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Signal Transduction in Human Macrophages by gp83 Ligand of *Trypanosoma cruzi:* Trypomastigote gp83 Ligand Up-Regulates Trypanosome Entry through the MAP Kinase Pathway

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We found that *Trypanosoma cruzi* trypomastigote cloned surface ligand (gp83 trans-sialidase) signals human macrophages to up-regulate parasite entry by inducing tyrosine phosphorylation of MAP kinase. Preincubation of human macrophages with r-gp83 trans-sialidase significantly enhanced both the percentage of phagocytosed trypanosomes and the number of trypanosomes per cell in a concentration dependent fashion. Incubation of r-gp83 with macrophages induced tyrosine phosphorylation of several macrophage proteins. This enhancement was inhibited by genistein, a tyrosine kinase inhibitor. The r-trypanosome ligand enhanced tyrosine phosphorylation of ERK1 and this enhancement was specifically inhibited by the inhibitor of MAP kinase phosphorylation, PD 98059, or by genistein. PD 98050 or genistein also inhibited the enhancement of trypomastigote uptake by macrophages induced by the r-ligand. These results indicate that *T. cruzi* uses a novel mechanism to signal cells in the process of trypanosome entry, via a secreted trypanosome ligand which signals macrophages through the MAP kinase pathway.

Trypanosoma cruzi, the protozoan which causes Chagas' disease and affects millions of people, must enter macrophages and other cells to establish infection (1). 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 faster death when associated to HIV-1 infections (2, 3). The possible emergence of *T. cruzi* as an opportunistic infection of HIV-1 infected individuals in the US has recently been considered (4). Trypomastigotes attach to and penetrate host cell membranes to multiply intracellularly as amastigotes and disseminate in the body. *T. cruzi* entry is a unique and remarkable process in membrane biology that is poorly understood (reviewed in 5, 6). An understanding of how *T. cruzi* uses ligands to signal mammalian host cells to enter them is critical to the development of molecular means of intervention.

Our group has identified a glycoprotein (gp83) trans-sialidase on the surface of *T. cruzi* trypomastigotes that binds to phagocytic and non phagocytic cells in a ligand-receptor interaction manner (7-10). This molecule is more expressed in highly than weakly infective trypomastigotes (7) and is secreted by trypanosomes, suggesting that the regulation of expression of this molecule modulates *T. cruzi* infection (7,10). A monoclonal antibody to the gp83 trans-sialidase of trypomastigotes (4A4) blocks trypomastigote binding to phagocytic and non-phagocytic cells and prevents trypanosome entry (9,10). Furthermore, Mab 4A4 strongly neutralizes *T. cruzi* infection in BALB/c mice by passive immunization (10). How *T. cruzi* trypomastigote ligands signal host cells to gain entry is unknown. In this paper we report the novel observation that the cloned surface ligand (gp83 trans-sialidase) of *T. cruzi* trypomastigotes, which is secreted by trypanosomes, signals human macrophages to up-regulate entry through the MAP kinase pathway.

MATERIALS AND METHODS

Organism. The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of *T. cruzi* was used in this work (7). Pure culture trypomastigotes were obtained from the supernatant of heart myoblast monolayers as described (8). Trypanosomes were washed with pyrogen free RMPI medium (RPMI) (Life Technology, NY) containing 100 μg streptomycin and 100 U penicillin/ml and resuspended at 1×10^7 organisms/ml in RPMI medium supplemented with

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1% crystallized bovine albumin (RPMI-BSA) (Bayer Corporation, Kankakee, IL) (11).

Human macrophages. Human monocytes were isolated from anonymous healthy individuals (American Red Cross, Portland, OR) by gradient centrifugation on Ficoll followed by Percoll gradient (Pharmacia Biotechnology, Piscataway, NJ) as described (11). Cells were washed in RPMI and resuspended in RPMI supplemented with 20% FBS (Hyclone, Logan UT), 50 μM 2-mercaptoethanol (Life Technology, Grand Island, NY), 100 μg/ml streptomycin and 100 U/ml penicillin. Monocytes were plated in Lab-Tek chambers (Nalge Nunc International, Naperville, IL) at 2×10^5 cells/ well. Purity was analyzed with OKM-1 monoclonal antibody. Mononuclear phagocytic cell monolayers consisted of >99% nonspecific esterase-positive cells. Monocytes were differentiated into macrophages for 4 weeks before exposure to trypanosome ligand and for *T. cruzi* infection assays. FACSCAN analysis of macrophage preparations showed 97-99% purity using the macrophage marker CD14.

r-T. cruzi gp83. Recombinant gp83 trans-sialidase of *T. cruzi* was purified as a fusion protein from IPTG induced *E. coli* (XL1-Blue) transformed with Tc-3 gene in λZAP II by preparative isoelectrofocusing followed by Mono-Q anion exchange chomatography using a fast pressure liquid chromatography system as described (8,12). The fusion protein is abbreviated in the text as r-*T. cruzi* gp83. The Tc-3 clone codes for a subset of the family 2 of trans-sialidases of *T. cruzi* and expresses a fusion protein that is recognized by the mAb 4A4. This monoclonal antibody recognizes a peptide epitope on the gp83 of *T. cruzi* required for binding of the gp83 to host cells (9). FPLC purified r-gp83 showed a single band when analyzed by SDS-PAGE and stained with Silver or Coomassie blue stain. The FPLC purified r-gp83 was recognized by the mAb 4A4 on a western blot, whereas an isotype control monoclonal antibody was not. FPLC purified r-gp83 was passed through a Detoxi-Gel endotoxin removing gel column (Pierce, Rockford, IL), extensively dialyzed against PBS free of pyrogen (Life Technology) and diluted in RPMI before exposure to human macrophage monolayers. This preparation was highly purified and did not contain any detectable levels of endotoxin. Endotoxin levels were assayed using the BioWhittaker Kinetic-QCL quantitative kinetic chromogenic assay (Biowhitaker, Inc. Walkersville, MD). Endotoxin reference standard 055:B5 was used to generate a duplicate standard curve. Endotoxin levels were calculated using BioWhittaker Win KQL software. Samples were transferred to pyrogen free glass tubes and diluted in BioWhittaker LAL reagent water. Each test dilution was assayed in duplicate, both net and with a 5 EU/ml positive product control spike. Endotoxin values were calculated from the lowest dilution which displayed a +/− 50% positive product control recovery. BioWhittaker LAL reagent water was run with each assay as a negative control. This standardized chromogenic assay is regularly used to test recombinant cytokines free of endotoxin by Genzyme (Cambridge, MA). The 36 aminoacid (3.1 kDa peptide) of β-galactosidase amino terminal region expressed by the λZAP II vector was purified from IPTG induced XL1-Blue cells by immunoaffinity chromatography using immobilized anti-β-galactosidase (Sigma), followed by Mono-Q-FPLC as described above. This purified 3.1 kDa peptide free of endotoxin, removed as above, was used as a control for the recombinant fusion protein. The 3.1 kDa peptide will be referred in the text as control peptide. Purified soluble gp83 was obtained from PLC-treated membranes of trypomastigotes as described (8) and dialyzed against PBS. Monovalent Fab' fragments of mAb 4A4 specific for gp83 were purified as described (10).

Uptake of trypomastigote by human macrophages. These procedures have been described in detail (13,14). The effect of r-gp83 on the uptake of *T. cruzi* trypomastigotes by human macrophages was evaluated using different concentrations of recombinant protein ranging from 0.1-100 μg/ml at a 10:1 ratio of trypomastigotes to macrophages. Briefly, macrophages were washed with RPMI and incubated with RPMI-BSA alone (control) or supplemented with r-gp83 or control peptide (control) for 30 min in 5% CO_2 at 37°C. After 30 minutes exposure, macrophage monolayers were washed three times with RPMI and cultures then received trypomastigotes in RPMI-BSA and were further incubated for 2 h. Under these conditions cell bound trypanosomes were ingested by macrophages (13) as further confirmed by electron microscopy (results not shown). Unbound trypomastigotes were removed by washing with RPMI; after fixing and staining with Giemsa, the percentage of cells containing *T. cruzi* and the number of trypanosomes internalized per 200 cells were microscopically determined (13,14).

Protein phosphorylation. The ability of r-gp83 to enhance tyrosine phosphorylation of human macrophage proteins was investigated by treating 1 x 10^6 human macrophages with 10 μg/ml of r-gp83 for 0, 5, 15 and 30 min. Control cultures did not receive r-gp83 or received control peptide. Experiments were also performed by preincubating human macrophage monolayers with the tyrosine kinase inhibitor genistein (100 μM) for 10 min. before exposure to r-gp83 or control peptide. After gp83 exposure, monolayers were solubilized with boiling non-reducing SDS lysis buffer (1% SDS, 10 mM Tris, pH 7.3) supplemented with 200 μM orthovanadate (11). Five μg protein from each sample were separated by SDS-PAGE (16), blotted onto nitrocellulose (16) and probed with horseradish peroxidase-conjugated anti-phosphotyrosine antibody (RC20) (Transduction Laboratories, Lexington, KY) and developed with ECL (Amersham, Arlington Heights, IL) (11). Enhancement of tyrosine phosphorylation of human macrophage proteins was determined by laser densitometry (17). Parallel blots on Immobilon P were stained with Coomassie blue. Nitrocellulose blots were also probed with a monoclonal antibody specific for β-actin (Sigma) and developed with A-P.

Tyrosine phosphorylation of MAP kinase. The ability of gp83 to enhance MAP kinase tyrosine phosphorylation was investigated by immunoprecipitating cell lysates with ERK1 monoclonal antibody and detecting tyrosine phosphorylation of MAP kinase on immunoblots probed with RC20, a monoclonal antibody specific for tyrosine phosphorylated residues (11). Briefly, 20 μg of cell lysates of human mock treated human macrophages, PD 98059 (PD), a specific MEK1 inhibitor (18), or genistein pre-treated macrophages that had been exposed or not to r-gp83 were mixed with immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.5% NP-40), pre-cleared, and incubated with monoclonal antibody to ERK1 (Transduction Laboratories) for 1 hr at 4°C followed by overnight incubation with 10 μl 50% Protein G-agarose at 4°C. Immunoprecipitated proteins were dissociated with sample buffer, separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with horseradish peroxidase-conjugated RC20 (Transduction Laboratories) and developed with ECL (Amersham) (11) as described above.

Effect of PD 98059 or genistein on the uptake of trypomastigotes by human macrophages. Human macrophage monolayers were preincubated in triplicate with several concentrations of PD 98059 (37-150 μM in RPMI) for 1 hr or with genistein (100-400 μM in RPMI) for 10 min. and then exposed to r-gp83 (10 μg/ml in RPMI) or RPMI for 30 min. Macrophage monolayers were washed and exposed to *T. cruzi* trypomastigotes at the ratio of 10 parasites per cell and the percentage of macrophages infected with *T. cruzi* and the number of trypanosomes per 200 cells was determined as described above (13,14).

Presentation of results and statistical analysis. Results in this work were obtained from triplicate values and represent three independent experiments with identical protocols using macrophages obtained from different healthy donors. Results are expressed as the mean ± one standard deviation. Differences were considered to be statistically significant if $p \leq 0.05$ as determined by the Student t test.

RESULTS

r-T. cruzi gp83 ligand enhances T. cruzi trypomastigote uptake by human macrophages. Fig. 1A shows that ex-

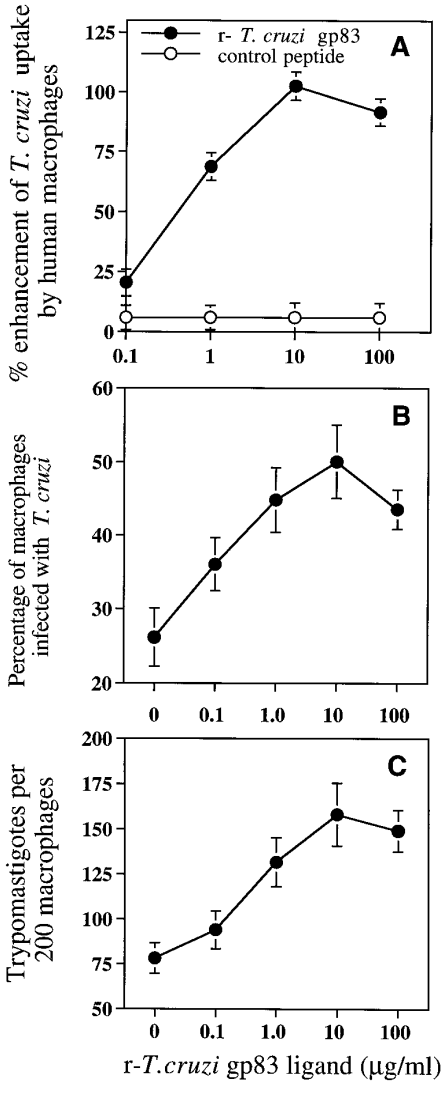

FIG. 1. Concentration dependent effect of r-*T. cruzi* gp83 on the percentage of enhancement of *T. cruzi* trypomastigote uptake by human macrophages (A), percentage of macrophages infected with trypanosomes (B), and the number trypanosomes per 200 cells (C). Several concentrations of r-*T. cruzi* gp83 were added to human macrophage monolayers in triplicate for 30 min, the unbound molecules were washed, and trypanosomes were added at the ratio 10:1. Trypomastigote uptake was evaluated at 2 h as described under Material and Methods. Controls included mock treated human macrophages and macrophages exposed to control peptide (3.1 kDa β-galactosidase peptide indicated in A). This is a set of a representative experiment of three performed with similar results. Each point represents the mean of triplicate determinations ± 1 SD.

posure of human macrophages to r-gp83 ligand significantly increases the percentage of *T. cruzi* trypomastigotes taken up by human macrophages in a concentration dependent fashion, showing a peak dose response at 10 μg /ml of gp83. A control peptide (3.1 kDa β-galactosidase) does not affect trypomastigote uptake (Fig.1A). A small enhancing effect is seen at 1 μg/ml of *T. cruzi* recombinant ligand. Treatment of human macrophages with r-gp83 for 30 min induced a significant increase in the uptake of *T. cruzi* trypomastigotes by human macrophages at 2 h with respect to RPMI mock-treated macrophages as evidenced by a significant increase in both the percentage of macrophages infected with *T. cruzi* trypomastigotes (Fig. 1B) and the number of trypomastigotes ingested by these cells (Fig. 1C). Furthermore, the gp83 induced increase in trypomastigote uptake was specifically blocked by the monovalent Fab' fragments of monoclonal antibody 4A4 which recognize a peptide epitope on the gp83 that is required for gp83 binding to host cells (results not shown), indicating the specificity of this effect. The ability of the r-gp83 ligand to increase trypanosome uptake by human macrophages varied occasionally in experiments with similar protocols but with cells from different healthy donors. However, a statistically significant increase in trypomastigote uptake by human macrophages was always observed when r-gp83 was added to human macrophages for 30 minutes, unbound r-gp83 was washed out and trypomastigotes were added for 2 hr.

r-T. cruzi gp83 enhances tyrosine phosphorylation of several human macrophage proteins. Results from these experiments show that exposure of human macrophages to r-*T. cruzi* gp83 ligand for 15 min induced significant tyrosine phosphorylation of several macrophage proteins (Fig. 2). The enhancement of tyrosine phosphorylation of these proteins could be reduced by the tyrosine kinase inhibitor genistein (Fig. 2). The same pattern of gp83-induced tyrosine phosphorylation of macrophage proteins was observed with macrophages obtained from different healthy volunteers. The enhancement of tyrosine phosphorylation induced by r-*T. cruzi* gp83 in different proteins of human macrophages with respect to basal tyrosine phosphorylation levels obtained in the absence of r-*T. cruzi* gp83 was analyzed by laser densitometry. r-*T. cruzi* gp83 induced a 2.3 fold increase in tyrosine phosphorylation of p94, a 2.4 fold increase of p54, a 2.1 fold increase of p43, a 2.4 fold increase of p42 and 2 fold increase of p35 (Fig. 2). Soluble native gp83 ligand purified from trypomastigotes induces the same pattern of tyrosine phosphorylation in human macrophages (results not shown). Control peptide at the same concentration of r-*T. cruzi* gp83 ligand does not cause any effect on tyrosine phosphorylation of macrophage proteins. Quantitative laser densitometric analysis of β-actin on the same blots probed with a monoclonal antibody against β-actin or total protein bands stained with Coomassie blue on all blots indicated that there were no intrinsic protein density changes in the bands (results not shown).

r-T. cruzi gp83 ligand enhance tyrosine phosphorylation of ERK1. The enhancement of tyrosine phosphorylation of MAP kinase was examined by immunoprecipitating cell lysates with ERK1 monoclonal antibody and detecting tyrosine phosphorylation of MAP kinase on immunoblots probed with RC20 monoclonal antibody.

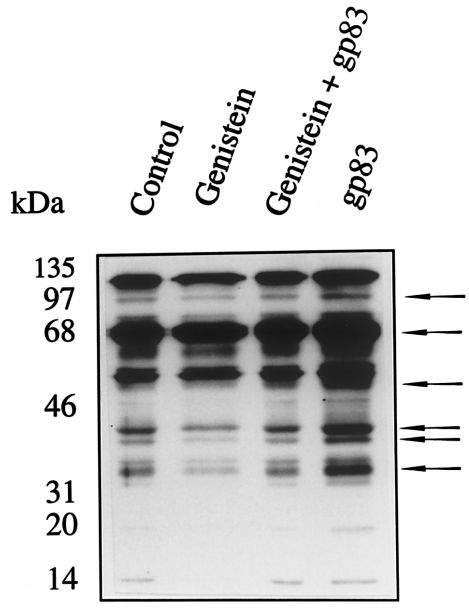

FIG. 2. r-*T. cruzi* gp83 enhances tyrosine phosphorylation of human macrophage proteins. Human macrophage monolayers (1×10^6) were incubated with genistein in RPMI or RPMI alone and then incubated with r-*T. cruzi* gp83 (10 μg in RPMI) or with RPMI for 5 min., and processed as described under Materials and Methods. Samples (5 μg) were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with anti-phosphotyrosine monoclonal antibody. Control human macrophages did not receive r-*T. cruzi* gp83. This is a representative experiment of three independently performed with similar results. Arrows point to enhanced protein tyrosine phosphorylation induced by r-gp83 with respect to control.

Addition of r-*T. cruzi* gp83 ligand to human macrophages for 15 min enhanced 4-16.8 fold tyrosine phosphorylation of ERK1 with respect to mock treated macrophages (Fig. 3 A and 3 B). Enhancement of tyrosine phosphorylation of ERK1 induced by r-gp83 ligand in human macrophages was significantly inhibited by either the tyrosine kinase inhibitor genistein (Fig. 3A) (77 %) or PD 98059 (72 %), a specific MEK1 inhibitor (Fig. 3B). These results encouraged us to examine whether PD 98059 or genistein at concentrations that inhibit ERK1 phosphorylation induced by r-*T. cruzi* gp83 could also inhibit the enhancement of *T. cruzi* trypomastigote uptake induced by the r-gp83 in human macrophages.

The specific MEK1 inhibitor PD 98059 or the tyrosine kinase inhibitor genistein abolishes the enhancement of T. cruzi uptake induced by r-gp83 ligand in human macrophages. Fig. 4 shows that pre-treatment of human macrophages with either genistein or PD 98059 followed by exposure to r-*T. cruzi* ligand abolished the enhancement of *T. cruzi* trypomastigote uptake induced by the trypanosome ligand as compared to mock treated human macrophages as evidenced by significant reductions in both the percentage of infected macrophages (Fig. 4 A and C) and the number of trypanosomes per 200 macrophages (Fig. 4 B and D). Genistein at 200μM or 75μM PD 98059 is sufficient to abolish the enhancement induced by gp83 in trypomastigote uptake by human macrophages (Fig. 4 A and C). Similarly, 100 μM genistein or 37 μM PD 98059 is enough to annul the increase in the number of trypanosomes ingested by 200 macrophages induced by gp83 (Fig. 4 B and D). Genistein at the concentrations of 100-400 μM or PD 98059 at the concentrations of 37-150 μM apparently do not affect the percentage of trypomastigotes taken up by human macrophages in the absence of *T. cruzi* gp83 (Fig. 4 A and C). Treatment of human macrophages with 100-400 μM genistein or 75-150 μM PD 98059 alone, reduced the number of trypomastigotes ingested by macrophages in the absence of gp83 stimuli below the levels seen in control mock treated macrophages (Fig. 4 B and D).

DISCUSSION

This report shows that *T. cruzi* uses a novel mechanism via the gp83 surface ligand to signal human mac-

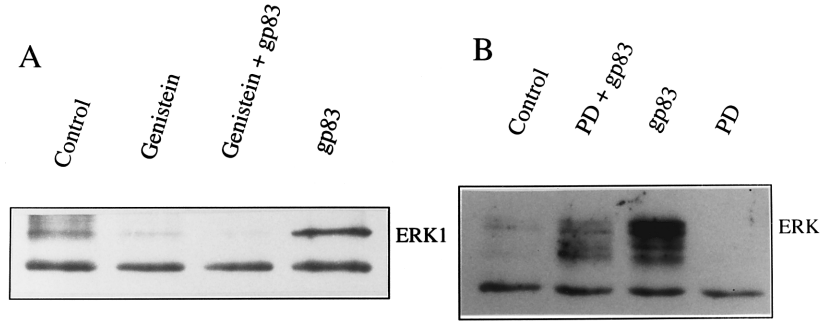

FIG. 3. r-*T cruzi* gp83 ligand enhances tyrosine phosphorylation of ERK1 in human macrophages. The enhancement of tyrosine phosphorylation of MAP kinase was examined by immunoprecipitating cell lysates with ERK1 monoclonal antibody and detecting tyrosine phosphorylation of MAP kinase on immunoblots probed with RC20 monoclonal antibody as described under Material and Methods. Samples in A were separated by 10% SDS-PAGE, whereas samples in B were separated by 7.5% SDS-PAGE. This is a representative experiment of three independently performed with macrophages from different healthy individuals with the same results.

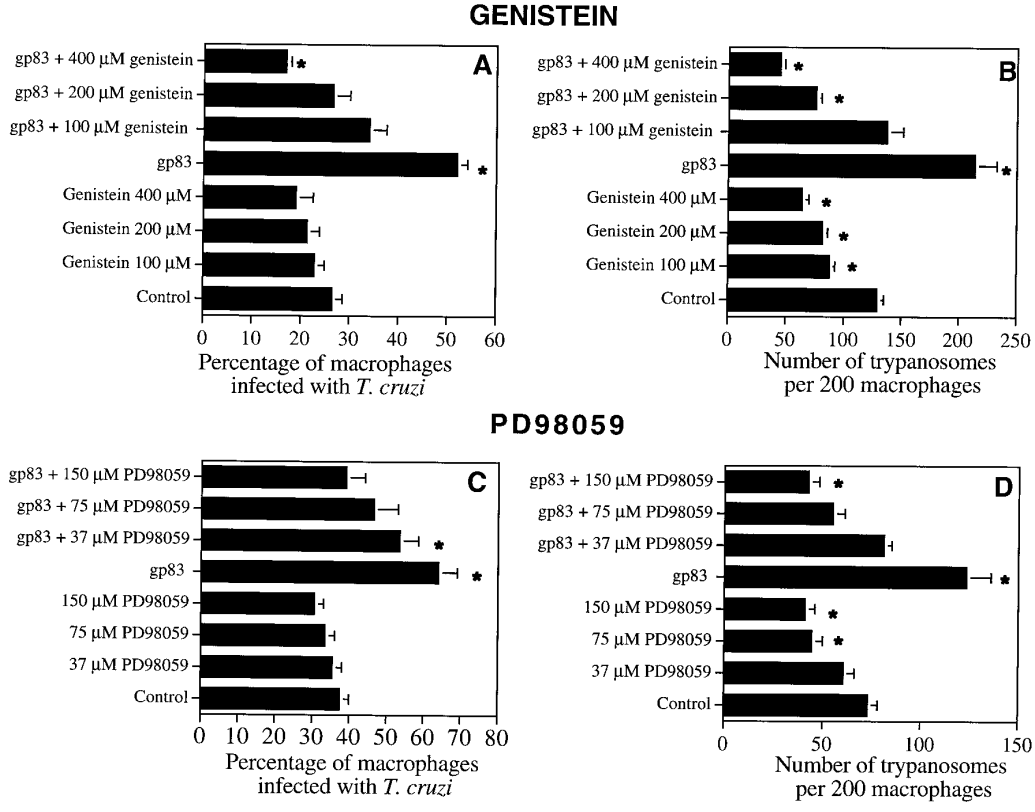

FIG. 4. Effect of PD 98059 or genistein on the enhancement of uptake of *T. cruzi* trypomastigotes induced by r-*T. cruzi* gp83 in human macrophages. Human macrophages were treated with or without PD 98059 or genistein and then exposed or not to r-*T. cruzi* ligand as described under Material and Methods. The percentage of macrophages infected with *T. cruzi* and the number of trypanosomes per 200 cells were determined as described under Material and Methods. This is a typical representative experiment of three independently performed in triplicate with similar results. Each bar represents the mean of triplicate determinations + 1 SD. *, represents a statistically significant change in the percentage of macrophages infected with *T. cruzi* or the number of trypanosomes per 200 macrophages with respect to their controls in the presence of media ($p<0.05$).

rophages utilizing the MAP kinase pathway for effective entry into host cells. In previous studies we have shown that the *T. cruzi* gp83, a GPI membrane anchored protein, mediates binding of invasive trypomastigotes to phagocytic and non phagocytic cells when the gp83 is anchored in the membrane of invasive trypanosomes (8-10). We report here that when this molecule is secreted by invasive trypanosomes it up-regulates the uptake of *T. cruzi* trypomastigotes by human macrophages. This novel signaling effect induced by the secreted gp83 ligand facilitates the invasion process and may lead to *T. cruzi* immune evasion. Therefore, this mechanism may facilitate occlusion of invasive forms of *T. cruzi* in the human host. Since the role of trans-sialidases in *T. cruzi*-host cell signaling is unknown and since the gp83 is a trans-sialidase belonging to the family 2 of trans-sialidases, this study defines a novel function for a trans-sialidase ligand in signaling the host during *T. cruzi* entry. Interestingly, the secreted *T. cruzi* gp83 of invasive trypomastigotes binds to all cells that *T. cruzi* can infect (unpublished results) and signals other types of cells to facilitate entry (unpublished results).

Our findings are in agreement with a previous report showing that the gp83 ligand is expressed more abundantly on the surface of highly than weakly invasive trypomastigote clones (7). Because expression of this molecule correlates with infectivity of trypomastigote clones, we hypothesize that its expression and secretion may modulate trypanosome invasiveness. This molecule is developmentally regulated in the cell cycle of *T. cruzi*, is expressed only in invasive trypomastigotes but not in non invasive epimastigotes or amastigote forms of *T. cruzi*, and carries a peptide epitope which is recognized by the neutralizing monoclonal antibody 4A4 which is required for gp83 binding to host cells (9,10). The fact that this molecule up-regulates cellular infection by signaling the host indicate that this trypanosome ligand is an attractive candidate for molecular intervention against *T. cruzi* infection.

The enhancing effect of the gp83 ligand seen on *T. cruzi* trypomastigote uptake by human macrophages is

specific, since monovalent Fab' fragments of the monoclonal antibody 4A4, that recognize a peptide epitope on the *T. cruzi* gp83 ligand that is required for gp83 binding to host cells, abolished these effects when compared to the lack of inhibition seen in the presence of the isotype control monoclonal antibody. The fact that the specific MEK1 inhibitor PD 98059 or the tyrosine kinase inhibitor genistein inhibit the enhancement of tyrosine phosphorylation of ERK1 induced by the trypanosome gp83 and that either genistein or PD 98059 inhibit the enhancement of trypomastigote uptake by human macrophages induced by gp83 supports the notion that *T. cruzi* gp83 signals human macrophages to up-regulate entry involving the MAP kinase pathway. The trypanosome gp83 ligand in addition to enhancing phosphorylation of ERK 1 induces tyrosine phosphorylation of other proteins in human macrophages. The latter are in the process of being characterized by our group.

In other protozoa such as *Plasmodium falciparum* and *Leishmania mexicana* the glycan component of glycosylphosphatidylinositols induces tyrosine phosphorylation and activates protein kinase C in mouse macrophages (19). Whether these effects are involved in microbial entry of these organisms are unknown. Moreover, whether the above glycan can induce the same effects in human macrophages is also unknown.

T. cruzi triggers a combination of events-a transient increase in cytosolic free calcium, rapid rearrangement of the cortical actin cytoskeleton, and lysosome recruitment and clustering during invasion of non phagocytic cells (20,21). Phospholipase C and inositol 1,4, 5-triphosphate (IP3) formation are responsible for the calcium release from intracellular stores and the subsequent microfilament rearrangement under the plasma membrane of non phagocytic cells (22). However, the trypanosome molecules that cause these signaling events are largely unknown. Moreover, the mechanisms used by *T. cruzi* ligands to signal phagocytic cells to gain intracellular entry are also unknown. Our findings show for the first time that a *T. cruzi* ligand, that is secreted by the invasive form of this organism, facilitates trypanosome entry into human macrophages and enhances tyrosine phosphorylation of MAP kinase. We suggest that the MAP kinase pathway is involved in the up regulation of *T. cruzi* entry by gp83 ligand. *T. cruzi* determinants that bind to host cell receptors (23) to signal the host to facilitate invasion process may be of interest for developing vaccines and receptor-blocking therapies.

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