Ministério da Saúde

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INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Parasitária

Atuação das proteínas que ligam à heparina no ciclo biológico do *Trypanosoma cruzi*

FRANCISCO ODENCIO RODRIGUES DE OLIVEIRA JUNIOR

Rio de Janeiro

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária.

Orientadores: Prof. Dr. Mirian Cláudia da Silva Pereira e
Prof. Dr. Carlos Roberto Alves

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RESUMO

A doença de Chagas, causada pelo protozoário *Trypanosoma cruzi*, é uma doença tropical negligenciada que representa um grave problema de saúde pública. Assim, compreender a biologia da interação *T. cruzi*-hospedeiros constitui um grande desafio, uma vez que o ciclo de vida deste parasito exige um repertório de adaptações para garantir sua dispersão em hospedeiros vertebrados e invertebrados. O presente estudo demonstra o potencial das proteínas com propriedade de ligação à heparina (PLHs) em atuar no ciclo biológico do *T. cruzi*.

Durante este trabalho foi oportuno isolar uma fração de proteínas hidrofóbicas com propriedade de ligação à heparina, com massas moleculares entre 70 kDa e 59 kDa em formas epimastigotas e tripomastigotas de *T. cruzi* por cromatografia de afinidade à heparina. A presença destas proteínas na superfície celular destes parasitos foi confirmada por ressonância plasmônica de superfície. Tais ensaios também foram decisivos na determinação da especificidade e estabilidade da ligação das PLHs a heparina, heparam sulfato (HS) e condroitim sulfato (CS). Os ensaios de competição realizados indicaram que a interação entre PLHs e GAGs pode influenciar a adesão dos epimastigotas à superfície de células epiteliais do trato intestinal de *Rhodnius prolixus*.

O envolvimento de GAGs na invasão de amastigotas em cardiomiócitos, célula alvo da infecção pelo *T. cruzi*, também foi demonstrado através de ensaios de competição com 20 µg/ml de GAGs solúveis, incluindo heparina, HS, CS, dermatam sulfato (DS) e queratam sulfato (KS). Uma drástica redução no nível de infecção foi evidenciada apenas com heparina e HS, atingindo 82% e 65% de redução da invasão, respectivamente. Ensaios com células deficientes em GAGs (CHO-745) corroboraram o importante papel destes componentes de matriz extracelular no processo de reconhecimento e invasão de amastigotas.

Na continuidade deste estudo, avançamos na caracterização bioquímica de PLHs, na determinação da expressão e distribuição espacial destas proteínas em tripomastigotas. As análises por citometria de fluxo revelaram que PLHs são abundantes na superfície de tripomastigotas, clone Dm28c, e a detecção destas proteínas por imunofluorescência indireta revelou uma localização predominante na membrana flagelar do parasito. Com os ensaios de zimografia realizados neste trabalho, revelamos que as PLHs de tripomastigotas tem atividade de protease sobre gelatina em uma ampla faixa de pH (5,5 - 8,0). A sensibilidade destas enzimas a presença de inibidores de serino protease indicam que as PLHs de tripomastigotas têm propriedades similares à tripsina. O conjunto de resultados deste trabalho aponta para o importante papel das PLHs em todas as etapas do ciclo biológico do *T. cruzi* a partir de eventos de adesão e invasão celular, através do reconhecimento de glicosaminoglicanos sulfatados.

Palavras-chave: PLHs, Glicosaminoglicanos, *Trypanosoma cruzi*, *Rhodnius prolixus*, interação parasita-hospedeiro.

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ABSTRACT

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is a neglected tropical disease that causes a serious public health problem. Thus, understanding the biology of the *T. cruzi*-hosts interaction is a major challenge, since the life cycle of this parasite requires a repertoire of adaptations to ensure their dispersion in vertebrate and invertebrate hosts. The present study demonstrates the potential of proteins with heparin-binding property (HBPs) to act in the biological cycle of *T. cruzi*.

During this study, it was opportune to isolate a hydrophobic protein fraction with property to bind to heparin, with molecular weights ranging from 70 kDa to 59 kDa, in epimastigotes and trypomastigotes of *T. cruzi* by heparin affinity chromatography. The presence of these proteins on the cell surface of these parasites was confirmed by surface plasmon resonance. These assays were also decisive in determining the specificity and stability of the binding of HBPs to heparin, heparan sulfate (HS) and chondroitin sulfate (CS). The competition assays indicated that the interaction between HBPs and GAGs can influence the adhesion of the epimastigote to the surface of epithelial cells of the intestinal tract of *Rhodnius prolixus*.

The involvement of GAGs in the invasion of amastigotes in cardiomyocytes, target cell of *T. cruzi* infection, was also demonstrated by competition assays with 20 µg/ml of soluble GAGs, including heparin, HS, CS, dermatan sulfate (DS) and keratan sulfate (KS). A drastic reduction in the level of infection was observed only with heparin and HS, reaching 82% and 65% reduction of the invasion, respectively. Experimental assays using cells deficient in GAGs (CHO-745) corroborated the important role of extracellular matrix components in the recognition and invasion of amastigotes.

In continuation of this study, we advanced in the biochemical characterization of HBPs and also determined the expression and spatial distribution of these proteins in trypomastigotes. The flow cytometric analysis revealed that HBPs are abundant at the surface of trypomastigotes, Dm28c clone, and the detection of these proteins by indirect immunofluorescence revealed a predominant location in the flagellar membrane of the parasite. With the zymography assays conducted in this work, we revealed that HBPs of trypomastigotes have proteinase activity on gelatin in a wide pH range (5.5 - 8.0). The sensitivity of these enzymes in the presence of serine proteinase inhibitors indicates that HBPs of trypomastigotes have properties similar to trypsin. All together, the results of this study points out to the important role of HBPs at all stages of the life cycle of *T. cruzi* from events of adhesion and cell invasion, through the recognition of sulfated glycosaminoglycans.

Keywords: PLHs, Glycosaminoglycans, *Trypanosoma cruzi*, *Rhodnius prolixus*, Parasite-host interaction.

LISTA DE SIGLAS E ABREVIATURAS

Apo – Aprotinina

BSA - Albumina de soro bovino

BZ – Benzinidazol

CHO - *chinese hamster ov*ary cells

CS – condroitim sulfato

DABCO - 1,4-diazabicyclo-(2,2,2)-octane

DAPI - 4,6-diamidino-2-phenylindole

D-GalNAc - *N*-acetil-D-galactosamina

D-GlcA - D-glucurônico

D-GlcNAc - *N*-acetil-D-glucosamina

DIC - Differential interference contrast

DMEM - meio Eagle modificado por Dulbecco

DO – densidade óptica

DS – Dermatam sulfato

DTT - Dithiothreitol

DTUs - Unidades discretas de tipagem

E64 - L-trans-epoxisuccinil-leucilamida-(4-guanidino) butano

ECL – luminol quimioluminescente

ERM - Proteínas erzina-radixina-moesina

EXT1- Exostosina 1

EXT2 – Exostosina 2

FAP - Fator de agregação plaquetária

FGF – Fator de crescimento de friblobastos

FITC – Fluorescein isothiocyanate

FN - Fibronectina

g - gravidade

GAG - Glicosaminoglicanos

Gal – Galactose

GIPLs – Glicoinositolfosfolipídeos

GlcNAc – N-acetil-glicosamina

GlcNAc – N-sulfo-glicosamina

GlcUA – ácido glicurônico

GPI - Glicosil Fosfatidil Inositol

HA – ácido hialurônico

Hep – heparina

HS – heparam sulfato

HSPG – proteoglicano de heparam sulfato

IdoUA – ácido idurônico

IFN-γ - Interferon gama

kDa – quilo Daltons

kDNA – DNA do cinetoplasto

KS – queratam sulfato

L-IdoA - L-idurônico

LN - laminina

MMP-9 - metalo proteinases de matriz-9

MP – Membrana perimicrovilar

NA – N-acetilado

NA/NS – N-acetilado/N-sulfatado

NS – N-sulfatado

OPBTc - Oligopeptidase B

o-phe - 1,10-phenanthroline

PBS – tampão fosfato de sódio

Pep A – pepstatina A

PFA – Paraformaldeido

PG – Proteoglicano

PGHS - proteoglicanos de heparam sulfato

PI3 – Inositol trisphosphate

PI3-K - fosfatidilinositol-3-quinase

PLC – Fofolipase C

PLH - Proteína de ligação à heparina

PMSF - Fluoreto de fenilmetil-l-sulfonil

RE – Reticulo endoplasmático

RGD – Seqüência de adesão celular da FN – arginina, glicina e ácido aspártico

RU – Unidade de ressonância

SDS-PAGE – *sodium dodecylsulfate-polyacrylamide gel electrophoresis*

SFB – Soro fetal bovino

SPR - *surface plasmon resonance*

Sulfo-NHS-LC-biotina - éster de sulfo-N-hidroxi-succinamidobiotina

TCI – *Trypanosoma cruzi* I

TCII – *Trypanosoma cruzi* II

TCIII – *Trypanosoma cruzi* III

TCIV - *Trypanosoma cruzi* IV

TCV - *Trypanosoma cruzi* V

TCVI - *Trypanosoma cruzi* VI

TGF-β – Fator de crescimento de transformação beta

Tris-HCl – tris (hidroximetil-amino) metano HCl

TS – Trans-sialidase

Tween 20 – laurado de sorbitan etoxilado 20

TX-100 – Triton X-100 (T-octilfenoxipoli-etoxietanol)

TX-114 – Triton X-114 (octilfenoxipoli-etoxietanol)

WHO – World Health Organization

β-ME - β-mercaptoetanol

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1. INTRODUÇÃO

1. Introdução

1.1. Breve histórico e relevância

A doença de Chagas ou tripanossomíase Americana é uma doença parasitária que permanece como um importante problema de saúde pública na América Latina. A doença foi primeiramente descrita pelo Dr. Carlos Justiniano Ribeiro Chagas (1909), um médico sanitarista, cientista e bacteriologista brasileiro, que trabalhou como clínico e pesquisador. Em missão de combate à malária no estado de Minas Gerais, em 1907, o Dr. Carlos Chagas despertou interesse sobre os insetos hematófagos (Triatomíneo), conhecidos como barbeiro, que se alimentavam de sangue humano durante a noite. Ao examinar o conteúdo intestinal destes insetos relatou a presença de numerosos protozoários flagelados, denominado *Trypanosoma cruzi* em homenagem ao Dr. Oswaldo Cruz. Em 1909, em Lassance (Minas Gerais), Dr. Carlos Chagas descreve o primeiro caso de infecção aguda pelo *T. cruzi* em humanos ao examinar a paciente Berenice, uma criança de três anos que apresentava um quadro de febre e anemia (Chagas, 1909). O coletivo dos seus estudos representa um marco na história da parasitologia e medicina, pois foi o primeiro e único cientista na história da medicina a descrever completamente uma doença infecciosa: o patógeno, o vetor, os hospedeiros, reservatório animal, as manifestações clínicas e a epidemiologia.

Não obstante a tripanossomíase ter sido identificada no início do século XX com os estudos do Dr. Carlos Chagas, existem indícios de que o *T. cruzi* circula na natureza a milhares de anos como enzootia de animais silvestres antes mesmo da chegada do homem às Américas (Zeledón & Rabinovich, 1981; Guhl et al., 2000; Aufderheide et al., 2004; Araújo et al., 2009). A hipótese clássica propõe que a doença de Chagas foi originada na região andina entre os povos pré-históricos quando eles começaram a domesticar animais, passaram a ter hábitos sedentários, e adotaram a agricultura. Essas mudanças no modo de vida aconteceram quase 6.000 anos atrás e com a invasão dos nichos enzoóticos deste parasita pelo homem, a doença teria emergido pela transmissão por antropozoonose destas infecções (Coura 2007; Prata et al., 2011).

Com o uso de métodos moleculares foi possível revelar a ocorrência do parasito em múmias de indivíduos procedentes de sítios arqueológicos no Atacama há 4.000 anos (Guhl et al., 1999) e há 9.000 anos (Aufderheide et al., 2004). Estudo realizado com múmias do sul dos Estados Unidos da America (EUA) revelou um indivíduo com DNA do parasita, que morrera há 1.150 anos, com intestino dilatado (Araújo et al., 2009). O DNA do parasito foi detectado em múmias humanas com sinais de megacolon do Vale do Peruaçu (Minas Gerais) datadas de 7.000 anos (Fernandes et al., 2008; Ferreira et al., 2011). O conjunto destes dados mostrou que a infecção pelo *T. cruzi* e doença de Chagas ocorreram em populações pré-históricas tanto no Sul e Norte da América, muito antes que o tempo sugerido e podem ser tão antigas quanto à presença humana no continente americano.

Embora a descoberta da doença Chagas seja centenária, esta enfermidade permanece uma zoonose tropical negligenciada pelas indústrias farmacêuticas. Apresenta uma prevalência estimada em 13 milhões de indivíduos infectados, com 50.000 novos casos/ano e cerca de 8 milhões de indivíduos com cardiomiopatias graves, interferindo na capacidade de trabalho de indivíduos infectados com impacto negativo na qualidade de vida (Marin-Neto e Rassi 2009). A doença se distribui geograficamente em regiões endêmicas do México a Argentina. A prevalência da infecção natural na América Latina ainda é destaque entre os diferentes mecanismos de transmissão, sendo responsável por 80-90% dos casos, seguido da infecção por transfusão sanguínea e transplante de órgãos (5-20% dos casos), transmissão congênita (0,5-8% dos casos), acidentes de laboratório e infecção por via oral (revisto por Dias, 2000; revisto por Rassi et al., 2010). No entanto, nas últimas décadas têm sido relatados casos de indivíduos infectados nos EUA, Canadá, Comunidade Européia (principalmente Bélgica, França, Itália, Espanha, Suíça e Inglaterra), Japão e Austrália (Fig. 1) (Coura & Viña 2010, Schmuñis & Yadon 2010).

O cenário atual de distribuição da doença ocorrer devido ao crescimento da movimentação de pessoas entre as áreas rurais e urbanas e ainda, em virtude das migrações internacionais (Albajar-Viñas & Jannin 2011). Aproximadamente 300.000 pessoas vivem com doença de Chagas nos EUA e mais 80.000 casos foram relatados na Espanha, constituindo um novo problema de saúde pública para estas regiões (Rassi et al., 2010; Schmunis & Yadon 2010; Parker & Sethi,

2011). Estudos recentes revelaram prevalência de casos de transmissão do *T. cruzi* na América do Norte e Espanha por transfusão de sangue e/ou componentes sanguíneos e transplante de órgãos por doadores provenientes de áreas endêmicas, demonstrando a necessidade de introdução emergencial de políticas de controles para bancos de sangue e transplante de órgãos e, ainda, investimento na detecção e tratamento da doença (Benjamin et al., 2012).

Doença de Chagas

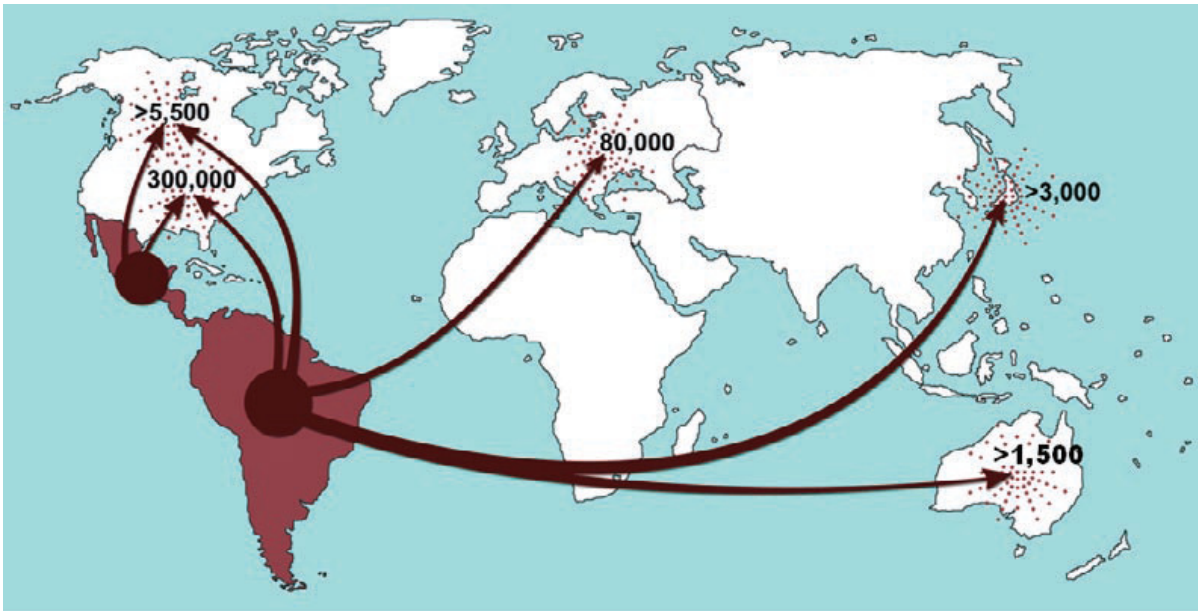

Figura 1. Distribuição da doença de chagas no mundo. (Coura and Viña, 2010).

No Brasil, os índices de novos casos da doença reduziram drasticamente com o rigoroso controle de bancos de sangue e efetivo controle da transmissão vetorial (Rassi et al., 2010). No entanto, as regiões norte e nordeste apresentam índices elevados de infecção aguda e mortalidade, provavelmente decorrente da domiciliação de outras espécies do triatomíneo e infecção por via oral (WHO 2010). Atualmente, a infecção por via oral representa um fator de emergência da doença de Chagas aguda e chama a atenção de pesquisadores e autoridades governamentais. Nos últimos anos, vários surtos da doença relacionados à ingestão de alimento contaminado com *T. cruzi* foram relatados em diferentes estados brasileiros, incluindo Santa Catarina, Pará, Amapá e Tocantins (Pereira et al., 2010; Shikanai-Yasuda & Carvalho 2012). Embora os

dados estatísticos apontem declínio de novos casos da doença, é importante ressaltar a necessidade de manutenção dos programas de monitoramento de bancos de sangue e transplantes de órgãos e ainda, educação para o uso correto dos alimentos, vigilância entomológica e reforço em programas de melhoria habitacional. Destacamos ainda, que a tripanossomíase silvestre persiste e atualmente representa a principal fonte de circulação do parasita e consequentemente um fator de risco para uma eventual reemergência da doença humana (Ceballos et al., 2011; Prata et al., 2011).

1.2. Patologia e patogenia

A doença de Chagas tem um curso clínico variável que pode apresentar-se sob a forma assintomática (forma indeterminada) ou doença crônica grave com distúrbios cardiovascular e/ou gastrointestinal (megaesôfago ou megacolon). Interessantemente, a progressão da doença para estas diferentes formas clínicas parece estar relacionada com a variabilidade genética do parasita em sua distribuição geográfica (revisto por Macedo et al., 2002; Ramírez et al., 2010). O fato de diferentes assinaturas do kDNA ocorrerem em tecidos como coração e esôfago é um indicativo de que o polimorfismo genético do *T. cruzi* que infecta as populações podem exercer uma influência sobre a patogênese da doença de Chagas crônica (Vago et al., 2000).

Após infecção, a doença se manifesta por um curto período de fase aguda, cerca de 4 a 8 semanas, caracterizada pela elevada parasitemia, a qual é rapidamente controlada pelo sistema imunológico que não é capaz de eliminar completamente o parasito, o qual persiste nos tecidos do hospedeiro (Nagajyothi et al., 2012). Em 90% dos casos a doença aguda se resolve espontaneamente sem manifestação clínica. Entretanto, 5 a 10% dos indivíduos sintomáticos nesta fase apresentam miocardite e meningoencefalite graves que pode acarretar em óbito (Rassi et al., 2010). Os sintomas mais frequentes observados nesta fase são: febre, mialgias, suor, hepatoesplenomegalia, falha no coração proveniente de miocardite, expansão do coração e menos frequentemente meningoencefalites (Rassi et al., 2010).

A maioria dos indivíduos se recupera desta fase aguda, e ingressa em uma fase crônica indeterminada, caracterizada por sorologia positiva, na qual 60 a 70% dos pacientes não desenvolvem sintomas clínicos. No entanto, cerca de

30% dos casos indeterminados pode evoluir para forma sintomática, apresentando complicações cardíacas graves, megaesôfago ou megacolon e/ou neurológicas. O comprometimento cardíaco caracteriza-se como a manifestação mais grave e frequente na fase crônica da doença, acarretando hipertrofia cardíaca, arritmias e aneurismas (Coura & Borges-Pereira 2011). Em áreas endêmicas da América Latina, a cardiopatia chagásica pode ser considerada a principal causa de morte associada a problemas cardíacos em pacientes na faixa etária entre 30-50 anos (Rassi *et al.*, 2000).

Um notável aspecto na cardiomiopatia chagásica são os danos progressivos do miocárdio decorrente do intenso infiltrado inflamatório (revisto por Fernandes & Andrews 2012; Gutierrez et al., 2009). O perfil de intensa resposta inflamatória e raros ninhos de amastigotas nos tecidos nesta fase levou alguns pesquisadores a sugerir a progressão de uma resposta autoimune (revisto por Cunha-Neto et al., 2011). No entanto, a detecção de antígenos do parasita nos tecidos de animais infectados (Jones et al., 1993; Vago et al., 1996) e reagudização da parasitemia em indivíduos imunocomprometidos (Braz et al., 2001; De Almeida et al., 2011) foi determinante para revelar a persistência do parasita e seu papel na exacerbação da resposta imune (Tarleton & Zhang 1999, Nagajyothi et al., 2012). Ainda, a presença do *T. cruzi* em tecido adiposo na fase crônica da doença sugere o papel do adipócito como um reservatório da infecção nesta fase (Ferreira et al., 2011). A persistência e liberação do parasita não contribuem apenas com a destruição direta dos cardiomiócitos e para a manutenção do processo inflamatório, mas também de forma indireta induzindo outros mecanismos adicionais para o dano cardíaco como as anormalidades microvasculares coronarianas, microtrombos, espasmos microvasculares e disfunções endoteliais (Rassi et al., 2010). Assim, a atual discussão refere-se à recomendação para tratamento quimioterápico em casos envolvendo crianças e pacientes na fase indeterminada na tentativa de eliminar o parasita e prevenir a manifestação clínica da doença (Coura & Borge-Pereira 2011).

Ainda hoje, mais de cem anos após a descoberta da doença de Chagas, existem apenas dois medicamentos efetivos no tratamento da doença, o nifurtimox e benzinidazol (BZ). O BZ é a droga de primeira escolha para o tratamento por ser mais tolerado pelos pacientes que o nifurtimox. O Nifurtimox

foi descontinuado na década de 80 no Brasil e em outros países da América do Sul, entretanto, sua produção foi reiniciada em 2000 pela Bayer Helthcare (Jannin e Villa, 2007). Tanto o Nifurtimox quanto o BZ são efetivos na fase aguda, mas possuem efeitos limitados na fase crônica. O uso de BZ durante a fase crônica é controverso, mas estudos demonstram que alguns pacientes deste grupo podem ser beneficiados (Urbina, 2010). Além disso, Garcia e colaboradores (2005) demonstraram que o tratamento com BZ na fase crônica reduz a carga parasitária, disfunção cardíaca e miocardite. Ambos compostos estão longe de ser o tratamento ideal, uma vez que apresentam longo tempo de terapia, limitada eficácia contra diferentes isolados de parasitas, baixa eficiência na fase crônica e sérios efeitos colaterais, que incluem anorexia, emagrecimento e manifestações digestivas, causadas pelo nifurtimox, e dermatites com erupção cutânea e polineuropatias, causadas pelo BZ (revisto por Salomon, 2012; Castro et al., 2006; Coura, 1996). Dessa forma, a busca por novos compostos para o tratamento da patogenia da doença de Chagas permanece alvo de intensa investigação.

O Posaconazol é um composto derivado do triazol que atua na inibição da síntese do ergosterol. Este quimioterápico tem se mostrado capaz de induzir a cura parasitológica em camundongos na fase aguda e crônica da infecção chagásica experimental (revisto por Urbina, 2010). Dados recentes demonstraram que posaconazol obteve eficácia superior quando comparado ao BZ na infecção crônica de um caso de lúpus eritematoso sistêmico, com resolução da infecção independente da terapia imunossupressora (Pinazo et al., 2010). Atualmente, o posaconazol se encontra na fase 2 de triagem clinica (kappagoda et al., 2011) e parece ser um potencial composto para o tratamento da doença.

Os avanços nos estudos sobre a biologia do *T. cruzi* têm contribuído para identificação de novos alvos permitindo o desenvolvimento de quimioterápicos, incluindo aqueles que atuem na biossíntese de esterol, na inibição de cisteína protease e no metabolismo de pirofosfato do parasito (revisto em Apt, 2010) ou mesmo, disparando o processo de apoptose no parasito (De Souza et al., 2006). Estratégias terapêuticas que têm sido sugeridas são a combinação de diferentes compostos antiparasitários, associação de antiparasitários e drogas

imunomoduladoras e re-tratamento, visando assim, encontrar uma alta eficácia e baixa toxicidade (Coura, 2009; Le Loup et al., 2011).

1.3. Agente etiológico, vetores e ciclo biológico

1.3.1. Trypanosoma cruzi

O *T. cruzi* pertence ao reino Protozoa, sub-reino Protozoa, filo Sarcomastigophora, subfilo Mastigophora, ordem Kinetoplastea, família Trypanosomatidae e gênero *Trypanosoma* (Levine *et al*., 1980). Uma característica dos membros da família Trypanosomatidae é a presença de cinetoplasto, que representa um compartimento especializado de uma única mitocôndria e contém grande quantidade de DNA (kDNA) (Maslov & Simpson, 1995), compartimentalização da via glicolítica em glicossomas (Opperdoes, 1995) e ainda um único flagelo. Este protozoário hemoflagelado é um parasito digenético, ou seja, necessita de dois hospedeiros distintos para o desenvolvimento de seu ciclo biológico: um hospedeiro vertebrado e outro hospedeiro invertebrado. Durante as diferentes etapas de seu ciclo de vida, o *T. cruzi* apresenta três formas evolutivas distintas: tripomastigotas, epimastigotas e amastigotas (Fig. 2). No hospedeiro vertebrado, encontramos as formas infectantes tripomastigotas, não replicativas, e amastigotas, replicativas, capazes de manter o ciclo intracelular (Ley et al., 1988), enquanto epimastigotas, formas replicativas, e tripomastigotas metacíclicos são encontrados no trato intestinal do inseto vetor. Estas formas evolutivas divergem em sua morfologia (Fig. 2) e podem apresentar (*i*) o corpo alongado com cinetoplasto circular e flagelo longo emergindo da bolsa flagelar na região posterior ao núcleo – tripomastigotas, (*ii*) corpo alongado com cinetoplasto em forma de barra e flagelo longo emergindo da região anterior ao núcleo – epimastigotas, ou (*iii*) corpo arredondado com cinetoplasto em forma de barra e flagelo curto emergindo da bolsa flagelar na região anterior ao núcleo – amastigotas (revisto por De Souza 2002).

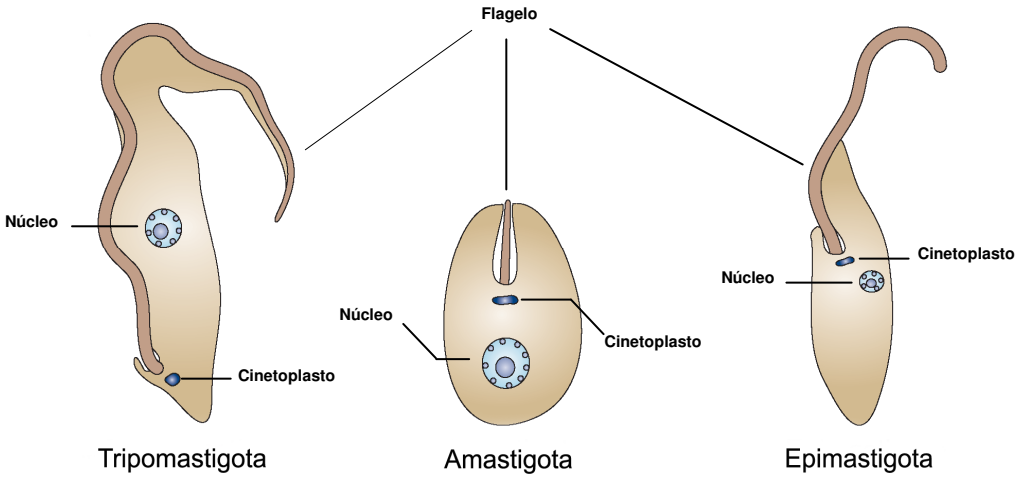

Figura 2. Desenhos esquemáticos de *T. cruzi.* Características morfológicas das formas tripomastigotas, amastigotas e epimastigotas. (adaptado de Docampo et al., 2005 – Nature Reviews Microbiology).

Além destas diferentes formas do parasito é possível estabelecer diferenças no comportamento biológico de populações naturais de *T. cruzi*. A população do *T. cruzi* apresenta alta diversidade genética, consistindo de diferentes cepas que circulam em seus hospedeiros. A variabilidade genética dos isolados de *T. cruzi* levaram a classificação de 2 linhagens filogenéticas divergentes: *T. cruzi* I (TCI) e *T. cruzi* II (TCII) (Anonymous, 1999, Zingales*, et al.*, 2009). Inicialmente, TCII foi subdividido em 5 subgrupos TCII$^{a\text{-}e}$ (Brisse et al., 2000) e, posteriormente a população de *T. cruzi* foi classificada em 3 linhagens – TCI, TCII e TCIII (de Freitas et al., 2006). No entanto, a estrutura e evolução das populações de *T. cruzi* é bastante complexa, sendo mais recentemente reclassificado em 6 unidades discretas de tipagem (DTUs), TcI, TcIV, TcII, TcIII, TcV e TcVI, baseados em diferentes marcadores genéticos (Zingales et al., 2009). O *T cruzi* I refere-se à maioria dos isolados da floresta amazônica e de transmissão doméstica ao norte da linha equatorial, enquanto o grupo II está associado com a maioria dos casos humanos e vinculado à transmissão por populações domésticas de *Triatoma infestans* no Cone Sul (revisto por Prata et al., 2011).

1.3.2. Triatomíneos

Dentre todos os organismos existentes na Terra, os insetos são os mais numerosos em espécies. Estes estão presentes em grande parte dos ecossistemas terrestres, nos quais desempenham um papel ecológico relevante: como polinização, controle natural de pragas entre outros. Além da importância ecológica mencionada anteriormente, muitos insetos são vetores de doenças importantes como: Dengue, Febre amarela, Leishmaniose, febre maculosa e doença de Chagas (Colwell et al., 2011; Gubler et al., 2011). No entanto, focaremos apenas na ordem hemíptera que é tema de estudo desta tese.

A ordem Hemíptera é constituída pelos percevejos. Os insetos triatomíneos, transmissores do *T. cruzi,* pertencem à família *Reduviidae* e subfamília *Triatominae*, com ampla distribuição em diferentes ecótopos naturais de regiões tropicais e subtropicais do continente americano. Seus ecótopos naturais são: tocas e cavidades de árvores, habitats rochosos terrestres ou tocas de roedores, tocas e ninhos de tatus, pacas e aves, no solo e especialmente nas palmeiras (Gaunt & Miles, 2000).

Desde a eclosão do ovo até atingir à fase adulta, os triatomíneos passam por cinco estádios ninfais (Figura 3). As principais diferenças entre ninfas e adultos são a ausência total das asas e ocelos, o caráter rudimentar dos hemélitros (pteroteca), os olhos menores em relação aos adultos e ausência de diferenciação sexual nas ninfas, dificultando o reconhecimento dos sexos antes do quinto estádio. Independente do estádio e do sexo, todos são hematófagos. Um fato importante que diferencia esta ordem de insetos refere-se à presença de uma matriz extracelular recobrindo as células epiteliais do intestino, denominada de Membrana Perimicrovilar (MP) por Terra (1988). A MP, também chamada por camada de membrana extracelular, recobre as microvilosidade das células epiteliais do intestino médio posterior do inseto. Esta estrutura é evidente 10 dias após a alimentação com sangue (Billingsley & Downe, 1983; Nogueira et al., 1997). As MP estão envolvidas com o processo digestivo, absorção de aminoácidos e nutrientes do sangue (Billingsley, 1988; Billingsley and Downe, 1986, 1989; Ferreira et al., 1988; Terra, 1990; Terra & Ferreira, 1994; Terra & Ferreira, 2005). Além disso, epimastigotas interagem

com a MP, sendo este processo de interação essencial para o desenvolvimento do parasita no intestino do inseto. Ainda, apresentam uma metamorfose incompleta (hemimetábolo) e podem ser classificados como ectoparasitas temporários, uma vez que mantêm contato com o hospedeiro unicamente durante o repasto (Guarneri *et al.*, 2000). Portanto, o tempo de defecação é importante na caracterização de potenciais vetores, uma vez que as espécies de tritomíneos que depositam suas fezes ainda sobre o hospedeiro são mais competentes na transmissão do parasito (Loza-Murguía and Noireau 2010). A dispersão é outro aspecto epidemiológico importante, podendo ocorrer através do mecanismo passivo, transportado pelo hospedeiro vertebrado, ou ativo, pelo próprio triatomíneo em busca de novas fontes de alimentação e abrigo (Galvão et al., 1998).

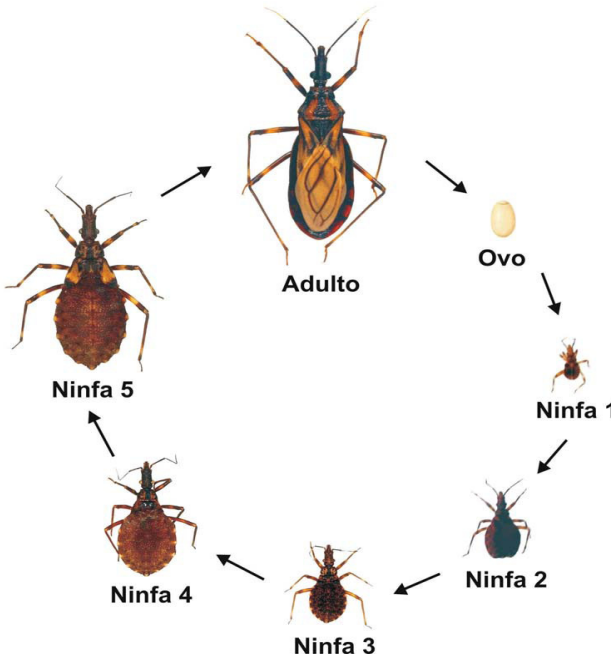

Figura 3. Fases do desenvolvimento do *Triatoma brasiliensis brasiliensis*. (Fotos: Rodrigo Méxas, IOC/Fiocruz). As figuras estão fora de escala.

Devido ao hábito hematofágico, a subfamília Triatominae se destaca por apresentar uma grande importância médica. Dentre as 143 espécies de triatomíneos descritas até o momento, 62 espécies estão presentes no Brasil (Galvão et al., 2009). Embora todas as espécies sejam vetores em potencial do *T. cruzi*, apenas aquelas que colonizam o domicílio e/ou peridomicílio reúnem

melhores condições para transmitir a doença de Chagas. Neste aspecto, os gêneros de maior importância epidemiológica são: *Panstrongylus, Triatoma e Rhodnius* (Garcia et al. 2010).

T. infestans é historicamente o principal vetor da infecção humana pelo *T. cruzi*, pois sua adaptação ao domicílio e peridomicílio data do período de colonização do Brasil (Ferreira et al., 2011). Mediante a importância de *T. infestans* na transmissão humana, este vetor foi alvo do Programa de eliminação nos países do Cone Sul (1991-2001) que interrompeu a transmissão natural por este vetor no Chile, Uruguay, Brasil, nordeste do Paraguai e partes da Argentina (Dias et al., 2002; Dias 2007). Embora este tipo de transmissão venha diminuindo no Brasil e na América latina (Costa e Lorenzo, 2009; Coura & Viñas, 2010), estudos recentes demonstram a existência de população de *Panstrongylus megistus* ingurgitados com protozoários semelhantes ao *T. cruzi* na região intradomiciliar e peridomiciliar (Villela et al., 2010), fato que demonstra os riscos de re-emergência da transmissão vetorial. Além disso, habitat silvestres escondem focos de *T. infestans* e *R. prolixus* que pode ser determinante na reinfestação das casas (Ceballos et al., 2011). Assim, espécies de triatomas silvestres e peridomésticos reconhecidos atualmente como alvos de programas de controle vetorial são *T. dimidiata* na América central, *Panstrongylus megistus*, *T. brasiliensis*, *T. pseudomaculata* no Brasil, *Rhodnius ecuadoriensis* no norte do Peru e Equador, *T. pallidipennis* no México.

Intervenções eficazes de controle do vetor têm adotado como estratégia principal a pulverização de casas com inseticidas de ação residual (piretróides). Entretanto, o desenvolvimento de resistência aos piretróides, elevado grau de toxicidade a mamíferos e impacto ambiental demonstram a necessidade de pesquisas em busca de novos inseticidas (Stevens et al., 2011). Ainda, a eliminação dos vetores em áreas endêmicas prevê novos desafios, sendo determinante a sustentabilidade dos níveis de controle de transmissão vetorial domiciliar através da eliminação dos focos residuais de *T. infestans* e manutenção das casas livres de colônias de espécies nativas, tanto reconhecidamente vetoras como as com potencial para atuar na transmissão domiciliar (Silveira 2011).

1.3.3. Ciclo Evolutivo

Entre os tripanosomatídeos, o *T. cruzi* apresenta o mais complexo ciclo de vida envolvendo diferentes estágios evolutivos em hospedeiros vertebrados e invertebrados. A aproximação do homem aos nichos naturais do triatomíneo foi determinante para a dispersão da doença. Assim, devido ao habito alimentar (hematófago) e adaptação domiciliar das diferentes espécies do vetor estabeleceu-se a infecção humana.

O ciclo biológico do *T. cruzi* abrange dois ciclos distintos, um silvestre e outro doméstico. O ciclo silvestre é bastante complexo e mantém-se através da infecção de uma variedade de mamíferos em seus ecótopos naturais, incluindo desertos, palmeirais, matas, caatinga, cerrado, pampa, charco, pantanal entre outros. Os mamíferos são considerados reservatórios, pois são responsáveis pela manutenção do parasito na natureza. Dentre estes reservatórios destacam-se: roedores, marsupiais, canídeos, felinos, edentados e primatas (Figura 4C) (Roque et al., 2008; Coronado et al., 2009; Galuppo et al., 2009; Pineda et al., 2010; Abolis et al., 2011).

O ciclo doméstico ocorre a partir da migração e instalação do homem no ambiente silvestre, geralmente sob condições precárias que favorecem a domiciliação dos triatomíneos. Neste ciclo, os reservatórios são o homem e pequenos mamíferos domésticos, como gatos, cães, ratos e coelhos. Um fato importante a ser comentado é o papel de mamíferos reservatórios na região peridomiciliar, principalmente cães, que constituem um elo para manutenção da transmissão vetorial (Gürtler et al., 2006).

A infecção de hospedeiros mamíferos pode ocorrer durante o repasto sanguíneo, quando um triatomíneo ingurgitado com *T. cruzi* elimina formas tripomastigotas metacíclicas juntamente com fezes e urina (Figura 4A). O parasito não é capaz de penetrar na pele íntegra, assim, formas tripomastigotas metacíclicas (Figura 4B) penetram através do ferimento causado pela picada do inseto vetor e/ou por pequenas fissuras presentes na pele do hospedeiro vertebrado, permitindo a invasão de macrófagos e/ou fibroblastos do tecido conjuntivo próximos a “porta de entrada” do parasito. De 7 a 10 dias após o período de incubação o sítio de entrada do parasito no homem é marcado por uma lesão de pele denominada de chagoma de

inoculação, frequentemente na face do indivíduo. Os tripomastigotas metacíclicos também podem penetrar pela conjuntiva ocular, gerando dessa forma uma inflamação local denominada sinal de Romaña (Romaña 1935) (Figura 4C).

No hospedeiro vertebrado, as formas tripomastigotas metacíclicas são capazes de invadir e desenvolver seu ciclo intracelular em diferentes tipos celulares (Brener, 1973). No interior de células de mamíferos, as formas tripomastigotas se diferenciam em amastigotas que se rediferenciam em tripomastigotas sendo liberadas após intensa multiplicação por ruptura da célula hospedeira (Figura 4D-E). Assim, os parasitos liberados podem alcançar a corrente sangüínea e infectar outros tipos celulares ou serem ingeridos pelo inseto vetor (Figura 4F).

Uma vez no interior das células, os tripomastigotas são observados em vacúolos parasitóforos que se fundem com endosomas tardios e lisosomas (Meirelles et al., 1986; Woolsey & Burleigh 2004). Os tripomastigotas escapam do vacúolo parasitóforo após acidificação e formação de poros, via ativação de Tc TOX (Andrews et al., 1990). Livres no citoplasma se diferenciam em formas amastigotas que iniciam o processo de divisão celular por fissão binária. Após sucessivas divisões, as formas amastigotas se diferenciam em tripomastigotas que serão liberadas no interstício após a ruptura da célula hospedeira (Brener, 1973; Chagas, 1909; 1911a,b; 1922).

As etapas do ciclo biológico no trato digestivo dos triatomíneos infectados iniciam-se com a ingestão de formas tripomastigotas sanguíneas (Figura 4G). Neste momento, os tripomastigotas alcançam o estomago do inseto onde se diferenciam em formas epimastigotas, forma multiplicativa no hospedeiro invertebrado, sendo também observadas esferomastigotas (Garcia et al., 2010). Ao alcançar a região posterior do intestino médio do triatomíneo, os epimastigotas sofrem sucessivas multiplicações (Figura 4H) e podem aderir a membrana perimicrovilar das células epiteliais (Gonzales et al., 1999; Garcia et al., 2010). Esta adesão é mediada por diferentes moléculas e mecanismos que serão discutidos posteriormente. Em seguida, os epimastigotas aderem à cutícula do reto, por interações hidrofóbicas e se diferenciam em tripomastigotas metacíclicos que são eliminados nas fezes e urina durante o repasto sanguíneo do inseto vetor (Figura 4I) (Garcia & Azambuja 1991, Kollien

& Schaub 2000; revisto por Garcia et al., 2010), dando continuidade ao ciclo biológico.

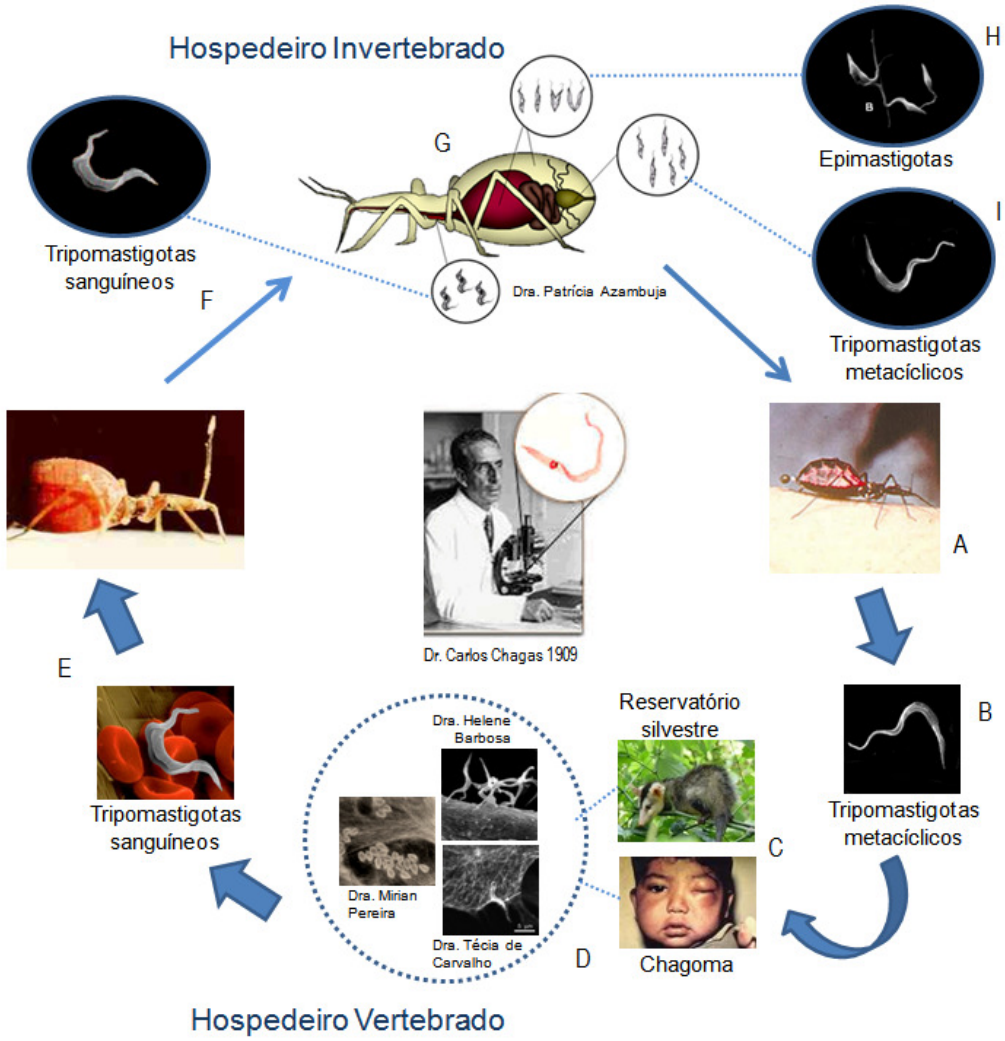

Figura 4. - Ciclo evolutivo do *Trypanosoma cruzi*.

Fontes: (A) http://www.fundep.ufmg.br/homepage/fotos_cases/barbeiro_interna.jpg (acessado em nov/2008). (B) http://www.cib.uaem.mx/agebiol/pr03_archivos/image016.jpg (acessado em nov/2008). (C) Dra. Mirian Pereira / Dra. Helene Barbosa / Dra. Técia de Carvalho. (d) http://www.ioc.fiocruz.br/pages/informerede /corpo/noticia/2006/fevereiro/img/chagas_2.jpg (acessado em out/2008). (E) www.fiocruz.br/ccs/media/chagas.jpg (acessado em nov/2008). (F-H) http://www.fiocruz.br/chagas/cgi/cgilua.exe/sys/start.htm?sid=1 (acessado em nov/2008). (*) Dra. Patrícia Azambuja.

A competência do vetor na transmissão do *T. cruzi* depende em parte do hábito alimentar (capacidade de ingestão de sangue e tempo médio de excreção) e maturação do parasita no intestino do triatomíneo. O processo de maturação do *T. cruzi*, conhecido como metaciclogênese, depende da interação de ligantes da superfície do parasito e moléculas na superfície de

células intestinais do inseto vetor. Estas interações podem ser alvos potenciais para o controle da transmissão do *T. cruzi* (Hurwitz et al., 2011), sendo um dos temas abordados nesta tese.

1.4. Componentes de superfície do T. cruzi X interação com os hospedeiros

Durante o ciclo de vida do *T. cruzi* a membrana de superfície desse protozoário atua nos diferentes micro-ambientes de seus hospedeiros, vertebrado e invertebrado, contribuindo para a permanência do parasito nesses ambientes. A fase intracelular obrigatória do *T. cruzi* no hospedeiro vertebrado é dirigida por eventos de adesão e interiorização, enquanto que em sua fase extracelular, no lúmen do trato digestivo do inseto vetor, apenas por eventos de adesão. Sendo assim, a expressão de certos componentes de superfície em cada uma das formas do parasito, pode ser relacionada aos mecanismos de adaptação nos seus diferentes micro-ambientes de vida. Deste modo, os componentes de superfície dos tripomastigotas, amastigotas e epimastigotas são determinantes na interação deste parasito com seus hospedeiros e podem significar alvos de ação sobre a cadeia de transmissão da doença. Uma série de proteínas tem sido identificada como chave para o desenvolvimento desse processo adaptativo, incluindo proteínas semelhantes às mucinas, trans-sialidases, receptores para fibronectina, proteases, proteínas ligantes de heparina, cruzipaína entre outras (Fig. 5).

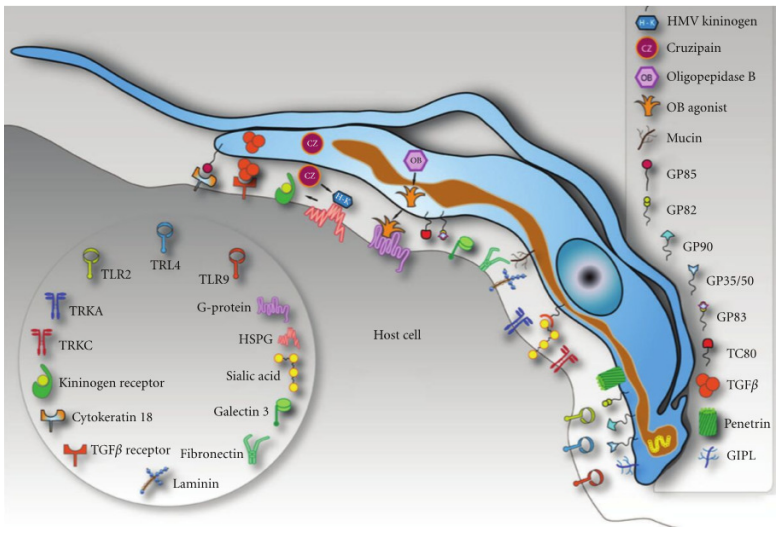

Figura 5. Modelo esquemático demonstrando as moléculas envolvidas no processo de interação *T. cruzi*-célula hospedeira (de Souza *et al.*, 2010).

1.4.1. Principais componentes do parasita que dirigem a interação com o hospedeiro vertebrado

A interação do *T. cruzi* com o hospedeiro vertebrado é orquestrada por uma variedade de moléculas protagonistas do processo de reconhecimento e invasão, entre as quais se destacam as proteínas codificadas por genes que compreendem famílias multigênicas, como as mucinas e as trans-sialidases (De Pablos & Osuna 2012).

A mucina é uma família de proteína rica em treonina, tirosina e serina altamente glicosiladas (Pereira-Chioccola et al., 2000). Inicialmente, estas proteínas foram descritas em mamíferos desempenhando um papel de proteção ao epitélio digestivo e respiratório destes organismos (Hollingsworth & Swanson, 2004). Posteriormente, glicoproteínas semelhantes às mucinas foram identificadas na superfície do *T. cruzi* ancoradas à membrana por glicosil fosfatidil inositol (GPI) (Schenkman et al., 1993). Estas proteínas estão presentes em ambas as formas, tripomastigotas (metaciclicas e derivadas de cultivo celular) e epimastigotas (Acosta-Serrano et al., 2001) e desempenham importante papel na interação parasita-hospedeiro. Em tripomastigotas metacíclicos, mucinas foram descritas como proteínas na faixa de 35 kDa a 50 kDa (gp35/50) e são resistentes à ação das proteases, enquanto em tripomastigotas derivadas de cultivo celular o perfil de expressão destas proteínas é mais amplo, atingindo massa molecular entre 60/70 kDa a 200 kDa, e são susceptíveis à ação de proteases (Schenkman et al., 1991; Di Noia et al., 1998). Além da função protetora descrita anteriormente, a gp35/50 também se relaciona à adesão e sinalização com mobilização de cálcio durante o processo de interação com a célula hospedeira (Mortara *et al.*, 1992; Schenkman *et al.*, 1993; Villalta *et al.*, 2008). No entanto, diferentemente de outras proteínas moduladoras do processo de invasão, a gp35/50 induz liberação transiente de Ca^{2+} de acidocalcissomas por um mecanismo dependente de AMP cíclico gerado por ativação de adenil ciclase (Neira *et al.*, 2002; revisto por Yoshida, 2006). Interessantemente, este sinal transiente de Ca^{2+} dispara um mecanismo

de invasão dependente do citoesqueleto da célula alvo, configurando uma invasão mediada por processo endocítico (Ferreira et al., 2006).

A superfamília das gp85/trans-sialidases (TcTS) constitui um dos poucos exemplos naturais de glicosiltransferases superficiais encontradas em eucariotos. O *T. cruzi* é incapaz de sintetizar ácido siálico e utiliza esta enzima para remover este monossacarídeo de glicoconjugados da superfície de células do hospedeiro vertebrado e trasnferi-los, através de ligação 2,3 com galactose (Agustí et al., 2007), para proteínas aceptoras, como mucina-GPI, presentes na membrana plasmática do parasita (Araya et al., 1994; Colli & Alves 1999). As mucinas sialiladas estão envolvidas no processo de aderência e subsequente penetração do parasita nas células do hospedeiro (Eugenia-Giorgi & Lederkremer 2011). Estudos pioneiros descreveram que células deficientes em ácido siálico apresentaram uma redução nos níveis de infecção do *T. cruzi* (Ming et al., 1993), o que claramente confirma a importância destas glicoproteínas na interação parasita-hospedeiro.

Dentre os membros constituintes desta superfamília, encontram-se glicoproteínas com potente atividade trans-sialidase ou inativas, como a gp82 e Tc85, sendo todas relacionadas com o processo de invasão do parasito (revisto por Alves e Colli 2008). O genoma do *T. cruzi* apresenta 3 principais grupos de gp82 (A, B e C) que apresentam 95-97% de homologia entre si (Songthamwat et al., 2007). A gp82 participa do processo de invasão de tripomastigotas metacíclicos através da ativação da proteína tirosina quinase (p175), fosfolipase C (PLC) com liberação de PI3 que disparam a cascata de sinalização de Ca^{2+} requerido para invasão do parasita (Yoshida et al., 2000). Neste processo, Ca^{2+} liberado do retículo endoplasmático induz a desorganização do citoesqueleto de actina cortical favorecendo a penetração do *T. cruzi* (Cortez et al., 2006) por um mecanismo dependente do recrutamento de lisossomos para o sítio de adesão do parasita (revisto por Yoshida 2006). Este processo de invasão configura um mecanismo de penetração ativa do *T. cruzi*, ou seja, independente do citoesqueleto da célula alvo. Entretanto, é importante destacar que existem diferenças evidentes na expressão de moléculas envolvidas na sinalização de Ca^{2+} entre cepas distintas e sua capacidade infectiva (Ruiz et al., 1998). Embora a expressão de gp82 em cepas altamente virulentas, como CL, e pouco infectivas, como G,

seja semelhante em ambas as cepas, a expressão de gp35/50 e gp90 é superior na cepa G, acarretando níveis inferiores de Ca^{2+} liberado quando comparado à cepa CL (Ruiz et al., 1998). A invasão mediada por gp82 ainda parece estar envolvida na rota de infecção por via oral (Yoshida 2009). A gp82, que apresenta capacidade de ligação a mucinas gástricas, é resistente a digestão peptídica em pH ácido, enquanto a gp90, que regula negativamente a invasão em células de mamíferos (Ruiz et al., 1998), é degradada pelo suco gástrico, aumentando a virulência do *T. cruzi* e exacerbando a gravidade da infecção em surtos de infecção oral (Staquicini et al., 2010; Yoshida 2009). Ainda, tripomastigotas metacíclicos secretam proteínas de 55 kDa enriquecida em serina, alanina e prolina (SAP) que também estão envolvidas na mobilização intracelular de Ca^{2+}, sugerindo que o parasita apresenta uma variedade de moléculas na superfície celular e proteínas secretadas capazes de disparar cascatas de sinalização envolvidas na invasão (Baida et al., 2006).

Ainda, o repertório de moléculas da família de gp85 inclui membros com sítio de ligação para citoqueratina-18 e laminina e, enzimas como cruzipaína e oligopeptidase B (revisto por Yoshida 2006). É cada vez mais evidente o papel da Tc85 na adesão do parasita às células de mamíferos mediando à invasão celular. Estas glicoproteínas ancoradas na superfície dos tripomastigotas via GPI (revisto por de Souza, 2010) possuem um domínio conservado (VTVXNVFLYNR) denominado FLY capaz de se ligar a citoqueratina 18 (CK18) na superfície de células LLC-MK2 e aumentar significantemente a invasão de tripomastigotas nestes tipos celulares. Esta propriedade da Tc85-11 foi descrita como um potencial do parasita em superar as barreiras impostas pelas membranas celulares, matrizes extracelulares e lâmina basal para atingir a célula hospedeira (Magdesian et al., 2001). O domínio FLY também promove a desfosforilação e reorganização de CK18 e, consequentemente, ativação de ERK1/2, resultando em um aumento na invasão de parasitas (Magdesian et al., 2007). Ainda, a administração de peptídeos sintéticos contendo o domínio FLY em animais experimentais antes do desafio com *T. cruzi* acarreta elevada parasitemia e mortalidade, sugerindo que FLY facilite a infecção in vivo e concorra com fatores que propiciem a sobrevivência (Tonelli et al., 2011).

Estudos pioneiros indicaram que o *T. cruzi* expressa receptores para componentes de matriz extracelular, como a glicoproteína fibronectina, que

auxiliam o processo de invasão. A adição de anticorpos anti-fibronectina no meio nutritivo inibe significativamente a infecção de fibroblastos de rato (FR 3T3) por tripomastigotas, sugerindo que fibronectina da superfície celular pode atuar como mediadores da ligação dos parasitas (Ouaissi et al., 1984). Estudos demonstraram que fibronectina participa na adesão e entrada de tripomastigotas em macrófagos e fibroblastos (Ouaissi et al., 1985; Wirth and Kierszenbaum, 1984) e amastigotas em macrófagos murinos ou humanos (Noisin e Villalta 1989). Posteriormente, a participação da sequência Arg-Gli-Asp (sequência RGD) de fibronectina foi evidenciada no processo de invasão de formas tripomastigotas de *T. cruzi* em cardiomiócitos (Calvet et al., 2004).

Além das moléculas presentes nas diferentes formas do parasita descritas anteriormente, o processo de adesão e reconhecimento pode ser facilitado por enzimas proteolíticas, incluindo TC80 (Santana *et al.*, 1997; Grellier *et al.*, 2001), cruzipaína (Cazzulo *et al.*, 1990) e oligopeptidades B (Burleigh & Andrews 1995b), e ainda, por outras proteínas como gp83 (Villalta *et al.*, 2008) e penetrina (Ortega-Barria & Pereira, 1991; Oliveira Jr *et al.*, 2008).

As proteases constituem outro grupo de proteínas envolvidas no processo de invasão do *T. cruzi* a células do hospedeiro vertebrado. Embora as principais classes de proteases (aspártico proteases, cisteíno proteases, serino proteases e metallo proteases) terem sido descritas nesse parasito (McKerrow et al., 2006; Alvarez et al., 2012), apenas as cisteíno proteases, serino proteases e metalo protease foram relacionadas com a interação entre parasito e o hospedeiro vertebrado.

Dentre as proteases, a cruzipaína, uma cisteíno protease, é a mais estudada, sendo a principal protease encontrada em todas as formas evolutivas *do T. cruzi*. Esta glicoproteína possui massa molecular variando entre 57 kDa e 51kDa (gp 57/51) com atividade proteolítica na faixa de pH 5 a pH 7,5 (revisto por Villalta, 2009). A cruzipaína foi relacionada com o processo de invasão do *T. cruzi,* uma vez que é capaz de induzir a síntese de bradiquinina que ativa os receptores para bradiquinina e assim, induzir a mobilização de Ca^{2+}. Estes achados foram descritos nos estudos de invasão de células endoteliais da veia umbilical e de ovário de hamster chinês superexpressando o receptor tipo B(2) de bradiquinina (CHO-B (2) R) (Scharfstein et al., 2000).

A atuação das serino proteases na interação entre o *T. cruzi* e seu hospedeiro vertebrado parece mediar: (1) eventos de transdução de sinal disparados pelas enzimas secretadas pelo parasito através do reconhecimento de receptores na superfície da célula hospedeira; (2) atividade catalítica sobre componentes da matriz extracelular do tecido do hospedeiro vertebrado. A Oligopeptidase B (OPBTc) é capaz de disparar um sinal transiente de Ca^{2+} intracelular e promover a invasão do parasito pelo mecanismo dependente de lisossomos (Burleigh et al., 1997; Caler et al., 1998; 2000). A Tc80 é capaz de hidrolisar colágeno tipo I e IV e fibronectina. Essa atividade proteolítica parece facilitar a invasão do parasita pela degradação de componentes da barreira de matriz extracelular ou mesmo, pela clivagem de colágeno e interação direta com receptores de integrinas (Grellier et al., 2001). Recentemente, foi proposto que a OPBTc é uma proteína de natureza dimérica, cuja atividade máxima ocorre em pH 8,0 e até 42 °C, conferindo estabilidade estrutural e funcional em ambientes com fatores químicos e físicos distintos e, portanto, facilitando o desenvolvimento do parasita nos diferentes hospedeiros (Motta et al., 2012).

Proteases homólogas a gp63 *Leishmania spp* foram detectadas tanto em tripomastigotas metacíclicos quanto em tripomastigotas derivados de cultura (Cuevas et al., 2003). Essa enzima foi associada ao processo de invasão do parasito a célula hospedeira, uma vez que a utilização de anticorpos contra elas reduziram em aproximadamente 50% a invasão de células Vero pela formas tripomastigotas do *T. cruzi* (Cuevas et al., 2003). Recentemente, enzimas semelhantes às metalo proteinases de matriz-9 (MMP-9) foram identificadas no *T. cruzi*. Estas enzimas foram descritas, por *Western blotting,* como uma proteína de 97 kDa e 85 kDa no extrato total do parasito, sendo apenas a de 85 kDa secretada pelo parasito. A possibilidade destas enzimas agirem sobre componentes da matriz extracelular no hospedeiro vertebrado é um indicativo de seu potencial na interação entre o *T. cruzi* e a célula hospedeira, durante o processo de invasão (Nogueira de Melo et al., 2010).

Ainda, o *T. cruzi* apresenta ligantes em sua superfície capazes de reconhecer proteoglicanos de heparam sulfato na superfície da célula hospedeira. A Proteína de ligação à heparina (PLH), denominada penetrina (60 kDa), foi primeiramente descrita em formas tripomastigotas de *T. cruzi* por Ortega-Barria e Pereira (1991). Inicialmente, os estudos reportaram a

capacidade de penetrina se ligar a heparina e componentes de matriz extracelular (heparam sulfato e colágeno do tipo I e IV), promovendo a entrada de parasitas em células de mamíferos (Ortega-Barria e Pereira 1992; Calvet et al., 2003) em um mecanismo distinto da via de ácido siálico-Trans-sialidase (Herrera et al., 1994). Aplicando a estratégia de Triton X-114 associada com a cromatografia de afinidade de heparina (Alves et al., 2012 – Anexo 2), identificamos duas bandas de proteínas, 59 kDa e 65,8 kDa, em formas tripomastigotas e amastigotas de *T. cruzi* com capacidade para se ligar à glicosaminoglicanos (GAGs) sulfatados, como heparina, heparam sulfato (HS) e condroitim sulfato (CS) (Oliveira-Jr et al., 2008), que são cadeias longas de polissacarídeos constituída de unidades repetidas de dissacarídeos (hexosamina e ácido urônico), cuja estrutura e função biológica será detalhadamente discutido posteriormente. Além disso, demonstramos o envolvimento dos proteoglicanos de heparam sulfato (PGHS) na adesão e invasão de cardiomiócitos por formas tripomastigotas (Calvet et al., 2003; Oliveira-Jr et al., 2008) e a participação do domínio N-acetilado/N-sulfatado da cadeia de heparam sulfato (HS) na ligação seletiva de HS para ligantes de *T. cruzi*, que induz a entrada do parasita (Oliveira-Jr et al., 2008), enquanto condroitim sulfato (CS) não apresentou efeito na invasão de células de mamíferos (Calvet et al., 2003). A capacidade de PLHs reconhecer diferentes GAGs e a presença de glycosaminoglicanos em células do hospedeiro vertebrado (Dreyfuss et al., 2009) e trato intestinal do inseto vetor (Costa filho et al., 2004; Souza et al., 2004) levantou a hipótese do possível papel de PLHs no ciclo biológico do *T. cruzi*.

1.4.2. Principais componentes do parasita que dirigem a interação com o hospedeiro invertebrado

Apesar dos epimastigotas não serem infectivos, estas formas desempenham um papel primordial para manutenção do ciclo biológico do *T. cruzi*, uma vez que são formas multiplicativas presentes no hospedeiro invertebrado. Para que o *T. cruzi* possa completar o seu ciclo biológico, as formas epimastigotas necessitam interagir com o trato intestinal do inseto vetor, principalmente em duas regiões: intestino médio posterior e reto. No intestino

médio posterior, a ligação de epimastigotas ocorre na MP e nas células do epitélio, e envolve a participação de diferentes componentes do parasito.

Semelhante ao que acontece em tripomastigotas metacíclicos, as mucinas podem atuar na proteção dos epimastigotas contra a ação de proteases presentes no trato gastrointestinal do inseto vetor (Revisto por Villalta, 2009), além de servirem como aceptores de ácido siálico. Além das mucinas, epimastigotas expressam trans-sialidases, as quais se diferenciam estruturalmente das enzimas encontradas em tripomastigotas (Chaves et al., 1993). Embora apresentem a capacidade de transferir ácido siálico às mucinas presentes em sua superfície, sua atividade em epimastigotas foi equivalente a 17% da atividade encontrada para tripomastigotas. Dessa forma, a transferência de ácido siálico parece estar envolvida com a adesão de epimastigotas a células epiteliais da ampola retal do inseto (Frasch et al., 2000). Além disso, o tratamento de epimastigotas com fator de agregação plaquetária (FAP) é capaz de promover um aumento no número de parasitos aderidos ao intestino do inseto vetor (Zimmermann et al., 2011). Esses dados corroboram os achados anteriores pelo fato de que FAP modula a carga de superfície do parasito tornando-a mais negativa, por alterar as quantidades de ácido siálico em sua superfície.

Além de ácido siálico, outros carboidratos estão envolvidos na adesão de epimastigotas no trato intestinal do triatomíneo (Pereira et al., 1981). Em estudos de interação de proteínas do *R. prolixus* com o *T. cruzi* foi demonstrado que proteínas hidrofóbicas de epimastigotas ligam a proteínas da membrana perimicrovilar com massas moleculares entre 47,7 kDa e 13 kDa. Além disso, o pré-tratamento de epimastigotas com N-acetilgalactosamina, N-acetilmannosamina, N-acetilglucosamina, *D*-galactose, *D*-manose ou ácido siálico foi capaz de inibir diferentemente a adesão de epimastigotas a membrana perimicrovilar em 28.9%, 30.3%, 52.5%, 62.2%, 88.2% e 89.9%, respectivamente, quando comparados com o grupo controle. O conjunto destes dados indica que a adesão de epimastigotas ao epitélio intestinal do inseto envolve a participação de proteínas hidrofóbicas do parasita e glicoconjugados da membrana perimicrovilar (Alves et al., 2007).

Glicoinositolfosfolipídeos (GIPLs) são os principais glicoconjugados encontrados na superfície de epimastigotas de *T. cruzi*. GIPLs estão envolvidas

na adesão do parasita a células epiteliais do intestino de *R. prolixus*, uma vez que, o tratamento do intestino posterior com 50 e 100 nM de GIPLs de epimastigotas (Dm28c) acarreta 95% de inibição da adesão ao trato intestinal. Além disso, esta parece ser mediada por resíduos de β-galactofuranose, sendo evidenciado que o tratamento da molécula de GIPLs com ácido trifluoroacético reduziu a adesão em apenas 50% (Nogueira et al., 2007).

Embora epimastigotas possam colonizar o intestino médio posterior e o reto, estes parasitas aderem preferencialmente ao reto dos triatomíneos. Estudos pioneiros identificaram a natureza do processo de adesão dos parasitas. Neste estudo foi proposto que a camada superficial do reto de *T. infestans* consiste principalmente de lipídeos, uma vez que a análise por microscopia de fluorescência do reto incubado com *Nile red,* corante específico de lipídios, revelou uma intensa marcação desta região do intestino (Schimidt et al., 1998). Além disso, preparações de microscopia eletrônica de transmissão demonstraram que epimastigotas estavam aderidos a camada superficial do reto pelo flagelo e que essa adesão seria mediada por uma interação hidrofóbica entre o flagelo do parasita e o reto do inseto (Schimidt et al., 1998; Schaub et al., 1998).

1.5. Glicosaminoglicanos

Glicosaminoglicanos (GAGs), juntamente com seus proteoglicanos, têm importância em uma variedade de eventos relacionados a interação célula-célula e célula-matriz extracelular, migração celular e proliferação, sequestro de fatores de crescimento, ativação de citocinas e quimiocinas, morfogênese durante o desenvolvimento embrionário e invasão de patógenos (Revisto por Li et al., 2012). GAGs são cadeias lineares de polissacarídeos compostas de unidades repetidas de dissacarídeos, os quais são divididos em: (i) ácido hialurônico (HA); (ii) queratam sulfato (KS); (iii) condroitim sulfato (CS); (iv) dermatam sulfato (DS); (v) heparam sulfato (HS) e (vi) heparina, sendo identificados por meio de suas estruturas, natureza e tipo de ligação entre resíduos de açúcares, número e localização de grupamentos sulfato (Jackson et al., 1991; Turnbull et al., 1995).

Os dissacarídeos são formados por um ácido hexurônico podendo ser *D*-glucurônico (*D*-GlcA) ou L-idurônico (*L*-IdoA) e por um resíduo de açúcar aminado (hexosamina) podendo ser N-acetil-*D*-glucosamina (*D*-GlcNAc) ou N-acetil-*D*-galactosamina (*D*-GalNAc), que podem ser sulfatadas. Esta estrutura é similar nas diferentes famílias, exceto no queratam sulfato que apresenta uma galactose no lugar do ácido urônico (Gallagher et al., 1986; Nasciuttii et al., 2006). A maioria dos GAGs esta ligado a um resíduo de aminoácido serina da proteína central, proteoglicano, por um tetrassacarídeo (Xilose-galactose-galactose-ácido glucurônico), sendo a xilose inicialmente adicionada pela atividade da xilosiltransferase I no retículo endoplasmático (RE) (Cuellar et al., 2007). A adição de dois resíduos de galactose (Gal) ocorre pela atividade de galactosiltransferases I e II no domínio cis/medial do complexo de Golgi e finalmente, a adição de ácido glucurônico pela atividade de glucuronosiltransferase I na região trans do Golgi (Silbert & Sugumaran, 2002). No entanto, a estrutura característica dos GAGs só ocorre após a adição de α-GlcNAc pela α-N-acetilglucosaminiltransferase I ou β-GalNAc por β-N-acetilgalactosaminiltransferase I, identificando as cadeias das famílias de heparam sulfato e condroitim sulfato, respectivamente. O número de repetições de dissacarídeos por cadeia de GAGs pode variar enormemente, desde cerca de 15 dissacarídeos em KS, até 50-200 em HS/CS, e mais de 4000 unidades no ácido hialurônico. As cadeias de GAGs são fortemente hidrofílicas, tendendo a adotar conformações que ocupam um grande espaço em relação à massa, e a formar géis mesmo em baixas concentrações.

KS é dividido em três diferentes classes de acordo com o tipo de ligação realizada com a proteína central. Dessa forma, a classe KSI inclui todos os KS ligados a uma asparagina; KSII se refere a todos os KS ligados a proteína pelos resíduos de aminoácidos serina ou treonina e o KSIII determina a ligação do KS à proteína dos mesmos aminoácidos via uma manose (Funderburgh, 2000). O KS é composto por cadeias curtas de dissacarídeos, sendo alongadas pela ação de glucosiltransferases que alternadamente adicionam resíduos de GlcNAc e Gal (Funderburgh, 2000). Durante a síntese do KS, ambos os resíduos de açúcares (GlcNAc e Gal) podem ser *O*-sulfatados no C6 por enzimas específicas. Duas sulfotransferases foram identificadas, sendo uma enzima com capacidade de adicionar sulfato aos resíduos de GalNAc do CS e

também a Gal em KS (Habuchi et al., 1996), enquanto a outra transfere sulfato para Gal de KS, mas não age sobre CS (Fukuta, 1997).

O CS e DS são ambos derivados do mesmo polímero, sendo constituídos por GalNAc e GlcA. O DS difere do CS por seus resíduos de GlcA sofrerem uma epimerização no carbono C5 para formar o IdoA (Nasciutti et al., 2006; Taylor & Gallo, 2006). A proporção de dissacarídeos contendo IdoA em DS é variável, podendo alcançar quase 100% da cadeia de polissacarídeo (Kusche-Gullberg & Kjellén, 2003). No DS, o sítio mais comum de sulfatação é no C4 do GalNAc, pela atividade de condroitim 4-*O*-sulfotranferase 1 ,2 e 3, as quais preferem domínios ricos em GlcA, e dermatam 4-*O*-sulfotransferase, que é específica para regiões de IdoA presentes, mas grupos sulfatos podem também ser encontrados no C6 da molécula GalNAc pela ação da condroitim 6-*O*-sulfotransferase (Turnbull et al., 1995). Além disso, a sulfatação no C2 do IdoA pode ser observada, sendo promovida pela enzima CS/DS 2-*O*-sulfotransferase (Taylor & Gallo, 2006).

HS e heparina são GAGs estruturalmente semelhantes e assim, classificados dentro de uma mesma família. Heparina é sintetizada exclusivamente em mastócitos e estocada em grânulos presentes em seu citoplasma. Por outro lado, HS é abundantemente encontrado na superfície celular e matriz extracelular em uma grande variedade de células de mamíferos (Lindahl et al., 1998, Sasisekharan & Venkataraman, 2000; Salmivirta et al., 1996). HS/heparina são caracterizados por apresentar uma cadeia linear composta de 10-200 unidades de dissacarídeos constituídos por resíduos alternados de *D*-GlcNAc e ácido *D*-Glc (Dietrich et al., 1983, 1998; Sasisekharan & Venkataraman, 2000). A biossíntese do HS e heparina inicia-se com a adição de quatro monossacarídeos ao resíduo de aminoácido serina na proteína central. Posteriormente, a cadeia é estendida com adição de uma GlcNAc e GlcA por duas glucosiltransferases – exostosina 1 e 2 (EXT1 e EXT2) (revisto por Li et al., 2010). Estas cadeias são modificadas através de etapas consecutivas de N-deacetilação e N-sulfatação do açúcar GlcNAc pelas ações das enzimas N-deacetilase e N-sulfotransferases (NDST), respectivamente, substituindo assim, um grupamento N-acetil por um grupamento sulfato. O próximo passo da modificação é a epimerização, onde o carbono C5 do GlcA resulta na formação de um IdoA, realizada pela enzima C5-epimerase

(Sasisekharan & Venkataraman, 2000; Taylor & Galo, 2006). Subsequentemente, ocorre a *O*-sulfatação do C2 do IdoA e a *O*-sulfatação do C6 e C3 do GlcNAc. Diferentemente do HS, uma alta proporção de GlcNAc na heparina são N-sulfatados, além disso, os polissacarídeos são altamente *O*-sulfatados e apresentam uma alta taxa de IdoA em relação a GlcA. Resumidamente, a heparina é distinguida do HS por apresentar um maior grau de modificações (epimerização e sulfatação) (Lindahl & Kjellén, 1991; Nakato & Kimata, 2002; Taylor & Galo, 2006).

1.6. Proteoglicanos

Proteoglicanos (PGs) são glicoconjugados presentes em praticamente todas as células animais, na membrana basal de vários tecidos e na matriz extracelular (Li et al., 2012). Estes são estruturas constituídas por uma variedade de proteínas centrais que contém uma ou mais cadeias de GAGs sulfatados covalentemente ligadas. Além disso, o tipo e o padrão das repetições dos dissacarídeos de cada GAG podem ser modificados pelo grau de sulfatação, aumentando ainda mais a heterogeneidade dos PGs. Assim, podemos definir os PGs como um grupo diversificado de glicoproteínas altamente glicosiladas cujas funções são mediadas tanto pelo núcleo protéico quanto pelas cadeias de GAGs (Hardingham & Fosang, 1992).

Os PGs são subdivididos em três grupos principais, baseados nos GAGs que os compõem, podendo ser proteoglicanos de heparam sulfato (PGHS), PGs de condroitim sulfato (PGCS) e PGs de queratam sulfato (PGKS) (Ly et al., 2010). Dentre estes, PGHS são amplamente exploradas por diversos microorganismos patogênicos durante o processo de adesão e invasão como observado para bactérias (Chang et al., 2011, Sava et al., 2009), vírus (Karasneh et al., 2011 ; Schowalter et al., 2011; Choudhary et al., 2011) e protozoários (Kobayashi et al., 2010; Calvet et al., 2003; Carruthers et al., 2000).

Os principais membros da família dos PGHS são: sindecans, glipicans, betaglicans, perlecans, agrin, serglicina e colágeno tipo XVIII. Dentre estes PGs apenas sindecans, betaglicans e serglicinas podem possuir HS e CS ligados a sua estrutura (Ly et al., 2010), sendo os sindecans a principal família

de proteoglicanos transmembranares presentes na superfície celular. Existem 4 diferentes tipos de sindecan conforme classificação a seguir: (i) sindecan-1, presente em células epiteliais, (ii) sindecan-2, encontrado principalmente em fibroblastos, (iii) sindecan-3, intensamente expresso no tecido nervoso e (vi) sindecan-4, encontrado em todos os tipos celulares (Contreras et al., 2010). A estrutura dos sindecans compreende três principais domínios: extracelular, transmembranar e citoplasmático.

O domínio extracelular é responsável pela ligação das cadeias de GAGs perto da região N-terminal. Os GAGs são inseridos na proteína central em uma região apresentando os aminoácidos serina ou treonina. Os resíduos de açúcares, que compõem os GAGs, não são inseridos diretamente na proteína central sendo necessária a participação de quatro monossacarídeos. Primeiramente, uma Xilosiltransferase insere uma xilose, seguido de duas galactoses e ácido glucurônico inseridos pela atividade de galactosiltransferases I e II e glucuroniltransferase, respectivamente (Silbert & Sugumaran, 2002; Taylor & Gallo, 2006). Após a formação do tetrassacarídeo, os resíduos de carboidrato são inseridos por enzimas específicas determinando assim, o tipo de PG sintetizado. Além disso, o domínio extracelular pode promover adesão celular em um mecanismo dependente de integrina (Multhaupt et al., 2009). Entretanto, o domínio transmembranar é altamente conservado entre os sindecans e é responsável por mediar a dimerização dos PGs (Alexopoulou et al., 2007).

O domínio citoplasmático possui três regiões envolvidas com a dinâmica de proteínas do citoesqueleto e ativação de vias de sinalização. Dentre elas as regiões C1 e C2 são altamente conservadas, enquanto que a região V, entre as regiões C1 e C2, é única para cada PG (Alexopoulou et al., 2007). A região C1 é a mais próxima da membrana plasmática e é capaz de se ligar a diversas proteínas como cortactina, beta-tubulina, tirosinas quinases (c-src e fyn) e ainda, interagir com erzina no complexo de proteínas erzina-radixina-moesina (ERM) que regula citoesqueleto de actina submembranar (Alexopoulou et al., 2007; Couchman, 2010). Dessa forma, PGs tem a capacidade de se associar ao citoesqueleto (filamentos de actina e microtúbulos), além de participar dos processos de sinalização celular. A região C2 de todos os sindecans termina em um motivo EFYA, o qual é capaz de se ligar a diferentes proteínas

contendo um domínio PDZ (*Postsynaptic density 95, Disk large, Zona occludens-1*), tal como sinectina, sinbindina, sintenina e CASK/LIN (Wilcox et al., 2002; Alexopoulou et al., 2007). Estas proteínas ao se ligarem ao domínio C2 irão desempenhar uma série de funções distintas. Dentre elas Sintenina parece estar envolvida com a reciclagem dos sindecans tal como ocorre com fator de crescimento de fibroblastos-2 (FGF-2) (Alexoupoulo et al. 2007). Sinectina juntamente com sindecan-4 parece ativar Rac promovendo dessa forma a migração celular (Lambaerts et al., 2009).

Dessa forma, proteoglicanos são proteínas complexas que possuem alta capacidade de ligação à componentes da região extracelular e citoplasmática, e assim mediar uma variedade de eventos celulares distintos.

2. OBJETIVOS

2.1. Objetivo geral

Caracterização parcial das proteínas com propriedade de ligação à heparina (PLHs) em *Trypanosoma cruzi* e análise de seu papel no reconhecimento de glicosaminoglicanos (GAGs) sulfatados da superfície da célula do hospedeiro mamífero e inseto vetor.

2.2. Objetivo específico

I. Isolar e identificar PLHs em formas epimastigotas de *T. cruzi* (Artigo 1);
II. Investigar o papel de PLHs de epimastigotas no reconhecimento de células do trato intestinal do *Rhodnius prolixus* (Artigo 1);
III. Analisar a participação de GAGs sulfatados no reconhecimento e invasão de formas amastigotas de *T. cruzi* em células de mamíferos (Artigo 2);
IV. Determinar a expressão e localização de PLHs em formas tripomastigotas de *T. cruzi* (Artigo 3);
V. Investigar a atividade proteolítica das PLHs de tripomastigotas de *T. cruzi* (Artigo 3);
VI. Avaliar a afinidade e estabilidade da ligação de PLHs de tripomastigotas e epimastigotas de *T. cruzi* com heparina (Artigo 1 e 3).

3. MÉTODOS e RESULTADOS

3.1. Artigo 1:

Oliveira FO, Alves CR, Souza-Silva F, Calvet CM, Côrtes LM, Gonzalez MS, Toma L, Bouças RI, Nader HB, Pereira MC 2012. *Trypanosoma cruzi* heparin-binding proteins mediate the adherence of epimastigotes to the midgut epithelial cells of *Rhodnius prolixus*. Parasitology 139:735-743.

Até o presente momento, as proteínas que ligam a heparina (PLHs) foram demonstradas apenas em formas tripomastigotas e amastigotas do *Trypanosoma cruzi* e fora descrito o envolvimento destas proteínas no reconhecimento e invasão de tripomastigotas em células de mamíferos (Calvet et al., 2003; Oliveira-Jr et al., 2008). Com o desenho experimental proposto neste estudo, descrevemos pela primeira vez que estas proteínas também estão presentes nas formas epimastigotas deste parasito, assim como determinamos seu potencial biológico na interação entre parasito e vetor.

Apresentamos dados de que PLHs majoritárias de epimastigotas possuem massas moleculares de 65,8 kDa e 59 kDa. Estas proteínas foram isoladas de fração hidrofóbica de epimatigotas submetidas à cromatografia de afinidade em coluna de heparina-agarose e identificadas por glicosaminoglicanos (GAGs) sulfatados conjugados a biotina. A presença das PLHs na superfície de epimastigotas foi confirmada por ensaios de biossensoriamento de superfície (*surface plasmon resonance* - SPR). Os ensaios de SPR também foram potentes em demonstrar que a ligação entre PLHs na superfície de epimastigotas e GAGs são específicas e estáveis. No decorrer do estudo, realizamos ensaios de competição com GAGs solúveis para avaliar a contribuição da interação PLHs-GAGs no reconhecimento e adesão de epimastigotas com células epiteliais do trato intestinal de *Rhodnius prolixus*. Nestes ensaios, observamos que células epiteliais pré-incubadas com PLHs produziram uma inibição de 3,8 vezes na adesão de epimastigotas ao trato intestinal do inseto vetor. O pré-tratamento de epimastigotas com heparina, heparam sulfato e condroitim sulfato inibiu significativamente a adesão do parasita a células epiteliais, sendo este evento confirmado por microscopia eletrônica de varredura.

Apresentamos evidências de que as PLHs de superfície de formas epimastigotas de *T. cruzi* têm um papel chave no reconhecimento de GAGs sulfatados presentes na superfície das células do epitélio do intestino médio do inseto vetor.

3.2 Artigo 2:

Artigo 2: Bambino-Medeiros R, Oliveira FO, Calvet CM, Vicente D, Toma L, Krieger MA, Meirelles MN, Pereira MC 2011. Involvement of host cell heparan sulfate proteoglycan in *Trypanosoma cruzi* amastigote attachment and invasion. Parasitology 138(5): 593-601.

Miócitos cardíacos, células alvo de infecção pelo *T. cruzi*, foram utilizados como modelo experimental para aprofundar o entendimento do papel de PLHs de *T. cruzi* no ciclo intracelular deste parasito. Considerando-se que amastigotas são capazes de invadir células do hospedeiro vertebrado (Ley et al., 1988) e apresentam importante papel na manutenção do foco inflamatório no tecido cardíaco (Scharfestein & Morrot 1999), nos propusemos a avaliar a participação de proteoglicanos sulfatados no processo de reconhecimento e interiorização de amastigotas em cardiomiócitos.

O desenho experimental proposto neste estudo inclui ensaios de competição com GAGs solúveis, os quais revelaram que o pré-tratamento de amastigotas de *T. cruzi* com heparina e heparam sulfato é capaz de acarretar uma redução na infecção, alcançando 82% e 65% de inibição da invasão, respectivamente. Outros GAGs sulfatados, tais como condroitim sulfato, dermatam sulfato e queratam sulfato, não apresentaram efeito no processo de invasão de amastigotas. A participação de GAGs na adesão e entrada de amastigotas em células hospedeiras foi confirmada pela utilização de células de ovário de hamster chinês (*chinese hamster ov*ary cells - CHO) deficientes em GAGs, atingindo uma redução da infecção de 67% após 4h de interação com amastigotas.

O conjunto destes resultados fornece subsídios que suportam o envolvimento de proteoglicanos de heparam sulfato na invasão de amastigotas em células de mamíferos.

3.3. Artigo 3:

Oliveira-JR FOR, Alves CR, Souza-SilvA F, Côrtes LMC, Toma L, Bouças RI, Aguilar T, Nader H. B., Pereira MCS 2012. *Trypanosoma cruzi* heparin-binding proteins: Subcellular localization and enzymatic property (artigo submetido)

As PLHs desempenham um papel chave na interação de tripomastigotas de *Trypanosoma cruzi* com células do hospedeiro. Estas proteínas reconhecem heparam sulfato (HS) na superfície da célula alvo e são capazes de induzir citoaderência e invasão deste parasito (Oliveira-Jr et al., 2008; Oliveira-Jr et al., 2011). Nesta etapa do nosso estudo, analisamos as propriedades bioquímicas, expressão e localização subcelular das PLHs em tripomastigotas de *T. cruzi*.

Os ensaios de citometria de fluxo revelaram que PLHs são altamente expressas na superfície de tripomastigotas e apresentam uma localização peculiar na membrana flagelar. A especificidade e estabilidade da ligação PLHs-GAGs sulfatados foi determinada por ensaios de ressonância plasmônica de superfície, sendo evidenciado maior estabilidade nas interações entre PLHs e GAGs constituídos de N-acetilglicosamina e ácido urônico (Heparina e HS). Outro dado relevante deste estudo refere-se à descrição da atividade de proteinase de PLHs sobre substrato complexo (colágeno tipo I). A atividade gelatinolítica, evidenciada por ensaios de zimografia, revelou proteinases, com massas moleculares de 70 kDa, 65,8 kDa e 59,0 kDa, em ampla faixa de pH (5,5 - 8,0). Estas atividades proteolíticas foram sensíveis a incubação com inibidores de serino proteinase, tais como aprotinina e fluoreto de phenylmethanesulfonyl, sugerindo que estas PLHs têm propriedade enzimática similar a tripsina.

Em conjunto, os dados deste trabalho indicam que PLHs de tripomastigotas, serino proteinases, localizam-se em um importante domínio de sinalização celular, e sugere que, além de favorecer a ligação do parasito a célula hospedeira, HS induz a invasão do parasito. Este achado é concordante com a ação de serino proteinases envolvidas no processo de invasão do *T. cruzi (Burleigh et al., 1997;* Grellier et al., 2001*)*.

Subcellular localization and enzymatic properties of *Trypanosoma cruzi* heparin-binding proteins

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ABSTRACT

Heparin-binding proteins (HBPs) play a key role in *Trypanosoma cruzi*-host cell interactions. HBPs recognize heparan sulfate (HS) at the host cell surface and are able to induce the cytoadherence and invasion of this parasite. Herein, we analyzed the biochemical properties of the HBPs and also evaluated the expression and subcellular localization of HBPs in *T. cruzi* trypomastigotes. A flow cytometry analysis revealed that HBPs are highly expressed at the surface of trypomastigotes, and their peculiar localization mainly at the flagellar membrane, which is known as an important signaling domain, may enhance their binding to HS and elicit the parasite invasion. The plasmon surface resonance results demonstrated the stability of HBPs and their affinity to HS and heparin. Additionally, gelatinolytic activities of 70 kDa, 65.8 kDa and 59 kDa HBPs over a broad pH range (5.5-8.0) were revealed using a zymography assay. These proteolytic activities were sensitive to serine proteinase inhibitors, such as aprotinin and phenylmethylsulfonyl fluoride, suggesting that HBPs have the properties of trypsin-like proteinases.

Keywords. *Trypanosoma cruzi*, heparin-binding protein, Plasmon surface resonance, serine proteinase

1. INTRODUCTION

Chronic chagasic cardiomyopathy, which is caused by *Trypanosoma cruzi*, is an important clinical manifestation of Chagas' disease and causes morbidity and mortality in Latin America (Tanowitz et al., 2009). In endemic areas, *T. cruzi* is transmitted to humans primarily via triatomine feces during blood meals, but food-borne transmission has also emerged as an important mechanism of *T. cruzi* infection (Kribs-Zaleta 2010; Toso et al., 2011; Yoshida et al., 2011). For a successful infection in mammalian hosts, infective forms of *T. cruzi* must recognize molecules on the surfaces of the host cells that trigger the invasion process of the parasite (reviewed by Caradonna and Burleigh, 2011). Many glycoproteins on the surface of the parasite have been demonstrated to participate in the recognition and invasion process (reviewed by De Souza et al., 2010). However, the expression of these molecules depends on the strain and developmental stage of *T. cruzi*. For example, variations in the expression of glycoproteins such as gp82, gp90 and gp35/50 in metacyclic trypomastigotes can define the invasiveness of the parasite in the host cell (Ruiz et al., 1998). The binding of these molecules to their receptors triggers signaling cascades involved in Ca^{2+} mobilization from different cellular compartments (Yoshida and Cortes, 2008), which leads to a mechanism of invasion dependent on or independent of the actin cytoskeleton (Ferreira et al., 2006). Members of the trans-sialidase (TS) superfamily also play a key role in the attachment and invasion of the parasite (Eugenia Giorgi and de Lederkremer 2011). Gp85/trans-sialidase, for instance, interacts with multiple ligands at the cell surface of the host. The highly conserved peptide sequence of this protein (FLY peptide) mediates binding to cytokeratin 18 on the surface of epithelial cells (Magdesian et al., 2007) and may also selectively promote parasite tissue tropism (Tonelli et al., 2010). Additionally, *T. cruzi* peptidases, such as members of the propyl oligopeptidase family of serine proteinase (oligopeptidase B and Tc-80), are also involved in the invasion of host cells (reviewed by Cazzulo, 2002). It has been shown that *T. cruzi* oligopeptidase B is engaged in the generation of the Ca^{2+} signaling agonist required for the lysosome-dependent mechanism of invasion in mammalian cells (Burleigh et al., 1997; Caler et al., 1998), whereas POP Tc-80 may degrade extracellular matrix (ECM) components and activate molecules on the parasite and/or host

cell ECM that are essential for *T. cruzi* invasion (Grellier et al., 2001). The invasion process also involves proteins that are able to bind to heparin (Ortega-Barria and Pereira 1991; Calvet et al., 2003; Oliveira-Jr et al., 2008), but little is known about these proteins identified in *T. cruzi*.

Heparin-binding protein (HBP) was first reported in trypomastigote forms of *T. cruzi* by Ortega-Barria and Pereira (1991). This 60 kDa protein named penetrin has the ability to bind to heparin and ECM components (heparan sulfate and collagen), promoting parasite entry into mammalian cells (Ortega-Barria and Pereira 1992; Calvet et al., 2003) in a mechanism distinct from the TS-sialic acid route (Herrera et al., 1994). After purification using Triton X-114 and heparin affinity chromatography, we have identified two protein bands (59 kDa and 65.8 kDa) in the trypomastigote and amastigote forms of *T. cruzi* that have the ability to bind sulfated GAGs (Oliveira-Jr et al., 2008). Additionally, we have demonstrated the involvement of the heparan sulfate proteoglycans (HSPG) in the attachment to and invasion of cardiomyocytes by trypomastigotes (Calvet et al., 2003; Oliveira-Jr et al., 2008) and amastigotes (Bambino-Medeiros et al., 2011). Our previous data also suggested that the N-acetylated/N-sulfated domain of the HS chain is involved in the selective binding of HS to *T. cruzi* ligands to trigger parasite entry, whereas chondroitin sulfate (CS) had no effect on the invasion of mammalian cells (Oliveira-Jr et al., 2008). Recently, we have also demonstrated the presence of HBPs in epimastigotes, suggesting that these HBPs play a role in vector-*T. cruzi* interaction (Oliveira-Jr et al., 2012).

Therefore, because HBPs play a key role in the host cell-parasite recognition process and are observed in different stages of the life cycle of *T. cruzi*, a better characterization of these proteins is essential for a more complete knowledge of the physiological function of HBPs and the potential of these proteins as drug target for the treatment of Chagas' disease. In the present study, we focused our attention on the spatial distribution and enzyme properties of HBPs. We provide evidence that the HBPs are located primarily at the flagellar membrane and bind to sulfated GAGs such as heparin and HS. Additionally, we provide evidence that the HBPs have characteristics of serine proteinases.

2. MATERIALS AND METHODS

2.1. Reagents

The detergents [Triton X-100 (TX-100), Triton X-114 (TX-114) and sodium dodecyl sulfate (SDS)], proteinase inhibitors [transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), aprotinin (Apo), 1,10-phenanthroline (o-phe), pepstatin A (Pep A) and phenylmethylsulfonyl fluoride (PMSF)], reducing reagents [dithiothreitol (DTT) and β-mercaptoethanol (β-ME)], gelatin, bovine serum albumin (BSA), penicillin and the aprotinin-agarose column (Sigma-Aldrich; 1.5 × 2.5 cm) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The heparin-sepharose column (HiTrap-Heparin; 1.5 × 2.5 cm) was purchased from GE Healthcare (Piscataway, NJ, USA). Fetal bovine serum (FBS) was purchased from Cultilab S/A (Brazil). Heparin (Hep) from bovine lungs was purchased from INORP Laboratories (Buenos Aires, Argentina). Heparan sulfate (HS) from the bovine pancreas was a kind gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). All biotinylated GAGs (Hep and HS) were prepared as previously described (Bouças et al., 2008).

2.2. Parasites

Vero cells were cultivated in Dulbecco's modified Eagle medium (DMEM; Sigma) and maintained at 37 °C in an atmosphere of 5% CO_2. The cells grown in 150 cm^2 tissue culture flasks were infected for 24 h with 10^7 *T. cruzi* trypomastigotes, clone Dm28c. Free trypomastigotes were harvested from the supernatant of the *T. cruzi*-infected cultures after 4 days of infection. The parasites were used in the biochemical and fluorescence assays.

2.3. Triton X-114 extraction and chromatographic procedures

The trypomastigotes were washed three times with phosphate-buffered saline (PBS), pH 7.2, and the detergent-soluble proteins were collected using the TX-114 phase separation technique. Briefly, $5x10^{10}$ trypomastigotes were extracted for 40 min on ice with 2% TX-114 in TBS (150 mM NaCl, 10 mM Tris, pH 7.4), and the soluble proteins were obtained after condensation at 37 °C and then centrifugation at 12,000×*g* for 15 min.

The hydrophobic phase was washed three times with PBS and subjected to a sequential affinity chromatography procedure on two columns. First, the hydrophobic proteins were applied to a heparin affinity column, as previously described (Oliveira-Jr et al., 2008). After washing, the retained proteins were eluted with 0.5 M NaCl in PBS. The proteins were dialyzed in a second equilibrium buffer (10 mM Tris-HCl, pH 7.5), concentrated using a Centriprep YM-10 and passed through an aprotinin-agarose column that was previously equilibrated with the second equilibrium buffer. The column was washed with the second equilibrium buffer, and the retained proteins were eluted using the same buffer with an increased concentration of NaCl (1.5 M).

All of the chromatography washes and elution steps were accompanied by spectrophotometric measurements at 280 nm (Ultrospec 1100 pro; Amersham Biosciences, UK) and were performed in a refrigerated room. The eluted proteins were concentrated by ultra-filtration in Centriprep 10 filters (Millipore Corporation, Bedford, USA), and the protein concentration was determined colorimetrically using a BCA-Protein assay kit (PIERCE) with BSA as a standard.

2.4. Electrophoresis assay

SDS-PAGE was performed at room temperature using 12% polyacrylamide gels in Laemmli´s buffer (Laemmli, 1970). The samples (10 µg) were dissolved in SDS-PAGE sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 12% glycerol and 0.015% bromophenol blue) that was supplemented with 5% β-mercaptoethanol and then boiled for 5 min. After electrophoresis, the protein bands were identified using silver staining (Gonçalves et al., 1990).

2.5. Zymographic assays with gelatin

The proteinase activity was determined using SDS-PAGE with gelatin copolymerized in the gel (substrate-SDS-PAGE), as previously described (Heussen and Dowdle, 1980; Alves et al., 1993). Briefly, the soluble proteins were subjected to electrophoresis under reducing conditions (in sample buffer) using 12% acrylamide gels that were copolymerized with 0.1% gelatin. Following electrophoresis, the gel was washed (1 h, 25 ℃) with 2.5% Triton X-100 and then incubated (16 h, 37 ℃) with activation buffers: 10 mM sodium

acetate, pH 3.5, for aspartic proteinases; 10 mM Tris–HCl, pH 5.5, containing 1.0 mM of DTT for cysteine proteinases; 10 mM Tris–HCl, pH 7.5, for serine proteinases; and 10 mM Tris–HCl, pH 8.0, for metalloproteinases. The inhibition assays were performed by adding the inhibitor of each proteinase into the activation buffer. The hydrolysis of the gelatin was detected by staining the gels with 0.1% (w/v) amide black, which was prepared in a methanol:acetic acid:water (3:1:6, v/v/v) solution.

2.6. Fluorescence microscopy and flow cytometry assays

The culture-derived trypomastigotes ($3x10^6$ cells) were incubated for 1 h on ice with 20 µg/mL sulfated glycosaminoglycans (GAGs) conjugated with biotin, including heparin (Hep) and heparan sulfate (HS) in DMEM supplemented with 0.5% BSA; Sigma). The incubation step was followed by fixation with 4% paraformaldehyde (PFA) in PBS, pH 7.2. After washing with PBS, the parasites were incubated for 1 h at room temperature with FITC-conjugated streptavidin (1:200) in PBS. The DNA was detected with 10 µg/ml 4,6-diamidino-2-phenylindole dye (DAPI; Sigma), and the cover slips were mounted with 2.5% 1,4-diazabicyclo-(2,2,2)-octane (DABCO; Sigma) in PBS, pH 7.2, containing 50% glycerol. The controls were prepared by omitting the biotinylated GAGs. Images of the samples were acquired using a Zeiss Axio Imager M1 epifluorescence microscope (Zeiss) equipped with an AxioCam HRm (AxioVision Digital Image Processing Software). Additionally, the samples were excited at 488 nm and quantified using a flow cytometer (FACSCalibur, BD Bioscience, USA) equipped with a 15 mW argon laser emitting at 488 nm. Each experimental population was then mapped using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population (n = 10,000) was analyzed for the log of green fluorescence using a single parameter histogram.

2.7. Binding assays using surface plasmon resonance (SPR)

The SPR assays were performed using a sensor chip with a carboxyl surface coated with neutravidin (Biocap; Nomadics, USA), as previously described (Oliveira et al., 2012; Côrtes et al., 2012). Briefly, the chip surface was covered with biotinylated heparin (0.5 µg) and used in the interaction with BSA (0.1 -

0.001 µg) or whole trypomastigotes (3×10^6 cells). To perform the inhibition assays, the trypomastigotes were pre-incubated for 1 h on ice with different concentrations of sulfated GAGs (0.1 µg - 0.001 µg). Prior to the interaction with the sensor chip surface, the trypomastigotes were fixed for 1 h at 4 °C with 1% PFA and washed three times by centrifugation (800×*g*, 10 min, 4 °C) in PBS. The SPR assays were performed at 25 °C with 100 µL of material injected at a flow rate of 10 µL/min. The binding assays were performed in PBS and registered in real time using a sensorgram. The resonance signals of the samples were analyzed after subtraction of the resonance unit (RU) values from the reference channel to avoid methodological artifacts. The SPR experiments were conducted in an optical biosensor SensiQ Pioneer instrument (Icx Nomadics, USA), and the data were analyzed using Qdat software (Icx Nomadics, USA). The dissociation RU values presented here are representative of the average response between 1,250 and 1,550 seconds in all assays.

2.8. Statistical analysis

To compare the results, Student's test was applied, assuming an equal variance between samples. The data matrices were considered statistically distinct when the p-value was lower than 0.05.

3. Results

Following the previous work in which we identified two proteins with heparin-binding properties in *T. cruzi* and discussed the ability of these proteins to recognize sulfated glycosaminoglycans (Oliveira-Jr et al., 2008), we now extend the study to investigate the expression, spatial distribution and other biochemical features of these proteins in this parasite. Fluorescence assays were performed to evaluate the expression and subcellular localization of these proteins in trypomastigotes. The availability of the HBPs at the surface of cultured-derived trypomastigotes was investigated by pre-incubation of living parasites with 20 µg/mL sulfated glycosaminoglycans (GAGs) conjugated with biotin, including heparin and heparan sulfate (HS), on ice prior to the fixation step, and this incubation was followed by detection using streptavidin-FITC. A flow cytometric analysis revealed a homogeneous profile in the expression of the HBPs on the surface of trypomastigotes that recognize heparin and HS (Fig.

1). The histogram of the fluorescence intensity indicates that 78% and 62% of the parasite population expresses proteins that are able to bind heparin and heparan sulfate, respectively. A small fraction (~5%) of this population displays high levels of GAGs that bind at the surface of the parasite.

Subsequently, the binding specificity of HBPs on the surface of trypomastigotes to GAGs was determined using an SPR analyses with a Biocap sensor chip. Heparin and HS were used in this competition assay. Thus, trypomastigotes were treated with different concentrations of GAGs and then passed through a biotinylated heparin-coated sensor chip to determine the parasite attachment onto the chip surface. In the SPR assay, in which parasites were not submitted to GAG treatment, the dissociation value was 83.3 ± 2.0 RU. Pre-incubation of trypomastigotes with sulfated GAGs led to an inhibition of parasite binding to immobilized heparin, and lower dissociation RU values obtained compared with those of the control assay. The significant residual binding RU was dose-independent and reached 15.7 ± 2.2 ($p = 0.00058$) for HS and 19.5 ± 2.5 ($p = 0.0013$) for heparin, corresponding to an inhibition of 81.1% and 76.6%, respectively (Fig. 2). Additionally, control assays using three different concentrations of BSA were processed in parallel using the same SPR assays, as previously described (Oliveira et al., 2012). The dissociation values were consistent with the lack of relevant binding between BSA and heparin (data not shown), demonstrating the specificity of the binding assays performed with trypomastigotes.

Because the HBPs of trypomastigotes play a key role in the recognition and invasion process in mammalian cells (Ortega-Barria and Pereira 1991, Calvet et al., 2003), we wondered whether these proteins localize to a particular membrane domain in trypomastigotes. Therefore, HBP-biotinylated-GAG binding at the parasite surface was investigated using streptavidin-FITC, and the samples were analyzed using fluorescence microscopy. Interestingly, the fluorescence images revealed an intense labeling mainly localized in the flagellar membrane after pretreatment of the parasites with heparin or HS, whereas no signal could be detected when the GAG treatment was omitted (Fig. 3).

Because many proteins that are involved in the invasion of *T. cruzi* exhibit enzymatic properties, we addressed the question of whether the HBPs have

proteinase activity. The strategy used in the present work allowed for the elution of protein fractions with hydrophobic properties that had previously been concentrated in the detergent Triton X-114. The first affinity chromatography step using a heparin-sepharose column yielded approximately 0.26 ± 0.04 mg of HBPs, corresponding to 10% of the total hydrophobic protein applied. As a result of this chromatography procedure, we observed a protein peak ranging from 250 kDa to 30 kDa, consistent with the protein band with molecular mass of 65.8 kDa that was revealed by SDS-PAGE after silver staining (Fig. 4).

The activities of the HBPs were determined using a proteolysis assay with gelatin in the gel matrix. A zymography analysis of the HBPs showed a gelatinolytic activity of 70 kDa, 65.8 kDa, 59 kDa and 30.0 kDa over a pH range from 5.5 to 8.0 (Fig. 5), but no activity was detected at pH 3.0 (data not shown). The activity of these bands was sensitive to inhibition by aprotinin at pH 7.5, and no inhibition was evident in the gels incubated with other proteinase inhibitors, suggesting a serine proteinase-like activity (Fig. 5).

In light of the fact that a predominant serine proteinase activity was observed in the zymography assays, a second affinity chromatography step was performed using an aprotinin-agarose column. This chromatography step yielded approximately 0.03 ± 0.001 mg of protein, corresponding to 12% of the HBP applied to the column. In general, a major band of 65.8 kDa was identified by SDS-PAGE after silver staining, regardless of the three protein bands of 70 kDa, 65.8 kDa and 59 kDa that have always been detected using the zymography assay (Fig. 6). All of the gelatinolytic bands were inhibited by Apo and PMSF inhibitor, but they were not inhibited by other proteinase inhibitors such as Pep A, E-64 and o-phe (Fig. 6).

4. Discussion

The establishment and persistence of intracellular pathogens in mammalian hosts relies on the recognition and invasion of the target cells. Multiple molecules at the surface of the parasites have been described to mediate cytoadhesion (reviewed by De Souza et al., 2010; Sahra et al., 2011). Heparin-binding proteins have been identified as potential parasite ligands implicated in the recognition of the host cell surface glycosaminoglycans (Tossavainen et al., 2006; Bosetto and Giorgio, 2007; Wu and Wang 2012). In *Trypanosoma cruzi*,

HBPs modulate the adhesion to both insect and mammalian cells and constitute an important protein in the parasite life cycle (Calvet et al., 2003; Bambino-Medeiros et al., 2011; Oliveira-Jr et al., 2012). However, the HBPs-GAG interaction is not completely understood. In this article, we have described the expression, subcellular localization and proteolytic activity of trypomastigote HBPs.

We assessed the expression of the HBPs on the surface of trypomastigotes by pre-incubating the parasites with soluble biotinylated-GAGs, which was followed by streptavidin-FITC labeling and a flow cytometry analysis. Our data demonstrated a high level of HPBs at the surface of trypomastigotes with the ability to bind heparin and HS, suggesting that these proteins may contribute to parasite invasiveness. The balance of surface protein expression implicated in *T. cruzi* invasion appears to be essential to induce signaling pathways that trigger parasite entry. A heterogeneous expression of cruzipain, a surface protein involved in parasite invasion, was observed between *T. cruzi* populations (TCI and TCII), suggesting that the level of cruzipain expression may interfere with the parasite virulence (Fampa et al., 2010). Additionally, the balance between cruzipain and chagasin appears to influence the ability of the parasite to invade human smooth muscle cells (Scharfstein and Lima, 2008). Similarly, the differential expression of glycoproteins involved in Ca^{2+} signaling, such as gp82, gp35/50 and gp90, appears to be directly responsible for the ability of *T. cruzi* strains to invade host cells (Ruiz et al., 1998). These proteins are involved in the Ca^{2+} signal that triggers a lysosome-dependent mechanism of invasion (reviewed by Yoshida 2006; Yoshida and Cortez, 2008). Although the role of HBPs in the parasite invasion of mammalian cells is well known (Calvet et al., 2003; Oliveira-Jr et al., 2008; Bambino-Medeiros et al., 2011), the signaling pathway triggered by this receptor-ligand recognition is still unclear.

One striking feature is the peculiar localization of HBPs mainly at the flagellar membrane. Interestingly, this membrane domain is enriched with lipid raft microdomains that are involved in protein sorting and signaling (Tyler at al., 2009). Additionally, another remarkable fact is the similar pattern of localization of HBPs and Ca^{2+}-binding protein, which was also demonstrated to be concentrated in the flagellar membrane of *T. cruzi* (Maric et al., 2011). Because the Ca^{2+}-binding protein may interact with scaffold proteins involved in signaling

pathways (Chakravart et al., 2012), it is possible that the localization of HBPs in this dynamic signaling site may underlie distinct signal outputs that coordinate the mechanisms engaged in the parasite entrance. This field is an interesting area of investigation and will be the focus of future research. Although most of the studies to date have demonstrated that surface proteins involved in the parasite invasion, including trans-sialidase, gp82, gp35/50 and gp90, are distributed throughout the parasite body (Cordero et al., 2008; Penã et al., 2009; Buschiazzo et al., 2012), this specific subcellular localization of HBPs may facilitate the interaction of the parasite ligand to its host cell surface receptor and other key cellular components to promote parasite invasion.

The analysis of HBP-heparin binding using SPR demonstrated that the *T. cruzi* HBPs strongly bind to GAG, thus reinforcing the putative function of these trypomastigote proteins in the life cycle of this parasite. The physicochemical assay effectively confirmed the strength and specificity of the binding of trypomastigote HBPs to heparin immobilized onto the sensor chip surface, as has also been demonstrated for the HBPs of epimastigotes (Oliveira et al., 2012). The biosensing surface procedures have been used to elucidate the adhesion between intracellular pathogens and host cells, with a focus on the parasite surface proteins and their interaction with glycosaminoglycans. In this context, the interaction induced by heparin has been assessed in the *Plasmodium falciparun* circumsporozoite protein during the invasion of liver cells (Rathore et al., 2001) and in the interaction between the measles virus in SLAM-negative cell lines (Terao-Muto et al., 2008). In addition, the localization of HBPs on the surface of *Leishmania (Viannia) braziliensis* promastigotes was identified using a biosensing assay in both flagellar and membrane protein fractions. However, the HBPs from the flagellar membrane have a higher affinity to bind to heparin (Cortês et al., 2012), supporting the involvement of the parasite flagellum in the adhesion to the host cells (Bates and Rogers, 2008; Bates, 2008).

In terms of the functionality of the HBPs, our data show that trypomastigote HBPs exhibit serine proteinase activity related with the 70 kDa, 65.8 kDa and 59 kDa protein bands that were responsible for the hydrolysis profile observed in the zymography assays. Serine proteinases are enzymes that catalyze the cleavage of peptide bonds and utilize a serine residue as the nucleophilic amino

acid in the active site (Hedstrom, 2002). Serine proteases are classified into two fundamental families: chymotrypsin-like (trypsin-like) and subtilisin-like (Madala et al., 2010). These enzymes have a broad spectrum of action including digestion, immune response, blood coagulation and reproduction (Hedstrom, 2002; Antalis et al., 2011). Because the proteinase activity of HPBs was sensitive to incubation with inhibitors such as aprotinin and PMSF, it is possible that these HBPs possess the properties of trypsin-like proteinases. Both inhibitors are described as appropriate for the identification of trypsin-like proteinases (James, 1978; Madala et al., 2010).

In *T. cruzi*, the most studied proteinases belong to the cysteine proteinase class (Rangel et al., 1981; Ashall, 1990; McKerrow et al., 2006), whereas metalloproteinases (Lowndes et al., 1996; Cuevas e al., 2003; Nogueria-Melo et al., 2010) and aspartyl proteinases (Pinho et al., 2009) are poorly studied. The serine proteinase is the second most studied proteinase in *T. cruzi*. The first report of this enzyme class in *T. cruzi* described a 200 kDa serine proteinase with esterase and transamidase activities (Bongertz and Hungerer, 1978). Only twenty years after its description, a cytosolic serine endopeptidase of 80 kDa was identified as a requirement for the generation of Ca^{2+} signaling in mammalian cells (Burleigh and Andrews, 1998). Subsequently, the extended substrate binding site of the recombinant 80 kDa oligopeptidase B enzymes (OPBTc) was characterized in *T. cruzi* and *Trypanosoma brucei*, and the specificity of their S3, S2, S1', S2' and S3' subsites was evaluated (Hemerly et al., 2003). Recently, it was proposed that the OPBTc enzyme has a dimeric structure and is fully active at temperatures up to 42 °C. OPBTc has a highly stable secondary structure over a broad range of pH values; it undergoes tertiary structural changes at low pH and is less stable under moderate ionic strength conditions (Motta et al., 2012). Some molecular, functional and structural properties of the 80 kDa prolyl oligopeptidase (Tc80) were proposed as requirements for parasite entry into mammalian cells (Bastos et al., 2005). Additionally, a 75 kDa protein was identified as an excretory product in epimastigotes and may be involved in metacyclogenesis (Silva-Lopez et al., 2008). In fact, serine proteinases have been described in many species of parasites, and these proteins are related to interesting biological aspects of the host-parasite interaction, including the invasion properties and degradation of

the extracellular matrix (McKerrow et al., 2006; Ghosh and Jacobs-Lorena 2011 Meyer-Hoffert and Schröder 2011). The hydrolysis of large substrates, such as fibronectin and native collagen, was also proposed for the 80 kDa *T. brucei* serine proteinase (Bastos et al., 2010), allowing the parasite to migrate through tissue barriers. These enzymes, which have been extensively studied in *Leishmania* spp., another protozoan parasite of the family Trypanosomatidae, have a broad molecular mass range of 115 kDa to 45 kDa (Silva-Lopez et al., 2004; Morgado-Diaz et al., 2005; Silva-Lopez et al., 2005; Guedes et al., 2007; Silva-Lopez et al., 2010) and may play a key role in the parasite life cycle.

Herein, we propose that *T. cruzi* trypomastigotes express 70 kDa, 65.8 kDa and 59.0 kDa serine proteinases on their surfaces, and these enzymes have the ability to bind to GAGs such as heparin and heparan sulfate. The HBPs may act as protagonists of the proteolytic activities triggered by the HBP-GAG interaction, which may be decisive for the penetration of the parasite. The signal transduction pathway involved in this mechanism of the *T. cruzi*-mammalian cell invasion will be the focus of a future investigation.

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Legends

Figure 1. Flow cytometric analysis showing the of expression of heparin-binding proteins in the culture-derived trypomastigotes of *T. cruzi* clone Dm28c. Live parasites were incubated with 20 µg/mL biotinylated-heparin and biotinylated-heparan sulfate on ice prior to fixation, and this incubation was followed by detection with streptavidin-FITC. The negative controls were prepared in the absence of sulfated glycosaminoglycans (GAGs). The results are expressed as the mean ± SD (n = 3).

Figure 2. Analysis of the interaction between trypomastigotes and heparin using surface plasmon resonance. These assays were performed with 10^6 parasites in a final volume of 100 µL and at a flow rate of 10 µL/s. The biocap sensor chips were covered with biotinylated heparin, and the parasites were passed over their surface. The inhibition assays were performed following the incubation of the parasites with glycosaminoglycans (GAGs), such as heparin (A) and heparan sulfate (B). The parasites were assayed either without pre-incubation or after pre-incubation with 0.001 µg/mL, 0.01 µg/mL or 0.1 µg/mL GAGs. The interaction assays were performed in PBS, and a significant inhibition was achieved: (*), $p < 0.05$. The resonance signals are represented by sensorgrams, which were analyzed after subtraction of a reference line using the Qdat software. The data are presented in arbitrary resonance units (RU) and are representative of four independent experiments.

Figure 3. Spatial distribution of heparin-binding proteins in *T. cruzi* trypomastigotes. (A-C) The negative controls were prepared in the absence of biotinylated-GAGs. The HBPs were detected by incubating living trypomastigotes with 20 µg/mL biotinylated-GAGs, such as heparin (D) and heparan sulfate (G), on ice followed by streptavidin-FITC and DAPI staining (B, E and H). Note the localization of the HPBs predominantly at the flagellar membrane of the trypomastigotes. (C, F and I) Differential interference contrast image (DIC). Bar = 10 µm.

Figure 4. Denaturant electrophoresis assays of trypomastigote proteins. The hydrophobic protein samples (10 µg) were collected prior to (A) or after separation on heparin-sepharose (B) and aprotinin-agarose (C) columns, which was a second step after heparin chromatography. The proteins were subsequently submitted to SDS-PAGE and then visualized using silver staining. These results are representative of four independent experiments. The molecular mass markers are indicated (kDa).

Figure 5. Proteinase activity in the trypomastigote proteins eluted from a heparin-affinity chromatography column. The proteinase activity was determined by hydrolysis of gelatin that was copolymerized with polyacrylamide. After electrophoresis of the samples eluted from a heparin-sepharose column, the gels were incubated with different buffers (pH 5.5, pH 7.5 and pH 8.0) in the absence (-) or presence (+) of specific inhibitors for different classes of proteinases: E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. These results are representative of four independent experiments. The molecular mass markers are indicated (kDa).

Figure 6. Proteinase activity in the trypomastigote proteins eluted from an aprotinin-affinity chromatography column. The proteins eluted from the heparin-sepharose column were submitted a second chromatography step on an aprotinin-agarose column, and the proteinase activity was measured by hydrolysis of gelatin that was copolymerized with polyacrylamide. After electrophoresis, the gels were incubated with 10 mM Tris-HCl, pH 7.5, in the absence (-) or presence (+) of specific inhibitors for different classes of proteinases: Pep A, E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. These results are representative of four independent experiments. The molecular mass markers are indicated (kDa).

Figure 1:

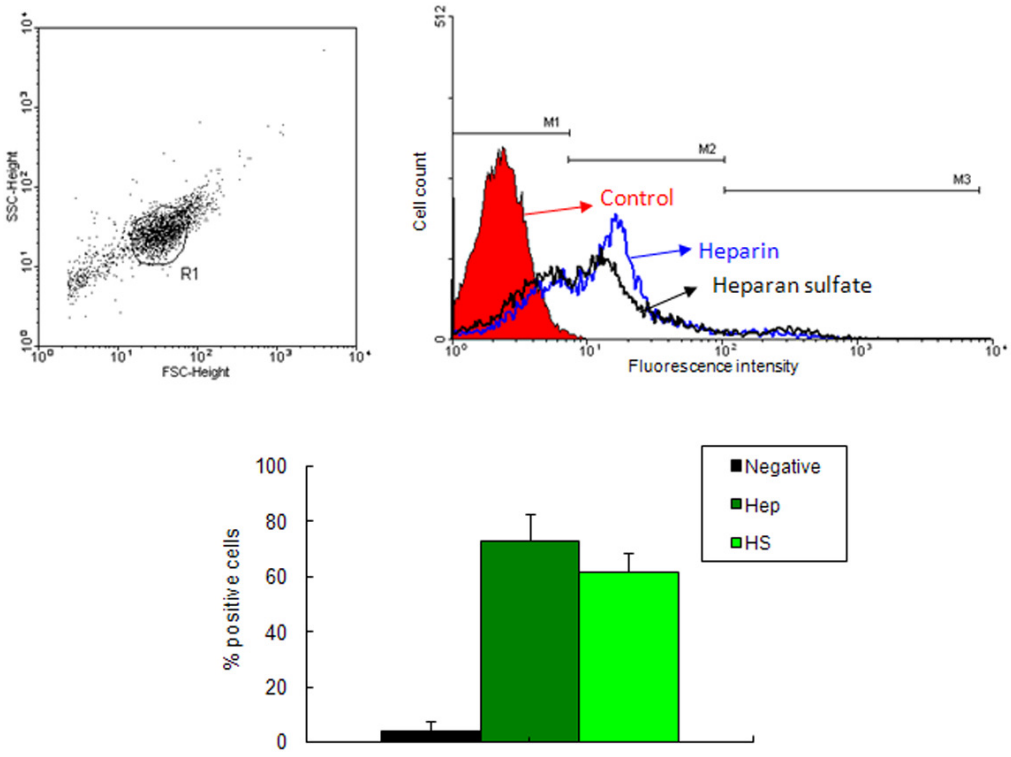
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Figure 2:

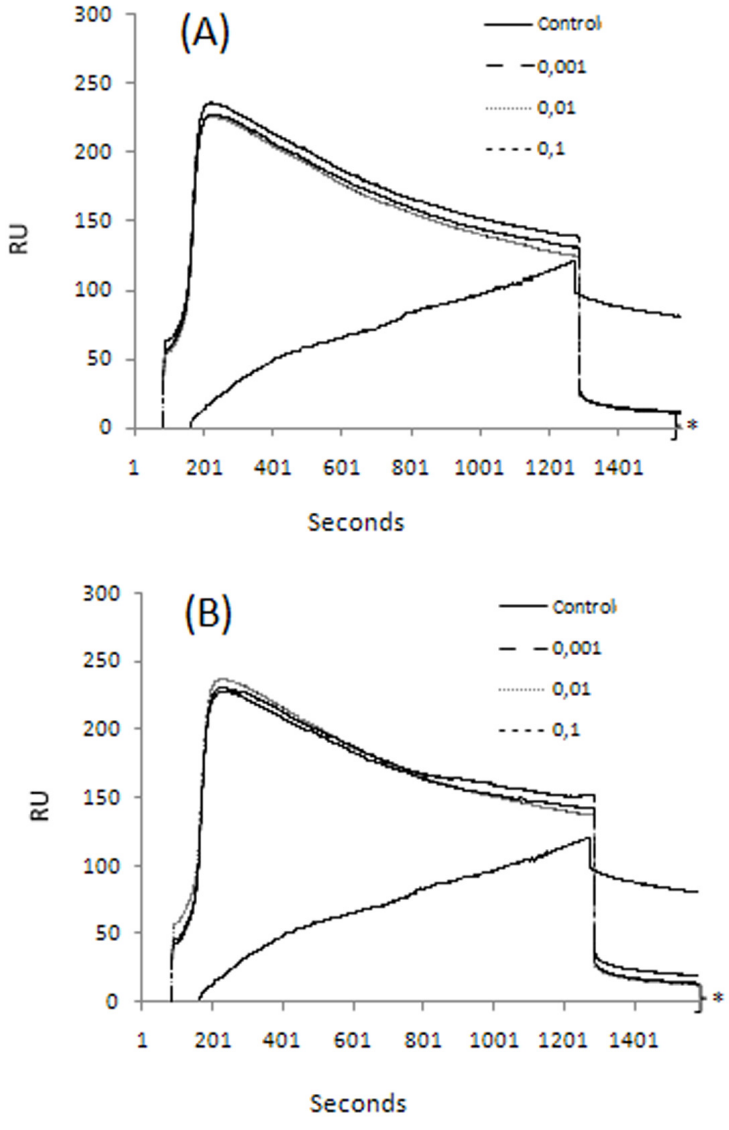
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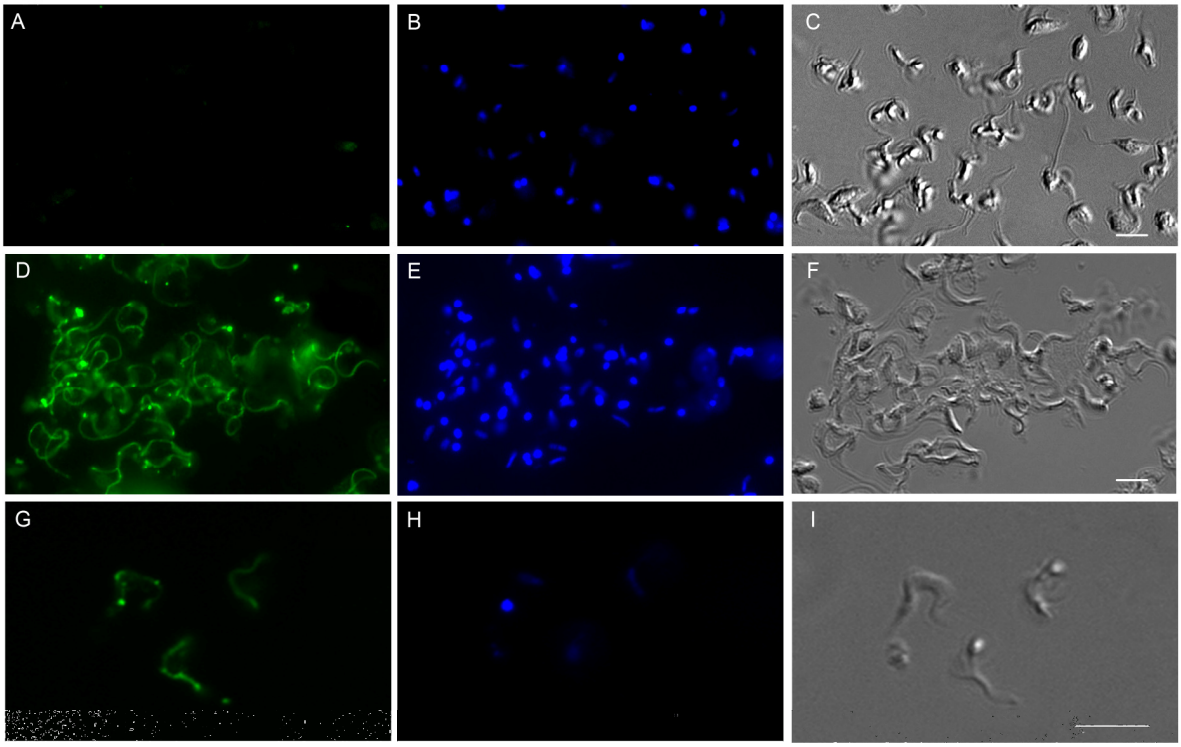
Figure 3:



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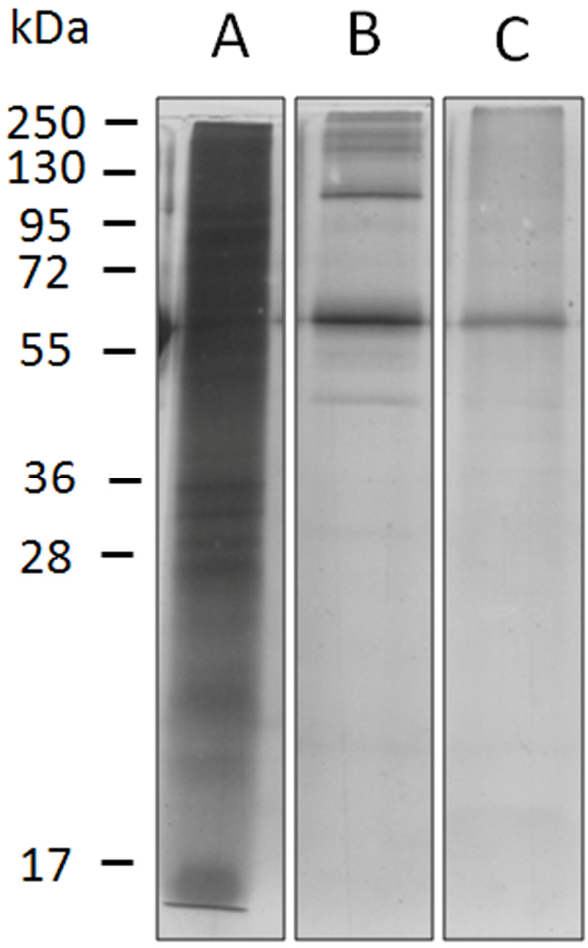

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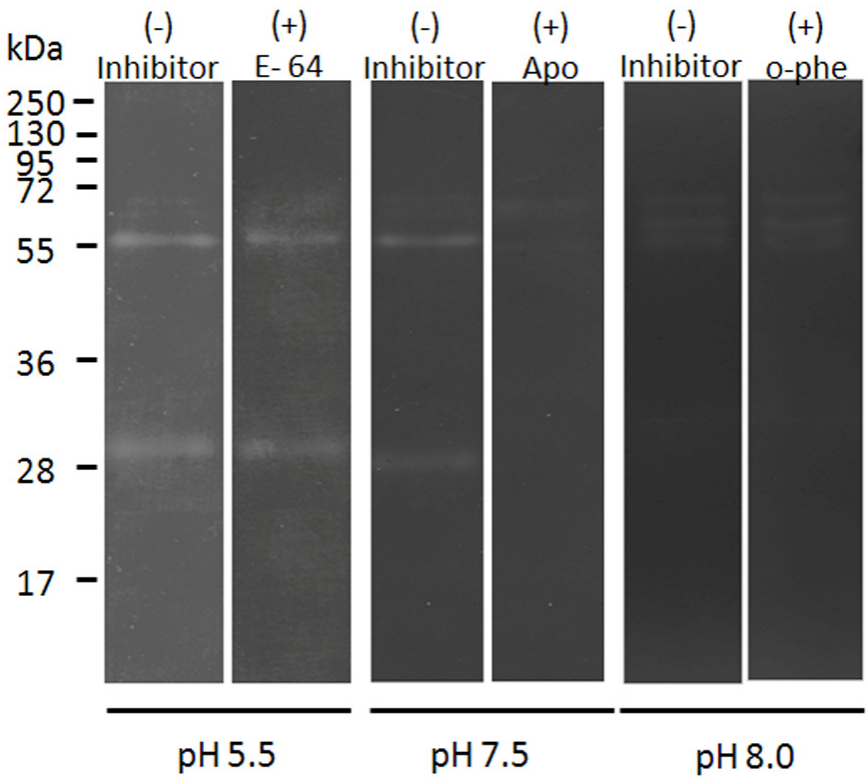
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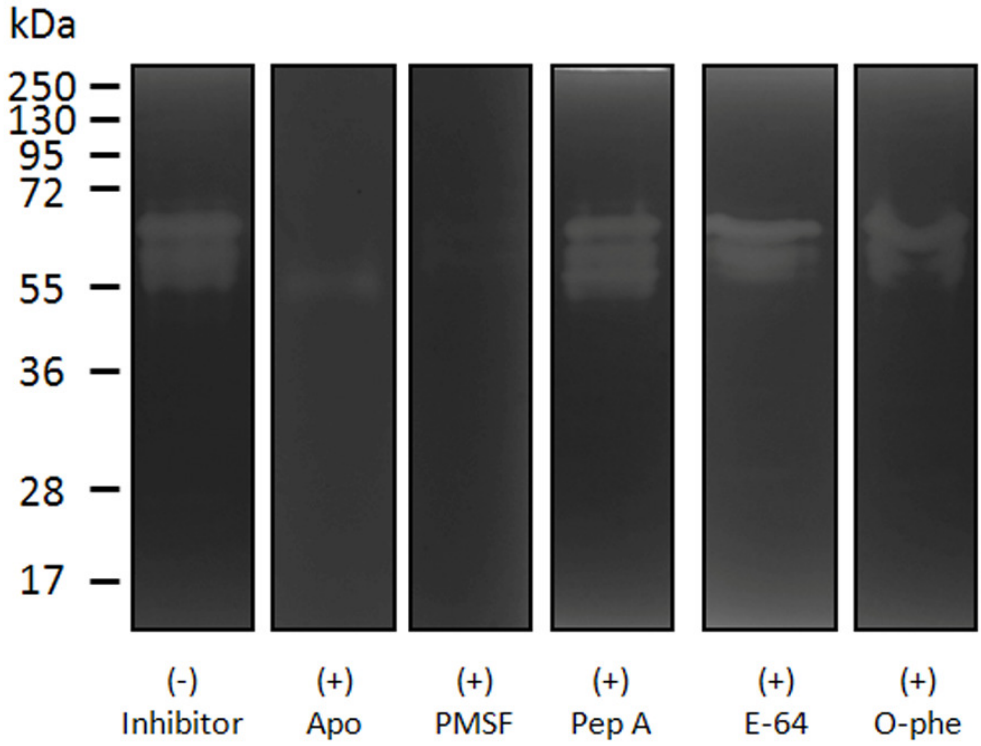
Figure 6:



4. Discussão

O programa nacional de controle da doença de Chagas é um dos maiores sucessos do sistema de saúde pública do Brasil. Mesmo assim, a doença continua sendo uma questão relevante, visto que 3,5 milhões de indivíduos apresentam a infecção crônica da doença. (Barreto et al., 2011). Esse fato denota que o diagnóstico e o tratamento desses indivíduos constituem uma carga permanente sobre os serviços de saúde (Costa-Lima et al., 2001). Sem dúvida alguma, parte do sucesso do controle dessa doença está relacionada ao domínio do conhecimento do ciclo de vida do *T. cruzi*, que tem um estilo de vida envolvendo processos e inter-relações ecológicas com seus hospedeiros triatomíneos e mamíferos; e novos conhecimentos nesta temática podem resultar em avanços no controle da doença de Chagas. Assim, um dos grandes desafios no estudo dessa doença é entender como a organização da superfície celular pode influenciar no sucesso adaptativo deste parasito a partir de eventos de reconhecimento e invasão celular, em ambos hospedeiros, sendo o foco deste trabalho de tese.

O estado da arte sobre o reconhecimento celular que se estabelece entre o *T. cruzi* e seus hospedeiros propõe o envolvimento de ligantes, predominantemente de natureza protéica, do parasita e seus receptores presentes nas células dos hospedeiros vertebrados ou invertebrados. Dessa forma, para que o parasita cumpra seu ciclo biológico todas as formas evolutivas, amastigotas, tripomastigotas e epimastigotas, necessitam interagir com componentes específicos presentes nos microambientes que ocorrem em seus hospedeiros. Como a superfície celular do parasito está em contato direto com esses microambientes é sensato propor que os componentes de superfície do parasito estejam direcionados ao sucesso do seu ciclo biológico, atuando na adesão e interiorização ou apenas na adesão do parasito à célula alvo.

Uma multiplicidade de proteínas destacam-se neste processo (revisto por De Souza et al., 2010) e o presente trabalho acrescenta novas informações nessa temática, uma vez que propõe que proteínas com propriedades de ligar à heparina (PLHs), com massas moleculares entre 70 kDa e 59 kDa, podem

atuar nas duas fases do ciclo biológico do *T. cruzi*, contribuindo com o sucesso do estilo de vida desse parasito. Reunimos evidências de que as PLHs das formas epimastigotas participam na adesão do parasito às células do intestino de triatomíneos (Artigo 1), da participação dessas proteínas de formas amastigotas na adesão e invasão de cardiomiócitos (Artigo 2), que PLHs apresentam distribuição espacial preponderante ao longo da membrana flagelar de tripomastigotas, e que, além de estabelecerem ligações estáveis com glicosaminoglicanos (GAGs), têm propriedades enzimáticas de serino proteinases (Artigo 3).

Uma fase crítica no ciclo de vida do *T. cruzi* é sua adaptação ao hospedeiro invertebrado. Nesta etapa do ciclo, os epimastigotas necessitam aderir ao trato intestinal do inseto vetor, sendo este evento essencial para a multiplicação e diferenciação do parasito, antes de sua eliminação nas fezes e urina do inseto durante o repasto sanguíneo. A colonização envolve a participação de diferentes componentes da superfície do parasito e do trato intestinal do hospedeiro invertebrado (Gonzalez et al., 2011; Gonzalez et al., 201; Alves et al., 2007). Assim, temos evidencia de que a adesão do epimastigota pode ser influenciada pela interação com heparam sulfato e condroitim sulfato com PLHs. Ressaltamos que estes GAGs foram identificados em diferentes órgãos, incluindo o trato intestinal, de *Triatoma brasiliensis* e *Rhodnius prolixus* (Costa-Filho et al., 2004; Souza et al., 2004) e de acordo com nossos resultados e dados recentemente publicados (Gonzalez et al., 2011), os GAGs sulfatados são importantes nesta etapa de reconhecimento e adesão do parasito.

A invasão bem sucedida de células de mamíferos por parasitas patogênicos é geralmente considerada a partir de evidências circunstanciais, por ser uma consequência de mecanismos específicos de reconhecimento dos componentes da superfície celular – ligantes/receptores. Vários estudos com tripanosomatídeos enfocam a biologia da interação do parasito com as células de mamíferos, visando à identificação de alvos para fins terapêuticos (Pinho et al., 2009; Da Silva Lopez & De Simone, 2004). No entanto, o conhecimento dos receptores da superfície celular dos mamíferos que interagem com o parasita é ainda limitado. Os proteoglicanos sulfatados, abundantemente expressos em células de mamíferos, têm sido descritos como uma classe de receptores

envolvidos no processo de adesão e invasão de uma variedade de parasitas intracelulares (Kobayashi et al., 2010; Carruthers et al., 2000; Lang et al., 2011), incluindo o *T. cruzi* (Calvet et al., 2003; Oliveira-Jr et al., 2008; Ortega-Barria e Pereira, 1991). Com os achados desse trabalho fica evidente que proteoglicanos de heparam sulfato (PGHS) desempenham importante papel no ciclo biológico do *T. cruzi*. Além do potencial de GAGs contribuírem com etapa do ciclo biológico no *R. prolixus* (Gonzalez et al., 2011), a participação de PGHS como mediadores da invasão de amastigotas do *T. cruzi* às células de mamífero (Artigo 2), da mesma forma que previamente descrito para tripomastigotas (Calvet et al., 2003; Oliveira-Jr et al., 2008; Ortega-Barria e Pereira, 1991) indica a ampla capacidade deste parasito se disseminar no hospedeiro mamífero, uma vez que heparam sulfato (HS) é encontrado em todos os tecidos (Dreyfuss et al., 2009). Outro aspecto interessante refere-se ao reconhecimento de um mesmo componente de superfície celular envolvido na invasão das formas evolutivas do parasito nos hospedeiro vertebrado. Até o momento, os dados da literatura apontavam para diferenças quanto a mobilização de ligantes e/ou receptores entre as formas amastigotas e tripomastigotas durante a entrada do parasito (Mortara, 1991), disparando mecanismos distintos de invasão (Mortara et al., 2008).

Não obstante, PLHs de *T. cruzi* sejam capazes de reconhecer heparam sulfato (HS) e condroitim sulfato (CS) na superfície celular, apenas HS é capaz de disparar a invasão do parasito em células de mamíferos (Calvet et al., 2003). No entanto, o mecanismo envolvido neste processo ainda não foi elucidado. É possível que as vias de sinalização desencadeadas pela ligação de amastigotas e tripomastigotas via heparam sulfato possam fornecer novas propostas para mecanismos subjacentes à infecção pelo *T. cruzi*. Assim, a via de sinalização que envolve PGHS das células de mamíferos e PLHs do *T. cruzi* torna-se de elevado interesse e pode ser considerada como um mecanismo adicional, junto a outros já estudados, sendo um potencial alvo terapêutico (Ulrich et al., 2002).

Com base na capacidade de HSPG interagir com uma diversidade de ligantes e receptores na superfície celular, incluindo fatores de crescimento, citocinas, quimiocinas, proteínas de matriz extracelular e glicoproteínas,

enzimas entre outros (Choi et al., 2011), a ligação PGHS-PLHs poderia disparar mecanismos de invasão dependente ou independente do citoesqueleto através do recrutamento de proteínas moduladoras de diferentes vias de sinalização.

Mecanismos de invasão envolvendo fosfatidilinositol-3-quinase (PI3-K) e Rho-GTPase, como Rac1, com participação de proteínas do citoesqueleto foram descritos na invasão de amastigotas (Fernandes & Mortara 2004; Fernandes et al., 2006), mas o ligante de superfície envolvido neste processo não foi elucidado. Ainda, se considerarmos os diferentes mecanismos de invasão descritos na interação tripomastigota-célula hospedeira, como mecanismos de invasão dependente (Vieira et al., 2002) ou independente (Woolsey et al., 2003) do citoesqueleto através da ativação de PI3-K e, ainda, dependente de lisossomos pela ativação de PLC (Rodriguéz et al., 1995), poderíamos especular o papel chave da interação PLHs-PGHS nos diferentes processos, atuando como receptores e/ou co-receptores da transdução de sinal. Assim, poderíamos propor que o reconhecimento e ligação de PLHs do *T. cruzi* à HSPGs na superfície das células alvo poderia disparar a fosforilação de FAK, uma tirosina quinase de adesão focal envolvida na migração celular, através da regulação da dinâmica do citoesqueleto. Esta hipótese estaria pautada na capacidade de PGHS regular diretamente a fosforilação de FAK em um mecanismo dependente de Rho-GTPase (Wilcox-Adelman et al. 2002) ou ativar FAK entre outras vias através de cooperação com integrinas (Woods & Couchman 2001). Esta ligação receptor/ligante poderia estimular agregação de PGHS e/ou integrinas que modulam a autofosforilação de FAK que, uma vez fosforilada, é capaz de se ligar a Src, a subunidade p85 da fosfatidilinositol-3-quinase (PI3-K) (Chen HC et al., 1996), fosfolipase C e proteína quinse C (Zhang et al., 1999) entre outras proteínas adaptadoras. No caso da ativação de FAK, o recrutamento de Src levaria a amplificação da fosforilação de FAK, acarretando polimerização de filamentos de actina para projeção da membrana celular e interiorização do parasita. Ainda, a associação do complexo de proteínas ezrina, radixina e moesima e, ainda, cortactina com o domínio citoplasmático C1 do PGHS (Couchman, 2010) poderia regular a dinâmica de actina através do disparo da via de sinalização de FAK, como demonstrado

com *Helicobacter pilory* (Tegtmeyer et al., 2011) ou através de eventos de sinalização com Rho-GTPase e tirosina quinase (Weed & Parsons 2001).

Com o achado deste trabalho que as PLHs hidrofóbicas de 70 kDa, 65.8 kDa and 59.0 kDa de tripomastigotas apresentam atividade de proteinase surge um vasto campo de estudo sobre o potencial funcional dessas proteínas na patogênese da doença de Chagas. Os questionamentos atuais sobre o dano tecidual, que ocorre no músculo cardíaco na doença de Chagas, têm sido avaliado sobre dois aspéctos: (1) persistência parasitária (Tarleton e Zhang 1999; Gutierrez et al., 2009) ou (2) por uma resposta autoimune (Cunha-Neto et al., 2011; Engman & Leon, 2002). Em ambos os modelos de progressão da doença, as proteinases do parasito vem sendo descritas como um fator indutor desse dano (McKerrow et al., 2006). Assim, ao descrevermos que as PLHs do *T. cruzi* apresentam propriedades de serino proteinase estamos propondo que essas enzimas poderiam atuar sobre a hidrólise de substratos complexos, como colágeno e fibronectina, facilitando a migração do parasito através do tecido, como fora proposto para serino proteinase de *T. brucei* (Bastos et al., 2010) e TC80 (Grellier et al., 2001), e dessa forma, contribuindo com o dano tecidual. Ainda, podemos sugerir que as serino proteinases hidrofóbicas dos tripomastigotas estariam associadas a indução da geração de Ca^{2+} intracelular nas células do hospederio vertebrado como fora descrito para Oligopeptidase B do *T. cruzi* (Burleigh & Andrews, 1998).

Além disso, a propriedade proteolítica das PLHs poderia ser importante na ativação da via de sinalização de TGF-β. A ativação desta via foi evidenciada durante o processo de invasão do *T. cruzi* (Ming et al., 1995), sendo demonstrado aumento da expressão de Smad2 fosforilada e inibição da interiorização do parasito pela ação de inibidores específicos da via (Waghabi et al., 2002). Assim, a ligação PLHs-PGHS poderia favorecer a proteólise do TGF-β latente, estocado na matriz extracelular através de sua associação com PGHS (Chen et al., 2007; Parsi et al., 2010), transformando-o em TGF-β ativo capaz de se ligar ao receptor TGF-β do tipo II (TGFβRII) e disparar a via de sinalização através da fosforilação do receptor tipo I (TGFβRI) e Smad2 para transcrição de genes envolvidos na modulação da síntese de matriz extracelular, citoesqueleto entre outros (Huang & Chen 2012).

A questão sobre a especificidade da interação da serino proteinase do *T. cruzi* e os GAGs permanecem em aberto neste estudo. O fato de várias proteínas ligarem a heparina (Casu & Lindahl 2001) levou um número de investigadores a examinar se existe uma sequência consenso de aminoácidos envolvidos nesta ligação. Estudos estruturais indicaram que algumas proteínas que ligam a GAGs podem interagir com diferentes sequências de oligossacarídeos (Raman et al., 2005). Destes estudos se concluiu que os sítios de ligação na proteína sempre contêm aminoácidos básicos (Lys e Arg) que ligam aos sulfatos carregados negativamente e carboxilas nas cadeias de heparina e HS. No entanto, o arranjo desses aminoácidos básicos pode ser bastante variável, de acordo com o posicionamento de grupos sulfato do GAG (Jeffrey et al., 2009). De certa forma, nossos dados apontam para uma diferença deste arranjo, uma vez que as PLHs hidrofóbicas de epimastigotas foram eluídas em 1,0 M de NaCl (Artigo 1), enquanto que as PLHs de tripomastigotas em 0,5 M de NaCl (Oliveira-Jr et al., 2008). Este fato é um indicativo de diferença de carga na superfície dessas proteínas que pode ser devido à quantidade ou ao grau de protonação dos aminoácidos básicos expostos na superfície das PLHs das duas formas do parasito.

A alteração da carga de superfície dessas proteínas é um fato importante a ser comentado e pode estar relacionado à microvariações estruturais relevantes para ligação e sinalização celular induzida pelas PLHs. Mesmo apresentando perfis de eluição diferente das formas tripomastigotas, as PLHs de epimastigotas são capazes de reconhecer GAGs (Artigo 1), o que é um indicativo de que os motivos de ligação de GAGs não estão alterados. Entretanto, estas alterações de carga poderiam ser suficientes para desorganizar o motivo mínimo envolvido no disparo de vias de sinalização impossibilitando, desta forma, a invasão celular por formas epimastigotas.

Os resultados do nosso estudo contribui para desvendar os eventos moleculares envolvidos na atividade das PLHs. Sugerimos um papel alostérico para os GAGs na regulação da estabilidade estrutural das PLHs de tal maneira que a proteína permitiria o estabelecimento dos contactos mais fortes e, assim, a manutenção da estrutura global para a sua função de acordo com o

microambente que se encontra. Estamos certos que estudos adicionais são necessários para confimar esta hipótese.

A aplicabilidade do método de biossensoriamento de superfície para detecção de estruturas bioativas e vias de reconhecimento celular em células vivas (Velasco-Garcia, 2009), foi avaliada neste trabalho (Artigo 1 e 3). Com esta abordagem, foi possível simular os eventos de interação molecular e indicar a presença de PLHs na superfície de epimastigotas e tripomastigotas do *T. cruzi*. Além disso, os nossos dados de bissensoriamento de superfície foram decisivos ao indicarem a especificidade e estabilidade das interações que se estabelecem entre as PLHs e os GAGs. O que nos permite afirmar, que tais interações não são aleatórias e podem desencadear sinais importantes para adesão e/ou invasão do parasito em conjunto com outras formas de interação, como campo eletrostático, que facilitam a aproximação do parasito e, consequentemente, favorece o reconhecimento de componentes da célula hospedeira, proporcionando então sua invasão. Este fato foi previamente relatado em nossos estudos de interação entre tripomatigotas e a célula muscular cardíaca, onde evidenciamos que o tratamento da célula hospedeira com clorato de sódio, que remove a sulfatação da superfície dos GAGs, interfere na aproximação eletrostática e, consequentemente no reconhecimento do domínio N-acetilado/N-sulfatado([IdoUA-GlcNAc]-[GlcUA-GlcNS]$_3$-[GlcUA-GlcNAc]$_4$[GlcUA]) da cadeia de HS reduzindo a interiorização do parasito (Oliveira-Jr et al., 2008).

O conjunto de resultados dos estudos físico-químicos apresentados, corresponde a uma primeira abordagem de biossensoriamento de superfície aplicada na detecção de proteínas de superfície de *T. cruzi* com potencial interação com células dos hospedeiros (Artigos 1 e 3), tendo como base a indução do transporte de massas na superfície do *sensor chip. Co*mo visto em estudos com células de mamíferos, esta abordagem se mostra promissora no rastreamento de ligantes na superfície de células com atividade biológica importante, como recentemente descrita para detecção de células osteogênicas (Kuo et al., 2011) e de PLHs de *L.(V). braziliensis* (de Castro Cortês et al., 2012).

Ressalta-se ainda, que uma das vantagens da aplicação desta metodologia neste trabalho foi a possibilidade de análise do fenômeno de ligação diretamente na superfície dos epimastigotas e tripomastigotas, em tempo real, ou seja, levando-se em conta, também, a quarta dimensão no estabelecimento da interação molecular (Van Regenmortel, 1996). Desta forma, os resultados de *SPR* que apresentamos são dinâmicos e simulam a realidade do fenômeno de interação que está sendo pesquisado. Estamos certos que estudos adicionais são necessários para aperfeiçoar estas análises em uma abordagem quantitativa dos epimastigotas e tripomastigotas, como proposto para quantificação de *Escherichia coli* (Wang et al., 2011). Com esta estratégia, que simula as condições dos microambientes dos hospedeiros, foi possível especular sobre a atuação das PLHs na adesão do parasito nas fases do ciclo de vida.

Os dados apresentados nessa tese ressaltam uma modalidade adaptativa do *T. cruzi,* na qual envolve as PLHs da superfície do parasito e os GAGs dos hospedeiros. A possibilidade do genoma desse parasito codificar para PLHs que sofrem influências nos seus diferentes hospedeiros reforça a hipótese de uma mesma proteína ter funções distintas durante o ciclo biológico do *T. cruzi*, envolvidas nos eventos de adesão no *R. prolixus* e reconhecimento e invasão em células de mamífero. Os achados desse trabalho indicam que as interações entre PLHs e GAGs contribuem ao estilo de vida do *T. cruzi* culminando em sua eficiência em manter o ciclo biológico.

5. Conclusões

- As PLHs estão presentes em todas as três formas evolutivas do *T. cruzi* e desempenham papel biológico importante no ciclo de vida do parasito. Em relação à diferentes formas evolutivas, os seguintes resultados foram obtidos:

1) Formas epimastigotas:

- Epimastigotas apresentam proteínas com propriedade de ligação à heparina (PLHs) em sua superfície, com massas moleculares majoritárias de 65,8 kDa e 59 kDa, capazes de estabelecer ligações específicas e estáveis com heparina, heparam sulfato e condroitim sulfato.

- PLHs desempenham importante papel no reconhecimento e adesão de epimastigotas em células epiteliais do trato intestinal de *Rhodnius prolixus*, sugerindo uma potencial participação destas proteínas no processo de metaciclogênese.

2) Formas Amastigotas:

- Glicosaminoglicanos sulfatados, constituídos de ácido glucurônico/ ácido idurônico–N-acetilglicosamina, modulam o reconhecimento e disparam a invasão de amastigotas em células de mamíferos. Outros GAGs sulfatados, como condroitim sulfato, dermatam sulfato e queratam sulfato não atuam na entrada deste parasito.

- A interação de amastigotas com GAGs sulfatados solúveis não afeta a viabilidade e estrutura morfológica dos parasitos nem a capacidade endocítica de miócitos cardíacos. A inibição da invasão é decorrente do bloqueio do reconhecimento de GAGs na superfície celular e não por efeitos tóxicos ao parasito ou distúrbios da via endocítica da célula hospedeira.

- Redução na invasão de amastigotas foi evidenciada em linhagens celulares derivadas de ovário de hamster chinês deficiente em GAGs

(CHO-745), corroborando a participação de GAGs como mediadores do processo de invasão de amastigotas.

3) Formas tripomastigotas:

- PLHs são abundantemente expressas em tripomastigotas e se localizam predominantemente na membrana flagelar do parasito, sítio de intensa transdução de sinal, sugerindo sua participação como moduladores da ativação de vias de sinalização envolvidas na invasão do parasito.

- As PLHs conferem ligações estáveis com heparam sulfato e heparina e apresentam propriedade de serino proteinase. Proteínas com massas moleculares de 70 kDa, 65,8 kDa e 59 kDa são capazes de hidrolisar gelatina e possuem sensibilidade à inibição com PMSF e aprotinina.

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7. Anexos

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Trypanosoma cruzi heparin-binding proteins and the nature of the host cell heparan sulfate-binding domain

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Abstract

Trypanosoma cruzi invasion is mediated by receptor–ligand recognition between the surfaces of both parasite and target cell. We have previously demonstrated the role of heparan sulfate proteoglycan in the attachment and invasion of *T. cruzi* in cardiomyocytes. Herein, we have isolated the *T. cruzi* heparin-binding proteins (HBP-Tc) and investigated the nature of cardiomyocyte heparan sulfate (HS)-binding site to the parasite surface ligand. Two major heparin-binding proteins with molecular masses of 65.8 and 59 kDa were observed in total extract of amastigote and trypomastigote forms of *T. cruzi*. Hydrophobic [S^{35}]methionine labeled proteins eluted from heparin–sepharose affinity chromatography also revealed both proteins in trypomastigotes but only the 59 kDa is strongly recognized by biotin-conjugated glycosaminoglycans. Competition assays were performed to analyze the role of sulfated proteoglycans, including heparin, keratan sulfate and both acetylated and highly sulfated domains of heparan sulfate, in the recognition and invasion process of *T. cruzi*. Significant inhibitions of 84% and 35% in the percentage of infection were revealed after treatment of the parasites with heparin and the N-acetylated/ N-sulfated heparan sulfate domain, respectively, suggesting the important role of the glycuronic acid and NS glucosamine domain of the HS chain in the recognition of the HBP-Tc during the *T. cruzi*–cardiomyocyte interaction.

Keywords: *Trypanosoma cruzi*; Cardiomyocytes; Glycosaminoglycans; Recognition process; Chromatography

1. Introduction

Chagas' disease, caused by the parasite *Trypanosoma cruzi*, is an important cause of irreversible cardiomyopathy which affects approximately 13 million people in Latin America [1]. In addition, this infection poses a potential hazard to many countries including those in North America and Europe because of blood transfusion and organ transplantation [2,3]. The pathogenesis of chronic Chagas' disease is not completely understood and may be related to parasite persistence, parasite antigens [4,5] and/or autoimmune mechanisms [6–8]. Invasion of mammalian cells by *T. cruzi* is mediated by receptor–ligand recognition between the surfaces of both parasite and target cells. Carbohydrate-binding protein in the parasite [9,10] and host cell galactose, manose, *N*-acetylgalactosamine residues are important for attachment and host cell invasion [11–13]. The parasite expresses a family of active and inactive *trans*-sialidase, a glycosylphosphatidylinositol anchored protein on its surface which has been demonstrated to be involved in host cell invasion and immunomodulation [14,15]. In addition to parasite ligands, several host cell surface molecules have been implicated

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in the recognition and invasion process of this parasite [16,17].

Glycosaminoglycans (GAGs) are considered potential receptors in the process of microbial pathogens attachment to the target cells. Most GAGs consist of disaccharide repeats of an amino sugar and an uronic acid, except keratan sulfate in which galactose replaces the uronic acid. Binding of sulfated proteoglycans to parasite appears to require specific GAGs chain in a variety of human pathogens adherence mechanism. Heparan sulfate proteoglycan (HSPG) consists of a repeating disaccharide with alternating hexuronic acid (D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)) and D-glucosamine units [18]. They are ubiquitously distributed on the surface and extracellular matrix of mammalian cells and are involved in the invasion process of many infectious microorganism, including virus [19–21], bacteria [22–24] and protozoan [25–27].

Although a heparin-binding protein (HBP) of 60 kDa, named penetrin, has previously been reported on the surface of the trypomastigote form of *T. cruzi* [28], the biochemical feature of this protein has not been completely characterized. Furthermore, even though *T. cruzi* HBP (HBP-Tc) plays an important role in the recognition of host cell surface HSPG [29,30], the molecular basis of *T. cruzi*–HSPG interaction has not been elucidated and may be a potent target for drug therapy. In this study, we have isolated HBP from trypomastigote forms of *T. cruzi* and also investigated the domain of the HS chain involved in the parasite–host cell invasion.

2. Results

2.1. HBPs characterization

The fact that many intracellular pathogens exploit sulfated proteoglycans to adhere at and/or gain into host cells lead us to investigate the presence of HBP in the infective stages of *T. cruzi*. To determine whether HBP is present in trypomastigote and amastigotes, total protein extracts were separated by sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE) and ligand detection was performed by *Western blotting* analysis using glycosaminoglycans-biotin conjugated. We demonstrated a complex protein profile with molecular masses ranging from 160 to 10 kDa when the parasites were revealed by heparin-biotin conjugated (Fig. 1). Interesting, two major bands with molecular masses of 65.8 and 59 kDa were detected by heparan sulfate and chondroitin sulfate-biotin conjugated.

We attempted to isolate the heparin-binding protein of trypomastigotes (HBP-Tc) by Triton X-114 extraction method associated with heparin–sepharose column. Initially, trypomastigotes were metabolically labeled with 100 μCi of [S^{35}]methionine and the hydrophobic proteins were eluted from heparin–sepharose affinity chromatography. HBP-Tc were preferentially eluted at 0.5 M of NaCl and some

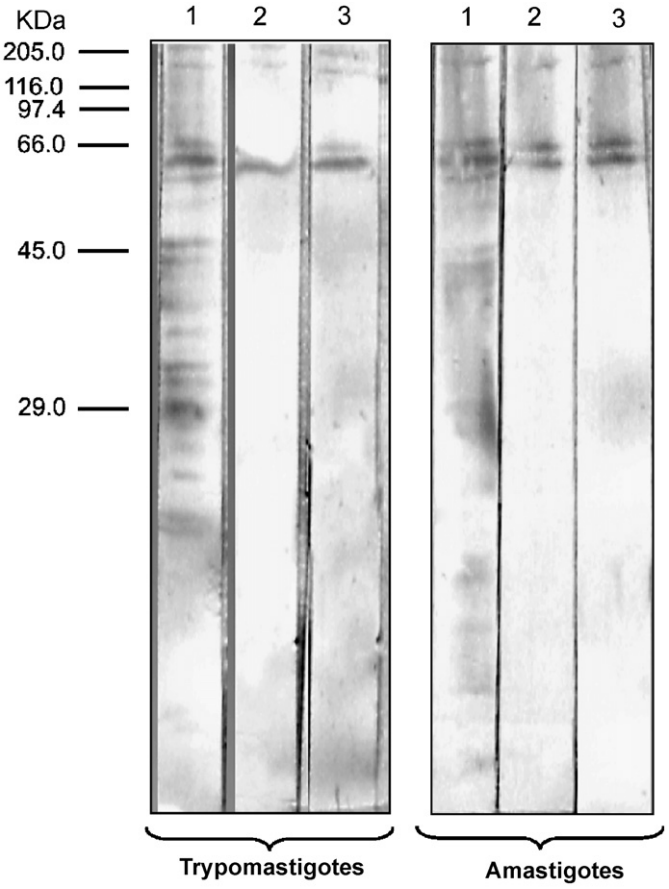

Fig. 1. Detection of proteins with properties to bind to sulfated glycosaminoglycans (GAGs). The assays were performed with trypomastigotes and amastigotes by *Western blotting*. Total proteins were separated by SDS–PAGE electrophoresis and transferred to the nitrocellulose membrane. The immobilized proteins were incubated with biotin-conjugated GAGs (2 μg/ml), such as heparin (1), chondrotin sulfate (2; CS) and heparan sulfate (3; HS). Note the presence of two major bands with molecular masses of 65.8 and 59 kDa in both CS and HS while heparin revealed a broad range of proteins, including the 65.8 and 59 kDa proteins. Molecular mass standard proteins are indicated on the left.

proteins bands profiles were detected, including the 65.8 and 59 kDa as the major bands (Fig. 2). Similar results were also obtained in non-isotopic assays, showing both protein bands profiles (65.8 and 59 kDa) eluted from affinity chromatography. This was compared with the whole hydrophobic fraction profile, as demonstrated by silver staining (data not shown). To address the question whether the protein bands present the property to bind to sulfated GAGs, heparin–sepharose affinity purified proteins from trypomastigotes were detected by heparin-, HS- and CS-biotin conjugated by *Western blotting*. Our results demonstrated the recognition of both protein bands (65.8 and 59 kDa) by all sulfated GAGs analyzed, but an intense labeling was mainly detected with the 59 kDa protein (Fig. 3).

2.2. The role of sulfated GAGs in T. cruzi invasion

T. cruzi infects many cultured cells type. Our previous data demonstrated the role of HSPG, but not chondroitin

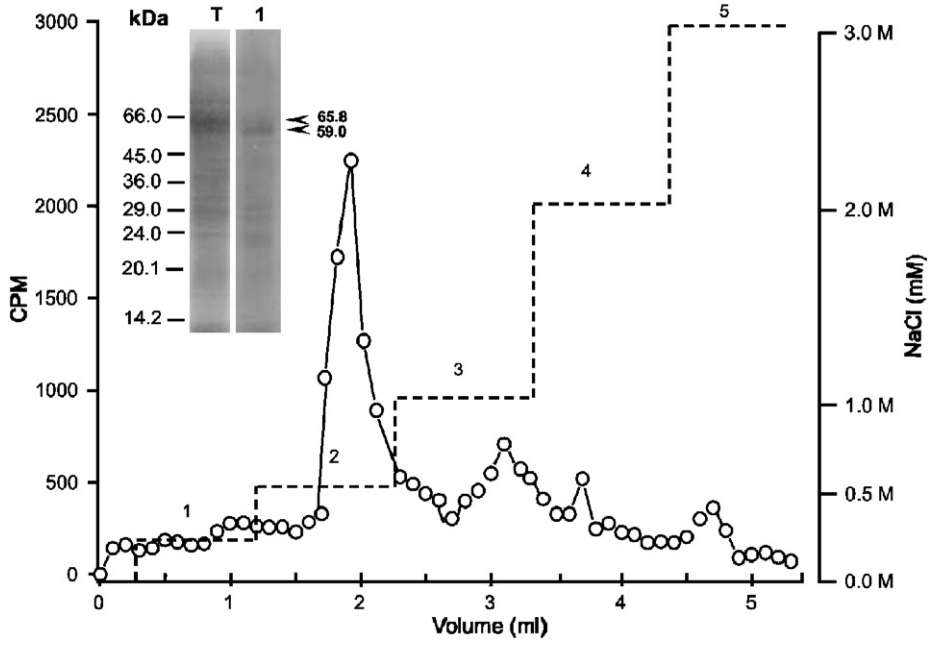

Fig. 2. Affinity chromatography of hydrophobic proteins from trypomastigotes labeled with [S^{35}]methionine. The total hydrophobic proteins (93000 cpm) were obtained by the Triton X-114 phase separation technique and applied to heparin–sepharose column previously equilibrated. The proteins were eluted with different concentrations of salt (0.25 M to 5.0 M) and the peak of protein elution, presenting 2300 cpm of radioactivity, was obtained at 0.5 M of NaCl (2) Inset: total hydrophobic proteins (T) and 65.8 and 59 kDa protein bands (1; arrowheads) from elution peak. Molecular mass standard proteins (SDS-7) are indicated on the left.

sulfate, in the invasion of the host cell by this parasite. [29]. Here we addressed the question whether other classes of sulfated proteoglycans may be involved in the parasite–host cell recognition. Additionally, we wanted to determine the domain of HS chain recognized by HBP-Tc and its implication in the parasite invasion. A GAG competition assay was performed and in this trypomastigotes were pre-treated for 1 h on ice with 20 μg/ml of soluble heparin, keratan sulfate or N-acetylated (NA), highly sulfated (hS) and N-acetylated and N-sulfated (NA/hS) domain of HS chain, obtained by nitrous acid treatment, heparitinase I and heparitinase II, prior to cardiomyocyte interaction. We found that heparin but not keratan sulfate is involved in the *T. cruzi*–cardiomyocyte recognition. The quantitative data revealed a significant decrease in the percentage of infection; there was a reduction from 38% in the control to 5% ($p \leqslant 1.65 \times 10^{-6}$) after heparin pre-treatment of the parasites, which represents an inhibition of parasite invasion of 84% (Figs. 4 and 5). No change in the invasion ratio was observed when trypomastigotes were pre-treated with 20 μg/ml of keratan sulfate (Figs. 4 and 5).

Since heparin and heparan sulfate are structurally similar, but only heparan sulfate is present in the host cell surface, we analyzed the nature of HS chain domain involved in the receptor–ligand binding. In the competition assay where trypomastigotes were pre-treated with different HS chain domains only the NA/hS domain was able to inhibit parasite invasion. A reduction of 45% in the infection level was obtained with NA/hS domain compared with the control ($p \leqslant 0.0031$). Treatment of the parasites with hS or NA domains did not alter the percentage of infected cardiomyocytes (Figs. 4 and 5).

Although hS domain did not participate in the recognition and binding of the HS chain to the parasite ligand, the high level of sulfation in this HS domain could be important to modulate the receptor–ligand proximity. To address this question, we treated cultured cardiomyocytes with 25–75 μM of sodium chlorate, which inhibits the sulfation of GAGs, 24 h before the parasite–host cell interaction and observed a dose-dependent effect in the infection ratio after sodium chlorate treatment, reducing significantly from 38% in the control to 28%, 19.6% ($p \leqslant 0.007$) and 10% ($p \leqslant 0.014$) after 25, 50 and 75 μM, respectively, representing a inhibition of 26%, 48.5% and 73.6% of infection (Figs. 6 and 7).

3. Discussion

Intracellular pathogens have developed a strategy to interact specifically with cell surface GAGs in order to recognize and gain entry into their target cells [19–27]. The ability of the parasites to recognize different GAGs at the host cell surface may contribute to amplify the spectrum of the target tissues. The aim of this study was to show (i) the *T. cruzi* heparin-binding proteins (HBP-Tc) of trypomastigotes and amastigotes, (ii) analyze the participation of heparin and keratan sulfate in *T. cruzi* invasion and (iii) determine the nature of host cell HS chain domain involved in the ligand recognition.

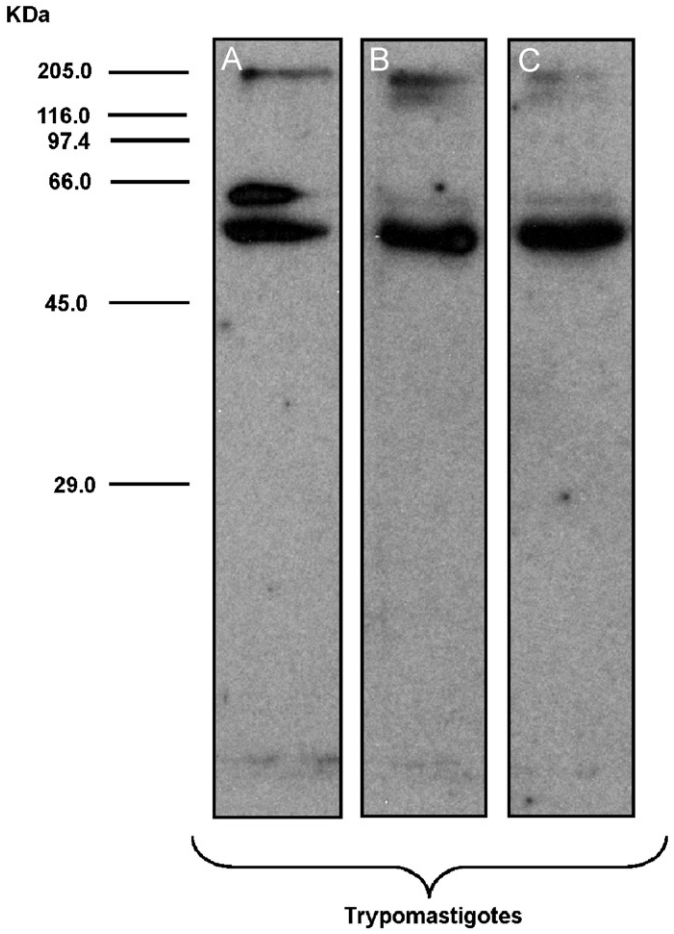

Fig. 3. Heparin-binding proteins isolated from trypomastigotes using Triton X-114 and heparin affinity chromatography methodologies. After chromatography the proteins were detected with 2 μg/ml of biotin-conjugated sulfated glycosaminoglycans by *Western blotting*. Subsequently, the proteins were revealed with streptavidin-peroxidase conjugated and the ECL system. Note that the 59 kDa band was mainly revealed by heparin (A), heparan sulfate (B) and chondroitin sulfate (C). Molecular mass standard proteins are indicated on the left.

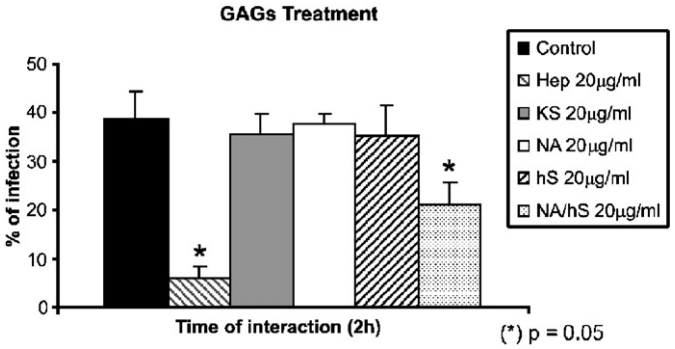

Fig. 4. Competition assay was performed to determine the involvement of sulfated glycosaminoglycans in *T. cruzi*–cardiomyocyte invasion. Trypomastigotes, were treated for 1 h on ice with 20 mg/ml of heparin, keratan sulfate (KS) and heparan sulfate (HS) domains (D–F), such as N-acetylated (NA), highly sulfated (hS) and N-acetylated/highly sulfated (NA/hS), prior to 2 h of interaction. The results showed a reduction in the infection ratio, decreasing from 38% of infection in control to 5.9% and 21% after heparin and N-acetylated/highly sulfated HS domain treatment, respectively.

Heparin-binding chromatography revealed two profiles of protein bands with a molecular mass of 59 and 65.8 kDa identified in trypomastigotes and amastigotes. Surprisingly, we detected HBPs in the amastigotes once these proteins had not yet been demonstrated in this evolutive form. In trypomastigotes, the HBP-Tc profile was detected in denaturant electrophoresis assays, suggesting that this isolated fraction may contain two proteins with distinct molecular masses. Our results contrast with a previous report that demonstrated only a 60 kDa HBP, penetrin, in trypomastigote forms of *T. cruzi* [28], since we have detected both 65.8 and 59 kDa HBPs in amastigotes and an additional band of 65.8 kDa in trypomastigotes. Furthermore, it is important to have in mind that the observation of two proteins profiles may not be an artifact caused by the proteolysis of 65.8 kDa protein since the chromatography assays were performed in the presence of proteinases inhibitors. As yet, the identity of these proteins has not been established. Further studies are underway to analyze the structural profiles of both proteins.

Recently, we reported that *Leishmania (V.) braziliensis* has proteins that bind to heparin with complex structural organization, probably with more than one subunit [31]. Such a complex protein has also been demonstrated in *Anadara granosa* that has a heparin-binding lectin with a native mass of 300 kDa composed of five identical subunits of 60 kDa [32]. Thus, with the present data set we hypothesize that the HBP-Tc is one fraction with two distinct proteins able to bind to heparin. These structural differences between HBPs from both trypanosomatids may reflect the involvement of these proteins in distinct physiological processes in their life cycles. Interesting, the 59 kDa protein was preferentially detected by heparin-biotin conjugated in the *Western blot* analysis, suggesting that this protein is responsible for the recognition and binding process. This observation may explain the fact that only a 60 kDa protein, called penetrin, has been previously reported as a HBP in trypomastigotes surface using isotopic assays [28]. In fact, our results revealed that HBP-Tc also has the ability to bind to heparan sulfate and chondroitin sulfate but only the former GAG is engaged in the invasion process of trypomastigotes [29].

Although a tropism for muscle cells have been reported in *T. cruzi* infection [33–35], the broad range of cell type recognition by *T. cruzi* in vitro may be attributed to the ability of this parasite to bind a variety of GAGs in the host cell surface as occurs with *Toxoplasma gondii* [36], *Encephalitozoon intestinalis* [26] and *Staphylococcus aureus* [37]. Therefore, the ability of *T. cruzi* to recognize multiple GAGs and the nature of HS-binding domain involved in the recognition process was investigated using heparin, keratan sulfate and different domains of HS chain (N-acetylated/N-sulfated). As expected, treatment of

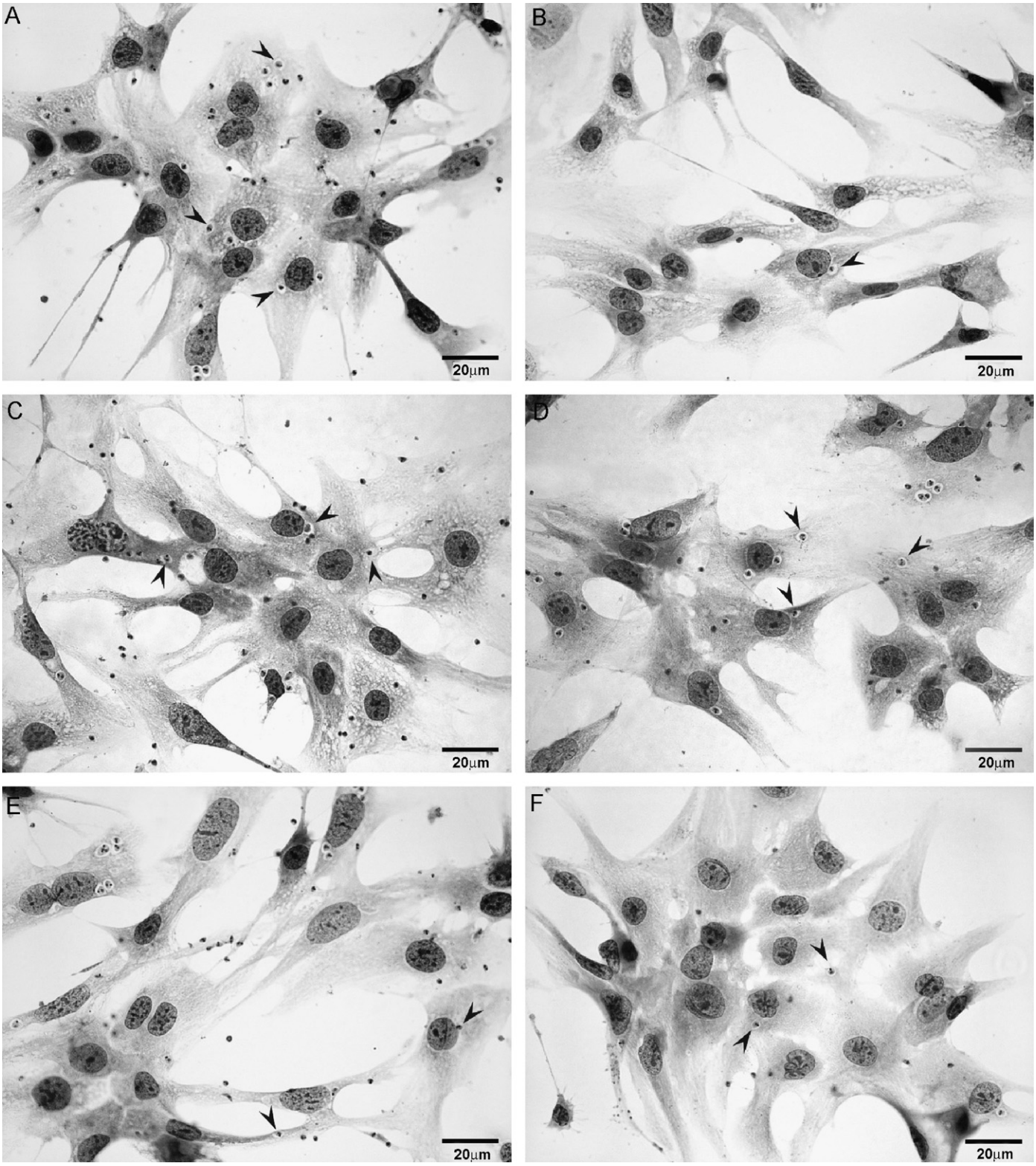

Fig. 5. Light microscopy image of cardiomyocytes infected with trypomastigotes of *T. cruzi*. The cells were untreated or treated with different GAGs and heparan sulfate (HS) domains, prior to infection. (A) Cardiomyocytes infected with trypomastigotes for 2 h (control). (B) Treatment of trypomastigotes for 1h on ice with 20 mg/ml of heparin, (C) keratan sulfate, (D) N-acetylated (NA) HS domain, (E) highly sulfated (hS) HS domain and (F) N-acetylated/highly sulfated (NA/hS) HS domain. Note the drastic reduction of infection after heparin treatment (B), whereas keratan sulfate (C), NA (D) and hS (E) did not alter the infection pattern. The infection was also reduced when trypomastigotes were pre-treated with the N-acetylated/highly sulfated domain (F). Arrowheads show the intracellular parasites. Barr 1/4 20 mm.

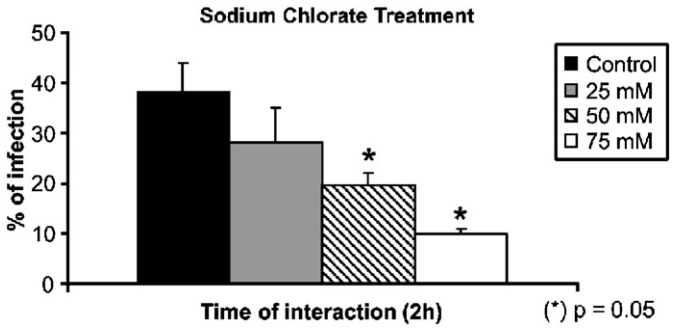

Fig. 6. Role of sulfation in the *T. cruzi*–cardiomyocyte invasion. Cardiomyocyte cultures were treated with different concentrations of sodium chlorate (25–75 mM) before trypomastigote interaction. A significant reduction was observed after high concentration of sodium chlorate (50 and 75 mM) when compared to control. The infection decreased from 38% in control to 28%, 19.6% and 10% after treatment of the cultures with 25, 50 and 75 mM of sodium chlorate, respectively.

trypomastigotes with heparin, a GAG structurally similar to HS with higher degree of sulfation, significantly decrease the ratio of infection in cardiomyocytes while keratan sulfate treatment had no effect in the invasion process, achieving similar infection ratios as control cells. Although we have observed that HBP-Tc binds to CS, our previous results revealed that this GAG is not implicated in the invasion of *T. cruzi* in cardiomyocytes [29], suggesting that only the specific binding of GlcA or IdoA and D-glucosamine disaccharide units in HSPGs may trigger a signaling cascade involved in the mechanism of invasion.

The structural diversity of HSPG allows recognition and selective binding of proteins to HS chain, modulating their biological activity or degradation. Ligand binding may occur by specific interaction with N-acetylated (NA domain), highly sulfated (hS domain) or alternated

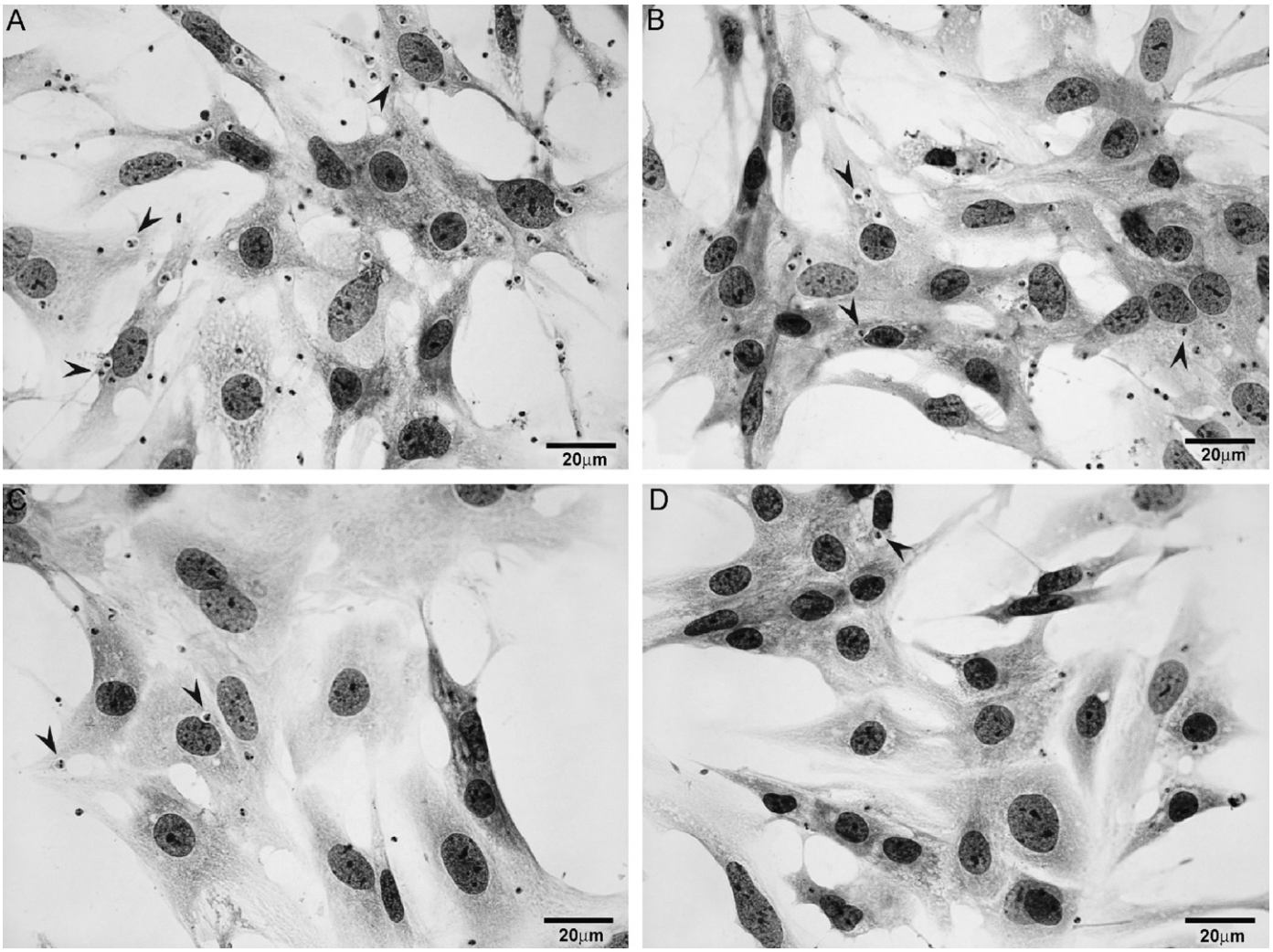

Fig. 7. Treatment of cardiomyocytes with sodium chlorate inhibits parasite invasion. Aspect of the *T. cruzi*-infected cardiomyocyte cultures (A; control) and after treatment with 25 mM (B), 50 mM (C) and 75 mM (D) of sodium chlorate. Note a dose-dependent reduction of the infection levels. Arrowheads show the intracellular parasites. Barr 1/4 20 mm.

N-acetylated and highly sulfated (NA/hS domain) disaccharide units [38,39]. Most growth factors have been thought to bind GAGs [40–42] but the nature of HS chain-binding domain differs markedly according to the ligand. Fibroblast growth factors (FGF) bind to N-sulfated sequence (L-iduronic acid 2-O-sulfated) [43], while platelet-derived growth factor (PDGF) recognition depends on N-sulfated saccharide domains containing both 2-O and 6-O-sulfated groups [44]. However, the protein–GAG interaction may be more complex, as in the case of interferon-γ-binding where two highly sulfated domains separated by N-acetylated domain are the target sequence in the HS chain [45]. Pathogens–HSPG interaction also seems to be mediated by a specific HS chain domain. Our data demonstrated an inhibition of 34% in the invasion of *T. cruzi* when trypomastigotes were pre-treated with a partially sulfated domain of heparan sulfate, suggesting the specific recognition of [GliUA-GlcNAc]-[GliUA-GliNS]$_3$-[GliUA-GlcNAc]$_4$ domain (NA/NS domain) by the HBP in the surface of the parasite. A specific-binding domain of heparan sulfate, the N-sulfated residues (IdoA2S-GlcN3S), has also been reported to be involved in the recognition of glycoprotein gD from *Herpes simplex* virus [46].

In addition, our data also revealed that treatment of cardiomyocytes cultures with sodium chlorate, an inhibitor of 3′-phosphoadenyl 5′-phosphosulfate (PAPS) that reduce sulfation and the presence of IdoA in HS structure [47], inhibits *T. cruzi* invasion process, suggesting that sulfation may modulate the invasion process, by acting as co-adjuvant molecule contributing to parasite–host cell association, but internalization of trypomastigotes may be triggered by the recognition and binding of the NA/NS domain by the parasite HBPs. This hypothesis is supported by the fact that chondroitin sulfate [29], keratan sulfate and also N-sulfated domain of HS do not affect the *T. cruzi* invasion process. In contrast, attachment and invasion of *Chlamydia trachomatis* was also inhibited by treatment with sodium chlorate, suggesting the role of N-sulfated residues of HS chain in the parasite–host cell interaction [48]. However, it seems that this mechanism of invasion depends on Chlamydia sp [49]. Microsporidian spore adherence to host cell is also dependent on a specific sulfated-glycan mechanism [26].

In summary, the results herein demonstrated that both trypomastigote and amastigote forms of *T. cruzi* presents a HBPs with molecular mass of 65.8 to 59 kDa, which binds to sulfated GAGs. The specific binding of the HBP-Tc to the N-acetylated/ N-sulfated domain of heparan sulfate chain may trigger the *T. cruzi* invasion in cardiomyocytes. Future structural analysis of the HS chain, such as minimum length of the disaccharide repeats and the level of sulfation as well as studies of signal transduction following ligand interaction is likely to yield information regarding the *T. cruzi*–HS specific domain interaction.

4. Materials and methods

4.1. Materials and chemicals

The following reagents were purchased from Sigma-Aldrich Chemical Co. (USA): Dulbeccos' modified Eagle medium (DMEM), detergents [Sodium dodecyl sulfate (SDS), Triton X-100 (TX-100), Triton X-114 (TX-114) and 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)], proteinases inhibitors [*trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane (E-64), 1,10-phenanthroline (o-phe), pepstatin A (pep A) and phenylmethylsulfonyl fluoride (PMSF)], molecular mass markers (SDS-6H and SDS-7), fetal bovine serum (FBS), gelatin, heparin–sepharose 4B column, horse serum (HS), L-glutamine, bovine serum albumin (BSA), methionine-free medium (RPMI), streptavidin conjugated to horseradish peroxidase and trypsin. SDS-PAGE reagents and nitrocellulose membrane were purchased from BioRad Laboratories Inc. The [^{35}S] methionine was purchase from Amersham Corp (UK) while the collagenase enzyme was obtained from Worthington (New Jersey, USA). The reagents for chemiluminescence detection (ECL-kit) were purchased from Santa Cruz Biotechnology, Inc. All other reagents were analytical grade or superior.

4.2. Cell culture

Primary cultures of cardiomyocytes were obtained from 18-day-old mouse embryos as previously described [50]. Briefly, cardiac fragments were dissociated in phosphate-buffered saline (PBS, pH 7.2) containing 0.025% trypsin plus 0.01% collagenase and plated (1×10^5 cells/ml) into 24-wells culture dishes containing glass coverslips previously coated with 0.01% gelatin. For biochemical analysis, the isolated cells were plated at a density of 2.5×10^6 cells in 60 mm culture dishes. The cells were cultivated in DMEM supplemented with 10% horse serum, 5% FBS, 2.5 mM $CaCl_2$, 1 mM L-glutamine, 2% chicken embryo extract and maintained at 37 °C in a 5% CO_2 atmosphere.

4.3. Parasite

Cell culture-derived trypomastigotes of *T. cruzi*, clones Dm28c, were obtained from infected Vero cells. Semi-confluent cultures were infected at a ratio of five parasites per host cell (5:1) and free trypomastigotes removed after 24 h of infection. After 4 days post-infection, trypomastigotes, released from the rupture of the cells, were removed and washed with DMEM supplemented with 0.5% BSA prior to GAG assay. A total of 10^{10} cells were harvested from *T. cruzi*-infected cultures and the protein extract was obtained using TX-114 methodology.

4.4. GAGs competition assay

To test the influence of GAGs in the parasite invasion, culture-derived trypomastigote forms were pre-treated for

1 h on ice with 5–20 μg/ml of heparin, keratan sulfate (KS) or three different domains of heparan sulfate (HS) chain in DMEM. The highly sulfated, N-acetylated/highly sulfated and N-acetylated domains of HS chain were obtained by heparitinase I and II activity and after treatment with nitrous acid at pH 1.5, respectively [51,52]. After pre-treatment with each compound, the trypomastigotes were incubated for 2 h at 37 °C with cardiomyocytes. After interaction, the cultures were fixed for 5 min at room temperature with Bouin (75 ml 1.2% aqueous picric acid solution, 25 ml formalin (40% formaldehyde) and 5 ml glacial acetic acid) and stained with Giemsa. A minimum of 200 cells in different microscopic fields was examined by light microscopy, and the percentage of infected cells was determined. The experimental assays were performed three times in duplicate.

4.5. Sodium chlorate treatment

To analyze the role of sulfation in the *T. cruzi*–cardiomyocyte recognition process, 24h-cardiomyocyte cultures were treated with 25, 50 or 75 mM of sodium chlorate in DMEM containing 0.5% BSA. After 24 h of sodium chlorate treatment, the cells were infected with trypomastigoteat a ratio of 10 parasites per host cell (10:1). After 2 h of interaction, the cultures were fixed with Bouin fixative and stained with Giemsa. A minimum of 200 cells in different microscopic fields were examined by light microscopy, and the percentage of infected cells was determined. The experimental assays were performed three times in duplicate. The sodium chlorate treatment neither affected the cardiomyocyte or the parasite viability, even after prolonged period. The cardiomyocytes retained their morphology and spontaneous contraction in all sodium chlorate concentrations. The parasite morphology and motility were not altered by sodium chlorate treatment.

4.6. Parasite-radiolabeled proteins

The metabolic radiolabeling assays were performed as previously described [53]. Briefly, trypomastigotes (2×10^7 cells/ml) were incubated for 1 h at 37 °C with 1 ml of methionine deficient RPMI prior to the addition of 100 μCi/ml of [^{35}S]methionine. After 2 h of incubation, fetal bovine serum (FBS) 1% was added and parasites were washed three times (3 000 × g, 15 min, 4 °C) and the pellet resuspended in PBS containing FBS 1%. The labeled parasites were washed three times with PBS without FBS and the aqueous and detergent soluble proteins were obtained by TX-114 methodology.

4.7. Protein extraction

Trypomastigote and amastigote forms (10^9 cells) were washed three times with phosphate buffered saline (PBS) pH 7.2 and incubated for 30 min at 4 °C with lysis buffer (10 mM Tris–HCl, pH 6.8 containing 1% TX-100 and proteinases inhibitors such as 10 mM of E-64, 1 mM of o-phe, 10 M of PMSF and 10 mM of pep A. Afterwards, the samples were centrifuged at $10\,000 \times g$ for 15 min at 4 °C and the total protein was stored at −20 °C. Alternatively, hydrophobic proteins of trypomastigotes were obtained by TX-114 phase separation technique [54,55]. Briefly, 10^{10} cells were extracted for 40 min on ice with 2% (w/v) TX-114 and soluble proteins were obtained after condensation at 37 °C followed by centrifugation ($12\,000 \times g$, 5 min, 25 °C) and treated with the proteinases inhibitors. Protein concentration was determined as previously described [56], using BSA as standard.

4.8. Affinity chromatography

After concentration of hydrophobic proteins, by centrifugation in Centripep 10 filters, the samples were submitted to a heparin affinity chromatography procedure [57]. Briefly, the hydrophobic proteins were dialyzed with equilibrium buffer (10 mM sodium phosphate solution pH 7.2, 0.15 M NaCl, 0.5% glycerol and 0.5% CHAPS and, then, passed through a heparin–sepharose 4B column (10 cm × 1.2 mm, ID) previously equilibrated in the same buffer (PBS containing 0.5% glycerol and 0.5% CHAPS). The column was washed with the equilibrium buffer until no further protein was detected by absorbance at 280 nm or liquid cintilography. The retained proteins were eluted using a stepwise gradient with the same buffer increased of different concentrations of NaCl (0.25 M to 3.0 M). The fraction eluted was concentrated by ultra filtration in Centripep 10 filters and stored at −20 °C until further analysis.

4.9. Electrophoresis and western blotting

SDS–PAGE was performed at room temperature in gels (12%) containing 0.1% SDS, as previously described [58]. Samples were dissolved in sample buffer (v/v; 80 mM Tris–HCl pH 6.8, 2% SDS, 12% glycerol, 5% β-mercaptoethanol and 0.015% bromophenol blue) and the electrophoresis was performed in running buffer (50 mM Tris–HCl pH 8.3, 0.19 M glycine and 0.1% SDS). After electrophoresis, the gels were stained by silver nitrate, as previously described [59]. The SDS-6H was used as molecular mass markers.

The total protein extract and heparin chromatography eluted proteins from trypomastigotes were separated by SDS–PAGE and transferred onto 0.2 μm nitrocellulose membranes in transfer buffer (25 mM Tris–HCl pH 8.3, 0.19 M glycine and 20% methanol), as previously described [60]. Non-specific-binding sites were blocked by incubating the membranes with PBS pH 7.2, containing 5% skim milk (w/v) and 0.5% Tween-20 (16 h, 4 °C). The nitrocellulose membranes were washed three times with PBS containing 0.05% Tween-20 (PBST) and incubated (1 h, 25 °C) with 2 μg/ml of biotin-conjugated heparin, heparan sulfate or chondroitin sulfate. After six washing cycles with PBST,

the blots were incubated (1 h, 25 °C) with a 1:100 dilution of streptavidin conjugated to horseradish peroxidase in PBST. After another cycle of six washes with PBST, the complex was revealed by ECL-kit.

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Regulation of extracellular matrix expression and distribution in *Trypanosoma cruzi*-infected cardiomyocytes

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Abstract

Alterations in the extracellular matrix have been observed in the cardiomyopathy of Chagas disease caused by *Trypanosoma cruz*i infection. However, the mechanism of extracellular matrix regulation in *T. cruzi*-infected cultured cardiomyocytes (CMs) is unclear. Using confocal laser microscopy, we demonstrated that treatment of these cultures with transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) leads to an enhancement of the fibronectin matrix only in uninfected CMs, while infected myocytes displayed low fibronectin expression. Digital image analysis also revealed low superposition of the fibronectin signal with parasite nests in cytokine treated and untreated cultures. Cytochalasin D treatment resulted in microfilament disarray that induced a disturbance in the fibronectin network of CMs, suggesting that cytoskeleton disruption caused by *T. cruzi* infection disorganizes the fibronectin matrix. Western blot analysis revealed a 2-fold increase in the fibronectin expression in CM cultures after cytokine treatment, whereas *T. cruzi* infection significantly reduced fibronectin levels in all conditions. In contrast, no change in the laminin expression was detected after cytokine treatment. Laminin distribution was altered in *T. cruzi*-infected CMs, with intense laminin labeling only at the cell periphery even after cytokine treatment. Our observations indicate that TGF-β and TNF-α stimulates fibronectin expression only in uninfected cells of the *T. cruzi*-infected cultures, whereas the cells harboring the parasites display low or no fibronectin fibrils.

Keywords: Cardiomyocyte; *Trypanosoma cruzi*; TGF-β; TNF-α; Fibronectin; Laminin

Introduction

Chagas disease, a progressive incapacitating illness affecting millions of people in Latin America (WHO, 2005), is caused by the flagellated protozoan *Trypanosoma cruzi* (Chagas, 1909). Chronic chagasic cardiomyopathy is the most devastating manifestation of Chagas disease, affecting approximately 30% of infected individuals (Higuchi et al., 2003). In experimental *T. cruzi* infection, several components of the extracellular matrix (ECM), such as collagen I, III, IV, fibronectin (FN), and laminin (LM), are enhanced in cardiac tissue and central nervous system during both the acute and chronic phases associated with

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inflammatory infiltrates (Andrade et al., 1989; Calvet et al., 2004; Garg et al., 2003; Mukherjee et al., 2003; Sanchez et al., 1993; Silva et al., 1999). Based on data from animal experiments, it is unclear if the ECM alterations during *T. cruzi* infection are a result of the parasitic infection or the subsequent inflammation. Evidence that the alterations are a direct result of the parasite per se comes from the observation that treatment of mice with trypanocidal agents leads to a reversion of the fibrosis process (Andrade et al., 1991). In addition, parasite-released antigens enhance ECM expression in uninfected cell lineages (Pinho et al., 2002). However, it has also been observed that ECM enhancement in the heart is associated with inflammatory infiltrates (Andrade et al., 1989; Calvet et al., 2004) and elevated cytokine levels in plasma (Araujo-Jorge et al., 2002; Waghabi et al., 2002), which may indicate that some inflammation process participates actively in this event.

Transforming growth factor beta (TGF-β) is a multifunctional cytokine synthesized during the immune response that controls proliferation, differentiation, and ECM transcription (Agrotis et al., 2005). The factor is secreted in a biologically inert form, the latent TGF-β. Once activated, mainly via proteolysis, it binds and activates 2 receptors (TGF-β receptors type I and type II), which phosphorylate TGF-β-specific intracellular effectors, the Smad proteins, resulting in signal transmission into the nucleus and activation of gene transcription (Lebrin et al., 2005). Several non-Smad signaling pathways are also activated by TGF-β and may influence the final outcome of TGF-β stimulation (Runyan et al., 2006). Recent studies have shown that *T. cruzi* directly triggers latent TGF-β activation (Waghabi et al., 2005), which may contribute to the pathogenesis of Chagas disease. It has been shown that alpha2-macroglobulin deficient mice display increased levels of TGF-β correlated with intense cardiac fibrosis (Waghabi et al., 2002). High levels of TGF-β in the plasma, ranging from 10 to 200 ng/ml, associated with fibrosis and phosphorylated Smad2 detection in the cardiomyocyte (CM) nuclei have also been demonstrated in the cardiac tissue of chronic chagasic patients, suggesting that TGF-β may represent a prognostic marker of Chagas disease progression (Araujo-Jorge et al., 2002).

Tumor necrosis factor alpha (TNF-α) is important in the pathogenesis of heart failure (Garza et al., 2002). TNF-α is synthesized as a transmembrane protein, and cleaved in the extracellular domain by a TNF-α converting enzyme (TACE) to release a mature soluble 17-kDa protein, which forms a biologically active homotrimer (Palladino et al., 2003). Binding of TNF-α to its two receptors (type 1 – TNFR1 and type II – TNFR2) results in activation of complex intracellular signaling processes that lead to differentiation, activation, release of pro-inflammatory mediators and apoptosis, through the recruitment and activation of several adaptor proteins, reviewed elsewhere (Wajant et al., 2001). Elevated levels of TNF-α in the plasma of Chagas patients suggest that chronic TNF-α production, prior to heart failure, may play a role in Chagas cardiomyopathy (Mocelin et al., 2005). TNF-α has been shown to be capable of stimulating ECM synthesis and elevating matrix metalloproteinase activity in the heart in several models of cardiomyopathy (Li et al., 2000; Sun et al., 2007).

In spite of all these observations, the mechanisms underlying ECM alterations and the role of the CM in this process have not been fully investigated. Previously, we have demonstrated a reduction in fibronectin (FN) expression and a redistribution of laminin (LM) in *T. cruzi*-infected, cultured cardiomyocytes (Calvet et al., 2004). We hypothesized that the addition of immune mediators, such as TGF-β and TNF-α, would be able to reverse the ECM reduction in vitro. Our results demonstrate that TGF-β and TNF-α stimulate FN expression only in uninfected cells of the *T. cruzi*-infected cultures and the low expression of FN remained unaltered in highly infected CMs.

Materials and methods

Primary cell culture

CMs were isolated from 18-day-old mouse embryos as previously described (Meirelles et al., 1986). Cardiac fragments were dissociated in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 0.88 mM KH_2PO_4, 6.4 mM Na_2HPO_4, pH 7.2) containing 0.025% trypsin and 0.01% collagenase (Worthington Co., Lakewood, NJ, USA) and plated into a 24-well plate (10^5 cells/ml) containing glass coverslips coated with 0.01% gelatin. Although cardiomyocytes constitute approximately 70% of the total cells, the culture still displayed minor myoblasts and fibroblasts. Cultures were cultivated in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma), 2.5 mM $CaCl_2$, 1 mM L-glutamine and 2% chicken embryo extract, and maintained at 37 °C in an atmosphere of 5% CO_2.

Parasites and cell culture infection

Bloodstream trypomastigote forms of *T. cruzi* (Y strain) were obtained from Swiss Webster mice at the peak of parasitemia as previously described (Meirelles et al., 1984). Muscle cell cultures were infected at a multiplicity of infection of 10 parasites per host cell. After 24 h of interaction, free trypanosomes in the

medium were removed by washing the cultures with Ringer's solution (154 mM NaCl, 56 mM KCl, 17 mM Na_2HPO_4, pH 7.0), and fresh medium was added to the culture. The time course of infection was interrupted after 48 and 72 h.

Treatment of the cultures with TGF-β or TNF-α

The uninfected and *T. cruzi*-infected (24 h) CMs were washed in Ringer's solution to remove FBS contained in the nutrient medium. Cytokine treatment was performed by diluting the cytokines in DMEM supplemented with 1% bovine serum albumin (BSA; Sigma) and 2.5 mM $CaCl_2$. TGF-β from bovine platelets (Promega, Madison, WI, USA) was added at a concentration of 1, 2, 5, 10, or 15 ng/ml; TNF-α (Sigma) was diluted to 50 or 100 ng/ml. The concentrations of cytokines used in the experiments were similar to the levels detected in the plasma of chagasic patients (Araujo-Jorge et al., 2002). The medium containing cytokines was replaced every 24 h, and cultures were fixed after 48 and 72 h of infection as described in the indirect immunofluorescence assay. Persistence of the TGF-β used in the experiments was assessed using Mv1Lu cells stably transfected with a TGF-β responsive reporter gene (Abe et al., 1994).

Cytoskeleton disruption assay

To inhibit polymerization of actin filaments and microtubules, 96 h-cultured CMs were incubated for 4 h at 37 °C with 12.5 μg/ml cytochalasin D (Sigma) and 15 μg/ml nocodazole (Sigma), respectively, in DMEM supplemented with 1% BSA and 2.5 mM $CaCl_2$. The long-term treatment was necessary to disrupt the well-organized myofibrils of CMs (Barbosa and Meirelles, 1995). After drug treatment, FN and LM distribution was analyzed by indirect immunofluorescence.

Indirect immunofluorescence

The uninfected and *T. cruzi*-infected CMs were fixed for 5 min at room temperature in 4% paraformaldehyde in phosphate buffered saline (PBS), followed by 3 rinses in PBS. To avoid nonspecific binding, the cells were submitted to 3 washes (20 min each) with PBS containing 4% BSA. Cells were then incubated overnight at 4 °C with anti-fibronectin (1:800) or anti-laminin (1:600) antibodies (Sigma). To control drug activity in the cytoskeleton disruption assay, the coverslips treated with nocodazole and cytochalasin D were incubated with an anti-tubulin antibody (1:100; Sigma) and phalloidin-FITC (1:300; Sigma), respectively. After rinsing, the cells were incubated for 1 h at 37 °C in anti-rabbit IgG-TRITC conjugated antibody (1:400; Sigma) or anti-mouse IgG FITC-conjugated antibody (1:200; Sigma). To stain nuclei, 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was used. The coverslips were mounted with 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO; Sigma) and 50% glycerol in PBS and examined with an Olympus Scanning Confocal laser or with a Zeiss LSM 510 Meta microscope. The images were submitted to digital processing using KS 400 3.0 (Zeiss) software.

Protein extraction and Western blotting

Total proteins were extracted using a lysis buffer (50 mM Tris–HCl, NaCl 150 mM, 1% Triton X-100, 1 mM ethylene glycol-bis-(2-amino-ethyl ether) *N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA; Sigma), 100 μg/ml phenylmethylsulfonyl fluoride (PMSF; Sigma), 1 μg/ml pepstatin (Sigma), and 1 μg aprotinin, pH 8.0). The amount of protein was determined by the Folin-Lowry method (Lowry et al., 1951). A total of 20 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked overnight at room temperature with PBS containing 5% non-fat dry milk and 0.1% Tween 20 (Sigma). Detection of fibronectin and laminin was performed by incubation of the membranes for 1 h at 37 °C with rabbit anti-fibronectin (1:5000) or anti-laminin (1:5000) antibodies diluted in blocking solution. Mouse anti-GAPDH (1:30,000; Ambion, Austin, TX, USA) antibodies combined with primary antibodies were used as the internal control. After several washes, the membranes were incubated with anti-mouse (1:30,000; Pierce Biotechnology, Rockford, IL, USA) or anti-rabbit (1:30,000; Pierce Biotechnology) antibody conjugated to horseradish peroxidase. The enzyme activity was revealed with a SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology). The densitometry of the bands was performed with Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/), and normalized to the GAPDH band (~36 kDa), which was used as the internal control. The immunoblotting experiments were performed independently at least 3 times.

Statistical analysis

For comparison of the mean values of pixel intensities obtained with KS 400 software and of the densitometry of bands generated by Western blots, the Student's *t*-test was used; values were considered statistically significant when $p \leqslant 0.05$.

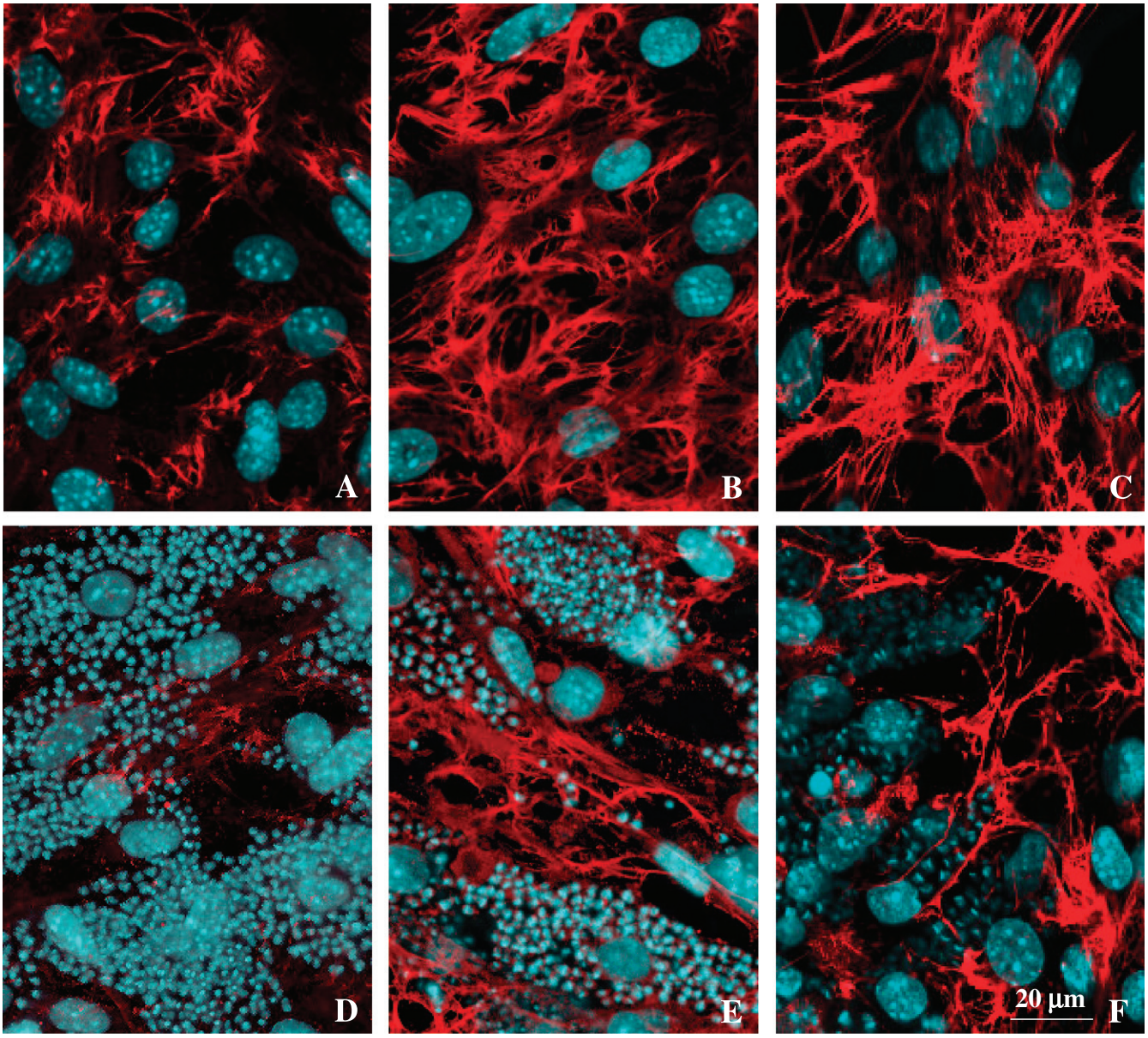

Fig. 1. Visualization of fibronectin (FN) distribution by confocal laser microscopy. Double labeling of uninfected and *T. cruzi*-infected cardiomyocytes with anti-fibronectin antibody (red) and DAPI (blue) to detect fibronectin and DNA, respectively. (A) Cardiomyocytes after 96 h of culture showing an interconnected net of FN fibrils. An enhancement of the FN matrix was noticed after treatment of the cardiomyocyte cultures with 15 ng/ml of TGF-β (B) and 100 ng/ml of TNF-α (C). *T. cruzi* infection (72 h) induced a decrease of FN fibrils over highly infected cells, whereas adjacent uninfected cells still display a typical FN matrix (D). The stimulation of the FN matrix by TGF-β (E) and TNF-α (F) was also observed in *T. cruzi*-infected cultures, but not in areas with prevalence of highly infected cells, which displayed a low FN signal. DAPI stained the nuclei of cells and the nuclei and the kinetoplast of parasites in the *T. cruzi*-infected cultures.

Results

Cytokine treatment triggers FN overexpression

FN matrix was visualized as an interconnected network arranged in fibrils at the surface of CMs by indirect immunofluorescence (Fig. 1A). Addition of 1, 2, and 5 ng/ml of TGF-β or 50 ng/ml of TNF-α in CM cultures did not elicit any effect in FN staining (data not shown). However, a remarkable change on the typical FN distribution was observed after treatment with 15 ng/ml of TGF-β, showing an expressive dose-dependent enhancement of FN that appears as highly interconnected and thick fibrils (Fig. 1B). The Mv1Lu cells, used as a positive control of the TGF-β effect, responded to TGF-β treatment in concentrations

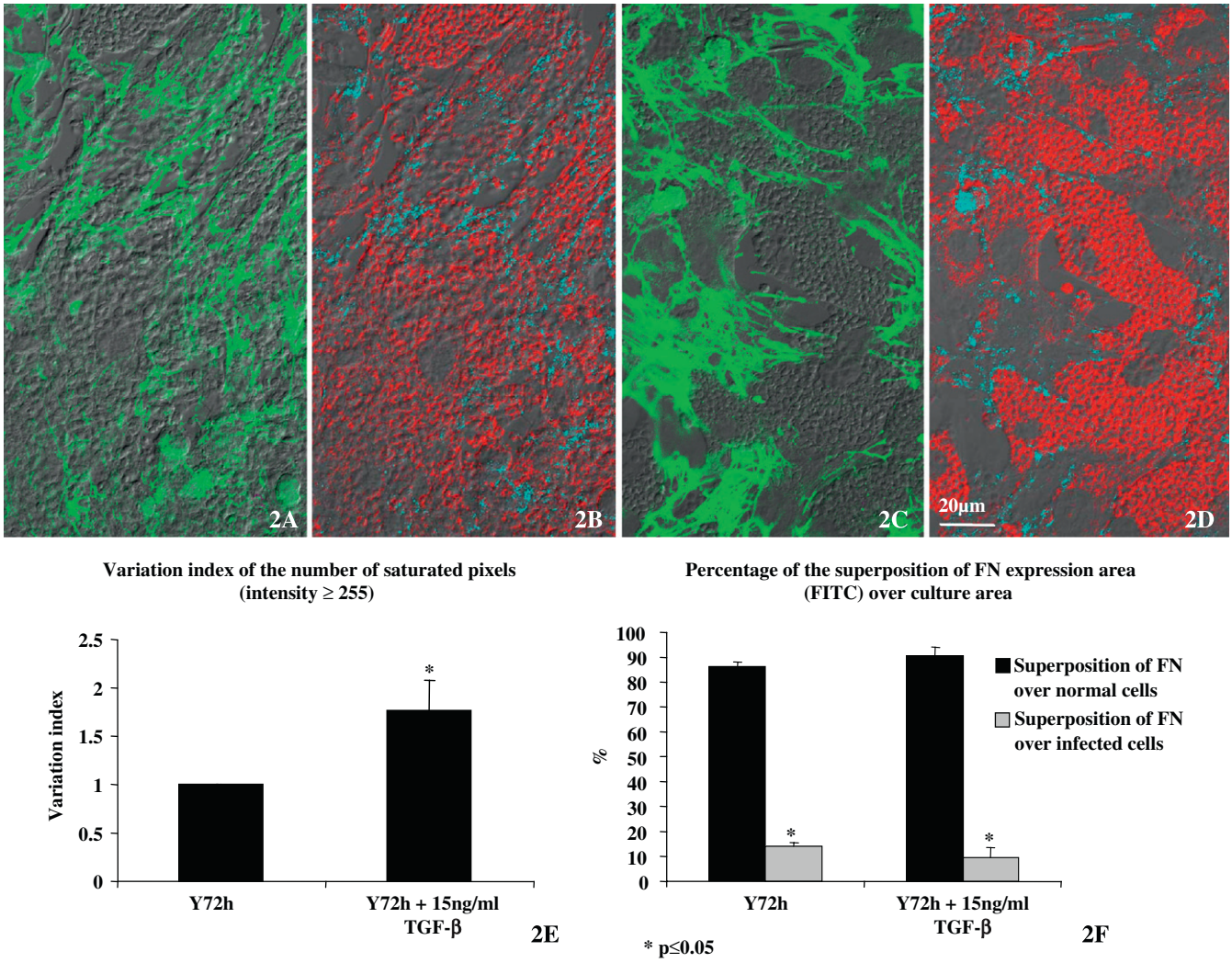

Fig. 2. Digital image processing using KS 400 software (Zeiss). Fibronectin (FN) immunodetection (green) in *T. cruzi*-infected cardiomyocyte cultures merged with DIC imaging, which allow the visualization of infected cells either untreated (A) or TGF-β-treated (C) cultures. KS 400 analysis images display the low superposed area (blue) of FN expression (green) on infected cells (red) (B), even after TGF-β treatment (D). Quantification of the number of pixels with saturated intensities from green channel (FN staining) obtained from untreated and TGF-β-treated culture images (E). Note the significant 1.76-fold increase ($p \leqslant 0.02$) in the number of saturated pixels after TGF-β treatment. Quantitative data of the area of fibronectin expression superposed over culture area obtained using KS 400 (F). Although TGF-β altered the FN intensity (E), it did not change the low superposition of FN staining over infected cells (F), showing that uninfected cells remained with more than 86% of the FN expression area both in untreated and treated cells. Student's *t* test: $p \leqslant 0.05$.

ranging from 0.1 to 10 ng/ml (data not shown). Treatment of CM cultures with 100 ng/ml of TNF-α also altered the FN network, resulting in an enlargement of the FN fibrils (Fig. 1C) compared to untreated CMs (Fig. 1A).

As previously described (Calvet et al., 2004), *T. cruzi* infection causes a reduction in FN fibrils over highly infected cells, whereas adjacent uninfected cells in the infected cultures display an FN organization similar to control cultures (Figs. 1D and 2A). The stimulation of the FN matrix was also observed in *T. cruzi*-infected cultures, but a low expression of FN was still detected in areas prevalent of highly infected cells, even after TGF-β treatment (Figs. 1E and 2C). Similarly, TNF-α treatment did not promote the FN remodeling in highly *T. cruzi*-infected CMs (Fig. 1F), and an enlargement of the FN network induced by TNF-α treatment was also clearly observed only in uninfected cells within infected cultures (Fig. 1F).

Localization and quantitation of FN enhancement by digital image analysis

We addressed the question whether the FN intensification induced by TGF-β treatment correlated with the

uninfected cells area in *T. cruzi*-infected cultures. To confirm the enhancement of FN expression in TGF-β-treated cultured CMs, KS 400 software was utilized to quantify the number of pixels with saturated intensities (value $\geqslant$255, indicating high fluorescence emission). First, we evaluated the expression of FN in untreated and in TGF-β-treated cultures. Our results demonstrated a significant increase of 1.76 fold ($p \leqslant 0.02$) in the number of pixels with saturated intensities in TGF-β-treated CM cultures, thus confirming the enhancement of FN expression revealed by indirect immunofluorescence (Fig. 2E).

We then addressed the question whether the FN intensification induced by TGF-β treatment correlated with the uninfected cells in *T. cruzi*-infected cultures. Therefore, to verify the precise localization of the FN meshwork in *T. cruzi*-infected cultures, KS 400 software identified the uninfected and infected areas in the CM cultures, considering the parasite granularity in differential interference contrast (DIC) images and the FN signal intensity demonstrated in green. Next, the software segmented the infected area of the culture images obtained by DIC using texture tools, showing the parasite nests in red (Fig. 2B and D). Then KS 400 calculated the percentage of superposition of the area occupied by FN expression (Fig. 2A and C, green) over parasite nest area (Fig. 2B and D, red). The superimposed area (blue) was very low, indicating that the majority of FN expression was localized over uninfected CMs (Fig. 2B and D). The fact that TGF-β treatment was not able to promote FN deposition in infected CMs was confirmed by image analysis, showing rare FN

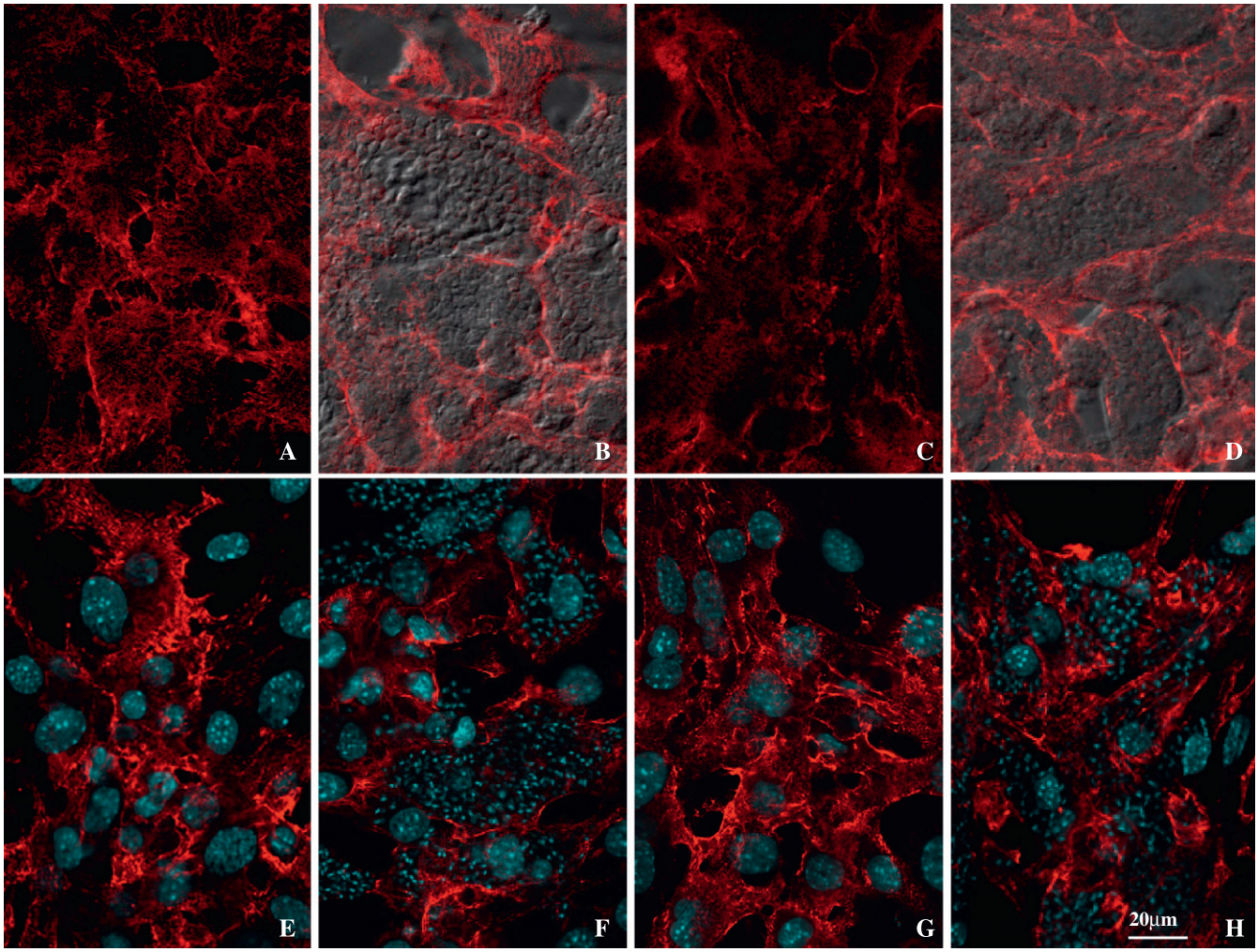

Fig. 3. Detection of laminin (LM) in uninfected (A, C, E, and G) and *T. cruzi*-infected cardiomyocyte cultures (B, D, F, and H) using confocal laser microscopy. LM is distributed in a widespread and a punctuate pattern in cardiomyocytes (A and E) without changing its localization or intensity after TGF-β (C) and TNF-α (G) treatment. After 72 h of infection, the LM was localized at the border of the cardiomyocytes (B and F) even after stimulation with TGF-β (D) and TNF-α (H). The treatment of both cytokines resulted in the same pattern of LM staining of its untreated peers. DIC images allowed the visualization of the intracellular parasites (B and D). DAPI (blue) stained host cell nuclei and also the kinetoplast and the nuclei of the parasites (E–H).

fibrils associated with highly infected cells (Fig. 2C and D). The KS 400 quantitative data revealed that only 13.89% of the FN expression area was localized over *T. cruzi*-infected cells in untreated CMs, while 86.11% of FN distribution was correlated to uninfected CM area. Although FN expression was intensified in *T. cruzi*-infected CM cultures after TGF-β treatment, the FN fibrils were preferentially detected in uninfected CMs, comprising 90.64% of the FN area, whereas only an average of 9.36% of the FN expression area was localized over *T. cruzi*-infected cells (Fig. 2F).

Effect of cytokines in laminin expression

We previously reported that the punctate and spread pattern of laminin in CMs (Fig. 3A and E) was altered after *T. cruzi* infection and was concentrated at the borders of infected cells (Fig. 3B and F) (Calvet et al., 2004). In order to determine whether cytokines can induce the rearrangement of LM distribution in *T. cruzi*-infected CMs, uninfected and infected CM cultures were treated with TGF-β and TNF-α. Confocal laser microscopy analysis revealed that neither TGF-β nor TNF-α affected LM distribution. Even high doses of TGF-β and TNF-α treatment, 15 and 100 ng/ml, respectively, did not induce the LM overexpression in CM cultures (Fig. 3C and G) or redistribution in highly infected cells. The distribution was maintained at the cell periphery (Fig. 3D and H).

Effect of TGF-β and TNF-α on extracellular matrix (ECM) expression

To determine if *T. cruzi* infection alters FN and LM expression, protein extracts of uninfected and *T. cruzi*-infected CMs were taken for FN detection by Western

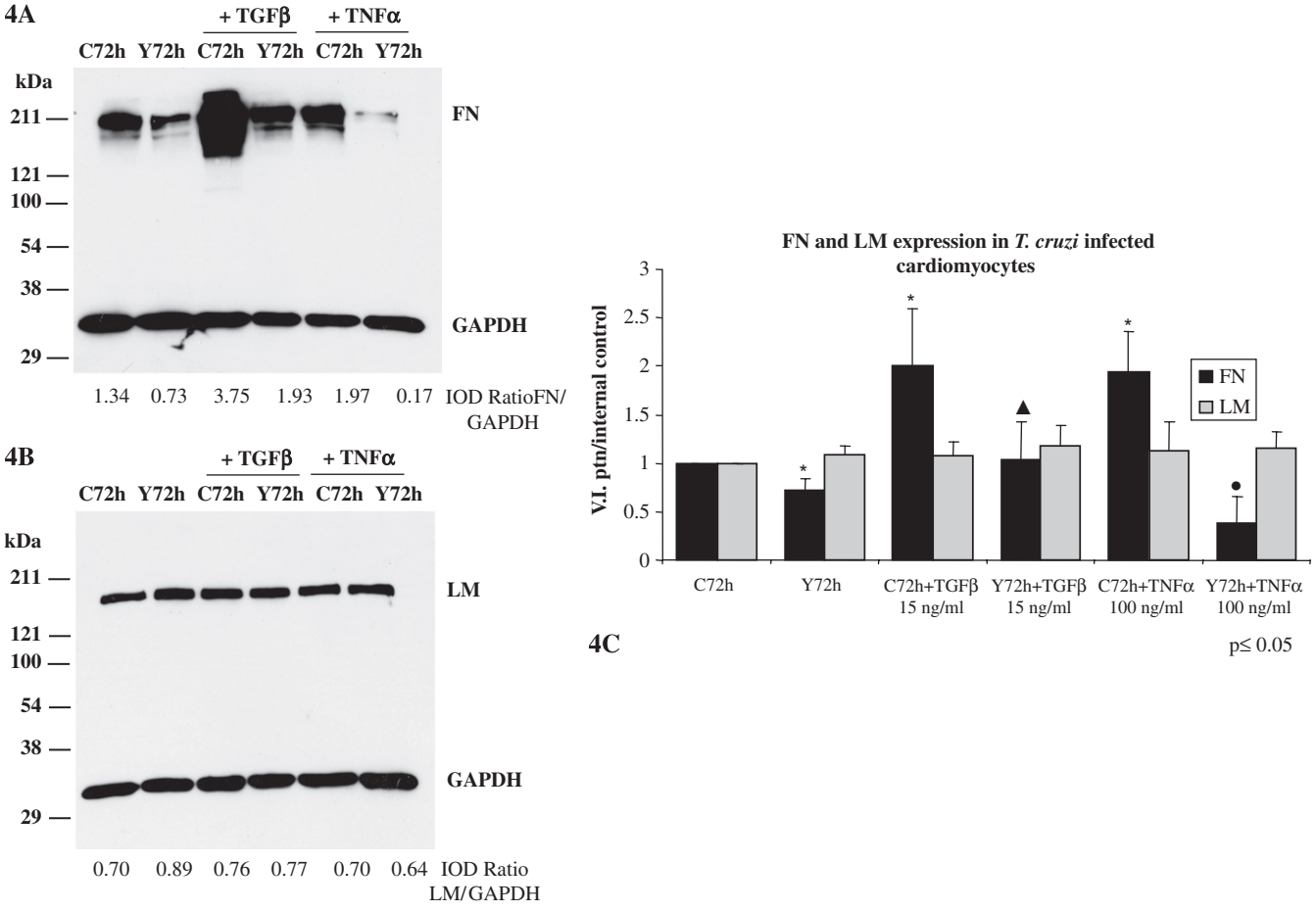

Fig. 4. Immunoblot detection of fibronectin (FN) (A) and laminin (LM) (B) in uninfected and *T. cruzi*-infected cardiomyocyte culture extracts. The integrated optical density (IOD) ratios were obtained from FN (A) and LM (B) values normalized with GAPDH values used as internal control. Note the reduction of FN expression after *T. cruzi* infection and its expressive augment after TGF-β and TNF-α treatment, which was significantly repressed by *T. cruzi* infection (A and C). LM levels remained unaltered in cardiomyocyte cultures either after *T. cruzi* infection or cytokine treatment (B and C). FN and LM levels obtained from 4 separated experiments show the statistically significant effects in FN expression (C). Student's *t* test: statistically significant * versus C72h, $p \leqslant 0.02$; ▲ versus C72h + TGF-β, $p \leqslant 0.04$; ● versus C72h + TNF-α, $p \leqslant 0.002$.

blotting. The immunoblots revealed that 72 h-infected cultures display a lower FN expression when compared with the controls (Fig. 4A). Densitometric analysis of the bands, using GAPDH as an internal control, demonstrated a significant decrease of 28% in FN levels ($p \leqslant 0.003$) in 72 h-infected CMs (Fig. 4A and C), a reduction that reached a maximum of 46% in a representative experiment (Fig. 4C). As expected, TGF-β and TNF-α treatment induced a significant enhancement of FN levels ($p \leqslant 0.02$), both corresponding to

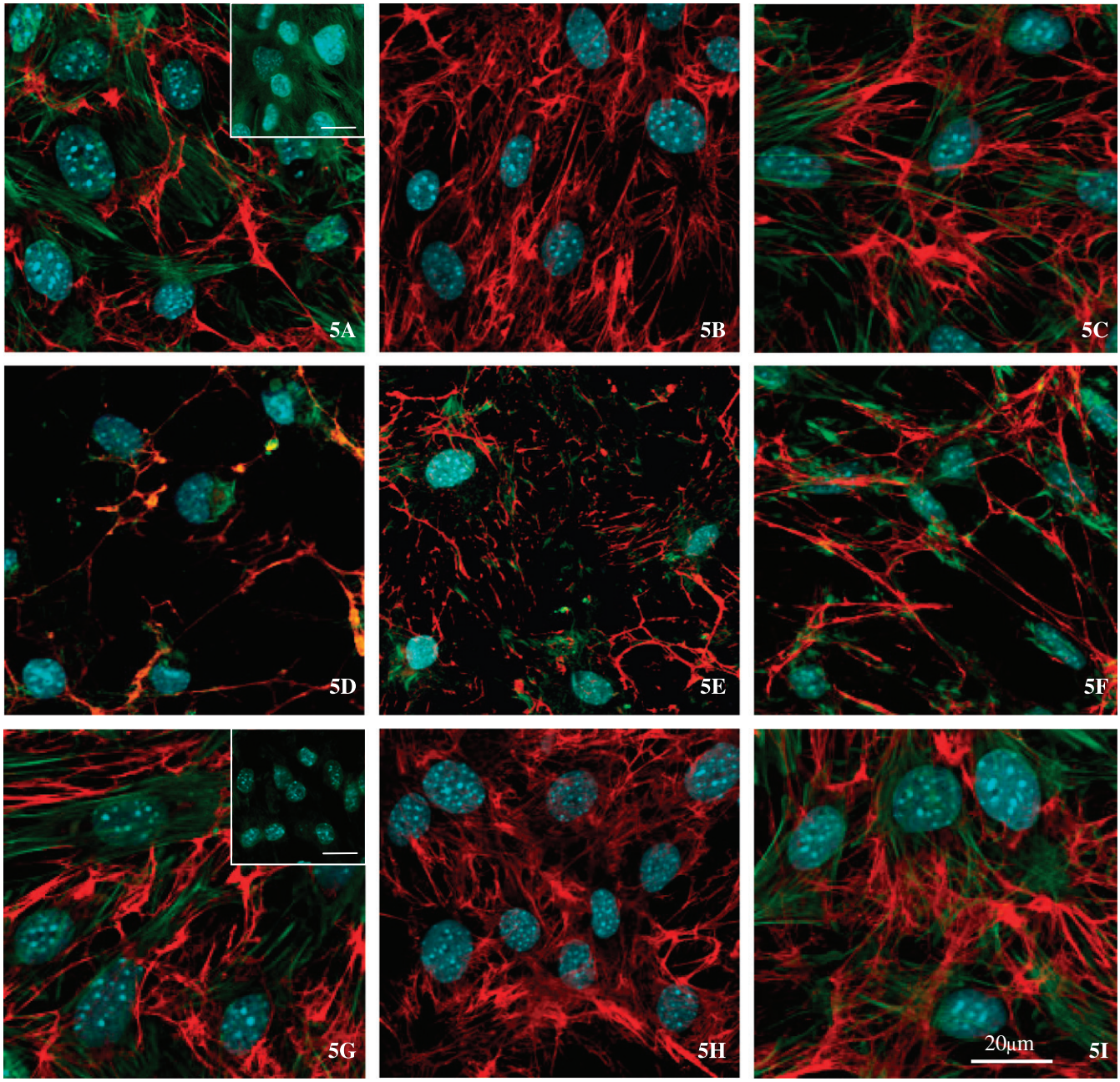

Fig. 5. Effect of cytoskeleton disrupting agents in the fibronectin (FN) matrix of cardiomyocytes. Triple labeling of cardiomyocytes with anti-fibronectin antibody (red), phalloidin-FITC or anti-tubulin antibody (green), and DAPI (blue) to reveal FN, actin filaments or microtubules, and DNA, respectively. (A) Control, untreated cardiomyocytes display normal FN network and actin microfilament distribution. The inset in (A) shows microtubule organization stained in green. An enhancement of fibronectin matrix was detected in cardiomyocyte cultures after TGF-β (B) and TNF-α (C) treatment. Cytochalasin D exposure resulted in microfilaments disruption and FN network disorganization (D–F). TGF-β (E) and TNF-α (F) pre-incubation did not prevent FN matrix disruption. Nocodazole treatment, which depolymerizes microtubules in cardiomyocyte cultures, as demonstrated by the low microtubule immunostaining in the inset in (G), neither affected microfilament (green) nor FN fibril (red) organization (G–I). The latter was intensified for TGF-β (H) and TNF-α (I) treatment, even in the presence of nocodazole.

approximately a two-fold increase, when compared with the densitometric values of FN expression in control cultures (Fig. 4). Although cytokine treatment resulted in a 30% increase in the FN expression levels of infected cultures, a reduction of 50% and 80% of FN levels was still observed after TGF-β and TNF-α treatment, respectively, compared to the level of cytokine-treated uninfected cultures (Fig. 4). These observations confirm the KS 400 image analysis (Fig. 4A and C).

Although both qualitative and quantitative data revealed that TGF-β and TNF-α treatment induced an enhancement of FN expression, immunodetection analysis demonstrated that neither cytokine was able to modify the LM deposition. To verify if cytokine treatment stimulates LM synthesis, we performed Western blotting to determine the effect of cytokines on uninfected and *T. cruzi*-infected CMs as well as cells stimulated with TGF-β and TNF-α. Our results demonstrated that LM expression is very stable in CMs. Neither *T. cruzi* infection nor TGF-β or TNF-α treatment altered LM levels in CM extracts in any concentration tested (Fig. 4B and C).

Cytoskeleton disruption affects FN organization

T. cruzi infection disrupts the cytoskeleton of CMs (Pereira et al., 1993), and the FN matrix is dependent on intact actin microfilaments in cell lineages (Wu et al., 1995). Therefore, we hypothesized that the cytoskeleton breakdown caused by the parasite may also be responsible for FN matrix disorganization in infected CMs. The cytoskeleton breakdown caused by the parasite was reproduced with drugs that interfere with actin and microtubule polymerization, cytochalasin D, and nocodazole, respectively, in cultures pre-treated with TGF-β and TNF-α. Confocal laser microscopy analysis of FN immunodetection demonstrated that actin cytoskeleton disruption resulted in FN matrix disorganization. Cytochalasin D-treated cells presented low or absent FN staining or fibrils (Fig. 5D). TGF-β (Fig. 5E) and TNF-α (Fig. 5F) pre-treatment did not prevent cytoskeleton disarray or orchestrate FN matrix rearrangement. Disturbance in the FN distribution was evident only after actin disorganization, while nocodazole treatment, which depolymerizes the microtubules, did not affect the FN matrix (Fig. 5G–I).

Discussion

TGF-β is a strong profibrogenic cytokine implicated in the genesis of fibrosis associated with chagasic cardiomyopathy fibrosis (Araujo-Jorge et al., 2002; Magalhaes-Santos and Andrade, 2005). It is also known to promote ECM accumulation. TNF-α also regulates ECM deposition and is associated with chagasic cardiomyopathy (Sun et al., 2007; Mocelin et al., 2005). The accumulation of FN and LM in vivo has previously been described during acute and chronic phase of Chagas disease (Andrade et al., 1989; Calvet et al., 2004; Garg et al., 2003; Mukherjee et al., 2003). However, little attention has been given to the mechanism of ECM regulation during *T. cruzi*–CM interaction.

Our results revealed that stimulation of CM cultures with TGF-β and TNF-α induced an enhancement of the FN network and also increased FN protein levels, suggesting that these cytokines are effective in stimulating FN gene expression. This observation is consistent with previous reports showing the fibrogenic effect of TGF-β in the heart and in cardiac fibroblasts (Leask, 2007; Leask and Abraham, 2004). In contrast, the role of TNF-α on ECM regulation is still controversial. While heart overexpression of TNF-α resulted in an increased collagen deposition in mice (Li et al., 2000), the treatment of fibroblasts with TNF-α in vitro resulted in a reduction in collagen synthesis (Siwik et al., 2000). Our data reinforce the idea that TNF-α contributes to cardiac fibrosis via the stimulation of FN synthesis.

Although cytokine treatment promotes FN overexpression, LM distribution and expression remained unaltered in CMs. This may be the result of different signaling mechanisms that lead to protein synthesis and regulation of its organization (Mao and Schwarzbauer, 2004; Wierzbicka-Patynowski and Schwarzbauer, 2003; Yurchenco et al., 2004). A distinct pathway of TGF-β activation without the requirement of Smad4 through the c-Jun N-terminal kinase (JNK) pathway has been demonstrated to induce FN remodeling in the human fibrosarcoma HT1080-derived cell line BAHgpt (Hocevar et al., 1999). Therefore, the JNK pathway or an alternative TGF-β signaling pathway may be immediately stimulated by TGF-β inducing a rapid FN, but not LM, remodeling in CMs. The stability of LM synthesis in CMs after cytokine stimulation differs from previous reports that demonstrated an enhancement of LM synthesis after TGF-β treatment (Jiang et al., 2005; Kawano et al., 2000). This observation may be related to a low response of CMs to TGF-β and TNF-α, as only doses of 15 and 100 ng/ml of these cytokines, respectively, were able to induce FN expression, whereas lower doses (1–2 ng/ml) stimulate other cell types (Vásquez et al., 2007). Additionally, even if LM expression was stimulated by the cytokines, the competency of different cells to assemble basement membranes is regulated by expression of sulfated glycolipids, which is the first step in the anchoring of LM molecules to the cell surface triggering LM matrix assembly (Li et al., 2005). It is possible that the expression of sulfated glycolipids may also be kept unchanged in CMs after TGF-β or TNF-α stimulation, resulting in no alteration in LM organization at the cell surface.

In the present study, we found that the *T. cruzi*-infected, cultured CMs display a reduced response to TGF-β and TNF-α stimulation so that they are unable to re-establish their FN and LM matrix. It appears that this is a direct, parasite-driven effect since adjacent uninfected cells are capable of responding to cytokine treatment. *T. cruzi* infection causes a down-regulation of cytokine receptors on the host cell (Sztein and Kierszenbaum, 1992; Majumder and Kierzenbaum, 1996), including a down-regulation of mannose receptors in CM primary culture (Soeiro et al., 1999). The possibility of down-modulation of TGF-β and TNF-α receptors, resulting from *T. cruzi* infection in CMs rendering the infected cells unresponsive to cytokine treatment cannot be ruled out and is currently under investigation.

T. cruzi can alter ECM expression and distribution at several levels. For example, the infection can influence protein synthesis and protein secretion and the disarray of ECM assembly process, which is a cell-mediated event dependent on ECM receptors (integrins and syndecans) and signaling mechanisms that orchestrate cytoskeleton rearrangement resulting in subsequent polymerization of ECM components (Mao and Schwarzbauer, 2004; Yurchenco et al., 2004). We found that *T. cruzi* inhibits FN synthesis but does not alter LM expression. Even after cytokine treatment, *T. cruzi*-infected cultures still displayed reduced FN expression when compared with uninfected cultures. FN reduction as a result of *T. cruzi* infection may be related to the inhibition of factors that regulate ECM transcription, such as connective tissue growth factor (CTGF, also nominated CCN2). CTGF was shown to be repressed by *T. cruzi* in dermal fibroblasts, which led to a down-regulation of FN and collagen. This repression was also present as a result of TGF-β treatment (Unnikrishnan and Burleigh, 2004), and it may occur in our model of *T. cruzi*–CM interaction, inhibiting FN stimulation. In addition, it has been shown that TNF-α induces CTGF expression and synergistically with TGF-β regulates the renal fibrosis (Cooker et al., 2007). Therefore, this *T. cruzi*-dependent repression of CTGF (Unnikrishnan and Burleigh, 2004) may also be responsible for the low FN expression in the infected/TNF-α-treated cultures.

Besides affecting a mechanism that interferes with ECM metabolism, the alteration in the FN distribution may also be related to the cytoskeleton disruption caused by *T. cruzi* infection. FN matrix assembly is dependent on an intact cytoskeleton (Wu et al., 1995), generating the tension required for expansion of compact FN dimers and exposure of FN–FN binding sites (Wierzbicka-Patynowski and Schwarzbauer, 2003). *T. cruzi*-infected CMs have a disorganized cytoskeleton with myofibrillar breakdown (Pereira et al., 1993; Melo et al., 2006; Taniwaki et al., 2006). Thus, we examined the effect of cytochalasin D and nocodazole on the FN matrix arrangement. We found that actin cytoskeleton disorganization resulted in a low or complete lack of FN fibrils on the CM surface, and TGF-β and TNF-α pre-treatment did not repair the FN network. These observations suggest that *T. cruzi*-associated cytoskeleton breakdown, together with a decreased FN synthesis, results in a reduction of FN matrix on the infected CM surface in the absence of cytokines. Nevertheless, the infection can alter other steps important to FN fibrillogenesis, such as integrin or syndecan expression, or signaling events that regulate this process. This is the focus of ongoing investigations.

Interestingly, *T. cruzi* infection affected only the LM distribution in CMs without affecting its expression. These observations are different from other in vitro models that showed an intensification of LM staining triggered by the parasite or its antigens (Sanchez et al., 1993; Pinho et al., 2002). The concentration of LM at the borders of the infected cells may be due to altered signaling events after *T. cruzi* infection, since aberrant localization of LM in dense patches at the cell periphery was also observed on epithelial cells treated with Y-27632, an inhibitor of Rho-kinase (deHart and Jones, 2004).

The tridimensional model of CM culture displayed a general enhancement of FN and LM expression after *T. cruzi* infection, whereas infected cells presented only low FN staining (Garzoni et al., 2007) similar to our observations. However, ECM expression in vivo during acute and chronic experimental infection is enhanced (Andrade et al., 1989; Calvet et al., 2004; Garg et al., 2003; Mukherjee et al., 2003; Sanchez et al., 1993; Silva et al., 1999) and is associated with inflammatory lesions. This suggests that the inflammatory process and its soluble mediators are involved in the ECM stimulation. In fact, there is no doubt that an enhancement is observed of ECM in the cardiac tissue. However, since the infection rate is generally very low in tissue, with scarce parasite nests, the specific ECM expression in the infected CM in the myocardium has never been addressed.

On the basis of our data, we suggest that cytokines synthesized by inflammatory cells stimulate FN expression only on uninfected cells, whereas the cell harboring the parasites presents low or no FN fibrils. Further studies will be performed to determine if this specific event in *T. cruzi*-infected CMs is related to cytokine receptor expression and distribution or associated with alterations in cytokines-induced signaling.

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Leishmania (Viannia) braziliensis: insights on subcellular distribution and biochemical properties of heparin-binding proteins

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SUMMARY

Leishmaniasis is a vector-borne disease and an important public health issue. Glycosaminoglycan ligands in *Leishmania* parasites are potential targets for new strategies to control this disease. We report the subcellular distribution of heparin-binding proteins (HBPs) in *Leishmania (Viannia) braziliensis* and specific biochemical characteristics of *L. (V.) braziliensis* HBPs. Promastigotes were fractionated, and flagella and membrane samples were applied to HiTrap Heparin affinity chromatography columns. Heparin-bound fractions from flagella and membrane samples were designated HBP F_f and HBP M_f, respectively. Fraction HBP F_f presented a higher concentration of HBPs relative to HBP M_f, and SDS-PAGE analyses showed 2 major protein bands in both fractions (65 and 55 kDa). The 65 kDa band showed gelatinolytic activity and was sensitive to inhibition by 1,10-phenanthroline. The localization of HBPs on the promastigote surfaces was confirmed using surface plasmon resonance (SPR) biosensor analysis by binding the parasites to a heparin-coated sensor chip; that was inhibited in a dose-dependent manner by pre-incubating the parasites with variable concentrations of heparin, thus indicating distinct heparin-binding capacities for the two fractions. In conclusion, protein fractions isolated from either the flagella or membranes of *L. (V.) braziliensis* promastigotes have characteristics of metallo-proteinases and are able to bind to glycosaminoglycans.

Key words: *Leishmania (Viannia) braziliensis*, glycosaminoglycans, heparin, heparin-binding protein, surface plasmon resonance, metallo-proteinase.

INTRODUCTION

The genus *Leishmania* (Kinetoplastida: Trypanosomatidae) includes species that are causative agents of leishmaniases, which are widely spread anthropozoonotic diseases in tropical and subtropical regions. In Brazil, *Leishmania (Viannia) braziliensis* is the main species involved in the cutaneous and mucocutaneous forms of the disease (Reithinger *et al*. 2007). In the southeast and northeast regions of Brazil, this species is mainly transmitted by the sand flies *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (Nyssomyia) whitmani* (Rangel and Lainson, 2003).

During its life cycle, *Leishmania* parasites alternate between promastigote and amastigote forms within the invertebrate and vertebrate hosts, respectively. The former forms survive in the sand fly digestive tract lumen, whereas the latter are found in the parasitophorous vacuoles of macrophages (Bates and Rogers, 2004). In the case of *L. (V.) braziliensis*, a peripylarian parasite, its development begins in the hindgut of the insect followed by migration to the anterior midgut and mouth parts (Lainson and Shaw, 1987).

The cell surfaces of *Leishmania* spp. present molecules that are related to specific activities during the life cycle of the parasite and contribute to survival in harsh surroundings (Descoteaux and Turco, 1999). These surface molecules not only afford protection to the parasite within the insect vectors and vertebrate hosts but also provide specificity for their interactions with cells in the sand fly gut and with mononuclear phagocytic cells of mammals. The most intensely studied molecules located on the surfaces of promastigotes and amastigotes are the glycoconjugate lipophosphoglycan (LPG) and the metallo-proteinases (Moody, 1993). *Leishmania* spp.

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also produce a number of less-abundant surface molecules: glycosylinositol phospholipids (McConville *et al*. 1990), a promastigote surface antigen-2 complex of glycoproteins (Murray *et al*. 1989; Symons *et al*. 1994), a glycoprotein of approximately 46 kDa (Kahl and McMahon-Pratt, 1987) and 2 cysteine-proteinases of 63 and 43 kDa (Rebello *et al*. 2009). These molecules share the common trait of attaching to the plasma membrane via glycosylphosphatidylinositol lipid anchors.

The activity of these metallo-proteinase surface components has been associated with the hydrolysis and inactivation of immunoglobulin G, the inactivation of factor C3b of the complement C3bi in the mammalian host (Brittingham *et al*. 1995), and the protection of promastigotes from trypsin and chymotrypsin in the gut of the insect vector (Pimenta *et al*. 1997). Although LPGs have commonly been identified as having a life cycle-maintaining role, they can also act on the vertebrate host (i.e., influencing the innate and acquired immune responses and subverting the functions of the macrophage – Becker *et al*. 2003; Brandonisio *et al*. 2004; Lodge and Descoteaux, 2008; Soong, 2008) and on the insect vector (i.e., protecting the parasite from the proteolytic activities of the blood-digesting midgut, participating in the attachment to the gut wall, inducing the production of chitinases that degrade the stomodeal valve of the sand fly and influencing the secretion of aneuropeptide that arrests midgut and hindgut peristalsis – Sacks and Kamhawi, 2001; Kamhawi, 2006).

Metacyclic forms of *L. (V.) braziliensis* LPGs have also been reported to display differential attachment to the midgut of *Lutomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia* sand flies (Soares *et al*. 2010). This observation has been related to an upregulation of β-glucose residues in the LPG repeated units during metacyclogenesis of this parasite (Soares *et al*. 2005).

There is evidence that glycosaminoglycans, such as heparin, influence the development of *L. (L.) major* in the gut of the vector, increasing the parasitic load in experimentally infected insects (Volf *et al*. 2001). It has also been reported that specific receptors on the surface of *Leishmania* spp. are able to bind glycosaminoglycans with structures similar to heparin present in host tissues. Such receptors are known as heparin-binding proteins (HBPs), and these molecules can influence the parasite-host interactions during infection.

Experiments performed using promastigotes of *Leishmania (Leishmania) donovani*, for example, have shown that HBPs can be found on the surface of the parasite and are related to the inhibition of protein kinase C (Mukhopadhyay *et al*. 1989). HBPs have been associated with the infective forms of *L. (L.) donovani*, as they predominate on the surface of stationary-phase promastigotes from recently isolated samples. However, subsequent passages of parasites in culture have led to the loss of the heparin-binding abilities of the parasites (Butcher *et al*. 1992; Kock *et al*. 1997).

HBPs have also been described in other *Leishmania* species, specifically *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Leishmania*) *major*; however, contrary to what has been observed for *L. (L.) donovani*, in these species those proteins appear to be predominant in amastigotes (Love *et al*. 1993).

In addition, HBPs can influence the parasitic attachment to the gut of the insect vector because hydrophobic HBPs from *L. (V.) braziliensis* have been shown to possess the physicochemical potential to bind to *L. (N.) whitmani* and *L. (N.) intermedia* gut cells (Azevedo-Pereira *et al*. 2007).

Due to the lack of information on HBPs in *L. (V.) braziliensis*, we present new data regarding the subcellular distribution, proteolytic activity and binding kinetics of HBPs from this parasite.

MATERIALS AND METHODS

Chemicals

Detergents {Tween 20, Triton X-100 (TX-100), and 3-[(3-cholamidopropyl)-dimethylammonium]-1-propanesulfonate (CHAPS)}, heparin sodium salt, biotinylated heparin, gelatin, bovine serum albumin (BSA), penicillin, proteinase inhibitors [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline (o-phe), pepstatin A (Pep A), and p-phenylenediamine], reducing agents [dithiothreitol (DTT) and β-mercaptoethanol (β-ME)] were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). A heparin-Sepharose column (HiTrap-Heparin; 1·5 × 2·5 cm) was purchased from GE Healthcare. Fetal calf serum (FCS) was purchased from Cultilab S/A (Brazil). Brain heart infusion (BHI) medium was purchased from Difco (Detroit, USA). Epon resin was purchased from Hexion Specialty Chemicals, Inc. (US). The electrophoresis reagents were purchased from Bio-Rad Laboratories Inc. (US). Pre-Stained™ Plus Protein Ladder was purchased from Fermentas Life Sciences (US). Amicon Centriprep YM-10 filter devices were purchased from Millipore (Billerica Inc, MA, USA). Chemiluminescence luminol reagent-ECL kit was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were of analytical grade or better.

Parasite strain and culture

Infective promastigotes of *L. (V.) braziliensis* (strain MCAN/BR/1998/619) were maintained at 28 °C as a stock culture in Novy, MacNeal and Nicolle medium and subcultured every 4 days. Promastigote cultures

were grown in Brain Heart infusion medium supplemented with 10% heat-inactivated FCS until a density of 1×10^8 cells/ml was obtained.

Subcellular fractionation

Subcellular fractions enriched for flagella or surface membranes were obtained by centrifugal fractionation as previously described (Morgado-Diaz *et al*. 2005). Briefly, after 4 days of culturing, promastigotes were washed twice (3800 ***g***, 10 min, 4 °C) in phosphate-buffered saline, pH 7·2, (PBS). The remaining pellet was then washed twice with 10 mM Tris-HCl (pH 7·2) buffer containing 1 M NaCl, 0·2 M K_2HPO_4 and 0·5 M $MgCl_2$. The pellet was then resuspended in 10 mM Tris-HCl, pH 7·5, containing 0·05 M sucrose (**S** buffer; 10 ml/g of cells) and disrupted in 1% CHAPS with 40–80 strokes of a Dounce-type homogenizer. Isotonic conditions were restored by adding sucrose to reach a final concentration of 0·25 M. Cell lysates were centrifuged (10 min, 4300 ***g***, 4 °C), and the supernatant was collected and centrifuged (15 min, 12 000 ***g***, 4 °C). The pellet from the final centrifugation constituted the flagellar fraction (F_f), whereas the supernatant was centrifuged again (45 min, 35 000 ***g***, 4 °C) to obtain the pellet that comprised the membrane fraction (M_f).

Electron microscopy

The ultrastructural composition of subcellular fractions was determined as previously described (Morgado-Diaz *et al*. 2005). Briefly, subcellular fractions were fixed with 2·5% glutaraldehyde in a 0·1 M sodium cacodylate buffer, pH 7·2, for 1 h at 4 °C and post-fixed with a 1% osmium tetroxide (OsO_4) solution for 1 h at 4 °C. Samples were dehydrated in a graded series of acetone and embedded in PolyBed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a JEOL-1011 (JEOL UK – Welwyn Garden City, Hertfordshire, UK) transmission electron microscope.

Affinity chromatography

The soluble proteins from F_f and M_f were dialysed against an equilibrium buffer (10 mM sodium phosphate, pH 7·0, and then passed through a HiTrap-Heparin column previously equilibrated in the same buffer. The column was washed with the equilibrium buffer adjusted with 0·5 M NaCl, and the bound proteins were eluted using the equilibrium buffer adjusted with 2·0 M NaCl at a flow rate of 2 ml/min. The eluted fractions were concentrated using a Centriprep filter device, and the protein concentration was determined as previously described (Lowry *et al*. 1951).

Electrophoresis and Western blot analysis

Soluble proteins (~10 μg) were resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and silver staining, as previously described. Electrophoresis was performed at 25 °C in a Mini Protean II system (Bio-Rad Laboratories, USA). For Western blot assays, proteins were resolved using SDS-PAGE and then transferred onto 0·22 μm nitrocellulose membranes, as previously described (Azevedo-Pereira *et al*. 2007). Non-specific binding sites were blocked (4 °C for 16 h) using a solution of 5% skimmed milk (w/v) in PBS containing 0·5% Tween 20. The blots were washed 3 times with PBS containing 0·05% Tween 20 (PBST) and incubated (37 °C, 1 h) with biotinylated heparin diluted 1:500 in PBST. After 6 washes with PBST, the blots were incubated (37 °C, 1 h) with 1:1000 streptavidin conjugated to horseradish peroxidase in PBST. After 6 washes with PBST, the complex was revealed by chemiluminescence.

Zymographic assays

Protease activities of the HBPs (in samples of 5 μg) were determined using a gelatin substrate (0·1%) in 12% polyacrylamide gels (Heussen and Dowdle, 1980; Alves *et al*. 1993). Electrophoresis under denaturing conditions (using SDS) was performed at 4 °C in a Mini Protean II system and, subsequently, the gels were washed with 2·5% Triton X-100 under agitation (60 min, 25 °C) and incubated (16 h, 37 °C) in the appropriate buffer for each proteinase class (aspartic-proteinase: 10 mM sodium acetate buffer, pH 3·0; cysteine-proteinase: 10 mM sodium acetate buffer, pH 5·5, containing 1 mM DTT; serine-proteinase: 10 mM Tris-HCl, pH 7·5; metallo-proteinase: 10 mM Tris-HCl, pH 8·0). Incubations were performed in the presence or absence of inhibitors for each proteinase class (aspartic-proteinase: 1·0 μM Pep A; cysteine-proteinase: 10·0 μM E-64; serine-proteinase: 1·0 mM PMSF; and metallo-proteinase: 1·0 mM o-phe). The hydrolysis of gelatin was detected by staining the gels with 0·1% (w/v) Coomassie blue prepared in a methanol:acetic acid: water (3:1:6, v/v/v) solution.

Surface plasmon resonance (SPR) assays

SPR assays were performed using sensor chips that presented a carboxyl surface (COOH) coated with immobilized neutravidin (Biocap; Nomadics, USA). The sensor chip surface was further covered with biotinylated heparin (0·5 μg) and used to detect the presence of HBPs in each fraction (2 μg), BSA (1·0–0·01 μg) or in whole promatigotes ($1{\cdot}4 \times 10^5$ cells). Alternatively, promastigotes were pre-incubated (2 h, 4 °C) with different concentrations of heparin

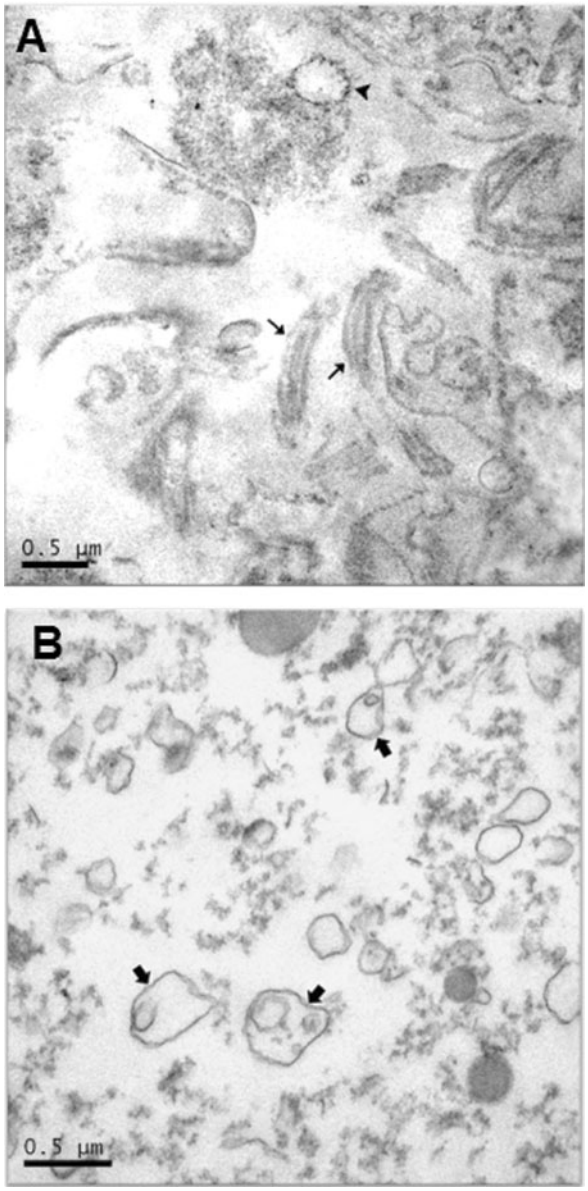

Fig. 1. Ultrastructural analysis of flagellar – F_f – (A) and membrane – M_f – (B) subcellular fractions of *Leishmania (V.) braziliensis* promastigotes obtained after differential centrifugation. The F_f was recognized by flagellum fragments (←) and flagellar membrane (▶), and the M_f by spherical membrane-bound vesicles (⬅). These images are representative of 3 independent experiments.

(1·0 μg/ml, 0·1 μg/ml and 0·01 μg/ml) in PBS to assess specific binding inhibition. Prior to interaction with the sensor chip surfaces the promastigotes were fixed (1 h, 4 °C) with 1% paraformaldehyde and washed 3 times by centrifugation (800 ***g***, 10 min, 4 °C) in PBS. SPR assays were performed at 25 °C with 100 μl of material injected under a flow rate of 10 μl/min. The binding assays were performed in PBS and registered in real time using a sensorgram, where changes in the SPR angle (θspr) were measured as resonance units (RU). Such changes were proportional to the concentration of bound proteins or the number of cells attached to the sensor chip surfaces. Resonance signals of the samples were analysed after subtraction of the RU values from the reference channel. Association (Ka) and dissociation (Kd) constants were measured and used to calculate the affinity constant of the fraction samples (KD). SPR experiments were conducted in an optical biosensor SensiQ Pioneer (Icx Nomadics, USA), and the data were analysed using Qdat software (Icx Nomadics, USA).

RESULTS

Ultrastructural characterization of the subcellular fractions of promastigotes

Subcellular fractions were obtained from promastigotes in the early stationary growth phase. Differential centrifugation was used for the enrichment of flagella (F_f) and membrane (M_f) fractions (Fig. 1A and B, respectively). F_f was assessed by the presence of typical flagellum fragments and flagellar membranes, whereas M_f was assessed by the predominance of spherical membrane-bound vesicles, using transmission electron microscopy.

Heparin-binding proteins from F_f and M_f fractions of promastigotes

We assessed the heparin-binding properties in proteins from both aforementioned isolated subcellular fractions. These assays yielded an estimative value of 0·04 ± 0·001 mg of HBPs in F_f, corresponding to 13·3% of the total fraction of proteins (0·3 ± 0·1 mg), whereas HBPs in M_f yielded 0·05 ± 0·001 mg of HBPs, corresponding to 8·3% of the total M_f proteins (0·6 ± 0·02 mg).

The protein profiles of F_f and M_f (and their correspondent heparin-binding fractions) were analysed by SDS-PAGE. Western blot analysis with F_f and M_f indicated that these fractions present complex and distinct band profiles ranging from 65·0 to 17·0 kDa. Surprisingly, denaturant electrophoresis assays with samples from both subcellular fractions after elution from the heparin-affinity columns showed 2 main protein bands with relative molecular masses of approximately 65·0 and 55 kDa, as revealed by silver staining (Fig. 2).

Proteinase properties of HBPs from M_f and F_f fractions

Our results indicate that the 65 kDa-protein band, from both HBP fractions, has a major gelatinolytic activity within a pH range between 5·5 and 8·0. A band with a minor gelatinolytic activity was also observed around 49 kDa in HBP M_f. The activity of these bands was sensitive to inhibition by 1,10-phenanthroline, and no inhibition was evident in gels incubated with other proteinase inhibitors, thus suggesting metallo-proteinase-like activity (Fig. 3).

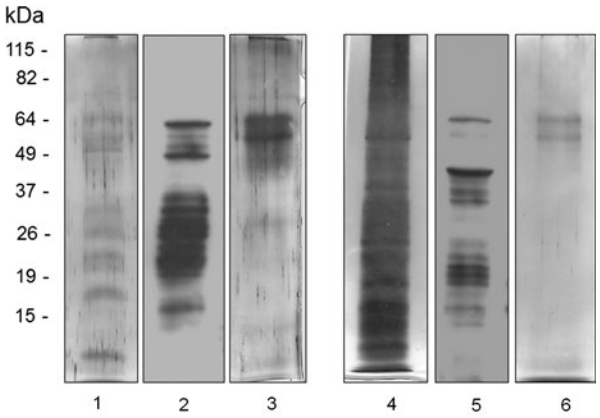

Fig. 2. Electrophoresis assays of samples from *Leishmania (V.) braziliensis*. Samples from flagellar (F_f) and membrane (M_f) fractions collected prior to (1 and 4, respectively) or after heparin-Sepharose column purification (HBP F_f (3) and HBP M_f (6)) were applied into each slot, submitted to SDS-PAGE and revealed by silver staining. In addition, Western blotting using biotinylated heparin as marker and revelation by chemiluminescence was performed with F_f (2) and M_f (5) to detect HBPs. Molecular mass markers (in kDa) are indicated on the left. These results are representative of 5 independent experiments.

Heparin-binding assays with Leishmania (V.) braziliensis *proteins*

SPR assays were designed to assess the presence of heparin ligands on *L. (V.) braziliensis* promastigote surfaces and to directly quantify these ligands in parasite subcellular fractions obtained by affinity chromatography. Kinetic constants obtained during the binding of biotinylated heparin onto a sensor chip surface characterized the specificity of this immobilization ($Ka = 2{\cdot}4 \times 10^5\ M^{-1}\ s^{-1}$; $Kd = 0{\cdot}079\ s^{-1}$; $KD = 320$ nM).

The presence of HBPs on the surface of promastigotes was confirmed by the sensorgram display of association and dissociation values of $33{\cdot}0 \pm 2{\cdot}0$ RU and $14 \pm 1{\cdot}0$ RU, respectively, following the injection of parasites onto the sensor chip surface. These values of association and dissociation are 1·6-fold higher than those observed after the injection of heparin (baseline), thus indicating binding of parasites to the immobilized heparin on the sensor chip surface (Fig. 4).

The specificity of parasite binding to heparin was confirmed by additional assays in which the promastigotes were incubated with increasing concentrations of heparin prior to being injected on the sensor chip surface. In these assays, it was possible to detect a dose-dependent inhibition of promastigotes binding to immobilized heparin with decreasing RU values of 48%, 39%, and 33% inhibition when parasites were incubated with 1·0, 0·1 and 0·01 μg/ml of heparin, respectively, as shown in Fig. 4.

We also analysed the capacity of proteins from HBP F_f and HBP M_f to bind to heparin at neutral pH. The analysis of the dissociation RU values indicated that HBP F_f contains more HBPs (445 ± 30 RU, equivalent to $0{\cdot}4$ ng/mm^2 of proteins on the sensor chip surface) than HBP M_f (175 ± 20 RU, equivalent to $0{\cdot}1$ ng/mm^2). The RU values observed for the HBP F_f and M_f fractions were 53-fold and 20-fold higher, respectively, than the RU value observed after the immobilization of heparin (baseline). The binding of HBP F_f samples to immobilized heparin was also observed to be stronger than that of HBP M_f with lower dissociation rates (Fig. 5). As expected, the negative controls using BSA showed very low dissociation RU values at 3 different concentrations ($1{\cdot}0\ \mu g = 2{\cdot}0 \pm 0{\cdot}3$ RU, $0{\cdot}1\ \mu g = 1{\cdot}2 \pm 0{\cdot}1$ RU and $0{\cdot}01\ \mu g = 0{\cdot}4 \pm 0{\cdot}3$ RU).

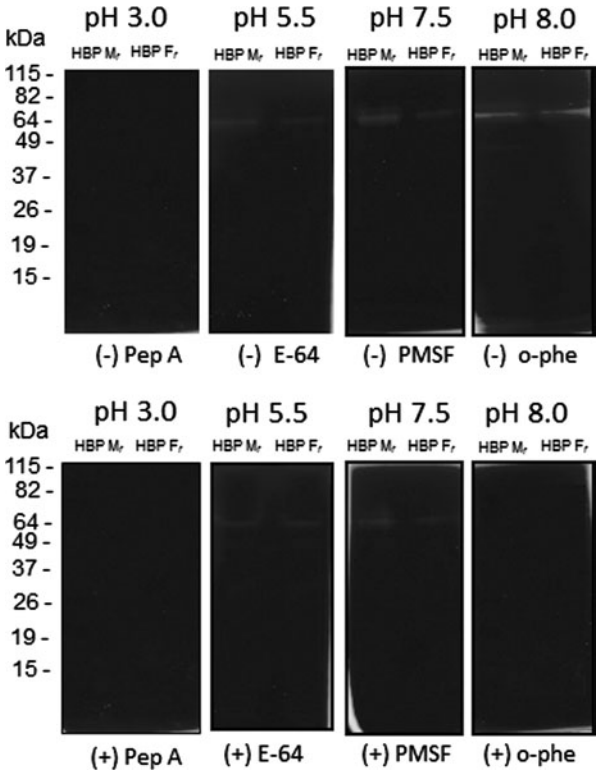

Fig. 3. Measurement of proteinase activity in *Leishmania (V.) braziliensis* HBPs by gelatin-SDS-PAGE. After electrophoresis of samples eluted from a heparin-Sepharose column (membrane proteins – HBP M_f; flagellum proteins – HBP F_f), gels were incubated with different buffers (pH 3·0, pH 5·5, pH 7·5 and pH 8·0) in the absence (−) or presence (+) of specific inhibitors for different classes of proteinases: Pep A, E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. Molecular mass markers are indicated (kDa). These results are representative of 4 independent experiments.

DISCUSSION

The surfaces of protozoan parasites, such as those belonging to the genus *Leishmania*, regulate interactions with the extracellular environment and are responsible for the uptake of nutrients and signalling

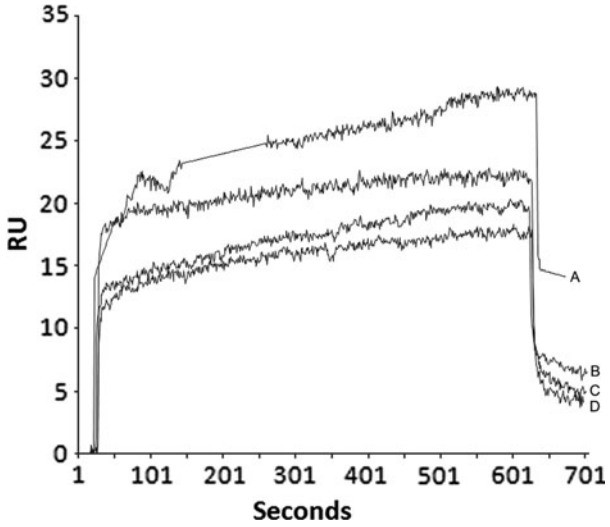

Fig. 4. Analysis of HBPs on the surfaces of *Leishmania (V.) braziliensis* promastigotes by surface plasmon resonance biosensing. Sensor chips were covered with biotinylated heparin, and promastigotes were passed onto the chip surface. The parasites were assayed without pre-incubation with heparin (A) or with pre-incubation with 0·01 μg (B), 0·1 μg (C) or 1·0 μg (D) of heparin. The data are presented as resonance units (RU) and are representative of 2 independent experiments.

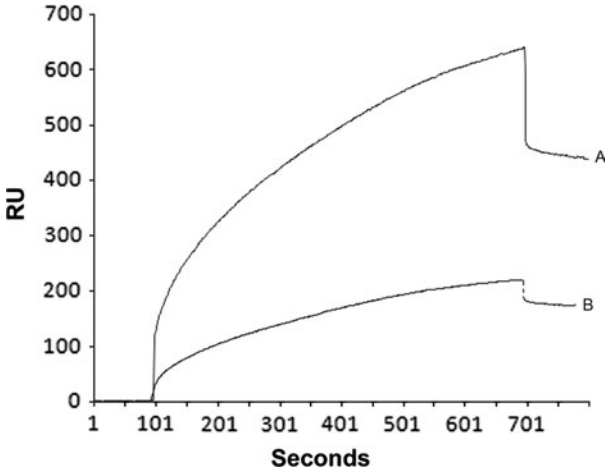

Fig. 5. Analysis of the interactions between heparin and HBP-enriched fractions from *Leishmania (V.) braziliensis* promastigotes by surface plasmon resonance biosensing. The interaction kinetics of HBP F_f (A) and HBP M_f (B) with biotinylated heparin immobilized on a sensor chip were assessed. The data are presented as resonance units (RU) and are representative of 2 independent experiments.

pathways. In this context, we have previously proposed the potential of HBPs from *L. (V) braziliensis* promastigotes to mediate parasitic adhesion to proteins from the guts of *Lutzomyia* sand flies (Azevedo-Pereira *et al*. 2007). Herein, we have confirmed that these proteins are components of surface membranes and flagella of *L. (V) braziliensis* promastigotes, reinforcing their potential roles in the parasite-vector interaction.

Cell fractionation paired with morphological and/or biochemical methodology has increasingly been used for parasite analyses (de Souza *et al*. 2003). In this study, this approach was applied to investigate HBPs from *L. (V) braziliensis* promastigotes to indicate that cellular membranes and flagella contain two main HBP bands (65 and 55 kDa) after elution from affinity chromatography, as previously described (Azevedo-Pereira *et al*. 2007).

The complex structural organization of heparin receptors has been previously described in other organisms. The mollusk *Anadara granosa* presents an HBP that has a native molecular mass of 300 kDa and is composed of several identical 60 kDa subunits (Dam *et al*. 1994). In *Trypanosoma cruzi*, a 60 kDa HBP was detected in trypomastigote membranes (Ortega-Barria and Pereira, 1991), and these receptors appear to be involved in the interaction of this parasite with cardiomyocytes (Oliveira Jr *et al*. 2008). Based on this evidence, it is possible that the heparin receptor detected in the present study also presents a complex structural organization corresponding to its functionality in *L. (V) braziliensis* promastigotes.

The cell surfaces of *Leishmania* parasites are the first sites of contact between these organisms and different microenvironments; thus, the regulation of surface macromolecule expression provides an important mechanism for adaptation during their life cycle. The surfaces of *Leishmania* spp. have an outer layer consisting of a latticework of carbohydrates covalently linked to lipids and proteins, known as the glycocalyx. This structure is rich in glycoconjugates with complex oligosaccharide structures that may be incorporated into the extracellular matrix or attached to secreted glycoproteins (Novozhilova and Bovin, 2010). Many components of the glycocalyx play roles in the interactions between protozoa and host cells (Chava *et al*. 2004; Naderer *et al*. 2004).

Glycosaminoglycans, such as heparin and heparan sulfate, are covalently attached to core proteins and have different cellular localizations in cells of animal species. Although heparin is found inside mastocytes (Nader *et al*. 1999; Strauss *et al*. 1982), heparan sulfate is found on the cell surface of vertebrates and invertebrates (Cassaro and Dietrich, 1977; Dietrich *et al*. 1980; Nader *et al*. 1984). It is important to note that heparan sulfate is the actual glycosaminoglycan that acts in the interaction between promastigotes and the sand fly gut epithelium; however, because heparin and heparan sulfate are both glycosaminoglycans that share a common chemical structure (Dreyfuss *et al*. 2009), the use of heparin in the binding assays of this study is an acceptable adaptation.

Our analyses show that HBPs from *L. (V.) braziliensis* promastigotes surface have biochemical properties of metallo-proteinases, which are well-known glycoproteins present on the surface and flagellar

pockets of *Leishmania* spp. and other trypanosomatids (Yao, 2010). In recent years, it has been shown that the molecular weights of metallo-proteinases of *Leishmania* spp. are not homogeneous, and enzymes of this class with molecular weights ranging from 50 kDa to values above 63 kDa have previously been detected (Alves *et al*. 2004; Cuervo *et al*. 2006).

For the first time, the interaction of HBPs from *L. (V.) braziliensis* promastigotes with heparin was assessed in real time using SPR. The use of this technology is a new trend in identifying cell surface proteins using a biosensing system (Velasco-Garcia, 2009) due to its flexible and powerful capacity for detecting biomolecular interactions (Tanious *et al*. 2008). SPR analysis enabled the real-time assessment of the interaction between HBPs from the promastigote surface and immobilized heparin (without specific markers). This method was performed in a similar manner to a previously proposed technique for the detection of ligands in mammalian cells (Quinn *et al*. 2000; Hide *et al*. 2002) and pathogenic microorganisms in the environment or in food (Bergwerff and van Knapen, 2006).

Biosensing surface technologies have been useful in elucidating the adhesion and invasion processes in parasite-host interactions that involve parasite proteins and binding with glycosaminoglycans. For example, biosensing surface methods have been used to assess the interactions between heparin and a malarian circumsporozoite protein and demonstrate the role of parasitic glycosaminoglycan receptors in the invasion of liver cells (Rathore *et al*. 2001). In addition, the direct measurement of the binding between the purified measles virus and heparin proved that this interaction prevents the infection of SLAM-negative cells lines (Terao-Muto *et al*. 2008). Therefore, our results that indicated the binding of promastigotes to immobilized heparin onto a biosensing surface confirm the presence of heparin receptors in the surface of the parasites and also support the effectiveness of this methodology.

It was also observed that the HBPs present in flagella are more efficient at binding to heparin than HBPs from the plasma membranes of promastigotes. However, further studies are necessary to clarify the reasons for this difference and to define the nature of the glycosaminoglycan binding site involved in this interaction while considering the quantity of saccharides of the different glycosaminoglycans.

Collectively, our results provide evidence that HBPs, which are the heparin receptors from the surface of *L. (V.) braziliensis* promastigotes, present characteristics of metallo-proteinases and are capable of forming stable complexes with glycosaminoglycans (similar to the activity of heparin). It is possible that HBPs modulate signalling activity in the cellular environment and play specific roles in parasite-host interactions.

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16

Affinity-Based Methods for the Separation of Parasite Proteins

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1. Introduction

Affinity chromatography-based techniques have been developed to purify parasite proteins and improve our understanding of the parasite life cycle. These advances can be translated into concrete proposals for new drugs, diagnostic methods and vaccines for parasite diseases and help to reduce social inequality.

Affinity chromatography has been demonstrated to be a powerful tool for the isolation and purification of parasite proteins and has potential applications for diagnosis and therapy. Many studies have focused on parasite proteins that modulate host cell defense, as gp63, a glycoprotein from *Leishmania spp.,* that is involved in the cleavage of the complement factor C3b to iC3b, which promotes adhesion of promastigotes to macrophages via complement receptor 3 (Brittgham et al., 1995). This route of internalization does not lead to production of oxygen radicals or NO and favors parasite subsistence within the host cell. Another example is the cysteine protease B (CPB), an important virulence factor of the *Leishmania (L.) mexicana* complex, that inhibits lymphocytes Th1 and/or promotes the Th2 response either through proteolytic activity or through epitopes derived from its COOH-terminal extension (Pereira et al., 2011).

Due to the important role of these molecules, many researchers seek to develop specific and potent inhibitors for therapeutic strategies. Aspartic protease, a potential target for antiparasitic therapies, has been isolated from *Trypanosoma cruzi* by affinity chromatography using a specific inhibitor of this enzyme (Pinho et al., 2009); this enzyme is target for treatment of infections caused by HIV (Wlodawer & Vondrasek, 1998) and Candida (Hoegl et al., 1999). This enzyme has also been reported in *Plasmodium* spp. and *Schistosoma mansoni*, where it plays an important role in host hemoglobin degradation (Klemba & Goldberg, 2002). Additionally, specific inhibitors of plasmepsins and renin are viable drugs for the treatment of patients with malaria and high blood pressure.

These parasite proteins, along with others, have been tested as new targets for chemo- and immunotherapies for parasite diseases. They have been assessed by lectins or protease inhibitor affinity chromatography. The separation of sugars based on lectin affinity is one of main procedure that has been used. This technique is based on the ability of lectins to bind

specifically to certain oligosaccharide structures on glycoconjugates isolated from parasites. Parasite proteins are processed through a multi-lectin affinity column, and they bind to the immobilized lectins through their sugar chains. Certain glycoconjugates are important for the parasite life cycle, and lectin affinity chromatography can help to reveal their roles (Guha-Niyogi et al., 2001).

The use of protease inhibitors in affinity chromatography is another important approach for assessing parasite proteins. Proteases hydrolyze peptide bonds and can therefore degrade proteins and peptides that influence a broad range of biological functions, including the process of parasite infection (Mackeron et al., 2006). The specificity of the protease inhibitor used is an important aspect of this methodology; L- trans-epoxy-succinylleucylamido-(4-guanidino) butane (specific to cysteine-protease), pepstatin A (to aspartyl-protease) and aprotinin (to serine-protease) are frequently immobilized on a solid matrix for this technique.

Glycosaminoglycan (GAG) affinity is the only affinity chromatography method that is based on the sugar chains of lectin-like proteins. Some of these molecules (such as heparin sulfate, heparan sulfate, dermatan sulfate, keratan sulfate and chondroitin sulfate) contain complex oligosaccharide structures, which may be displayed on cell surfaces, incorporated into the extracellular matrix or attached to secreted glycoproteins, suggesting that they play structural roles (Dreyfuss et al., 2010). GAGs have been reported as potential candidates for therapeutic intervention against parasitic infections, such as leishmaniasis and Chagas diseases (Azevedo-Pereira et al., 2007; Oliveira-Jr et al., 2008).

According to the general principle of affinity chromatography (Fig. 1), a protein of interest is recovered based on its capacity to bind a specific functional group (ligand) that is immobilized on a bead material (matrix) that has been packed into a solid support (column). Although many ligands (enzymatic substrates, inhibitors of an enzyme, lectin, sugar residues, vitamins, enzyme cofactors, monoclonal antibodies) have been used to isolate proteins based on affinity, only lectin, an enzyme inhibitor and glycosaminoglycans have been used to obtain parasite proteins. The most commonly used matrix materials for the attachment of the ligand are polysaccharide derivatives (cellulose, dextran and agarose) and polyacrylamide.

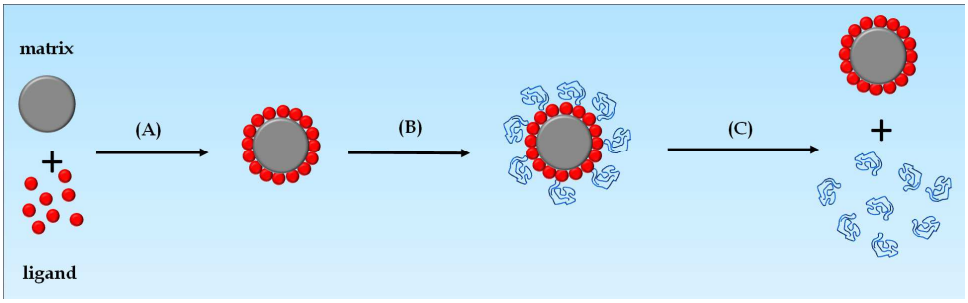

Fig. 1. The principle of affinity chromatography. The ligand is covalently bound to a matrix (A). The functionalized matrix is then able to bind to a target protein aided by a binding buffer (B). Afterwards, the bound proteins are eluted with a different buffer (C).

In these procedures, the soluble proteins are prepared from crude parasite lysates (or subcellular fractions) and loaded onto a column under chemical (buffer) and physical (temperature and pressure) conditions that promote the specific binding of the protein to the immobilized ligand (affinity) in what is known as the binding phase. Proteins that do not bind to the immobilized ligand under these conditions are removed from the solid phase by application of a constant liquid phase, which is referred to as the wash phase. Then, the bound protein can be recovered by changing the buffer conditions to favor desorption during the elution phase.

In this chapter, we describe the use of affinity chromatography to assess parasite proteins and the importance of these methods for public health. Several affinity chromatography protocols are considered. Additionally, we discuss our experience using affinity chromatography to obtain parasite proteins, and we include some unpublished results related to *Dermatobia hominis* third (L3) instar larvae proteases.

2. The use of affinity chromatography in parasite protein studies

2.1 Lectin affinity-based separation of parasite proteins

There are relatively few studies available in the current literature describing the use of lectins to affinity-purify glycosylated proteins from parasites. However, the reports on this subject demonstrate that this technique is useful for the retrieval of putative virulence factors or potential protective immunogens from a large array of parasites, including apicomplexan, trypanosomatids and nematodes (e.g., Fauquenoy et al., 2008, Gardiner et al., 1996, Smith et al., 2000). In addition to its utility in the isolation of parasite factors, lectin-based affinity chromatography is also a valuable resource for characterization of the structure of carbohydrates bound to proteins from these organisms due to the distinct specificities of the lectins that are available for this type of analysis.

Lectins are proteins that specifically bind to sugars, and they have been used for many types of studies, ranging from blood typing to immune regulation analysis (Rüdiger & Gabius, 2001). These proteins are generally isolated from plants (mostly legume seeds), where they can be found in abundance. Their usage is determined by the particular sugar structures that they are able to bind (Rüdiger & Gabius, 2001). The surveyed literature the use of six plant lectins [concanavalin A (Con A), ricin, jacalin, peanut agglutinin (PNA), wheat germ agglutinin (WGA) and Wisteria floribunda agglutinin (WFA)] in studies of parasites glycoproteins. Furthermore, one report described the use of Biomphalaria alexandrina lectin (BaSII), which in contrast to the others is a lectin obtained from an animal.

Con A is a lectin that can be extracted from jack beans of the species *Canavalia ensiformis* (family Fabaceae). It binds to mannose or glucose residues and is thus characterized as a mannose-binding lectin. This lectin presents a high affinity for the oligosaccharide GlcNAcβ2Manα6(GlcNAcβ2Manα3)-Manβ4GlcNAc. It is also known to be a potent mitogen (Beckert & Sharkey, 1970; Rüdiger & Gabius, 2001).

Ricin, along with jacalin and PNA, is a lectin that binds to galactose. Specifically, it binds with high affinity to the motif Galβ4GlcNAcβ2Manα6 (Galβ4-GlcNAcβ2Manα3) Manβ4GlcNAc. Ricin is highly toxic because it can impair ribosome activity through cleavage of the nucleobases of ribosomal RNA, and it has potential to be used as a biological

weapon. This lectin is extracted from *Ricinus communis* (Family Euphorbiaceae) (Rüdiger & Gabius, 2001; Lord et al., 2003).

Jacalin binds to galactose and N-acetylgalactosamine, and presents a high affinity for the motif Galβ3GalNAcα. It is obtained from *Artocarpus integrifolia* (Family Moraceae). It is commonly used to isolate IgA from human plasma (Kabir, 1998, André et al., 2007).

Like Con A, PNA is a legume lectin and is isolated from plants that belong to the family Fabaceae. It is extracted from *Arachis hypogea* and binds specifically to the monosaccharide galactose and to the motif Galβ3GalNAcα, similarly to the binding motif of jacalin. PNA is used as a marker of T-cell subpopulations and to differentiate between the stages of the Leishmania parasites life cycle (Dumont & Nardelli, 1979, Wilson & Pearson, 1984, Rüdiger & Gabius, 2001).

WGA is obtained from the species *Triticum vulgare*. It presents a low affinity for N-acetylgalactosamine, but it binds to the sialic acid N-acetylneuraminic and to the motif GlcNAcβ4GlcNAcβ4GlcNAcβ4-GlcNAcβ4GlcNAc. This lectin has been shown to bind more avidly to activated human T lymphocytes (Hellström *et al.*, 1976, Rüdiger & Gabius, 2001).

WFA is isolated from *Wisteria floribunda*, a woody liana of the family Fabaceae. Although some uncertainty regarding its binding specificity remains, it seems that this agglutinin binds preferentially to the monosaccharide N-acetylgalactosamine and to the motif GalNAcα6GalNAc. WFA is used to fractionate lymphocyte populations, and although it is not mitogenic like Con A, it can induce lymphokine production in murine splenocytes (Jacobs & Poretz, 1980; Rüdiger & Gabius, 2001).

BaSII is a lectin that can be isolated from the snail *B. alexandrina*, an intermediate host of the trematoda parasite *Schistosoma mansoni*, the causative agent of schistosomiasis. It specifically binds to the motif Fucα1,2Galβ1,4Glc (Mansour, 1996).

2.1.1 General procedures for the isolation of parasite proteins by lectin affinity

The rational for lectin-based affinity chromatography is the same as for other types of affinity-based fractionation: a sample is exposed to a solid phase that has been coupled to an affinity separation molecule (a lectin, in this case) under conditions that are adequate for binding (Fig. 2A). The unbound material from the sample is washed away (generally using the same buffer applied to equilibrate the solid-phase), and in the final step, the affinity-bound fraction is recovered by altering the equilibrium conditions of the solid phase (by changing the system pH or salt concentration) or by adding a molecules that competes for the binding site of the ligand.

To provide several practical examples, a collection of lectin affinity-based methodologies used to isolate and/or characterize glycoproteins from distinct parasites is listed in the Table 1.

It is important to note that some techniques, such as metabolic radioactive labeling (by [3H]-myristic acid or [3H]-glucosamine, for example) and cell disruption (by Triton X-100, dioxane or hypotonic solution), must be applied prior to lectin chromatography to allow for the identification of molecules eluted from the column or the preparation of suitable samples for the chromatography column, respectively.

Ligand	Organism	Isolated protein	First phase Matrix	Bind	Wash	Elution	Second phase Methods	References
WGA	*Trypanosoma cruzi*	85 kDa surface glycoprotein	Sepharose	10mM Tris-Hcl (pH 7.2), 150 mM NaCl	10mM Tris-Hcl (pH 7.2), 150 mM NaCl	0.1 M N-acetyl-D-glucosamine in 10mM Tris-Hcl (pH 7.2), 150 mM NaCl	None	Couto et al., 1990
Ricin	*Trypanosoma brucei rhodesiense*	Membrane Glycoprotein	Agarose	10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	0.2 M acetic acid in 10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	None	Brickman & Balber, 1993
BaSII	*Schistosoma mansoni*	37 kDa glycoprotein	Sepharose	20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM PMSF, 1% (v/v) Triton X-100, 1 mM PCMB, 1 mM o-phen, 1 mM iodoacetamide	20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$	300 mM L-fucose in 20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$	HPLC	Mansour, 1996
Concanavalin A or ricin	*Trypanosoma brucei*	Small Variable Surface Glycoprotein	Sepharose (Con A) or agarose (ricin)	10 mM Tris-HC (pH 7.4) ,150 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 2% NP-40, 100 µg/ml of antipain, leupeptin and E-64	10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 2% NP-40, 100 µg/ml of antipain, leupeptin and E-64	0.5 M alpha-methyl mannoside (Con A) or 0.5 M galactose (ricin) in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, and 50 µg/ml antipain, leupeptin and E-64	Dylasis	Gardiner *et al.*, 1996
Concanavalin A	*Trypanosoma congolense*	Variant Surface Glycoprotein	Sepharose	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM $CaCl_2$, 1mM $MnCl_2$	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM $CaCl_2$, 1mM $MnCl_2$	Isoelectric focusing	Gel filtration (Biogel P 30)	Gerold *et al.*, 1996
WFA	*Trypanosoma congolense*	Variant Surface Glycoprotein	Agarose	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide, 100 mM GalNAc	None	Gerold *et al.*, 1996
Concanavalin A	*Trypanosoma brucei*	Invariant surface glycoprotein heavily N-glycosylated	Sepharose	50 mM Tris buffer (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100	10mM Tris buffer (pH 6.5), 0.1% (w/v) Triton X-100	0.5 M α-methylmannoside in 10mM Tris buffer (pH 6.5) containing 0.1% (w/v) Triton X-100	Ion exchange chromatography (DEAE-52)	Nolan *et al.*, 1997

Table 1. Lectin affinity-based

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
PNA or jacalin	*Haemonchus contortus*	Glycoprotein fractions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca^{2+}, 10 mM Mg^{2+}	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca_2, 10 mM Mg^{2+}	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q – jacalin-binding material only)	Smith *et al.*, 2000
Concanavalin A	*Caenorhabditis elegans*	Glycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$ 0.25% CHAPS	None	Redmond *et al.*, 2004
Ricin	*Trypanosoma brucei*	Glycoproteins presenting giant poly-N-acetyllactosamine carbohydrate chains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	30 mg/ml lactose and 30 mg/ml galactose in 12,5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X-100, 25 mM TLCK, 0.25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih *et al.*, 2005
Concanavalin A	*Toxoplasma gondii*	N-linked glycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M α-methyl-α-Dmannose in 20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	None	Fauquenoy et al., 2008
Concanavalin A	*Leishmania (V.) braziliensis*	Cysteine proteinases	Sheparose	20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM α-Dmannose in 20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009

Table 1. (continued)

During the affinity chromatography procedure, other methods, such as isoelectric focusing, may be used instead of the application of competing carbohydrates to elude the column-bound material. Furthermore, distinct affinity columns can be used in sequence to purify fractions with specific characteristics from a single sample.

As for the handling of the material that is eluted from an affinity column, many options for further purification are available, depending on the analysis method chosen for the study. Some of these options include: anion exchange chromatography, size exclusion chromatography and dialysis.

The combination of these accessible approaches allows for a vast array of study possibilities. Several examples of the results obtained by applying lectin-affinity chromatography in association with other techniques are described in the following paragraphs.

2.1.2 Parasite proteins isolated by lectin affinity chromatography

The structure of an N-linked oligosaccharide from a surface glycoprotein of *Trypanosoma cruzi*, an important human parasite that causes Chagas disease, was defined in a study using lectin chromatography (Couto et al., 1990). It was determined that the structure of this oligosaccharide is comprised of complex carbohydrate chains that possess a terminal sialic acid, α-L-fucose and a galactosyl(α1,3)galactose unit.

The cellular localization of glycoproteins of *Trypanosoma brucei rodhesiense*, a subspecies of the parasite responsible for the African sleeping sickness, was analyzed using ricin-based chromatrography (Brickman & Balber, 1993). It was observed that the ricin-binding proteins were primarily located in the vesicles of the lysosomal /endosomal system.

Gardiner et al., (1996) characterized small glycoproteins isolated from the surface of *Trypanosoma vivax*, which causes bovine trypanosomiasis. That study was the first to detail the characteristics of a *T. vivax* Variable Surface Glycoprotein (VSG). The isolated protein, designated ILDat 2.1 VSG, presented a molecular mass of 40 kDa and contained mannose (or a derivative sugar) in small quantities, and it was poorly retained by the lectin affinity column. It is possible that carbohydrates comprise only the C-terminal anchoring structure of this protein.

The characteristics of a fucosyllactose determinant of a *S. mansoni* glycoprotein were identified using affinity chromatography based on a lectin that was isolated from a host of this parasite, *B. alexandrina*. This determinant is expressed in the outer chain of a single unit of complex type N-linked oligosaccharides (Mansour, 1996).

Additionally, the VSG glycosyl-phosphatidylinositol membrane anchors of *Trypanosoma congolense*, another trypanosomatide species that causes bovine trypanosomiasis, were studied by lectin affinity (Gerold et al., 1996) using a modification of the technique in which the bound proteins are electrophoretically desorbed (Reinwald et al., 1981). This analysis allowed for description of the VSG GPI-anchor in this parasite: it contains a β1,6-linked galactose as the terminal hexose of the branch and an N-acetyl-glucosamine residue. Also, it was observed that *T. congolense* synthesizes two potential GPI-anchor precursors, one of which is insensitive to phospholipase C activity.

Nolan et al., (1997) identified a new invariant surface glycoprotein that is heavily N-glycosylated in the bloodstream forms of *Trypanosoma brucei* and designated it as ISG_{100}. This glycoprotein presents a large internal domain composed of a serine-rich repetitive motif, which was previously undescribed, and N-glycosylation sites on the N-terminal domain. Additionally, ISG_{100} is encoded by a single gene, whereas the trypanosomal plasma membrane proteins are commonly encoded by tandemly repeated genes that are part of a multigene family.

Potentially protective glycoprotein fractions from *Haemonchus contortus*, a parasitic nematode in ruminants, were also obtained by lectin chromatography (Smith *et al.*, 2000). The findings from that study confirmed the potential of the *H. contortus* PNA-binding glycoprotein fraction as an efficacious antigen against this parasite infection in sheep. Furthermore, this study identified another highly protective fraction that binds to jacalin. This second protective fraction presents sialyted versions of the same oligosaccharides that bound to the PNA column.

Another study on the protective properties of the glycoproteins of *H. contortus* was performed by the same group (Smith et al., 2003). The results showed that the four purified glycosylated zinc metalloproteinases from this parasite were such an efficacious antigen that, to an extent, they could account for most of the protection conferred by the urea-dissociated whole glycoproteins fraction. However, the role for the glycan moieties of these enzymes in the protection process was not clear.

The capacity of glycoproteins from *Caenorhabditis elegans*, a free living nematode, to induce protection from a challenge with *H. contortus* in sheep was assayed by Redmond et al. (2004). The lectin affinity methodology was able to identify glycoproteins with molecular masses between 25 and 200 kDa in extracts prepared from *C. elegans*, but the fractionated glycoproteins were not able to confer protection against an *H. contortus* challenge. These findings suggest that the conserved glycan moieties between these two species of worm are not solely responsible for the protections levels observed when native *H. contortus* antigens are used.

Trypanosoma brucei glycoproteins were shown to present distinctive structural features, such as the presence of giant poly-N-acetyllactosamine carbohydrate chains (Atrih et al., 2005). The recovered affinity-bound molecules were predominantly, but not exclusively, from the flagellar pocket. These glycoproteins carry massive glycans, representing the largest poly-LacNAc structures reported to that date, and they may produce a gel-like matrix in the lumen of the flagellar pocket and/or the endosomal/lysosomal system. Despite their remarkable size, these glycans present a very simple neutral structure, containing only mannose, galactose and N-acetylglycosamine.

Important glycoproteins from the apicomplexan parasite *Toxoplasma gondii* have also been analyzed by lectin affinity methods. It was shown that these components are pivotal factors for host invasion and intracellular development of parasites (Fauquenoy et al., *2008*).

Cysteine proteinases from promastigostes of *Leishmania (Viannia) braziliensis* were shown to be anchored to the membrane by glysoylphosphatidylinositol structures in an analysis of the hydrophobic fraction of promastigote forms. These enzymes are suggested to play a role in the process of parasite survival inside its hosts (Rebello et al., 2009).

2.1.3 Remarks on the isolation of proteins by lectin affinity chromatography

These reports provide examples of the uses of lectin affinity chromatography to identify potentially antigenic fractions of parasites that could be used for vaccine development. Also, they point to the potential of this method to characterize glyconjugates, such as the glycoproteins that are present on the parasite surface or secreted by these organisms. However, apart from these purely structural or clinically oriented applications, this method may also be relevant in other investigations, including studies of host-parasite interactions. This hypothesis is reinforced by reports indicating that lectin-glycan binding is important for the infection and virulence processes of some parasites, *e.g. Acanthamoeba castellanii* (Garate et al., 2006), *H. contortus* (Turner et al., 2008), *L. (V.) braziliensis* (Rebello et al., 2009) and *T. gondii* (Fauquenoy et al., 2008)

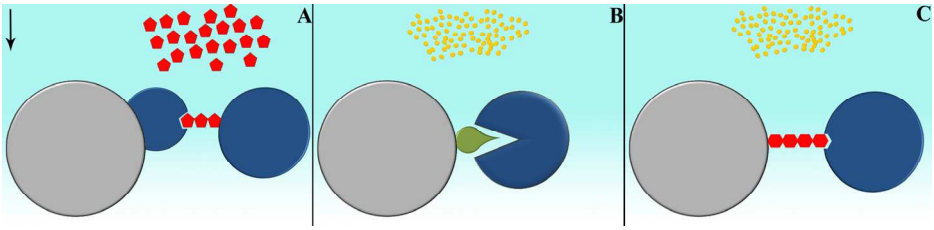

Fig. 2. Illustration of the affinity chromatography methodologies. The target molecules are bound to their ligands immobilized on a solid phase matrix. (A) Lectin affinity chromatography, (B) Protease inhibitor affinity chromatography and (C) Glycosaminoglycan affinity chromatography. Proteins = blue circle; carbohydrates = red pentagon and hexagon; protease inhibitors = green drop-like form; ions =yellow circles; and solid phase matrix beads = gray circle.

2.2 Protease inhibitors affinity-based separation of parasite proteins

Methodologies for the purification of parasite proteases have been applied in studies investigating the biological roles of these enzymes in parasite, including their participation in the infection process and in the survival of the parasites inside their hosts (McKerrow et al, 2006). Inhibitor affinity chromatography consists of the fractionation of parasite samples based on the reversible interactions between proteases and their specific inhibitors while the latter are covalently attached to a matrix (Fig. 2B). This technique can also be performed using irreversible inhibitors under particular conditions that will be described further in this section.

It is also interesting to note that, based on the specificity of the inhibitor used in the affinity chromatography, it is possible to suggest the enzyme class of the isolated protein. However, complementary analyses, such as characterization of the proteolytic activity, are often necessary to confirm these findings. Nevertheless, this purification strategy presents an initial advantage when compared to other methodologies.

In this section, fractionation approaches for serine-, aspartic acid- and cysteine proteases in specific parasites will be described. These approaches must take the class of the studied enzyme into consideration, as well as the inhibitor to be used and the characteristics of the mobile phase used for chromatography.

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
PNA or jacalin	*Haemonchus contortus*	Glycoprotein fractions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca^{2+}, 10 mM Mg^{2+}	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca_2, 10 mM Mg^{2+}	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q – jacalin-binding material only)	Smith *et al.*, 2000
Concanavalin A	*Caenorhabditis elegans*	Glycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$ 0.25% CHAPS	None	Redmond *et al.*, 2004
Ricin	*Trypanosoma brucei*	Glycoproteins presenting giant poly-N-acetyllactosamine carbohydrate chains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	30 mg/ml lactose and 30 mg/ml galactose in 12,5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X-100, 25 mM TLCK, 0.25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih *et al.*, 2005
Concanavalin A	*Toxoplasma gondii*	N-linked glycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M α-methyl-α-D-mannose in 20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	None	Fauquenoy et al., 2008
Concanavalin A	*Leishmania (V.) braziliensis*	Cysteine proteinases	Sheparose	20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM Tris–HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009

Table 2. Protease Inhibitors affinity-based

Table 2. (continued)

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
Pepstatin A	*Neospora caninum*	52 kDa	Agarose	5 mM NaOAc, (pH 5.5)	5 mM NaOAc, (pH 5.5)	0.1 M Tris–HCl, 0.5 M NaCl, (pH 8.5)	None	Naguleswaran *et al*, 2005
Aprotinin	*Leishmania amazonensis*	115 and 56 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez et al; 2005
Bacitracin	*Trichomonas vaginalis*	60 and 30 kDa	Agarose	20 mM NaOAc, (pH 4.0)	20 mM NaOAc, (pH 4.0)	0.1 M Tris-HCl, 1.0 M NaCl 25% 2-propanol, (pH 7.0)	Bio-Gel P-60	Sommer *et al*; 2005
Aprotinin	*Leishmania braziliensis*	60 and45 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Guedes HL *et al*; 2007
Aprotinin	*Trypanosoma cruzi*	75 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez *et al*; 2008
Aprotinin	*Leishmania donovani*	115 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH 7.5)	10 mM Tris–HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris–HCl, 1.5 M NaCl, (pH7,5)	None	Choudhury *et al;* 2009
Pepstatin A	*Trypanosoma cruzi*	120, 48 and 56 kDa	Agarose	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M Tris–HCl, 1.0 M NaCl, (pH 8.6)	HPLC gel filtration	Pinho *et al*; 2009
Aprotinin	*Leishmania donovani*	58 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,4)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,4)	10 mM Tris-HCl, 1 M NaCl, (pH7,5)	DEAE	Choudhury *et al*; 2010
Pepstatin A	*Rhipicephalus (B.) microplus*	42 kDa	Agarose	20 mM NaOAc, 1 M NaCl, (pH 5.3)	20 mM NaOAc, 1 M NaCl, (pH 5.3)	100 mM Tris-HCl, 1 M NaCl, (pH 8.6)	Mono-Q	Cruz *et al; 2010*
Benzamidine	*Plasmodium ookinete*	37 kDa	Sepharose	0.1% Triton X-100 in PBS	0.1% Triton X-100 in PBS	PBS	None	Li *et al;* 2010
Aprotinin	*Leishmania chagasi*	LCSII (105, 66, 60 kDa); LCSI (60, 58 kDa) and LCSIII (76, 68 kDa)	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	HPLC	Silva-Lopez *et al*; 2010
E-64	*Trypanosoma cruzi*	60 kDa	Sepharose	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris–HCl, 150 mM NaCl, 100 M PMSF, 2.0 M NaCl, (pH 7.4)	None	Bourguignon *et al* 2011

Aprotinin and pepstatin A are examples of inhibitors that are frequently used in the isolation of serine- and aspartic acid proteases, respectively, from many parasite species (Bond & Beynon). Other inhibitors that have been previously described in the isolation of serine proteases include soybean trypsin inhibitor (SBTI) and chloromethylketone (CMK). As for the purification of cysteine proteases, the use of three other inhibitors has been reported: L-transepoxysuccinyl-leucylamido-[4-guanidino]butane (E-64), bacitracin and glycyl-phenylalanyl-glycyl-semicarbazone (Table 2). It must be emphasized that these inhibitors cannot be used to isolate all of the proteases classes from parasites, as they present distinct affinities for members of different groups and families within these enzyme classes. Therefore, investigation of the possible variations present in the active site of these enzymes may prove useful.

The features of the buffer (temperature, pH and ionic strength) to be used may vary according to the ligand's physicochemical characteristics, the chemical environment of the parasite enzyme and the analyzed species of parasite. For example, distinct buffers were used for the purification of serine proteases from *S. mansoni* and *Trichinella spiralis* using benzamidine. It is also noteworthy that for each organism, a different matrix was used to immobilize the inhibitor, sepharose for *S. mansoni* and celite for *T. spiralis*. The use of distinct buffers in studies that are based on the same inhibitor is also noted in reports of SBTI, E-64, bacitracin and glycyl-phenyalanyl-glycyl-semicarbazone, all of which are cysteine protease inhibitors.

Affinity chromatography with an irreversible inhibitor has also been described previously; the cysteine-protease inhibitor is an example of this strong binding. In the interaction between E-64 and cysteine-protease, a covalent bond is established (Matsumoto, 1989). Therefore, a reaction between the epoxy groups of the inhibitor and the thiopropyl group of the sepharose matrix is necessary to bind E-64 to a solid support. This reaction prevents the reaction of E-64 with the cysteine residue at the protease catalytic center. However, this does not affect the bond between the inhibitor and cysteine-protease; instead, it only results in inhibition of the proteolytic activity (Govrin, 1999).

2.2.1 Parasite proteins isolated by cysteine-protease inhibitors affinity chromatography

There is only one published example of the use of E-64 affinity chromatography to assess cysteine-protease isolated from a parasite, and this study was conducted with the *T. cruzi* epimastigote. In this study, chromatography was useful for assessing the effects of β-Lapachone naphthoquinones on a 60 kDa cysteine-protease activity present in *T. cruzi*. The results demonstrated the potential of this protease inhibitor as a new antichagasic compound (Bourguignon et al., 2011). Another example of a cysteine-protease isolated by inhibitor affinity chromatography in parasites was described for *Plasmodium falciparum*. In this case, a glycyl-phenyalanyl-glycyl-semicarbazone-based column was used to isolate a protease with a molecular weight of 27 kDa, as determined by SDS-PAGE (Shenai et al, 2000).

2.2.2 Parasite proteins isolated by serine-protease inhibitors affinity chromatography

Aprotinin affinity-based chromatography was useful for the isolation of a serine-protease of 115 kDa (Silva-Lopez et al., 2005), a 68 kDa (Morgado- Diaz et al., 2004; Silva-Lopez et al., 2004) and a 56kDa (Silva-Lopez et al., 2004) from *L.(L.) amazonensis* compared to other

purification procedures that were used to isolate parasite serine peptidase enzymes (Kong et al., 2000; Ribeiro de Andrade et al., 1998). In *Leishmania (V) braziliensis* promastigotes, 60 kDa and 45 kDa enzymes were purified using the aprotinin affinity-based and activity esterase assessed against N-alpha-benzoyl-L-arginine ethyl ester hydrochloride and Nalpha-p-tosyl-L-arginine methyl ester hydrochloride (Guedes et al., 2007). Furthermore, three protein profiles were isolated from *Leishmania chagasi* promastigotes, including LCSI (58 and 60 kDa), LCSII (60, 66, 105 and kDa) and LCSIII (68 and 76 kDa), which were characterized as serine-protease enzymes based on their activity toward α-N-ρ-tosyl-L-arginine methyl ester substrate (Silva-Lopez et al., 2010). Furthermore, serine proteases with molecular weights of 75 kDa (Silva-Lopez et al., 2008) and 115 kDa (Choudhury et al., 2009) were identified as excretory products of *T. cruzi* and components of the sub-cellular environment in *Leishmania donovani*, respectively, although the chromatography step was not able to produce a homogeneous fraction. Furthermore, a intracellular serine protease of 58 kDa was were purified from *Leishmania donovani* (Choudhury et al., 2010).

In addition, the aprotinin affinity-based chromatography was useful for the isolation of serine-proteases of 35 kDa and 26 kDa from *Anisakis simpZex* (Morris et al, 1994), 43 kDa from *Candida albicans* (Morrison et al, 1993), 15 kDa from *Schistosoma mansoni* (Salter et al, 2000), 42 kDa from *Rhipicephalus (B.) microplus* (Cruz et al, *2010*), 60 kDa and 30 kDa from *Trichomonas vaginalis* (Sommer *et al*; 2005) and 35 to 52 from *Caenorhabditis elegans* (Geier et al; 1999).

Benzamidine-celite was applied in the isolation of serine proteases among the excreted or secreted proteins of *T. spirali*. The recovered proteases were not purified to homogeneity, and they showed molecular masses of 18 kDa, 40 kDa and 50 kDa (Todorova & Stoyanov). A similar finding was reported for the serine-proteases of *Chrysomya bezziana* larvae by using an SBTI-based column to purify four proteins with molecular masses of 13 kDa, 16 kDa, 26 kDa and 28 kDa (Muharsini et al., 2000).

Because it is possible to isolate heterogeneous products using inhibitors for affinity-based chromatography, we assessed a serine-protease from the third (L3) instar larvae of *D. hominis*. This ectoparasite causes dermatobiose in vertebrates, including humans, and it is particularly relevant in cattle, where it can cause a drop in production of meat and milk, leather as well depreciation (Maia & Guimarães, 1985).

Due to the association of DEAE-Sephacel with aprotinin agarose, it was possible to assess a serine protease from L3 larvae (Fig. 3). The fractions obtained by ion change chromatography containing estearasic activity were pooled and then fractionated on an aprotinin-agarose column. This fraction showed a profile with multiple bands by SDS-PAGE and silver staining, and only one band of enzyme activity (50 kDa) was detected by gelatin-SDS-PAGE at pH 7.5 (Fig. 3). Interestingly, this band of 50 kDa was not initially detected in the extracts from L3 by gelatin-SDS-PAGE. The expression of this enzyme is likely low in these larvae, and it can only be detected after concentration by chromatographic methods. The proposed strategy to isolate a serine protease allowed for the detection of a band of 50 kDa in extracts of L3 larvae, and this band had not been previously detected in the direct analysis of the total extract by gelatin-SDS-PAGE. Additionally, this fraction was found to have esterase activity (data not shown).

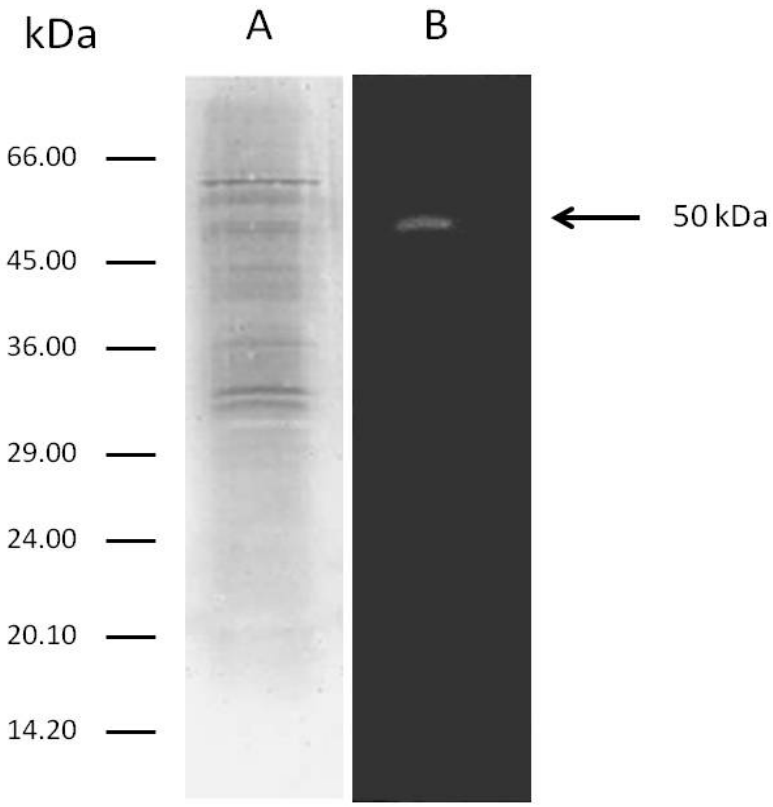

Fig. 3. Electrophoresis of proteins from L3 instar larvae of *Dermatobia hominis* eluted from a column of aprotinin-agarose. A total of 20 µg of protein from each fraction was resolved by SDS-PAGE (A) and gelatin-SDS-PAGE (B) and the bands were detected by silver staining and negative coloration, respectively. The arrow indicates a serine protease of 50 kDa. The molecular mass markers are indicated (kDa). These results are representative of two independent assays

2.2.3 Parasite proteins isolated by aspartyl-protease inhibitors affinity chromatography

Affinity-based chromatography based on pepstatine A was used to isolate a 52 kDa aspartyl protease from *Neospora caninum* tachyzoites (Naguleswaran et al., 2005) and a 45 kDa enzyme from *S.mansoni* (Valdivieso et al., 2003). In *Trypanosoma cruzi* epimastigotes, two aspartyl proteases were isolated (cruzipsin-I and cruzipsin-II). The molecular mass was estimated to be 120kDa by high performance liquid chromatography gel filtration, and the activities of these enzymes were detected in a doublet of bands (56 kDa and 48 kDa). These findings demonstrate that both proteases are novel *T. cruzi* acidic proteases. The physiological function of these enzymes in *T. cruzi* is not completely defined (Pinho et al., 2009).

An aspartyl protease with molecular mass of 37 kDa (plasmepsin) was isolated from the surface of *Plasmodium ookinete*, and its sequence was determined by mass spectrometry (Li et al., 2010). This protease was purified by using a benzamidine affinity-based column, which is typically used for the isolation of serine proteases. Structural similarity between the active site residues of the serine- and aspartyl proteases is possible, as some hydrogen-bonded residues can are arranged without any strain, such as in the formation of an oxyanion hole, in a manner that resembles the active site of a serine protease (Andreeva et al., 2004)

2.2.4 Remarks on the isolation of proteins by protease inhibitors affinity chromatography

Although the studies that have been conducted to isolate parasite proteases are of great medical interest, no parasiticide drug has been proposed thus far. In general, the chromatography methods involving inhibitor-based affinity-capture have been useful only to describe these enzymes in parasites and to establish their biochemical properties, their functions and their application in drugs tests.

Furthermore, the heterogeneous material obtained from affinity-based chromatography may require additional procedures for purification of the enzyme. Thus, other techniques must be applied to obtain proteases with greater purity, including molecular exclusion and ion exchange chromatography.

2.3 Glycosaminoglycans affinity-based separation of parasite proteins

Microbes have developed different strategies to gain access into mammalian cells (Bermúdez et al., 2010; Caradonna & Burleigh 2011; Soong et al., 2011). The first step involves the recognition of molecules at the surface of the target cell, which triggers the activation of signaling pathways that are implicated in the parasite internalization (Abban & Meneses 2010; Epting et al., 2011). Host cell surface sulfated proteoglycans have been implicated as key molecules at the host cell-parasite interface, mediating the adhesion and invasion of numerous parasitic microorganisms (Jacquet et al., 2001; Kobayashi et al., 2010; O'Donnell & Shukla 2010).

2.3.1 Structure of glycosaminoglycans

Proteoglycans (PGs) are composed of core proteins that are covalently linked to glycosaminoglycan (GAG) chains. As components of the extracellular matrix, the structural diversity of PGs depends on the identity of the core protein and the GAG composition. GAGs are linear polysaccharides comprised of disaccharide repeats containing uronic acid and hexosamine. GAGs vary in the type of hexosamine, hexose or hexuronic acid unit. The sulfated GAGs are classified as heparin [2-O-sulfo-β-D-glucuronic acid (GlcUA-2S) or 2-O-sulfo-α-L-iduronic acid (IdoUA-2S) and N-acetylglucosamine (GlcNAc) or N-sulfoglucosamine (GlcNS)], heparan sulfate [GlcUA, IdoUA or IdoUA-2S and GlcNAc or GlcNS], chondroitin sulfate [GlcUA and N-acetylgalactosamine (GalNAc)], dermatan sulfate [GlcUA or IdoUA and GalNAc] and keratan sulfate [galactose (Gal) and GlcNAc]. In fact, the structural diversity of PGs may provide sites of affinity for different ligands and, therefore, function as co-receptors or receptors for GAG-binding proteins (Dreyfuss et al., 2009; Ly et al., 2010). Although heparin is not found on the cell surface, this GAG has being

commonly used as tool for pathogen-host cell interaction assays. Heparins are negatively charged structures and native heparin presents molecular weights in the range of 5 to 30 KDa, whereas commercial heparin preparations are in the range of 12 kDa to 15 kDa.

2.3.2 Role of heparin-binding proteins in pathogen-host cell

Many pathogens express surface proteins that interact with GAGs in different stages of their life cycle. Although some parasites can bind to multiple GAGs (Coppi et al., 2007; Fallgren et al., 2001), heparan sulfate proteoglycan (HSPG) has been implicated in the recognition and/or invasion process of a wide range of pathogens, including viruses, bacteria and protozoan parasites (Bambino-Medeiros et al., 2011; Dalrymple & Mackow 2011; Yan et al., 2006;). Despite the role of heparin-binding proteins in many physiological and pathological processes, the basis of the heparin-protein interaction at the molecular level is still unclear.

Thus, efforts have been concentrated to enhance methods for the isolation and characterization of heparin-binding proteins, and, in parallel, to determine the role of this GAG in pathogen-host cell interaction. Currently, heparin affinity chromatography has been applied to the purification of GAG-binding proteins from different pathogens (Table 3). In these chromatography assays, the heparin is covalently coupled to agarose or sepharose beads and its sulfates and carboxylates chains are able to bind many proteins by basic amino acids (Fig. 2C).

This technique has been used to isolate heparin-binding proteins without loss of their biological activity, leading to a better understanding of the mechanism involved in the parasite invasion process. For example, chlamydial outer membrane complex (OmcB), a 60 kDa cysteine-rich protein, displays a protein motif (50-70OmcB peptide) that acts as an acceptor molecule to bind heparan sulfate (HS) and promote Chlamydia invasion in eukaryotic cells (Stephens et al., 2001). Attachment of *Helicobacter pylori* to gastric epithelial cells also involves HS recognition. Two major proteins, one with a molecular mass of 71.5 kDa and pI 5.0 (HSBP50) and the other with a molecular mass of 66.2 kDa and pI 5.4 (HSBP54), have been identified on the surface of bacterial cells that are able to bind HS. The amino acid sequences of these proteins (HSBP50 – VPERAVRAHT; HSBP54 - VHLPADKTNV) are not homologous with bacterial adhesins or other HS-binding proteins (Ruzi-Bustos et al., 2001). Other proteins with the ability to bind heparin (66 and 60 kDa) have been identified in *Staphylococcus aureus*. The partial characterization of the amino acid sequences, which consist of DWTGWLAAA for the 66 kDa protein and MLVT for the 60 kDa protein, revealed no identity with HBPs from Chlamydia or *Helicobacter pylori*. HBPs from *S. aureus* have been demonstrated to be sensitive to heat and proteases, such as pronase E, proteinase K, pepsin and chymotrypsin (Liang et al., 1992). Interestingly, a 17-kDa heparin-binding protein with pI 4.6 has also been isolated from *S. epidermis* and *S. haemolyticus*, but the amino acid sequence similarity is low between these two organisms (MXTAHSYTXKYNGYTAN and MATQTKGYYYSYNGYV, respectively) and other bacterial HBPs (Fallgren et al., 2001).

Trypanosomatidaes also exploit HS for successful parasite attachment to and/or invasion of the mammalian and vector hosts. The adhesion of Leishmania amastigotes to macrophages is mediated by HS, but not other sulfated polysaccharides (Love et al., 1993). Two heparin-

binding proteins, (65 and 54.5 kDa) from *L. (V.) braziliensis* promastigotes (HBP-Lb) recognize several molecules in the gut of *Lutzomyia intermedia* and *Lutzomyia whitmani* (Azevedo-Pereira et al., 2007). The biochemical characterization of these proteins revealed that only the 65-kDa HBP-Lb has metallo-proteinase activity, and this protein is primarily localized at the flagellar domain of the promastigotes. Surface plasmon resonance (spr) also demonstrated high-affinity binding at the flagellar domain, which forms a stable binding complex (Côrtes et al., 2011). In *T. cruzi*, HBPs also mediate parasite adhesion by recognition of PGHS on the surface of the target cells (Bambino-Medeiros et al., 2011; Calvet et al., 2003; Oliveira-Jr et al., 2008; Ortega-Barria & Pereira, 1991). Currently, three HBPs have been described in this parasite: a 60-kDa protein named penetrin (Ortega-Barria & Pereira, 1991) and two other proteins of 65.8 and 59 kDa that bind heparin, HS and chondroitin sulfate (CS). These proteins have been identified in both trypomastigotes and amastigotes (Oliveira-Jr et al., 2008). Interestingly, the HBP-HS binding is related to a specific region of the HS chain, the N-acetylated/N-sulfated HS domain, which promotes parasite attachment and invasion (Oliveira-Jr et al., 2008). Although only HS binding triggers *T. cruzi* invasion of mammalian cells (Ortega-Barria & Pereira, 1991; Calvet et al., 2003; Oliveira-Jr et al., 2008; Bambino-Medeiros et al., 2003), the multiple GAG recognition may provide an efficient association with other GAGs within the parasite life cycle. Recently, it has been demonstrated that sulfated proteoglycans are involved in the adhesion of epimastigotes to the luminal midgut epithelial cells of Rhodnius prolixus (Gonzalez et al., 2011).

2.3.3 Remarks on the isolation of proteins by glycosaminoglycans affinity chromatography

While the application of affinity chromatography has provided advances in our understanding of heparin-binding proteins, a large number of studies have focused on the parasite-host cell interface to improve our comprehension of the mechanisms that are activated by the receptor-ligand interaction (reviewed by Chen et al., 2008). The binding of Dengue virus to HS, for example, seems to result in the accumulation of virions at the surface of the human hepatoma cell line HuH-7 and elicit clathrin-dependent endocytosis (Hilgard & Stockert 2000). In addition to promote attachment and parasite invasion, HSPG also seems to be involved in the tropism of pathogen to specific tissues. The degree of HSPG sulfation guides the migration of Plasmodium sporozoites and the invasion of hepatocytes. Highly sulfated heparan sulfate at the surface of hepatocytes seems to regulate the proteolytic activity of the calcium-dependent protein kinase-6 on the CSP, which triggers the invasion of the parasite (Coppi et al., 2007).

Another interesting phenomenon is the release of syndecan-1, a transmembrane PGHS, as a mechanism of host defense inhibition. *Pseudomonas aeruginosa* induces syndecan-1 shedding through the enzymatic activity of LasA, leading to an enhancement of bacterial virulence (Park et al., 2001). A similar mechanism has been described for *Staphylococcus aureus* in which β-toxin, a secreted virulence factor, also induces syndecan-1 shedding by activating a metallo-proteinase involved in the host cell shedding mechanism, leading to enhancement of bacterial virulence due to the recruitment of inflammatory cells (Hayashida et al., 2009). Because heparan sulfate has been shown to be a receptor for a variety of pathogens, HS-binding polypeptides have been the subject of intense research and provide possibilities for drug intervention.

Organism	Isolated proteins	First phase				Second phase	References
		Matrix	Bind	Wash	Elution	Methods	
Trypanosoma cruzi	Penetrin – 60 kDa	Sepharose	PBS, pH 7.2	PBS containing 0.05% Triton X-100, pH7.2	1.5M guanidine-HCl 2.0M potassium thiocyanate and NaCl gradient (0.25-3.0M)	None	Ortega-Barria and Pereira 1991
Staphylococcus aureus	*S. aureus*-HBP – 66 kDa and 60 kDa	Sepharose	PBS containing 100mM PMSF, pH6.0	PBS containing 100mM PMSF, pH6.0	NaCl gradient (0-1M), followed by 2M NaCl in 200mM Tris-HCl pH 8.0	None	Liang et al., 1992
Trypanosoma cruzi	Penetrin – 60 kDa	Sepharose	PBS, pH 7.2	PBS containing 0.05% Triton X-100, pH7.2	1.5M guanidine-HCl 2.0M potassium thiocyanate and NaCl gradient (0.25-3.0M)	None	Ortega-Barria and Pereira 1991
Chlamydial	Outer membrane complex (COMC) - 60 kDa	Agarose	50 mM DTT and 2% Triton X-100	PBS	2% SDS in PBS	None	Stephens et al., 2001
Helicobacter pylori	Outer membrane protein (OMP) - 71.5 kDa and 66.2 kDa	Sepharose	0.05M sodium acetate pH5.0	0.1M Sodium acetate, pH5.0	NaCl gradient (0-2M)	None	Ruzi-Bustos et al., 2001
Staphyloccocal	HBP – 17 kDa	Sepharose	PBS	None	NaCl gradient (0-2M)	None	Fallgren et al., 2001
Leishmania (V.) braziliensis	HBP-Lb – 65 kDa and 54.5 kDa	Sepharose	PBS containing 0.5% glycerol and 0.5% Chaps, pH7.2	PBS containing, 0.5% glycerol and 0.5% Chaps, pH7.2	PBS containing 0.5% glycerol, 0.5% Chaps and 2M NaCl, pH7.2	None	Azevedo-Pereira et al., 2007
Trypanosoma cruzi	HBP-Tc – 65.8 kDa and 59 kDa	Sepharose	PBS and 0.5% glycerol, pH 7.2	PBS, pH 7.2	NaCl gradient (0-1M)	None	Oliveira-Jr et al., 2008

Table 3. Heparin affinity-based

3. Conclusion

The chromatographic procedures described here maintain the minimal amount of native folding necessary for proteins to retain their biological and biochemical activities. Thus, the materials used as supports for packed affinity columns, including agarose, sepharose and celite (from diatomaceous earth), to immobilize ligands, such as lectins, protease inhibitors and glycosaminoglycans, do not interfere with the functional properties of these proteins.

Furthermore, proteins obtained by affinity-based procedure have been useful in understanding the biological processes related to the life cycles of parasites and in the interaction with hosts. These studies are essential to developing strategies, such as the use of vaccines and drugs, to control the parasite diseases.

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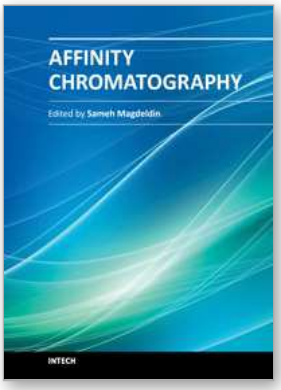

Affinity Chromatography

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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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Trypanosoma cruzi heparin-binding proteins mediate the adherence of epimastigotes to the midgut epithelial cells of *Rhodnius prolixus*

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SUMMARY

Heparin-binding proteins (HBPs) have been demonstrated in both infective forms of *Trypanosoma cruzi* and are involved in the recognition and invasion of mammalian cells. In this study, we evaluated the potential biological function of these proteins during the parasite-vector interaction. HBPs, with molecular masses of 65·8 kDa and 59 kDa, were isolated from epimastigotes by heparin affinity chromatography and identified by biotin-conjugated sulfated glycosaminoglycans (GAGs). Surface plasmon resonance biosensor analysis demonstrated stable receptor-ligand binding based on the association and dissociation values. Pre-incubation of epimastigotes with GAGs led to an inhibition of parasite binding to immobilized heparin. Competition assays were performed to evaluate the role of the HBP-GAG interaction in the recognition and adhesion of epimastigotes to midgut epithelial cells of *Rhodnius prolixus*. Epithelial cells pre-incubated with HBPs yielded a 3·8-fold inhibition in the adhesion of epimastigotes. The pre-treatment of epimastigotes with heparin, heparan sulfate and chondroitin sulfate significantly inhibited parasite adhesion to midgut epithelial cells, which was confirmed by scanning electron microscopy. We provide evidence that heparin-binding proteins are found on the surface of *T. cruzi* epimastigotes and demonstrate their key role in the recognition of sulfated GAGs on the surface of midgut epithelial cells of the insect vector.

Key words: heparin-binding proteins, *Trypanosoma cruzi*, epimastigotes, Rhodnius prolixus parasite-vector interaction.

INTRODUCTION

Chagas disease is a neglected tropical disease caused by an infection with the protozoan *Trypanosoma cruzi* that affects ~13 million people in the American continent (World Health Organization, 2005). Currently, the geographical distribution of this infectious disease is not limited to the Americas, as cases of infection have been reported in Europe, Australia and Japan, which has been attributed to organ transplants and immigration from Central and South America (Schmunis, 2007; Develoux *et al*. 2010). In Latin America, *T. cruzi* is mainly transmitted to humans by the feces of triatomine bugs (family *Reduviidae*, subfamily *Triatominae*) during blood feeding, but the occurrence of acute Chagas disease outbreaks has also been associated with infection by oral routes (Coura, 2006). Thus, determining the biological aspects of the interaction of *T. cruzi* with vertebrate and invertebrate hosts may provide potential new targets for vector control intervention and Chagas disease therapy.

T. cruzi has a complex life cycle that involves the interactions of distinct evolutive forms with both the vector digestive tract and mammalian cells. Therefore, the parasite exploits strategies to survive within the host and sustain the infection (Tyler and Engman, 2001). One essential event required for the bona fide parasite-host interface is the recognition of molecules between parasite and host cells. Lectins and carbohydrates have been reported to mediate receptor-ligand binding during vector- and mammalian cell-*T. cruzi* interplay (Alves *et al*. 2007; Villalta *et al*. 2009). Retention of epimastigotes in the triatomine midgut has been associated with the recognition of high-mannose glycans by carbohydrate-binding proteins of the parasite (Bonay and Fresno, 1995; Bonay *et al*. 2001). Glycoinositolphospholipids at the surface of epimastigotes are also involved in adhesion to the insect epithelial cells (Nogueira *et al*. 2007). In fact, it has been demonstrated that the successful attachment of *T. cruzi* to the perimicrovillar membrane (PMM) is crucial for

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the establishment of Reduviidae insect infection (Gonzalez *et al*. 1998, 2006; Alves *et al*. 2007). This initial step seems to coordinate biological events, such as epimastigote growth and differentiation into trypomastigotes and, therefore, is a focus of intervention strategies. The characterization of the PMM has revealed several potential molecules, mainly carbohydrates and carbohydrate-binding molecules that may be implicated in the mechanism of interaction with *T. cruzi* (Albuquerque-Cunha *et al*. 2009). Glycosaminoglycans (GAGs), such as heparan sulfate (HS) and chondroitin sulfate (CS), have also been detected in different organs of *Rhodnius prolixus*, including the intestinal tract (Costa-Filho *et al*. 2004; Souza *et al*. 2004).

In fact, sulfated GAGs have emerged as key molecules involved in the attachment and invasion processes of several microorganisms (Wadström and Ljungh, 1999; Sava *et al*. 2009; Boyle *et al*. 2010). GAGs are linear polysaccharides composed of disaccharide repeats that consist of uronic acid (glucuronic acid and iduronic acid) or galactose and an amino sugar (N-acetylglucosamine and N-acetylgalactosamine) (Dreyfuss *et al*. 2009; Ly *et al*. 2010). The ubiquitous distribution of GAGs at the surface of mammalian and insect cells (Souza *et al*. 2004; Dreyfuss *et al*. 2009) has indicated their putative role in *T. cruzi* interaction. We have previously demonstrated that trypomastigotes and amastigotes possess HBPs that mediate parasite attachment and invasion in mammalian cells (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008; Bambino-Medeiros *et al*. 2011). Therefore, we postulated that sulfated GAGs at the surface of midgut epithelial cells may also play a role in the adhesion of epimastigotes to the triatomine midgut.

In this study, we attempted to isolate and characterize the heparin-binding proteins (HBPs) of the epimastigote forms of *T. cruzi*. We confirmed the specificity and stability of protein binding between HBPs and GAGs and determined their potential biological function in the parasite-vector interaction.

MATERIALS AND METHODS

Glycosaminoglycans

Heparin (Hep), from bovine lung, was purchased from INORP Laboratories (Buenos Aires, Argentina). Heparan sulfate (HS), from bovine pancreas, was a kind gift from Dr P. Bianchini (Opocrin Research Laboratories, Modena, Italy). Chondroitin 4-sulfate (C4S), from whale cartilage, was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All biotinylated GAGs (Hep, HS, C4S) were prepared as previously described (Bouças *et al*. 2008).

Rhodnius prolixus

Triatomines were reared and maintained in the laboratory as previously described (Garcia *et al*. 1984). Fifth-instar larvae, which were starved for 30 days after the last ecdysis, were allowed to feed on citrated human blood through a membrane feeder. Insects were dissected 10 days after feeding, and the interaction of parasites and midgut epithelial cells was performed as previously described (Alves *et al*. 2007).

Parasites

An epimastigote form of *T. cruzi*, clone Dm28c, was grown at 28 °C in LIT medium (Camargo *et al*. 1964) containing 10% FBS. For the biochemical assays and insect-parasite interaction studies, the parasites were cultivated at a density of 10^4 parasites/ml and harvested during the exponential growth phase.

Metabolic radio-isotope labelling of the parasite proteins

Epimastigotes (2×10^7 cells/ml) were starved (1 h) of methionine and incubated (1 h, 37 °C) with 100 mCi/ml of [^{35}S]methionine. The metabolic labelling was stopped by the addition of 1% FBS. After washing in PBS, the proteins of labelled parasites were obtained using Triton X-114 (TX-114) (Bordier, 1981).

Protein extraction

Parasites (10^{11}) were washed with PBS, and detergent-soluble proteins were obtained by TX-114 phase separation. After parasite extraction, soluble proteins were obtained after condensation at 37 °C followed by centrifugation (12 000 ***g***, 15 min, 25 °C). The hydrophobic phase was subjected to affinity chromatography.

Affinity chromatography

Hydrophobic proteins were applied to a heparin affinity column as previously described (Oliveira-Jr *et al*. 2008). After washing, the retained proteins were eluted with a stepwise application of increasing NaCl concentrations (0·25 M, 0·5 M, 1·0 M, 2·0 M and 3·0 M) in the equilibrium buffer. The proteins were dialysed and concentrated using a Centriprep YM-10 (Millipore, MA, USA). Finally, the eluted fractions were stored at −20 °C until further analysis.

SDS-PAGE and Western Blot

The total extracted protein (40 μg) and proteins eluted from the heparin column (20 μg) from epimastigotes were separated by SDS-PAGE (12%). After electrophoresis, the gels were stained with silver nitrate and Coomassie blue. SDS-6H (Sigma-Aldrich Chemical Co., St Louis, MO, USA) was used as a molecular mass marker.

For Western blot, the separated proteins were transferred to a nitrocellulose membrane and blocked (16 h, 4 °C) with PBS containing 5% skim milk and 0·5% Tween-20. The membranes were incubated with 2 μg/ml of biotinylated GAGs (heparin, HS or C4S) for 1 h at 25 °C and with streptavidin-conjugated horseradish peroxidase (1:100) for 1 h at 25 °C. The complex was visualized using an ECL-kit (Santa Cruz Biotechnology, CA, USA).

Rhodnius prolixus-*epimastigote interaction*

The *in vitro* interaction of insect midguts and epimastigotes was performed as described previously (Alves *et al*. 2007). The dissected insect midguts were mixed with 10^6 epimastigotes in 200 μl of PBS. The involvement of HBPs and sulfated GAGs in the insect-parasite interaction was analysed by incubating the insect midguts with HBPs isolated from epimastigotes or parasites treated with 1·0–0·01 μg/ml (1 h, 4 °C) heparin, HS or CS prior to interaction. The midguts were spread onto glass slides and the number of attached parasites was randomly quantified by light microscopy. Ten insect midguts were used for each experimental group.

Surface plasmon resonance (SPR) assay

The SPR assays were performed on a carboxyl sensor chip surface (COOH) coated with immobilized neutravidin (Biocap; Nomadics, USA) that had been previously activated with biotinylated heparin (1 μg/ml). The HBPs were assessed in whole epimastigotes (10^6 cells) that had previously been washed in PBS. The assays were performed at 25 °C in 100 μl of running buffer (PBS) and at a flow rate of 10 μl/min. The binding specificity was assessed by incubation (1 h, 4 °C) of epimastigotes with 0·1–0·001 μg/ml sulfated GAGs (heparin, HS or CS). After the parasites were fixed in 4% paraformaldehyde, the binding assays were monitored in real time by a sensorgram and recorded as resonance units (RU). In these cases, the dissociation RU values represent the average of response between 633 and 925 seconds in all assays. A negative control was performed by flowing different concentrations of bovine serum albumin (BSA) directly across the heparin-coated sensor chip. Constants for association (Ka) and dissociation (Kd) were measured. These experiments were conducted in an optical biosensor apparatus, SensiQ Pioneer, and the data were analysed using the Qdat software (Nomadics, USA).

Scanning electron microscopy

After interaction of *R. prolixus* with untreated and GAG-treated epimastigotes, the insect midgut was fixed (1 h, 4 °C) with 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer, pH 7·2, followed by post-fixation with 1% osmium tetroxide in a similar buffer. The samples were dehydrated in a graded series of acetone, critical-point dried, coated with gold in a sputter coater and analysed with a JEOL scanning microscope (JSM6390LV).

Statistical analysis

The results were analysed for significance using ANOVA and Tukey's test according to the StatsDirect statistical software, version 2.2.7 for Windows 98 (Armitage *et al*. 2002). Differences between treated and control insects were considered not statistically significant when $P > 0{\cdot}05$. The probability levels are specified in the text.

RESULTS

The [^{35}S]methionine metabolic labelling assay combined with Triton X-114 phase separation and heparin-affinity chromatography was performed to evaluate the expression of heparin-binding proteins in the epimastigote forms of *T. cruzi*. This strategy yielded an enriched fraction of hydrophobic proteins that bound to heparin, which were eluted from an affinity column with a stepwise NaCl concentration gradient. Using this procedure, the peak of protein elution was obtained with 1·0 M NaCl, where 2 protein bands with molecular masses of 65·8 kDa and 59 kDa were mainly identified by SDS-PAGE (Fig. 1). Proteins with similar molecular masses were observed when unlabelled hydrophobic proteins were subjected to the same purification steps used for the labelled proteins. These unlabelled proteins were analysed by Western blot using biotinylated GAGs and are shown in Fig. 2. Our results demonstrated that both the 65·8 kDa and 59 kDa proteins are recognized by GAGs that consist of N-acetylglucosamine and uronic acid (heparin and HS) as well as N-acetylgalactosamine and glucuronic acid (C4S) (Fig. 2). Although both proteins interacted with the biotinylated GAGs, the 59 kDa protein seemed to be preferentially recognized by all GAGs evaluated (Fig. 2).

In addition, SPR assays were designed to directly evaluate the HBPs at the surface of epimastigotes and to characterize the receptor-ligand interaction. The presence of HBPs at the parasite surface was confirmed by the injection of the parasites onto the sensor chip surface, previously recovered with biotinylated heparin, and the sensorgram displayed dissociation values of 61·62 ± 2·87 RU (Fig. 3). These disassociation values were approximately 7·3-fold higher than the values obtained after injection of heparin (dissociation 8·4 ± 1·7 RU), indicating parasite attachment to the immobilized heparin on the sensor chip surface.

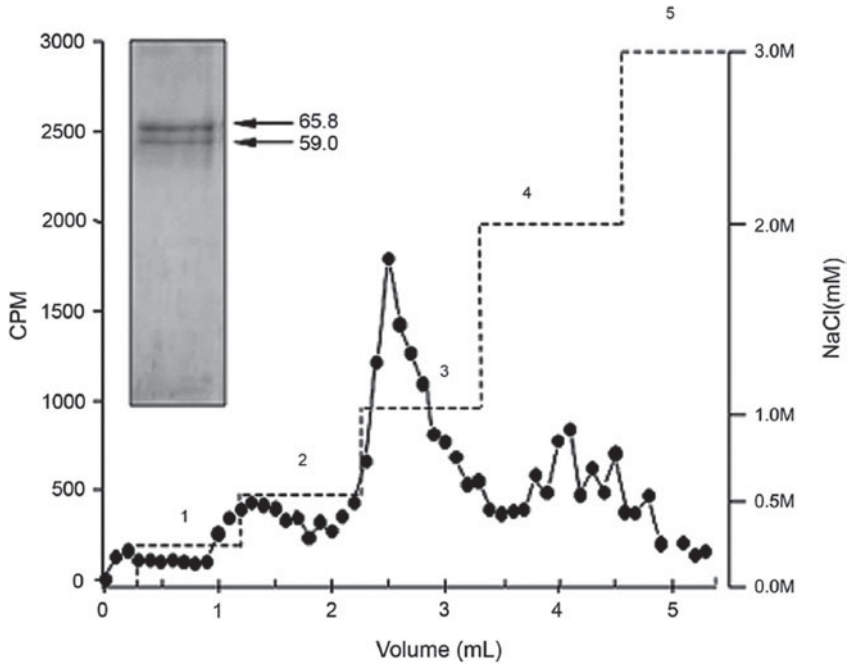

Fig. 1. Affinity chromatography of heparin-binding proteins (HBPs) from epimastigotes labelled with [^{35}S]methionine. Whole hydrophobic proteins were obtained by the TX-114 method. Proteins were eluted using different concentrations of NaCl – 0·25 M (1), 0·5 M (2), 1·0 M (3), 2·0 M (4) and 3·0 M (5), showing a peak of elution at 1·0 M. The data are presented as counts per minute (cpm). Analysis by SDS-PAGE revealed 2 bands with molecular masses of 65·8 and 59 kDa (insert).

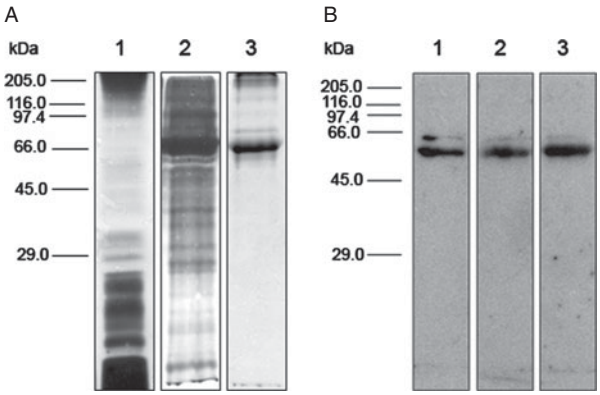

Fig. 2. Electrophoresis analysis of *Trypanosoma cruzi* epimastigote proteins. (A) Total hydrophobic proteins (1) or HBPs (2 and 3) were separated in each lane in the SDS-PAGE and revealed by silver (1 and 2) or Coomassie blue (3) staining. (B) HBPs were also separated by SDS-PAGE, transferred to a nitrocellulose membrane and revealed after incubation with biotinylated heparin (1), heparan sulfate (2) and chondroitin sulfate (3) by chemiluminescence. Molecular mass standard proteins (kDa) are indicated on the left.

The specificity of epimastigote binding to heparin was confirmed by GAG competition assays, where the parasite was previously incubated with increasing concentrations of heparin, HS and CS. Pre-incubation of epimastigotes with these sulfated GAGs led to an inhibition of parasite binding to immobilized heparin, which was measured by the association and dissociation RU values (Fig. 3). The inhibition of parasite binding was assessed by low dissociation RU values as compared to the control assay. The significant binding of RU was $37{\cdot}6 \pm 9{\cdot}49$ ($P \leqslant 0{\cdot}00002$) for heparin, $37{\cdot}14 \pm 8{\cdot}07$ ($P \leqslant 0{\cdot}00009$) for HS and $48{\cdot}3 \pm 3{\cdot}14$ ($P \leqslant 0{\cdot}0013$) for CS, achieving a binding inhibition of 39%, 40% and 22%, respectively. Additionally, control assays using 3 different concentrations (0·1, 0·01 and 0·001 μg/ml) of BSA were processed in parallel in the same SPR assays. The assessed dissociation ($2{\cdot}0 \pm 0{\cdot}3$, $1{\cdot}2 \pm 0{\cdot}0$ and $0{\cdot}4 \pm 0{\cdot}3$ RU, respectively) values were consistent with the lack of relevant binding between BSA and heparin, showing the specificity of the binding assays performed with epimastigotes.

Because GAGs, specifically HS and CS, have been identified in different tissues of *Triatoma brasiliensis* and *R. prolixus* (Souza *et al*. 2004), including the intestinal tract where epimastigotes attach, multiply and produce free, infective, metacyclic forms, we evaluated the role of HBPs in the epimastigote-vector interaction. Insects were dissected 10 days after feeding, and the luminal face of the midgut was exposed to facilitate the *in vitro* interaction of epithelial midgut cells with epimastigotes. Pre-incubation of the epithelial cells with an enriched fraction of HBPs, obtained by affinity chromatography, yielded a 3·8-fold inhibition in the adhesion of epimastigotes to posterior midgut epithelial cells. Specifically, the adhesion rate of $192{\cdot}5 \pm 27{\cdot}4$ epimastigotes/100 midgut epithelial cells in the controls (untreated parasites) was reduced to $50{\cdot}3 \pm 24{\cdot}4$ in pre-treated epithelial cells (Fig. 4). In preparations

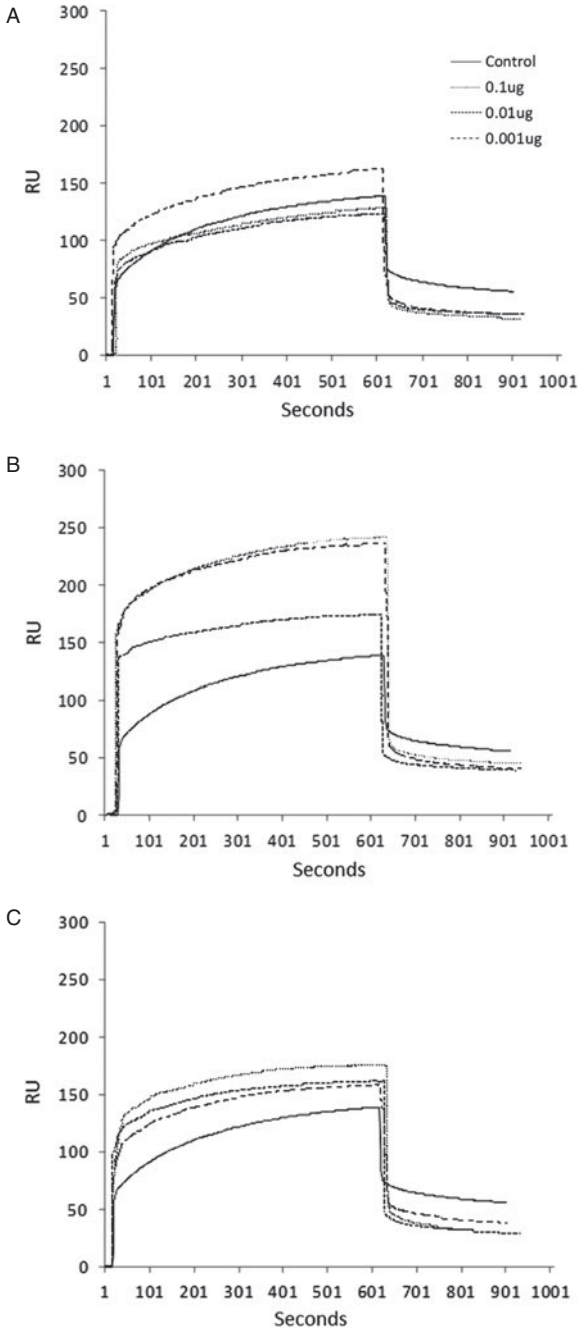

Fig. 3. Sensorgrams showing the presence of HBPs on the surface of *Trypanosoma cruzi* epimastigotes by surface plasmon resonance. SPR assays were performed with 10^6 epimastigotes in a final volume of 100 μl and a flow rate of 10 μl/s. Biocap sensor chips were covered with biotinylated heparin, and epimastigotes were passed over its surface. The inhibition assays were performed following incubation of the epimastigotes with glycosaminoglycans (GAGs), i.e., heparin (A), heparan sulfate (B) and chondroitin sulfate (C). The parasites were assayed without pre-incubation with GAGs or after a pre-incubation with 0·1 μg/ml, 0·01 μg/ml or 0·001 μg/ml GAGs. The interaction assays were performed in PBS. The resonance signals were analysed after subtraction of a reference line using the Qdat software. The assays were performed in triplicate, and the mean value for resonance units was obtained based on the results of 3 independent assays.

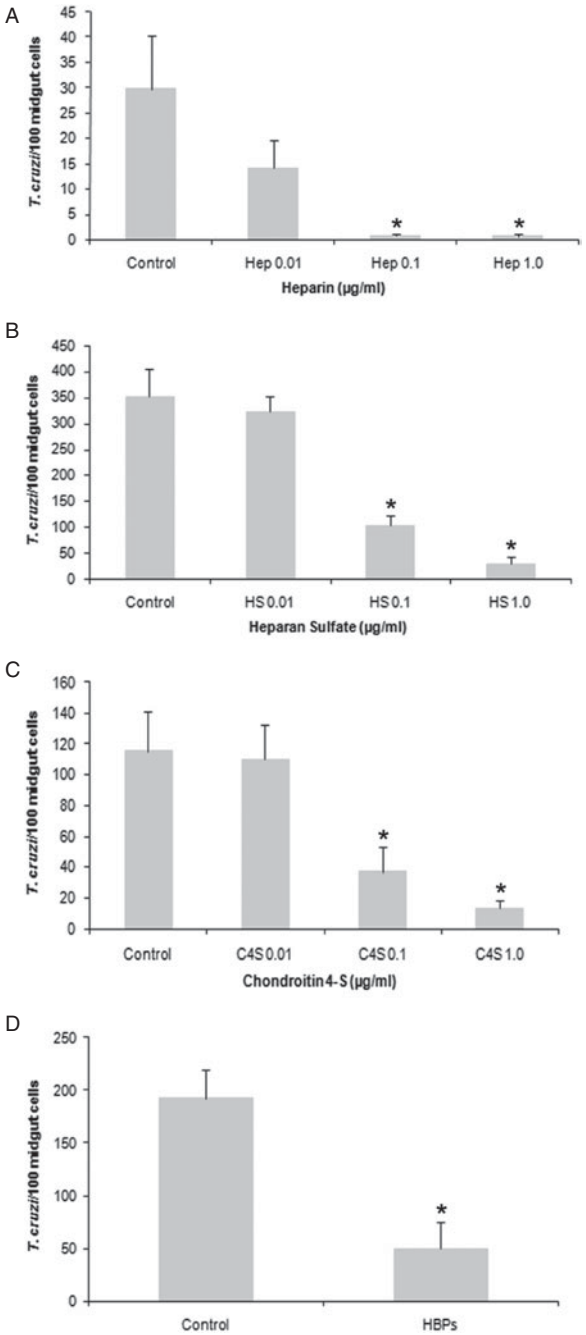

Fig. 4. Role of epimastigote HBPs and sulfated GAGs on the *in vitro* attachment of *Trypanosoma cruzi* to the posterior midgut epithelium of *Rhodnius prolixus*. Epimastigotes were previously incubated with different concentrations of GAGs (0·01, 0·1 and 1·0 μg/ml) in LIT medium containing $2{\cdot}0 \times 10^7$ parasites/ml, washed and added to the interaction medium. Alternatively, posterior midguts were incubated with isolated HBPs from 10^{11} epimastigotes prior to interaction with *T. cruzi*. Adhered epimastigotes were counted per 100 epithelial cells in 10 different fields of each midgut preparation using a Zeiss microscope with a reticulated ocular. Each group shows the mean ± S.E. of parasites attached in 10 midguts. Non-treated (Control), heparin (Hep; A), heparan sulfate (HS; B), chondroitin 4-sulfate (C-4S; C) and HBPs (D). Non-treated (control) experiments were performed without GAGs or HBPs in the same pre-incubation conditions. (*) $P \leqslant 0{\cdot}05$.

obtained from the controls and epimastigotes pre-treated with 0·01 μg/ml of heparin, HS or C4S, similar rates of parasite adhesion per 100 midgut cells were observed ($P > 0·05$). In contrast, attachment of only 102·08 ± 18·08 ($P < 0·0001$) and 29·8 ± 12·02 ($P < 0·0001$) parasites per 100 midgut epithelial cells were recorded when the flagellates were incubated with either 0·1 or 1·0 μg/ml HS, respectively, while the control group exhibited an adherence of 351·14 ± 53·34 parasites under the same conditions. Similar results were observed after parasite incubation with 0·1 μg/ml (37·3 ± 15·57) or 1·0 μg/ml (13·3 ± 5·43) C4S compared to controls (115·5 ± 27·71). Similarly, parasite incubation with either 0·1 or 1·0 μg/ml heparin strongly reduced the attachment of *T. cruzi* to only 0·84 ± 0·29 and 0·67 ± 0·46 epimastigotes per 100 midgut cells ($P < 0·001$), respectively (Fig. 4). This inhibition was clearly visualized by scanning electron microscopy (SEM). As expected, SEM micrographs revealed a large number of microvilli on the surface of the midgut epithelial cells. After the interaction between parasite and midgut cells *in vitro*, epimastigotes were mostly attached by the flagellum to the epithelium of the intestinal tract, though an association with the parasite body was also observed. Large numbers of epimastigotes were found to be associated with midgut epithelial cells in the control. In contrast, few epimastigotes were found to be adhered to the epithelial cells after pre-incubation of the parasites with GAGs (Fig. 5).

DISCUSSION

Arthropods are considered to be important vectors in the transmission of human infectious diseases (Romi, 2010; Williams *et al*. 2010). The transmission of infection by the parasite is mediated through the control and manipulation of biological and molecular events in the vector and mammalian hosts (Lefèvre and Thomas, 2008; Matthews, 2011). Therefore, a large number of investigations have focused on understanding the biology of host-parasite interactions. In this context, studies analysing the relationship between *T. cruzi* and its vector, triatomines (Hemíptera: Reduviidae: Triatominae), have been emphasized in recent years (Zimmermann *et al*. 2010; Garcia *et al*. 2010; Ennes-Vidal *et al*. 2011). An important stage in the developmental cycle of *T. cruzi* within the invertebrate host is the interaction between the surface of the parasite and molecules that are present in the intestinal tract of triatomines. In this article, we report the presence of HBPs in epimastigotes and their important role in the recognition and adherence of the parasite to the epithelium of the intestinal tract of *R. prolixus*.

Structures that bind to host cell GAGs have been identified in many pathogens (Herrera *et al*. 1994; Scagliarini *et al*. 2004; Azevedo-Pereira *et al*. 2007; Linder *et al*. 2010). In *T. cruzi*, HBPs have been reported to promote attachment to and invasion of mammalian host cells (Ortega-Barria and Pereira, 1991; Herrera *et al*. 1994; Calvet *et al*. 2003). We have previously demonstrated that trypomastigotes and amastigotes present HBPs that modulate parasite adhesion and invasion in cardiomyocytes (Oliveira-Jr *et al*. 2008; Bambino-Medeiros *et al*. 2011). However, these proteins had not been detected in epimastigotes, parasites found in the invertebrate host, and their involvement in the parasite-vector interaction had not been evaluated thus far. Our data show that epimastigotes also possess proteins with heparin-binding properties. Heparin-affinity chromatography revealed 2 major hydrophobic proteins capable of recognizing not only heparin but also HS and CS. Interestingly, HBPs have been previously identified in trypomastigotes and amastigotes (Oliveira-Jr *et al*. 2008), suggesting that these proteins are constitutively produced throughout the *T. cruzi* life cycle.

Thus, considering that HBPs have a key role in adhesion and invasion of mammalian cells, we evaluated their participation in the attachment of epimastigotes to midgut epithelial cells of *R. prolixus*, as GAGs, such as HS and CS, have been identified in the intestinal tract of this vector (Costa-Filho *et al*. 2004; Souza *et al*. 2004). Inhibition of epimastigote adhesion was achieved after pre-incubation of the midgut cells with HBPs isolated from epimastigotes. In addition, the inhibition of parasite adhesion after pre-treatment of epimastigotes with GAGs corroborates the competence of ligand-receptor binding. SEM showed that GAG treatment abrogates parasite adhesion, as a substantial reduction in the levels of epimastigote adhesion was observed after GAG pre-incubation.

The most striking find was the participation of CS in the adherence of epimastigotes to midgut epithelial cells. The involvement of CS in host-parasite recognition seems to be specific for epimastigotes because this GAG does not interfere with the adhesion and invasion of *T. cruzi* in mammalian cells (Calvet *et al*. 2003; Bambino-Medeiros *et al*. 2011). Therefore, the recognition of multiple GAGs by HBPs, evidenced by Western blot assay, may improve the success of the infection in the invertebrate host by promoting a tight association between the parasite and vector midgut cells. The involvement of GAGs in the modulation of cell adhesion has also been reported for the *Plasmodium* sp. life cycle. Highly sulfated HSPGs mediate the invasion of *Plasmodium* sporozoites in hepatocytes through the major surface proteins CSP and TRAP (Pradel *et al*. 2002; Coppi *et al*. 2007). In addition, the participation of chondroitin sulfate A (CSA) has been reported in the adhesion of *Plasmodium* sp.-infected erythrocytes to the placenta, which causes severe pregnancy-associated malaria (Nunes and Scherf, 2007). In the *Anopheles* mosquitoes, both HS and CS

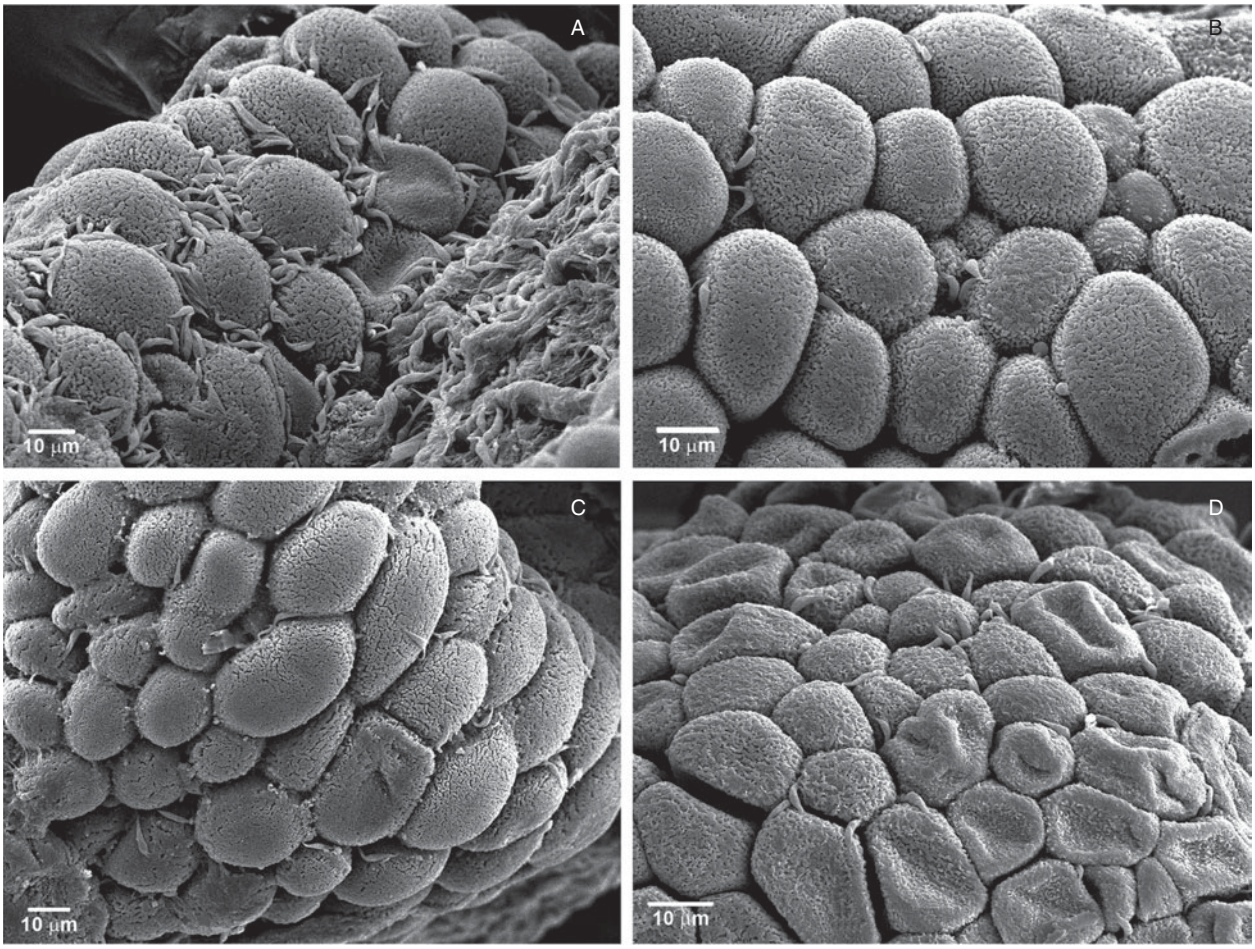

Fig. 5. Scanning electron microscopy of the epimastigote-*Rhodnius prolixus* interaction. Competition assays were performed to evaluate the participation of sulfated GAGs in the parasite-host cell recognition. (A) Midgut epithelial cells were incubated with epimastigotes (control). Pre-treatment of epimastigotes with 1 μg/ml heparin (B), heparan sulfate (C) or chondroitin sulfate (D) led to an inhibition of parasite attachment to the midgut epithelial cells. Scale bar = 10 μm.

have been characterized in the salivary gland (Sinnis *et al*. 2007). Interestingly, it has been demonstrated that ookinetes recognize the chondroitin sulfate proteoglycans at the apical midgut microvilli during epithelial cell invasion (Dinglasan *et al*. 2007), suggesting that distinct proteoglycans may have different binding affinities.

Besides demonstrating the role of HBPs in *R. prolixus*-epimastigote recognition, we also examined the stability of the receptor-ligand interaction by biosensor analysis. The SPR data effectively showed the binding of epimastigotes to immobilized heparin on the Biocap chip, indicating the surface localization of proteins with the ability to bind heparin. In addition, the reduction in the association/dissociation values in the GAG competition assay reinforced the conclusion that the HBPs recognize GAGs, which is in good agreement with the SEM data. Furthermore, the maintenance of the dissociation rates above the sensorgram baseline is evidence of the stable interaction between epimastigote proteins and heparin. Although whole-cell biosensor analysis has already been applied to evaluate receptor-ligand interactions (Fang *et al*. 2006; Oli *et al*. 2006), this is the first time that this technology has been proposed to detect specific proteins on the surface of epimastigotes and correlate the physiological and stable interaction that occurs at the interface of epimastigotes to the midgut cells of the insect.

Biosensing surface procedures have been widely used to elucidate the adhesion and invasion phenomena in parasite-host relationships, especially concerning parasite proteins that recognize and bind to GAGs. Direct interaction between the measles virus and heparin was assessed by the biosensor method to prove that GAG binding to the haemagglutinin protein of this virus prevents the infection of SLAM-negative cells lines (Terao-Muto *et al*. 2008). In addition, this technology was useful for the direct measurement of the interactions between GAGs and the *Plasmodium* circumsporozoite protein, which supported the hypothesis of the mechanism of invasion in liver cells. Heparin bound most strongly among the GAGs tested, and heparin decasaccharide is the smallest circumsporozoite protein-binding sequence (Rathore *et al*. 2001).

In conclusion, our data demonstrate the presence of HBPs (59 kDa and 65·8 kDa) on the surface of epimastigotes that have the ability to recognize GAGs, such as heparin, HS and C4S. We also provide evidence of a stable receptor-ligand interaction and highlight the role of HBPs in targeting epimastigotes to the midgut of *R. prolixus*. Additional studies are needed to elucidate the nature of the GAG binding sites involved in the epimastigote-vector interaction.

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Involvement of host cell heparan sulfate proteoglycan in *Trypanosoma cruzi* amastigote attachment and invasion

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SUMMARY

Cell surface glycosaminoglycans (GAGs) play an important role in the attachment and invasion process of a variety of intracellular pathogens. We have previously demonstrated that heparan sulfate proteoglycans (HSPG) mediate the invasion of trypomastigote forms of *Trypanosoma cruzi* in cardiomyocytes. Herein, we analysed whether GAGs are also implicated in amastigote invasion. Competition assays with soluble GAGs revealed that treatment of *T. cruzi* amastigotes with heparin and heparan sulfate leads to a reduction in the infection ratio, achieving 82% and 65% inhibition of invasion, respectively. Other sulfated GAGs, such as chondroitin sulfate, dermatan sulfate and keratan sulfate, had no effect on the invasion process. In addition, a significant decrease in infection occurred after interaction of amastigotes with GAG-deficient Chinese Hamster Ovary (CHO) cells, decreasing from 20% and 28% in wild-type CHO cells to 5% and 9% in the mutant cells after 2 h and 4 h of infection, respectively. These findings suggest that amastigote invasion also involves host cell surface heparan sulfate proteoglycans. The knowledge of the mechanism triggered by heparan sulfate-binding *T. cruzi* proteins may provide new potential candidates for Chagas disease therapy.

Key words: cardiomyocytes, *Trypanosoma cruzi*, amastigotes, heparan sulfate, recognition process.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease, exploits different strategies to invade mammalian cells. The invasion process is complex and seems to depend on the molecules engaged in the recognition process during *T. cruzi*-host cell interaction, triggering distinct signalling pathways (Fernandes *et al*. 2006; Ferreira *et al*. 2006; Yoshida and Cortez 2008). Although trypomastigotes are classic infective forms, amastigotes are also able to invade mammalian cells (Ley *et al*. 1988) and are crucial for the maintenance of tissue infection and inflammation (Scharfstein and Morrot, 1999).

Molecules at the host cell surface, including carbohydrates (Barbosa and Meirelles, 1992; Araújo-Jorge and De Souza, 1998), extracellular matrix components (Calvet *et al*. 2003, 2004), and also specialized microdomains at the plasma membrane (Barrias *et al*. 2007; Fernandes *et al*. 2007), play key roles in the *T. cruzi* invasion process. Binding of amastigotes mainly at the surface microvilli of HeLa cells, while trypomastigotes invade preferentially by the cell edges, is suggestive of the participation of distinct membrane molecules in the recognition and invasion process of infective forms of *T. cruzi* (Mortara, 1991). Although efforts have been made to determine the molecules involved in the receptor-ligand recognition during trypomastigote-host cell interaction, little is known about the surface receptors and extracellular matrix molecules that orchestrate adhesion and invasion of amastigotes.

Evidence has demonstrated the involvement of the mannose receptor in the recognition of *T. cruzi* amastigotes by macrophages (Kahn *et al*. 1995). Also, the participation of caveolae and lipid rafts has been recently demonstrated in the internalization of amastigotes (Barrias *et al*. 2007; Fernandes *et al*. 2007). The localization of cell surface heparan sulfate proteoglycans (HSPG) in signalling membrane microdomains, including lipid rafts (Chu *et al*. 2004) and caveolae (Stan, 2002), which concentrates protein- and lipid-signalling molecules (Kawamura *et al*. 2003), lead us to postulate its involvement in amastigote invasion.

HSPG consist of heparan sulfate (HS) glycosaminoglycan (GAG), composed of alternating units of hexuronic acid [D-glucuronic acid (GlcA) or

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L-iduronic acid (IdoA)] and D-N-acetylglucosamine, covalently linked to specific core proteins, syndecans and glypicans (Bernfield *et al*. 1999; Dietrich *et al*. 1998). This molecule is ubiquitously distributed in the surface of mammalian cells and participates in many biological processes (Ori *et al*. 2008; Yung and Chan, 2007). A wide variety of bioactive proteins, including growth factors, chemokines and cytokines, are modulated by HSPG, which act as key regulators of mitogenic factors at the cell surface (Chen *et al*. 2007; Yung and Chan, 2007). Furthermore, studies have demonstrated that HSPG mediate the invasion process of many intracellular pathogenic microorganisms (Jacquet *et al*. 2001; Plotkowski *et al*. 2001; Reddi and Lipton, 2002; Chen *et al*. 2008), including *Trypanosoma cruzi* (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008; Ortega-Barria and Pereira, 1991). The presence of a heparin-binding protein has been demonstrated in both infective forms of *T. cruzi* (Oliveira-Jr *et al*. 2008; Ortega-Barria and Pereira, 1991), but its role in the amastigote invasion has not been demonstrated yet. In this study, we evaluated the participation of sulfated proteoglycans in the invasion process of amastigote forms of *T. cruzi* in cardiomyocytes.

MATERIALS AND METHODS

Cardiomyocyte cultures

Primary cultures of cardiomyocytes were obtained from 18-day-old mouse embryos (Meirelles *et al*. 1986). Briefly, ventricular cardiac cells were isolated by enzymatic dissociation using 0·025% trypsin (Sigma Chemical Co., Missouri, USA) and 0·01% collagenase (Worthington Co., New Jersey, USA). The isolated cells were plated at a density of 8×10^4 cells/ml into 24-well culture dishes containing glass cover-slips coated with 0·01% gelatin (Sigma). The cells were cultivated in Dulbeccos' modified Eagle medium (DMEM; Sigma) supplemented with 5% fetal bovine serum (FBS; Sigma), 2·5 mM $CaCl_2$, 1 mM L-glutamine (Sigma), 2% chicken embryo extract plus antibiotics and maintained at 37 °C in a 5% CO_2 atmosphere. The animal procedures were approved by the animal care and use committee (license LW-11/09, Oswaldo Cruz Foundation).

Amastigogenesis in vitro

Culture-derived trypomastigotes, clone Dm28c, were obtained from Vero cultures at day 4 post-infection. The *in vitro* amastigogenesis procedure was modified from Tomlinson and co-workers (1995). Trypomastigotes (5×10^6 parasites/ml) were incubated for 4 h at 37 °C in RPMI 1640 medium, pH 5·0, plus 1% FBS. Afterwards, acid-induced parasites were centrifuged at 2100 ***g*** for 10 min and the sediment was resuspended in RPMI, pH 7·2, plus 1% FBS. The parasites were transferred to 25 cm^2 flask culture (5×10^7/10 ml) and maintained at 37 °C in a 5% CO_2 atmosphere. Parasite morphology was observed daily under a phase-contrast inverted microscope (Axiovert Zeiss).

Parasite-host cell interaction

Forty-eight hour cardiomyocyte cultures were infected with extracellular amastigote forms of *T. cruzi*, Dm28c, at a ratio of 30:1, 50:1 and 65:1 parasites/host cell. After 2 h and 4 h of interaction, the cultures were fixed with Bouin fixative and stained with Giemsa. The infection level was determined by quantifying a minimum of 200 cells in random selected microscopic fields. The invasion assay was performed in duplicate in 3 independent experiments. Amastigote differentiation indices lower than 95% were not employed in the parasite-host cell interaction assays.

Glycosaminoglycan competition assay

To investigate the participation of GAGs on parasite invasion, extracellular amastigotes (Dm28c) were pre-treated for 1 h on ice with 20 μg/ml of heparin from bovine intestine, heparan sulfate (HS) from bovine pancreas, chondroitin 4-sulfate (CS) from whale cartilage (Seikagaku America, USA), dermatan sulfate (DS) from bovine mucosa (Sigma) or keratan sulfate (KS) from bovine cornea (Sigma). Untreated amastigotes were used as control. After treatment, the amastigotes were incubated, at a ratio of 65:1 parasites/host cell, for 2 h at 37 °C with the target cells followed by fixation with Bouin and Giemsa staining. The percentage of infection was determined as described above.

As a control of the endocytic process, we interacted Zymosan (Zy) particles (Sigma) with cardiomyocyte cultures. Similar to amastigotes, the Zy particles were pre-treated or not with 20 μg/ml of GAGs prior to interaction, at a ratio of 65:1 Zy particles/host cell, for 2 h at 37 °C with cardiomyocytes, fixed and processed as described. The assay was performed 3 times in duplicate.

Chinese hamster ovary cells (CHO cells)

CHO (CHO-k1, wild-type) and glycosaminoglycan-deficient (CHO-745) cells were plated at 5×10^4 cells/ml in Ham's F-12 medium (GIBCO, Grand Island, NY, USA) plus 7% FBS on 24-well culture dishes containing glass cover-slips and grown for 24 h at 37 °C in a 5% CO_2 atmosphere. The cells were infected with extracellular amastigotes at a ratio of 65:1 parasites/host cell. After 2 h and 4 h of interaction, the cells were fixed with Bouin and stained with Giemsa. The percentage of infection was determined as described above. Also, an experimental assay was performed adding GAGs to CHO-745 cultures during amastigote infection.

Indirect immunofluorescence

Amastigotes were adhered to poly-L-lysine-coated glass cover-slips and fixed for 20 min at 4 °C with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7·2. Following blockage, the parasites were incubated overnight at 4 °C with anti-Ssp4 (1:100) monoclonal antibody, which was kindly supplied by Dr Norma Andrews. After rinsing, the cells were incubated for 1 h at 37 °C with FITC-conjugated anti-mouse IgG (1:200; Sigma). The DNA was detected with 10 μg/ml 4,6-diamidino-2-phenylindole dye (DAPI; Sigma) and the cover-slips were mounted with 2·5% 1,4-diazabicyclo-(2,2,2)-octane (DABCO; Sigma) in PBS containing 50% glycerol, pH 7·2. Controls were performed by incubation with homologous serum or by omission of the primary antibody. Images were acquired using a Zeiss Axioplan epifluorescence microscope (Zeiss) equipped with a cooled CCD camera (Color View Soft Imaging System).

Flow cytometry study

To evaluate the effect of glycosaminoglycans on parasite viability, amastigotes were incubated for 1 h on ice with 20 μg/ml of heparin or HS followed by 2 h of treatment at 37 °C. Afterwards, the parasites were stained with 30 μg/ml propidium iodide (PI) for 15 min as previously described (Menna-Barreto *et al*. 2007). As positive control, amastigotes were permeabilized with 0·1% saponin for 30 min and PI stained. Untreated amastigotes were used as negative control. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). In total 10 000 events were acquired in the regions previously established as those corresponding to each form of *T. cruzi*.

Transmission electron microscopy

Acid-induced amastigotes were treated with 20 μg/ml of HS or heparin for 48 h and then fixed for 1 h at 4 °C with 2·5% glutaraldehyde in 0·1M sodium cacodylate buffer, pH 7·2, containing 3·5% sucrose. Afterward, the parasites were post-fixed for 1 h at 4 °C with 1% osmium tetroxide (OsO_4) in 0·1M sodium cacodylate buffer, pH 7·2, containing 3·5% sucrose. The samples were dehydrated in an acetone series and embedded in Epoxy resin. Ultrathin sections were stained with 5% uranyl acetate and 2% lead citrate and then observed in a JEOL transmission electron microscope (JEM/1011). Untreated amastigotes were used as control.

Statistical analysis

Student's *t*-test was used to determine the significance of differences between means in the invasion assays; a P value $\leqslant 0{\cdot}05$ was considered significant.

RESULTS

To analyse the participation of host cell sulfated proteoglycans in the invasion process by *T. cruzi* amastigotes, we performed a competition assay using soluble sulfated glycosaminoglycans (GAGs). First, we evaluated the level of infection of extracellular amastigotes, Ssp4 positive (Fig. 1), in cardiomyocyte cultures at early time-points (2 h and 4 h). The kinetic study with different host cell/parasite ratios (30:1, 50:1 and 65:1) revealed levels of infection varying between 10% and 30% after 2 h and 4 h of interaction at ratios of 30:1 and 50:1, respectively (Fig. 1). Thus, in order to avoid shedding of bound molecules from the parasite surface in the invasion assay, we performed the competition experiments at the shorter time of interaction (2 h) using a ratio of 65:1 parasite/host cell, which achieved approximately 25% infection.

Next, we analysed the involvement of sulfated GAGs in the recognition and invasion process of amastigotes in cardiomyocytes. The amastigotes were pre-treated with 20 μg/ml of soluble sulfated GAGs, including heparan sulfate (HS), heparin, dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS). Our results revealed that treatment of amastigotes with HS or heparin, GAGs consisting of repeated alfa-linked disaccharides, inhibit amastigote invasion in cardiomyocytes. A drastic reduction in the level of infection was noticed when the amastigotes were treated with heparin, decreasing from 25% in the control to 4·5% infection ($P \leqslant 0{\cdot}0001$), which represents 82% inhibition of parasite invasion (Fig. 2). A significant reduction was also observed after pre-treatment of the parasites with HS, achieving 8·7% ($P \leqslant 0{\cdot}0004$) infection that corresponds to 65% inhibition of invasion (Fig. 2). An approximately 10-fold decrease in the number of attached and intracellular parasites was observed when amastigotes were pre-treated with heparin and heparan sulfate (Fig. 2), indicating that blockage of heparin-binding protein impairs amastigote invasion. The effect of heparin and HS in *T. cruzi* invasion is clearly visualized by light microscopy, showing only a few cells with attached and interiorized parasites (Fig. 2). In contrast, the other sulfated GAGs examined herein, CS, DS and KS, which are GAGs consisting of beta-linked disaccharides, did not inhibit amastigote entry, reaching infection levels similar to the control (Fig. 2). However, to address the question of whether the GAGs are either involved in amastigote invasion or interfere with the endocytic process, we interacted an inert particle, zymosan (Zy), with the cardiomyocyte culture in the presence of HS or heparin. Neither treatment with HS nor heparin affected the cardiomyocyte endocytic process. The level of Zy particle

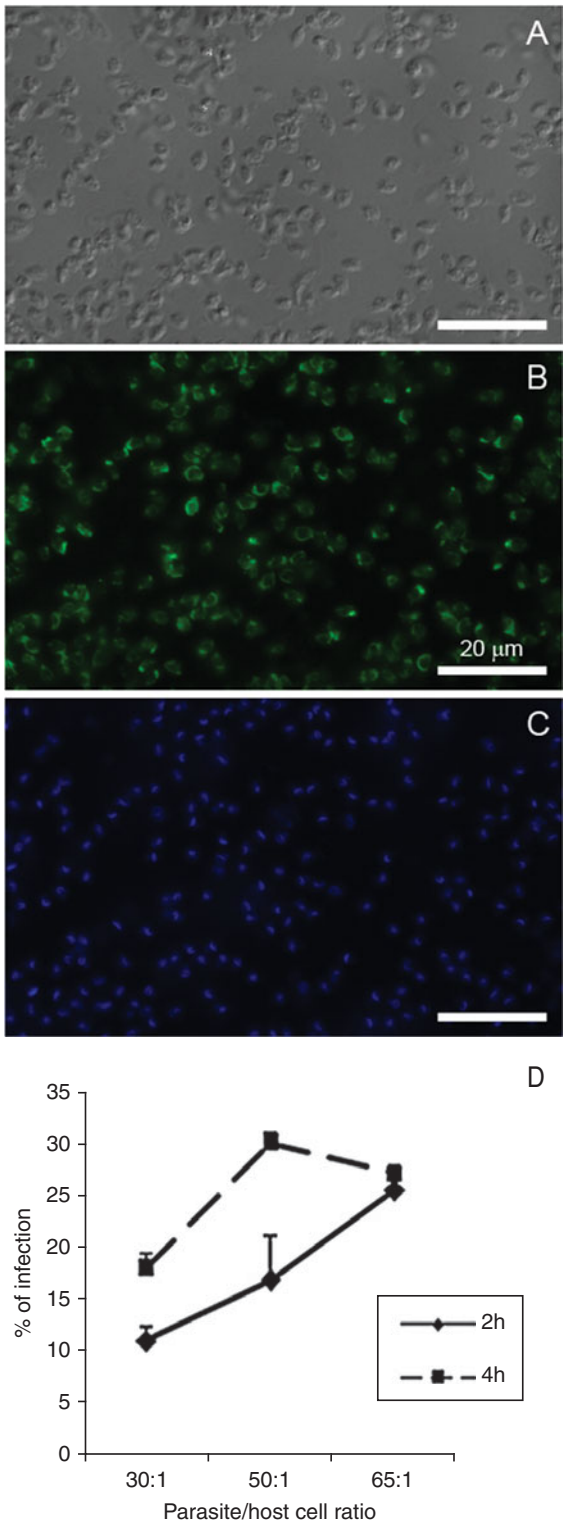

Fig. 1. Kinetic study of amastigote-host cell interaction. (A) Differential interferential contrast (DIC) image of extracellular amastigotes. (B) Amastigotes revealed an intense staining of Ssp4 (green), the stage-specific antigen of amastigotes, at the surface of the parasite. (C) DNA labelling with DAPI (blue)-stained nuclei and kinetoplast of parasites. (D) Cardiomyocytes were infected at the ratio of 30:1, 50:1 and 65:1 amastigotes/ host cell for 2 h and 4 h of interaction. A high level of infection, 25%, was achieved after 2 h of interaction at the ratio of 65:1 amastigotes/ host cell. Scale bar = 20 μm.

internalization reached 13% after 2 h of interaction with cardiomyocyte cultures and remained unaltered in the presence of GAGs (Fig. 2). The involvement of GAGs in amastigote invasion was also evaluated using proteoglycan-deficient Chinese hamster ovary (CHO) cells. Wild-type CHO cells (CHO-K1) and GAG-deficient cells (CHO-745), a mutant defective in GAG biosynthesis due to the xylosyl transferase depletion, were infected for 2 h and 4 h with amastigotes at a ratio of 65:1 parasite-host cell. Our results demonstrate that amastigote invasion is impaired in CHO-745 mutant cells when compared to the wild-type CHO-K1 cells. The percentage of infection reduced significantly from 20·3% and 28% in CHO-K1 to 5·3% ($P \leqslant 0{\cdot}0009$) and 9% ($P \leqslant 0{\cdot}013$) in CHO-745 after 2 h and 4 h of infection, respectively (Fig. 3). Addition of soluble HS and heparin to CHO-745 cultures during amastigote infection did not revert the infection level (data not shown). The failure of soluble GAGs to restore the infection may be related to the fact that binding of GAGs to the core protein of proteoglycans is carried out by activity of specific enzymes (glycosyltransferases – EXT1 and EXT2) in the Golgi apparatus.

To ensure that the alterations observed in amastigote invasion were not related to toxic effects of soluble GAGs on amastigotes, we analysed the viability of heparin- and HS-treated amastigotes using propidium iodide (PI) labelling. The flow cytometry analysis demonstrated no significant alteration in the amastigote viability with GAGs treatment (Fig. 4). Membrane permeabilization was not observed after heparin or HS treatment, which exhibited a similar amount of PI uptake compared to control cells. Also, ultrastructural analysis revealed no evidence of morphological changes in amastigotes or signs of parasite death. In addition, amastigotes in the process of division were visualized after GAG treatment by transmission electron microscopy (Fig. 4).

DISCUSSION

Intracellular pathogen-host cell interactions are dynamic and complex processes, in which parasite attachment is a primary requisite to establish infection. Glycosaminoglycans interact with cell surface molecules of a variety of intracellular pathogens, which enable attachment and trigger parasite invasion (Tossavainen *et al*. 2006; Yokoyama *et al*. 2006; Moelleken and Hegemann, 2008; Schuksz *et al*. 2008). Therefore, efforts have been made to identify the parasite's heparin-binding proteins involved in the parasite-host cell recognition and their application as a prospective target for diagnostics or therapy.

In *T. cruzi* infection, both trypomastigotes and amastigotes are able to invade mammalian cells and sustain the intracellular cycle (Ley *et al*. 1988). Although distinct mechanisms and signalling

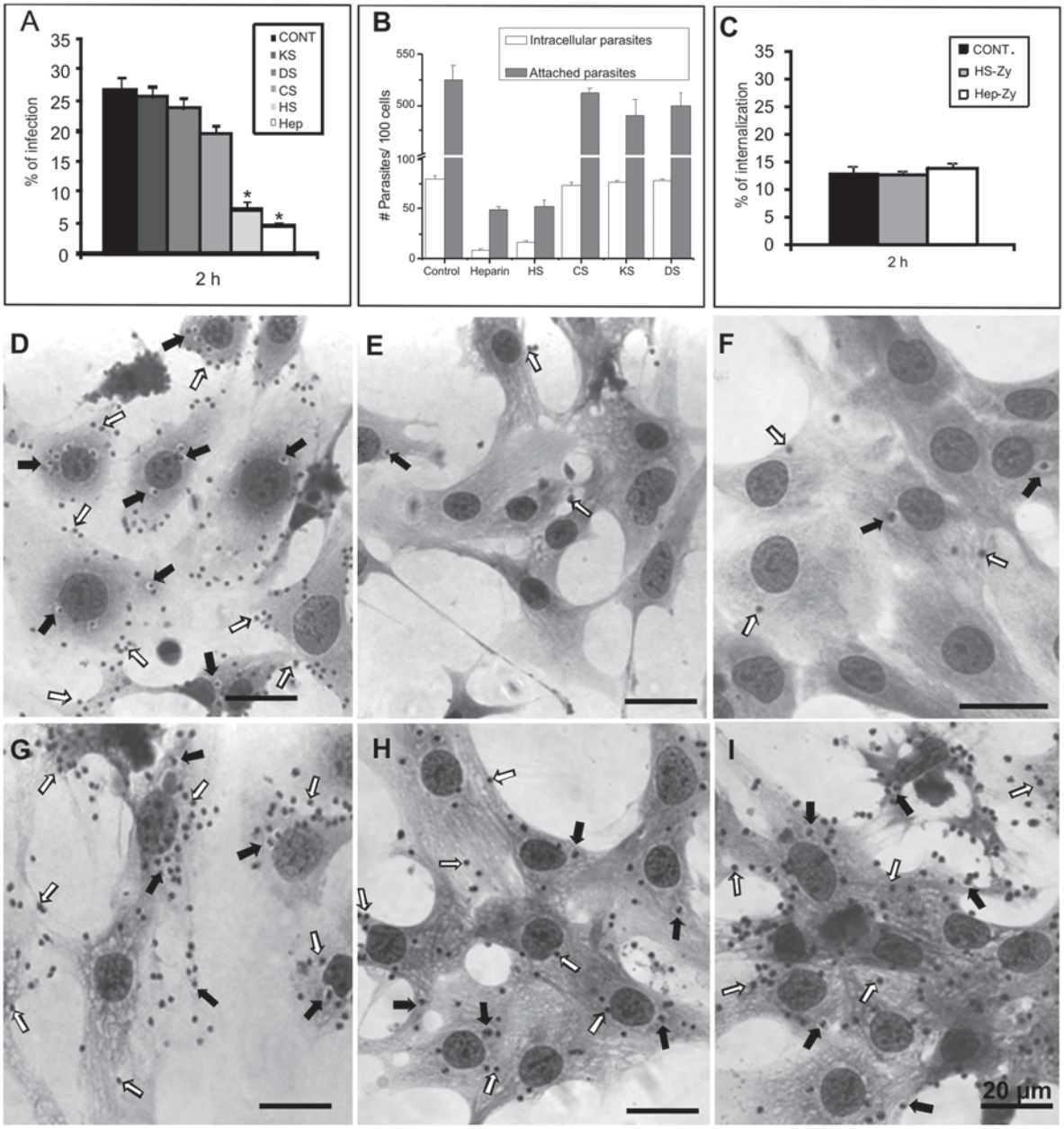

Fig. 2. Sulfated glycosaminoglycans (GAGs) are involved in host cell invasion by *Trypanosoma cruzi* amastigotes. (A) Pre-treatment of amastigotes with 20 μg/ml of heparin and heparan sulfate (HS) prior to cardiomyocyte interaction resulted in a significant inhibition of 82% and 65% in the infection levels (2 h). No inhibition was detected with chondroitin sulfate (CS), keratan sulfate (KS) and dermatan sulfate (DS). (B) The relative number of attached and internalized parasites after treatment with GAGs. (C) Effect of GAGs on the endocytic process. Note that zymosan (Zy) particle internalization is not altered after GAGs treatment. (D) General aspect of amastigote-cardiomyocyte interaction (2 h of infection) and after treatment of amastigotes with 20 μg/ml of heparin (E), HS (F), CS (G), KS (H) and DS (I). Arrows indicate interiorized (➞) or attached (⇨) parasites. (*) Student's *t*-test: statistically significant $P \leqslant 0{\cdot}05$. Scale bar = 20 μm.

pathways involved in the invasion process have been reported between these infective forms (Mortara *et al*. 2008; Alves and Mortara, 2009), the molecules required for amastigote invasion have been poorly investigated.

The fact that HSPG can activate signalling cascades involved in cytoskeleton remodelling (Dreyfuss *et al*. 2009) associated with the detection of glycosaminoglycan-binding proteins in amastigote forms of *T. cruzi* (Oliveira-Jr *et al*. 2008) opened the question as to whether sulfated proteoglycans would also mediate amastigote invasion. Our results showed that treatment of amastigotes with heparin and heparan sulfate prevented cardiomyocyte invasion, achieving 82% and 65% inhibition, respectively. Similar to trypomastigotes, amastigote recognition is mediated by GAGs containing alpha-linked disaccharides, while the other negatively charged

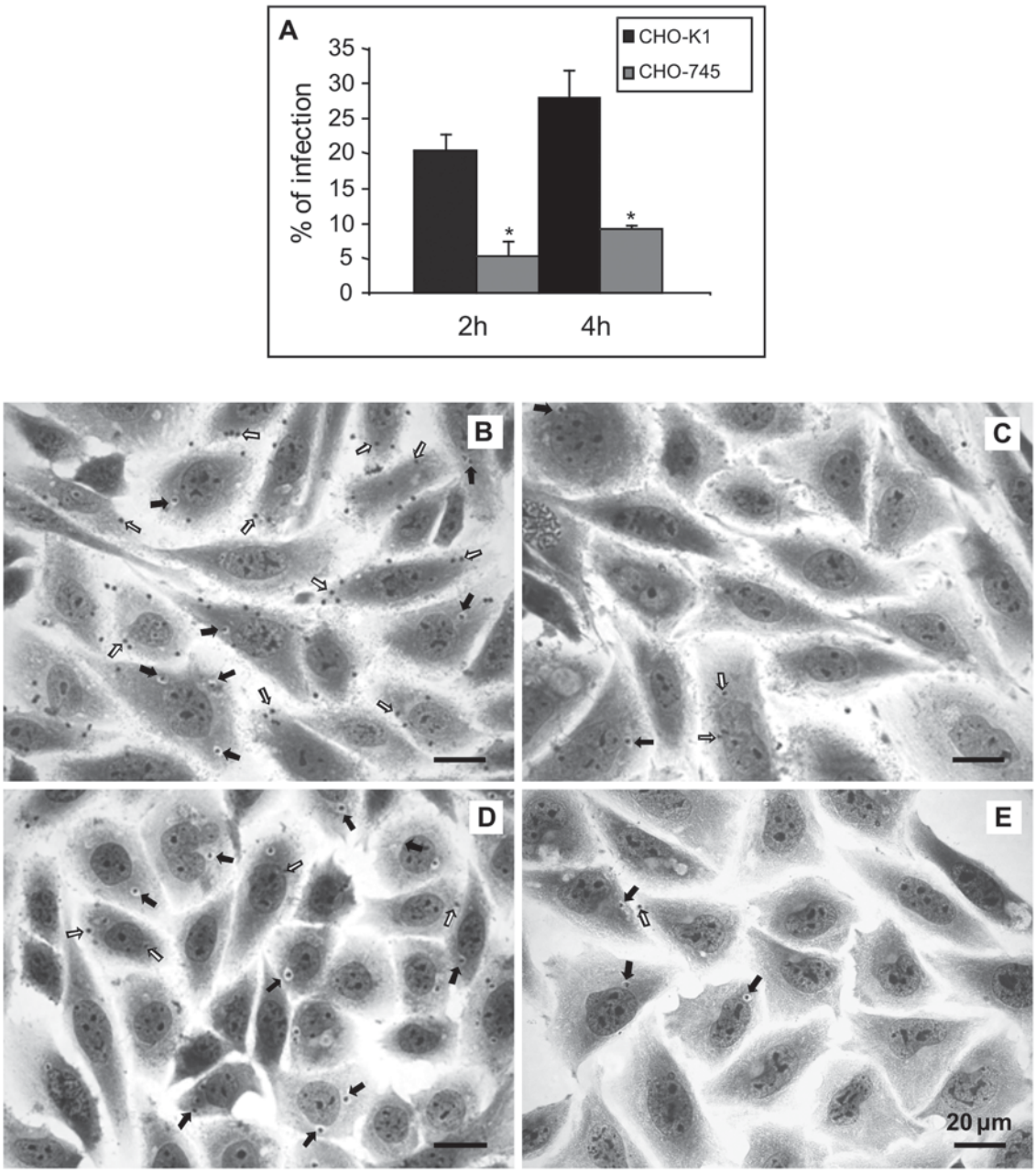

Fig. 3. Amastigote invasion in Chinese hamster ovary (CHO) cells. CHO-K1 (wild-type) and CHO-745 (proteoglycan-deficient mutant) cultures were infected with amastigotes of *Trypanosoma cruzi* for 2 h and 4 h (A). Amastigote invasion was impaired in the CHO-745 mutant when compared to the wild-type CHO-K1, with a statistically significant inhibition in the infection level of 74% and 67% after 2 h and 4 h of interaction, respectively. (B–E) Aspect of Chinese hamster ovary (CHO) cell cultures infected with *T. cruzi* amastigotes. (B–D) CHO-K1 wild-type and (C–E) CHO-745, proteoglycan-deficient cells, after 2 h (B–C) and 4 h (D–E) of infection. Note the low levels of infection in CHO-745 cultures (C–E), with few interiorized (➞) and attached (⇨) parasites, either after 2 h or 4 h of interaction, when compared with the wild-type, CHO-K1. Scale bar = 20 μm. (*) Student's *t*-test: statistically significant $P \leqslant 0{\cdot}05$.

GAGs, such as chondroitin sulfate, dermatan sulfate and keratan sulfate, did not mediate amastigote invasion. Although the *T. cruzi* heparin-binding ligand has the ability to bind most sulfated glycosaminoglycan (Oliveira-Jr *et al*. 2008), only heparan sulfate recognition triggers trypomastigote (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008) and amastigote invasion. The inability of GAGs to alter the uptake of Zy internalization by cardiomyocytes reinforces the important role of heparan sulfate in amastigote entry, suggesting that HS may be a key molecule in this process.

Few molecules have been demonstrated to mediate amastigote-host cell recognition. Mammalian lectins, such as mannose-binding proteins (MBP) and mannose receptor, are involved in the *T. cruzi* uptake by

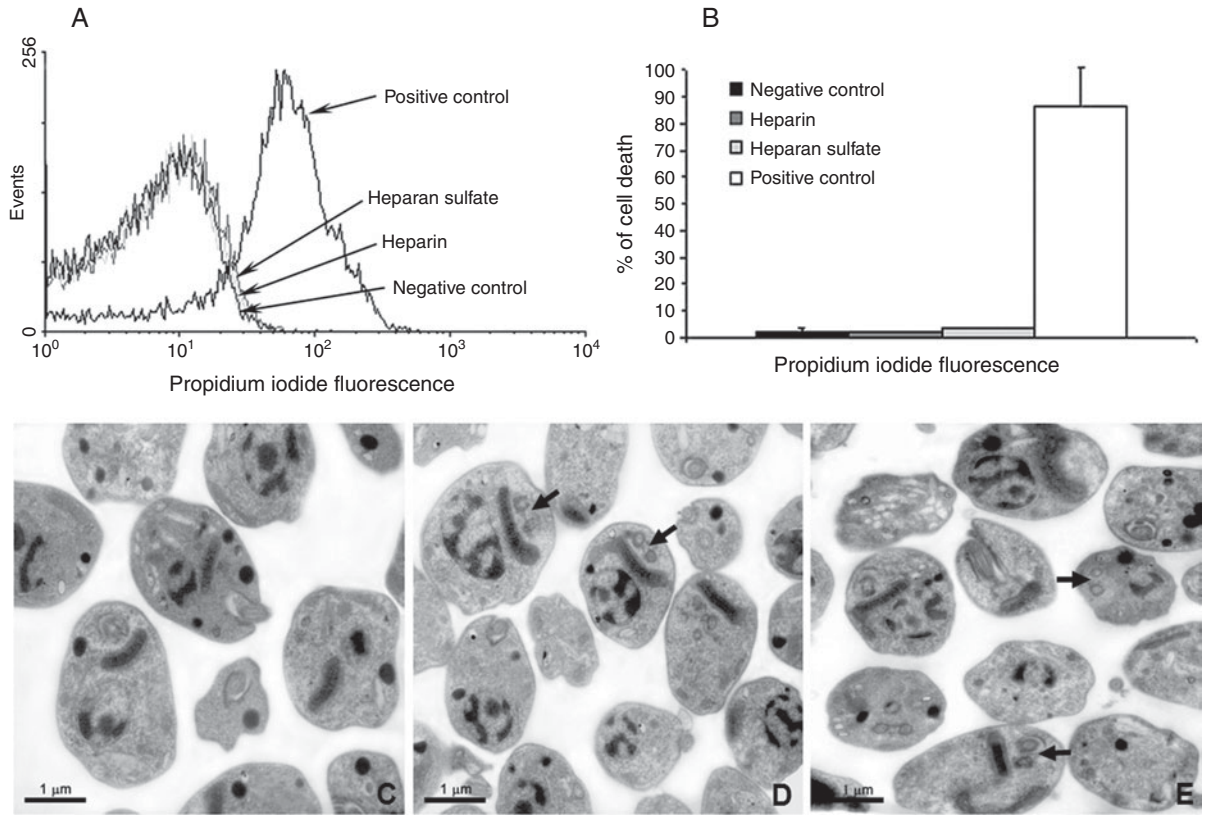

Fig. 4. Flow cytometry analysis of amastigotes treated with heparin and heparan sulfate (A). Note that treatment with GAGs did not induce cell death as compared to the positive control, i.e. parasites permeabilized with saponin, that reached 86·7% PI incorporation (B). Ultrastructural analysis of the glycosaminoglycan effect on amastigote forms of *Trypanosoma cruzi*. Extracellular amastigotes presented bar-shaped kinetoplasts positioned between the nucleus and flagellum (C). Heparin (D) and heparan sulfate (E) treatment neither altered parasite morphology nor their proliferation. Arrows show signs of a cellular division process in all conditions analysed. Scale bar = 1 μm.

phagocytic cells (Kahn *et al*. 1995, 1996). The protein SA85-1 expressed by amastigotes interacts strongly with MBP, but does not mediate MBP binding to trypomastigotes, suggesting a stage-specific recognition involved in amastigote attachment and invasion (Kahn *et al*. 1996). In addition, it has recently been shown that membrane rafts, enriched in sphingolipids, cholesterol, ganglioside GM1, are implicated in the internalization of both extracellular amastigote and trypomastigote infective forms (Barrias *et al*. 2007; Fernandes *et al*. 2007). Interestingly, evidence has demonstrated that heparan sulfate proteoglycans are localized in cholesterol-rich lipid raft domains (Chu *et al*. 2004) and modulate signal transduction events activated by fibroblast growth factor-2 at specialized membrane microdomains, favouring an effective sensitivity to diluted ligands (Fuki *et al*. 2000).

Since it has also been demonstrated that RhoA GTPases play a key role in the invasion of extracellular amastigotes (Fernandes and Mortara, 2004; Mortara *et al*. 2005) and that HSPG-microbe interaction activates several signalling pathways, including PI3-kinase, focal adhesion kinase (FAK) and RhoA GTPases, triggering their internalization by endocytosis (Chen *et al*. 2008), we postulated that sulfated proteoglycans would mediate amastigote-cardiomyocyte invasion. Based on the fact that the cytoplasmic region of HSPG has a conserved domain, C1 domain, that binds to intracellular proteins, such as erzin, tubulin, Src kinase and cortactin (Granés *et al*. 2000; Tehrani *et al*. 2007), it is possible that the binding of amastigote heparin-binding protein to host cell HS orchestrates different signalling transduction pathways, including (*i*) ezrin interaction eliciting actin remodelling by Rho GTPases, (*ii*) FAK/Src complex activation or (*iii*) phosphorylation of cortactin by Src activation, leading to actin polymerization and amastigote entry. Therefore, the investigation of signalling pathways triggered by binding of amastigotes to heparan sulfate may provide new insights into the mechanisms underlying *T. cruzi* amastigote infection.

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