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Differential Expression and Characterization of a Member of the Mucin-Associated Surface Protein Family Secreted by *Trypanosoma cruzi*▽†

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We describe the characterization, purification, expression, and location of a 52-kDa protein secreted during interaction between the metacyclic form of *Trypanosoma cruzi* and its target host cell. The protein, which we have named MASP52, belongs to the family of mucin-associated surface proteins (MASPs). The highest levels of expression of both the protein and mRNA occur during the metacyclic and bloodstream trypomastigote stages, the forms that infect the vertebrate host cells. The protein is located in the plasma membrane and in the flagellar pockets of the epimastigote, metacyclic, and trypomastigote forms and is secreted into the medium at the point of contact between the parasite and the cell membrane, as well as into the host-cell cytosol during the amastigote stage. IgG antibodies specific against a synthetic peptide corresponding to the catalytic zone of MASP52 significantly reduce the parasite's capacity to infect the host cells. Furthermore, when the protein is adsorbed onto inert particles of bentonite and incubated with a nonphagocytic cell culture, the particles are able to induce endocytosis in the cells, which seems to demonstrate that MASP52 plays a role in a process whereby the trypomastigote forms of the parasite invade the host cell.

Trypanosoma cruzi is a flagellate protozoon belonging to the order *Kinetoplastidae* and is the etiological agent of American trypanosomiasis, or Chagas' disease. It is estimated that some 16 to 18 million people suffer from the disease and that between 50,000 and 200,000 new cases are reported annually (42), mainly in central and south America, where around 21,000 people die of the disease every year (1). Although it has in the past been confined to Latin America, cases have recently been diagnosed outside this area due to human migration from the traditional endemic zones (37).

This flagellate has a life cycle involving mammalian and insect hosts. In insects (Reduviidae, Hemiptera) the cycle of development takes place in the intestinal tract of the host. Bloodstream trypomastigote (T) forms ingested from the mammalian host by the insect become epimastigote forms (E), which undergo division. After about 8 to 15 days, metacyclic trypomastigotes (M), developed from E, appear in the rectum. These M forms, which do not replicate, pass into the feces and urine and from there go on to infect mammalian host cells, where they transform before replication into amastigotes (A). The A forms multiply and differentiate into T forms, which are liberated into the intercellular spaces and the bloodstream.

Some of the *T. cruzi* cell invasion mechanisms have been described by various authors, who have studied it at the ultrastructural level and have also investigated some of the biochemical strategies involved in the interaction between the parasite and its host cell (12, 16, 20, 28, 30, 40, 44). Among these mechanisms are certain alterations that take place in the host cell while it is being invaded by *T. cruzi*, including a rise in cytosolic calcium levels caused by its excretion from intracellular deposits (12, 29, 31, 33), followed by the depolymerization of actin filaments and the recruitment of lysosomes toward the plasma membrane in a kinesin-dependent process, which results in the cytoskeleton of the host cell's participating in the parasite's invasion (29, 33, 34, 40, 41, 44, 46).

Glycoproteins deriving from the glycocalyx of the parasitic trypomastigote forms intervene in the initial recognition stage previous to the parasite's invasion of the host cell. These proteins belong to the TcMUCII family and have glycosylphosphatidylinositol (GPI) motifs with which they anchor themselves to the membrane of *T. cruzi* (17) and a wide variety of epitopes that facilitate the adhesion of the trypomastigote to the host cell and its subsequent invasion and also help it evade any immune response (2, 32). The TcMUC proteins appear to be related to the trans-sialidases (TSs), another protein family essential to the invasion process. The TSs transfer sialic acid from the host cell membrane to the parasite's glycoproteins and bind the parasite to the host cell during the invasion process (27, 45). gp82 is another glycoprotein known to be involved in the invasion process; it forms an integral part of the surface of the parasite, where it plays a role in freeing calcium from the intracellular deposits and the disassembly of F-actin (4, 15, 21, 25, 47).

The mucin-associated surface protein (MASP) gene family came to light during a study of the genome of *T. cruzi*; it

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comprises a family of 1,377 genes located among clusters of TS and mucin genes (9). They all have highly conserved N- and C-terminal regions that encode a peptide signal, *N*- and *O*-glycosylated regions, a GPI anchor addition site, and a hypervariable central region (9). Proteomic assays have shown that MASP expression is quite low compared to the number of genes found and that up to 14 genes of the MASP family are expressed simultaneously in the T and/or A stages of *T. cruzi* (7, 19). Some of these proteins have recently been found in the membrane of the trypomastigotes, and members of this family are secreted into the culture medium by the infectious forms obtained from cell cultures, but these proteins have never been characterized (9). MASPs may undergo posttranslational modifications similar to those shown by mucins, which have been identified as *N*-linked glycoproteins (6). Although the mechanism behind the expansion of the MASP family in *T. cruzi* remains unexplained, immune pressure may well constitute the driving force that has given rise to the wide presence of *masp* genes in its genome (9).

We describe here the purification and characterization of an MASP, which we named MASP52, according to the data obtained from its sequence. Our study suggests that it is involved in the invasion of the host cell by the infective forms of *T. cruzi*.

MATERIALS AND METHODS

Cell culture, parasite strain, and parasite culture. Host Vero cells (ECACC 84113001) were incubated at 37°C in a moist atmosphere with 5% CO_2 in 75-cm^2 plastic flasks (Costar) containing Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% (vol/vol) heat-inactivated (30 min at 56°C) fetal calf serum (IFCS; Gibco).

We used the CL-Brener strain of *T. cruzi*. The E forms were cultured at 28°C in MTL medium supplemented with 10% IFCS, as described elsewhere (35). The infective M forms were obtained and purified according to the method described by Osuna et al. (29). Host Vero cells were infected with the M forms as described previously (29). After 96 h of infection, the medium containing the T forms was centrifuged at 300 × *g* for 10 min. The pellet was resuspended in DMEM and centrifuged twice to obtain the T forms. The infected cultures were kept for 8 days, after which A forms were collected and purified by centrifugation in discontinuous Percoll density gradient (1.100, 1.090, 1.080, and 1.070 g/ml) prepared as described elsewhere (13). The purified A forms were collected from the 1.070/1.080-g/ml interphase. Cell debris appeared in the 1.060/1.070-g/ml interphase. All of the forms of the biological cycle of *T. cruzi* used in the assays were at least 95% pure, as tested by Giemsa staining.

Purification and sequencing. MASP52 was isolated and purified from the excretion-secretion product (ESP) during parasite-cell interaction. To this end, the medium was removed from semiconfluent cultures of Vero cells, after which they were washed several times in DMEM without serum (DMEMwS) and infected with a suspension of M forms of the parasite in DMEMwS at 5:1 parasite/host cell ratio, conditions, and infection time as described above. After a 24-h reaction period, the medium was removed and centrifuged at 1,500 × *g* for 10 min at 4°C to eliminate any forms of the parasite that had not penetrated. The supernatant was filtered through a 0.22-μm-pore-size filter (Sartolab 20; Sartorius).

The proteins in the ESP were concentrated with molecular exclusion filters of 5 kDa (Amicon Ultra; Millipore) and then subjected to chromatography through a wheat germ lectin-agarose column (Sigma) to purify the *N*-glycosylated proteins. The wash buffer was 0.1 M carbonate (pH 9), and the same buffer containing 0.5 M *N*-acetylglucosamine (Sigma) was used as the elution buffer. The eluted fraction was chromatographed through a Mono P 5/200 GL column (GE health care) eluted with Polybuffer 74 (GE) as the elution buffer. The fractions between isoelectric point (IP) 5.2 and IP 4.6 were collected. Subsequent purifications were undertaken using immunoprecipitation with magnetic beads (Dynabeads) bound to the polyclonal antibodies (anti-CR) obtained as described below, following the instructions of the bead manufacturers.

All of the chromatographic analyses were undertaken using the fast-protein liquid chromatography Äkta Purifier 10 system (Amersham Biosciences). The results were evaluated by SDS–12.5% PAGE as described below.

MASP52 was sequenced and identified at the Servicio de Proteómica del Centro de Biología Molecular Severo Ochoa (CBMSO) in Madrid, Spain. The relevant band from the SDS-PAGE was excised manually, along with the least possible quantity of gel, and digested automatically *in situ* with a robot digester (Bruker) using trypsin according to a previously described protocol (38). The supernatant from the digestion (containing the peptides) was acidified with trifluoroacetic acid (0.1% final concentration) and dried in a Speed Vac (Thermo) before being resuspended in TA (0.1% trifluoroacetic acid, 33% acetonitrile). A 0.5-ml aliquot was placed on an anchor-chip plate (Bruker), using 2.5-dihydroxybenzoic acid (DHB) as a matrix, to a concentration of 5 g/liter via the "fast evaporation" method. The plate was measured in an Autoflex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Bruker) equipped with a reflector. The mass spectra thus obtained were used as a peptide fingerprint to identify proteins in the database, using search engines available on the internet (Mascot, Profound).

Search for homologies and structural motifs. The homology of the amino acid and nucleotide sequences was studied via the database of the *T. cruzi* genome in *Tcruzi*DB (http://tritrypdb.org/tritrypdb/) using the BLASTP and BLASTN algorithms in GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The search for structural motifs was undertaken via the proteomic tools server of Expasy (http://expasy.org) using the programs Motif Scan (to find structural motifs), SignalP 3.0 (to look for the signal sequence), TMPred (to find transmembrane sequences), and Protscale (to predict the hydrophobicity of our sequence based on the Kyte-Doolittle hydrophobicity scale).

Synthesis of peptides and production of polyclonal antibodies. We designed a peptide corresponding to the catalytic zone of MASP52 (ATP/GTP binding motif A), corresponding to residues 159 to 168 (AEEAAGKT). The designed peptides were synthesized at the CBMSO. The antiserum against the peptide was prepared by intraperitoneal injection of BALB/c mice with 50 μg of peptide linked to keyhole limpet hemocyanin with Freund complete adjuvant as described elsewhere (39), followed by boosters 2 and 4 weeks later with Freund incomplete adjuvant. The mice were bled 2 weeks after the final booster. Test bleed sera were checked by indirect enzyme-linked immunosorbent assay in multiwell plates coated with 10 μg of the synthetic peptide/well in 0.1 M bicarbonate coating buffer (pH 8.6). Sera with titers higher than 1:1,600 were pooled and stored at −80°C until used.

We then purified the IgGs both from the polyclonal serum against the synthetic peptide and from the nonimmunized control mice using the Protein A HP Spin Trap kit (GE Healthcare). The specific IgGs from the mice immunized against the catalytic region was then purified by affinity chromatography using Sepharose (GE) linked to the synthetic peptide. The IgGs were called anti-CR. The proteins were determined by the Bradford method (11).

Cell invasion assay. The M forms obtained were incubated for 30 min at 27°C with a 1:50, 1:100, or 1:200 dilution of specific immunoglobulin anti-CR to MASP52, obtained and purified as described above. The treated forms, together with their media, were used to infect semiconfluent Vero cells (3×10^5 cells/well) at a parasite/cell ratio of 5:1 for 2 h at 37°C in DMEMwS. Immunoglobulins from nonimmunized mice at the same dilution were used as controls. At the end of the interaction period, the cultures were washed, fixed in methanol, and stained with Diff-Quick (Medion Diagnostics, GmbH, Düdingen, Switzerland). After staining, the cells were studied under a light microscope to determine the number of adhering, penetrating and intracellular parasites. We defined adhering parasites as those that were touching the surfaces of the cells and penetrating parasites as those whose kinetoplast zone has already passed through the cell membrane or entered into the cytoplasm of the potential host. A minimum of 500 cells per slide were examined, and each experiment was repeated at least four times.

Excretion/secretion, membrane fractionation, and Western blot analysis. The expression of MASP52 during the different stages of the parasite's life cycle was studied by Western blotting. To this end, 5×10^7 organisms of each stage were resuspended in 2 ml of lysis buffer (20 mM phosphate-buffered saline [PBS], 0.25 mM saccharose, 1 mM EDTA, 0.145 mM KCl, 0.01% NP-40, 1 mM dithiothreitol [DTT]) at pH 7.4 plus Complete Mini inhibitor protease cocktail (Roche Molecular Biochemicals). After treatment for 10 min, the parasites were sonicated at 0°C for three 30-s cycles, adjusted to the same protein concentration, and electrophoresed using SDS–12.5% PAGE (23).

Any secretion of the protein into the culture medium by the M forms was investigated by studying the ESPs of the infective forms of the parasite incubated for 24 h with different treatments, i.e., alone in the media, with live Vero cells, with cell membrane fractions of host cells, or with dead Vero cells, as described below. The ESPs derived from the different interactions were treated by immunoblotting the possible excretion of the MASP52 protein into the interaction media.

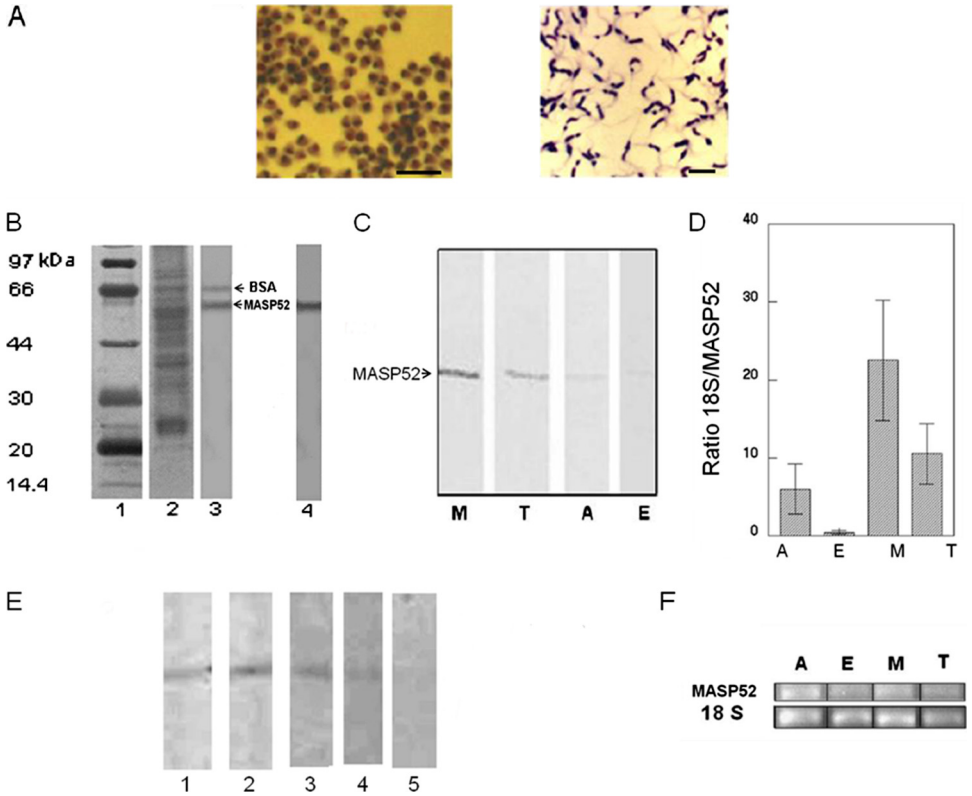

FIG. 1. (A) Purified amastigote and metacyclic trypomastigote forms. Bar, 10 μm. (B) SDS–12.5% PAGE and silver-nitrate staining of the purified proteins of the ESP. Lane 1, marker LMW; lane 2, ESP; lane 3, semipurified fraction; lane 4, MASP52 immunoprecipitated. (C) Western blots of M, T, A, and E forms of *T. cruzi* using anti-CR IgGs. (D) qRT-PCR analysis of the expression of MASP52. To calculate the relative quantity of the MASP52 mRNA transcribed, we compared it to the expression of the ribosomal 18S gene according to the ΔC_T method, as described in Materials and Methods. We measured the relative quantities for the M, T, A, and E stages. (E) Western blots of different samples. Lane 1, hydrophilic fraction of *T. cruzi* membrane; lane 2, ESP of M forms with live Vero cells; lane 3, ESP of M forms with purified membranes of Vero cells; lane 4, ESP of M forms with dead Vero cells, lane 5, ESP of free metacyclic forms. All ESPs were collected after 24 h of incubation. (F) Agarose gel (1%) of the amplicon of the 18S gene and MASP52 from the A, E, M, and T forms of *T. cruzi*.

To obtain ESPs from the interaction between M and dead cells, the cultures were washed with PBS three times and treated for 2 h in a fixative solution composed of PBS 1% glutaraldehyde, washed three times in PBS, treated for 30 min with blocking buffer (20 mM glycine in PBS), and incubated with the M forms in medium without serum, as we have described above for the live cultures,. All of the incubations were performed at 37°C.

To find out whether the protein was secreted after interaction of the parasite with the cell membrane, the M forms were incubated with cell membrane suspensions. These were obtained from a Vero cell culture (1.5×10^7) separated from the surface of the flask by treatment with 0.02% PBS-EDTA. They were then centrifuged at $300 \times g$ for 10 min and treated by hypotonic shock in a Tris-HCl buffer (pH 8) with 2 mM EDTA–1 mM DTT before being lysed in a homogenizer for 30 s at 0°C. Cell breakage was checked under a light microscope, and the membranes were concentrated by centrifugation at $10,000 \times g$ for 10 min at 4°C. After repeated washing using centrifugation, the pellet containing the membranes was purified by centrifugation at $10,000 \times g$ in a 0.5 M sucrose solution at 4°C. The membranes, found in the interphase between the buffer and the saccharose, were washed by centrifugation repeatedly at $10,000 \times g$ in PBS at 4°C and resuspension in DMEMwS culture medium, in which the parasite-membrane interaction took place, as described above.

To obtain the hydrophilic fraction of the M membrane, we used the method described by Atayde et al. (4).

The protein samples secreted and those deriving from the different forms of the parasite were adjusted to the same concentration prior to electrophoretic separation and immunoblotting so that all of the electrophoresis lanes contained the same quantity of proteins (18 μg per line). All of the samples were subject to SDS–12.5% PAGE using Miniprotean 3 (Bio-Rad). After electrophoresis the separated proteins were transferred to nitrocellulose membrane (Hybond-C Extra; GE Healthcare) according to the method described by Tyler et al. (43). Blots were exposed to anti-CR (tested at 1:50) for 2 h at 37°C, followed by peroxidase-conjugated (polyclonal goat anti-mouse immunoglobulin-HRP [Dako Cytomation]) for 2 h at 37°C. The reaction was developed with 3-3′diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl buffer (pH 7.2). To quantify protein expression in the different forms of the parasite, the gels were digitized and processed by using Quantity One software (Bio-Rad).

mRNA isolation and qRT-PCR. Total RNA of the different parasite stages was isolated with a SV total RNA isolation kit (Promega). For reverse transcription (RT), we used an iScript cDNA synthesis kit (Bio-Rad), which contains random primers, to obtain as representative an RNA mold as possible. The expression of MASP52 was quantified by quantitative RT-PCR (qRT-PCR) using the Sensimix dT kit (Quantace). Expression levels were quantified for all of the stages of the parasite's life cycle studied. The primers MASP.220F (5′-CCA GTT GCG AGA TTG AAG GT-3′) and MASP.220R (5′-TGC AGA TGC TTC AAC TGC TG-3′), which give rise to an amplicon of 198 bp belonging to the hypervariable central zone of the *masp*52 gene (GenBank accession number XP_820015.1), were used throughout the present study. To normalize the quantity of *masp*52 cDNA present in each sample, we used the primers V1 (5′-CAA GCG GCT GGG TGG TTA TTC CA-3′) and V2 (5′-TTG AGG GAA GGC ATG ACA CAT GT-3′) for the 18S rRNA ribosomal gene (14) of *T. cruzi*, thus obtaining an amplicon of 179 bp (GenBank accession number X53917). The PCR bands were confirmed by sequencing with a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). The samples were quantified according to the ΔC_T method, in which the 18S/MASP52 ratio = $2^{CT\ 18S\ -\ CT\ MASP52}$. All of the assays were carried out in triplicate.

Immunocytochemistry and immunofluorescence. The protein was located in the various forms of the life cycle of *T. cruzi*, obtained as described above. The different pellets containing the four forms were washed three times with 5 ml of 0.125 M PBS and fixed for 12 h at 4°C in 1% glutaraldehyde and 2% formalde-

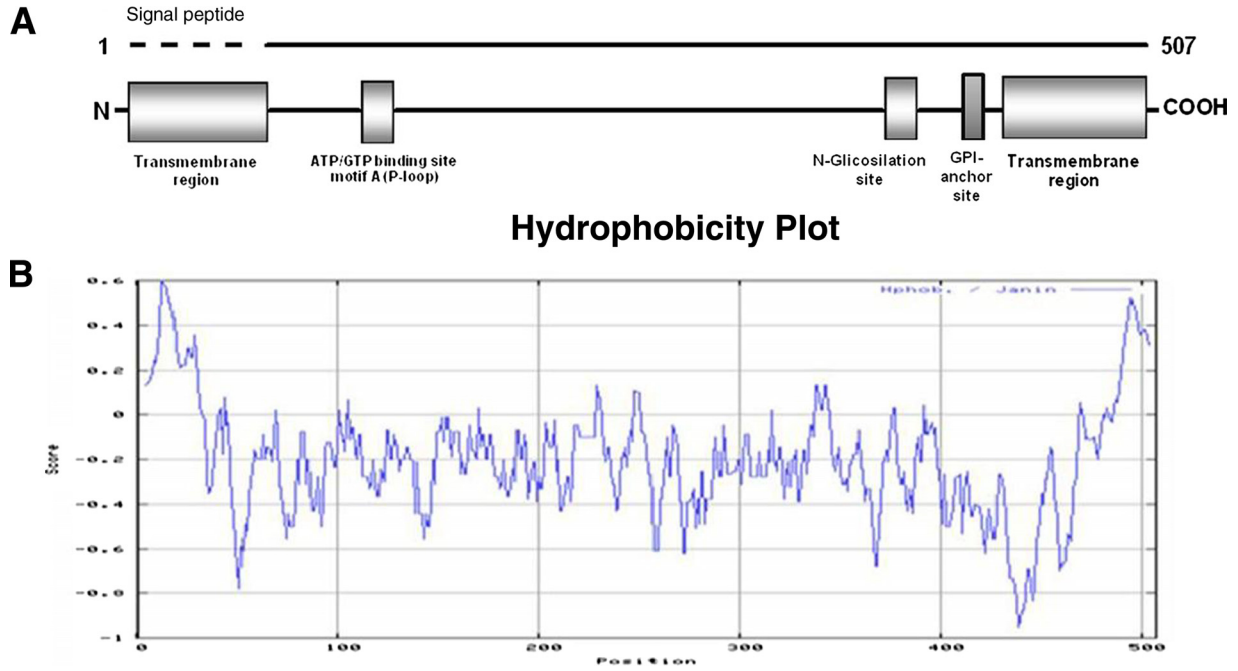

FIG. 2. (A) Structural motifs and their compositions determined using the Expasy proteomic tools server (http://expasy.org). There are two transmembrane regions at the N and C terminals, a peptide signal (MAMMMTGRVLLVCALCVLWSVAADG) from amino acids 1 to 25, ATP/GTP binding-site motif A (P-loop; AEEAAGKT) from amino acids 159 to 166, an *N*-glycosylation site (ANGTNATAI) from amino acids 465 to 473, and a GPI anchor site from amino acids 482 to 484. (B) Hydrophobicity plot for MASP52 according to the Kyte-Doolittle hydrophobicity scale.

hyde in cacodylate buffer with 0.1 M saccharose (pH 7). They were then embedded in LRWhite resin, sliced, and incubated for 1 h at 37°C with the anti-CR IgGs at a dilution of 1/30 in blocking buffer (PBS [pH 7.2], 1% bovine serum albumin [BSA]). As a secondary antibody, we used an anti-mouse coupled to 10-nm gold particles (Sigma) incubated at a dilution of 1/125 for 1 h at 37°C. A negative control was made by incubation under the same conditions with serum from the nonimmunized mice. Finally, ultrathin sections were stained with uranyl acetate and examined under a Zeiss electron microscope (model 6EM902).

In the immunofluorescence experiments, both the parasites and the Vero host cells were fixed with 2% formaldehyde for 10 min and then permeabilized with 0.1% Triton X-100. The parasites were fixed during the various stages of their life cycle and also during the process of recognition and entry of the M forms into the cells so as to observe the presence and location of MASP52 in the A forms and in the infected cells. Once they were fixed and washed with PBS, the different preparations were incubated for 30 min in blocking buffer (PBS [pH 7.2], 1% BSA) and then treated for 1 h with the anti-CR IgGs at a dilution of 1/100 in blocking buffer at room temperature. As a secondary antibody, we used an anti-mouse labeled with fluorescein isothiocyanate (Sigma) diluted in blocking buffer according to the manufacturer's recommendations, to which the preparations were also exposed for 1 h at room temperature. To stain the DNA, the samples were finally treated for 15 min in a 10-μg/ml PBS solution of DAPI (4′,6′-diamidino-2-phenylindole). The samples were then preserved and mounted in mounting medium (Prolong antifade kit; Molecular Probes) and examined under a Leica DMI6000 confocal laser microscope.

Adsorption of MASP52 onto bentonite particles. The size of the bentonite particles and the adsorption of MASP52 were chosen as described previously (22). A 100-μl suspension of particles ranging in diameter from 1 to 2 μm incubated for 12 h at 4°C with 50 μl of a 20-μg/ml solution of protein (BSA) adsorbed onto bentonite was used as a control at the same concentration and under the same conditions as those described above. The bentonite particles coated with either MASP52 or BSA were incubated with Vero cells (3×10^5) at 37°C for 4 h. The cells were then washed with PBS and fixed in acetone for study by fluorescence microscopy. The fixed preparations were treated with a 1:100 dilution of the anti-CR antibody and with the secondary anti-mouse antibody labeled with fluorescein, as described above. In the control cells, which were incubated with BSA-coated bentonite, we used an anti-BSA polyclonal anti-mouse serum (Sigma) at the same dilution and incubation time prior to incubation with the same secondary antibody. We used a 0.01% solution of Evans blue in carbonate buffer (pH 8.6) as a counterstain and studied the samples under a confocal fluorescence microscope, as described above.

Statistical analysis. A Bonferroni test was used to estimate the significance of the difference between means. The results are indicated as mean values (standard errors of the mean of the different groups at different times for each experiment performed were determined). All of the experiments were repeated three times; $P \leq 0.001$ was considered to be extremely significant. GraphPad Instat v3.05 software was used for the statistical testing.

RESULTS

Purification, expression, sequencing, and search for structural motifs in MASP52. The two bands obtained between IP 5.2 and IP 4.6 (66 and 52 kDa, respectively) (Fig. 1B) were analyzed by MALDI-TOF-MS and tandem mass spectrometry (MS/MS) to reveal that the 66-kDa band corresponded to BSA deriving from the remains of the culture medium. The 52-kDa band had an IP of 4.75, and when we undertook peptide mass fingerprinting with the Mascot program, we found six matches, with a score of 47.9 and 17% coverage for the sequence (see Fig. S1 in the supplemental material). This sequence appears in the databases as an MASP (EMBL accession number XP_820015.1), and we have named it MASP52.

An analysis by SDS-PAGE of the expression of MASP52 during the different stages of *T. cruzi* (Fig. 1A) and its subsequent analysis by Western blotting using anti-CR IgGs revealed that the protein was recognizable as a single band and showed different levels of expression during the various stages of the parasite's life cycle (Fig. 1C). The expression in M was 12-fold higher than in E, 5-fold higher than in A, and twice as high as that found in T. A study of mRNA by qRT-PCR

showed similar results. All of the stages transcribed different levels of mRNA specific to MASP52. Transcription in the M forms was 3-fold higher than in T, 11-fold higher than in A, and 50-fold higher than in E (Fig. 1D). Analysis of the ESP revealed that MASP52 was secreted during interaction between the M form and Vero cells, the membrane fraction of these cells, and fixed dead cells (Fig. 1E). We found no secretion whatsoever in the secretion media of M forms (Fig. 1E). Western blots of the hydrophilic fraction of membrane from the M forms (Fig. 1E) revealed the presence of MASP52.

We then undertook a search for possible structural motifs in MASP52 using the Expasy proteomic tools server (http://expasy.org). We obtained a peptide signal of 1 to 25 amino acids (MAMMMTGRVLLVCALCVLWSVAADG), an ATP/GTP binding site A-motif (P-loop) which may correspond to the catalytic center (amino acids 159 to 166) with the sequence AEEAAGKT, a *N*-glycosylation site (amino acids 465 to 470) with the sequence ANGTNA, and a GPI anchor site (amino acids 482 to 484) with the sequence DGS (Fig. 2). We also found a repetitive sequence characteristic of this type of proteins (amino acids 106 to 132 and amino acids 171 to 197) with the sequence EASAKAAEAAAKAKTLAETAETATEAA.

Location of MASP52 in the different life cycle stages of *T. cruzi* and adsorbed onto bentonite particles. Confocal laser microscopy studies with anti-CR IgGs revealed the presence of MASP52 both in the cytosol and bound to plasma membrane in all of the various stages (Fig. 3). In cells that had transformed and multiplied as A forms, MASP52 surrounded the intracellular A forms between the parasite and the cytoplasm of the host cell (Fig. 4A; see also Fig. S2 in the supplemental material).

A study of the immunolocation of MASP52 during the interaction of the M forms with the host cells led to its detection in the cytosol of the trypomastigotes and at the point of contact between the parasite and its target host cell. There was also some dispersed fluorescence in the cell cytoplasm in the vicinity of the parasite-cell interaction (Fig. 4B).

Ultrastructural studies in the M, T, and E parasite stages using anti-CR IgGs showed how the gold labels were located in the parasite membrane, either dispersed or in clusters in the M and T forms (Fig. 5B and D). These clusters tended to be situated inside vacuoles (Fig. 5B and C) in the flagellar pocket and root of the flagellum in the E and T forms (Fig. 5A and C).

Confocal laser microscopy studies of the interaction between MASP52 adsorbed onto inert particles of bentonite and cultures of nonphagocytic Vero cells and BSA-coated bentonite particles, using anti-CR IgGs or anti-BSA antibodies as control, revealed that the cells endocytosed the MASP52-coated particles, whereas the BSA-adsorbed control particles remained free and were eliminated in the washes after the interaction process, as could be seen by the immunofluorescence when they were revealed with anti-BSA antibodies (Fig. 6). As shown in the orthogonal projection of images, the MASP52 particles were located inside vacuoles in the cytoplasm of the cells that had been invaded (Fig. 6).

Effects of MASP52 antiserum on the invasion of host cells by *T. cruzi*. Figure 7 summarizes the results of treating parasite-cell interactions with anti-CR in the presence of different dilutions (1:50, 1:100, and 1:200) of specific immunoglobulin. The percent inhibition compared to the controls was significant, ranging from 17.14% for the 1:200 dilution to 61.9% for the 1:100 dilution to 77.14% for the 1:50 dilution for intracellular parasites in Vero cells. The levels of penetration fell by 30.7% for the 1:200 dilution, 74.38% for the 1:100 dilution, and 78.9% for the 1:50 dilution, and the percent inhibition of M adhering to cells fell by 49.92% for the 1:200 dilution, 74.35% for the 1:100 dilution, and 74.48% for the 1:50 dilution (Fig. 7).

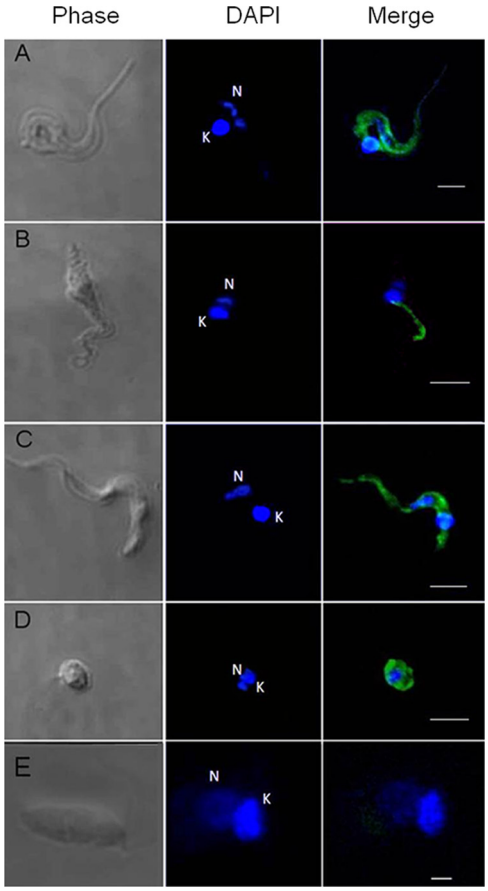

FIG. 3. Confocal laser microscope images of the location of MASP52. We used anti-CR IgGs (green) and DAPI to label the nuclei (blue). The images show different stages of the parasite as follows: trypomastigotes from cell cultures (A), epimastigotes (B), metacyclic trypomastigotes (C), amastigotes (D), and negative control (E). Bar, 2 μm. N, nucleus; K, kinetoplast.

DISCUSSION

The MASP gene family (~1,300 genes) accounts for ca. 6% of the total parasite diploid genome (9). Nevertheless, only 14 MASP proteins have been identified to date by proteomic identification made via a single peptide match (5, 7, 19), which suggests that this gene family either does not express itself as abundantly as the TS proteins or that they undergo posttranscriptional modifications, as do the mucins, which makes their detection difficult by shotgun proteomics. Subsequent sequencing and proteomic approaches to MASP52 resulted in seven matches, which corresponded to a putative MASP, with the EMBL accession number XP_820015.1 (see Fig. S1 in the

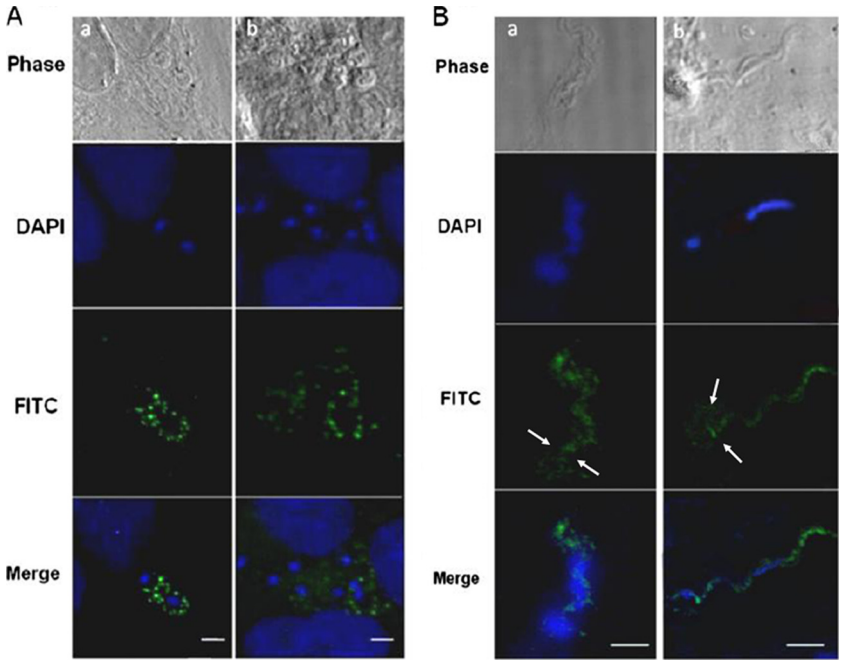

FIG. 4. (A) Labeling of cells infected with amastigotes secreting protein into the cytoplasm (bar, 5 μm). (B) Labeling of metacyclic trypomastigotes interacting with the host cell. The moment of cell-parasite interaction can be seen. (a) bar, 5 μm; (b) bar, 10 μm. The arrows indicate the interaction point between the parasite metacyclic forms and the cultured cells.

supplemental material). This then is the first of the MASP proteins to have been isolated and purified in its native state.

MASP52 was purified from the interaction medium between the infectious M forms and prospective host cells by affinity chromatography with WGA lectin. That the protein showed affinity for these lectins confirms that it was glycosylated with *N*-acetylglucosamine terminal residues capable of binding to lectin (26). In accordance with this, we deduced that the sequence of the purified protein contained an *N*-glycosylation site with the sequence ANGTNA, corresponding to the posi-

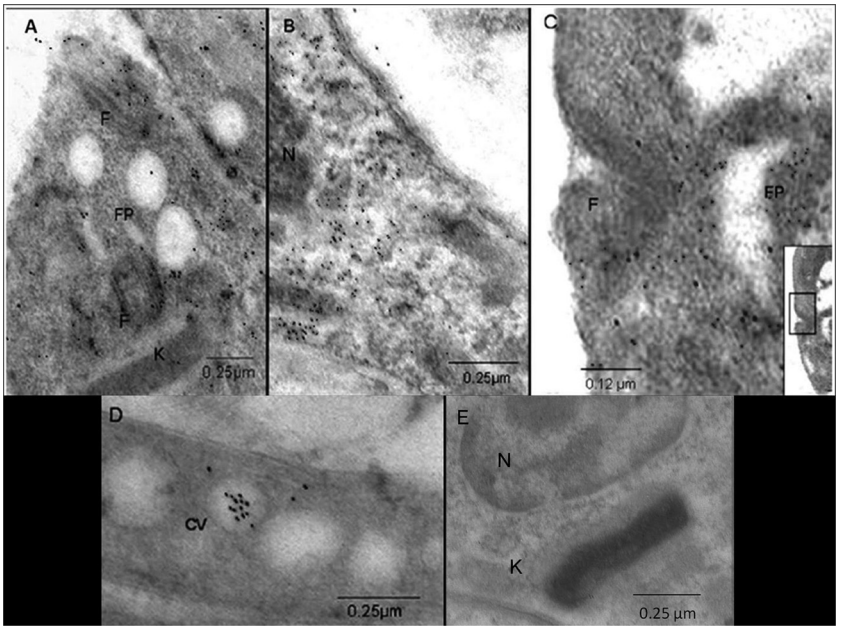

FIG. 5. Immunocytochemistry of MASP52 using anti-CR IgGs labeled with gold (10 nm). (A) Epimastigote IgG located in flagellar pocket; (B) trypomastigotes from cell cultures located in the membrane, cytosol, and outside the cell; (C) flagellar pocket and root of a cell culture-derived trypomastigote; (D) cytosolic vesicles of a metacyclic trypomastigote; (E) negative control. K, kinetoplast; F, flagellum; FP, flagellar pocket; CV, cytoplasmic vesicles; N, nucleus.

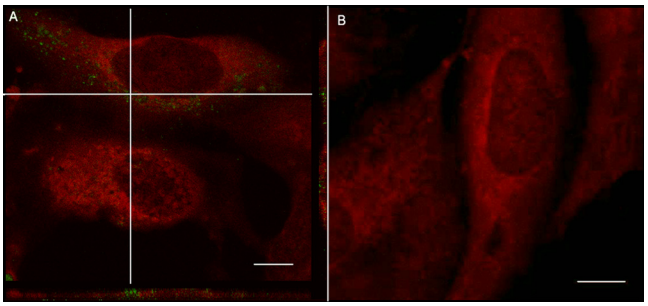

FIG. 6. Bentonite particles coated with MASP52 observed under a confocal microscope in orthogonal projection (A) and BSA as control (B) after 4 h of interaction. The particles were located using anti-CR IgG antibodies and an anti-BSA polyclonal serum (bar, 10 μm).

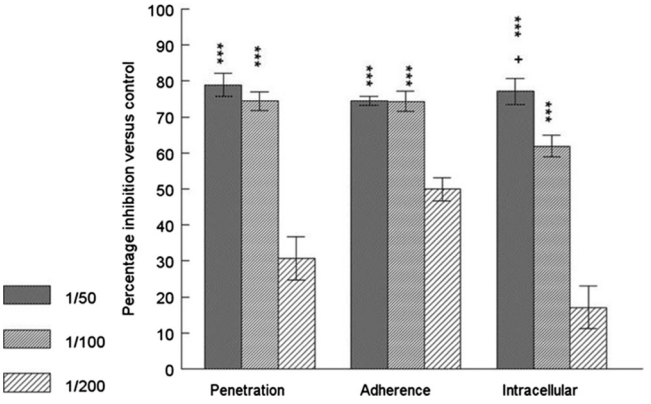

FIG. 7. Inhibition of the invasion of the Vero cells by metacyclic trypomastigotes caused by IgG antibodies to the catalytic region of MASP52. The anti-CR IgG dilutions (1:50, 1:100, and 1:200) were incubated for 30 min with the metacyclic trypomastigotes. The parasites were then washed and left to interact with Vero cells for 2 h. The bars represent the means ± the standard error of three experiments. Asterisks indicate $P \leq 0.001$ versus 1/200; a plus sign indicates $P \leq 0.05$ versus 1/100 by as determined by the Bonferroni test.

tions of amino acids 465 to 470 (Fig. 2A). There was also an ATP/GTP binding-site motif A (P-loop), with the sequence AEEAAGKT (consensus pattern [AG]-x(4)-G-K-[ST]) situated between positions 159 and 166 (Fig. 2A), which is a catalytic center related to a powerful enzyme activity. The results of analyses performed using the TritrypDB database (http://tcruzidb.org/tcruzidb/) revealed that this sequence is only found in MASP52 within the *T. cruzi* genome.

In a similar way, a study of the MASP52 sequence indicates that it contains an extremely hydrophobic C-terminal transmembrane region and GPI anchor site (Fig. 2A), a zone that is highly conserved in the MASP family (9), which would allow it to anchor to the membrane. This anchorage to the membrane is confirmed by the presence of MASP52 in the protein fraction of the hydrophilic membrane of the M forms (Fig. 1E), as shown by Western blotting and immunological studies after recognition with the antibody directed against the catalytic center (anti-CR IgGs) (Fig. 4 and 5). Immunochemical studies confirm the location of MASP52 in the plasma membrane and cytosol of all of the parasite stages studied. Some authors, using proteomic techniques, have identified the presence of other proteins belonging to the MASP family in the plasma membrane of *T. cruzi* trypomastigotes (9, 19), which supports our finding of one of these proteins on the surface of the parasite.

According to our quantification of the protein's expression via Western blotting (Fig. 1C) and quantification of its mRNAs by qRT-PCR (Fig. 1D), MASP52 is expressed in all of the various stages of the life cycle of *T. cruzi*, although at different levels. The M and T forms are those that show the highest levels of mRNA and protein expression, the A somewhat less, and the amastigote forms only basal levels. The fact that the protein is expressed most highly in the infective forms of the parasite points to its possible role in the invasion process into the host cell. Within this context, other authors have also described the higher expression of members of the MASP family in the T stage of *T. cruzi* (6, 7).

Some of the electron-microscope immunolocation images show MASP52 clusters inside vacuoles and in the flagellar pockets of the M forms (Fig. 5A and C). The MASP proteins have been described as being anchored to the membrane (9, 18), but more recently it has also been recognized that they are secreted into the medium (9). Our results confirm this latter idea that MASP proteins are secreted by the M forms during the process of interaction between the parasite and the host cell. The absence of MASP52 in the cell-free incubation media of the M forms would seem to confirm that the secretion of the protein by the M forms is induced by contact between the parasite and cell membranes. This secretion is mediated by *T. cruzi* rather than the host cell, as is confirmed by our observations that MASP52 is secreted when the parasite is exposed to fixed dead mammalian cells (Fig. 1E).

Fluorescence imaging shows how MASP52 appears at the point of contact between the M form and the host cell and that the protein enters into the cell during the internalization process just before or at the moment of invasion (Fig. 4B). The fluorescence distribution patterns observed in the A forms vary according to whether they are intracellular A forms present in the infected cells or purified free forms. In the latter case, MASP52 is situated in the cytosol of the parasite membrane (Fig. 3), whereas with intracellular amastigotes it is situated in the cell cytoplasm surrounding the parasite. Although some *T. cruzi* proteins, such as TcTox (3) and TS (24), have been identified as playing a part in the infection and liberation process from the cells in which A forms have multiplied, we are still unaware whether MASP52 plays a similar role in the transformation mechanism of amastigote to trypomastigote and the rupture of the cell to release the parasites into the medium.

The capacity of *T. cruzi* to invade and survive in a wide variety of cell types distinguishes it from other kinetoplastid protozoan parasites. The specificity of this family for *T. cruzi*, the diversity of the *masp* genes, and their expression by the infective stages of the parasite, together with the existence of hypervariable regions, all lead us to believe that some of the proteins encoded by these genes may well be involved in the invasion process and establishment of the parasite within the host cell (9, 18). Just as the TS, gp82, Tc-1, and POPTc80 proteins have been identified through the use of antibodies as being essential to cell invasion (5, 8, 10, 36, 45), the results of our assays using specific antibodies against the catalytic region

demonstrate the participation of MASP52 in the invasion of the host cell by *T. cruzi* (Fig. 7) by reducing significantly the percentages of parasitization. To corroborate the participation of MASP52 in the invasion process and ascertain whether the results obtained with the antibodies could be due to some indirect effect brought about by the antibodies themselves, we adsorbed the native protein onto inert particles of bentonite and confirmed that the bentonite coated with MASP52 was located in the cytosol of the nonphagocytic cells used in our assays. It can be seen in Fig. 6 that after 4 h of interaction with the cells the MASP52-coated bentonite was recognized by the anti-CR IgGs, whereas particles coated with BSA did not fluoresce after treatment with anti-BSA antibodies, all of which suggests that MASP52 participates in an active endocytic process on the part of the cell similar to the invasion of the M forms into the host cell.

This evidence, together with the higher mRNA expression in the infective stages of the parasite and the inhibition of cell invasion capacity by antibodies against the catalytic center of the protein, indicates that MASP52 may well be one of the proteins involved in the process of infection by *T. cruzi*.

Our work provides the first evidence of different levels of expression of a MASP protein in the various forms of *T. cruzi* and is thus the first indication of the role played by such a protein in the parasite's life cycle. We are currently studying the molecular mechanisms by which the protein acts to enhance the parasite's entry into nonphagocytic cells.

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