase (TcHIP) no complexo de Golgi em diferentes formas evolutivas de *T. cruzi* (amastigotas, tripomastigotas e epimastigotas), mas não nos reservossomos **(ARTIGO 1);**

2. Soro policlonal murino contra a cruzipaína recombinante (TcCruzipaína) reconheceu a enzima no complexo de Golgi e reservossomos **(ARTIGO 2);**

3. Anticorpo monoclonal (mAb CZP-315.D9) contra a cruzipaína recombinante reconheceu exclusivamente a cruzipaína nativa presente nos reservossomos por microscopia de fluorescência e eletrônica de transmissão, co-localizando fortemente com transferrina ingerida por epimastigotas. Este anticorpo monoclonal é, portanto, um marcador específico de reservossomos **(ARTIGO 2);**

4. Ensaios com complexos transferrina-AlexaFluor analisados por citometria de fluxo e imunofluorescência com amastigotas intracelulares ou axênicos demonstraram a ocorrência de endocitose por estas formas evolutivas de *T. cruzi* **(ARTIGO 3);**

5. Em amastigotas, transferrina ingerida co-localiza com a marcação de cruzipaína, indicando internalização e direcionamento da proteína para as estruturas reservossomos-*like* destas formas **(ARTIGO 3).**

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