Chagasin, the endogenous cysteine-protease inhibitor of *Trypanosoma cruzi*, modulates parasite differentiation and invasion of mammalian cells

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Summary

Chagasin is a *Trypanosoma cruzi* protein that was recently characterized as a tight-binding inhibitor of papain-like cysteine proteases (CPs). Considering that parasite virulence and morphogenesis depend on the endogenous activity of lysosomal CPs of the cruzipain family, we sought to determine whether chagasin and cruzipain interact in the living cell. Ultrastructural studies showed that chagasin and cruzipain both localize to the Golgi complex and reservosomes (lysosome-like organelles), whereas free chagasin was found in small intracellular vesicles, suggesting that chagasin trafficking pathways might intersect with those of cruzipain. Taking advantage of the fact that sodium dodecyl sulphate and β-mercaptoethanol prevent binding between the isolated proteins but do not dismantle preformed cruzipain-chagasin complexes, we obtained direct evidence that chagasin-cruzipain complexes are indeed formed in epimastigotes. Chagasin transfectants (fourfold increase in CP inhibitory activity) displayed low rates of differentiation (metacyclogenesis) and exhibited increased resistance to a synthetic CP inhibitor. These phenotypic changes were accompanied by a drastic reduction of soluble cruzipain activity and by upregulated secretion of cruzipain-chagasin molecular complexes. Analysis of six *T. cruzi* strains revealed that expression levels of cruzipain and chagasin are variable, but the molar ratios are fairly stable (~50:1) in most strains, with the exception of the G strain (5:1), which is poorly infective. On the same vein, we found that trypomastigotes overexpressing chagasin are less infective than wild-type parasites in vitro. The deficiency of chagasin overexpressers is caused by lower activity of membrane-associated CPs, because membranes recovered from wild-type trypomastigotes restored infectivity and this effect was nullified by the CP inhibitor E-64. In summary, our studies suggest that chagasin regulates the endogenous activity of CP, thus indirectly modulating proteolytic functions that are essential for parasite differentiation and invasion of mammalian cells.

Key words: Trypanosome, Cysteine protease, Chagasin, Inhibitor, Infectivity

Introduction

Cysteine peptidases (CPs) belonging to the C1 family of the papain superfamily have been implicated in the control of growth, differentiation and survival of several pathogenic protozoa (for a review, see Sajid and McKerrow, 2002). In *Trypanosoma cruzi*, the causative agent of human Chagas' disease, studies with synthetic irreversible CP inhibitors suggested that *T. cruzi* infectivity and intracellular growth depend on the activity of cruzipain, the main lysosomal CP (Meirelles et al., 1992; Harth et al., 1993; Engel et al., 1998a). Encoded by a large polymorphic gene family (Eakin et al., 1992; Campetella et al., 1992), the mature form of cruzipain has a central domain homologous to mammalian lysosomal CPs but this is linked to a unique 130-residue C-terminal extension with unknown function (for a review, see Cazzulo et al., 1997). Cloning and heterologous expression of a few polymorphic cruzipain-encoding genes revealed that some of these isoenzymes display significant differences in their substrate specificities and kinetic properties (Lima et al., 1994; Lima et al., 2001). The solution of the crystal structure of the catalytic domain (McGrath et al., 1995) paved the way for the development of new generations of irreversible synthetic inhibitors, some of which could protect mice from lethal *T. cruzi* infection (Engel et al., 1998b). Recent clues to cruzipain function have come from analyses of the Ca^{2+}-dependent signalling pathways driving *T. cruzi* invasion of endothelial cells and cardiomyocytes (Scharfstein, 2000; Todorov et al., 2003). These studies revealed that parasite infectivity is increased by activation of G-protein-coupled kinin receptors (for a review, see Scharfstein, 2003). Of further interest, recent studies suggested that alternative signalling routes, also dependent on cruzipain-mediated proteolysis, drive *T. cruzi* invasion of human smooth-muscle cells (Aparicio et al., 2004).

Although cruzipain has been extensively characterized at the biochemical (Serveau et al., 1996; Del Nery et al., 1997; Meldal et al., 1998; Serveau et al., 1999; Lima et al., 2001; Lima et al., 2002) and structural (McGrath et al., 1995; Gillmor et al., 1997) levels, little is known about the mechanisms

controlling its activity in vivo. Cruzipain is post-transcriptionally regulated during the parasite's life cycle (Tomas and Kelly, 1996). *T. cruzi* replicative stages (epimastigotes and amastigotes) exhibit high contents of cruzipain, whereas the nondividing infective forms (trypomastigotes) express this enzyme at low levels (Bonaldo et al., 1991). As is true for other trypanosomatid CPs (Sanderson et al., 2000; Caffrey et al., 2001), the proteolytic excision of the N-terminal pro-domain of the zymogen precursor, pro-cruzipain, generates the enzymatically active protease (Eakin et al., 1992). The precise location where cruzipain is activated is still unclear. The discovery of lysosomal targeting sequences in the pro-domain of cruzipain, similarly to leishmania CPs (Huete-Perez et al., 1999) suggests that pro-cruzipain processing might occur in late compartments of the endosomal system. However, chimeras of pro-cruzipain and the green fluorescent protein (GFP) containing a mutation that prevents autocatalytic processing tend to accumulate in the Golgi, suggesting that maturation might normally occur in this compartment (Huete-Perez et al., 1999). Consistent with this notion, studies of the cellular toxicity induced by synthetic CP inhibitors revealed the presence of more unprocessed pro-cruzipain molecules in the Golgi complex (Engel et al., 1998a).

Cysteine proteinase inhibitors of the cystatin superfamily are fairly ubiquitous in nature (for a review, see Abrahamson et al., 2003) but are apparently absent from the genomes of trypanosomatids. Early studies described the presence of a CP inhibitory activity in the extracts of *Leishmania* (Irvine et al., 1992). More recently, we have cloned and expressed chagasin, a single-chained *T. cruzi* protein (109 amino acid residues) that was characterized as a novel type of tight-binding inhibitor of papain-like CPs (Monteiro et al., 2001). Interestingly, chagasin displays similar physicochemical properties (heat stability) to CP inhibitors of the cystatin family (Monteiro et al., 2001), although their primary sequences are not similar. Of further interest, computational screens suggested that chagasin orthologues might be present in some prokaryotes and lower eukaryotes (Ridgen et al., 2002). More recently, the cloning and expression of chagasin-like genes from *Trypanosoma brucei*, *Leishmania mexicana* and *Pseudomonas aeruginosa* (Sanderson et al., 2003) offered definitive proof that these proteins are members of a new family of cysteine peptidase inhibitors, provisionally termed ICPs (inhibitors of cysteine peptidases).

In spite of progress made in the molecular characterization of chagasin, there is no direct evidence that this protein acts as an endogenous regulator of *T. cruzi* cysteine peptidases. The possibility that chagasin is targeted to the endosomal or lysosomal compartments must be reconciled with the fact that its gene sequence does not predict any obvious signal peptide motif and precludes the formation of disulfide bridges, a structural feature that distinguishes secretory cystatins (type II) from their cytoplasmic counterparts (type I). The complexity of this issue is highlighted by the recent finding that mammalian cystatin F (type II) is not targeted to the endosomal/lysosomal system, revealing that, in this case, the inhibitor is segregated from the endogenous cysteine peptidases (Cappello et al., 2004).

Here, we investigated the biological role of chagasin in wild-type (WT) and genetically manipulated *T. cruzi*. Our results suggest that chagasin might act as an endogenous modulator of cruzipain function in *T. cruzi* and that chagasin-mediated regulation plays an important role in parasite differentiation and infectivity.

Materials and Methods

Parasite culture and lysates

Epimastigotes from the strains Dm28c, G, Y, Sylvio X10/6, Brazil and CL were grown in liver infusion tryptose (LIT) medium supplemented with 10% foetal calf serum (FCS) at 28°C. Lysates were obtained by repeated cycles of freeze and thaw of the epimastigotes in 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2 (PBS), followed by the addition of Triton X-100 to 1% final concentration. After 10 minutes on ice, the samples were cleared by centrifugation at 13,000 ***g*** for 15 minutes and the protein concentration of the supernatant was determined using the Dc-Bioassay kit (Bio-Rad).

Parasite transfection

The chagasin-encoding gene was cloned into the *Bam*HI site of the pTEX episomal shuttle vector (Kelly et al., 1992). Log-phase epimastigotes (5×10^8) were washed twice in PBS and resuspended in 25 mM HEPES, 140 mM NaCl, 0.4 mM Na_2HPO_4, pH 7.0. The cells were then transferred to a 0.2 cm electroporation cuvette, incubated with 30 μg purified plasmid (empty vector, MOCK; pTEX-chagasin, pCHAG) on ice for 5 minutes and subsequently electroporated with one pulse at 500 μF, 350 kV. The cells were cultivated overnight in LIT medium containing 10% FCS and subsequently selected for 4 weeks with 200 μg ml^{-1} geneticin (G418, Life Technologies), followed by a second round of selection in medium supplemented with 800 μg ml^{-1} G418 for additional 4 weeks. The transfected cell populations were subsequently analysed by Southern blot. Briefly, DNA (15 μg) from epimastigotes was digested with *Hin*dIII, separated on a 0.8% agarose gel and transferred to a nylon membrane. A *Pst*I/*Kpn*I 900 bp fragment of pTEX containing the neomicin-encoding gene was used as a probe. Single fragments of the expected sizes were observed in MOCK (5.6 kb) and pCHAG parasites (6 kb).

Antibodies and western blotting

Approximately 100 μg purified n-cruzipain [obtained as described by Murta et al. (Murta et al., 1990)] was mixed 1:1 with complete Freund's adjuvant and inoculated intraperitoneally in mice. After 21 days, the animals received three consecutive boosts at 2-week intervals with additional 100 μg each of the antigen, until an adequate response was achieved. Monoclonal antibody (mAb 212BH6) against cruzipain (Murta et al., 1990) and rabbit antiserum against chagasin (Monteiro et al., 2001) were obtained as described. Monoclonal antibodies against the cathepsin-B-like protease (mAb 2AD3) were obtained as previously described (Yong et al., 2000). Monoclonal antibodies to *T. cruzi* calreticulin were used as a loading control for western blots, at 1:2000 dilutions (provided by A. Ferreira, University of Chile, Santiago). Western blots for CPs were performed using 20 μg epimastigote lysate loaded onto a 11% polyacrylamide gel, and the membranes were blocked with 9% non-fat milk in PBS, 0.05% Tween-20. For detection of chagasin, 100 μg samples were subjected to 14% sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and western blots was performed as described above, using antiserum against chagasin (1:1000 dilution). For biotin-N-Pip-F-hF-VSPh blots, 20 μg samples of the lysates were incubated with 10 μM biotin-N-Pip-F-hF-VSPh for 2 hours at 37°C. Reactions were stopped by the addition of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol (βME), 0.012% bromophenol blue) and subsequently boiled for 3 minutes. Samples were loaded into 11% SDS-PAGE gel, transferred to nitrocellulose membranes, blocked with skimmed milk and incubated

for 60 minutes with alkaline-phosphatase-conjugated streptavidin. The reactive bands were visualized using the BCIP-NBT solution provided by the manufacturer.

Stability of the cruzipain-chagasin complex

The effect of SDS plus βME on preformed cruzipain-chagasin complexes was studied using purified Dm28c cruzipain (Murta et al., 1990) and recombinant chagasin (Monteiro et al., 2001) obtained as described. The complexes were formed by incubating the isolated molecules in approximately equal molar ratios for 20 minutes in PBS. The molecular complexes were then subjected to the following treatments: buffer alone (62.5 mM Tris-HCl, pH 6.8); buffer supplemented with 2% SDS; buffer supplemented with 2% SDS and 5% βME; buffer supplemented 2% SDS and 5% βME, and boiling for 5 minutes. For the detection of cruzipain-chagasin complexes formed in vivo, mid-log-phase epimastigotes (10^7) were lysed in 100 mM Tris-HCl buffer, pH 6.8, 2% (w/v) SDS, 5% (w/v) βME, 10% (v/v) glycerol, 0.012% (w/v) bromophenol blue and subjected to 11% SDS-PAGE and a western blot. The membrane was further incubated with anti-chagasin antiserum (1:1000) and the reactive bands were visualized as described in the previous section.

Enzymatic assays and titrations of cruzipain and chagasin

Lysates from epimastigotes were tested for CP activity in 50 mM Na_2PO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM dithiothreitol (DTT), 5% dimethyl sulfoxide (DMSO) at room temperature, using of 5 μM fluorogenic synthetic peptide Z-Phe-Arg-MCA (Sigma) as a substrate. Substrate hydrolysis was monitored in continuous assays in a Hitachi F-4500 spectrofluorimeter at 380 nm excitation and 440 nm emission. The linear regression of the substrate hydrolysis curves was used to calculate initial velocities as described (Lima et al., 2001). The titration of epimastigote lysates to calculate cruzipain molar concentration was performed using the irreversible synthetic inhibitor N-Pip-F-hF-VSPh (J. McKerrow, UCSF, San Francisco, CA, USA), which binds preferentially to the active site of cruzipain (equilibrium constant for inactivation K_i=0.360 μM) compared with cathepsin B (K_i=11 μM) (Palmer et al., 1995). To ensure selectivity for cruzipain in the titrations, the lysates were diluted to keep the enzyme concentration in the low nanomolar range. The titration was performed upon incubation of the normalized and diluted lysates with various inhibitor concentrations at room temperature for 30 minutes, followed by the determination of residual peptidase activity as described above. Enzyme concentration was calculated by linear regression of the initial velocity (V_0) × inhibitor concentration ([I]) plots, assuming [I] at which V_0=zero. In order to determine the chagasin concentration, epimastigote lysates were boiled for 5 minutes and submitted to centrifugation at 10,000 ***g*** for 10 minutes at 4°C. The soluble material was collected and different dilutions were incubated with 3.32 nM papain that had been previously titrated with E-64, as described (Abrahamson, 1994), in 50 mM Na_2PO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, for 20 minutes at room temperature. The remaining activity was measured by the addition of Z-Phe-Arg-MCA to a final concentration of 5 μM as described above.

In vivo affinity labelling of active CPs

Log-phase epimastigotes were incubated with 10 μM CP synthetic inhibitor biotin-N-Pip-F-hF-VSPh for 1 hour at 28°C in LIT. The cells were washed twice in PBS and submitted to five cycles of freeze and thaw. Soluble and membrane fractions from epimastigote lysates were fractionated by centrifugation at 100,000 ***g*** for 1 hour at 4°C. The pellet (membrane fraction) was resuspended in PBS containing 1% Triton X-100 to the original volume. The samples (20 μg) were resolved by 11% SDS-PAGE, transferred to nitrocellulose membrane and probed with streptavidin/alkaline-phosphatase.

Analysis of epimastigote secretion products

For secretion experiments, parasites were washed twice in PBS, resuspended in this buffer (5×10^8 cells ml^{-1}) and incubated for 1 hour at 28°C. Culture supernatants were collected by centrifugation at 3000 ***g*** for 10 minutes, filtered in a 0.2 μm membrane (Millipore) and the cysteine peptidase activity of supernatants (80 μl) were measured as described in the previous section. For the detection of the inhibitory activity, the supernatants were boiled for 5 minutes and the soluble fraction was collected by centrifugation (10,000 ***g*** for 10 minutes). The inhibitory activity was subsequently measured by preincubating the soluble fraction (150 μl) with papain for 20 minutes at room temperature, followed by the determination of residual peptidase activity as described above. For the detection of the cruzipain-chagasin complex, 50 μl samples of supernatants were diluted in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% βME, 0.012% bromophenol blue (no boiling), resolved by 11% SDS-PAGE and submitted to a western blot using the anti-chagasin antiserum as described above.

Estimating the IC_{50} of N-Pip-F-hF-VSPh for the growth of epimastigotes

Epimastigotes were inoculated in LIT containing 10% FCS at 10^6 cells ml^{-1} supplemented with 1% DMSO or with 1% DMSO and variable concentrations (2 μM, 4 μM, 6 μM or 8 μM) of the synthetic irreversible cysteine peptidase inhibitor N-Pip-F-hF-VSPh. The parasites were cultivated for 7 days at 28°C and the growth was estimated by daily counts of the cultures using a haemocytometer chamber. The experiments were performed in triplicate. The 50% inhibitory concentration (IC_{50}) is the drug concentration at which a 50% reduction of growth was observed.

Metacyclogenesis

Stationary-phase epimastigotes (8×10^7 cells ml^{-1}) were inoculated at 1:5 (vol/vol) dilution in Grace's insect medium, pH 6.5 (Gibco-BRL) and cultivated for 10 days at 28°C. The metacyclogenesis rate was estimated by counting the number of trypomastigotes per 500 epimastigotes from day 5 to day 8 of the culture. At day 9, cell death started to occur. The experiments were performed in triplicate.

Mammalian cell invasion by tissue-culture trypomastigotes

Stationary-phase epimastigotes (WT, MOCK and pCHAG) were differentiated to metacyclic trypomastigotes upon cultivation for 5-8 days in Grace's insect medium, pH 6.5 at 28°C, supplemented with 800 μg ml^{-1} geneticin. The culture was used to infect monolayers of LLCMK2 cells, cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% FCS at 37°C in a 5% CO_2 humidified atmosphere. After 5 hours, the extracellular parasites were removed by repeated washes and the cells were cultivated for additional 4-5 days in DMEM containing 2% FCS. Trypomastigotes released to the supernatant were collected by a 3000 ***g*** centrifugation for 15 minutes, washed twice in Hank's balanced salt saline supplemented with 1 mM glucose (HBSS) and used in invasion assays. Because the culture could not be maintained in the presence of geneticin owing to its toxicity to the mammalian cells, the presence of the plasmid in tissue-culture trypomastigotes was verified by Southern blot using the *neo* gene as a probe, as described above. Chagasin overexpression was determined by measuring the inhibition of papain activity upon incubation with boiled parasite lysates, as described above; pCHAG-containing tissue-culture trypomastigotes maintained a fourfold overexpression of chagasin for 5 weeks in the absence of geneticin. Human primary cultures of smooth-muscle cells (<20 passages) were purchased at the Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil) and cultivated in DMEM containing 10% FCS at 37°C, as described (Aparicio et al., 2004). Invasion assays were

performed as described (Scharfstein et al., 2000). Briefly, the cells were plated on coverslips in 24-well plates and cultivated until semiconfluent. Freshly released trypomastigotes were washed twice in HBSS and incubated with host cells at a 5:1 parasite:host cell ratio (unless otherwise stated in the figure legends) in DMEM, 0.1% bovine serum albumen (BSA) for 3 hours, at 37°C in 0.6 ml. Extracellular parasites were removed by extensive washes, the cells were fixed with Bouin, stained with Giemsa and the coverslips were mounted on entellan (Merck). The number of intracellular parasites was estimated by counting under the light microscope. All assays were performed in triplicates and in at least three independent experiments.

Statistical analysis

The analysis of significance of the data was performed by analysis of variance (ANOVA) using GraphPad Prism 4.0. The data were analysed by one-way ANOVA using the Bonferroni post-test comparing all pairs of columns at a significance level of 5% for Fig. 3B,C, Fig. 6A,C, Fig. 7 and Fig. 11C,D. The scores showing statistical significance are indicated in the figures with asterisks and the *P* values are indicated in the legends. The analysis of significance on Fig. 11A,B were performed using one-way ANOVA and the linear trend post-test. The analyses of significance of parasite growth in the presence of the CP inhibitor (Fig. 8) were performed using two-way ANOVA and the Bonferroni post-test. The analyses of variance were performed on the estimated growth rate between days 4 and 5, because it is at day 4 that the effect of the drug on growth can be safely observed. The growth rate was calculated as the difference in the number of parasites per ml between days 4 and 5. For Fig. 7, ANOVA was performed on the rate of parasite differentiation that was calculated as the difference between the number of trypomastigotes per 500 epimastigotes at each day in culture, using the results of days 5-8. In Table 1, the error of each titration curve (linear regressions) was corrected for error propagation considering the individual errors of the slopes and of the constants. The errors of the ratios were subsequently corrected using the appropriate equation for the calculation of error propagation and found to be below 20% in all cases.

Enzymatic characterization of trypomastigote supernatants

Trypomastigotes were washed three times in HBSS, resuspended at 2×10^7 ml^{-1} in this solution and incubated for 2 hours at 37°C to allow the secretion of soluble components and the shedding of plasma membrane vesicles (Gonçalves et al., 1991). The suspension was centrifuged at 3000 ***g*** for 15 minutes and parasite-free supernatants were filtered through 0.2 μm pore-size membranes (Millipore). The membrane fraction was recovered by centrifugation at 100,000 ***g*** for 1 hour at 4°C, and the pellet (of the membrane fraction) was resuspended to the original volume in HBSS containing 0.1% BSA. For invasion assays, 100 μl of the membrane fraction was incubated with 2.5 mM DTT for 5 minutes at 37°C in order to activate CPs; where indicated, E-64 was added at 10 μM. The samples were subsequently added to the invasion assays. Controls were performed by addition of HBSS containing 2.5 mM DTT. The peptidase activity present in the sample was determined by hydrolysis of 5 μM Z-Phe-Arg-MCA in 50 mM Na_2PO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, 5% DMSO at room temperature.

Effect of chagasin on mammalian CPs

Smooth-muscle cells were cultivated to semiconfluence in DMEM containing 10% FCS, washed twice in HBSS and incubated in DMEM containing 2% FCS with PBS buffer alone or with PBS containing either E-64 or different concentrations of recombinant chagasin for 1 hour at 37°C. Chagasin concentration was estimated by titration using papain (previously active-site titrated with E-64) as a reference enzyme. The cells were washed three times with HBSS and lysed in 100 mM sodium acetate, 150 mM NaCl, pH 5.5, 1% Triton X-100 and incubated for 10 minutes on ice. The soluble fraction was recovered by centrifugation at 10,000 ***g*** for 10 minutes and 0.25 μg ml^{-1} lysates were assayed for peptidase activity as described above.

Table 1. Cruzipain-chagasin molar ratios in different *T. cruzi* isolates

Isolates	Cruzipain (nM)	Chagasin (nM)	Ratio
Dm28c	4500	118	38
G	410	85.7	4.8
Y	3500	80.3	45
Silvio X10/6	4000	81.4	48.8
CL	1500	25.7	57.7
Brazil	2500	53.3	46.7

Epimastigote lysates were normalised to 1 μg ml^{-1} and submitted to serial dilution to perform active-site titration of cruzipain using N-Pip-F-hF-VSPh. Fixed aliquots of lysates were incubated with increasing concentrations of the compound for 1 hour at room temperature in 50 mM Na_2HPO_4, 200 mM NaCl, 5 mM EDTA, pH 6.5, 5 mM DTT and the residual peptidase activity was determined by the hydrolysis of CBZ-Phe-Arg-AMC. The enzyme concentration in the titration experiments was always in the nanomolar range. For the titration of chagasin, normalized lysates were boiled and the soluble fraction was collected. Different aliquot volumes of these samples were incubated with 3.32 nM papain in the same buffer as above for 20 minutes at room temperature and the residual activity was measured as described above. The error of each titration experiment was within 5-15% and the errors of the ratios were corrected for error propagation using the appropriate equation. The ratio errors were kept below 20% and the errors of the 95% confidence interval overlapped in all cases except the G strain, indicating that the cruzipain:chagasin ratio of the G strain is the significantly different ratio.

Immunofluorescence

Mid-log-phase epimastigotes were washed twice in PBS and fixed in PBS containing 4% paraformaldehyde for 30 minutes at room temperature. The cells were washed twice in PBS, adhered to poly-L-lysine (Sigma) coated glass coverslips and permeabilized by treatment with PBS containing 1% NP-40 for 1 minute, followed by extensive washes in PBS. The parasites were incubated with 50 mM ammonium chloride and unspecific sites were blocked with PBS containing 3% BSA for 30 minutes. The incubations with antibodies were performed for 1 hour at room temperature with rabbit anti-chagasin antiserum and mouse anti-cruzipain antiserum (diluted 1:100 and 1:500, respectively) in the blocking buffer, followed by two washes in PBS and incubation with Alexa-488-conjugated anti-mouse antibody and Alexa-546-conjugated anti-rabbit antibody for 1 hour at room temperature. Glass coverslips were examined under a Zeiss confocal laser scan microscope LSM 510.

Cryoimmunoelectron microscopy

Epimastigotes were fixed in PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde, washed, infiltrated with 2.3 M sucrose, transferred to specimen freezing supports and quickly frozen by immersion into liquid nitrogen. Supports were transferred to a cryoultramicrotome (Reichert), with which cryosections were obtained at –70°C, collected in 2.3 M sucrose, placed on Formvar/carbon-coated nickel grids and transferred to PBS containing 3% BSA. Sections were blocked with PBS containing 50 mM ammonium chloride and 3% BSA before incubation with rabbit anti-chagasin antiserum (1:100) and mouse anti-cruzipain antiserum (1:500), followed by goat anti-rabbit IgG coupled to 5 nm gold particles and goat anti-mouse IgG coupled to 15 nm gold particles (at 1:100), respectively. Labelling was observed under a Jeol 1200 EX electron microscope after uranyl-acetate and lead-citrate staining.

Results

Evidence that chagasin and cruzipain form molecular complexes in vivo

Studies of the intracellular trafficking pathways of mammalian cystatin F provided evidence for the physical segregation between an endogenous inhibitor of papain-like enzymes and the endosomal/lysosomal CPs (Cappello et al., 2004). Given that there is no predictable signal peptide in the chagasin sequence, we asked whether the trafficking pathways of chagasin and cruzipain could intersect in epimastigotes. Immunofluorescence showed a punctate pattern of chagasin distribution throughout the cell, compatible with vesicular localization (Fig. 1C). Consistent with ultrastructural data (Murta et al., 1990; Souto-Padron et al., 1990), we confirmed that cruzipain accumulates in the reservosome, a lysosome-like organelle (Soares et al., 1992) located at the posterior end of epimastigotes (Fig. 1B, arrow). Discrete cruzipain staining was observed at the anterior end (Fig. 1B, arrowhead), an area that is close to the Golgi complex in this parasite (De Souza, 2002). Merged images revealed intense colocalization of cruzipain and chagasin at both the posterior and the anterior ends of the cell (Fig. 1D). The organelles where cruzipain and chagasin colocalise were subsequently identified as the reservosome (Fig. 1E,F) and the Golgi complex (Fig. 1G) by electron microscopy. We frequently observed chagasin and cruzipain in close proximity (Fig. 1F) in the reservosomes. Chagasin was also frequently observed in small vesicles free of cruzipain (Fig. 1E,G, white arrows). Because proteolytically active cruzipain is primarily found at reservosomes and presumably also at the Golgi stacks (Souto-Padron et al., 1990; Engel et al., 1998a), the present data suggest that the protease might be interacting with chagasin at these compartments. The detection of cruzipain-chagasin molecular complexes formed in vivo is not trivial because, upon cell lysis, one would expect free chagasin molecules to bind rapidly to mature forms of endogenous cysteine proteases, the latter present in high excess over chagasin. We therefore searched for conditions that would not cause dissociation of preformed complexes while preventing the association between free enzyme and inhibitor molecules. To this end, isolated cruzipain and chagasin were mixed at an equal molar ratio and the resulting molecular complexes then diluted in various buffers. The identification of high-molecular-mass complexes containing chagasin was identified by immunoblotting. As shown in Fig. 2A, high-molecular-mass complexes between cruzipain and chagasin persisted after sample treatment with Tris buffer alone (Fig. 2A, lane 1), 2% SDS (Fig. 2A, lane 2) or 2% SDS and 5% βME (Fig. 2A, lane 3). By contrast, chagasin-cruzipain complexes were completely dissociated upon boiling in the SDS/βME buffer (Fig. 2A, lane 4). These data indicated that cruzipain-chagasin complexes remain stable in the presence of SDS-βME, only being dismantled upon heating. Another important observation was that free cruzipain was rendered catalytically inactive upon SDS-βME treatment and was thus incapable to associate with free chagasin (Fig. 2B, lane 2 vs lane 1).

Based on these findings, we predicted that, upon disruption of living cells by addition of SDS-βME, preformed enzyme-inhibitor complexes would remain bound, whereas the free cruzipain would not associate with chagasin. We therefore added SDS-βME to living epimastigotes (Fig. 2C) and subjected the resulting cell lysates to immunoblotting with anti-chagasin antibodies. Importantly, the samples were not boiled before loading, because this would promote chagasin dissociation from CPs. Our results revealed the presence of high-molecular-mass enzyme-inhibitor complexes in

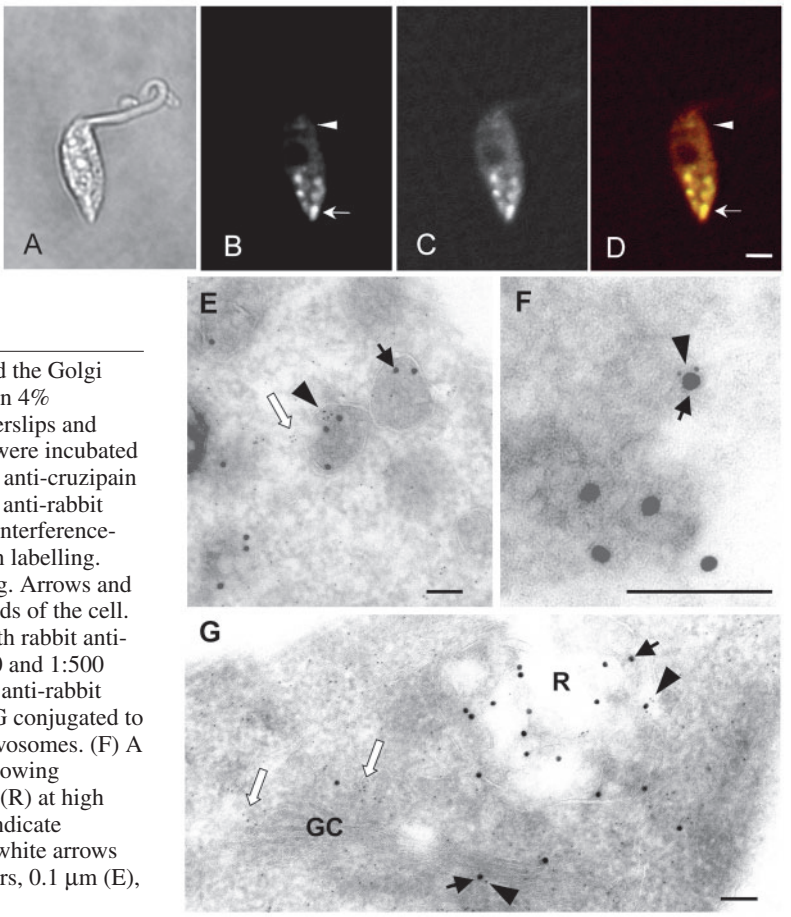

Fig. 1. Cruzipain and chagasin co-localize to reservosomes and the Golgi apparatus of *T. cruzi* epimastigotes. Epimastigotes were fixed in 4% paraformaldehyde, adhered on poly-L-lysine-coated glass coverslips and permeabilized with PBS containing 1% NP-40. The parasites were incubated overnight at 4°C with anti-chagasin rabbit antiserum or mouse anti-cruzipain antiserum, followed by incubation with Alexa-543-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies. (A) Differential-interference-contrast image. (B) Anti-cruzipain labelling. (C) Anti-chagasin labelling. (D) Merged image of anti-cruzipain and anti-chagasin labelling. Arrows and arrowheads, respectively, indicate the posterior and anterior ends of the cell. Scale bar, 2 μm. Epimastigote cryosections were incubated with rabbit anti-chagasin antiserum or mouse anti-cruzipain antiserum at 1:100 and 1:500 dilutions. The sections were subsequently incubated with goat anti-rabbit IgG conjugated to 5 nm gold particles and goat anti-mouse IgG conjugated to 15 nm gold particles (at 1:100), respectively. (E) *T. cruzi* reservosomes. (F) A higher-magnification image of the interior of a reservosome showing cruzipain and chagasin in close proximity. (G) A reservosome (R) at high magnification, next to the Golgi complex (GC). Dark arrows indicate cruzipain staining, arrowheads indicate chagasin staining and white arrows point to chagasin inside cruzipain-free small vesicles. Scale bars, 0.1 μm (E), 0.4 μm (F) and 0.1 μm (G).

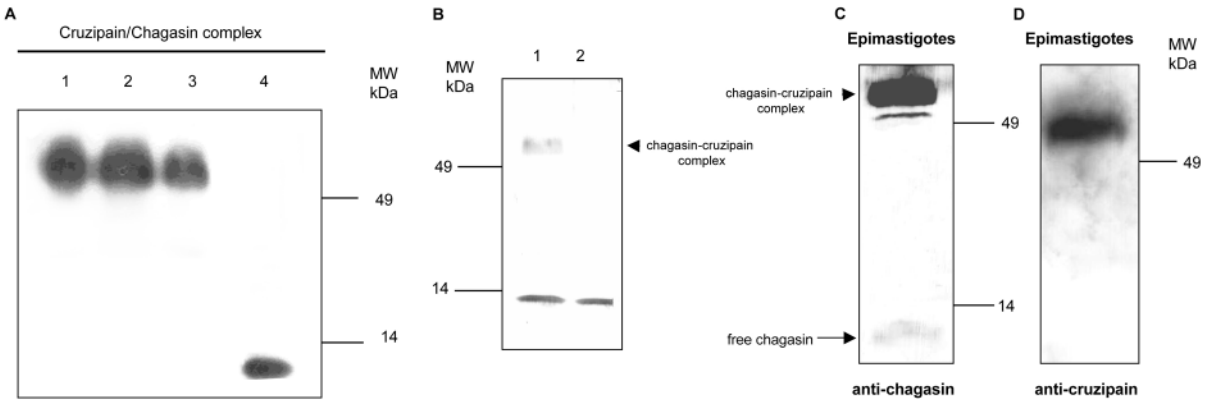

Fig. 2. Detection of the cruzipain-chagasin molecular complex in vitro and in vivo. (A) The cruzipain-chagasin complex is stable upon treatment with SDS-βME. Purified cruzipain and recombinant chagasin were incubated at equal concentrations in PBS, pH 7.2, for 20 minutes and subsequently diluted in different solutions: buffer alone (62.5 mM Tris-HCl pH 6.8, 10% glycerol) (lane 1); buffer containing 2% SDS; buffer containing 2% SDS and 5% βME; and buffer containing 2% SDS and 5% βME, and boiled for 5 minutes. The samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and incubated with rabbit anti-chagasin antiserum (1:1000). (B) Free cruzipain is inactivated by SDS-βME. The cruzipain-chagasin complex was formed as described in A and incubated in buffer containing 2% SDS and 5% βME (lane 1) or cruzipain was diluted in the SDS-βME buffer immediately before the addition of chagasin (lane 2). The samples were not boiled before loading in SDS-PAGE. Western blots with anti-chagasin antibodies were performed as described above. (C,D) Detection of the cruzipain-chagasin complex in living parasites. Epimastigotes (5×10^6) were washed twice in PBS and lysed directly by addition of a solution containing 2% SDS and 5% βME. The samples (not boiled) were resolved by SDS-PAGE and submitted to western blot with anti-chagasin antibodies (C) or anti-cruzipain anti-serum (1:1000) (D).

epimastigote lysates obtained with SDS-βME, indicating that the molecular complexes were indeed preformed in the living cell. (Fig. 2D). Importantly, a small proportion of free chagasin was also detected in such lysates (Fig. 2C). Because cruzipain is present in large molar excess over chagasin in epimastigotes, the detection of a small proportion of free chagasin supports the premise that SDS-βME has prevented the binding of free chagasin to excess CPs upon cell lysis. Moreover, the detection of free chagasin in epimastigotes is consistent with ultrastructural data showing the presence of chagasin in vesicles devoid of cruzipain (Fig. 1, white arrows).

Chagasin overexpression leads to downregulation of functional cruzipain

The control of cruzipain expression is primarily post-transcriptional, but the underlying mechanism is not well understood (Tomas et al., 1996). Given indications that chagasin associates with mature cruzipain in epimastigotes, we asked whether cruzipain enzymatic activity was sensitive to changes in the expression levels of chagasin. We took advantage of the fact that chagasin is expressed at very low levels in epimastigotes (Monteiro et al., 2001), this developmental stage being a convenient model system to study the effect of chagasin overexpression through parasite transfection. This goal was accomplished using the episomal pTEX vector containing the chagasin-encoding gene, followed by prolonged selection of the transfected populations in the presence of geneticin (>8 weeks). Stable parasite populations bearing empty vector (MOCK) or vector containing the chagasin-encoding gene (pCHAG) were analysed by Southern blot using the *neo* gene as a probe. The probe hybridized with DNA fragments of the expected sizes (data not shown). Both transfected parasites grew similarly to the WT in culture and presented no visible morphological alteration (data not shown). As expected, epimastigotes that received the pCHAG construct had increased levels of this protein (Fig. 3A, lane 3), whereas no detectable changes in the chagasin contents were seen in parasites transfected with empty vector (MOCK) (Fig. 3A, lane 2), compared with the WT (Fig. 3A, lane 1). Chagasin overexpression was confirmed by the detection of its inhibitory activity in epimastigotes lysates using papain as a target enzyme (Fig. 3B). We found that pCHAG lysates had higher inhibitory activity than lysates from MOCK or WT cells (Fig. 3B). The inhibitory activity present in the lysates of mock-transfected parasites was undistinguishable from that of untransfected cells. Importantly, in order to detect chagasin activity, we boiled the parasite lysates, a procedure that: (i) inactivates endogenous CPs and/or other peptidases, thus precluding their involvement in the enzymatic assays; and (ii) dismantles preformed cruzipain-chagasin complexes without disturbing chagasin function, because chagasin is a heat-stable protein (Monteiro et al., 2001). Inhibitor titration with papain further indicated that pCHAG contained approximately four times more CP-inhibitory activity (537 nM) than MOCK (122 nM) or WT (118 nM).

The content of functional peptidases in parasite lysates was then assessed by enzymatic assays using fluorogenic substrates. Our data revealed that chagasin overexpression resulted in a marked reduction in the levels of functional CPs (Fig. 3C). MOCK parasites presented a slightly lower CP activity than WT (17% drop) but much less than the decrease in CP activity observed in pCHAG cells (85% drop in activity). However, pCHAG parasites did not show altered levels of cruzipain expression at both protein and RNA levels (data not shown). A similar result was obtained when we checked for the expression of the parasite's cathepsin-B-like 30 kDa cysteine peptidase (TcCB) at the protein level (data not shown). Combined, these results indicate that chagasin overexpression did not induce changes in the expression and/or turnover of

these enzymes. Similarly, *T. cruzi* epimastigotes transfected with pTEX-cruzipain did not display any alterations in the contents of chagasin at the protein level (F. C. G. Reis et al., unpublished). To confirm that the marked reduction in CP activity observed in pCHAG was not an artefact resulting from enzyme-inhibitor association after parasite lysis, we sought to label active cruzipain in living parasites by incubating the cells with biotin-N-Pip-F-hF-VSPh, the assumption being that this membrane-permeable irreversible synthetic inhibitor would only bind to free enzyme molecules. The amount of active-site-labelled enzyme present either in the soluble or membrane fractions was subsequently evaluated by western blot using avidin-peroxidase. Our data showed significantly reduced labelling of soluble forms of cruzipain by the biotinylated inhibitor in pCHAG (Fig. 4A, lane 3) compared with MOCK (Fig. 4A, lane 2) and WT (Fig. 4A, lane 1). The small (5%) reduction in CP labelling in MOCK cells was not considered to be relevant compared with the reduction of CP labelling observed in pCHAG cells (70%). Surprisingly, there was no change in the labelling of membrane-associated protease from pCHAG (Fig. 4B), suggesting that chagasin overexpression

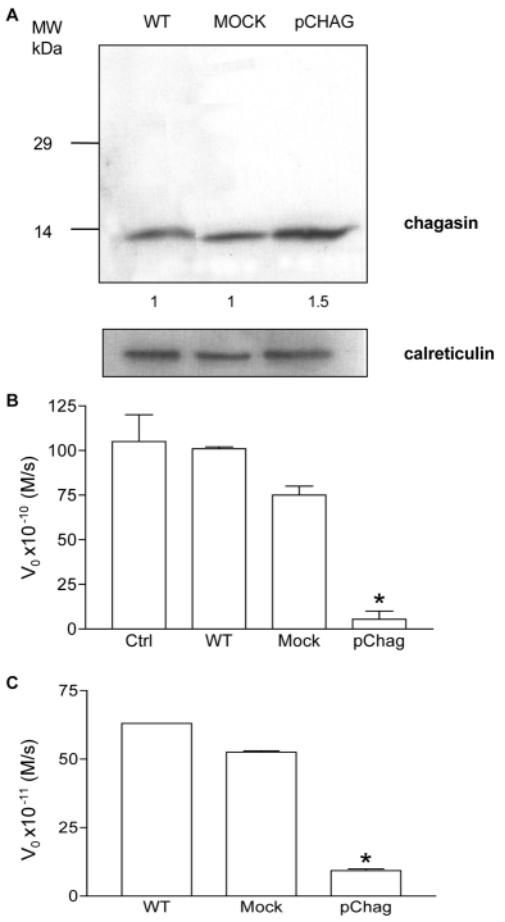

Fig. 3. Overexpression of functional chagasin in transfected *T. cruzi*. (A) Epimastigotes were washed twice in PBS and lysed in PBS containing 1% Triton X-100. The lysates (100 µg) were resolved by SDS-PAGE and submitted to western blot using anti-chagasin antiserum (1:1000). Anti-calreticulin monoclonal antibodies were used as a control for sample loading in the gels. (B) Inhibitory activity in transfected epimastigotes. Lysates (2.4 µg) were boiled for 20 minutes and the soluble fraction was collected after 10,000 ***g*** centrifugation. Equal volumes of the soluble fraction were incubated with papain (3.32 nM) in 50 mM Na_2PO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT for 15 minutes at room temperature and the residual activity was subsequently monitored by the hydrolysis of 5 µM CBZ-Phe-Arg-AMC in the same buffer containing 5% DMSO. As a control, papain was incubated for the same period in buffer and the peptidase activity was measured in the same conditions. (C) Chagasin overexpression leads to a reduction in CP activity. The peptidase activity contained in normalized epimastigote lysates (1 µg) was determined in 50 mM Na_2HPO_4, 200 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, 5% DMSO at room temperature, using 5 µM CBZ-Phe-Arg-AMC as a substrate. Substrate hydrolysis was fully inhibited by 10 µM E-64 (data not shown), confirming that the activity detected corresponds to that of CPs. The graph shows the initial velocities for the substrate hydrolysis curves. The experiments were performed in triplicate and are represented as mean values with standard deviations (SD). The analysis of variance was performed using ANOVA and the asterisk indicates the scores that are statistically significant at $P<0.05$ (B) or $P<0.001$ (C).

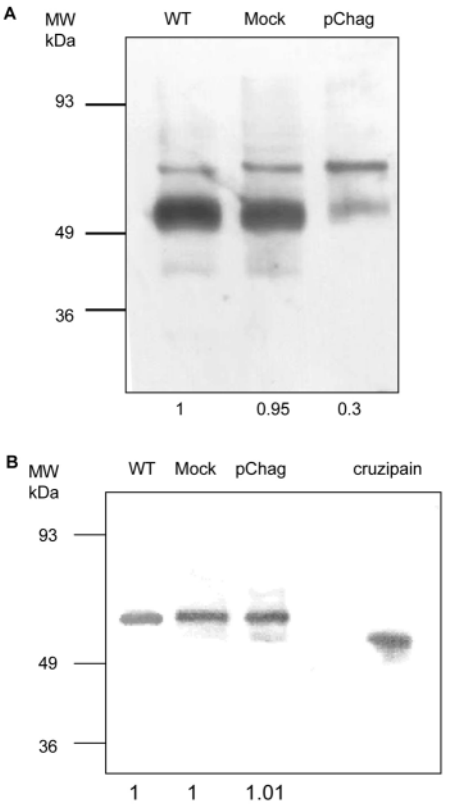

Fig. 4. Chagasin overexpression results in downregulation of soluble, but not of membrane-associated, cruzipain. In-vivo active-site labelling of CPs: epimastigotes were incubated in LIT medium containing 10 µM biotin-N-Pip-F-hF-VSPh (an irreversible CP inhibitor) for 1 hour at 28°C. The cells were washed twice in ice-cold PBS, lysed in this buffer by freeze-thaw and fractionated by 100,000 ***g*** centrifugation. The pellet was resuspended in PBS containing 1% Triton X-100 to the original volume. The samples were submitted to SDS-PAGE, transferred to nitrocellulose and the reactive bands visualized upon incubation with streptavidin/alkaline-phosphatase. The relative intensities of the reactive bands were estimated by scanning densitometry and normalised to the WT, and are indicated below each lane. (A) Soluble fraction. (B) Membrane fraction.

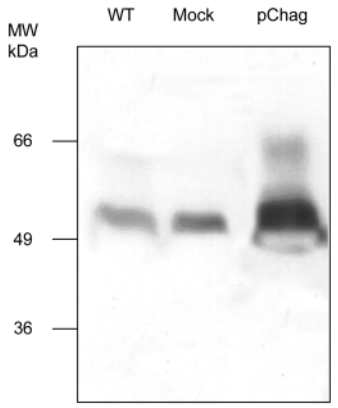

Fig. 5. Epimastigotes overexpressing chagasin accumulate more of the cruzipain-chagasin complex. Epimastigotes (5×10^6) were lysed directly in the SDS-βME buffer (not boiled) and submitted to a western blot. The high-molecular-weight complex was visualized using anti-chagasin antiserum.

reduces cruzipain activity in vivo by interacting preferentially, if not exclusively, with soluble forms of the enzyme. To test this hypothesis further, we lysed the cells in an SDS-βME solution and checked by western blotting the contents of cruzipain-chagasin complexes in living cells (Fig. 5), as described in Fig. 2. The reactivity pattern of anti-chagasin antibodies confirmed that pCHAG parasites accumulated more high-molecular-mass complexes (Fig. 5, lane 3) than MOCK (Fig. 5, lane 2) or WT (Fig. 5, lane 1).

Because a substantial amount of active cruzipain is normally secreted through the flagellar pocket, the levels of CP activity present in culture supernatants were also analysed. As observed in parasite lysates, the supernatant of pCHAG cells displayed a 65% reduction in the peptidase activity compared with MOCK and WT (Fig. 6A). The small (15%) reduction in peptidase activity observed in MOCK-transfected cells was considered to be minor compared with the large decrease in activity observed in the supernatants for pCHAG. After boiling the supernatants, we found that pCHAG parasites displayed correspondingly higher levels of chagasin (Fig. 6C), whereas the levels present in MOCK-transfected cells were equivalent to those found in untransfected cells. The secretion of cruzipain-chagasin complexes was analysed by diluting the supernatants in SDS-βME (without boiling), followed by western blotting with anti-chagasin antibodies (Fig. 6B). Interestingly, this analysis suggested that the decrease in extracellular peptidase activity of pCHAG resulted from increased secretion of cruzipain/chagasin molecular complexes.

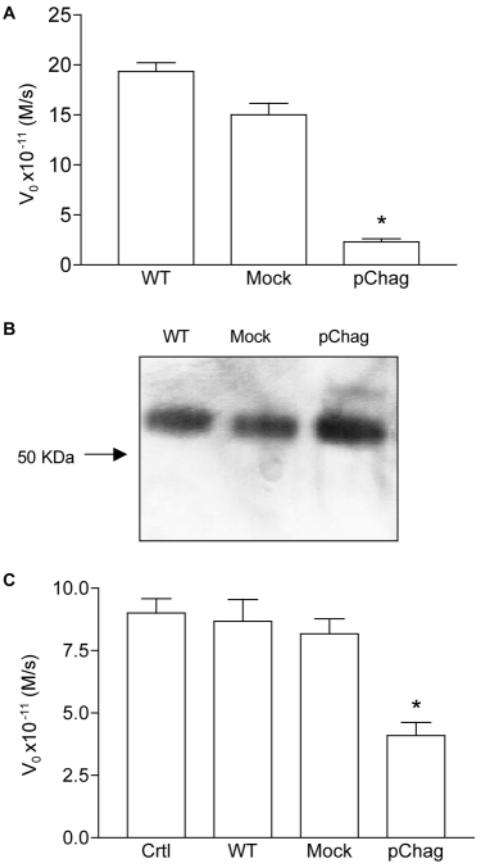

Fig. 6. Chagasin overexpression leads to reduced extracellular CP activity. The secretion products of epimastigotes were obtained by resuspending washed cells in PBS and maintaining them for 3 hours at 28°C. The supernatants were collected by centrifugation at 3000 ***g*** and filtered through 0.2 μm membranes. (A) The peptidase activity present in the supernatants were measured in 50 mM Na_2HPO_4, 200 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, 5% DMSO using 5 μM CBZ-Phe-Arg-AMC. Substrate hydrolysis was fully inhibited by E-64 (not shown). (B) Detection of the cruzipain-chagasin complex in supernatants. Supernatants were diluted in SDS-βME buffer, not boiled, resolved by SDS-PAGE, blotted onto nitrocellulose and probed with anti-chagasin antiserum. (C) Supernatants were boiled for 20 minutes centrifuged at 10,000 ***g*** and the inhibitory activity present in the soluble fraction was determined upon incubation with papain in 50 mM Na_2HPO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT for 15 minutes at room temperature. After incubation, the residual activity of papain was measured by the hydrolysis of CBZ-Phe-Arg-AMC. The graphs represent the initial velocities for the substrate hydrolysis curves. The experiments were performed in triplicates and are represented as the mean values with the standard deviations (SD). The analysis of variance was performed using ANOVA and the asterisks indicate the scores that are statistically significant at $P<0.001$ (A) and $P<0.01$ (C).

Chagasin overexpression in epimastigotes impairs parasite differentiation and increases resistance to synthetic CP inhibitors

We then asked whether chagasin-mediated regulation of CPs was relevant to *T. cruzi* biology and sought to evaluate two biological parameters that had been previously reported to depend on cruzipain activity. First, we examined whether chagasin overexpression could impair the differentiation of epimastigotes to the infective insect stages (metacyclic trypomastigotes) (Tomás et al., 1997). Second, we asked whether pCHAG caused altered sensitivity to toxicity of synthetic inhibitors of CP (Yong et al., 2000). In conditions of induced differentiation in Grace's insect medium, epimastigotes overexpressing chagasin had impaired rates of metacyclogenesis (Fig. 7). Although MOCK parasites also displayed a somewhat reduced differentiation (30%), the proportion of trypomastigotes in the cultures after 8 days was approximately the same as observed with the untransfected cells, in contrast to the 70% reduction in differentiation observed with pCHAG. The statistical analyses indicated that the rate of differentiation of pCHAG presents significant variance compared with WT and MOCK. Small numbers of metacyclic trypomastigotes were also observed in stationary-

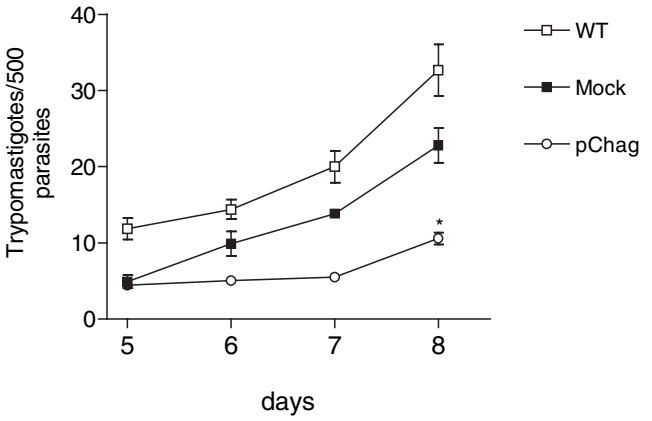

Fig. 7. Chagasin overexpression affects parasite differentiation. Stationary-phase epimastigotes were inoculated in 1:5 dilution (vol:vol) in Grace's insect medium, pH 6.5, and cultivated at 28°C for 10 days. The proportion of trypomastigotes in the cultures was estimated by daily counting under the light microscope starting at day 5. The experiments were performed in triplicate and are represented as the mean values with standard deviations (SD). The graph is representative of three independent experiments. The rate of parasite differentiation was analysed using ANOVA and the asterisk indicates statistical significance at $P<0.05$.

phase cultures (LIT medium) of pCHAG compared with cultures from MOCK and WT (data not shown). The second biological parameter was examined by cultivating the parasites in LIT medium supplemented with increasing concentrations of the synthetic CP inhibitor N-Pip-F-hP-VSPh. Under these conditions, pCHAG epimastigotes were less susceptible to the toxic effects of this drug (Fig. 8C) than MOCK (Fig. 8B) or WT (Fig. 8A) parasites. The estimation of the IC_{50} for growth in the presence of this synthetic inhibitor revealed that pCHAG displayed a twofold increase in the IC_{50} (8 μM) compared with WT (4 μM). At day 5, the culture densities of WT and MOCK parasites were similar at the various drug concentrations. The statistical analyses of the growth rate in the presence of the inhibitor indicated that WT and MOCK display significant differences in growth at 2 μM in relation to the control in DMSO, and there is no subsequent significant variation at the higher inhibitor concentrations compared with 2 μM. However, the growth rate of pCHAG was significantly different from the control at DMSO and in relation to one another at all drug concentrations. These analyses revealed that the decreased susceptibility of chagasin overexpressing parasites to the effect of the synthetic CP inhibitor was extremely significant compared with WT and MOCK. Taken together, these results indicate that chagasin decreases the levels of cruzipain enzymatic activity in *T. cruzi* epimastigotes through the formation of tight molecular complexes, with consequent changes in some biological properties.

Balanced levels of chagasin and cruzipain in *T. cruzi* strains

In the previous section, our data suggest that changes in chagasin content might result in drastic changes in cruzipain activity, perhaps contributing to the biological heterogeneity of *T. cruzi* strains and/or isolates (Souto et al., 1996; Fernandes

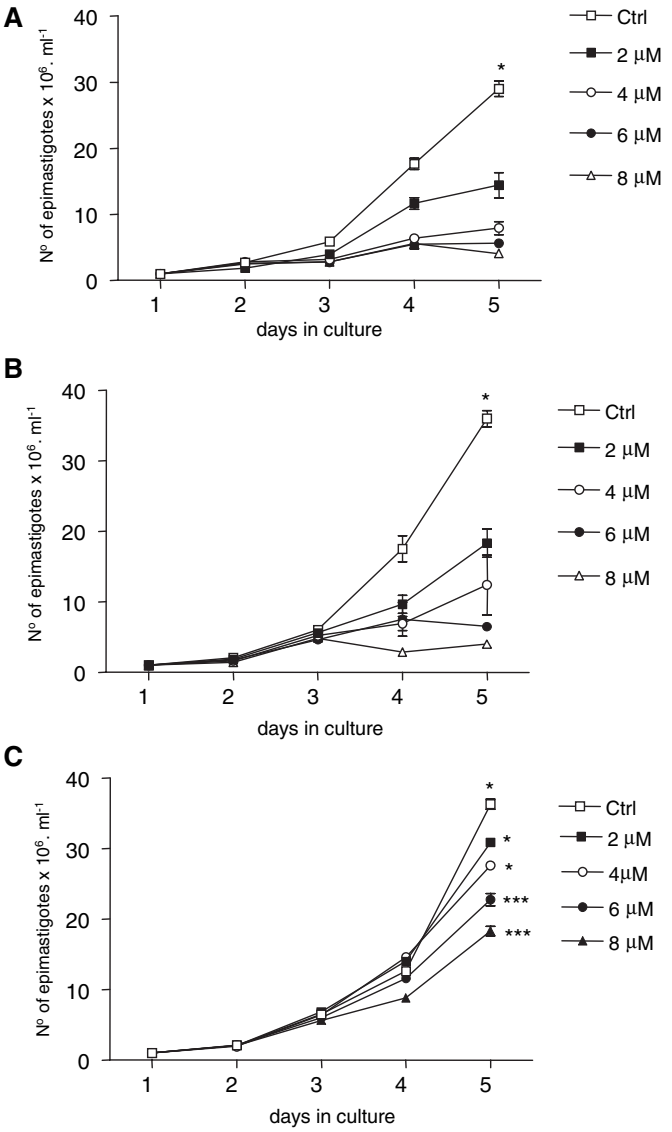

Fig. 8. Chagasin overexpression partially protects *T. cruzi* epimastigotes against the toxicity of the synthetic CP inhibitor N-Pip-F-hF-VSPh. Epimastigotes were inoculated at 10^6 ml^{-1} in LIT medium containing 10% FCS and 0.5% DMSO in the presence of 2 μM, 4 μM, 6 μM or 8 μM N-Pip-F-hF-VSPh, and cultivated for 5 days at 28°C. The controls were cultivated solely in the presence of the DMSO diluent. (A) WT. (B) MOCK. (C) pCHAG. The experiments were performed in triplicate and are reported as mean values with standard deviations (SD). The graphs are representative of two independent experiments. The analyses of variance were performed using two-way ANOVA and the Bonferroni post-test at a significance level of 5%. The single asterisks show the scores that are statistically significant at $P<0.05$ and the triple asterisks show the scores that are statistically significant at $P<0.01$. In the WT and MOCK individual graphs, the variation in the growth rate in the presence of all drug concentrations were equally significant in relation to growth in the presence of DMSO. The variances in the growth rates of pCHAG were significant in relation to the control and among the various drug concentrations. The variance in the growth rates of pCHAG in the presence of 6 μM and 8 μM N-Pip-F-hF-VSPh was extremely significant ($P<0.0001$) in relation to WT and MOCK.

et al., 1998; Miles et al., 2003). In order to approach this question, we measured the levels of functionally active CPs as well as of CP inhibitory activity in six different laboratory strains. Western blotting analysis indicated that the expression of cruzipain at the protein level varied considerably among the isolates, being higher in Dm28c, X10/6 and Y, and lower in CL, Brazil (Br) and G parasites, whereas the expression of *T. cruzi* cathepsin B (TcCB) seemed to be fairly constant (Fig. 9A). The amount of functional CP in parasite lysates, detected by enzymatic assays, showed a similar pattern (Fig. 9B), with the notable exception of G parasites, which express cruzipain at levels similar to those of CL but bear three to five times less active CP. Western blots revealed that G epimastigotes present a twofold higher chagasin content than CL and Br, which could account for the reduction in the levels of active CP in the former (Fig. 9C). Interestingly, the higher levels of cruzipain expression observed in Dm28c, X10/6 and Y are accompanied by higher chagasin levels.

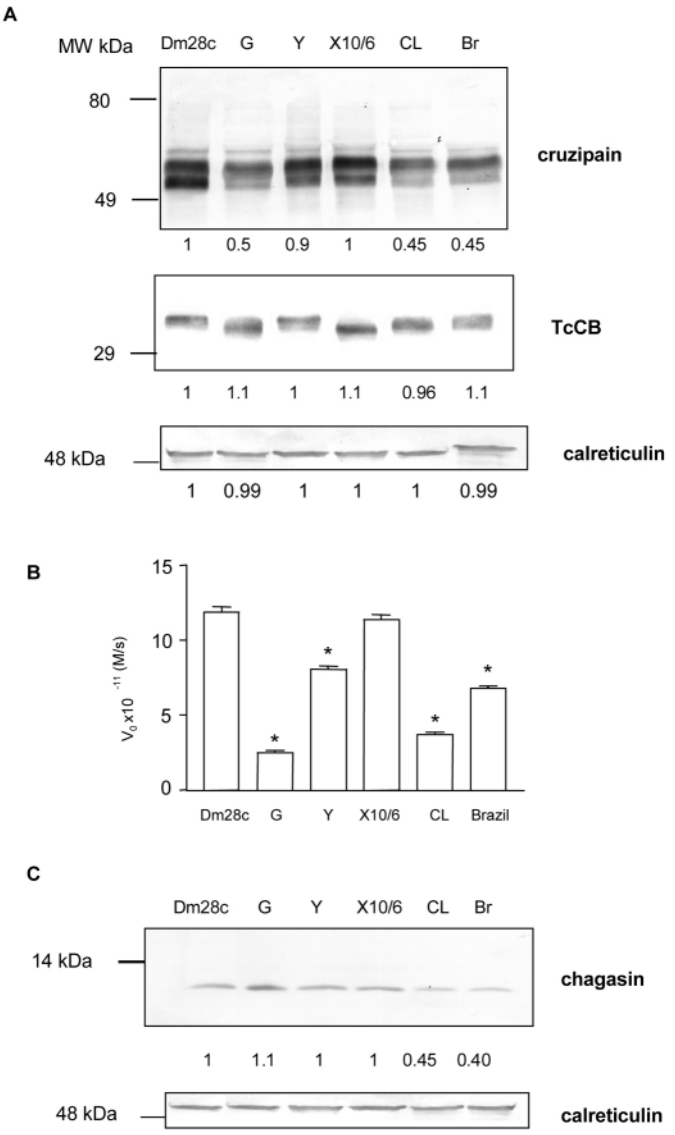

Fig. 9. CP and chagasin expression in different *T. cruzi* isolates. (A) Immunoblotting of epimastigote lysates probed with monoclonal antibody against cruzipain, monoclonal antibodies against the cathepsin-B-like enzyme of *T. cruzi* (TcCB) or monoclonal antibodies against calreticulin. (B) Detection of CP activity in epimastigote lysates (1 μg) in 50 mM Na_2HPO_4, 200 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, 5% DMSO at room temperature using 5 μM CBZ-Phe-Arg-AMC as a substrate. Substrate hydrolysis was fully inhibited by E-64 (not shown). The experiments were performed in triplicate and are represented as mean values with standard deviations (SD). The variance was analysed using ANOVA and the asterisks indicate the scores that are statistically significant at $P<0.001$. (C) Immunoblotting of epimastigote lysates probed with anti-chagasin antibodies or with monoclonal antibodies against calreticulin. The relative intensities of the reactive bands were estimated by scanning densitometry normalized to Dm28c and are indicated below each lane.

In order to determine accurately the relative amounts of functional cruzipain in these isolates, we performed active-site titration of the enzyme. Because epimastigotes express at least two papain-like CPs [cruzipain and the cathepsin-B-like CP (TcCB)], we first tested the selectivity of the irreversible synthetic inhibitor N-Pip-F-hF-VSPh for cruzipain (Fig. 10). Although the inactivation constant of this compound with TcCB was not determined, it should bind preferentially to cruzipain given that the K_i value for recombinant cruzipain (cruzain) is 360 nM, whereas the K_i for mammalian cathepsin B is 11 μM (Palmer et al., 1995). Active-site labelling of peptidases in parasite lysates with biotin-coupled N-Pip-F-hF-VSPh used at 10 μM showed exclusive interaction with cruzipain (Fig. 10, lane 2), whereas biotin-LVG-CHN_2 interacted with both cruzipain and TcCB. The broad reactivity profile of biotin-LVG-CHN_2 was expected (Fig. 10, lane 1) because its peptidyl core is based on the sequence of cystatin C, a wide-ranged inhibitor of papain-like peptidases (Lalmanach et al., 1996). This result indicates that N-Pip-F-hF-VSPh could be used safely to estimate the concentration of cruzipain in crude parasite lysates, given that we performed titration experiments with CP concentration at the nanomolar range. Titration of free cruzipain using this compound confirmed that the G isolate bears three to five times less active enzyme than CL and Br, whereas Dm28c, X10/6 and Y present two to three times more functional cruzipain than the others (Table 1). The relative levels of chagasin were likewise evaluated upon inhibitor titration in boiled parasite lysates (Table 1). Interestingly, although Dm28c, X10/6 and Y present higher chagasin contents, CL and Br parasites (which express

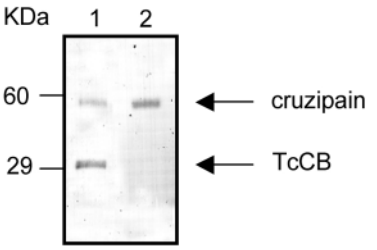

Fig. 10. Selectivity of the CP inhibitor N-Pip-F-hF-VSPh to cruzipain over TcCB. Epimastigote lysates were incubated with 10 μM biotin-N-Pip-F-hF-VSh (lane 2) or biotin-LVG-CHN_2 (lane 1) in 50 mM Na_2HPO_4, 200 mM NaCl, 5 mM EDTA, pH 6.5, 5 mM DTT for 2 hours at room temperature. Reactions were stopped by the addition of SDS-PAGE sample buffer, boiled for 3 minutes and analysed by western blot upon incubation with phosphatase-conjugated streptavidin.

lower cruzipain levels) display, respectively, four and two times less chagasin than Dm28c. The titration showed that chagasin levels in G parasites are comparable to those of Dm28c, although G epimastigotes display ten times less cruzipain activity than Dm28c. The calculation of the cruzipain:chagasin molar ratios revealed that the excess enzyme over the inhibitor was similar among all tested isolates (approximately 50:1 enzyme:inhibitor), with the exception of G parasites, which presented a 5:1 enzyme:inhibitor ratio. The correction of error propagation for the ratios revealed that all errors were kept below 20% and that, in a confidence interval of 95%, only the ratio of the G strain was significantly different among the tested strains. Importantly, the titration of cruzipain and chagasin in pCHAG strains revealed a 2.5:1 enzyme:inhibitor ratio (data not shown), suggesting that, in our model, Dm28c overexpressing chagasin might have similar characteristics to G-strain parasites. Importantly, the reduction in the chagasin levels of CL and Br strains enabled parasites with reduced cruzipain expression to maintain similar enzyme:inhibitor ratios, suggesting that the levels of cruzipain-chagasin complexes depend primarily on chagasin expression. This possibility was confirmed upon detection of cruzipain-chagasin complexes through SDS-βME lysis of living parasites, which demonstrated lower accumulation of these complexes in CL and Br, but not in the G isolate (data not shown). Taken together, these data support the notion that chagasin might act as a regulator of cruzipain function in epimastigotes of natural *T. cruzi* populations.

Chagasin impairs host-cell invasion by *T. cruzi* trypomastigotes

We then sought to investigate whether chagasin interfered with the infectivity of trypomastigotes, the assumption being that it could modulate cruzipain ability to generate activation signals that promote cellular invasion (Meirelles et al., 1992; Harth et al., 1993; Scharfstein et al., 2000; Aparicio et al., 2004). Addition of recombinant chagasin to cell cultures significantly impaired the invasion of human smooth-muscle cells by WT tissue-culture trypomastigotes, in a dose-dependent manner (Fig. 11A). We then tested whether recombinant chagasin could inactivate host-cell CPs. This was evaluated by adding chagasin or E-64 to smooth-muscle cell cultures, followed by determining the peptidase activity in cell lysates (Fig. 11B). We observed that the incubation of the cells with 5.5 nM chagasin led to a 55% drop in the peptidase activity, whereas 55 nM chagasin or 10 μM E-64 led to a ~62% drop in activity. It is worth mentioning that the peptidase activity detected in smooth-muscle cell lysates was fully inhibited by E-64 in vitro, demonstrating that substrate hydrolysis was strictly dependent on CPs. Next, we carried out invasion assays by incubating smooth-muscle cells with pCHAG tissue-culture trypomastigotes. Consistent with the inhibition of cell invasion observed with recombinant chagasin, pCHAG trypomastigotes displayed reduced infectivity compared with WT or MOCK in vitro (Fig. 11C).

It is well known that trypomastigotes continuously shed part of their plasma membranes, in the form of small vesicles, into the culture medium (Gonçalves et al., 1991). We have recently reported that the supernatants recovered from WT tissue-culture trypomastigotes contain active cruzipain molecules (Aparicio et al., 2004). Although most of the peptidase is found in the soluble fraction of supernatants, a minor proportion of the active CP activity is associated with the membrane-rich 100,000 ***g*** pellet (Aparicio et al., 2004). Because chagasin is found at the surface of trypomastigotes (Monteiro et al., 2001), we reasoned that the CP associated with shed membranes from pCHAG trypomastigotes could be reduced. The peptidase activity present in the 100,000 ***g*** fraction of pCHAG trypomastigotes supernatants was fully inhibited by E-64, indicating that it was mediated by papain-like CPs (data not shown). Consistent with our hypothesis, the CP activity of pCHAG membranes was approximately three times lower than that of the membranes of WT and MOCK parasites (Fig. 11D, inset). We then asked whether the decrease in membrane-associated CP activity of pCHAG trypomastigotes could account for their reduced infectivity. To test this hypothesis, invasion assays of smooth-muscle cell by pCHAG trypomastigotes were supplemented with a 100,000 ***g*** pellet (i.e. membranes) derived from the supernatant of WT tissue-culture trypomastigotes. As shown in Fig. 12D, this procedure significantly increased pCHAG infectivity. Importantly, this effect was nullified by E-64, indicating that CP activity present in the WT membrane fraction is crucial for the recovery of pCHAG infectivity. Interestingly, exogenously added E-64 did not inhibit basal invasion by pCHAG trypomastigotes (Fig. 11D), although it was capable of inhibiting host CPs (Fig. 11B). These results militate against an obvious involvement of host lysosomal CPs in the mechanisms of smooth-muscle invasion by tissue-culture trypomastigotes.

Discussion

It is well known that CPs (e.g. cruzipain) are crucially involved in several biological functions in *T. cruzi*. However, there are no reports of how the expression and/or activity of these peptidases are controlled by these parasites. Although chagasin is a natural candidate for regulating cruzipain function within the cells, there was no evidence that its trafficking pathways intersect those of cruzipain.

Using immunofluorescence and electron microscopy, we showed here that chagasin and cruzipain co-localize in at least two compartments of the secretory pathway of epimastigotes: the Golgi complex and the reservosome, an enlarged lysosome-like organelle present exclusively at this developmental stage. Considering that the autocatalytic processing of cruzipain zymogens is thought to occur in the Golgi (Engel et al., 1998a), our data suggest that chagasin might associate with mature CPs (e.g. cruzipain) in this compartment. Because cruzipain is present at high molar excess to chagasin, it is likely that chagasin associates with a minor proportion of active cruzipain molecules in the Golgi complex before sorting to reservosomes and/or the flagellar pocket. Although not directly addressed here, it is also possible that the association of chagasin with mature enzymes in the Golgi might prevent the massive autocatalytic processing of cruzipain zymogens.

The analysis of chagasin-cruzipain complexes in vitro revealed that they are extremely resistant, remaining intact in the presence of SDS and βME. This property enabled us to show that cruzipain-chagasin complexes are formed in living parasites. We were unable to detect molecular complexes between chagasin and the 30 kDa cathepsin-B-like TcCB,

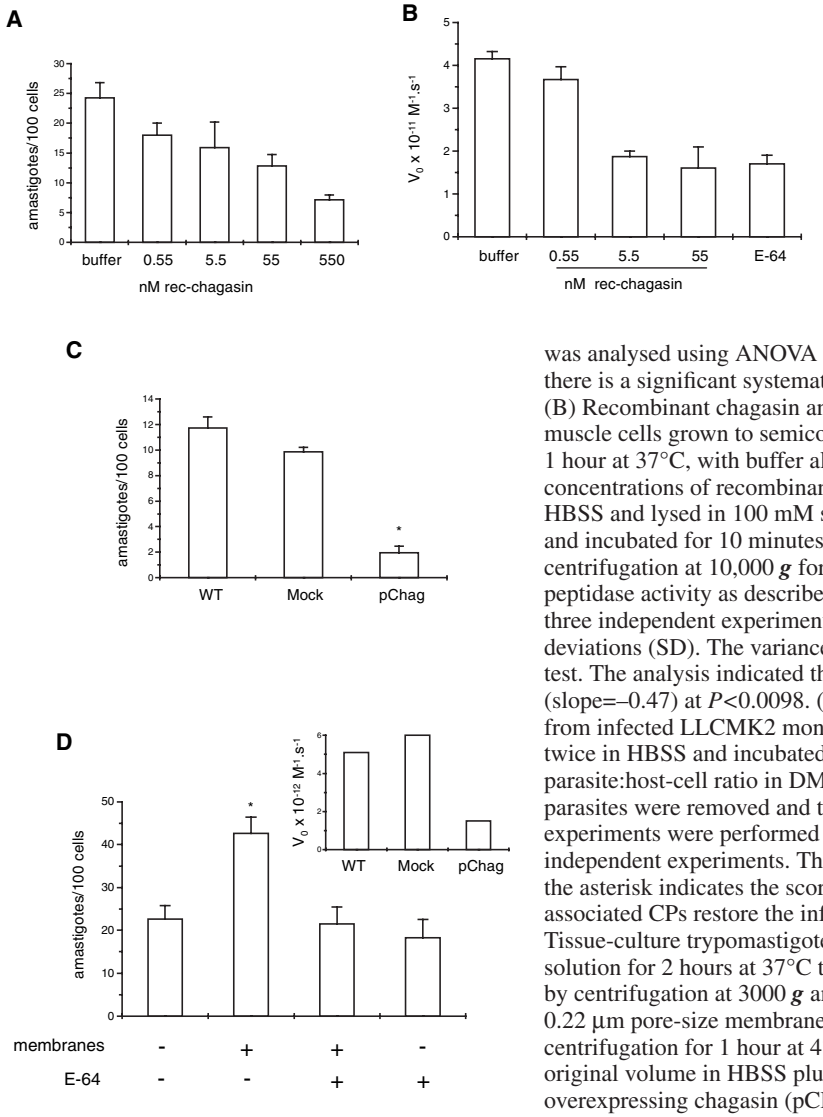

Fig. 11. Chagasin impairs mammalian-cell invasion by trypomastigotes. (A) Mammalian-host-cell invasion by WT tissue-culture trypomastigotes. Human primary culture of smooth-muscle cells plated on glass coverslips were incubated with *T. cruzi* tissue-culture trypomastigotes at a 5:1 parasite:host-cell ratio for 3 hours at 37°C in DMEM supplemented with 0.1% BSA in the presence of buffer or of recombinant chagasin at different concentrations. The number of intracellular parasites was estimated by counting under the light microscope. All the assays were performed in triplicate in three independent experiments and are represented as mean values with standard deviations (SD). The variance was analysed using ANOVA and the linear trend post-test. The analysis indicated that there is a significant systematic decrease of invasion (slope=–3.94), at $P<0.0001$. (B) Recombinant chagasin and E-64 inactivate host-cell CPs in living cells. Smooth-muscle cells grown to semiconfluence were incubated in DMEM plus 2% FCS for 1 hour at 37°C, with buffer alone, buffer containing 10 μM E-64 or different concentrations of recombinant chagasin. The cells were washed three times with HBSS and lysed in 100 mM sodium acetate, 150 mM NaCl, pH 5.5, 1% Triton X-100 and incubated for 10 minutes on ice. The soluble fraction was recovered by centrifugation at 10,000 ***g*** for 10 minutes and 0.25 μg ml^{-1} lysates were assayed for peptidase activity as described above. All the assays were performed in triplicate, in three independent experiments and are represented as mean values with standard deviations (SD). The variance was analysed using ANOVA and the linear trend post-test. The analysis indicated that there is a significant systematic decrease of activity (slope=–0.47) at $P<0.0098$. (C) *T. cruzi* tissue-culture trypomastigotes were obtained from infected LLCMK2 monolayers. Freshly released trypomastigotes were washed twice in HBSS and incubated with human smooth-muscle cultures at a 3:1 parasite:host-cell ratio in DMEM plus 0.1% BSA for 3 hours at 37°C. Extracellular parasites were removed and the cells were fixed and stained as described in A. All experiments were performed in triplicate. The graph is representative of three independent experiments. The analysis of variance was performed using ANOVA and the asterisk indicates the score with statistical significance at $P<0.001$. (D) Membrane-associated CPs restore the infectivity of trypomastigotes overexpressing chagasin. Tissue-culture trypomastigotes were washed twice in HBSS and incubated in this solution for 2 hours at 37°C to allow membrane shedding. The parasites were removed by centrifugation at 3000 ***g*** and the cell-free supernatant was filtered through a 0.22 μm pore-size membrane. The filtered sample was submitted to 100,000 ***g*** centrifugation for 1 hour at 4°C and the pellet (membranes) was resuspended to the original volume in HBSS plus 0.1% BSA. Tissue-culture trypomastigotes overexpressing chagasin (pCHAG) were incubated with smooth-muscle cells at a 15:1 parasite:host-cell ratio for 3 hours at 37°C in DMEM plus 0.1% BSA in the presence of HBSS supplemented with 2.5 mM DTT or in the presence of 100 μl membrane fraction recovered from WT trypomastigotes supplemented with 2.5 mM DTT. Where indicated, E-64 was added at a 10 μM final concentration. The variance was analysed using ANOVA and the asterisks indicate the scores showing statistical significance at $P<0.01$. (Inset) The peptidase activity present in the membrane fraction (100 μl) recovered from different trypomastigotes was assayed in 50 mM Na_2PO_4, 100 mM NaCl, 5 mM EDTA, pH 6.5, 2.5 mM DTT, 5% DMSO by the hydrolysis of 5 μM CBZ-Phe-Arg-AMC. Substrate hydrolysis was fully inhibited by E-64 (not shown). The graph represents initial velocities.

neither in WT parasites nor in chagasin-overexpressing cells. Recombinant chagasin inhibits mammalian cathepsin B, albeit with lower affinity than with papain or mammalian cathepsin L (M. Abrahamson et al., unpublished), so it is likely that it would associate with active TcCB. It is possible that TcCB-chagasin complexes are easily dismantled upon treatment with SDS-βME and thus could not be detected by the methods used here.

The biochemical changes resulting from chagasin overexpression in epimastigotes suggested that it regulates cruzipain post-translationally, because (i) we did not observe alterations in cruzipain expression at the RNA and protein levels, (ii) their effects are selective – the activity of soluble cruzipain, but not of membrane-associated enzyme, is reduced in parasites overexpressing chagasin, and (iii) there is increased secretion of cruzipain-chagasin complexes by these cells, a process that results in considerably less accumulation of extracellular peptidase activity in the transfected parasites. Importantly, parasites overexpressing chagasin presented alterations in three biological parameters associated with cruzipain activity: (i) epimastigote differentiation to metacyclic trypomastigotes; (ii) epimastigote susceptibility to the toxic effects of synthetic CP inhibitors; and (iii) in vitro infectivity of tissue culture trypomastigotes.

It was reported that cruzipain levels are significantly increased at early stages of parasite differentiation (Bonaldo et al., 1991) and that enzyme overexpression in epimastigotes (Silvio X10/6) increased metacyclogenesis (Tomás et al., 1997). Considering that the growth rate was not affected in chagasin-overexpressing cells, it is conceivable that parasite

differentiation depends critically on cruzipain-mediated breakdown of proteins. A second interesting phenotype observed in our study was that pCHAG parasites grown in the presence of the synthetic CP inhibitor N-Pip-F-hF-VSPh (Engel et al., 1998b) showed a twofold increase in its IC_{50}. The deleterious effect of this drug was previously attributed to a disruption of intracellular trafficking caused by massive accumulation of cruzipain in the Golgi (Engel et al., 1998b). In our study, the treatment of chagasin-overexpressing cells with biotinylated-N-Pip-F-hF-VSPh revealed that enzymatically active cruzipain is drastically reduced (Fig. 4A). This finding, together with evidence of increased formation of chagasin-cruzipain complexes, suggest that reduced target availability might account for the increased drug resistance displayed by the chagasin-overexpressing cells. In a previous study (Yong et al., 2000), we described a parasite cell line, R-Dm28, that showed a 13-fold higher resistance to another irreversible synthetic CP inhibitor. Among other phenotypic features, R-Dm28 epimastigotes have sharply reduced contents of mature cruzipain (Yong et al., 2000) and we proposed that diminished cruzipain target spared the cells from inhibitor-induced toxicity caused by the excessive accumulation of unprocessed enzyme in the Golgi. The reduction in the levels of mature cruzipain induced by chagasin overexpression could function in the same way, conferring increased resistance to the synthetic CP inhibitor.

As previously alluded to, chagasin preferentially interacts in vivo with soluble cruzipain, rather than membrane-bound enzyme forms (Fresno et al., 1994; Parussini et al., 1998). It is possible that soluble and membrane-bound enzyme forms circulate through different routes, but only the former intersects with chagasin-loaded vesicles. Importantly, our electron-microscopy studies revealed that epimastigotes bear a significant number of vesicular structures loaded with chagasin but seemingly devoid of cruzipain (Fig. 1E,G, white arrows). Given that chagasin is also found in reservosomes, it is possible that vesicles containing free chagasin are in transit to this organelle. We also detected cruzipain-chagasin molecular complexes in culture supernatants of wild-type epimastigotes, their contents being increased in pCHAG. The biological implications of secreted chagasin-cruzipain complexes are not clear. Epimastigotes replicate in the gut of the insect vector, a protease-rich environment (Garcia et al., 1978; Ferreira-da Silva et al., 2000). In this scenario, it is not obvious how chagasin would dissociate from cruzipain, thus becoming available to inhibit the vector-derived CPs.

In view of the remarkable genetic and biological diversity that exist in the *T. cruzi* species (Souto et al., 1996; Fernandes et al., 1998; Miles et al., 2003), we determined the concentration of functionally active cruzipain and chagasin in a panel of established *T. cruzi* strains. Interestingly, the strains that have reduced levels of cruzipain usually display a proportional drop in the levels of chagasin, suggesting that enzyme/inhibitor balance is somehow preserved. Consistent with this notion, most of the tested parasite strains exhibited a fairly constant enzyme:inhibitor molar ratio (approximately 50:1). Importantly, the G strain was peculiar because the stoichiometry was shifted to 5:1 and it displayed higher contents of cruzipain-chagasin complexes and diminished overall CP activity. The phenotype of the genetically manipulated pCHAG is reminiscent of that observed in the G strain, suggesting that increased chagasin expression modifies the balance of enzyme-inhibitor within the cell, thus negatively modulating CP activity. This might be significant to *T. cruzi* biology in view of our recent findings that tissue-culture trypomastigotes from the G strain are much less infective than Dm28c or X10/6, primarily because of their reduced cruzipain activity (Aparicio et al., 2004).

The role of chagasin in modulating mammalian-cell invasion by tissue-culture trypomastigotes was also evaluated. We observed that recombinant chagasin drastically impairs the invasion of mammalian cells. Consistent with this, trypomastigotes overexpressing chagasin were significantly less infective and presented a threefold reduction in membrane-associated CP activity. Infectivity of pCHAG parasites was restored by addition of membranes isolated from WT trypomastigotes, an effect that was nullified by E-64, is thus dependent on CP activity. Collectively, these data indicate that differences in the levels of chagasin expression are coupled to changes in cell-surface CP activity of trypomastigotes, thereby modulating their infectivity. Cruzipain was previously implicated in the mechanisms of host-cell invasion by *T. cruzi*, acting through at least two alternative cellular activation pathways: (i) a route that depends on cruzipain-mediated release of bradykinin from kininogens, followed by the activation of G-protein-coupled kinin receptors (Scharfstein et al., 2000; Todorov et al., 2003); and (ii) a kinin-receptor-independent route that requires cruzipain-mediated proteolysis of a hitherto unknown substrate present on trypomastigote membranes (Aparicio et al., 2004). As reported, the invasion assay conditions used in the present study did not favour the activation of the kinin-receptor-mediated route (Aparicio et al., 2004). At present, it is not clear how the changes in cruzipain/chagasin balance affect the efficiency of parasite infectivity. In addition to the inactivation of the trypanosome CPs, we showed that recombinant chagasin is capable of inactivating host peptidases when incubated with living cells. Because most, if not all, chagasin secreted by trypomastigotes is bound to parasite CPs, it is unlikely that the extracellular chagasin would be immediately available to inactivate host CPs. It remains to be determined whether the CP-chagasin complex is internalized by the mammalian cell and, if so, whether the intracellular environment (e.g. endosomes) favours chagasin dissociation from parasite CPs, enabling the inhibitor to inactivate host enzymes. However, the participation of host CPs in our model of mammalian-cell invasion by *T. cruzi* seems unlikely because: (i) although E-64 inactivated host CPs to the same extent as recombinant chagasin, it did not affect the basal invasion of pCHAG-transformed strains; (ii) the loss of infectivity displayed by pCHAG-transformed strains could be restored by the membranes of WT trypomastigotes. The discrepancy with the potent inhibitory effect of recombinant chagasin in cellular invasion might be explained by the lower affinity of E-64 for the different cruzipain isoforms expressed by trypomastigotes (Lima et al., 2001).

Taken together, our data indicate that regulation of endogenous CPs by chagasin influences several aspects of *T. cruzi* biology, such as morphogenesis, sensitivity to synthetic CP inhibitors and parasite infectivity. Future studies might clarify whether changes in chagasin/cruzipain expression levels and or in their trafficking routes contribute to the wide biological diversity of *T. cruzi* clones (Miles, 2003). Further

investigations of the role of chagasin in host-parasite interaction might offer additional insight onto the function of this new family of cysteine protease inhibitors.

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