Arrested growth of *Trypanosoma cruzi* by the calpain inhibitor MDL28170 and detection of calpain homologues in epimastigote forms

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SUMMARY

In this paper, we aimed to explore the effects of the calpain inhibitor III (MDL28170) and to detect calpain-like molecules (CALPs) in epimastigote forms of *Trypanosoma cruzi* isolate Dm28c. MDL28170 at 70 μM promoted a powerful reduction in the growth rate after 48 h. The IC_{50} value was calculated to be 31·7 μM. This inhibitor promoted an increase in the cellular volume, but not cell lysis, resulting in a trypanostatic effect. *T. cruzi* CALPs presented a strong cross-reactivity with anti-*Drosophila melanogaster* calpain and anti-cytoskeleton-associated protein from *Trypanosoma brucei* antibodies, and labelling was found mainly intracellularly. Furthermore, an 80 kDa reactive protein was detected by Western blotting assays. No significant cross-reactivity was found with anti-human brain calpain antibody. The expression of CALPs was decreased in cells kept for long periods in axenic cultures in comparison to a strain recently isolated from mice, as well as in MDL28170-treated cells, the latter being paralleled by an increased expression of cruzipain. Different levels of CALPs expression were also detected in distinct phylogenetic lineages, like Y strain (lineage TCI), Dm28c (TCII) and INPA6147 strain (Z3 zymodeme). These results may contribute for the investigation of the functions of CALPs in trypanosomatids.

Key words: *Trypanosoma cruzi*, calpain, peptidase, inhibitor.

INTRODUCTION

Trypanosoma cruzi is the aetiological agent of Chagas disease, also known as American trypanosomiasis. Chagas disease affects from 16 to 18 million people in the Americas, causing 21 000 deaths yearly and 40 million are at risk of infection throughout Central and South America (WHO, 2005). The life cycle of the parasite involves several stages of differentiation; in the mammalian host, the parasite has an obligate intracellular replicative form, the amastigote, and a non-replicative form, the bloodstream trypomastigote. The major forms present in the insect vector are also a replicative stage, the epimastigote, and a non-replicative stage, the infective metacyclic trypomastigote. The epimastigote form can be readily obtained in axenic culture, and has been used for this reason in most biochemical studies performed on the parasite (Cazzulo *et al*. 2001).

Peptidases of microbial pathogens, including *T. cruzi*, have attracted the attention of many laboratories because of their roles in pathogenesis (Cazzulo *et al*. 2001; Vermelho *et al*. 2007), but less is known about the presence of a group of peptidases, the calpains. These enzymes constitute a large family of calcium-dependent cytosolic cysteine peptidases that have been characterized mainly in humans and that exist in 2 major isoforms, m-calpain and μ-calpain, which require millimolar and micromolar concentrations of Ca^{2+}, respectively, for their activation. The role of calpain remains poorly understood, but it may participate in a variety of cellular processes, including the rearrangement of cytoskeletal proteins, different signal transduction pathways and apoptosis (Goll *et al*. 2003). Calpain activation in humans seems to be increased during normal ageing and in muscular dystrophy, cataract, arthritis and Alzheimer's disease, and in many acute traumas. A variety of calpain inhibitors are under development and the potential clinical utility of these

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inhibitors has been shown to treat Alzheimer's disease (Battaglia *et al*. 2003) and to minimize neuronal death after ischaemia (Hayes *et al*. 1998).

The presence of large numbers of calpain-related proteins (CALPs) in trypanosomatids has been revealed by some groups, although there are no data as yet on the specific functions of the calpain-like proteins in these microorganisms (Hertz-Fowler *et al*. 2001; Ramos *et al*. 2004; Ersfeld *et al*. 2005; Salotra *et al*. 2006; Vergnes *et al*. 2007; Andrade *et al*. 2008). Our group became involved in the study of these proteins though the purification of a proteolytically active CALP in the culture supernatant of the insect trypanosomatid *Crithidia deanei*, yet its function was not addressed (d'Avila-Levy *et al*. 2003). More recently, our group investigated the effect of calpain inhibitor III (MDL28170), a potent calpain inhibitor, on the growth of *Leishmania amazonensis*. This inhibitor promoted cellular alterations and arrested the cellular growth of the parasite, and a CALP was identified and detected on the cell surface of the flagellate (d'Avila-Levy *et al*. 2006*a*).

In this paper, we extended our studies on the presence of CALPs in trypanosomatids by investigating the inhibitory capability of MDL28170 against *T. cruzi*, aiming to show its effects on the growth and morphology of epimastigote forms. In addition, we have demonstrated the presence of calpain homologues in 3 distinct *T. cruzi* strains.

MATERIALS AND METHODS

Parasites and cultivation

Epimastigotes of *Trypanosoma cruzi* Dm28c (lineage TCII), Y (lineage TCI) and INPA 4167 (Z3 zymodeme) strains were grown in 3·7% brain heart infusion medium supplemented with 10% heat-inactivated fetal bovine serum at 28 °C for 4 days to reach late-log phase growth. Alternatively, epimastigote forms of Dm28c were obtained from the differentiation of trypomastigotes after a blood passage in mice; in this case, epimastigotes were kept for no more than 3 passages in the axenic culture.

Effects of the calpain inhibitor on growth rate and cell morphology

The effects of calpain inhibitor III (MDL28170; Z-Val-Phe-CHO; purchased from Calbiochem, San Diego, CA, USA) on epimastigotes of *T. cruzi* were assessed by a method similar to that previously described elsewhere (d'Avila-Levy *et al*. 2006*a*). Briefly, epimastigotes were counted using a Neubauer chamber and resuspended in fresh medium to a final concentration of 5×10^6 viable epimastigotes per ml. The viability was assessed by mobility and lack of staining after challenging with trypan blue. MDL28170 was added to the culture at final concentrations in the 10–70 μM range (starting from a 5 mM solution in dimethylsulfoxide (DMSO) that was serially diluted in culture medium). A dilution of DMSO corresponding to that used to prepare the highest drug concentration was assessed in parallel. After incubation for 24, 48, 72 and 96 h at 28 °C, the number of viable, motile epimastigotes was quantified by counting the flagellates in a Neubauer chamber. Alternatively, parasites grown for 72 h in the absence and in the presence of the calpain inhibitor were washed 5 times in cold phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7·2) prior to resuspension in a drug-free fresh medium and allowed to grow for another 72 h, in order to evaluate the trypanocidal or trypanostatic effect. The number of live epimastigotes was evaluated as well as cell morphology (after Giemsa staining) under optical microscopy at 24-h intervals (Mendonça-Filho *et al*. 2004). The 50% inhibitory concentration (IC_{50}) was evaluated after 72 h. This value was determined by linear regression analysis, by plotting the number of viable epimastigotes versus log drug concentration by use of Origin Pro 7.5 computer software.

Identification of CALPs by Western blotting

T. cruzi Dm28c cells were collected by centrifugation (500 ***g***/5 min/4 °C) and washed 3 times with cold PBS. Trypanosomes (10^8 cells) were resuspended in 100 μl of PBS and lysed by the addition of 1% SDS (Santos *et al*. 2006). The *Drosophila* extracts (kindly provided by Dr Alexandre Afranio Peixoto, FIOCRUZ, RJ, Brazil) were prepared in the extraction buffer (25 mM Hepes pH 7·2, 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol and 250 mM sucrose). About 10 frozen flies were crushed, suspended with 200 ml of cold extraction buffer and homogenized in a Teflon/glass homogenizer with 10–12 strokes at 4 °C. The homogenate was carefully mixed and centrifuged at 100 000 ***g*** for 1 h at 4 °C. The supernatant was stored at −80 °C. Protein concentration was determined by the method described by Lowry *et al*. (1951), using BSA as standard.

Immunoblot analysis was performed with total cellular extracts equivalent to 100 μg of protein, as previously described (d'Avila-Levy *et al*. 2005). The primary antibody used was a rabbit antiserum raised against *Drosophila melanogaster* calpain (anti-Dm-calpain; Emori and Saigo, 1994), at a dilution of 1 : 500. The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1 : 25 000 followed by chemiluminescence immunodetection after reaction with ECL reagents (d'Avila-Levy *et al*. 2005). The relative molecular mass of the reactive polypeptide was calculated by comparison with the mobility of SDS–PAGE standards.

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Flow cytometry and immunofluorescence microscopy for calpain detection

Epimastigotes (1×10^7 cells) used for these experiments were fixed at 4 °C in 0·4% paraformaldehyde in PBS (pH 7·2) for 30 min, followed by extensive washing in the same buffer. Alternatively, the fixed cells were permeabilized by 0·01% Triton X-100 in PBS for 15 min at room temperature and then washed twice in PBS. The fixed and permeabilized cells maintained their morphological integrity, as verified by optical microscopic observation. After this step, the cells were incubated for 1 h at room temperature with a 1 : 500 dilution of the following polyclonal antibodies: rabbit anti-Dm-calpain polyclonal antibody (Emori and Saigo, 1994); anti-C21, raised against the whole molecule of human brain m-calpain (Grynspan *et al*. 1997); and anti-CAP5.5, raised against the cytoskeleton-associated protein from *Trypanosoma brucei* (Hertz-Fowler *et al*. 2001). Cells were then incubated for an additional hour with a 1 : 200 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (d'Avila-Levy *et al*. 2006*b*). The cells were then washed 3 times in PBS and observed in a Zeiss epifluorescence microscope (Axioplan 2). Alternatively, the parasite-associated fluorescence was excited at 488 nm and quantified on a flow cytometer (FACSCalibur, BD Bioscience, USA) equipped with a 15 mW argon laser emitting at 488 nm. Non-treated cells and those treated with the secondary antibody alone were run in parallel as controls. Each experimental population was then mapped by using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population ($n = 10\,000$) was then analysed for log green fluorescence by using a single-parameter histogram.

Effects of the calpain inhibitor on peptidase expression

Epimastigotes (5×10^6 cells) of Dm28c were incubated with MDL28170 at the IC_{50} concentration for 24 h at 28 °C. Thereafter, cells were fixed and processed for flow cytometry analysis as previously described, employing a 1 : 500 dilution of the polyclonal antibodies anti-Dm-calpain and anti-cruzipain, the latter raised against the major cysteine peptidase of *T. cruzi* epimastigote forms (Cazzulo *et al*. 2001).

Statistical analysis

All experiments were performed in triplicate, in 3 independent experimental sets. The data were analysed statistically by means of Student's *t*-test using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software. *P* values of 0·05 or less were considered statistically significant.

Sequence data analysis

A search for Dm-calpain homologous proteins in *T. cruzi* was performed using the BlastP algorithm and the nr database at NCBI (GenBank). The theoretical molecular mass of homologous proteins was calculated using the ExPASy Server facilities (http://expasy.org). Identification of conserved domains was performed using the CD-Search Tool and Conserved Domain Database (CDD 27036 PSSMs) at NCBI.

RESULTS

The calpain inhibitor III (MDL28170) was added to *T. cruzi* clone Dm28c epimastigote forms in concentrations ranging from 20 to 70 μM, and the cellular growth was monitored for 4 days *in vitro*. Our results showed that MDL28170 arrested the growth in a dose-dependent manner (Fig. 1A). The lowest concentration of the drug (20 μM) presented a significant inhibitory effect only after 72 h of growth, but the remaining concentrations promoted reduced levels of growth that were statistically significant after 48 h (Fig. 1A). The calpain inhibitor at 50 μM decreased the growth rate by approximately 70–75% in the 48–96 h interval, and at 70 μM promoted a reduction in the cellular growth rate of around 91% after 48 h, and this ratio was also maintained up to 96 h of growth (Fig. 1A). Conversely, DMSO did not significantly affect the parasite growth behaviour (Fig. 1A). The same conditions performed for Dm28c were employed for the analysis of the effects of MDL28170 upon Y and INPA4167 strains, and similar profiles of growth inhibition were observed (data not shown). These strains were chosen to represent members of distinct *T. cruzi* lineages: TCI (Y strain), TCII (Dm28c) and Z3 zymodeme (INPA4167 strain). The IC_{50} was calculated for each strain, and similar values were detected: 31·7 μM (Dm28c), 34·3 μM (Y) and 37·4 μM (INPA4167).

The anti-trypanosomal activity of this inhibitor was reversible, since *T. cruzi* cells pre-treated for 72 h with the calpain inhibitor at 70 μM resumed growth when subcultured in a drug-free fresh medium (data not shown). Optical microscopy observations of *T. cruzi* Dm28c cells treated with the calpain inhibitor in the 20–70 μM range revealed, irrespective to the drug concentration, an increase in the cell volume, with the flagellates becoming round and some of them presenting no detectable flagellum (Fig. 1C, *b–i*), but no cell lysis was observed, corroborating the trypanostatic effect on the epimastigote growth. Cells cultured in the presence of DMSO at a dose used to dissolve the highest concentration of MDL28170 did not present any detectable morphological alterations (data not shown).

As demonstrated by flow cytometry and fluorescence microscopy analyses, the anti-Dm-calpain

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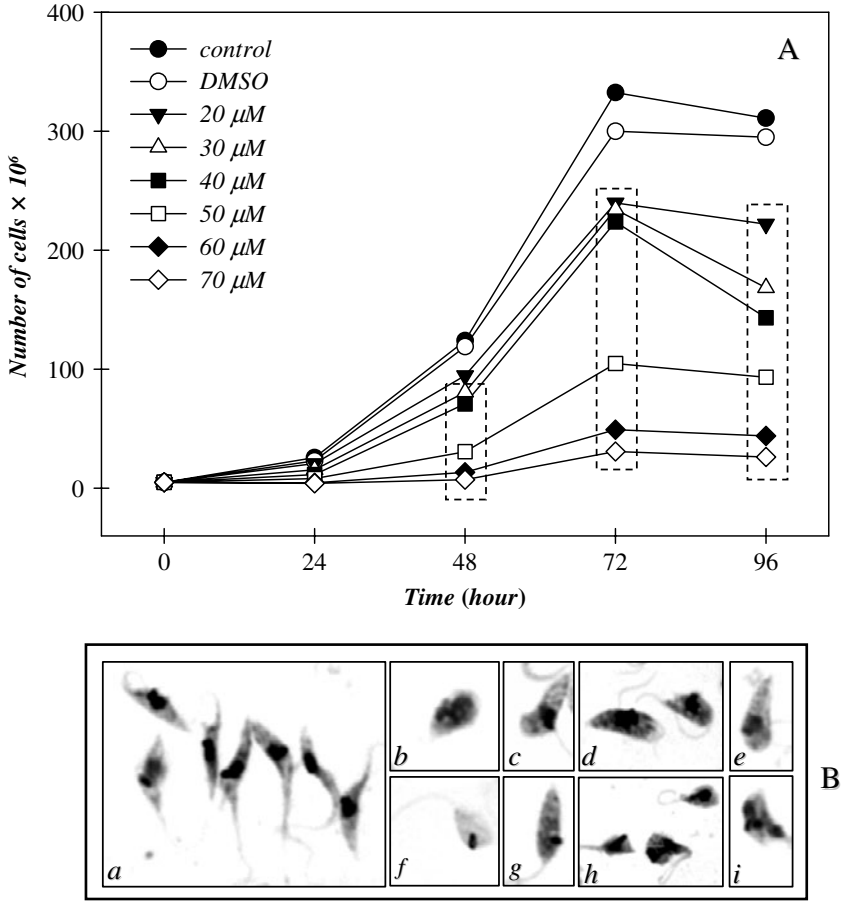

Fig. 1. Effect of calpain inhibitor III (MDL28170) on the growth rate and cellular morphology of *Trypanosoma cruzi* Dm28c. The growth pattern of the parasite was followed at 28 °C in the absence (control) or in the presence of MDL28170 (A) at concentrations ranging from 20 and 70 μM. The inhibitor was added to the cultures at 0 h, and the cells were counted daily. DMSO, used as the drug diluent, did not interfere with growth behaviour. Data shown are the mean of 3 independent experiments performed in triplicate. The dashed boxes highlight growth rates significantly different from control (P values $\leqslant 0{\cdot}05$). (B) Microscopic observations of the viability of epimastigote forms when incubated in the absence or in the presence of MDL28170. Control cells were cultured in BHI medium (*a*). Cells cultured in the presence of MDL28170 at 20–70 μM showed similar morphological alterations (*b-i*).

antibody was found to react with the cell surface of *T. cruzi* (Fig. 2A, B). In fixed/permeabilized cells, the antibody binding was stronger, being intensely detected in the whole cell body but not in the flagellum (Fig. 2A, B). In addition, a polypeptide band migrating at approximately 80 kDa was detected in the trypanosomatid cell lysate by cross-reactivity with the same antibody (Fig. 2A, inset). As a positive control, lysates of *D. melanogaster* cells presented a polypeptide with the same molecular mass that was recognized by anti-Dm-calpain antibody (Fig. 2A, inset).

Since we cannot exclude the possibility that the anti-Dm-calpain polyclonal antibody may cross-react with proteins unrelated to CALPs in *T. cruzi*, a search for homologues of *D. melanogaster* CALP in *T. cruzi* genome was performed. The fragment of the *D. melanogaster* protein CAA55297.1 that was employed to generate the antibody used in this work (Emori and Saigo, 1994) was compared in a BlastP analysis with *T. cruzi* proteins found in GenBank data base (data not shown). The first 15 hits (homologues with e-value ranging from 2e-8 to 0·003) all corresponded to calcium-dependent cysteine peptidases and had their theoretical molecular mass determined, and 4 out of these 15 homologues presented a molecular mass around 80 kDa: XP_816697.1 (78·3 kDa), XP_803757.1 (80·8 kDa) kDa), XP_820102.1 (82·4 kDa) and XP_816696.1 (82·6 kDa). These 4 proteins presented only 2 conserved domains (cd00044 and pfam09149) and showed high similarity to each other, which indicates the possibility of being isoenzymes. The conserved domain cd00044 is shared with the fragment of the protein CAA55297.1, which supports the recognition of *T. cruzi* 80 kDa CALPS by the anti-Dm-calpain antibody.

Parasite cells, either grown in axenic cultures without passages *in vivo* or obtained after the differentiation of trypomastigotes recently isolated

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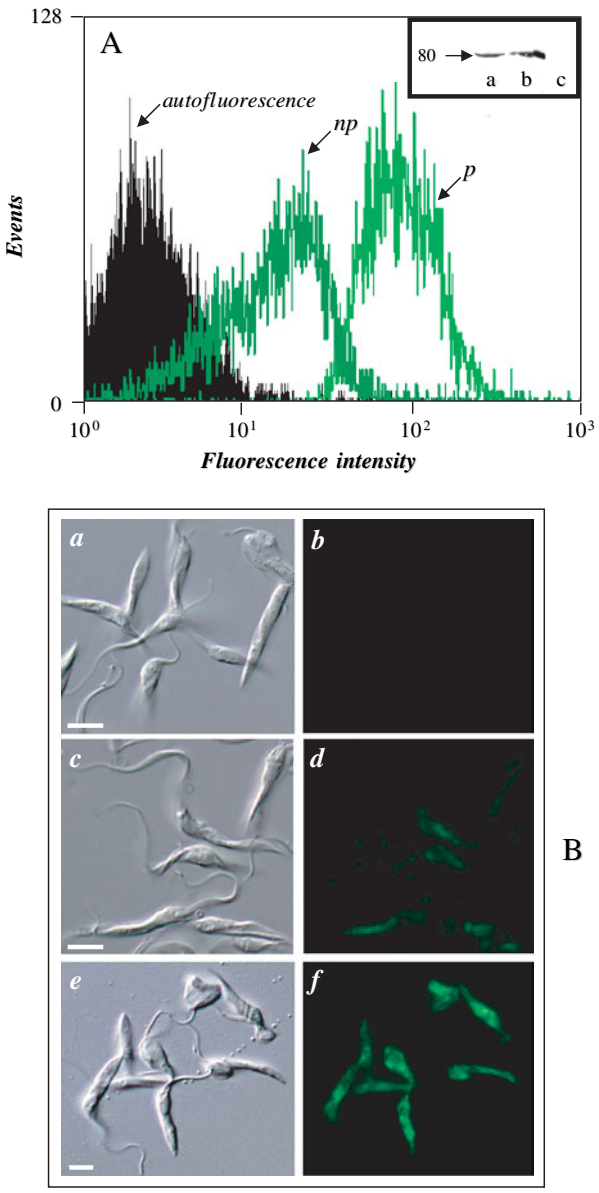

Fig. 2. Detection of cross-reactivity between a calpain-like protein from *Trypanosoma cruzi* Dm28c and *Drosophila melanogaster* calpain (Dm-calpain). (A) Flow cytometric analysis showing the anti-Dm-calpain antibody binding to *T. cruzi*. Paraformaldehyde-fixed cells were permeabilized (*p*) or not (*np*) with Triton X-100 and then incubated in the absence (autofluorescence) or in the presence of anti-Dm-calpain antibody at 1 : 500 dilution, in order to be analysed by flow cytometry. For simplicity, only the autofluorescence of non-permeabilized cells is shown, since the permeabilized cells presented similar values (data not shown). When treated only with the secondary-FITC antibody, both permeabilized and non-permeabilized cells generated similar curves to that observed in the autofluorescence of cells (not shown). Representative data of the analysis of 10 000 cells from 1 of 3 experiments are shown. The inset shows the Western blotting analysis of polypeptides from *T. cruzi* (lane a) and *D. melanogaster* cell extract (lane b) probed with anti-Dm-calpain antibody. In lane c, the blotted proteins from *T. cruzi* were incubated only with the secondary-peroxidase antibody, and no reactive polypeptide was detected. The number on the left indicates the apparent molecular mass, expressed in kDa, of the reactive polypeptide. (B) Fluorescence microscopy showing the labelling of *T. cruzi* with anti-Dm-calpain antibody. Fixed cells (*a–d*) and fixed-permeabilized cells (*e, f*) were analysed under differential interferential contrast images (*a, c, e*) and immunofluorescence (*b, d, f*). Parasites treated only with the secondary antibody presented no fluorescence intensity (*b*). The bars represent 1 μm.

from infected mice, were processed for flow cytometric analysis using anti-Dm-calpain in order to estimate the influence of *in vitro* maintenance of *T. cruzi* Dm28c epimastigote forms upon the expression of CALPs. A decrease in the relative CALPs expression between newly isolated and *in vitro*-maintained cells did take place, as demonstrated in Fig. 3A. In addition, 2 anti-calpain antibodies with distinct specificities were also employed in order to detect the possible binding of different anti-calpain antibodies to calpain-like molecules of *T. cruzi* Dm28c: anti-C21, raised against the whole molecule of human brain m-calpain (Grynspan *et al.* 1997); and anti-CAP5.5, raised against the cytoskeleton-associated protein from *T. brucei* (Hertz-Fowler *et al.* 2001). In both cases, cells were fixed and permeabilized, or not, with Triton X-100. When anti-C21 antibodies were employed, there were no significant differences in labelling among fixed and permeabilized cells and the labelling itself is very close to the autofluorescence of cells, which indicates the lack of immunological similarity between human brain m-calpain and *T. cruzi* CALPs. Conversely, fixed/permeabilized *T. cruzi* cells showed an intense labelling with anti-CAP5.5; the antibody binding was drastically diminished when fixed cells were not permeabilized (Fig. 3B).

A flow cytometric analysis was also performed in order to compare the relative levels of expression of CALPs in *T. cruzi* strains belonging to different phylogenetic lineages. To accomplish this, anti-Dm calpain antibodies were employed in fixed and/or permeabilized cells from members of each parasite lineage. In the 3 strains studied, the permeabilization with Triton X-100 raised significantly the mean of fluorescence intensity (MFI) values, which indicates that CALPs are located mainly intracellularly but also on the cell surface. In addition, distinct levels of expression of CALPs were detected: higher levels (twice as many) were found for Y strain in comparison to Dm28c, and a reduced labelling was found in INPA4167 strain (Fig. 4).

We also aimed to assess whether the MDL28170-treated cells of Dm28c presented any change in the levels of expression of CALPs and cruzipain in comparison to non-treated cells. Flow cytometry

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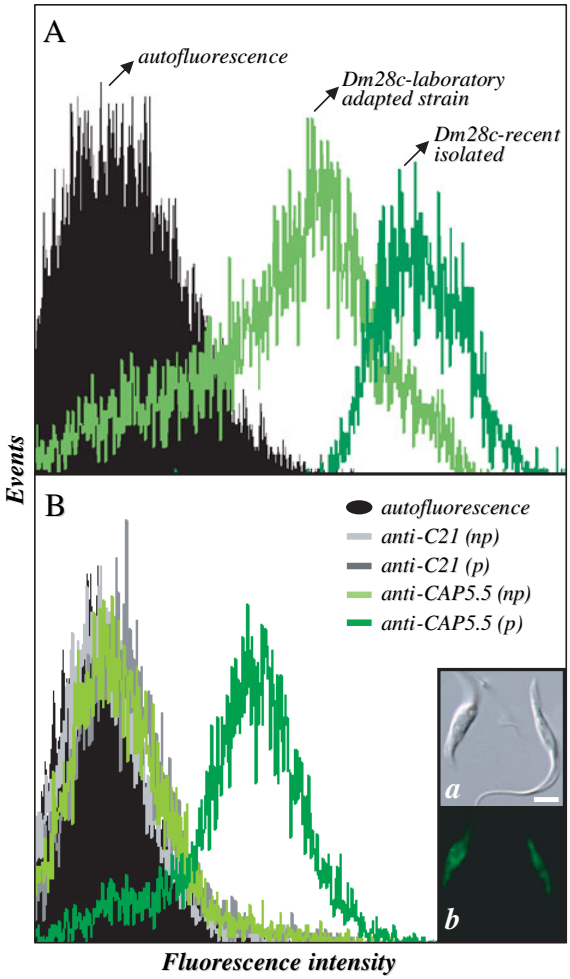

Fig. 3. Detection of binding of anti-calpain antibodies to *Trypanosoma cruzi* Dm28c epimastigote forms by flow cytometric analysis. (A) Cells regularly kept in BHI axenic culture (*Dm28c-laboratory adapted strain*) and cells obtained from the differentiation of trypomastigotes after a blood passage in mice (*Dm28c-recent isolated*) were fixed with paraformaldehyde and incubated in the absence (autofluorescence) or in the presence of anti-Dm-calpain antibody at 1 : 500 dilution. (B) Cells regularly kept in BHI axenic culture were fixed with paraformaldehyde, permeabilized (*p*) or not (*np*) with Triton X-100 and then incubated in the absence (autofluorescence) or in the presence of anti-C21 or anti-CAP5.5 antibodies at 1 : 500 dilution. Representative data of the analysis of 10 000 cells from 1 of 3 experiments are shown. The data from autofluorescence, fixed/permeabilized cells incubated with anti-C21 and fixed cells incubated with anti-CAP5.5 overlapped. The inset shows the fluorescence microscopy of *T. cruzi* permeabilized cells labelled with anti-CAP5.5 antibody. Note the fluorescence all over the cell body, but not in the flagellar region. The bar represents 1 μm.

analyses revealed that both peptidases had their expression altered significantly when parasites were subjected to the presence of the calpain inhibitor: a reduction in the contents of CALPs was paralleled by an increased expression of cruzipain (Table 1).

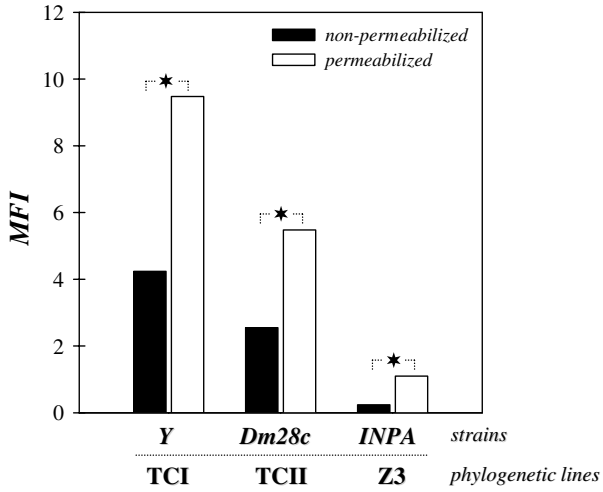

Fig. 4. The mean of the fluorescence intensity (MFI) calculated from the flow cytometric analysis of the anti-Dm-calpain antibody binding to *Trypanosoma cruzi* strains belonging to lineage TCI (Y strain), lineage TCII (Dm28c) and zymodeme Z3 (INPA4167 strain). Paraformaldehyde-fixed cells were permeabilized or not with Triton X-100 and incubated in the absence or in the presence of anti-Dm-calpain antibody at 1 : 500 dilution, and then analysed by flow cytometry. Representative data of the analysis of 10 000 cells from 1 of 3 experiments are shown. The asterisks highlight that MFI values from fixed and permeabilized cells are significantly different from each other (P values $\leqslant 0{\cdot}05$). Note that the MFI was also distinct among each strain.

DISCUSSION

In a previous study from our group, we showed that MDL28170, which is a potent, reversible and cell-permeable human calpain inhibitor (Rami *et al.* 1997), arrested the growth of *L. amazonensis* in a dose-dependent manner (d'Avila-Levy *et al.* 2006*a*). In this paper, a similar approach was employed in order to explore the effects of this calpain inhibitor upon *T. cruzi* growth and to establish the differences amongst both trypanosomatids with respect to the parasites' growth behaviour. Our results showed that the calpain inhibitor also arrested the growth of *T. cruzi* in a dose-dependent manner. When the IC_{50} values of MDL28170 for *T. cruzi* strains are compared to the calculated value for *L. amazonensis*, it is clear that the latter parasite, to which the IC_{50} value is 19 μM (d'Avila-Levy *et al.* 2006*a*), is more sensitive to MDL28170 than *T. cruzi*. These results are validated through the observation that the anti-trypanosomal activity of this inhibitor was reversible, while the anti-leishmanial activity of this compound at 25 μM is irreversible (d'Avila-Levy *et al.* 2006*a*). Similar optical microscopy observations (round-shaped cells) were described in other trypanosomatids when treated with cysteine peptidase inhibitors, due to osmotic stress caused by the peptidase inhibitor (Troeberg *et al.* 1999; Santos *et al.* 2006).

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Table 1. Detection of binding of anti-Dm-calpain and anti-cruzipain antibodies to untreated and MDL28170-treated *Trypanosoma cruzi* Dm28c epimastigote forms by flow cytometric analysis

	% Fluorescent cells		
Antibodies	Control cells	MDL28170-treated cells	Statistics
Anti-Dm-calpain	25·4±0·6	12·6±1·1	$P \leqslant 0{\cdot}01$
Anti-cruzipain	65·7±2·4	98·0±0·9	$P \leqslant 0{\cdot}05$

Although MDL28170 is a relatively specific calpain inhibitor, it cannot be ruled out that this inhibitor acts on other cysteine peptidases to a lesser extent, mainly cathepsins B (Rami *et al*. 1997). Nevertheless, our results suggest that, as it occurs with *L. amazonensis* (d'Avila-Levy *et al*. 2006*a*), *T. cruzi* possesses molecules that share antigens with invertebrate calpain-related enzymes, which suggests that CALPs may be the major target of MDL28170. The cross-reactivity of anti-Dm calpain antibodies and trypanosomatid proteins was already detected in *C. deanei* extracellular calpain-like peptidase (d'Avila-Levy *et al*. 2003). Recently, our group has demonstrated that a CALP that cross-reacted with the same antibody was detected in both the cellular body and flagellum of *Herpetomonas samuelpessoai* promastigote cells, and its presence is enhanced in DMSO-induced paramastigote cells (Pereira *et al*. 2009). Based on sequence analyses, our study identified 4 *T. cruzi* CALPs of around 80 kDa that share the same conserved domain (cd00044) with the fragment of the protein CAA55297.1 that was employed to generate the anti-Dm-calpain antibody. For this reason, it is reasonable to presume that this antibody is capable of recognizing these 4 *T. cruzi* proteins. In this sense, it is already known that anti-Dm-calpain antibody does not recognize mammalian m-calpain and μ-calpain (Emori and Saigo, 1994). Recently, a member of the family of CALPs in *T. cruzi* was characterized (Giese *et al*. 2008). The gene encoding TcCALPx11 corresponds to the protein XP_816697.1 and it is epimastigote-specific, being expressed mainly in epimastigotes subjected to the nutritional stress that precedes metacyclogenesis. No proteolytic activity was detected for this 80 kDa protein, and its differential expression may suggest that this protein may have a role in the *T. cruzi* stress response and/or in signal transduction.

In this study, we have also presented evidence that the maintenance of *T. cruzi* in axenic culture for a long time led to a decrease in the expression of CALPs. It has been reported that the long-term maintenance of *T. cruzi* cells in axenic cultures can select subpopulations less virulent than the parental strains, and the loss of virulence is associated with modification of the biological properties of this parasite that might lead to changes in the expression of some proteins (Contreras *et al*. 1998). Although a direct relationship between the expression of CALPs and parasite virulence cannot be established, our results raise the interesting possibility that this correlation might occur. Simultaneously, the absence of cross-reactivity between CALPs in *T. cruzi* and anti-human m-calpain is suggestive of substantial structural differences among these molecules. In this sense, Ersfeld *et al*. (2005) showed the presence of a large and diverse family of CALPs in *T. brucei*, *Leishmania major* and in *T. cruzi* displaying altered amino acid residues essential for catalytic activity and a moderate overall degree of sequence identity, which suggests that most CALPs do not have proteolytic activity. This large family exceeds the numbers found in most other organisms, which may point to organism-specific functions for these proteins.

Our results also indicate that CALPs in *T. cruzi* that cross-react with anti-CAP5.5 are located mainly in the intracellular milieu. CAP5.5 protein was the first characterized member of calpain-related genes in a trypanosomatid, specifically in *T. brucei* (Hertz-Fowler *et al*. 2001). This protein is characterized by the similarity to the catalytic region of calpain-type peptidases and it is detected exclusively in procyclic forms. CAP5.5 has been shown to be both myristoylated and palmitoylated, suggesting a stable interaction with the cell membrane through interactions with the underlying microtubule cytoskeleton as well. Interestingly, in our study the anti-CAP5.5 antibody labelled the whole cell body excluding the flagellum, as it occurs with *T. brucei*. Our results may indicate a possible correlation regarding the location of a subset of CALPs in both trypanosomes.

It is well established that many *T. cruzi* strains display a high level of biological divergence, which led to the development of many criteria for the grouping of these strains; early studies revealed substantial isozymic variability among isolates, defining 3 major groups or zymodemes – Z1, Z2 and Z3 (Miles *et al*. 1978). Subsequently, the use of DNA markers (Souto *et al*. 1996) proved the division of *T. cruzi* into 2 major phylogenetic lineages: lineage TCI (to which Z2 stocks belong) and lineage TCII (into which Z1 stocks fall). On the other hand, the position of Z3 zymodeme is still under review. In this sense, our results demonstrate a direct correlation between relative levels of CALP detection and *T. cruzi* strain classification, so it is possible that modulation in the expression of these proteins does take place. Nevertheless, the lineage-associated differences in CALPs expression must be confirmed by the use of a larger number of strains.

Fluctuations of the expression levels of CALPs were also observed when Dm28c cells were treated

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for 24 h with MDL28170 at IC_{50}: reduced labelling was found in treated cells, and a simultaneous increase in cruzipain expression was detected. Cruzipain is the major cysteine peptidase in *T. cruzi*, being expressed at different levels by distinct parasite stages, and much evidence points to its participation in penetration into the host cell, escape from the immune system and in the differentiation processes (Cazzulo *et al*. 2001). The lethal effect of cruzipain inhibitors was due to inhibition of the auto-catalytic processing of the enzyme, leading to a decrease of its amount in lysosomes and its accumulation in the Golgi (Engel *et al*. 1998). Resistance of *T. cruzi* strains to cruzipain inhibitors is correlated with negligible levels of cruzipain activity and secretion of cruzipain precursors into the medium, with the concomitant upregulation of expression of a distinct 30 kDa cysteine peptidase (Engel *et al*. 2000; Yong *et al*. 2000). In this case, it was supposed that the metabolic needs for cruzipain may be partly compensated by the increased expression of the 30 kDa enzyme (Yong *et al*. 2000). Interestingly, overexpression of cruzipain in *T. cruzi* transfectants has been associated with enhanced metacyclogenesis but not with increased infectivity (Tomas *et al*. 1997). Collectively, these biochemical changes may demonstrate a correlation of the expression levels of cysteine peptidases in *T. cruzi*, and it is tempting to speculate that, as previously suggested by Yong *et al*. (2000), the overexpression of a certain cysteine peptidase may be necessary in order to titrate out the toxic levels of the inhibitor compound that is active against a different peptidase from the same class.

There are no data as yet on the specific functions of any CALPs in trypanosomatids. The investigation of changes in parasite gene expression between drug resistant/sensitive strains and in the up-regulation of virulence-related genes in infective forms has brought to the fore the involvement of calpain-like proteins in distinct processes in trypanosomatids (Salotra *et al*. 2006; Vergnes *et al*. 2007; Andrade *et al*. 2008). Life cycle-specific expression may also demarcate the search for specific functions of these CALPs (Saxena *et al*. 2003; Giese *et al*. 2008). Altogether, the results presented herein may contribute to the investigation of the presence and the functions of CALPs in trypanosomatids. The global analyses concerning the functions of these CALPs may provide new understandings on the mechanisms that gave rise to such a diverse family of proteins in these organisms.

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