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Selective Binding of *Trypanosoma cruzi* to Host Cell Membrane Polypeptides

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An adaptation of the immunoblotting technique was used to investigate binding interactions between *Trypanosoma cruzi* and mammalian host cells at the molecular level. A specific binding interaction was observed between *T. cruzi* and two host cell membrane polypeptides with molecular masses of approximately 32 and 34 kilodaltons. This molecular interaction was observed with antigen extracts of *T. cruzi* and with live, infective trypomastigote stages of the parasite, suggesting that the observed phenomenon may have relevance to the initial attachment of the parasite to the host cell membrane before invasion.

Increasing evidence suggests that plasma membrane-associated molecules play an important role in the invasion of mammalian cells by *Trypanosoma cruzi*, the protozoan parasite which causes Chagas' disease (American trypanosomiasis). It has been well documented that the initial attachment and subsequent invasion of host cells by *T. cruzi* can be affected either positively or negatively by a variety of different chemical perturbations of either the host cell or parasite plasma membrane, including treatment with proteases (10, 16, 19), lectins (15, 26), and specific sugars (6, 27). The infection of mammalian cells also appears to be strongly dependent upon the phase of the host cell cycle (7, 17), suggesting there may be cell cycle-related alterations in the expression of cell surface molecules involved in the invasion process. In addition, Kierszenbaum and Stiles (12) have shown that membrane preparations from trypomastigote stages, but not epimastigote stages, can block the attachment of live parasites to rat heart myoblasts in vitro.

More direct evidence for a specific molecular interaction between *T. cruzi* and host cells has come from the analysis of specific molecular determinants on the surface of *T. cruzi* in an effort to determine which of these molecules might play a role in the invasion of host cells (22). Several of the identified membrane glycoproteins, including Tc-85 of Zingales and Colli (27), the 83-kilodalton (kDa) glycoprotein of Boschetti et al. (2), and the fibronectin-collagen receptor described by Ouassi and co-workers (18, 24), have been hypothesized to play a role in the attachment of the parasite to the host cell membrane. In addition, it has been suggested that molecules of *T. cruzi* possessing neuraminidase activity may play a regulatory role in the invasion process (5). The relationship between these various glycoproteins is unclear, and there is no consensus among investigators as to which one of these parasite molecules (or which combination of molecules) is most relevant to the invasion process. Also, there is little specific information available concerning host cell surface molecules which may be involved in putative receptor-ligand interactions.

In the present study, we have examined binding interactions between *T. cruzi* and host cell membrane polypeptides by using a modification of the immunoblotting technique and have identified host cell membrane-associated polypeptides with approximate molecular masses of 32 and 34 kDa which bind parasite antigens and live parasites in a highly specific manner.

MATERIALS AND METHODS

Parasites. Parasites used in this study were primarily of a Brazil strain of *T. cruzi*. This strain is routinely maintained in our laboratory as a stock infection in C3HeB/FeJ mice (13), as culture forms (CFs) in an undefined liver infusion tryptose medium (20), and as fibroblast-derived trypomastigotes (FDTs) in murine fibroblast cultures (23).

The World Health Organization reference strain of *Leishmania braziliensis panamensis* (WR676) was also maintained in in vitro culture (21) for use in comparative studies.

Cell cultures. Two murine fibroblast cell lines, P5C3H and SVB6KH, and one murine macrophage cell line, P388D1, were used in this study. All cells were maintained in RPMI 1640 (GIBCO, Grand Island, N.Y.) adjusted to pH 7.2 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and supplemented with 10% fetal bovine serum (Flow Laboratories, Maclean, Va.), gentamicin sulfate (50 μg/ml), penicillin G (100 U/ml), and streptomycin (100 μg/ml) (complete RPMI 1640). Cells were maintained at 37°C in a 5% CO_2 incubator at 70 to 80% relative humidity.

Parasite antigen extracts. Trypomastigotes were harvested from cultures of P5C3H fibroblasts infected with the Brazil strain of *T. cruzi*. Culture supernatants containing FDTs were combined and centrifuged at 1,300 × *g* for 30 min at 4°C. The supernatant fluid was discarded, and pelleted FDTs were suspended in complete RPMI 1640. The FDTs were subsequently separated from contaminating host cell debris by centrifuging 10-ml portions of the parasite suspension over 5 ml of Histopaque (Sigma Chemical Co., St. Louis, Mo.) at 350 × g for 20 min. FDTs accumulating as a band at the interface were collected and washed three times by centrifugation at 1,300 × *g* in Dulbecco phosphate-buffered saline (DPBS) before being suspended in 50 mM Tris hydrochloride (pH 7.4) containing 10 mM CHAPSO (Pierce Chemical Co., Rockford, Ill.). The parasite suspension was placed in a Pyrex tube on ice and was sonicated in repeated 10-s pulses at a setting of 20 kHz on a Biosonic III sonicator (Bronwill Scientific, Rochester, N.Y.). Protease inhibitors (100 U of aprotinin per ml, 2 mM pepstatin A, 2 mM phenylmethylsulfonyl fluoride [all from Sigma]) were added to the extract, and the resulting suspension was rotated overnight at 4°C to allow for maximum solubilization. The parasite extract was then centrifuged at 85,000 × *g* for 1 h at

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4°C, and the supernatant fluid containing solubilized parasite antigens was dialyzed overnight against a large volume of DPBS at 4°C.

The CF extract (~80% epimastigotes and 20% trypomastigotes) was prepared in the same manner after parasites in stationary-phase growth had been harvested and washed three times in DPBS by centrifugation (1,000 × *g* for 30 min). The protein concentration was determined by Bradford analysis (3), and aliquots of the parasite extracts were stored at −70°C until use.

Parasite-specific antisera. New Zealand White rabbits were immunized by a primary injection of the FDT antigen extract in an equal volume of Freund incomplete adjuvant (2 mg of total antigen). The primary immunization consisted of 10 subcutaneous injections of 100 μl along the back of each animal. The rabbits were boosted intravenously with 1 mg of total antigen in DPBS 5 to 6 weeks after the primary immunization. After 7 to 10 days, the rabbits were bled by venipuncture, and the serum was tested by immunoblot analysis. Each animal was given booster injections and bled periodically to obtain large quantities of parasite-specific antiserum. Control serum was also obtained from each rabbit before immunization.

Rabbit antisera elicited against antigen extracts of *Leishmania mexicana amazonensis* (strain WR669) and *L. braziliensis panamensis* (strain WR676) were also utilized where indicated.

Plasma membrane isolation. Plasma membranes were isolated from murine cells according to an adaptation of the polycationic bead technique of Jacobson (11) and Cohen et al. (4). Affigel 731 beads (Bio-Rad Laboratories, Richmond, Calif.) were used as a polycationic solid support. The beads were hydrated in distilled H_2O, washed five to six times in 0.2 M NaCl, twice in 100 mM sorbitol–40 mM sodium acetate buffer (pH 5.0) and twice in 140 mM sorbitol–20 mM sodium acetate (pH 5.0) (attachment buffer). After the final wash in attachment buffer, the beads were allowed to settle, the supernatant solution was removed, and enough attachment buffer was added back to make a 50% bead suspension.

Cultures of murine cells were allowed to reach confluency. Cells were harvested in a solution consisting of 0.6 mM EDTA, 8.3 mM Na_2HPO_4, 2.77 mM KCl, 1.5 mM KH_2PO_4, and 0.14 M NaCl (pH 7.4). Detached cells were suspended in complete RPMI 1640 and centrifuged at 700 × *g* for 10 min. The cells were then suspended and washed twice in DPBS by centrifugation (700 × *g* for 10 min), followed by two washes in 100 mM sorbitol–40 mM sodium acetate buffer (pH 5.0) and two washes in attachment buffer. After the final wash in attachment buffer, the supernatant fluid was removed and enough attachment buffer was added back to make a 25% cell suspension.

Aliquots of the 50% bead suspension were added dropwise to the 25% cell suspension as the tube was gently swirled. The attachment of cells was monitored microscopically. Beads with attached cells were allowed to settle, and the supernatant fluid containing unbound cells was removed from the tube. The settled beads were then suspended in an equal volume of attachment buffer (pH 7.0) containing 1 mg of dextran sulfate (M_w, approximately 5,000; Sigma) per ml. The purpose of this step was to block the unbound, positively charged sites remaining on the beads. The tube was then immediately filled with final attachment buffer, and again the beads were allowed to settle. The beads containing attached cells were washed two additional times in the final attachment buffer. After the last wash, the beads were allowed to settle, the supernatant fluid was removed, and the beads were vortexed vigorously for 10 s. The tube was then placed on ice and was immediately filled with ice-cold 10 mM Tris hydrochloride (pH 7.4). After the beads were allowed to settle, the supernatant solution was removed and the beads were vortexed and washed with 10 mM Tris hydrochloride four additional times by centrifugation at 40 × *g* to completely lyse the cells and to remove all cell debris. The final wash with attachment buffer was supplemented with the protease inhibitor cocktail described above. Beads containing membrane patches were placed immediately at −70°C for later use.

Membrane proteins were eluted from the beads by adding an equal volume of sample buffer (0.08 M Tris hydrochloride [pH 6.8], 0.1 M dithiothreitol, 2.0% sodium dodecyl sulfate (SDS), 10% glycerol) to the bead preparation, followed by a 10-min incubation in a 75°C water bath with intermittent vortexing at high speed. The beads were then sonicated at 20 kHz in five 5-s pulses, and the supernatant fluid containing solubilized membrane proteins was subjected directly to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Tracking dye (0.2% bromophenol blue) was added to the sample before electrophoresis.

Electrophoresis and protein blotting. Cell membrane extracts and parasite antigen extracts were analyzed by electrophoresis in polyacrylamide slab gels containing 0.1% SDS with a 5% (wt/vol) polyacrylamide stacking gel and a 12.5% (wt/vol) polyacrylamide resolving gel in buffered Tris-glycine by the method of Laemmli (14). Samples containing parasite components were reduced and denatured by boiling in an equal volume of sample buffer (described above) for 2 min before application to the gels. The following proteins were used as molecular weight markers: myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor (Bio-Rad Laboratories, Richmond, Calif.).

Host cell membrane preparations and parasite antigen extracts resolved by SDS-PAGE were transferred to nitrocellulose membrane strips. Nitrocellulose membranes (0.45-μm pore size; Bio-Rad), sponges, and filter papers, were preequilibrated in cold transfer buffer (25 mM Tris hydrochloride [pH 8.3], 192 mM glycine, 20% [vol/vol] methanol) for at least 2 h before use. Proteins were electrophoretically transferred from the gel onto 8-mm-wide strips of nitrocellulose at 30 V for 12 to 14 h at 4°C. The efficiency of transfer was determined by staining selected strips containing antigen and strips containing molecular weight markers in 0.1% (wt/vol) amido black in 40% (vol/vol) methanol–2% (vol/vol) acetic acid. Strips were destained in 70% (vol/vol) methanol–7% (vol/vol) acetic acid. Under these conditions, protein transfers were reproducible over a wide range of molecular weights.

Parasite antigen detection. Nitrocellulose strips containing parasite proteins resolved by SDS-PAGE were analyzed by an indirect enzyme-labeled antibody assay. The strips were rinsed in DPBS after the electrophoretic transfer and incubated in DPBS containing 3% bovine serum albumin, 1% ovalbumin, 3% normal goat serum, and 0.1% Tween 20 (all from Sigma) for 2 h as a blocking step. All incubations in the assay were performed in 25-well acrylic incubation trays (Bio-Rad) placed in a high-humidity shaking water bath set at 37°C. The primary antiserum employed in the assay was rabbit antiserum elicited against the detergent extract of FDTs (diluted 1:50 in DPBS containing 10% fetal bovine serum). Horseradish peroxidase-conjugated goat anti-rabbit total immunoglobulin (Fisher Scientific Co., Springfield, N.J.), diluted 1:1,000 in DPBS was used as a second anti-

body. Strips were incubated in 10 ml of each of the antiserum preparations for a period of 1 h with three 10-min washes in DPBS containing 0.1% Tween 20 after each incubation. The strips were then developed in 120 ml of substrate solution (0.34 mM 4-chloro-1-napthol dissolved in 20 ml of cold methanol and diluted in 100 ml Tris-saline buffer consisting of 0.04 M Trizma hydrochloride, 9.7 mM Trizma base, and 0.2 M NaCl [pH 7.4]; all from Sigma) activated by the addition of 60 μl of 30% H_2O_2.

Binding assay. After electrophoretic transfer, nitrocellulose strips containing separated host cell membrane proteins were rinsed in DPBS and incubated for 2 h at 37°C in the blocking solution described above. Each nitrocellulose strip was then incubated in 10 ml of the appropriate detergent-solubilized parasite extract (100 μg of antigen per 10 ml of DPBS) for 6 h at 37°C in a high-humidity water bath with gentle shaking. After the incubation period, the strips were washed three times for a total of 30 min in DPBS containing 0.1% Tween 20. Specific binding of parasite antigens to cell membrane proteins was then detected by the enzyme-labeled antibody assay described above. Controls included strips incubated in DPBS during the antigen incubation period and strips exposed to the parasite antigen extract but probed with normal rabbit serum as a primary antiserum. Areas of specific binding of parasite proteins to host cell membrane polypeptides were apparent as darkly staining bands after incubation of the strips in substrate activated by the addition of H_2O_2.

Binding experiments were also performed using detergent-solubilized FDT and CF antigen extracts, biotinylated by the method of Bayer et al. (1). Nitrocellulose strips containing separated host cell membrane proteins were incubated in blocking solution as described above and exposed to the biotinylated antigen preparations (100 μg/10 ml of DPBS containing 0.1% Tween 20) for 6 h at 37°C. After three 10-min washes in DPBS containing 0.1% Tween 20, the strips were incubated with avidin-peroxidase (diluted 1:1,000 in DPBS; Sigma) for 1 h. Regions of specific binding of parasite antigen were visualized as darkly staining bands after incubation of the treated strips in H_2O_2-activated substrate solution.

Alternatively, the nitrocellulose strips containing the P5C3H membrane proteins were used as solid affinity supports to assay for the binding of live FDTs derived from infected P5C3H fibroblasts, live CFs in the stationary phase of growth (~80% epimastigotes and ~20% trypomastigotes), and stationary-phase CFs of *L. braziliensis panamensis* (strain WR676). Parasites were exposed to the nitrocellulose strips in 10 ml of medium. Control strips were incubated in 10 ml of fresh medium. Nitrocellulose strips were incubated with the parasite suspensions for a period of 8 h at 37°C. At the end of this period, each strip was rinsed gently in DPBS and was placed on a sheet of filter paper to air dry. The binding of parasites to the nitrocellulose strips was then detected by using the indirect enzyme-labeled antibody assay described above. The primary antisera employed in these experiments were rabbit anti-FDT serum or rabbit anti-*Leishmania* sp. serum, each diluted 1:50 in DPBS containing 10% fetal bovine serum.

RESULTS

Immunoblot analysis of parasite antigen extracts. Detergent extracts of *T. cruzi* (FDTs and CFs) were resolved by SDS-PAGE and electrophoretically transferred onto strips of nitrocellulose. Indirect enzyme-labeled antibody analysis

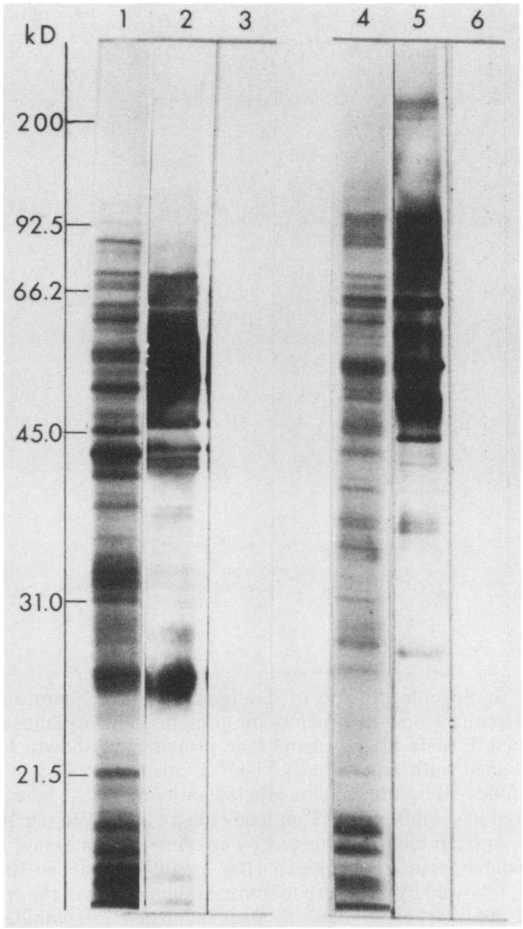

FIG. 1. Detection of *T. cruzi* polypeptides by immunoblot analysis. Lane 1, CF antigen stained with amido black; lane 2, CF antigen probed in enzyme-labeled antibody assay; lane 3, CF antigen and normal serum control; lane 4, FDT antigen stained with amido black; lane 5, FDT antigen probed in enzyme-labeled antibody assay; lane 6, FDT antigen and normal serum control.

indicated extensive cross-reactivity between CFs (Fig. 1, lane 2) and FDTs (Fig. 1, lane 5), as detected by rabbit antiserum elicited against the trypomastigote stage. This antiserum was used as the primary antiserum in subsequent experiments investigating molecular interactions between parasite extracts and molecules of the host cell membrane.

Specific molecular interactions between parasite antigens and murine cell membrane components. Nitrocellulose strips containing P5C3H membrane proteins were used as solid affinity supports to assay for binding activity with parasite antigen extracts prepared from either FDTs or CFs in the stationary phase of growth. The FDT extract and the CF extract both exhibited a specific binding interaction with membrane polypeptides purified from P5C3H murine fibroblasts (Fig. 2, lanes 3 and 5). The reactive host cell membrane polypeptides had relative mobilities corresponding to molecular masses of approximately 32 and 34 kDa. Faint reactivity was also occasionally observed with a third component of approximately 30 kDa. The binding of parasite antigens to specific host cell membrane polypeptides was also demonstrated by a biotin-avidin detection system. Biotinylated FDT and CF extracts exhibited the same binding specificity as nonconjugated parasite antigen extracts (Fig. 2, lanes 7 and 8). This molecular interaction appears to be

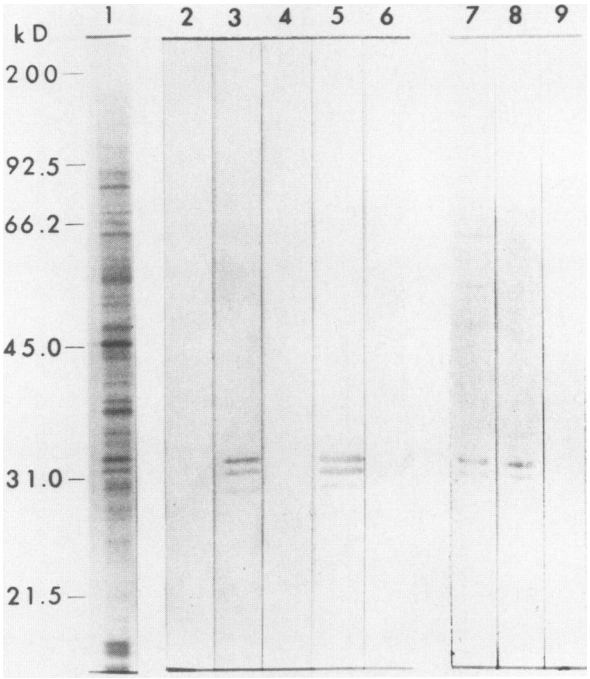

FIG. 2. Specific binding of detergent-solubilized parasite antigens to purified host cell membrane proteins. Nitrocellulose strips containing P5C3H plasma membrane proteins are shown. Lane 1, strip stained with amido black; lane 2, strip incubated in DPBS alone, followed by the enzyme-labeled antibody assay; lane 3, strip incubated in solubilized FDT antigen (100 μg of antigen per 10 ml of DPBS), antigen binding detected by enzyme-labeled assay; lane 4, strip incubated in FDT antigen (100 μg of antigen per 10 ml of DPBS), followed by exposure to normal rabbit serum in the enzyme-labeled antibody assay; lane 5, strip incubated in solubilized CF antigen (100 μg of antigen per 10 ml of DPBS), followed by the enzyme-labeled antibody assay; lane 6, strip incubated in CF antigen (100 μg of antigen per 10 ml of DPBS), followed by exposure to normal rabbit serum in the enzyme-labeled antibody assay; lane 7, strip incubated in biotin-FDT, followed by avidin-peroxidase assay; lane 8, strip incubated in biotin-CF, followed by avidin-peroxidase assay; lane 9, strip incubated in DPBS alone, followed by avidin-peroxidase assay.

highly specific, and similar results have been obtained in several repetitions of the experiment.

Selective binding of live *T. cruzi* to host cell membrane polypeptides. Because the initial results suggested that the 32- and 34-kDa host cell membrane proteins were serving as specific receptors for parasite antigens, it was of interest to determine whether live parasites would demonstrate a similar binding specificity to the host cell membrane molecules. In these experiments, live FDTs and CFs exhibited specific binding to two host cell membrane polypeptides of approximately 32 and 34 kDa (Fig. 3A, lanes 1 and 3). As in previous experiments, some binding was also observed to occur with a polypeptide of approximately 30 kDa. The interaction of live parasites with the host cell membrane polypeptides was observed by the indirect enzyme-labeled antibody assay. No binding was observed when CFs of *L. braziliensis panamensis* were exposed to host cell membrane proteins under the same experimental conditions (Fig. 3A, lane 5).

Similar experiments as described above were performed with mammalian cell membrane preparations from the SVB6KH mouse fibroblast cell line and the P388D1 mouse macrophage cell line. Live FDTs and CFs interacted in a similar manner with these two membrane preparations, exhibiting specific binding to membrane polypeptides of 32 and 34 kDa (Fig. 3B and C, lanes 1 and 3). No evidence was found to specific binding between these host cell membrane proteins and live *L. braziliensis panamensis* (Fig. 3B and C, lane 5).

DISCUSSION

Many investigators have presented experimental evidence that the invasion of mammalian cells by *T. cruzi* depends upon the ability of the parasite to recognize specific receptor molecules on the host cell membrane (22, 27). Although several surface molecules of *T. cruzi* have been implicated in the invasion process (2, 18, 24, 27), the precise role that these individual molecules play in the infection of host cells by *T. cruzi* has not been established. In addition, very little information exists concerning host cell glycoproteins involved in putative receptor-ligand interactions.

The objective of the present study was to investigate molecular interactions between *T. cruzi* and components of the plasma membrane of murine cells by using an adaptation of the immunoblotting technique. Plasma membranes from murine cell lines were purified on polycationic beads by the method of Jacobson (11) and Cohen et al. (4). The isolation of plasma membranes on polycationic beads is now a well-established technique which allows for rapid membrane purifications, yielding preparations which are of equal or greater purity to those obtained by more traditional methods. Membrane proteins isolated by this technique were subjected to SDS-PAGE, and resolved proteins were electrophoretically transferred to strips of nitrocellulose. Nitrocellulose strips containing plasma membrane proteins were then used to solid affinity supports to assay for the binding of solubilized parasite antigens and live parasites. Using this technique, we have identified two host cell polypeptides (32 and 34 kDa) which bind antigens of *T. cruzi* and live parasites in a highly specific manner. If the molecular interaction observed with the 32- and 34-kDa polypeptides has relevance to the invasion process, then a similar interaction should occur with a variety of cell lines permissive to infection by *T. cruzi*. For this reason, binding experiments were performed with three different murine cell lines, the P5C3H mouse fibroblast cell line, the SVB6KH mouse fibroblast cell line, and the P388D1 mouse macrophage cell line. Live *T. cruzi* interacted in an identical manner with these three membrane preparations, exhibiting a specific binding affinity for host cell polypeptides of 32 and 34 kDa. No evidence was found for specific binding between these host cell membrane proteins and live *L. braziliensis panamensis*, suggesting that the binding of *T. cruzi* to the 32- and 34-kDa host cell polypeptides is not simply a generalized binding phenomenon.

Velge and co-workers (24) have proposed that cell-bound fibronectin or collagen located on the extracellular matrix of mammalian cells may play a role in the attachment of *T. cruzi* to host cells via interactions with a fibronectin-collagen receptor located on the parasite surface. These researchers hypothesize that such interactions could be important in the initial phase of attachment which occurs between infective stages of *T. cruzi* and host cells before invasion. It is unlikely that fibronectin is serving as a bridging ligand for *T. cruzi* in the present study because the 32- and 34-kDa host cell molecules implicated as receptor molecules do not correspond in molecular mass to the polypeptide subunits of mammalian host cell receptors for fibronectin (25). How-

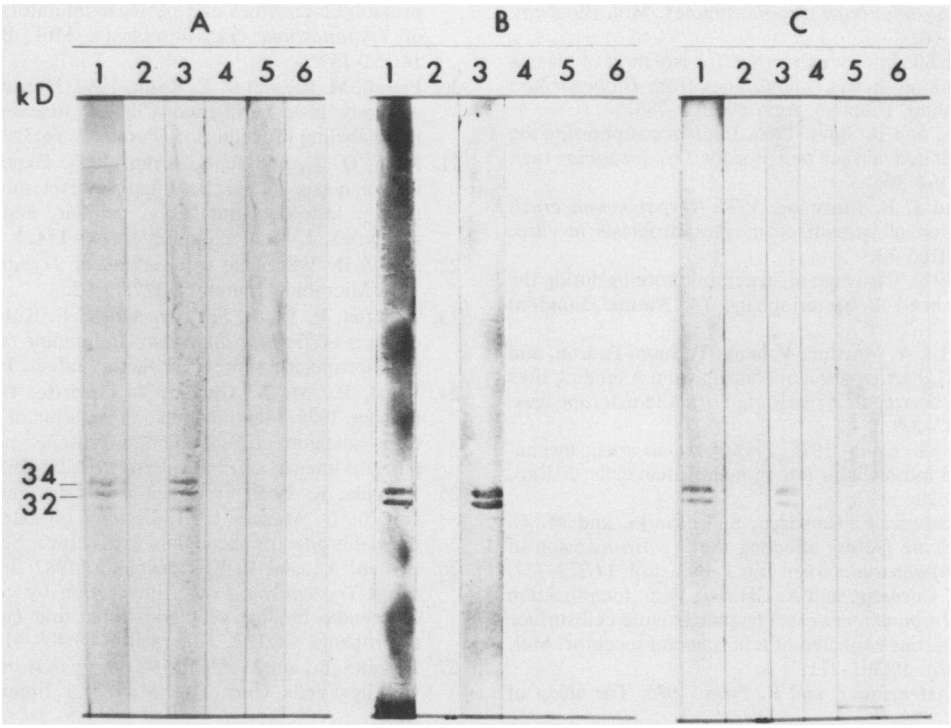

FIG. 3. Specific binding of live *T. cruzi* to membrane polypeptides isolated from P5C3H mouse fibroblasts (A), SVB6KH mouse fibroblasts (B), and P388D1 mouse macrophages (C). Binding of live parasites to nitrocellulose strips containing host cell membrane proteins was detected with the parasite-specific enzyme-labeled antibody assay. The primary antiserum employed in these experiments was rabbit anti-FDT serum or a rabbit anti-*Leishmania* sp. serum, each diluted 1:50 in DPBS containing 10% fetal bovine serum. Lanes 1, strips incubated with live CFs ($\sim 10^8$/ml) in the presence of LIT medium; lanes 2, strips incubated with fresh LIT medium (20) alone; lanes 3, strips incubated with live FDTs ($\sim 10^7$/ml) in the presence of complete RPMI 1640; lanes 4, strips incubated with complete RPMI 1640 alone; lanes 5, strips incubated with live *L. braziliensis panamensis* ($\sim 10^8$/ml) in Schneider medium (21); lanes 6, strips incubated in fresh Schneider medium alone.

ever, it is becoming increasingly apparent that the invasion of host cells by *T. cruzi* is a complex process potentially involving several different molecular interactions (2, 18, 24, 27). A major objective of future experimentation will be to determine whether the molecular interaction observed between *T. cruzi* and murine host cells in this study plays a role in this invasion process. Clearly, the identification and isolation of surface components of *T. cruzi* necessary for the invasion of host cells could have major importance in the development of immunoprophylactic methods to prevent Chagas' disease.

The technique of immunoblotting has been used effectively in a variety of studies involving the analysis of molecular interactions (8). Of particular relevance to the approach used in the present study is the work of Hayman and co-workers (9), in which the technique of protein binding was used to demonstrate the binding of normal rat kidney cells to plasma proteins. To our knowledge, however, this report is the first application of the technique to the investigation of molecular interactions between live protozoan parasites and host cell membrane proteins. Such an approach may be useful for investigating receptor-ligand interactions between other obligate intracellular parasites and their host cells.

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