Novel mechanism that *Trypanosoma cruzi* uses to adhere to the extracellular matrix mediated by human galectin-3

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Abstract Binding of *Trypanosoma cruzi* trypomastigotes to laminin is enhanced by galectin-3, a β-galactoside binding lectin. The galectin-3 enhanced binding of trypanosomes to laminin is inhibited by lactose. Co-immunoprecipitations indicate that galectin-3 binds to the 45, 32 and 30 kDa trypanosome surface proteins. Binding of galectin-3 to the 45, 32 and 30 kDa surface proteins is inhibited by lactose. Polyclonal and a monoclonal antibodies to galectin-3 immunoprecipitated a major 64 kDa trypanosome surface protein. *T. cruzi* monoclonal antibody to mucin recognized the 45 kDa surface protein. The 45, 32 and 30 kDa surface proteins interact with galectin-3 in order to enhance trypanosome adhesion to laminin.

Key words: Human galectin-3; Extracellular matrix; Laminin; Mucin; Adhesion; *Trypanosoma cruzi* surface protein

1. Introduction

Trypanosoma cruzi, the protozoon that causes Chagas' disease and affects millions of people, must move through the extracellular matrix before binding and entering host cells to establish infection [1]. The molecules that facilitate *T. cruzi* mobilization through the extracellular matrix are largely unknown. In this study, we have explored the role of human galectin-3 in enhancing the adhesion of invasive forms of *T. cruzi* to extracellular matrix proteins. The disease is acquired by entry of invasive trypomastigotes which are transmitted by insect vectors, or by blood infected with trypomastigotes during blood transfusion. This organism is now viewed as an emerging human pathogen of HIV-1 infected individuals, since it induces dramatic brain pathology and earlier death when associated with HIV-1 infection [2]. It has been reported that *T. cruzi* trypomastigotes bind to extracellular matrix components such as laminin [3], fibronectin [4], and collagen [5]. However, the mechanisms of these interactions are poorly understood. An understanding of how *T. cruzi* surface molecules interact with galectin-3 to facilitate trypanosome mobilization throughout the body may be critical for the development of a molecular means of intervention against this organism.

Human galectin-3 is a β-galactoside binding protein that interacts with glycoproteins containing polylactosamine residues such as laminin [6]. Galectins have been found in several species, including nematodes [7], mammals [6] and fungi [8]. Galectin-3 has been implicated in the regulation of adhesion of human breast carcinoma cells to the extracellular matrix [9]. Because galectin-3 is found on the cell surface of mammalian cells, secreted and known to interact with extracellular matrix proteins [6], we have investigated the role of galectin-3 in the process of trypomastigote adhesion to the extracellular matrix.

In this paper we report the novel observation that the cloned human galectin-3 specifically binds to *T. cruzi* trypomastigote surface proteins in order to enhance trypanosome adhesion to laminin.

2. Materials and methods

2.1. Organism

The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of *T. cruzi* was used [10]. Pure culture trypomastigotes were obtained from the supernatant of heart myoblast monolayers [11]. Trypanosomes were washed with pyrogen free phosphate buffered saline (PBS) and resuspended at 1×10^8 organisms/ml in PBS [12].

2.2. Reagents

Purified recombinant human galectin-3 was obtained as described [13]. Polyclonal antibodies to purified galectin-3 were produced in rabbits. A rat IgG monoclonal antibody (mAb) TIB 166 to galectin-3 [14] was used. A C10 mAb to a surface *T. cruzi* trypomastigote mucin and its isotype control [15] was kindly provided by Dr. M. Fresno from Universidad Autonoma de Madrid, Spain.

2.3. T. cruzi trypomastigote adhesion assay

The effect of galectin-3 in promoting adhesion of trypomastigotes to laminin or collagen was studied in 8 well Lab-Tek chambers coated with human laminin or collagen IV (1 μg/well) in PBS overnight at 4°C [16]. The free sites were blocked with bovine serum albumin [16]. Trypomastigotes (2×10^6) resuspended in PBS were added to wells coated with laminin or collagen in triplicate for 30 min at 37°C in either the presence or absence of galectin-3 (100 μg/ml). In control assays, trypomastigotes were incubated with 100 μg/ml of bovine serum albumin. Wells were washed, fixed, stained with Giemsa stain [17], and trypomastigotes were microscopically determined [18].

2.4. Sugar inhibition assay

In order to determine the specificity of galectin-3 mediating the interaction between trypomastigotes and laminin, trypanosomes were incubated in triplicate with galectin-3 in the presence of 50 mM lactose or 50 mM sucrose (a control) as described in Section 2.3. To evaluate the ability of lactose to inhibit the adherence of trypomastigotes to laminin, increasing concentrations of lactose (1–100 μg/ml) were added to laminin coated wells in the presence of trypomastigotes (2×10^6) and galectin-3 (100 μg/ml).

2.5. Interactions between galectin-3 and T. cruzi surface proteins analyzed by co-immunoprecipitations

The ability of galectin-3 to interact with trypomastigote surface

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proteins was investigated by co-immunoprecipitating solubilized biotinylated trypomastigotes in the presence of galectin-3 using anti-galactin-3 polyclonal antibodies or pre-immune antibodies. The specificity of galectin-3 binding to *T. cruzi* surface proteins was investigated by supplementing the reaction with either lactose or sucrose at a concentration of 50 μg/ml. Briefly, 100 μg of biotinylated trypomastigotes [19] were pre-cleared with protein A Sepharose beads in immunoprecipitation buffer [20] and supernatants were incubated with 100 μg/ml of galectin-3 in the same immunoprecipitation buffer containing half the amount of detergents, in the presence of 50 mM lactose or 50 mM sucrose for 12 h at 4°C. Immunoprecipitation controls using polyclonal antibodies to galectin-3 and biotinylated trypanosome surface proteins in the absence of galectin-3 were performed. Polyclonal antibodies to galactin-3 or pre-immune antibodies were added to the reaction at the final dilution of 1:50 followed by protein A Sepharose. The reaction was incubated overnight at 4°C. Immunoprecipitated proteins were separated by SDS–PAGE, blotted onto nitrocellulose membranes, probed with avidin–horseradish peroxidase (HRP) and developed by ECL (Amersham) [21]. To identify surface trypomastigote proteins that interact with galectin-3, the blot of Fig. 4A, lane 1 was stripped and probed with either anti-*T. cruzi* mucin mouse mAb or its isotype mAb control followed by incubation with goat anti-mouse IgG mAb-HRP and developed by ECL.

2.6. Immunoprecipitation of T. cruzi surface proteins with anti-galectin-3 mAb and immunoblots

For immunoprecipitations, 20 μg of solubilized biotinylated trypomastigotes were precleared with protein A Sepharose and supernatants were incubated with either a rat IgG mAb to galectin-3 or its isotype control (both diluted 1:10) in immunoprecipitation buffer overnight at 4°C [20]. Immunoprecipitates were separated by SDS–PAGE, blotted on nitrocellulose, incubated with avidin–HRP and developed by ECL. For immunoblotting, 5 μg of trypomastigote lysates were separated by SDS–PAGE, blotted onto nitrocellulose membranes and strips were probed with either a mAb to human galectin-3 or its isotype mAb control followed by incubation with goat anti-rat IgG mAb–HRP and developed by ECL.

2.7. Presentation of results and statistical analysis

Results in this work were obtained from triplicate values and represent three independent experiments with identical protocols. Results are expressed as the mean ± 1 standard deviation. Differences were considered to be statistically significant if $P < 0.05$ as determined by Student's *t*-test.

3. Results

3.1. Human galectin-3 enhanced T. cruzi trypomastigote adhesion to laminin but not to collagen

The ability of galectin-3 in promoting adhesion of trypomastigotes to laminin or collagen was investigated. Fig. 1 shows that addition of galectin-3 to coated wells with laminin

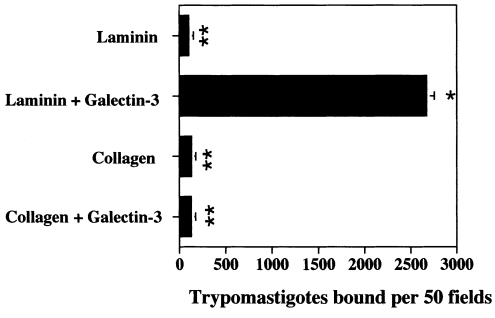

Fig. 1. Human galectin-3 dramatically enhances *T. cruzi* trypomastigote adhesion to laminin. Trypomastigotes (2×10^6) were added to micro wells coated with either laminin or collagen in the presence of galectin-3. The number of trypomastigotes bound per 50 fields was microscopically determined. This is a representative experiment of three performed. Differences between * and **, $P < 0.05$.

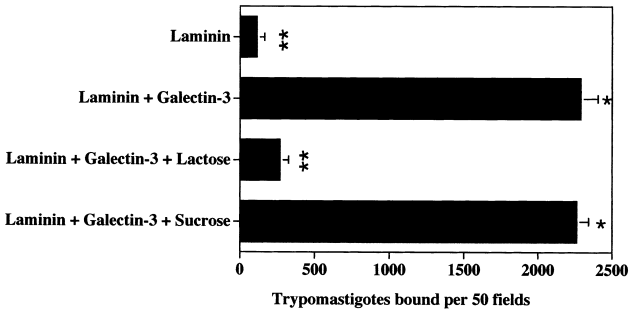

Fig. 2. The galectin-3 enhanced binding of trypanosomes to laminin is specifically inhibited by lactose, but not by sucrose. Trypomastigotes (2×10^6) were added to micro wells coated with laminin in the presence of galectin-3 supplemented with either 50 μg/ml of lactose or sucrose. The number of bound trypanosomes was microscopically determined. This is a representative experiment of three performed. Differences between * and **, $P < 0.05$.

dramatically enhanced the adhesion of trypomastigotes to laminin, whereas galectin-3 did not affect trypomastigote adhesion to collagen. Galectin-3 increased the normal adhesion of trypanosomes to laminin 20 times and this effect is specific since it is inhibited by lactose but not by sucrose (Fig. 2). Furthermore, the lactose induced inhibition of trypanosome adhesion to laminin, in the presence of exogenous galectin-3, is concentration dependent and saturable (Fig. 3), thus indicating that the specificity of this effect is mediated by the β-galactoside binding galectin-3. Lactose inhibits the enhancement effect seen at concentrations of 1 μg/ml, attaining maximal inhibition at 25–100 μg/ml (Fig. 3).

3.2. Human galectin-3 interacts with several surface proteins of invasive forms of T. cruzi and this interaction is inhibited by lactose

The ability of galectin-3 to interact with trypomastigote surface proteins was investigated by incubating solubilized

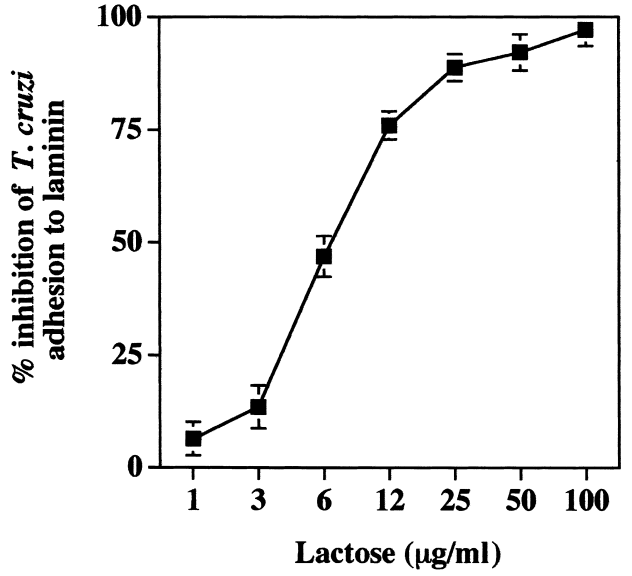

Fig. 3. Lactose inhibits trypanosome adhesion to laminin in the presence of exogenous galectin-3 in a concentration dependent and saturable manner. Increasing concentrations of lactose (1–100 μg/ml) were added to laminin coated wells in the presence of trypomastigotes (2×10^6) and galectin-3 (100 μg/ml) and the number of bound trypanosomes was determined.

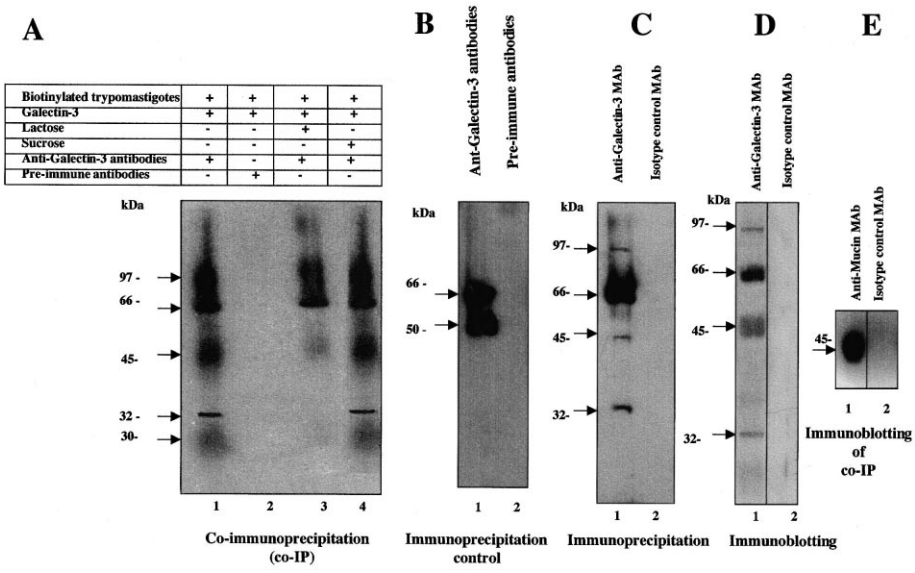

Fig. 4. Interactions between galectin-3 and trypomastigote surface proteins, and recognition of trypanosome surface proteins by mAb to galectin-3 or by mAb to *T. cruzi* mucin. A: Co-immunoprecipitations of trypomastigote surface proteins with galectin-3 using anti-galectin-3 polyclonal antibodies. Solubilized biotinylated trypomastigotes (100 μg) and galectin-3 (100 μg) were incubated together, as well as in the presence of either lactose or sucrose. The trypanosome surface proteins bound to galectin-3 were co-immunoprecipitated using polyclonal antibodies to galectin-3 or pre-immune antibodies. Biotinylated trypanosome surface proteins bound to galectin-3 were separated by SDS–PAGE, blotted onto nitrocellulose membranes and developed by ECL. B: Immunoprecipitation controls of solubilized biotinylated trypomastigotes (20 μg) using either anti-galectin-3 polyclonal antibodies or pre-immune antibodies in the absence of galectin-3. Immunoprecipitated proteins were blotted and developed by ECL. C: Immunoprecipitations of *T. cruzi* surface proteins using anti-galectin-3 mAb. Solubilized biotinylated trypomastigotes (20 μg) were incubated with either a mAb to galectin-3 or a mAb isotype control. Immunoprecipitated proteins were blotted and developed by ECL. D: Immunoblotting of *T. cruzi* lysates probed with anti-galectin-3 mAb. 5 μg of trypomastigote lysates were separated, blotted and probed with either anti-human galectin-3 mAb or its isotype control mAb and developed by ECL. E: An immunoblot prepared by stripping the blot in lane 1 of A and probing with either anti-*T. cruzi* mucin mAb or its isotype control mAb and developing by ECL.

biotinylated trypomastigotes with galectin-3 in the absence and presence of lactose or sucrose and co-immunoprecipitating trypanosome surface proteins bound to galectin-3 using anti-galectin-3 polyclonal antibodies. Co-immunoprecipitation assays revealed that galectin-3 binds to the 45, 32 and 30 kDa surface proteins of trypomastigotes (Fig. 4A, lane 1). Control pre-immune antibodies did not recruit proteins (Fig. 4A, lane 2). The interaction between galectin-3 and the 45, 32 and 30 kDa trypanosome surface proteins was inhibited by lactose (Fig. 4A, lane 3), but not by sucrose (Fig. 4A, lane 4). Immunoprecipitation controls using polyclonal antibodies to galectin-3 and solubilized biotinylated trypanosomes indicated that polyclonal antibodies to galectin-3 recognized the surface 64 and 50 kDa proteins (Fig. 4B, lane 1), whereas pre-immune antibodies did not (Fig. 4B, lane 2). These results indicate that the interaction between galectin-3 and the trypanosome 45, 32 and 30 kDa proteins is galectin specific, since galectin–mammalian protein interactions are specifically inhibited by lactose.

3.3. Trypanosome surface proteins that interact with galectin-3 are recognized by a mAb to galectin-3

Based on the facts that (a) human galectin-3 molecules can homodimerize to modulate biological functions in mammalian hosts [22], and (b) our results indicate that galectin-3 interacts with several surface molecules of trypanosomes (Fig. 4A, lane 1), we decided to explore the possibility that the trypanosome surface molecules that interacted with galectin-3 could be galectin-3 homologues by studying their ability to be immunoprecipitated by an anti-galectin-3 mAb. Fig. 4C, lane 1, shows that a mAb to galectin-3 immunoprecipitated surface proteins of 45 and 32 kDa of the same molecular weight as those that interacted with galectin-3 (Fig. 4A, lane 1), with the exception of the 30 kDa protein, whereas an isotype control mAb did not (Fig. 4C, lane 2). Fig. 4D, lane 1, shows an immunoblot of trypomastigote lysates probed with an anti-galectin-3 mAb recognizing proteins of the same molecular weights as those previously identified by immunoprecipitations (Fig. 4C) or by co-immunoprecipitations (Fig. 4A). An isotype control mAb did not recognize any *T. cruzi* proteins (Fig. 4D, lane 2). Both polyclonal antibodies to galectin-3 and a mAb to galectin-3 strongly recognized a major 64 kDa surface protein of trypomastigotes by immunoprecipitations (Fig. 4B, lane 1 and Fig. 4C, lane 1) as well as by immunoblotting (Fig. 4D, lane 1). These results suggest that the trypanosome surface proteins (45, 32 and 30 kDa) interact with galectin-3 and present galactosyl binding sites for galectin-3. To identify surface trypomastigote proteins that interact with galectin-3, the blot presented in lane 1 of Fig. 4A was stripped and probed with either anti-*T. cruzi* mucin mAb or its isotype control. Fig. 4E, lane 1, shows that a mAb to *T. cruzi* mucin recognized the *T. cruzi* 45 kDa surface protein, whereas an isotype control mAb did not (Fig. 4E, lane 2). This indicates that galectin-3 interacts with a 45 kDa surface *T. cruzi* mucin.

4. Discussion

This study reports a novel mechanism where a human parasite, *T. cruzi*, utilizes human galectin-3 to effectively interact with laminin. Our working model proposes that the exogenously supplied galectin-3 forms bridges between *T. cruzi* and laminin. Galectin-3 molecules interact with *T. cruzi*

45 (mucin), 32 and 30 kDa surface proteins on one hand and with laminin on the other, via their carbohydrate recognition domains and are joined together using the R-domains [13] in a concentration dependent manner. Therefore, depending on the level of secretion, *T. cruzi* galectin-3 homologues may also directly ligate trypanosomes to laminin. Since nearly all the tissues which *T. cruzi* infects are surrounded by basement membranes of which laminin is a major constituent, its ability to effectively interact with laminin is critically important for passage through the membrane barrier. Our studies suggest that this is a trypanosome trapping mechanism which enables the organisms to accumulate in the basement membrane prior to invasion, making galectin-3 a candidate molecule which enhances the pathogenesis of *T. cruzi*.

Whereas it was expected for the anti-galectin-3 mAb to recognize one protein band (~30 kDa) in the immunoblot, several minor bands were detected and a major band at around 64 kDa. Additional evidence supporting the finding that the 64 kDa band was the major band recognized was obtained from immunoprecipitations using both anti-galectin-3 mAb and polyclonal antibodies. It is likely that the 32 kDa band is the *T. cruzi* homologue of the human galectin-3. The 64 kDa band may represent the dimerized form of a galectin-3 homologue or the heterodimer formed between a galectin-3 homologue and a homologue of the 37 kDa laminin binding protein [23]. Similarly the 97 kDa band may represent a multimeric form of galectin-3. Although the heterogeneity resulting from galectin-3 cross-reacting molecules is not common in mammalian cells, the ~64 kDa and the 45 kDa band are occasionally observed as minor cross-reacting bands with this mAb in Western blots of mammalian cell lysates (unpublished observations). This may be a reason why this mAb reacts with the *T. cruzi* 45 kDa mucin. Interestingly, exogenously supplied galectin-3 was able to co-precipitate three trypanosome surface proteins in the immunoprecipitation experiments. This study also shows that one of the surface molecules of *T. cruzi* that interacts with galectin-3 is a *T. cruzi* 45 kDa mucin. *T. cruzi* complex family of mucins has been reported [15]. However, the genes, the identity and biochemical properties of the 32 and 30 kDa surface receptors for galectin-3 in *T. cruzi* are unknown and therefore warrant extensive investigation. So far, galectin genes in *T. cruzi* have not been reported. The interaction of galectin-3 with mammalian cell surface mucins has been demonstrated. Studies by Bresalier et al. [24] demonstrated that galectin-3 binds to colon cancer mucins in a lactose dependent manner.

Galectins have long been suspected of modulating cell to extracellular matrix interactions in a novel fashion [6,25,26]. Data from various laboratories suggest that one mechanism used involves the ligation of mammalian cells to extracellular matrix proteins which also interact with galectin-3 such as laminin and elastin [27,28] according to the model suggested. The other mechanism involves the interaction of galectins with the polylactosamine residues in integrins, resulting in the modulation of cellular adhesion to extracellular matrix proteins [16,29]. Whereas most of these studies have been done in mammalian systems, it has been suggested that galectins expressed by *Entamoeba* may be critical in their interactions with host cells [30]. It is likely that in parasitic organisms, the ligation of the organisms to the extracellular matrix proteins or cell surface glycoconjugates may be the primary adhesion mechanisms mediated by galectins.

In summary, we have demonstrated that human galectin-3 modulates the interaction of *T. cruzi* with laminin in a lactose dependent manner. There are several surface molecules in *T. cruzi* that interact with galectin-3 which are involved in the ligation of the parasite to laminin.

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