Biochemical and Biophysical Research Communications **290,** 29–34 (2002)
doi:10.1006/bbrc.2001.6189, available online at http://www.idealibrary.com on IDEAL®

Trypanosoma cruzi Uses a 45-kDa Mucin for Adhesion to Mammalian Cells

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Received November 26, 2001

A set of monoclonal antibodies that recognizes a *Trypanosoma cruzi* 45-kDa protein was produced and used to characterize this molecule and study its role in trypanosome adhesion to heart myoblasts. We found that the 45-kDa protein is a surface mucin, is expressed only in invasive trypomastigotes, but not in noninvasive epimastigotes or amastigotes, and is released by the trypanosome in culture medium. One of the monoclonal antibodies (Mab B5) from this set inhibits the attachment of trypomastigotes to heart myoblasts preventing trypanosome entry, whereas the others (Mabs B4 and F1) do not. This inhibition was seen with the B5 hybridoma culture supernatant, with the purified Mab B5 IgG or with Mab B5 Fab fragments. These novel findings identify the 45-kDa mucin as a new *T. cruzi* ligand that is used by invasive forms of this organism to adhere to heart myoblasts.

Trypanosoma cruzi, the protozoan that causes Chagas' disease affecting millions of people, must bind to mammalian cells before entry to establish infection (1). Chagas' disease is acquired by binding and 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 early death when associated with HIV-1 infection (2).

The identification of *T. cruzi* surface molecules that function as ligands to mediate trypanosome adhesion to mammalian cells to promote entry may be critical to molecular means of intervention against this organism. The surface of invasive *T. cruzi* trypomastigotes is partially covered with *trans*-sialidases and mucin molecules, with yet undefined functions in trypanosome–host cell interactions (3, 4). *T. cruzi* is unable to synthesize sialic acid and acquires this molecule from the host via surface *trans*-sialidases which transfer sialic acid from host glycoproteins to mucin molecules located on the surface of the trypanosome (3). Trypanosome surface mucins are very diverse and present variable sizes ranging from glycoproteins of 35–50 to 60–200 kDa (3). Their individual role in *T. cruzi* binding to host cells is unknown. The few previous studies with trypanosome mucins have been performed using partially purified chromatographic fractions, each containing several trypanosome mucins (4, 5) and mucin-like molecules which have been shown to stimulate proinflammatory cytokine production by macrophages (6). Current thoughts suggest that surface trypanosome mucins may be involved in *T. cruzi* adhesion to mammalian cells, since it has been shown that mammalian mucins participate in cell–cell interactions (7, 8). However, there are no reports to date showing that a single trypanosome mucin molecule is involved in *T. cruzi* attachment to mammalian cells. To fill this gap we have produced a set of monoclonal antibodies to a surface *T. cruzi* 45-kDa protein to characterize this molecule, and studied its role in trypanosome adhesion to heart myoblasts.

In this paper we report the novel observations that the Mab B5 recognizes an epitope on the *T. cruzi* surface 45-kDa mucin specific for invasive trypomastigotes that is required for trypanosome binding to heart myoblasts to promote parasite entry.

MATERIALS AND METHODS

Organism. The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of *T. cruzi* was used (9). Pure culture trypomastigotes were obtained from the supernatant of heart myoblast monolayers (10). Pure amastigotes were produced and purified under conditions previously reported (11) and epimastigotes were obtained as described (12). Parasites were washed with DMEM and resuspended at the concentrations described below. In some experiments the Y, Cl and Brazil strains of *T. cruzi* were used.

Production of hybridomas. Monoclonal antibodies(Mabs) to a *T. cruzi* 45-kDa protein, present in an isoelectrofocusing fraction of pH

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0006-291X/02 $35.00

5.5 obtained by separating trypomastigote membrane native proteins by preparative isoelectrofocusing (Rotofor) (13), were produced as described (14). Briefly, Balb/c mice were immunized with the *T. cruzi* 45-kDa protein using a protocol previously described (15). Spleen cells of immunized Balb/c mice were fused with the plasmacytoma cell line P3X63-Ag8.653, resuspended in MEM/HAT media supplemented with 20% fetal bovine serum and cloned on 96-well plates. Supernatants were tested for the presence of antibodies by ELISA (15) with the isoelectrofocusing fraction used for immunization, indirect immunofluorescence using pure trypomastigotes as antigens and by Western blots of solubilized trypomastigotes. Supernatants were also tested for the ability to block the attachment and internalization of trypomastigotes to heart myoblasts as described below. Selected hybridomas were recloned twice. Isotyping of Mabs was performed by ELISA using an isotyping kit (Amersham) following the recommendations described by the manufacturer. The concentration of Mabs in the hybridoma supernatants was determined using the Easy-Titer Mouse IgG Assay Kit (Pierce).

Purification of monoclonal antibodies and Fab fragments. Culture supernatants of selected hybridomas were precipitated with 50% ammonium sulfate, resuspended in 50 mM Tris buffer saline, pH 7.5 and dialyzed against the same buffer. IgG of Mabs and an isotype control were purified using an UltraLink Immunobilized Protein G column kit (Pierce). To obtain monovalent Fab fragments from purified Mabs (Fab), the ImmunePure Fab Preparation Kit (Pierce) was used. The purity of purified immunoglobulins or Fab was determined by separating IgG or Fab by SDS–PAGE and staining the gel with Coomassie blue, and by immunoblotting (15).

Characterization of the T. cruzi 45-kDa protein. The ability of the produced Mabs to recognize different epitopes on the 45-kDa protein of *T. cruzi* trypomastigotes and the expression of the protein in developmental stages of *T. cruzi* was investigated by immunoblots. Briefly, 10 μg of solubilized trypomastigotes, amastigotes or epimastigotes in Chaps supplemented with protease inhibitors (Calbiochem) (16) were separated by SDS–PAGE, blotted onto nitrocellulose membranes and probed with Mabs followed by incubation with goat anti-mouse IgG labeled with horseradish peroxidase (Pierce) and developed with ECL (Amersham) as described (17). The ability of purified Mabs to recognize the *T. cruzi* 45-kDa protein as a surface protein was investigated by immunoprecipitating biotinylated surface proteins of trypomastigotes with Mab antibodies. Briefly, 250 μg of labeled trypomastigote cell lysates were mixed with immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40), precleared, and incubated with a 1:200 dilution of mouse Mabs to the *T. cruzi* 45-kDa protein or an isotype control supplemented with protein G-agarose as described (18). Samples were incubated overnight at 4°C. Immunoprecipitated proteins were dissociated with sample buffer, separated by SDS–PAGE (15), blotted onto nitrocellulose membranes, probed with streptavidin conjugated to horseradish peroxidase (Pierce) and developed with ECL (Amersham) as described (18). The ability of Mabs to the *T. cruzi* 45-kDa protein to recognize the released 45-kDa protein in supernatants of cultured trypomastigotes was evaluated by immunoblots. Briefly, pure trypomastigotes (10^8 cells) were washed three times in DMEM, and resuspended in 200 μl of DMEM for 4 h at 37°C. Cells were centrifuged and 10 μl of supernatant were separated by SDS–PAGE, blotted onto nitrocellulose and strips were probed with Mab B5 or isotype control diluted 1:200. Nitrocellulose membranes were probed with mouse anti-goat IgG labeled with horseradish peroxidase (Pierce) and developed with ECL. We explored whether the 45-kDa protein is a glycoprotein by subjecting the immunoprecipitated 45-kDa protein recognized by Mabs to an enzymatic deglycosylation kit containing NANase II, *O*-glycosidase DS and PNGase F (Bio-Rad). Briefly, 800 μg of solubilized trypomastigotes were immunoprecipitated with purified Mab B5 IgG as described above. The immunoprecipitated protein was dissociated from MabB5 IgG with 0.1 M citric acid, pH 3.0, immediately neutralized, and subjected to the enzymatic deglycosylation kit (Bio-Rad) according to the manufacturer recommendations. Treated or untreated samples (20 μl) were separated by SDS–PAGE, blotted onto nitrocellulose membranes, probed with Mab B5, followed by incubation with goat anti-mouse IgG labeled with horseradish peroxidase and developed with ECL as described above. To explore whether the *T. cruzi* 45-kDa protein is a mucin, we immunoprecipitated lysed *T. cruzi* trypomastigotes with Mabs to the *T. cruzi* 45-kDa protein under conditions described above, separated the immunoprecipitated protein by SDS–PAGE, blotted onto nitrocellulose membranes and probed the blots with the Mab C20 which recognizes *T. cruzi* mucins ranging from 35 to 50 kDa (5). Dr. M. Fresno kindly provided Mab C20 from Universidad Autonoma de Madrid, Spain.

T. cruzi binding assays. *T. cruzi* trypomastigote–cell interactions were performed using a trypanosome: cell ratio of 10:1. The ability of Mabs in hybridoma supernatants, purified IgG Mab or purified Fab to inhibit the attachment of live trypomastigotes to 0.1% paraformaldehyde fixed rat heart myoblasts in Labtek chambers was evaluated as described (9). Briefly, trypomastigotes were incubated with increasing concentrations of Mabs, isotype control of Mab, Fab, Fab fragment isotype control or binding medium alone (DMEM supplemented with 1% bovine serum albumin) for 1 h at 4°C and then exposed to fixed myoblasts in binding medium at 37°C for 2 h. After removing unbound trypanosomes, the cells were fixed, stained with Giemsa and the number of bound trypanosomes were microscopically determined. The ability of Mab or Fab to inhibit trypanosome entry into heart myoblasts was performed using live myoblasts as described (9, 10). In some experiments Vero cell fibroblasts and mouse peritoneal macrophages were used as host cells as described (9, 16).

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 $\pm$ 1 standard deviation. Differences were considered to be statistically significant if $p < 0.05$ as determined by the Student t test.

RESULTS

The T. cruzi 45-kDa protein is a surface mucin that is expressed in invasive trypomastigotes but not in non-invasive epimastigotes or amastigotes and it is released by the trypanosome. We have produced a set of Mabs of IgG_1 isotype that recognizes a *T. cruzi* 45-kDa protein and used it to characterize this molecule and study its function in trypanosome adhesion to heart myoblasts. The set of Mabs B5, B4 and F1 recognizes the *T. cruzi* 45-kDa protein in lysed trypomastigotes by immunoblots, whereas an isotype control does not (Fig. 1A). Mabs B5, B4 and F1 are highly specific for the 45-kDa protein, since they did not cross-react with other proteins of trypomastigotes (Fig. 1). Immunoblots of solubilized trypomastigotes, epimastigotes or amastigotes probed with the set of Mabs B5, B4 and F1 show recognition of the 45-kDa molecule in invasive trypomastigotes, but not in noninvasive epimastigotes or amastigotes (Fig. 1B). Similar results were obtained when the Y, CL, and Brazil strains of *T. cruzi* were used as antigens for immunoblotting (results not shown). Immunoprecipitation of lysed biotinylated trypomastigotes with Mabs B5, B4 and F1 indicate that this set of Mabs immunoprecipitates only the surface 45-kDa of *T. cruzi* trypomastigotes, whereas an isotype control does not immunoprecipitate any pro-

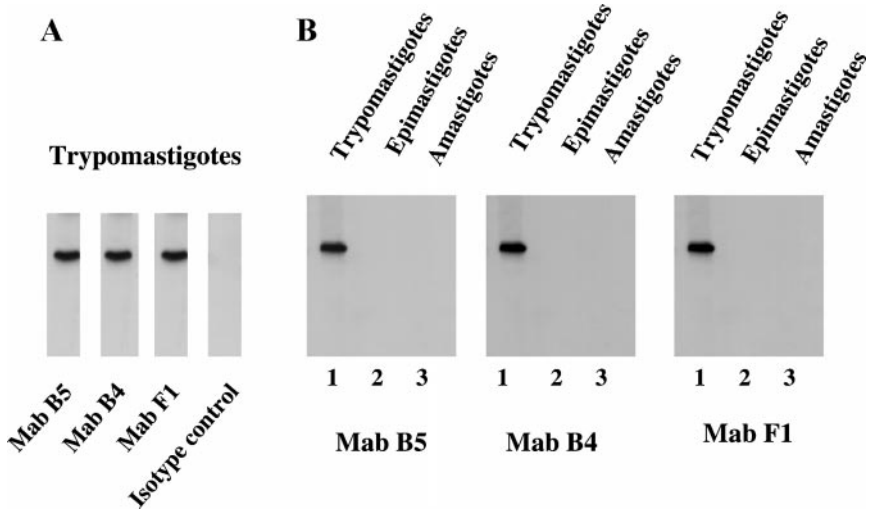

FIG. 1. A set of monoclonal antibodies to the *T. cruzi* 45-kDa protein recognizes the 45-kDa protein in invasive trypomastigotes, but not in noninvasive epimastigotes or intracellular amastigotes by immunoblots. 10 μg of solubilized trypomastigotes, epimastigotes, or amastigotes was separated by SDS–PAGE, blotted onto nitrocellulose membranes, probed with a set of Mabs of the isotype IgG_1 (B5, B4, F1 or an istotype control), incubated with goat anti-mouse IgG labeled with horseradish peroxidase, and developed by ECL as described under Materials and Methods.

teins on the parasite (Fig. 2A). These results indicate that the 45-kDa protein of *T. cruzi* is a surface protein which is expressed only in invasive trypomastigotes. Analysis of the immunoprecipitated 45-kDa protein with a protein deglycosylation kit resulted in an electrophoretic mobility shift with respect to the mocked treated sample (Fig. 2B), indicating that the surface *T. cruzi* 45-kDa protein is a glycoprotein. The fact that the deglycosylated protein is recognized by the Mab B5 suggests that this Mab recognizes a peptide epitope on the surface 45-kDa molecule. We explored whether the 45-kDa surface protein of trypomastigotes would be released in culture supernatants by immunoblotting assays. Our results indicate that Mab B5 recognizes the released 45-kDa surface protein in the supernatant of trypomastigotes that were incubated in DMEM for 4 h at 37°C, as evidenced by immunoblots (Fig. 2C, lane 2), whereas Mab B5 present negative reactivity in medium alone (Fig. 2C, lane 3). We also explored whether the 45-kDa protein immunoprecipitated by the set of Mabs B5, B4, and F1 is recognized by a highly specific anti-*T. cruzi* mucin Mab C20. Our results indicate that the anti-*T. cruzi* mucin Mab C20 strongly recognizes the surface glycoprotein 45 kDa of trypomastigotes on immunoblots of immunoprecipitated lysed trypomastigotes with Mabs B5, B4 and F1 (Fig. 2D). These results indicate that the 45-kDa surface protein is a mucin. Taken together these results indicate that the set of Mabs B5, B4 and F1 recognize a trypomastigote specific surface 45-kDa mucin which is released into culture supernatants by the trypanosome.

Mab B5 or its Fab fragments bind to the *T. cruzi* surface 45-kDa mucin to neutralize trypanosome attachment to heart myoblasts. To explore the role of the surface 45-kDa mucin of invasive trypomastigotes in trypanosome attachment to host cells, we reasoned that if trypomastigotes use the 45-kDa surface mucin to bind to heart myoblasts, then a Mab or its Fab specific for the 45-kDa mucin should inhibit the attachment of trypomastigotes to host cells to prevent trypanosome entry. Indeed, of the Mabs recognizing epitopes on the 45-kDa surface mucin, only the Mab B5 inhibits the attachment of trypomastigotes to heart myoblasts. Initially, trypomastigotes were pretreated with selected hybridoma supernatants containing the Mabs B5, B4, F1 or isotype control. Only pretreatment of trypomastigotes with the hybridoma supernatant containing the Mab B5 inhibited the attachment of trypomastigotes to heart myoblasts (Fig. 3) as evidenced by both the significant increase in the percent of inhibition of trypomastigote binding to myoblasts (Fig. 3A) and the reduction in the number of trypanosomes bound per 200 myoblasts in an antibody concentration dependent manner (Fig. 3B). Pretreatment of trypomastigotes with hybridoma supernatants containing the Mabs B4, F1 or isotype control did not affect the attachment of trypomastigotes to heart myoblasts (Fig. 3A). Similar inhibition was obtained when trypomastigotes obtained from other strains of *T. cruzi* (Y, CL, and Brazil), Vero cell fibroblasts or mouse peritoneal macrophages were used (results not shown). Pre-treatment of trypomastigotes with purified Mab B5 IgG significantly reduced the number of trypanosomes bound per 200 myoblasts in a concentration dependent manner (Fig. 4A), and inhibits *T. cruzi* binding to these cells in a similar fashion (Fig. 4B) at very low μg concentrations. Pretreatment of trypanosomes with isotype control IgG does not affect their ability to

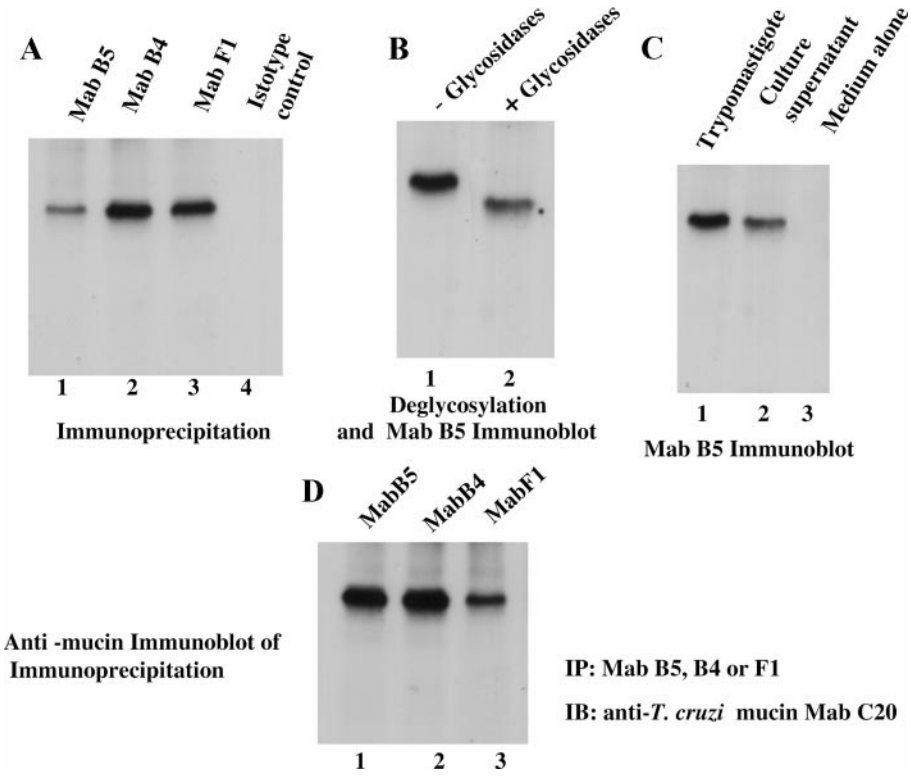

FIG. 2. The *T. cruzi* 45-kDa protein specific for invasive trypomastigotes is a surface mucin that is released by the trypanosome. (A) Mabs B5, B4, and F1 immunoprecipitate the 45-kDa surface protein of trypomastigotes. Solubilized biotinylated trypomastigotes were immunoprecipitated with Mabs B5, B4, F1, or isotype control, separated by SDS–PAGE, blotted on nitrocellulose membranes and probed with avidin labeled with horseradish peroxidase, and developed by ECL as described under Materials and Methods. (B) The trypomastigote 45-kDa protein is a glycoprotein as indicated by its mobility shift after deglycosylation. The immunoprecipitated 45-kDa protein was treated (B, lane 2) or mock-treated (B, lane 1) with a Bio-Rad protein deglysosylation kit, separated by SDS–PAGE, blotted onto nitrocellulose, probed with the Mab B5, and developed by ECL as described under Materials and Methods. (C) The Mab B5 recognizes the 45-kDa surface glycoprotein of trypomastigotes that is released into the medium. 10 μg of lysed trypomastigotes (C, lane 1) or 10 μl of supernatant of trypomastigotes (1×10^8 cells) incubated in DMEM for 4 h (C, lane 2) or DMEM alone (C, lane 3) was separated by SDS–PAGE, blotted onto nitrocellulose membranes, and probed with Mab B5 and developed by ECL. (D) The 45-kDa surface glycoprotein immunoprecipitated by Mabs B5, B4, and F1 is recognized by an anti-*T. cruzi* mucin Mab C20 by immunoblots. Solubilized trypomastigotes were immunoprecipitated with Mab B5 (D, lane 1), Mab B4 (D, lane 2), Mab F1 (D, lane 3), separated by SDS–PAGE, blotted onto nitrocellulose membranes and probed with anti-*T. cruzi* mucin Mab C20, and developed by ECL as described under Materials and Methods.

bind to myoblasts (Figs. 4A and 4B). Inhibition of trypanosome binding by Mab B5 IgG prevented trypanosome entry into heart myoblasts (results not shown). Similarly, treatment of trypomastigotes with purified monovalent Fab of Mab B5 significantly reduced the number of trypanosomes bound per heart myoblasts in a concentration-dependent manner, whereas purified Fab of an isotype control did not affect binding (Fig. 4C). The inhibition reaches 75% at the concentration of 1 μg of Fab/ml (Fig. 4D). Trypanosome binding in the presence of isotype Mab control or control Fab was similar to DMEM controls (data not shown). Taken together these results shown that the Mab B5 neutralizes the attachment of invasive *T. cruzi* trypomastigotes to heart myoblasts by blocking an epitope on the surface 45-kDa mucin specific for trypomastigotes that is required for trypanosome binding to heart myoblast cells.

DISCUSSION

These results indicate for the first time that invasive *T. cruzi* trypomastigotes use the surface 45-kDa mucin to attach to heart myoblasts to promote entry. Furthermore, these results also indicate that an epitope recognized by the Mab B5 on the surface 45-kDa mucin of invasive trypomastigotes is required for trypanosome binding to heart cells to initiate cellular infection. Moreover, we show for the first time that the surface 45-kDa mucin of *T. cruzi* is developmentally regulated in the cell cycle of *T. cruzi*, since this molecule is expressed in invasive epimastigotes but not in noninvasive insect epimastigotes or intracellular amastigotes.

Heart cells are a major target for *T. cruzi* and infection of these cells is involved in cardiac arrest followed by death [1]. Elucidation of the molecular interactions

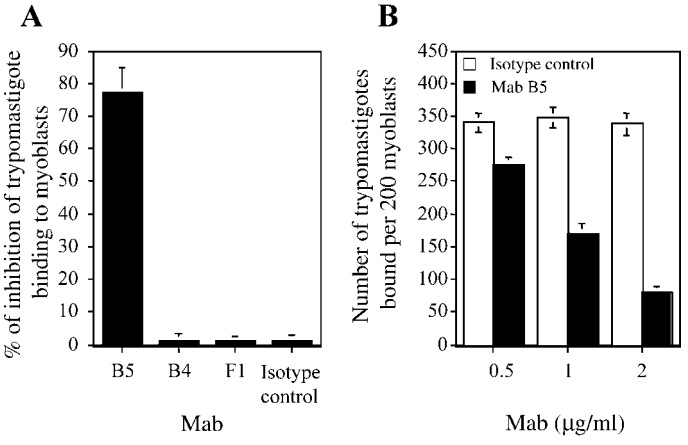

FIG. 3. Effect of hybridoma supernatants containing Mabs to the surface 45-kDa mucin of trypomastigotes on the attachment of trypomastigotes to heart myoblasts. (A) Trypomastigotes (10^7) were treated with the same concentration of Mabs B5, B4, F1, or its isotype control at 4°C for 1 h then exposed to paraformaldehyde-fixed heart myoblast monolayers for 1 h. After removing unbound trypanosomes, the monolayers were fixed, stained and the number of bound trypanosomes per 200 cells was microscopically determined as described under Materials and Methods. (B) Hybridoma culture supernatants containing Mab B5 inhibit binding of trypomastigotes to heart myoblasts in a concentration-dependent manner. Trypomastigotes were incubated with different concentrations of the Mab B5 and exposed to heart myoblast monolayers and the number of attached trypomastigotes per 200 myoblasts was determined as described above. Each column represents the mean of triplicate determinations ± SD. Differences between test and control in A and in B at each Mab concentration, $P < 0.05$.

between *T. cruzi* molecules and their receptors on heart cells may be important not only to understand how *T. cruzi* enters heart cells to alter their cardiophysiology, but also for the development of molecular means of intervention against Chagas' heart disease. Host cell invasion by *T. cruzi* involves activation of signaling pathways, cytoskeletal reorganization and targeted recruitment of host cell lysosomes (19). However, very little is known about *T. cruzi* ligands that are used by the trypanosome to bind to host cells receptors to trigger signal transduction events (17, 18). Knowledge of the role of *T. cruzi* mucins in trypanosome–host interactions is very limited (3, 4). A previous study from our group showed that a surface 45-kDa mucin of *T. cruzi* trypomastigotes interacts with human galectin-3 in order to promote trypomastigote adhesion to laminin (20). Other groups using a *T. cruzi* preparation containing 35- to 50-kDa mucins have shown that together these mucins trigger macrophage production of pro-inflammatory cytokines (5).

The fact that the surface 45-kDa mucin is also expressed in invasive trypomastigotes of other strains of *T. cruzi* and that the MabB5 inhibits the binding of trypomastigote to cells other than heart cells, which are able to be infected by *T. cruzi* such as macrophages and fibroblasts, strongly suggests that the 45-kDa mucin of *T. cruzi* is a common ligand that this organism uses to bind to and enter different types of host cells to initiate infection in the body. The fact that the 45-kDa surface mucin is released from the surface of trypanosomes is consistent with the fact that *T. cruzi* mucins are GPI anchored membrane molecules and are released into culture supernatant (3, 4). That fact that the surface 45-kDa mucin is released from the trypanosome raises the possibility that invasive trypomastigotes might up regulate their infectivity to mammalian cells by modulating expression and the release of this molecule. In fact, recent reports have shown that another *T. cruzi* ligand (21) released by the trypanosome, up-regulates entry of the parasite into phagocytic cells to activate host tyrosine kinases in a PKC and MAP kinase dependent pathways, which are required for trypanosome entry into these cells (17, 18).

The fact that monovalent Fab of Mab B5 specific for the *T. cruzi* 45-kDa surface mucin inhibit binding of trypanosomes to heart myoblasts, indicates that this effect is specific and is not due to steric hindrance of the

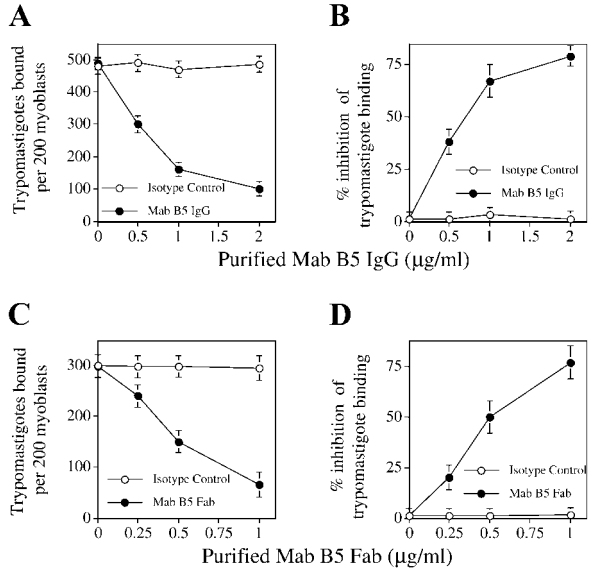

FIG. 4. Purified Mab B5 IgG or its Fab to the surface 45-kDa mucin of invasive forms of *T. cruzi* inhibit trypomastigote attachment to heart myoblasts in a concentration-dependent manner. (A and B) Purified Mab B5 IgG inhibits trypanosome attachment to myoblasts. Trypomastigotes were preincubated with several concentrations of purified Mab B5 IgG, exposed to heart myoblasts and the number of bound trypanosomes per 200 myoblasts (A) or the percentage of inhibition of trypomastigote binding to myoblasts (B) was determined as described under Materials and Methods. (C and D) Purified Mab B5 Fab inhibits trypanosome attachment to heart myoblasts. Trypomastigotes were preincubated with several concentrations of purified Mab B5 Fab, exposed to heart myoblasts and the number of bound trypanosomes per 200 myoblasts (C) or the percentage of inhibition of trypomastigote binding to myoblasts (D) was determined as described under Materials and Methods. Each point in the figures represent the mean of triplicate determinations ± SD. Differences between test and control in all points of the figure set, $P < 0.05$.

Mab B5 or due to possible agglutination of trypanosomes. These observations also strongly support the notion that the 45-kDa surface mucin is a new ligand that *T. cruzi* uses to attach to host cells to promote entry. Since Mab B5 or its Fab strongly but not completely neutralize *T. cruzi* binding to mammalian cells, we do not exclude the possibility that this organism may use alternative mechanisms to invade host cells. Consistent with this notion, two very recent reports have implicated the gp83 and the 85-kDa *trans*-sialidases of *T. cruzi* in binding to mammalian cells (21, 22). *T. cruzi* surface molecules that bind to host cell receptors during invasion may be of interest for developing vaccines and receptor-blocking therapies. In summary, we identified a new ligand on invasive forms of *T. cruzi* that is used by this organism to adhere to heart myoblasts to promote cellular entry.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grants AI-25637, AI-07281, HL-03149 and RR 03031 (to F.V.), GM 08037 (to F.V. and M.F.L.), and HL-03156 (to M.F.L.). C.W.T. was supported by a NIH fellowship grant (GM 18266). We thank Dr. F. Hatcher and the MMC Hybridoma Facility for the initial support.

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