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# Identification, characterization and localization of chagasin, a tight-binding cysteine protease inhibitor in *Trypanosoma cruzi*

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## SUMMARY

Lysosomal cysteine proteases from mammalian cells and plants are regulated by endogenous tight-binding inhibitors from the cystatin superfamily. The presence of cystatin-like inhibitors in lower eukaryotes such as protozoan parasites has not yet been demonstrated, although these cells express large quantities of cysteine proteases and may also count on endogenous inhibitors to regulate cellular proteolysis. *Trypanosoma cruzi*, the causative agent of Chagas' heart disease, is a relevant model to explore this possibility because these intracellular parasites rely on their major lysosomal cysteine protease (cruzipain) to invade and multiply in mammalian host cells. Here we report the isolation, biochemical characterization, developmental stage distribution and subcellular localization of chagasin, an endogenous cysteine protease inhibitor in *T. cruzi*. We used high temperature induced denaturation to isolate a heat-stable cruzipain-binding protein (apparent molecular mass, 12 kDa) from

epimastigote lysates. This protein was subsequently characterized as a tight-binding and reversible inhibitor of papain-like cysteine proteases. Immunoblotting indicated that the expression of chagasin is developmentally regulated and inversely correlated with that of cruzipain. Gold-labeled antibodies localized chagasin to the flagellar pocket and cytoplasmic vesicles of trypomastigotes and to the cell surface of amastigotes. Binding assays performed by probing living parasites with fluorescein (FITC)-cruzipain or FITC-chagasin revealed the presence of both inhibitor and protease at the cell surface of amastigotes. The intersection of chagasin and cruzipain trafficking pathways may represent a checkpoint for downstream regulation of proteolysis in trypanosomatid protozoa.

Key words: Cysteine protease, Chagasin, Cruzipain, Cystatin, Lysosomes, *Trypanosoma cruzi*

## INTRODUCTION

Transmitted to mammals by blood-sucking triatomine insects, the parasitic protozoa *Trypanosoma cruzi* is the etiological agent of Chagas' heart disease, a chronic infection affecting approximately 16 million people in Central and South America (WHO, Division of Control of Tropical Diseases, Chagas Disease Elimination: Burdens and Trends <http://www.who.int/ctd/chagas/index.html>). During its development, *T. cruzi* undergoes drastic changes in cellular shape, size and organelle localization (De Souza, 1995). Epimastigotes are non-infective dividing forms that later transform into infective metacyclic trypomastigotes in the insect gut. Metacyclic trypomastigotes released on wounded tissues can invade a wide range of host cells, where they gain access to the cytoplasm and transform into round-shaped amastigotes. These forms undergo several cycles of binary division before transforming into infective trypomastigotes. Upon host cell burst, the trypomastigotes fall into the bloodstream from where the infection is rapidly disseminated to multiple tissues.

Efforts to characterize the role of proteases in *T. cruzi* development converged to a heterogeneous group of papain-like cysteine proteases (Murta et al., 1990; Cazzulo et al., 1990a; Eakin et al., 1992), collectively termed 'cruzipain' (also called cruzain or GP57/51). Cruzipain is a developmentally regulated protease (Tomas and Kelly, 1996) that is encoded by numerous polymorphic genes organized in tandem units (Campetella et al., 1992; Lima et al., 1994). These enzymes are synthesized as zymogens (pre-pro-enzymes) that are activated by cleavage of the pro-domain to generate mature proteases (Eakin et al., 1992). Similarly to the type 1 cysteine proteases from *Leishmania* and *T. brucei* (Mottram et al., 1989), cruzipain has a long and highly glycosylated C-terminal extension (CTE) (Aslund et al., 1991; Mendonça-Previato et al., 1983; Parodi et al., 1995) that is absent from mammalian cathepsins. Recent studies have implicated a pro-region motif (Huete-Perez et al., 1999) in the targeting of cruzipain to endolysosomal-like vesicles of epimastigotes (Murta et al., 1990; Soares et al., 1992).

Early studies with synthetic cysteine protease inhibitors have suggested that the activity of cruzipain is essential for parasite

growth and/or differentiation (Meirelles et al., 1992; Harth et al., 1993; Franke de Cazzulo et al., 1994; Engel et al., 1998a). Later, it became clear that the inhibition of cruzipain blocks pro-enzyme maturation, thereby causing prohibitive accumulation of unprocessed pro-cruzipain in late Golgi vesicles, at least so in epimastigotes (Engel et al., 1998b). Some polymorphic cruzipain genes (Lima et al., 1994) encode iso-enzymes that differ with respect to substrate specificity and susceptibility to inactivation by natural or synthetic inhibitors of cysteine proteases (Lima et al., 2001), suggesting that *T. cruzi* relies on these versatile proteolytic enzymes to survive in a wide range of hosts. More recently, cruzipain was implicated in the activation of the kinin cascade system by *T. cruzi* trypomastigotes (Scharfstein et al., 2000). Accordingly, the parasites mobilize cruzipain to release kinin peptides from kininogen molecules that are displayed at the cell surface of target cells. Once released, the kinin molecules activate the heterotrimeric G-protein-coupled kinin receptors, thus rendering the host cells increasingly susceptible to parasite invasion (Scharfstein et al., 2000).

At least for lysosomal cathepsins of mammalian cells, there are indications that their activity is regulated by endogenous cysteine protease inhibitors from the cystatin superfamily (Rawlings and Barrett, 1990). For example, cystatin C regulates cell surface expression of MHC class II molecules in dendritic cells (Pierre and Mellman, 1998). In the context of infection, there is precedent for the involvement of cystatin C control of viral replication (Björck et al., 1990). Efforts to identify putative cystatin ancestors in parasitic protozoa (e.g. *Leishmania*) have met only partial success (Irvine et al., 1992), presumably because cysteine proteases are often present in stoichiometric excess over endogenous inhibitors. In the present study, we took advantage of the heat-lability properties of cruzipain, to isolate and subsequently characterize an endogenous inhibitor protein of 12 kDa that differs from cystatins with respect to molecular properties, developmental stage distribution and subcellular localization.

## MATERIALS AND METHODS

### Cells

*T. cruzi* epimastigotes (Dm28c clone) were cultivated at 28°C in liver infusion tryptose medium (LIT) containing 10% fetal calf serum (FCS) as described (Yong et al., 2000). Tissue culture trypomastigotes (TCTs) and amastigotes were harvested from the supernatants of infected Vero cell cultures maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2% FCS in a 5% CO<sub>2</sub> humidified atmosphere, at 37°C, as described (Scharfstein et al., 2000).

### Purified proteins

Natural chagasin (n-chagasin) was isolated from *T. cruzi* epimastigote lysates as follows:  $5 \times 10^{10}$  cells were resuspended in 40 ml of 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.2 (PBS), 2 mM EDTA, 0.5% Triton X-100 (v/v), supplemented with a protease inhibitor cocktail (5 mM benzamidinium chloride, 10 mM EDTA) and 0.1% (w/v) Na<sub>3</sub>. After subjecting the parasites to freeze and thaw cycles (five times), the suspension was centrifuged (13,000 g for 30 minutes) and the supernatant was collected. This sample (55 mg protein/ml) was boiled for 15 minutes, and precipitates were cleared by centrifugation at 13,000 g for 20 minutes. The supernatant (20 mg/ml) was filtered in a Centriprep 30 concentrator (Amicon Inc., Beverly, USA) and the

effluent was collected and applied to a Centriprep 3 concentrator (Amicon). The final solution containing n-chagasin (5 µg protein/ml) was then used to titrate the activity of papain, as described below. Recombinant chagasin (r-chagasin) was produced in *E. coli* and purified as described elsewhere (M.A. et al., unpublished data). Briefly, the insert of one of several clones identified in an epimastigote λgt11 cDNA expression library after screening by ligand binding to carboxymethylated papain and cruzipain (clone Tc18; GenBank/EMBL accession no. AJ299433) was subcloned in the pHD313 plasmid (Dalboge et al., 1989) for high-level expression in *E. coli*. The construct was composed of: (1) the Omp A signal sequence; (2) 14 residues of the N-terminus of human cystatin C (SSPGKPPRLVGGPM) introduced to improve the expression yields (Abrahamson et al., 1988); (3) a 7-residue linker (ASVSAEF); and (4) the Tc18 clone sequence (starting at nt 61 of the chagasin gene/AJ299433). The Omp A peptide, the cystatin C and the linker peptides were removed from the purified recombinant protein (126 residues, M<sub>r</sub> 13,854) during the isolation procedure, since N-terminal sequencing (FKGTR) revealed that it started at residue 2 of the open reading frame predicted in the Tc18 cDNA. RT-PCR of *T. cruzi* mRNA was performed using an upstream primer based on the mini-exon sequence and a chagasin downstream primer. This analysis indicated that the predicted 110-residue (M<sub>r</sub> 12,031) sequence of natural chagasin starts at residue 17 of the recombinant Tc18 protein. The recombinant protein (r-chagasin/Tc18; here called r-chagasin) was isolated by a two step ion-exchange/gel filtration procedure, essentially as described for similar production of human cystatin C (Abrahamson et al., 1988), and kept frozen as a 1 mg/ml solution in 50 mM ammonium bicarbonate buffer, pH 7.8, containing 100 mM NaCl until use. Recombinant human cystatin C (r-cystatin C) was produced as described (Abrahamson et al., 1988). Natural cruzipain (n-cruzipain) was isolated from crude aqueous extracts of Dm28c epimastigotes as described (Lima et al., 1992). Recombinant cruzain (r-cruzain), expressed in *E. coli* (Eakin et al., 1992), was a gift from J. H. McKerrow (UCSF, San Francisco, CA). Recombinant cruzipain 2 (r-cruzipain 2), expressed in *S. cerevisiae*, was obtained as described (Lima et al., 2001). GPI-PLC (*Bacillus cereus* GPI-PLC, Boehringer Mannheim, Germany) was a gift from Maria Lúcia Cardoso de Almeida (UNIFESP, Sao Paulo, Brazil).

### Antibodies

An antiserum against r-chagasin (rabαchagasin) was raised by immunizing rabbits subcutaneously with 0.2 mg of the recombinant protein emulsified with incomplete Freund's adjuvant (Difco Laboratories). The injection was repeated at 3 and 6 weeks, and the animal was bled every third week. The specificity of the antiserum was tested by ELISA and by western blotting using r-chagasin, human plasma and *E. coli* extracts as antigen. The IgG fraction of 100 ml of antiserum was isolated by absorption to protein A-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden) and the antibodies were subsequently eluted with 0.1 M glycine buffer, pH 2.2. Affinity-purified antibodies were obtained by loading the IgG fraction on CNBr-activated Sepharose 4B (Amersham-Pharmacia Biotech) coupled with 10 mg of r-chagasin under conditions recommended by the manufacturer. After loading the IgG fraction, the resin was washed with the equilibrating buffer (50 mM Tris-HCl buffer, pH 7.4, 500 mM NaCl and the bound antibodies were eluted in 0.1 M glycine buffer, pH 2.2. The affinity-purified antibodies were dialyzed against PBS and concentrated to 0.1 mg/ml by the use of Centriprep 30 (Amicon). Monoclonal antibody to cruzipain (mAb212BH6) was obtained as previously described (Murta et al., 1990). Peroxidase-labeled anti-mouse IgG and peroxidase-labeled anti-rabbit IgG conjugates were both purchased from Bio-Rad Laboratories, Hercules, CA.

### Immunoblotting

For the preparation of *T. cruzi* lysates the parasites were washed twice

in PBS, 2 mM EDTA and lysed in the same buffer supplemented with 0.5% (v/v) Triton X-100, 5 mM benzamidine chloride and 0.1% (w/v) NaN<sub>3</sub>. The suspension was centrifuged at 13,000 *g* for 20 minutes, and the supernatant was frozen at -20°C. Protein concentration was determined by the DC-protein kit (Bio-Rad Laboratories). Lysates containing 20 µg protein from each developmental stage were mixed (1:1) with 100 mM Tris-HCl buffer, pH 6.8, 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.012% (w/v) bromophenol blue, boiled for 5 minutes and subjected to 16% SDS-PAGE. After electroblotting the proteins onto nitrocellulose (Bio-Rad Trans-Blot cell, Bio-Rad Laboratories), the membrane was blocked with 3% (w/v) nonfat powdered milk diluted in PBS, 2 mM EDTA, 0.05% Tween 20 for 1 hour at room temperature. Membranes were incubated for 1 hour at room temperature with rabαchagasin diluted 1:1000 in the blocking buffer, washed three times and incubated with peroxidase-labeled anti-rabbit IgG conjugate according to the manufacturer's instructions. The immunoreactive bands were visualized by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech).

### Cruzipain ligand blotting assay

Soluble epimastigote extracts (200 µg) were diluted in 375 mM Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 0.012% (w/v) bromophenol blue and separated in 16% SDS-PAGE gels. After electroblotting, the membrane was blocked with 1% (w/v) BSA diluted in PBS, 2 mM EDTA and 0.05% Tween 20 for 1 hour at room temperature and subsequently incubated with purified n-cruzipain (10 µg/ml) for 3 hours at room temperature, in the blocking buffer. After three wash cycles, the membrane was incubated with mAb 212BH6, followed by reaction with the peroxidase-labeled anti-mouse IgG conjugate as described above. The bands reacting with the cruzipain probe were then visualized by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech).

### Northern blot

*T. cruzi* from the three developmental stages were obtained as described above and the total RNA of each sample was purified using the RNA purification kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). 20 µg of each RNA sample was loaded into a 2% agarose gel in 20 mM (3-[N-morpholino] propanesulfonic acid) (MOPS), 1 mM EDTA, 5 mM NaOAc, pH 7.0, 1% formaldehyde and run at 5 Volts/cm. The samples were transferred by capillarity to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) in 3 M NaCl, 300 mM sodium citrate pH 7.2 (20×SSC), overnight and baked at 80°C for 2 hours. The membranes were blocked with 5× SSC, 5× Denhardt's (Sigma), 1% SDS, 50 µg/ml salmon sperm DNA (Sigma), 50% formamide for 1 hour, at 42°C and subsequently probed overnight with radiolabeled Tc18 cDNA (described below) under the same conditions as the pre-hybridization. For the preparation of the probe, a 400 bp *Bam*HI-*Bam*HI fragment from the expression plasmid pTc18D (M.A. et al., unpublished data) corresponding to the entire coding sequence for chagasin was purified from a 0.8% agarose gel using the Gene Clean kit (BIO-101), according to the manufacturer's instructions. 25 ng of the insert were labeled by random priming (Rediprime II kit, Amersham Pharmacia Biotech) using [<sup>32</sup>P]dCTP (ICN). The blot was washed three times (15 minutes each) with 0.2× SSC, 0.1% SDS and exposed overnight.

### ELISA

Purified recombinant chagasin (10 µg/ml) or BSA (10 µg/ml), used as a specificity control, were diluted in PBS and plated on ELISA 96-well microtiter plates (Nunc) overnight, at 4°C. The wells were blocked with PBS, 0.5% Tween 20 (v/v) for 90 minutes and then incubated for 1 hour at room temperature with control human sera (*n*=25) or with sera from 25 chronic chagasic patients (a gift from J. Santana, University of Brasilia, Brazil) diluted 1:50 in PBS, 0.05% Tween 20 (v/v). The wells were subsequently washed three times (5

minutes each) with PBS, 0.05% Tween 20 (v/v) and incubated for 1 hour at room temperature with anti-human IgG antibodies conjugated to peroxidase (Sigma, St Louis, MO) diluted 1:2000 in the same buffer. After washing as described above, the reaction was developed according to manufacturer's instructions, using *o*-phenylenediamine (Bio Rad, Richmond, CA) as the substrate for color development. The optical density at 492 nm was read in a plate spectrophotometer reader.

### Triton X-114 phase partition

Amastigotes, tissue culture trypomastigotes and epimastigotes (5×10<sup>8</sup> cells) were lysed at 4°C in 100 µl of 100 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl containing 0.2% of precondensed Triton X-114 (GPI-PLC buffer), 0.1% (w/v) NaN<sub>3</sub> and a protease inhibitor cocktail (5 mM benzamidine chloride, 10 mM EDTA). Prior to condensation, a separate group of lysate samples (50 µl) were treated with 100 units (13 ng) of GPI-PLC at 30°C for 30 minutes. A stock of 10% Triton X-114 was added to all lysates to a final concentration of 1.25% Triton X-114. Phase partition was carried out incubating the samples at 4°C for 30 minutes and then at 37°C for 5 minutes, as described (Bordier, 1981). After centrifugation (13,000 *g* for 5 minutes at room temperature), a second cycle of phase partitioning was carried out by adding 10% Triton X-114 to the aqueous phase, while Tris-buffered saline was added to the detergent phase, to reach a final concentration of 1.25% of the detergent, in both cases. After pooling the aqueous or detergent fractions separately, the samples were diluted in complete SDS lysis buffer, boiled and run on a 16% SDS-PAGE gel. Immunochemical detection of chagasin or cruzipain was performed as described above.

### Enzyme inhibition assays

The molar concentration of chagasin (natural and recombinant) was determined by titration with papain, which had been previously titrated with *L-trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64) (Abrahamson, 1994). Sequential dilutions of chagasin were incubated with papain in 100 mM sodium phosphate buffer, pH 6.5, 2 mM EDTA, 1 mM dithiothreitol (DTT) for 30 minutes at room temperature. The substrate Bz-DL-Arg-pNA was added to 2.5 mM final concentration and the residual catalytic activity of papain was detected by measuring product generation as a function of the absorbance at 410 nm in a Hitachi U2000 spectrophotometer. The equilibrium constants for dissociation (*K<sub>i</sub>*) of chagasin complexed with natural cruzipain, recombinant cruzipain isoforms, and papain were determined in continuous rate assays, as previously described (Abrahamson, 1994; Bieth, 1984). Assays with n-cruzipain, r-cruzain and papain were performed by incubating these enzymes at 37°C with the substrate CBZ-Phe-Arg-AMC (10 µM), in 100 mM sodium phosphate buffer, pH 6.5, 2 mM EDTA, 1 mM DTT; r-cruzipain 2 was assayed with 10 µM ε-NH<sub>2</sub>(Cap)-Leu-S(Bz)Cys-AMC, in the same buffer (Lima et al., 2001). Substrate hydrolysis was monitored in a Hitachi F4500 fluorimeter at 380 nm excitation and 440 nm emission wavelengths. Steady-state velocities before (*v<sub>o</sub>*) and after (*v<sub>i</sub>*) addition of inhibitor were obtained by linear regression of the substrate hydrolysis curves. Apparent *K<sub>i</sub>* values [*K<sub>i</sub>(app)*] were calculated as the slope of the plot of [I]/(1-*v<sub>i</sub>*/*v<sub>o</sub>*) versus *v<sub>o</sub>*/*v<sub>i</sub>* (Henderson, 1972). The substrate-independent *K<sub>i</sub>* was then calculated from *K<sub>i</sub>(app)*=*K<sub>i</sub>*(1+[S]/*K<sub>M</sub>*), using *K<sub>M</sub>* values described in the literature (Lima et al., 2001). All determinations of *v<sub>o</sub>* and *v<sub>i</sub>* were based on assays with less than 2% substrate hydrolysis and a linear regression coefficient at steady-state greater than 0.990.

### Ultrastructural immunocytochemistry

Epimastigotes, tissue culture trypomastigotes and amastigotes were fixed overnight at 4°C in 1% glutaraldehyde (grade I), 4% paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.2, and free aldehyde groups were quenched in 100 mM glycine. Lowicryl K4M processing was performed as described (Bendayan et al., 1987).

Thin sections were incubated with affinity purified rab $\alpha$ chagasin followed by gold-conjugated goat anti-rabbit IgG (EY Labs, San Mateo, CA). Preparations were observed under a Zeiss CEM 902 electron microscope after uranyl acetate staining. Omission of primary antibody resulted in no staining.

### Confocal laser scanning microscopy

Isolated r-chagasin and purified cruzipain were conjugated to fluorescein (FITC) using standard methods (The and Feltkamp, 1970). Tissue culture trypomastigotes and amastigotes were harvested from the supernatants of infected Vero cell cultures, washed twice in PBS, 2 mM EDTA and resuspended in the same buffer. The parasites were then incubated with either FITC-chagasin (500  $\mu$ g/ml) or FITC-cruzipain (500  $\mu$ g/ml) for 30 minutes at 4°C. Labeled cells were washed by centrifugation in the above buffer, and fixed with 3% paraformaldehyde in 100 mM phosphate buffer, pH 7.0. Fixed parasites were spread on poly-L-lysine coated glass coverslips and processed for confocal microscopy. As specificity controls for cell binding assays with either FITC-chagasin or FITC-cruzipain, the fluoresceinated probes were preincubated with an excess of unlabeled n-cruzipain and r-cystatin C, respectively. Glass coverslips were examined under a Zeiss confocal laser scan microscope.

## RESULTS

### Identification of a functionally active inhibitor of cysteine proteases in *T. cruzi*

Given that cysteine protease inhibitors of the cystatin superfamily regulate proteolysis in mammalian cells, we set out to investigate if such endogenous inhibitors may exist in lower eukaryotes using *T. cruzi* as a model. Initially, the detection of a functionally active inhibitor of cysteine proteases in *T. cruzi* epimastigotes was not feasible, because cruzipain is expressed at very high levels by this developmental form (Tomas and Kelly, 1996; Campetella et al., 1990). We reasoned that endogenous inhibitors, if present in epimastigotes, should be segregated from the lysosomal-like vesicles where active forms of cruzipain accumulate (Murta et al., 1990; Soares et al., 1992; Engel et al., 1998b). Upon cellular lysis, it seemed plausible that tight molecular complexes between such an inhibitor and cruzipain could be formed. Since cystatins from other organisms are often thermoresistant (Lenney et al., 1979), we checked if heat-induced denaturation of cruzipain would allow the detection of an inhibitory activity. The data shown in

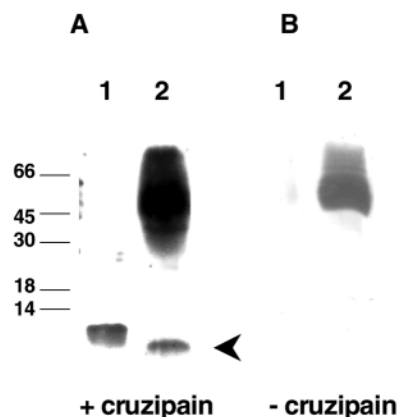
**Table 1. Functional detection of a *T. cruzi* cysteine protease inhibitor**

	Activity ( $V_0 \times 10^{-10} \text{ M s}^{-1}$ )	% Inhibition
Cruzipain	7.8	0
Cruzipain+lysate	12.6	0
Cruzipain+boiled lysate	1	87
Cruzipain+E-64	0.2	97

The activity of purified cruzipain (0.2 nM) was measured in 50 mM  $\text{Na}_2\text{HPO}_4$ , 200 mM NaCl, 5 mM EDTA, pH 6.5, supplemented with 2.5 mM DTT, at 37°C, using CBZ-Phe-Arg-MCA (10  $\mu$ M) as a substrate.  $V_0$  represents the initial velocity for the hydrolysis of the substrate before and after the addition of epimastigote lysates. Epimastigotes (Dm28c) were lysed in PBS pH 7.2, 2 mM EDTA, 0.5% Triton X-100, 0.1%  $\text{NaN}_3$ , the lysate was boiled (or not) and the soluble fraction was tested for the inhibition of cruzipain. E-64 (10  $\mu$ M) was used as a specificity control under the same conditions.

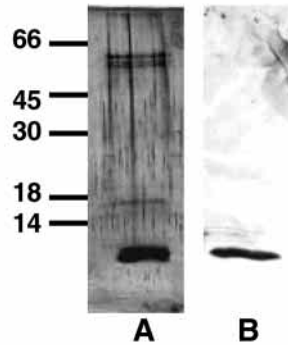
Table 1 indicate that a cysteine protease inhibitory activity is indeed detectable in the boiled epimastigote lysates.

The molecular characterization of cysteine protease-binding proteins present in epimastigote extracts began by using soluble cruzipain as a probe in ligand blotting assays. To this end, epimastigote aqueous extracts were mixed with non-reducing SDS-sample buffer but the sample was not heat-denatured, the premise being that molecular complexes formed between the endogenous inhibitor and cruzipain would possibly dissociate in the presence of SDS. After separating the proteins by SDS-PAGE, they were electroblotted onto a membrane and then probed with purified cruzipain. An anti-cruzipain monoclonal antibody (mAb212) was subsequently used to identify the cruzipain-binding proteins. Assays performed with immobilized human cystatin C (~13 kDa) indicated that it was recognized by the soluble cruzipain probe (Fig. 1A, lane 1), thus validating the functionality of this assay. As expected, mAb212 did not react with immobilized cystatin C when the soluble cruzipain probe was omitted from the first incubation step (Fig. 1B, lane 1). The profile obtained for *T. cruzi* extracts revealed the presence of a cruzipain-binding protein of low molecular mass (~12 kDa) (Fig. 1A, lane 2, arrowhead). Rather than representing a group of cruzipain-binding proteins, the diffused 50-57 kDa antigens recognized by mAb212 (Fig. 1A,B, lane 2) correspond to the endogenous cruzipain from epimastigotes (Scharfstein et al., 1986; Bonaldo et al., 1991); it is noteworthy that the same bands were seen in controls where the protease was omitted from the first incubation step (Fig. 1B, lane 2). In conclusion, a cruzipain-binding protein migrating with an apparent molecular mass



**Fig. 1.** Detection of a cruzipain binding protein in *T. cruzi* epimastigotes lysates. Proteins from epimastigotes lysates, or recombinant human cystatin C used as a control, were diluted in SDS-sample buffer (without boiling) under non-reducing conditions. After running a 16% SDS-PAGE, the polypeptides were transferred to nitrocellulose membranes. Proteins that were capable of binding to cysteine proteases were identified by incubating the membranes with (A) cruzipain (10  $\mu$ g/ml) in PBS, 2 mM EDTA, 1% BSA (w/v), 0.05% (v/v) Tween 20 for 1 hour at room temperature. After washing, the bound cruzipain probe was identified by treating the membrane with mAb $\alpha$ cruzipain. Peroxidase-labeled anti-mouse IgG was used to visualize the immuno-reactive bands (B), membrane treated with buffer (instead of the cruzipain probe) followed by incubation with mAb $\alpha$ cruzipain. Lane 1: recombinant human cystatin C (10 ng/ml); lane 2: epimastigote lysate.

**Fig. 2.** Purification of n-chagasin. (A) Silver staining of natural chagasin (arrow) purified from epimastigote lysates as described in Materials and Methods. (B) Immunoblotting of the purified protein, carried out with a rabbit antiserum directed against r-chagasin. Electrophoresis run on 16% SDS-PAGE under denaturing conditions.



similar to that of mammalian cystatins (11–13 kDa) was identified in the parasite extracts.

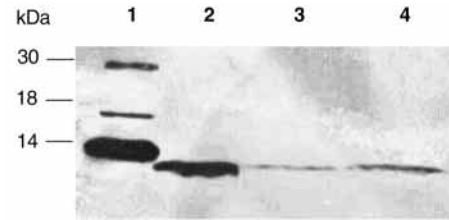
### Kinetic properties of chagasin

In order to verify if the ~12 kDa molecule corresponded to the heat-resistant inhibitor, the soluble fraction of a previously boiled epimastigote lysate was subjected to differential ultrafiltration, using Centriprep 30 and Centriprep 3 filters, resulting in the purification of a ~12 kDa protein (Fig. 2A) with capacity to inhibit both papain and cruzipain in enzymatic assays (below). Based on papain titration results, we estimated that the yield of the heat-resistant inhibitor recovered from 100 ml of stationary phase epimastigote culture was approximately 1.2 nanomoles. In parallel, the screening of a *T. cruzi* cDNA expression library using purified cruzipain and papain as probes led to the identification of a clone (Tc18) containing a 378 base pair open reading frame (ORF) of unidentified sequence (Tc18; GenBank/EMBL accession no. AJ299433). As reported elsewhere, r-chagasin (i.e. the recombinant product expressed in *E. coli*) presented inhibitory activity against cruzipain and papain. Polyclonal antibodies raised against r-chagasin reacted with the ~12 kDa cruzipain inhibitor purified from epimastigotes (Fig. 2B), indicating that we had isolated its natural counterpart, n-chagasin. The determination of the equilibrium dissociation constants ( $K_i$ ) with members of the cruzipain family and papain showed that both natural and recombinant chagasin are extremely potent cysteine protease inhibitors, displaying  $K_i$  values in the picomolar range (Table 2). Consistent with the results obtained with human cystatin C, the  $K_i$  value obtained for r-cruzipain 2 (Lima et al., 2001) was 4.5-fold higher than that with r-cruzain (Eakin et al., 1992). The inhibitory activity of chagasin was not limited to molecules from the cruzipain family, since it inactivated papain at high efficiency ( $K_i$  0.013 nM).

**Table 2. Equilibrium constants for dissociation ( $K_i$ ) of chagasin from enzyme complexes**

	$K_i$ (nM)	
	N-chagasin	R-chagasin
Papain	0.013±0.00078	0.021±0.0015
N-cruzipain	0.024±0.00216	0.0067±0.0004
R-cruzipain 1 (cruzain)	0.095±0.0076	0.012±0.0096
R-cruzipain 2	0.039±0.0023	0.075±0.00675

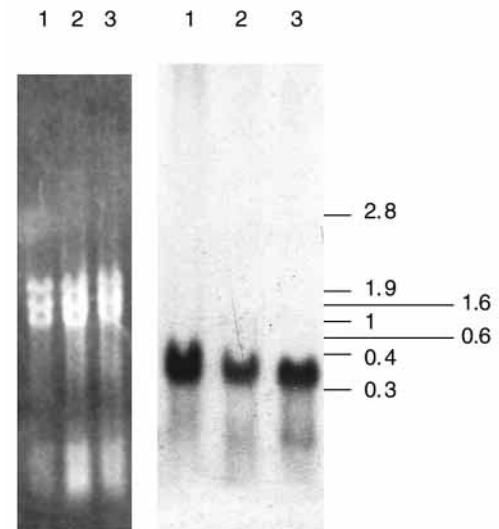
The values were calculated from continuous rate assay experiments as the slope of the plot of  $[I]/(1-v_i/v_0)$  versus  $v_0/v_i$  and corrected for substrate competition as described in Materials and Methods.



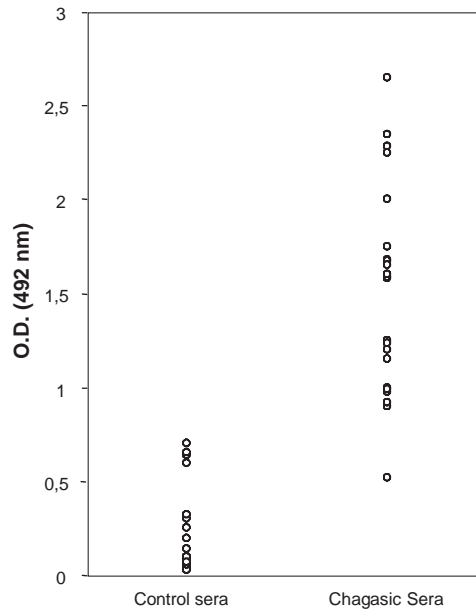
**Fig. 3.** Expression of chagasin during the life cycle of *T. cruzi*. Western blotting of Triton X-100 lysates (10 µg protein) from the three developmental stages of Dm28c *T. cruzi*. The membranes were incubated with rabbit anti-chagasin (1:1000) in PBS, 2 mM EDTA, 3% (w/v) nonfat dried milk, 0.05% (v/v) Tween 20 for 1 hour at room temperature. Lane 1, r-chagasin as a reference (100 ng); lane 2, trypomastigotes; lane 3, epimastigotes; and lane 4, amastigotes.

### Developmental expression of chagasin protein

The expression of chagasin in *T. cruzi* was investigated by immunoblotting using antibodies raised against r-chagasin. In agreement with the ligand blotting assays (Fig. 1), this antiserum specifically recognized a ~12 kDa polypeptide in lysates from all developmental forms of the parasite (Fig. 3). Our data showed that chagasin is present at higher levels in trypomastigotes than in amastigotes and epimastigotes, thus indicating an inverse correlation with the cruzipain contents, which are likewise developmentally regulated (Campetella et al., 1990; Bonaldo et al., 1991; Tomás and Kelly, 1996). Higher molecular mass r-chagasin aggregates detected here (Fig. 3, lane 1) are often observed when this inhibitor is overexpressed in *E. coli* (M.A. et al., unpublished). Northern blots using the

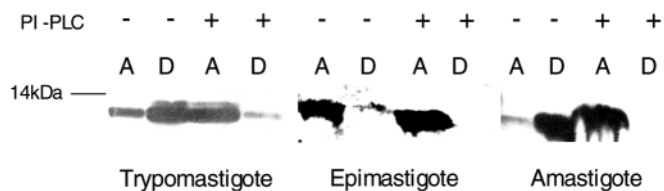


**Fig. 4.** Expression of chagasin RNA in *T. cruzi*. Total RNA (20 µg) from trypomastigotes (lane 1), epimastigotes (lane 2) or amastigotes (lane 3) were separated in a 2% agarose-formaldehyde gel, transferred onto nylon membranes and probed with  $[\gamma\text{-}^{32}\text{P}]$ chagasin cDNA in 5× SSC, 5× Denhardt's, 1% SDS, 50 µg/ml salmon sperm DNA, 50% formamide at 42°C, overnight, washed three times with 0.2×SSPE, 0.1% SDS, and exposed overnight at -70°C. Left panel: Ethidium bromide staining of the gel. Right panel: Autoradiograph. The approximate position of co-electrophoresed RNA molecular weight marker is indicated on the right.



**Fig. 5.** Screening of anti-chagasin antibodies in human sera. A panel of 25 sera from chronic chagasic patients, and 25 normal control human sera, were tested at 1:50 dilution against immobilized r-chagasin by ELISA. Following incubation with anti-human IgG conjugated to peroxidase, the color development was detected at 492 nm. Control wells coated with BSA and exposed to serum samples did not yield reactions (not shown).

Tc18 cDNA clone as a probe revealed one band in the range of 300-600 nucleotides (Fig. 4) and showed slightly less chagasin RNA levels in the epimastigote stage (Fig. 4, lane 2). Seeking for additional evidence of the expression of chagasin and/or related antigens during the parasite's life cycle, we investigated the possibility that anti-chagasin antibodies could be present in sera from chronically infected patients. When tested by ELISA, approximately 50% of the chagasic sera gave strong immunoreactivity against r-chagasin, while only weak reactions were observed with sera from normal individuals (Fig. 5). Combined with the western blotting profile, these data indicate that chagasin and/or antigenically related products are expressed by the parasites during *T. cruzi* infection.



**Fig. 6.** Phase partition of n-chagasin following Triton X-114 extraction. Cells representing the three developmental stages of *T. cruzi* (epimastigotes, amastigotes and trypomastigotes) were lysed in 100 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% of pre-condensed Triton X-114, 0.1% (w/v) NaN<sub>3</sub>, 5 mM benzamidine chloride and incubated with GPI-PLC (+) or not (-), for 30 minutes at 30°C. After subjecting these lysates to phase partition in Triton X-114, the samples were run on 15% SDS-PAGE and analyzed by western blotting, using rab $\alpha$ chagasin serum. A, aqueous phase; D, detergent phase.

### Phase partition distribution of chagasin during parasite development

The molecular properties of chagasin were further characterized in Triton X-114 phase partition assays. Western blotting indicated that most of the chagasin molecules detected in trypomastigote and amastigote lysates accumulate in the organic phase, and must, therefore, be associated to the parasite membranes (Fig. 6, D). A contrasting pattern was observed with the insect-stage epimastigotes, since the protein accumulated preferentially in the aqueous phase (Fig. 6, A). The treatment of trypomastigote and amastigote lysates with glycosylphosphatidylinositol phospholipase C (GPI-PLC) completely converted membrane-bound forms of chagasin into soluble forms (Fig. 6, D versus A), suggesting that GPI-anchors may be directly or indirectly responsible for chagasin association to cell membranes.

### Sub-cellular localization of chagasin

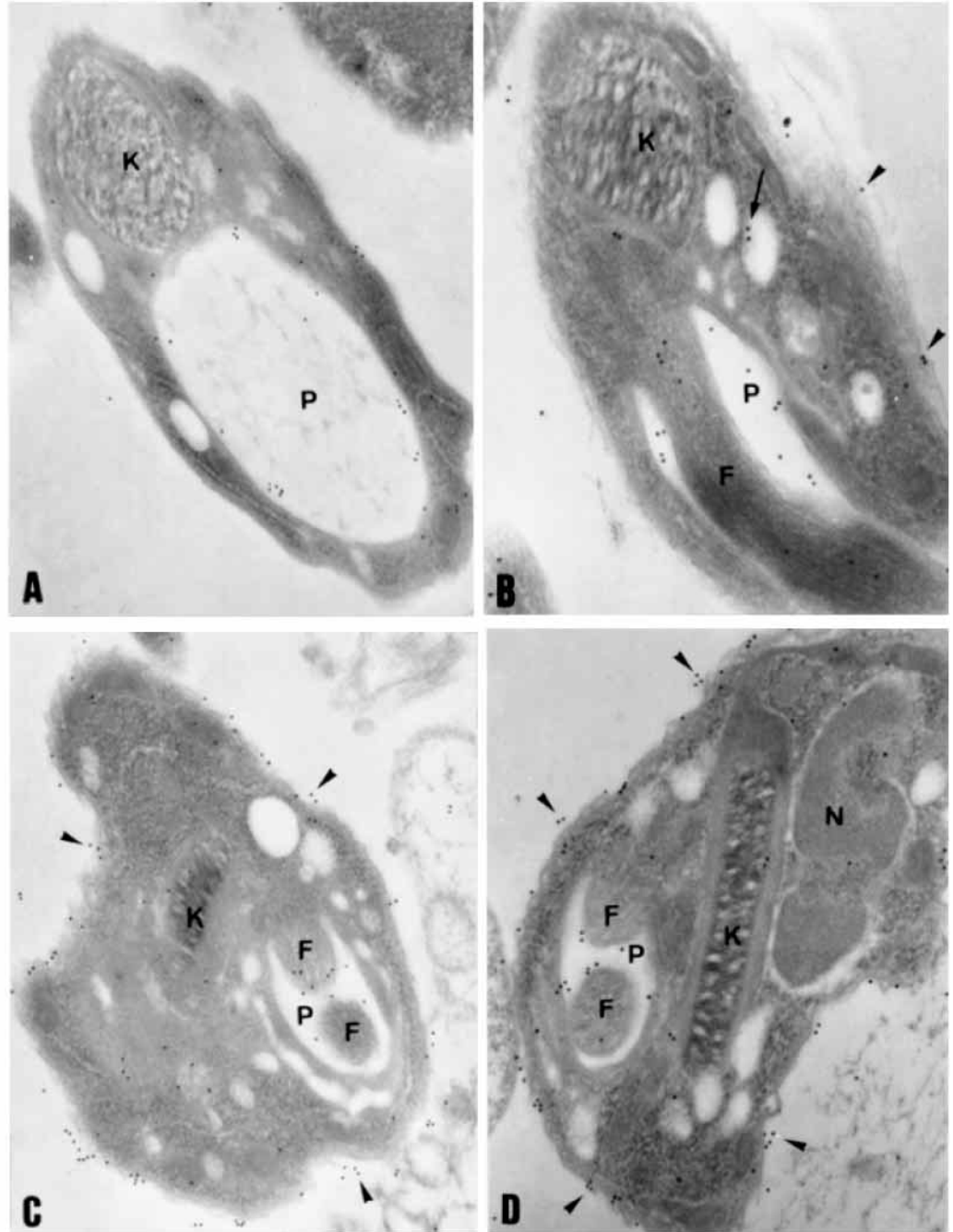
Immunocytochemistry using affinity purified rab $\alpha$ chagasin localized the protein to the flagellar pocket and cytoplasmic vesicles of trypomastigotes (Fig. 7A,B), and to a lesser extent to the cell surface. By contrast, amastigotes (Fig. 7D) and intermediate differentiating parasite forms (Fig. 7C) showed prominent cell surface labeling, which also involved the flagellar pockets' structures. These data further suggest that the cell surface display of chagasin is regulated during morphogenesis.

### Characterization of the cell surface binding sites for chagasin and cruzipain

The availability of functionally active chagasin at the cell surface was checked by incubating living trypomastigotes and amastigotes with FITC-cruzipain at low (4°C) temperature. Confocal microscopy revealed intense labeling of the amastigote cell surface, while trypomastigotes showed a discrete staining pattern (Fig. 8C,D). Controls run by incubating FITC-cruzipain with excess of r-cystatin C or r-chagasin (not shown) prior to parasite addition completely abrogated the surface labeling, suggesting that the binding of the protease probe to the cell surface is mediated through its active site. Conversely, treatment of the parasites with FITC-chagasin led to prominent surface labeling of amastigotes and a weak reaction with trypomastigotes (Fig. 8A,B). The binding of the FITC-chagasin to the cell surface of amastigotes was completely prevented by the addition of excess soluble cruzipain to the incubation medium (Fig. 8E,F). Collectively, these results suggest that amastigotes display functionally active chagasin and cruzipain molecules at their cell surface. The interactions seem to occur to a less extent, in trypomastigotes.

## DISCUSSION

In studies of plants and mammalian cells, it has been shown that regulation of mature lysosomal proteases depends on the activity of tight-binding endogenous inhibitors from the cystatin superfamily (Abrahamson, 1994). To date, the presence of cystatin-like inhibitors in protozoan parasites was not demonstrated, although previous studies in *Leishmania* suggested that trypanosomatids rely on similar molecules to

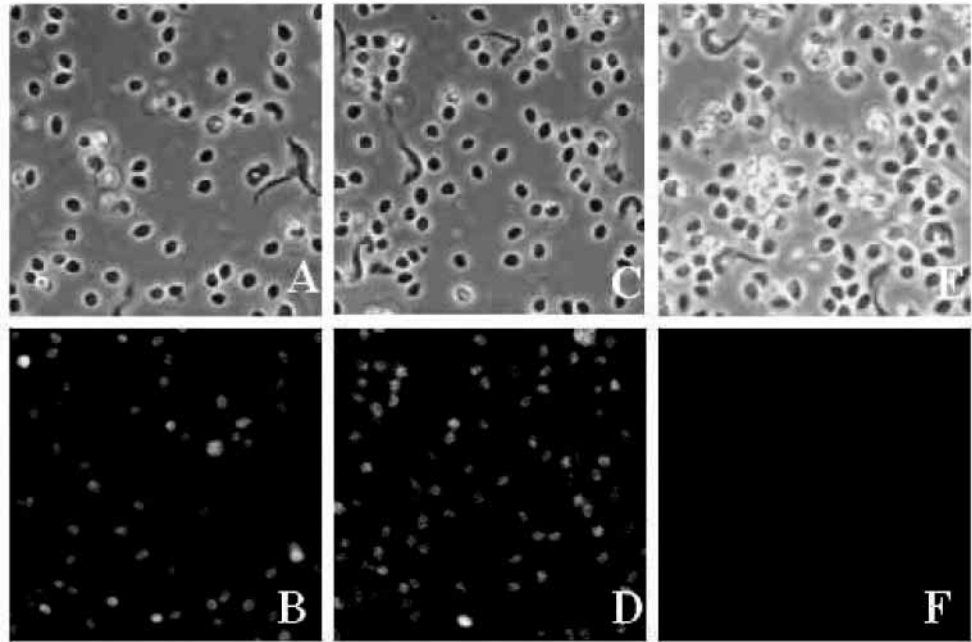


**Fig. 7.** Sub-cellular localization of chagasin. Trypomastigotes (A,B) displayed intense and moderate labeling in flagellar pocket (A) and cytoplasmic vesicles (B, arrow), respectively, but cell surface was poorly labeled (arrowheads). Intermediate forms (C) presented intensely labeled cell surface (arrowheads) as well as flagellar pocket. *T. cruzi* amastigote (D) showing intense immunolabeling at both cell surface (arrowheads) and flagellar pocket. Flagellar pocket (P), flagellum (F), kinetoplast (K) and nucleus (N).

regulate lysosomal cysteine proteases (Irvine et al., 1992). Using *T. cruzi* as a model, we have in the present report identified chagasin, a protein that: (1) acts as a tight-binding reversible inhibitor of papain-like proteases; (2) has a molecular mass similar to those of type 1 and 2 cystatins; and (3) is thermo-resistant. Although the biochemical properties of chagasin is reminiscent of those of cystatins, the cloning of the chagasin gene has revealed that its primary structure presents no homology with members of the cystatin superfamily or with other published sequences in data banks. These properties suggest that trypanosomatids depend on a different class of proteins to regulate the activity of lysosomal cysteine proteases, a function that cystatins have overtaken

later in cellular evolution. As mentioned, both natural and recombinant chagasin are remarkably resistant to heat treatment, a property that served well our attempts to purify it from boiled cell lysates. The purified endogenous protein displayed crossreactivity with r-chagasin and the  $K_i$  values with papain and with different cruzipain isoforms (natural and recombinant) were in the picomolar range, hence similar to those obtained for r-chagasin. Notably, n-chagasin exhibited a somewhat reduced affinity for r-cruzipain 2, an isoform that is also inhibited at lower efficiency by human cystatin C (Lima et al., 2001). Evidence for developmentally regulated expression of chagasin was obtained by immunoblotting of epimastigotes, amastigotes and

**Fig. 8.** Chagasin and cruzipain bind to the cell surface of *T. cruzi* amastigotes and trypomastigotes. Living parasites were washed twice in PBS, 2 mM EDTA, incubated in the same buffer containing FITC-chagasin or FITC-cruzipain, for 30 minutes at 4°C, washed and fixed with 3% paraformaldehyde. Upper panels are phase-contrast and the lower ones are fluorescence microscopy. (A,B) Cell surface binding of FITC-chagasin (E,F) Cell surface binding of FITC-chagasin preincubated with unlabeled n-cruzipain. (C,D) Cell surface binding of FITC-cruzipain.



trypomastigotes. In all parasite forms, chagasin molecules migrating faster (~12 kDa) than r-chagasin were identified; the data revealed that the larger size of the recombinant protein was due to a 16 amino acid residue (1.8 kDa) N-terminal extension giving a molecular weight of 13.8 kDa for this construct. Interestingly, the levels of chagasin were higher in the trypomastigotes (i.e. non-replicating forms) than in the amastigotes and epimastigotes (dividing forms). Given that trypomastigotes are poorly endocytic (De Souza, 1995), it is possible that high levels of chagasin expression in these metabolically quiescent parasitic forms are required to downregulate lysosomal protein catabolism. Conversely, the low level production of chagasin in the epimastigotes may allow for unrestricted lysosomal protein catabolism by these actively dividing and highly endocytic cells (De Souza, 1995).

In addition to the evidence for quantitative difference discussed above, changes in the physicochemical properties of the protein were distinguished between different developmental stages. For example, the majority of chagasin molecules from insect-stage epimastigotes partitioned to the hydrophilic phase, whereas those from amastigotes and trypomastigotes concentrated into the hydrophobic phase after separation in Triton X-114. When treated with GPI-PLC, the latter were converted into hydrophilic molecules, suggesting that GPI-anchors directly and/or indirectly contribute to chagasin anchorage to the plasma membrane of amastigotes and trypomastigotes, as reported for other developmentally regulated molecules (Garg et al., 1997).

Our ultrastructural studies revealed the presence of chagasin in cytoplasmic vesicles and flagellar pocket of trypomastigotes. Similarly to the pattern observed with cruzipain (Murta et al., 1990; Souto-Padrón et al., 1990), antibodies to chagasin reacted weakly with the cell surface of trypomastigotes. Although not explored in this study, it is possible that trypomastigotes may shed and/or secrete chagasin molecules once they adhere to host cells. It is well known that  $[Ca^{2+}]_i$

transients that trypomastigotes induce in the host cells promote the recruitment of peripheral lysosomes to sites of parasite attachment (Rodríguez et al., 1996). Because the host lysosomes fuse with the plasma membrane, the inactivation of released cathepsins by chagasin molecules may spare the internalized trypomastigotes from the potentially detrimental effects of excessive proteolysis.

The finding of prominent cell surface expression of chagasin on amastigotes was surprising, since active forms of cysteine proteases are abundantly expressed at such sites (Engel et al., 1998a). In accordance with this, FITC-chagasin reacted strongly to the amastigote surface. Intriguingly, a strong staining was likewise observed when the amastigotes were treated with FITC-cruzipain. In both assays, the cell surface staining reaction was abolished by addition of an excess of either cystatin C or r-chagasin to the parasite suspension, thus suggesting that chagasin binding involved interactions with the active site pocket of cruzipain.

Although not excluding the possibility that a fraction of the membrane forms of chagasin and cruzipain may combine with each other, our data suggest that extracellular proteolysis in amastigotes may be subjected to spatial and/or temporal constraints. The possibility that free (unoccupied) forms of chagasin and cruzipain are independently sorted from specialized vesicles to the amastigote cell surface is worth exploring, in view of previous findings showing that zymogen molecules from *L. mexicana* are sorted to the flagellar pocket prior to their conversion into mature cysteine proteases by trans-activation mechanisms (Brooks et al., 2000). Although the maturation of procrucipain in insect-stage epimastigotes seems to occur exclusively in the Golgi complex (Engel et al., 1999), the trafficking routes underlying zymogen activation in amastigotes and trypomastigotes have not been characterized. Recent studies indicate that the repertoire of cruzipain isoforms expressed by amastigotes and/or trypomastigotes is broader than in epimastigotes (Lima et al., 2001). It is thus conceivable that *Leishmania* may not be the only

trypanosomatid species in which cysteine protease maturation occurs by trans-activation mechanisms. If true, the coordinated display of chagasin inhibitors at the cell surface may be a checkpoint for physiological regulation of cysteine protease activity. Taken together, the description of this novel inhibitor of cysteine proteases in a trypanosomatid protozoan highlights the peculiarities of protease regulation in primitive eukaryotes.

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