Localization and Developmental Regulation of a Dispersed Gene Family 1 Protein in *Trypanosoma cruzi*†‡

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The dispersed gene family 1 (DGF-1) is the fifth largest gene family in the *Trypanosoma cruzi* genome, with over 500 members (11). Many of the predicted DGF-1 protein products have several transmembrane domains and N-glycosylation and phosphorylation sites and were thought to localize in the plasma membrane. Here, we report that affinity-purified antibodies against a region of one of these proteins (DGF-1.2) localized it intracellularly in different stages of the parasite. DGF-1.2 is more abundant in the amastigote stage than in trypomastigotes and epimastigotes, as detected by immunofluorescence and Western blot analyses. The protein changed localization during intracellular or extracellular differentiation from the trypomastigote to the amastigote stage, where it finally localized to small bodies in close contact with the inner side of the amastigote plasma membrane. DGF-1.2 did not colocalize with markers of other subcellular organelles, such as acidocalcisomes, glycosomes, reservosomes, lipid droplets, or endocytic vesicles. During extracellular differentiation, the protein was detected in the culture medium from 0 to 22 h, peaking at 14 h. The presence of DGF-1.2 in the differentiation culture medium was confirmed by mass spectrometry analysis. Finally, when epimastigotes were subjected to starvation, there was a decrease in the labeling of the cells and, in Western blots, the appearance of bands of lower molecular mass, suggesting its cleavage. These results represent the first report of direct immunodetection and developmental expression and secretion of a DGF-1 protein.

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*Trypanosoma cruzi* is the causative agent of Chagas disease, an endemic illness affecting between 16 and 18 million people in North, Central, and South America for which no vaccine or satisfactory treatment is available (22). During its life cycle, the parasite goes through different stages in the vector (epimastigotes and metacyclic trypomastigotes) and in the mammalian host (amastigotes and bloodstream trypomastigotes). As part of its survival strategy in these varying environments, the parasite has developed a large repertoire of multigene families (9, 11, 12, 16). Among these families, the dispersed gene family 1 (DGF-1) has approximately 565 copies, ranging from 6 to 10 kb, dispersed throughout the parasite genome (11). The members of the DGF-1 family encode proteins that share 85 to 95% sequence identity (11). Wincker and colleagues first identified clones bearing a common repeated sequence from a *T. cruzi* genome library (24) and later described the nucleotide sequence of a representative gene (DGF-1.1) (23). They concluded, from in silico studies, that DGF-1 genes encoded putative cell surface proteins (23). In 2005, Kim and colleagues (16) described a new member of this family (DGF-1.2) located in the subtelomeric region of a *T. cruzi* chromosome surrounded by mainly two kinds of sequences: genes encoding the trans-sialidase (TS) and retrotransposon hot spot (RHS) protein families. The sizes of the open reading frames (ORFs) of DGF-1 genes and their abundance in the *T. cruzi* genome suggested that they are essential sequences for parasite survival. Furthermore, the existence of some telomeric DGF-1 copies that were always flanked by pseudogenes suggested that these genes have been subjected to strong selective pressure and, as a consequence, that they should be expressed at some life cycle stage of the parasite (16).

A glycopeptide shared by several members of the DGF-1 family was recently detected in a glycoproteomic study of *T. cruzi* trypomastigotes, demonstrating that at least a DGF-1 family member protein is actually expressed and N-glycosylated (3). We also detected a number of peptides corresponding to several DGF-1 family member proteins in a proteomic study of acidocalcisome fractions of epimastigotes of *T. cruzi* (R. Docampo, J. A. Atwood, R. Tarleton, and R. Orlando, unpublished data). However, this family of proteins has no known orthologs in other species, even in trypanosomatids, and little is known about their localization, expression patterns, and functions in *T. cruzi*.

In the present work, we prepared affinity-purified antibodies against a peptide of the DGF-1.2 protein and investigated its subcellular localization by immunofluorescence microscopy. We also used mass spectrometry (MS) to identify specific peptides recognized by anti-DGF-1.2 antibodies by using fingerprinting analysis. We found that the antibodies preferentially labeled amastigotes. The localization of the protein was in intracellular bodies and not on the cell surface and changed during amastigote development. During the in vitro trypomastigote-to-amastigote transition, we detected continuous secretion of DGF-1.2...
into the medium, peaking at 14 h. Anti DGF-1 antibodies that recognized the intracellular protein in both differentiation forms also recognized the secreted protein from trypanomastigotes and amastigotes. Finally, when epimastigotes were subjected to starvation, there was a decrease in labeling of the cells and the appearance of defined bands of smaller molecular mass in Western blots, suggesting its cleavage.

MATERIALS AND METHODS

Materials. Anti-Trypanosoma brucei pyruvate phosphate dikinase (TbPPDK)-producing mouse hybridoma culture supernatant was a gift from Frederique Bringaud (University of Bordeaux, Bordeaux, France); anti-T. brucei vacuolar pyrophosphatase (TVP1) was a gift from Norbert Bakala (École Normale Supérieure de Chimie de Montpellier, France); anti-T. cruzi serine carboxypeptidase (TeSCP) was a gift from Vanina Álvarez (University of General San Martín, Argentina). Alexa Fluor 488-conjugated goat anti-rabbit antibodies, Alexa Fluor 546-conjugated goat anti-mouse antibodies, fluorescein isothiocyanate-bovine serum albumin (FITC-BSA), tetramethyl rhodamine isothiocyanate-concanavalin A (TRITC-ConA), dinitroctyl triphosphate (dNTP) mix, Bodipy 493/503, and Escherichia coli BL21(DE3) pLysS cells were from Invitrogen. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies were from Santa Cruz Biotechnology. Tag DNA polymerase in storage buffer B, magnesium-free 10× PCR buffer, restriction enzymes, and Wizard PCR Prep DNA Purification System were from Promega. The Set VII protease inhibitors and DNase I were from Calbiochem. Newborn and fetal bovine sera, Dulbecco's phosphate-buffered saline (PBS), Dulbecco's Hank's solution, 4,6-diamidino-2-phenylindole (DAPI), anti-α-tubulin monoclonal antibody, and other protease inhibitors and reagents were from Sigma. The bichromatic acid (BCA) Protein Assay Reagent and the enhanced chemiluminescence (ECL) detection kit were from Pierce Biotechnology. The pGEX-5X-2 vector was from Amersham Biosciences.

Culture methods. T. cruzi epimastigotes (strain Y) were grown at 28°C in liver infusion tryptose (LIT) medium (7) supplemented with 10% heat-inactivated newborn bovine serum. T. cruzi amastigote and trypanomastigote forms (strain Y) were collected from the culture medium of infected myoblasts (L6/E6 cell line), using a modification of the method of Schmutz and Murray (19), as described previously (10).

Sequence analysis. A teleomeric copy of DGF-1 (DGF-1.2; GenBank accession number AY551440) from T. cruzi strain CL Brener was used in this work. Sequence analysis and primer design were performed using DNAMAN v. 5.2.2 software (Lynnon BioSoft). Prediction of signal peptides was done with SignalP 3.0 software (Technical University of Denmark).

PCR and cloning. A recombinant 915-bp fragment (RE1; nucleotides [nt] 2638 to 3553) (Fig. 1) was amplified from a bacterial artificial chromosome (BAC) DnC recombinant (8) using the following primers: forward, 5′-GAT CGG ATC CGT GGT CTG TGC TTT GTC AAC-3′; and reverse, 5′-GTA CGT CCA CTA CGA CAC CTC CTC ATC GT3′- (BamHI and Sall restriction sites are in boldface). PCR was performed by mixing 10 to 20 ng of DNA, 1.5 mM MgCl2, 10× MgCl2-free buffer (10 mM Tris HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 200 μM dNTP mix, 0.2 μM forward and reverse primers (Bioneer), and 4 μl of Tag DNA polymerase in storage buffer B in a final volume of 100 μl. PCR was carried out in a PTC 200 thermocycler (MJ Research) under the following conditions: initial denaturation at 95°C for 5 min. 30 amplification cycles (94°C for 30 s, 50°C for 30 s, and 74°C for 30 s), and a final extension cycle at 74°C for 10 min. The PCR product size was confirmed by agarose gel electrophoresis and then purified using a Wizard PCR Prep DNA Purification System following the manufacturer’s instructions. For expression of the recombinant fragment fused to Schistosoma japonicum glutathione S-transferase (GST), the PCR product was cloned into the pGEX-5X-2 vector through its BamHI and Sall restriction sites. For expression of the RE1 peptide, the molecular construct was transformed into E. coli BL21(DE3) pLysS cells.

Expression and purification of RE1 recombinant protein. Bacteria harboring the recombinant RE1 fragment were grown in Luria-Bertani (LB) medium supplemented with 30 μg ml−1 chloramphenicol and 100 μg ml−1 ampicillin, and RE1 protein expression (amino acids [aa] 880 to 1177 from DGF-1.2 deduced protein) was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Thereafter, the cells were incubated for 6 h at 37°C. For protein extraction, cells were collected by centrifugation; washed once in PBS, pH 7.4; resuspended in lysis buffer (10 mM imidazole, 0.2% Triton X-100 in PBS) plus Set VII protease inhibitors and 1 U ml−1 DNase I; and then incubated for 30 min at 4°C. After centrifugation of the lysate at 10,000 × g for 10 min at 4°C, the recombinant protein was recovered from the pellet and its expression was confirmed by SDS-PAGE. Finally, the protein was purified from acrylamide strips by passive dialysis using elution buffer (50 mM NaHCO3, 0.1% SDS, and protease inhibitors) under constant shaking for 24 h at 4°C. The purified fractions were reanalyzed by SDS-PAGE (mass, ~57 kDa). The same procedure was performed for GST purification.

Antibody generation and purification. Anti-DGF-1.2 polyclonal antibodies were obtained by immunizing New Zealand rabbits with four doses (15 days apart) of the purified RE1 recombinant protein. Each rabbit received a first dose consisting of 200 μg of antigen with Freund's complete adjuvant (1:1) and another three doses of 100 μg each with Freund's incomplete adjuvant. After the rabbits were bled, the sera were separated by centrifugation at 3,000 × g, and the pellet was discarded. Anti-DGF-1.2 serum was affinity purified using a protein G-Sepharose column and then by immunoadsorption to the RE1 recombinant protein immobilized on nitrocellulose strips. The adsorbed antibodies were recovered with 0.1 M glycine, pH 2.5, and neutral pH was restored immediately by adding 1 M Tris-HCl buffer, pH 8.0.

Western blot analysis. The three stages of the T. cruzi life cycle (epimastigotes, trypanomastigotes, and amastigotes) were separately harvested, washed twice in PBS, and resuspended in PBS containing 1% SDS and protease inhibitors (1 μg ml−1 aprotinin, 1 μg ml−1 leupeptin, 1 μg ml−1 pepstatin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were lysed by five cycles of freezing and thawing, and the DNA was mechanically fragmented by passing the cell lysates through a 20-gauge needle five times. The protein concentration was estimated by spectrophotometry, using the BCA Protein Assay Reagent. Twenty micromgrams of protein from each total cell lysate was mixed with 2× Laemml sample buffer, boiled for 5 min, and analyzed by SDS-PAGE in gradient gels (4 to 10%). The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad) using a Bio-Rad transblot apparatus. The membranes were blocked with 5% nonfat dried skim milk in PBS (PBS containing 0.1% [vol/vol] Tween 20) overnight at 4°C. The blots were incubated with the purified rabbit anti-DGF-1.2 polyclonal antibody at a dilution of 1:1,000 overnight at 4°C or with an anti-α-tubulin monoclonal antibody at a dilution of 1:15,000 for 1 h at room temperature. After five washes with PBS, the blots were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG antibody at a dilution of 1:15,000 for 1 h. After washing five times with PBS, the immunoblots were visualized using the ECL detection kit according to the instructions of the manufacturer.

Fluorescence microscopy. For immunofluorescence assays (IFA), the parasites were sedimented by centrifugation at 1,700 × g for 10 min at 25°C, washed twice with PBS, and fixed with 4% paraformaldehyde in PBS for 1 h at 4°C. After being washed with PBS, the parasites were adhered to poly-L-lysine-coated coverslips, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with PBS containing 3% BSA, 1% fish gelatin, 50 mM NaHCO3, and 5% goat serum for 1 h. The
cells were incubated for 1 h at room temperature with the polyedral rabbit anti-DGF-1.2 antibody (1:200) and/or mouse anti-DGF-1.2 antibodies as described above. Protein bands of interest were excised from SDS-PAGE gels and placed in a tube for (i) reduction with 10 mM DTT, (ii) alkylation with 55 mM iodoacetamide (IAA), (iii) overnight tryptic digestion (6 ng/μl enzyme), and (iv) peptide extraction with 1% formic acid in 2% acetonitrile (ACN), and samples were loaded onto a matrix-assisted laser desorption ionization (MALDI) plate using 1 μl of the tryptic digests mixed in a 1:1 proportion with a solution of matrix (a saturated solution of 2-nitro-4-cyanocinnamic acid in 0.1% TFA). Protein identification by mass spectrometry. Protein bands of interest were excised from SDS-PAGE gels and placed in a tube for (i) reduction with 10 mM diithiothreitol (DTT), (ii) alkylation with 55 mM iodoacetamide (IAA), (iii) overnight tryptic digestion (6 ng/μl enzyme), and (iv) peptide extraction with 1% formic acid in 2% acetonitrile (ACN), and samples were loaded onto a matrix-assisted laser desorption ionization (MALDI) plate using 1 μl of the tryptic digests mixed in a 1:1 proportion with a solution of matrix (a saturated solution of a matrix of 1-cyano-4-hydroxy-cinnamic acid in 0.1% TFA). A ProTOF 2000 matrix-assisted laser desorption ionization–orthogonal time-of-flight (MALDI O-TOF) mass spectrometer (PerkinElmer Science) was used for peptide analysis. Calibration was performed using a commercial mixture of peptides with known molecular masses, PepMix (PerkinElmer Science). TOF-works 1.0 software (PerkinElmer Science) was used for data processing and for peak list generation. Peak lists containing the most intense peaks of the spectrum were sent to MASCOT PMF search using NCBI and SwissProt databases. Sequences of peptides that were captured by MASCOT search were used as queries in a BLAST (Basic Local Alignment Search Tool) search (http://blast.ncbi.nlm.nih.gov) against T. cruzi translated proteins. Results. DGF-1.2 sequence analysis. Figure 1 shows the general characteristics of the predicted protein product of DGF-1.2. In the ORF, there are two possible methionine start codons, and the complete coding region was established as described in Materials and Methods. The translation of this ORF yielded a polypeptide of 3,474 amino acid residues with a predicted molecular mass of 359.6 kDa. The analysis of the sequence by SignalP 3.0 software (CBS, University of Denmark) identified a possible cleavable 51-amino-acid N-terminal signal peptide with high scores for the S mean score (0.631) and D score (0.607). S is the probability of a site to be the cleavage site for a signal peptidase, whereas D determines the secretory or nonsecretory performance of a protein. These parameters are used to discriminate between an N terminus that is a signal peptide (a high score) or part of the mature protein (a low score). The results of a topology analysis suggested the presence of nine membrane-spanning domains near the C terminus, which suggests that this protein is anchored to a membrane. According to the hydrophilic profile, the rest of the protein appears to be exposed outside the membrane. In addition, there are six putative nonidentical epidermal growth factor 1 (EGF-1)-like and one EGF-2-like signatures, all of them spaced approximately every 400 amino acids along the hydrophilic portion of the protein. The putative protein lacks a site for glycosylphosphatidylinositol (GPI) anchoring. The 298-amino-acid region (RE1) used for overexpression in bacterial cells and the generation of polyclonal anti DGF-1.2 antibodies are also marked in Fig. 1. Finally, the locations of peptides found by mass spectrometry that matched DGF-1.2 (Table 1) are indicated by green bars in Fig. 1.

DGF-1.2 expression in T. cruzi stages. Polyclonal anti-DGF-1.2 antibodies were purified and used for Western blot analyses of lysates from epimastigotes, tissue culture-derived trypomastigotes, and amastigotes of T. cruzi. The results are shown in Fig. 2A. As expected, a broad band expanding from around 250 to higher values was detected in all three life cycle stages. The strongest signal was observed in amastigotes, followed by a lower level of expression in trypomastigotes, while the weakest signal was present in the epimastigote stage. No reaction was observed when preimmune serum was used under similar conditions (data not shown). The presence of a broad band is in agreement with the expression of several members of this family of proteins with different apparent molecular masses. In order to confirm the Western blot results, we carried out immunofluorescence microscopy assays of all three forms. As shown in Fig. 2B, discrete fluorescence bodies were observed in the cytoplasm of all parasite stages, although they were more abundant in the amastigotes.
DGF-1.2 does not colocalize with any described organelle. As DGF-1.2 showed a particulate cytosolic localization (Fig. 2B), and peptides corresponding to several DGF-1 proteins were detected in a proteomic analysis of acidocalcisome fractions (Docampo et al., unpublished), we investigated whether anti-DGF-1.2 antibodies colocalized with markers of different organelles. There was no colocalization of antibodies against DGF-1.2 with markers for acidocalcisomes (vacuolar proton pyrophosphatase, TbVP1 [20] [Fig. 3D and E]), glycosomes (phosphate pyruvate dikinase, TbPPDK [1] [Fig. 3I and J]), or reservosomes (serine carboxypeptidase, TcSCP [18] [Fig. 3N and O]). Concanavalin A and BSA endocytosis assays were
used to label endocytic vesicles (Fig. 3S, T, W, and Y), and the dye Bodipy was used to label lipid droplets (data not shown), also with negative results. We also performed a time course experiment of endocytosis with concanavalin A and similar experiments with all three developmental stages of *T. cruzi* with similar negative results (data not shown).

**DGF-1.2 expression during the differentiation of trypomastigotes into amastigotes.** As the expression of DGF-1.2 appeared to be more noticeable in amastigotes (Fig. 2), we investigated the time course of its expression during the intracellular differentiation of trypomastigotes into amastigotes in myoblasts and HeLa cells. The results for HeLa cells are shown in Fig. 4; they revealed that maximal DGF-1.2 expression occurred around 18 h postinfection, when the protein appears to be localized close to the inner side of the plasma membrane. At 24 h postinfection, the fluorescent signal started to diminish, disappearing completely by 40 h. Similar results were obtained using L6E9 myoblasts (data not shown). We also followed the expression of DGF-1.2 during the extracellular trypomastigote-to-amastigote in vitro differentiation by incubating trypomastigotes at acidic pH. As shown in Fig. 5, although the DGF-1.2 peak of expression was earlier than in the intracellular experiments, its localization at 12 to 22 h was similar to that of the parasites differentiated intracellularly, i.e., close to the inner side of the plasma membrane.

**DGF-1 secretion during trypomastigote-to-amastigote extracellular differentiation.** SDS-PAGE gels of extracellular-differentiation supernatants (Fig. 6A) revealed protein bands of high molecular weight (above 220,000) and an additional band of \(M_r\) 70,000 at all time points of the follow-up. Although samples for all time points were processed simultaneously and similar volumes were loaded on the gel, differences in protein loading could be observed. When cells present in the pellets were examined for any disruption, they seemed to be in good condition. When a Western blot of a similar gel was probed with anti-DGF-1.2 antibodies, they reacted with two of the high-molecular-weight bands and the smaller \(M_r\) 70,000 band at all time points, except at 22 h and in the culture medium control (Fig. 6B). In this blot, the intensities of the bands seem to be proportional to the amount of protein loaded on the gel (Fig. 6A), except for the time point corresponding to 22 h and the negative control. The loss of recognition observed at 22 h could be explained by changes in the protein structure or degradation caused by the long period of exposure to the acidic pH of the culture media. To assess the identities of the different bands, SDS-PAGE was repeated with supernatant samples from 2 and 14 h, and the bands marked in Fig. 6C were excised and analyzed by mass spectrometry. Table 1 shows the sequences of peptides with high scores for DGF-1, the masses of these peptides, the GeneIDs for DGF-1 sequences with the highest match, the bands where the peptides were detected, and the locations of the peptides on the deduced sequence of the DGF-1.2 protein. Peptides with high scores for DGF-1 were observed for all bands, and when we located these peptides along the sequence in Fig. 1, we confirmed that they mostly originated from the first 2,000 amino acids of the sequence. However, when we included sequences with lower scores for DGF-1 (see Table S1 in the supplemental material), the coverage was extended to 3,000 amino acids. No match was found for any peptide from the region that includes the nine C-terminal transmembrane domains.

**Cell starvation and protein expression.** A final assay was performed in order to elucidate the possible function of DGF-1.2; we studied its role as an amino acid reserve protein in epimastigotes. Therefore, we examined whether there was any change in DGF-1.2 expression when cells were subjected to starvation conditions. Epimastigotes were incubated overnight in PBS, and the protein was detected by Western blotting (Fig. 7A) and immunofluorescence analysis (Fig. 7B). As shown in Fig. 7A, at the beginning of the starvation experiment, the anti-DGF-1.2 antibody recognized the high-molecular-mass bands of the native DGF-1.2 protein, a second band of approximately 150 kDa, and a smaller diffused band of around 70 to 80 kDa. At 18 h of starvation conditions, we observed, in addition to the native protein bands, a very strong band of 150 kDa and three additional bands between 65 and 80 kDa. This

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FIG. 2. Western blot and immunofluorescence analyses of DGF-1.2 in different stages of *T. cruzi*. (A) Western blot analysis of total proteins from epimastigotes (e), trypomastigotes (t), and amastigotes (a), using affinity-purified antibodies against the recombinant RE1 protein. \(\alpha\)-Tubulin is shown at the bottom. (B) DIC and immunofluorescence analyses of epimastigotes (e), trypomastigotes (t), and amastigotes (a) using affinity-purified antibodies against the recombinant RE1 protein. Scale bars, 5 \(\mu\)m.
FIG. 3. Lack of colocalization of DGF-1.2 with different subcellular markers in epimastigotes. (A, F, K, P, and U) DIC images. (B, G, L, Q, and V) DAPI staining (blue) of the nucleus (n) and kinetoplast (k). (C, H, M, R [green], and X [red]) Immunodetection of DGF-1.2 using polyclonal antibodies against RE1 recombinant protein. (D, I, N, S [red], and W [green]) Labeling of acidocalcisomes (anti-PPase), glycosomes (anti-PPDK), reservosomes (anti-SCP), and ConA and BSA endocytic vesicles (BSA), respectively. (E, J, O, T, and Y) Merged images of DAPI staining and immunofluorescence images. Scale bars, 5 μm.
pattern suggests that during starvation DGF-1.2 is degraded following a discrete pattern. The appearance of a 150-kDa band at time zero could be explained by fast protein hydrolysis under the conditions of this experiment. A parallel IFA analysis during the starvation experiment revealed a decrease in immunofluorescence labeling of epimastigotes (Fig. 7B).

DISCUSSION

At least 50% of the T. cruzi genome is made of repetitive sequences, consisting mostly of large gene families of surface proteins, retrotransposons, and subtelomeric repeats (11). The DGF-1 family is the fifth largest gene family in the T. cruzi genome, after mucin-associated surface proteins (MASPs), members of the TS superfamily, mucins, and the surface glycoprotein gp63 protease (11), and the presence of a large number of pseudogenes, the large size of the predicted proteins, and the lack of orthology with any other known genes have been puzzling. As predicted DGF-1 proteins have spaced cysteine-rich domains, which potentially may serve as targets for interaction with other proteins and a number of transmembrane domains, Wincker and colleagues (23) suggested that DGF-1 proteins were cell surface proteins with adhesive properties. However, recent glycoproteomic studies using concanavalin A-mediated affinity chromatography have found peptides corresponding to DGF-1 amino acid sequences in trypomastigote organelle fractions but not in plasma membrane fractions, although using PSORT II software, this work predicted a 74% probability of plasma membrane localization for DGF-1 (3). Also, our own unpublished proteomic studies (Docampo et al., unpublished) found DGF-1 peptide matches in acidocalcisome-enriched fractions from epimastigote forms. These data agree with our IFA experiments with permeabilized T. cruzi cells, in which no fluorescence was observed at the cell surface (Fig. 2B), although we found no colocalization with acidocalcisomes or any of the other organelles examined here (Fig. 3). These results indicate that the vesicles containing DGF-1.2 copurify with acidocalcisomes. The fact that this protein is predicted to be anchored to a membrane and the presence of the protein in discrete fluorescent bodies led us to believe that DGF-1.2 is localized in a new and different type of vesicle whose role needs to be determined.

FIG. 4. Immunodetection of DGF-1.2 during in vivo differentiation of T. cruzi trypomastigotes into amastigotes after infection of HeLa cells. A time course assay was performed, and the hours postinfection are indicated in each image. DIC and immunofluorescence images using polyclonal antibodies against the RE1 region of DGF-1.2 are shown. The green labeling corresponds to DGF-1.2 detection. Scale bars, 10 μm.
The large sizes of DGF-1 open reading frames have raised speculation that perhaps these proteins may be precursors that undergo a cleavage process after or during their synthesis to give rise to mature functional polypeptides. However, in Western blot analyses, we showed for the first time that expressed and secreted DGF-1.2 retained a large size, although in all in vitro differentiation experiments, a band of 70 kDa recognized by anti-RE1 antibodies and identified by mass spectrometry.

**FIG. 5.** Immunodetection of DGF-1.2 during in vitro differentiation of *T. cruzi* trypomastigotes into amastigotes. A time course assay was performed, and the hours after the change in extracellular pH are indicated in each image. DIC and immunofluorescence images using polyclonal antibodies against the RE1 region of DGF-1.2 are shown. Green labeling corresponds to DGF-1.2 detection. Scale bars, 5 μm.

**FIG. 6.** Protein analysis of supernatants collected during *T. cruzi* trypomastigote-to-amastigote extracellular differentiation. (A) SDS-PAGE assay of a 4 to 10% polyacrylamide gradient gel. (B) Western blot assay using anti-RE1 polyclonal antibodies. (C) SDS-PAGE for MALDI-time of flight (TOF) MS band analysis. Bands 2A, 2B, 14A, and 14B, marked in the gel, were used for MS analysis. Collection times (in hours) are indicated above each image. Coomassie blue without methanol was used for staining. Molecular mass marker sizes are shown at the left of each gel.
analysis was also observed. Peptides identified by MS covered the first two-thirds of the protein, suggesting that the last part of DGF-1.2 (which includes the helix-rich C terminal) is not secreted. Also, active cleavage of the protein was induced by starvation conditions (Fig. 7B), which suggests that under nutritional stress this protein could also serve as an amino acid reserve (2).

In our experiments, we demonstrated the very important fact that during the T. cruzi life cycle, DGF-1.2 was differentially expressed, being predominant in amastigote forms. In this sense, it is very interesting that the protein not only has a higher expression level in amastigote forms, but also undergoes relocalization within the cell, accumulating against the internal side of the plasma membrane. This accumulation peaked around 18 to 24 h postinfection or postdifferentiation. Thereafter, the number of fluorescent bodies decreased (Fig. 4), suggesting that the protein is secreted into the host cell, in which case the fluorescent bodies could be secretory vesicles. In agreement with this suggestion, during the trypomastigote-to-amastigote extracellular differentiation throughout the process, the protein was detected in the culture medium, peaking at 14 h.

These facts and the absence of DGF-1 hits in the T. cruzi plasma membrane proteome (unpublished data) apparently contradict a recent report (15) suggesting a cell surface localization for DGF-1 in T. cruzi strain Y and G trypomastigotes. The authors of this work biotinylated trypomastigote cell surface proteins and used anti DGF-1 antibodies to detect streptavidin affinity-purified proteins. However, antibodies recognized both streptavidin-bound and unbound fractions. Moreover, the authors’ attempts to block trypomastigote infection by a previous incubation with anti-DGF-1 antibodies failed.

Interestingly, a large-scale screening using a yeast two-hybrid system identified several DGF-1 proteins that interacted with the T. cruzi protein kinase A (PKA) catalytic subunit, and one of them had a potential cyclic AMP (cAMP)-dependent protein kinase phosphorylation site (6). Although the deduced DGF-1.2 protein sequence lacks PKA phosphorylation sites, it contains one of the peptides rescued in the two-hybrid experiments, namely, from nucleotides 4555 to 5451 in gene Tc00.1047053416809.10. Therefore DGF-1.2 has the potential to interact with T. cruzi or perhaps with host PKA, where it could participate in signal transduction pathways or mediate the regulation of T. cruzi stage differentiation (14, 17).

In regard to DGF-1 expression under starvation conditions, for the assay, the number of parasites was determined at the beginning and at the end of the starvation period. Because the number of parasites did not change during the assay, we suggest that most proteins detected after 18 h of starvation came from the long starving parasites shown in Fig. 7B, which were physiologically affected but still alive. However, we cannot completely rule out the possibility that some degraded DGF-1 proteins could come from dying parasites as a consequence of damage caused by starvation conditions. The results shown in Fig. 7 also suggest that during the starvation period DGF-1 could be redistributed, because it could be detected only by Western blotting and not by IFA. The secretion of the protein could be a better explanation, in agreement with the results shown in Fig. 6 for DGF-1 release during the differentiation process. In fact, deprivation of nutrients has been described as an inducing factor for autophagy and cell differentiation in T. cruzi (2).

In silico studies of the robustness/volatility of DGF-1 and group 2 TS superfamily members have suggested that DGF-1 is a robust gene family and, compared with TS genes, has low codon volatility (4, 5). This fact, together with the lack of any enzymatic activity and its adhesive-like properties (13), may suggest that it has some structural role, e.g., as a receptor or effector for other proteins.

In conclusion, this is the first report of direct immunodetection and developmental expression and secretion of a DGF-1 protein. The preferential expression of DGF-1.2 in amastigotes (the intracellular replicative forms that develop in the mammalian host) and the absence of orthologs in other species suggest a unique role, perhaps related to the interaction of the parasite with the host cell, that needs to be further explored to assess its potential as a target for chemotherapy.

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