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HOST-CELL ATTACHMENT BY Trypanosoma cruzi: IDENTIFICATION OF AN ADHESION MOLECULE

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We have identified an 83 kDa surface glycoprotein in T. cruzi trypomastigotes which specifically binds to rat heart myoblasts. The binding of this molecule to myoblasts is inhibited by excess unlabeled material and saturable. Antibodies against the cell surface of insect trypomastigotes, blood trypomastigotes and produced during human infection recognize the 83 kDa glycoprotein adhesion molecule by immunoblotting, indicating that this molecule that mediates this critical step is immunogenic and is a candidate for vaccination against Chagas' disease.

Trypanosoma cruzi, the protozoan which causes Chagas' disease and affects millions of people in South and Central America (1), must attach to mammalian cells before it can invade them. The disease is acquired by infection with metacyclic trypomastigotes which are transmitted by insect vectors or by bloodstream trypomastigotes during blood transfusion. These trypomastigotes attach to and penetrate mammalian host cell membranes to multiply intracellularly and disseminate in the body. An understanding of how the parasite attaches to mammalian host cells becomes critical to the development of effective means to prevent the infection. However, present knowledge of this process is limited. The attachment of T. cruzi trypomastigotes to mammalian cells is thought to be mediated by a membrane phenomenon, since trypomastigote membranes inhibit this process (2), but it is unknown what parasite molecule binds to muscle cells, a major target for T. cruzi.

Previous work from several laboratories has shown that addition of lectins (3), or free sugars (4,5), removal of sugar residues from trypomastigotes (6-9), inhibition of parasite glycoprotein biosynthesis (10) and parasite glycoprotein processing (11) can affect the attachment of the parasite to mammalian cells. Although these observations indicate that glycoproteins on the surface of invasive trypomastigotes are involved in the attachment process, specific parasite molecules that mediate parasite attachment to muscle cells have not been identified. In this paper we identify an immunogenic 83 kDa glycoprotein on T. cruzi trypomastigote membranes capable of binding to heart muscle cells.

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MATERIAL AND METHODS

The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of T. cruzi (Lima, M.F., and Villalta, F., unpublished observations) was used in this work. Blood trypomastigotes were isolated from infected Crl/CD-1(ICR)BR Swiss mice (Charles River, Raleigh, NC) by chromatography on a diethylaminoethyl-cellulose column (12). Pure culture trypomastigotes were obtained from the supernatant of infected rat heart myoblasts monolayers (6). Insect-derived, metacyclic trypomastigotes were isolated from the hindgut of 4-week infected Rhodnius prolixus, from our insect colony at Meharry Medical College, through diethylaminoethyl cellulose (12).

Surface proteins of culture T. cruzi trypomastigotes were analyzed by binding of biotinylated parasites. Briefly, 27 ul of 75 mM N-hydroxisuccinimide (NHS)-biotin (Bio-Rad, Richmond, CA) were added to one milliliter containing 5 x 10^8 trypomastigotes in phosphate buffer saline, pH 7.2, (PBS), for 15 min at room temperature. Biotinylation does not affect morphology, motility and parasite numbers when observed microscopically. Parasites were then washed 3 times with cold PBS, solubilized with 0.8% (3-[(3-Cholamidopropyl)dimethyl-ammonio] 1-propane-sulfonate), (CHAPS), (13) in PBS in the presence of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 mM N-p-tosyl-L-lysine-chloro-methyl ketone, and 2.8 ug/ml aprotinin). Solubilized parasites were centrifuged at 13,000 x g for 5 min at 4°C to remove debris. Samples containing 5 ug of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (14), electroblotted onto nitrocellulose membranes, reacted with avidin-horseradish peroxidase (Bio-Rad), and developed with 4-chloro-1-napthol/H_2O_2. Biotinylated standards (Bio-Rad) were included to determine the relative molecular weight of parasite proteins. Nitrocellulose membranes were scanned with an LKB Ultroscan XL Laser Densitometer.

To identify the T. cruzi trypomastigote ligand which binds to myoblasts we modified the technique described by Baseman and Hayes (15) to identify ligands of Treponema pallidum that bind to epithelial cells. Biotinylation of the cell surface of trypomastigotes does not affect the capacity of the parasite to attach to rat heart myoblasts (Lima, M.F., and Villalta, F., unpublished observations). Pure culture trypomastigotes were biotinylated and solubilized with 0.8% CHAPS (13) as described above. After removing the detergent by extensive dialysis against PBS at 4°C, 1 mg of protein from the preparation was incubated with rat heart monolayers (2 x 10^6 cells/60 mm culture dish) at 4°C to avoid ligand internalization. Unbound parasite material was removed by washing the cell monolayers with cold PBS. Myoblasts containing the bound parasite attachment molecule were solubilized with 0.8% CHAPS (13) and the same protease inhibitors as described above. Samples were centrifuged at 13,000 x g at 4°C and the supernatants were dialyzed against water at 4°C and lyophilized. Samples of 120 ug of protein were then subjected to SDS-PAGE, blotted onto nitrocellulose membranes, reacted with avidin-horseradish peroxidase and developed with 4-chloro-1-napthol/H_2O_2. In addition, SDS-PAGE samples of the dissociated parasite ligand were stained by silver stain (Bio-Rad), periodic acid schiff (16) and silver-Commassie blue (17).

Inhibition was studied by pre-incubating heart myoblasts (3 x 10^5 cells/well) in 24 well plates with 200 ul of different concentrations of detergent-free CHAPS-solubilized preparations of unlabeled trypomastigotes (ranging from 0.6 to 2.5 mg of protein) at 4°C for 4 hours. After this period unbound parasite material was removed by washing and the monolayers were incubated with 200 ul of a constant concentration of biotinylated solubilized trypomastigotes (90 ug) as described above. After extensive washing, the monolayers were solubilized with CHAPS and samples (14 ug) were resolved by SDS-PAGE, blotted onto nitrocellulose and stained with streptavidin horseradish peroxidase (Bio-Genex, Dublin CA). Nitrocellulose membranes were also scanned with a laser densitometer.

Pure culture trypomastigotes (2 x 10^8 organisms) were radioiodinated with 2 mCi of Na[^{125}I] (specific activity, 17 Ci/mg I, ICN Radiochemicals, Irvine,CA) in Iodogen (Pierce Chemical Co., Rockford,IL) coated tubes (18). Unbound radioactivity was removed by five washes with Hanks' balanced salt solution (HBSS). This labeling procedure does not affect parasite morphology, motility and ability of trypomastigotes to bind and internalize host cells. Labeled parasites were solubilized with CHAPS in the presence of protease inhibitors and detergent was removed through an Extracti-gel column (Pierce Chemical Co.) prior to binding experiments.

The binding of solubilized ^{125}I-trypomastigotes (specific activity, 1.31 x 10^5 cpm/ug protein) to heart myoblasts was carried out by adding, in triplicate, increasing concentrations of labeled solubilized parasite material to myoblast monolayers (1 x 10^5 cells/well) in 96 well plates. Cultures were incubated for 4 hours at 4°C. After removing unbound parasite material by washing three times with HBSS, the monolayers were solubilized with 1% SDS in the presence of protease inhibitors as described above and the radioactivity in 30 ul aliquots was measured with a gamma counter. Non-specific binding was determined by adding an excess of 30 times unlabeled solubilized trypomastigotes to each point. Specific binding was determined by subtracting the non-specific binding from the total binding. Aliquots (30 ug) of SDS solubilized myoblasts containing the bound radioiodinated parasite material were resolved by SDS-PAGE and autoradiographed.

Antibodies against purified insect-derived uncloned T. cruzi metacyclic trypomastigotes and blood trypomastigotes were prepared by injecting New Zealand rabbits with 0.25% glutaraldehyde fixed parasites, conditions that do not significantly alter surface antigenic determinants (19). Parasites (3-5 x 10^7) were inoculated with complete Freund's adjuvant (Difco, Detroit, MI) three times in a period of 4 weeks. The presence of specific antibodies in the sera was verified by indirect immunofluorescence (20). Human serum from infected individuals was obtained from the Institute of Microbiology at the Federal University of Rio de Janeiro, Brazil. This serum presented an immunofluorescence titer of 1:128.

The immunogenicity of the 83 kDa glycoprotein trypomastigote attachment molecule was evaluated by immunoblot analysis of the dissociated parasite ligand from myoblasts, according to Burnette (21). The dissociated parasite ligand was subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Biotinylated molecular weight standards (Bio-Rad) were also included. The nitrocellulose strips were then reacted with a 3% solution of gelatin (Bio-Rad) for 1 hour at room temperature, and incubated with dilutions of anti-insect trypomastigote, anti-blood trypomastigote and human chagasic serum in a 1% gelatin solution for 1 hour at room temperature. Control lanes received pre-immune rabbit serum and normal human serum from healthy volunteers. Membranes were washed three times for 10 min with Tris Buffered Saline containing 0.2% Tween 20, pH 9.3 (TTBS) and incubated with a 1:100 dilution of biotinylated goat anti-human IgG or biotinylated goat anti-rabbit IgG (Cappel, West Chester, PA) for 1 hour at room temperature. Strips were washed three times with TTBS, incubated with Streptavidin-horseradish peroxidase for 10 min at room temperature, and revealed by incubation with 4-chloro-1-naphtol/H_2O_2.

All sets of results presented in this paper are typically representative of three independent repeat experiments.

RESULTS

Analysis of biotinylated surface proteins of the T. cruzi trypomastigote clone MMC 20A indicated that several labeled proteins ranging from 178 to 22 kDa are present in the cell surface of this invasive form of the parasite as shown in Fig. 1., right panel A. Laser scanning analysis of the blotted surface proteins of T. cruzi showed that the 83 kDa is the major surface protein as indicated in Fig.1., left panel A, and that this major surface protein is expressed 8.7 times more than the least expressed protein and 4.8 times more than the next highly expressed protein. To identify the surface trypomastigote component that binds to heart myoblasts we incubated detergent- free parasite surface proteins with live myoblast monolayers for 4 hours at 4°C. The interaction was carried out at 4°C to avoid ligand internalization. As can be seen in Fig. 1., right panel B and left panel B, our results indicate that the 83 kDa major component of virulent trypomastigotes binds to rat heart myoblasts. The specificity of the binding of the biotinylated 83 kDa trypomastigote molecule to rat heart myoblasts was indicated by the concentration dependent inhibitory effect of unlabeled solubilized trypomastigotes. Laser densitometry

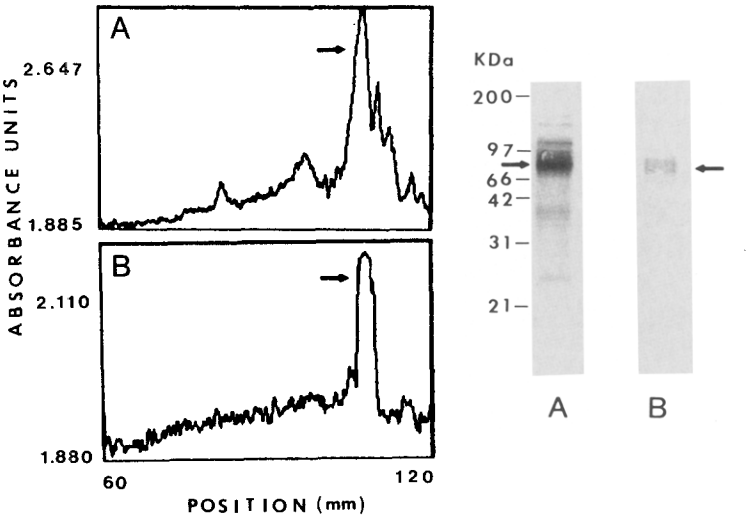

Figure 1. Identification of an 83 kDa adhesion molecule of T. cruzi trypomastigotes that binds to rat heart myoblasts. Right panel Lane A. Nitrocellulose blot of a SDS-PAGE gel of the biotinylated cell surface of T. cruzi trypomastigote clone MMC 20A revealed by avidin-Horseradish Peroxidase/H_2O_2. Right panel Lane B. Molecular identification on nitrocellulose membranes of a biotinylated trypomastigote 83 kDa adhesion molecule that binds to heart myoblasts, as described in materials and methods, and revealed as indicated above. Left panel A. Laser scan of the biotinylated cell surface of T. cruzi trypomastigotes on nitrocellulose membranes (right panel A) with an LKB Ultroscan Laser Densitometer. Left panel B. Laser scan of nitrocellulose membranes containing the 83 kDa trypomastigote molecule that binds to myoblasts (right panel B). Arrows point to the 83 kDa glycoprotein adhesion molecule. Blots were scanned from top to bottom. Molecular weight standards (Bio-Rad) are indicated in kilodaltons.

analysis of nitrocellulose membranes containing the blotted 83 kDa showed that at the highest concentration unlabeled solubilized trypomastigotes inhibited the binding of the labeled molecule to heart myoblasts by 87% (Fig. 2). The binding of solubilized ^{125}I-trypomastigotes to heart myoblasts is concentration dependent and saturable as indicated in Fig. 3 and the trypomastigote molecule mediating this interaction is the 83 kDa as seen in the insert of Fig. 3.

SDS-PAGE of the dissociated parasite adhesion molecule under reducing or non-reducing conditions gives the same relative molecular weight of 83 kDa. In addition, SDS-PAGE gels of either CHAPS solubilized trypomastigotes or the dissociated parasite adhesion molecule stained with periodic acid schiff and silver-Comassie blue (Dzandzu) indicated that the 83 kDa molecule is a glycoprotein (results not shown).

The immunoblot presented in Table I indicated that antibodies against (a) the cell surface of insect and (b) blood trypomastigotes and (c) parasites produced during human infection recognize with similar intensity the 83 kDa glycoprotein attachment molecule of culture trypomastigotes. In addition, sera from pre-immunized animals or sera from healthy volunteers did not react with the parasite 83 kDa.

DISCUSSION

These results show that T. cruzi trypomastigotes attach to rat heart myoblasts through an 83 kDa glycoprotein adhesion molecule which is present on the cell surface of virulent

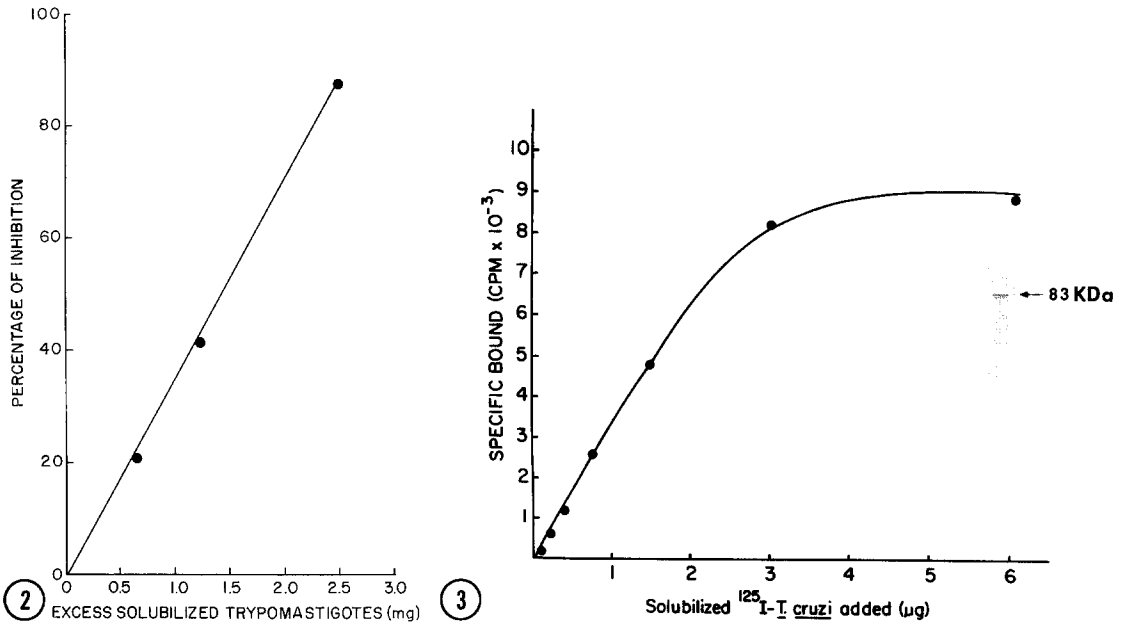

Figure 2. Inhibition of the binding of biotinylated 83 kDa to myoblasts by excess solubilized unlabeled trypomastigotes analyzed by laser densitometry. Full myoblast monolayers (24 well plates) were pre-incubated with increasing concentrations of unlabeled solubilized trypomastigotes and after removing unbound material the monolayers were incubated with a constant concentration of solubilized biotinylated trypomastigotes for 4 hours at 4°C. After extensive washing, the monolayers were solubilized with CHAPS and samples (10 ug) were blotted onto nitrocellulose membranes and stained as described in Figure 1. Nitrocellulose membranes showing only the 83 kDa band were scanned with a laser densitometer. The percentage of inhibition was determined applying the following formula: % I = 100 (1-A/B) where, B is the area of the 83 kDa band in the absence of unlabeled counterpart and A is the area of the 83 kDa band in the presence of different concentrations of unlabeled counterpart.

Figure 3. Binding of solubilized ^{125}I-T. cruzi trypomastigotes to heart myoblasts monolayers. Increasing concentrations of solubilized radioiodinated trypomastigotes were incubated with full myoblast monolayers in 96 well plates for 4 hours at 4°C. Specific binding was determined as described in material and methods. Insert is an autoradiogram of SDS-PAGE of SDS solubilized myoblast monolayer that was incubated with solubilized radioiodinated trypomastigotes showing the 83 kDa adhesion molecule (arrow).

trypomastigotes. This molecule, which appears to be the major protein component of the cell surface of infective trypomastigotes, specifically binds to the membrane of live rat heart myoblasts, since its binding is inhibited in a concentration dependent manner by unlabeled solubilized trypomastigotes. This molecule appears to bind to heart myoblasts in a ligand receptor interaction manner since the specific binding is concentration dependent and saturable, as can be seen in Fig. 3. Since either biotinylation or iodination of surface proteins of trypomastigotes does not affect the morphology, motility, parasite numbers and the ability of the parasite to attach and invade myoblasts monolayers, these labeling procedures did not modify the capacity of the parasite adhesion molecule to bind to host cells.

Our preliminary results indicate that the 83 kDa trypomastigote binding molecule is a glycoprotein. Several observations have suggested that sugar moieties on the parasite surface may play a role in the attachment of the parasite to its host cells (3-11). The possibility that the carbohydrate portion of the 83 kDa molecule is involved in the binding of the parasite to the host cell is under current investigation in our laboratory.

TABLE I

ANTIBODIES AGAINST THE CELL SURFACE OF INSECT, BLOOD TRYPOMASTIGOTE FORMS OF T. cruzi OR PRODUCED DURING HUMAN INFECTION RECOGNIZE THE 83 kDa GLYCOPROTEIN ADHESION MOLECULE OF CULTURE TRYPOMASTIGOTES BY IMMUNOBLOT ANALYSIS

Source of antibodies	Immunization or infection	Recognition of the 83 kDa adhesion molecule by immunoblot analysis*
Immunized rabbit	Insect trypomastigote	+
Pre-immune rabbit	None	-
Immunized rabbit	Blood trypomastigote	+
Pre-immune rabbit	None	-
Infected Human	Natural infection	+
Normal human serum	None	-

This is a representative experiment of three performed showing the same results.

* Antibodies against insect trypomastigotes, blood trypomastigotes or produced during human infection against the parasite recognize with similar intensity the 83 kDa attachment molecule of culture trypomastigotes.

The fact that the 83 kDa glycoprotein adhesion molecule of culture T. cruzi trypomastigotes is recognized by antibodies against the cell surface of insect-derived trypomastigotes and blood trypomastigotes indicates that this molecule may be present on the cell surface of both insect-derived trypomastigotes and blood trypomastigotes. These observations suggest that these two invasive life cycle stages of T. cruzi may both use the 83 kDa glycoprotein to attach to the host cell. If so, culture trypomastigotes, readily available in the laboratory in relative large numbers, may represent a suitable substitute to study the mechanism of host cell attachment by the forms which are responsible for the production of Chagas' disease in the nature and accidental infection by blood transfusion. In addition, the fact that the 83 kDa trypomastigote adhesion molecule is recognized by antibodies produced during human infection, but not from sera of uninfected individuals, indicates that this adhesion molecule is immunogenic during the course of human infection and is a candidate for human vaccination.

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REFERENCES

1. Brener, Z. (1973) Annu. Rev. Microbiol. 27, 347-382.
2. Kierszenbaum, F., and Stiles, B. (1985) J. Protozool. 32, 364-366.
3. Henriquez, D., Piras, R., and Piras, M.M. (1981) Mol.Biochem. Parasitol. 2, 359-366.

4. Crane, M. St. J., and Dvorak, W. (1982) Mol. Biochem. Parasitol. 5, 333-341.
5. de Araujo Jorge, T.C., and de Souza, W. (1984) Acta Trop. 41, 17-28.
6. Villalta, F., and Kierszenbaum, F. (1983) Biochim. Biophys. Acta 736, 39-44.
7. Villalta, F., and Kierszenbaum, F. (1984) Biochem. Biophys. Res. Commun. 119, 228-235.
8. Villalta, F., and Kierszenbaum, F. (1985) Biochim. Biophys. Acta. 845, 216-222.
9. Villalta, F., and Kierszenbaum, F. (1987) Mol. Biochem. Parasitol. 22, 109-114.
10. Piras, R., Piras, M.M., Henriquez, D., and Negri,S. (1982) Mol. Biochem. Parasitol. 6, 83-92.
11. Villalta, F., and Kierszenbaum, F. (1985) Mol. Biochem. Parasitol. 16, 1-10.
12. Villalta, F., and Leon, W. (1979) J. Parasitol. 29, 188-189.
13. Hazum, E., Schvartz, I., Wasksman, Y., and Keinan, D. (1986) J. Biol. Chem. 261, 13043-13048.
14. Laemmli, V.K. (1970) Nature 227, 680-685.
15. Baseman, J.B., and Hayes, E.C. (1980) J. Exp. Med. 151, 573-586.
16. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
17. Dzandzu, J.K., Deh, M.E., Barrat,D.L., and Wise, G.E. (1984) Proc. Natl. Acad. Sci. USA 81, 1733-1737.
18. Fraker, P.J., and Speck, J.C. (1982) Biochem. Biophys. Res. Commun. 80, 849-857.
19. Williams, A.F. (1973) Eur. J. Immunol. 3, 628-632.
20. Villalta, F., and Kierszenbaum, F. (1983) J. Protozool. 30, 329-331.
21. Burnette, W.N. (1981) Anal. Biochem. 112, 195-203.