Upregulation of the secretory pathway in cysteine protease inhibitor-resistant *Trypanosoma cruzi*

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SUMMARY

A novel chemotherapy in development for Chagas’ disease targets cruzain, the major cysteine protease of *Trypanosoma cruzi*. Peptidomimetic inhibitors disrupt the intracellular cycle of the parasite and rescue animals from a lethal infection. Inhibitor killing of parasites results from interruption of autocatalytic cruzain processing and transport to lysosomes, and massive accumulation of precursor protein in the Golgi complex. To further understand the mechanisms of protease processing and transport in this primitive eukaryote, and uncover potential mechanisms for resistance to these drugs, we generated cysteine-protease inhibitor (CPI)-resistant epimastigotes in vitro and investigated the mechanisms involved at the biochemical and structural levels. Resistance to 20-fold the lethal CPI concentration, achieved after a year of gradual drug increase, was accompanied by a modest decrease in growth rate. A marked increase in the number of vesicles trafficking from the Golgi complex to the flagellar pocket occurs in resistant cells. No mature protease reaches lysosomes though accumulation of endocytosed gold particles in lysosomes appears to be normal. Higher molecular mass cruzain species, consistent with complexes of cruzain precursors and inhibitor, are secreted by CPI-resistant parasites into the culture supernatant. Release of these cruzain precursors may be facilitated by an enhanced acidification of trans-Golgi cisternae in resistant parasites. The pH within Golgi cisternae is higher in control epimastigotes and most mature cruzain is lysosomal. Cruzain activity is negligible in CPI-resistant epimastigote extracts compared to the parental clone. Activity is restored following withdrawal of the inhibitor. No cross-resistance to the therapeutic drugs nifurtimox and benznidazole occurred and, conversely, parasites resistant to these drugs were sensitive to CPI. Protease inhibitors are thus potential therapeutical alternatives in cases of nifurtimox/benznidazole resistance. Cumulatively, these results suggest that CPI-resistance induces upregulation of Golgi complex function and post-Golgi secretory pathway, and release of precursors before the enzyme reaches its site of biologic activity.

Key words: *Trypanosoma cruzi*, Cysteine protease inhibitor, Drug resistance, Exocytosis

INTRODUCTION

*Trypanosoma cruzi* is the parasitic agent of Chagas’ disease or American trypanosomiasis. Nifurtimox and benznidazole are currently the only drugs available for the treatment of Chagas’ disease. Drug toxicity (Docampo, 1990) has restricted treatment mainly to the acute phase of the disease (Brener, 1979; Kirchhoff, 1993). There is variability in efficacy of treatment (Brener, 1979; Filardi and Brener, 1987; Andrade et al., 1985; Neal and van Bueren, 1988; The National Foundation of Brazil, 1996) and drug-related genomic abnormalities in treated children (Gorla et al., 1988). In vivo resistance to benznidazole has been generated experimentally, and these parasites are cross-resistant to a variety of anti-chagasic drugs including nifurtimox (Murta and Romanha, 1998; Murta et al., 1998). At the same time, new evidence on the pathogenesis of Chagas’ cardiomyopathy has highlighted the importance of chemotherapy in chronic Chagas’ disease (Andrade et al., 1991; Viotti et al., 1994; Apt et al., 1998; Sosa Estani et al., 1998; Tarleton and Zhang, 1998). Thus, the development of more effective chemotherapy for both acute infection and chronic disease is crucial (McKerrow et al., 1995; Urbina et al., 1996).

*Trypanosoma cruzi* belongs to a primitive lineage of eukaryotic cells. Replicative forms (epimastigotes and amastigotes) are highly polarized cells with a single nucleus, Golgi apparatus, mitochondrion, and a flagellar pocket, the site for exo/endocytosis. Little is known about protein trafficking through their secretory pathway but trypanosomes are unique experimental models for analyzing M6P receptor-independent protein sorting (Cazzulo et al., 1990). The major protease, cruzain, is a lysosomal hydrolase with significant homology to...
cathepsin L. Trypanosome cysteine proteases are synthesized as precursor proteins with a hydrophobic signal peptide, a prodomain, a catalytic domain and an unusual carboxy-terminal domain of as yet unknown function (Eakin et al., 1993). Cruzain transport to epimastigote lysosomes is mediated by the carbohydrate-free prodomain (Huete-Pérez et al., 1999) but the presence of complex type oligosaccharides suggests the protease transverses the entire Golgi complex up to the trans-Golgi network before delivery to lysosomes (Parodi et al., 1995).

Cysteine protease inhibitors (CPI) are an emerging therapy amenable for the treatment of acute and chronic Chagas’ disease. Some peptidomimetic inhibitors targeting cruzain (Cazzulo et al., 1997; Serveau et al., 1996; Eakin et al., 1993; Harth et al., 1993; McGrath et al., 1995; Engel et al., 1998a,b), are able to block the life cycle of the parasite in experimental animals (Engel et al., 1998a). The mechanism of action leading to the lethal effect on the parasite is by interruption of autocatalytic processing of cruzain and accumulation of cruzain precursors in the Golgi complex (Engel et al., 1998a,b). To evaluate the potential for resistance to cysteine protease inhibitors in T. cruzi populations we generated CPI-resistant parasites in vitro. In this report, we describe the mechanism of resistance at the biochemical and ultrastructural levels in epimastigotes. CPI-resistance results from upregulation of the trypanosomal secretory pathway. While control epimastigotes secrete mature cruzain into culture supernatants, CPI-resistant parasites secrete higher M Ter cruzain species, consistent with unprocessed cruzain precursors.

MATERIALS AND METHODS

Drug resistant T. cruzi

The cysteine protease inhibitors 4-morpholine carbonyl-phenylalanine-homophenylalanine-vinyl sulphone phenyl (Mu-F-hF-VSphenyl) and N-Pip-phenylalanine-homophenylalanine-vinyl sulphone phenyl (N-Pip-F-hF-VSphenyl) (Axys Pharmaceuticals, San Francisco, CA) arrest epimastigote growth and interrupt the intracellular amastigote cycle at 20 μM (Engel et al., 1998a,b) and 10 μM, respectively. By exposing epimastigotes of a sensitive parental T. cruzi clone (CA-I/72; Engel et al., 1982) to a gradual (2-10 μM) increase in drug concentration for over a year, we generated T. cruzi parasites resistant to 20-fold the lethal concentration of N-Pip-F-hF-VSphenyl (CA-I/KR). As expected, these parasites are cross-resistant to Mu-F-hF-VSphenyl. Parasites are now routinely cultured in 200 μM N-Pip-F-hF-VSphenyl. As N-Pip-F-hF-VSphenyl is water soluble and amenable to oral dosing, results reported here are with this inhibitor only. CPI-resistant epimastigotes of the Sylvio X10/7 clone of T. cruzi and Leishmania promastigotes were similarly generated (not shown).

Nifurtimox (N) [3-methyl-4 (5-nitrofurylidiene-amino) tetrahydro-4H-1, 4-thiazine-1, 1-dioxide] (Lampit, Bayer, Germany)-resistant trypomastigotes and epimastigotes of several T. cruzi strains including parasites derived from the nifurtimox-sensitive CA-I/72 clone (CA-I/NR) have been described previously (Nowaki et al., 1996).

Effect of drugs on epimastigote growth and analyses of cross-resistance

CPI-sensitive parental (CA-I/72), CPI-resistant (CA-I/KR), and nifurtimox resistant (CA-I/NR) T. cruzi epimastigotes were cultured in BHT medium with 10% heat-inactivated fetal calf serum at 26°C (Engel et al., 1987), with and without the addition of a lethal dose of 3 μM nifurtimox and/or 30 μM N-Pip-F-hF-VSphenyl. This CPI concentration was chosen based on the observation that 30 μM is 3-fold the lethal dose for CA-I/72 epimastigotes but does not affect CA-I/KR parasites. Similarly, 3 μM nifurtimox is lethal for CA-I/72 but not for CA-I/NR epimastigotes. To analyze cross-resistance of nifurtimox-resistant epimastigotes to benznidazole, CA-I/72 (controls) and CA-I/NR epimastigotes were cultured with 5 and 10 μM benznidazole. Parasites were counted daily in a hemocytometer. Doubling times and growth inhibition with the different treatment regimes were estimated from growth curves.

Ultrastructure, cytochemistry and immunocytochemistry

CA-I/72 and CA-I/KR epimastigotes were treated overnight with or without 10 μM and 200 μM N-Pip-F-hF-VSphenyl, respectively. For ultrastructure, parasites were fixed in 1.5% glutaraldehyde/0.06 M sodium cacodylate buffer/1% sucrose (pH 7.4) at room temperature for 2 hours. Samples were post-fixed in osmium tetroxide and uranyl acetate prior to embedding in Epoxy resin. Ultrathin sections were observed in a Phillips CM10 electron microscope.

For endocytosis studies, parasites were incubated with 1.4 nm positively charged Nanogold particles (Nanoprobes, NY) for 3 hours at 4°C followed by 0-60 minutes at room temperature. Epimastigotes were fixed and embedded in LR gold resin (London Resin, England) and thin sections enhanced with HQ Silver (Nanoprobes). Subsequent procedures were as described by Prescianotto-Baschong and Riezman (1998).

For immunocytochemistry, cells were fixed in 2% paraformaldehyde/0.1% glutaraldehyde/0.1 M phosphate buffer (pH 7.4). Sections were processed by cryoultramicrotomy and labeling with a specific anti-cruzain antibody (Engel et al., 1998a,b).

Ultramicrotomy was used for EM localization of enzymes to determine pH within subcellular compartments (Babai, 1977) in sections of CA-I/72, CA-I/72 treated with 10 μM N-Pip-F-hF-VSphenyl, and CA-I/KR cells. Epimastigotes were fixed in 1.5% glutaraldehyde-0.1 M sodium cacodylate-1% sucrose (pH 7.4) for 1 hour at room temperature, washed and stored in 0.1 M sodium cacodylate buffer-5% sucrose (pH 7.4) at 4°C. Three incubation procedures specific for alkaline phosphatase (Alk-Pase; pH 9.2), acid phosphatase (Ac-Pase; pH 5), and trimetaphosphatase (T-Pase; pH 3.9) activities were used to detect pH within subcellular structures (Westen and Bainton, 1979). Cells were incubated at 37°C for 150 minutes for Ac-Pase and 90 minutes for the other enzymes. Substrates were Naphthol AS-TR phosphate for Alk-Pase (Westen and Bainton, 1979); β-glycerophosphate for Ac-Pase (Quan et al., 1980; Poore et al., 1981); and Na-trimetaphosphate for T-Pase (Doty et al., 1977). Cells were fixed again in 1.5% glutaraldehyde and processed for EM.

Western blotting, autoradiography, and protein sequencing

For western blotting, CA-I/72 epimastigotes were cultured in BHT medium with and without 30 μM N-Pip-F-hF-VSphenyl overnight. CA-I/KR epimastigotes were similarly cultured except with 150 or 200 μM N-Pip-F-hF-VSphenyl as indicated. To investigate the effect of drug withdrawal, CA-I/KR epimastigotes were also cultured without CPI for 3 days. Only cultures maintained in the exponential phase of growth and with ≥99% cell viability were used. In some experiments, epimastigotes were metabolically labeled with L-[35S] methionine (Amersham, IL: >800 Ci mmol⁻¹) (Doyle and Dwyer, 1993). Epimastigotes (1×10⁹ cells/ml) were washed three times in phosphate buffered saline (PBS), frozen-thawed 5 times, and centrifuged at 14,000 g for 1 hour at 4°C. Soluble fractions were collected, and membrane pellets solubilized by vortexing in buffer containing 1% Triton X-100 for 1 hour at 4°C (Gardiner and Dwyer, 1983). For secretion experiments, parasites were washed and resuspended in PBS (1×10⁹ cells/ml), prior to incubation for 3 hours at 26°C (Engel et al., 1990). Culture supernatants were collected by centrifugation. All samples were aliquoted and stored at -70°C until used. Samples (1×10⁷ epimastigotes/lane) were electrophoresed in 4-12% gradient gels (Novek, CA), blotted onto PVDF membranes (Millipore), and...
developed with anti-cruzain antibody (dilution 1/2000; Engel et al., 1998a,b) followed by goat anti-rabbit antibody bound to Alk-Pase (Zymed) or ECL (Amersham) as indicated. All procedures were according to the manufacturer’s instructions.

Samples were also immunoprecipitated with the anti-cruzain antibody, electrophoresed, and blotted onto PVDF (Matsudaira, 1987). Amino acid sequencing was performed at the Protein and Nucleic Acid Facility; Beckman Center; Stanford Medical Center, Stanford, CA.

**Activity assays**

CA-I/72 and CA-I/KR epimastigotes were sonicated and used for cruzain assays. As additional controls, CA-I/72 cells were also cultured overnight with 20 μM N-Pip-F-hF- VSf, 100 μM E64 (Sigma), and 100 μM/ml protease inhibitor cocktail (P 8340, Sigma). Protease activity comes from 1x10^6 parasites/μl for Table 1 and 1x10^5 parasites/μl for Table 2. Activity assays were in a reaction buffer containing 100 mM sodium acetate, pH 5.5, 10 mM dithiothreitol. Proteolytic activity was monitored by the hydrolysis of 7-amino-4 methyl coumarin from the substrate Z-F-R-AMC (Sigma). Rates were measured in microtitre plates using a Fluoroskan II spectrofluorometer at 460 nm emission and an excitation wavelength of 355 nm for 10 minutes at 25°C (Roush et al., 1999; Chan et al., 1999). Data were fitted using the Ultrafit non-linear regression analysis software (Biosoft, Ferguson, MO). Results (± s.d.) from at least two independent experiments (n=2-3) were normalized to the corresponding controls. Recombinant cruzain was used as an additional control (Eakin et al., 1993).

**RESULTS**

**Development of CPI-resistant *T. cruzi* and epimastigote growth rate**

CA-I/72 epimastigotes were subjected to a gradual increase from an initial 2 μM to a final 200 μM N-Pip-F-hF-VSf. Adaptation of the parasites to the inhibitor was slow and difficult, and required over a year; but once established resistance has been stable. Drug withdrawal for 4 months did not affect subsequent epimastigote growth in the presence of 200 μM CPI. Epimastigote doubling times were 28 hours for CA-I/72 controls, 30 hours for CA-I/KR without N-Pip-F-hF-VSf for 4 months, and 34 hours for CA-I/KR with 200 μM N-Pip-F-hF-VSf. CA-I/KR epimastigotes were sensitive to 3 μM nifurtimox. Conversely, CA-I/NR epimastigotes were cross-resistant to 10 μM benznidazole but sensitive to N-Pip-F-hF-VSf. Three μM nifurtimox or benznidazole and 10 μM CPI are lethal for parental CA-I/72 cells.

**Ultrastructure of CPI-resistant *T. cruzi***

When the ultrastructure of CA-I/KR epimastigotes was compared to that of parental sensitive parasites (Fig. 1A), a marked increase in size and number of Golgi cisternae and associated vesicles trafficking to the flagellar pocket became evident (Fig. 1B-D). Immunoelectron microscopy with a specific anti-cruzain antibody showed no significant labeling in the flagellar pocket and cell surface of untreated controls (Fig. 2A). In contrast, labeled cruzain was present both in the lumen of the flagellar pocket and within vesicles trafficking towards it in CPI-resistant parasites (Fig. 2B), and concomitantly decreased in lysosomes (Fig. 2C-E).

**Electrophoresis and western blotting**

The cruzain profile of CA-I/KR epimastigotes cultured without the inhibitor for 3 days (Fig. 3, lanes 1 and 5) was similar to that of controls (lanes 4 and 8) and showed a major cruzain species of approximately 45 kDa in their soluble fractions (lanes 1 and 4). In contrast, CA-I/KR epimastigotes cultured with 150 μM N-Pip-F-hF-VSf (lanes 2 and 6) showed an increase in higher Mr (51/57 kDa) cruzain species in the soluble

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**Table 1. Cruzain activity (± s.d.) of CPI-sensitive and -resistant *T. cruzi***

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CA-I/72</th>
<th>CA-I/72+20 μM CPI*</th>
<th>CA-I/KR</th>
<th>CA-I/KR+200 μM CPI‡</th>
<th>CA-I/KR-CPI§</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>405±60¶</td>
<td>12.9±1.2</td>
<td>13±0.7</td>
<td>32.6±14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(100%)</td>
<td>(3.1%)</td>
<td>(3.2%)</td>
<td>(8.4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CA-I/72 epimastigotes were cultured with 20 μM N-Pip-F-hF-VSf for 16 hours.
‡CA-I/KR epimastigotes were cultured with 200 μM N-Pip-F-hF-VSf for 1 year.
§CA-I/KR epimastigotes were cultured with 200 μM N-Pip-F-hF-VSf for 1 year and without CPI for 16-24 hours.
¶Activities are mean fluorescence units/minute (± s.d.) of 3 independent experiments (n=3).
||Relative activities with the substrate Z-F-R-AMC were normalized to CA-I/72 controls.

**Table 2. Inhibition of proteolytic activities from CPI-sensitive and -resistant *T. cruzi***

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activity (± s.d.) (% relative inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA-I/72‡</td>
</tr>
<tr>
<td>None</td>
<td>1330±56</td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
</tr>
<tr>
<td>E64 (100 μM)</td>
<td>19.8±1.7</td>
</tr>
<tr>
<td></td>
<td>(98%)</td>
</tr>
<tr>
<td>N-Pip-F-hF-VSf (100 μM)</td>
<td>17.4±1</td>
</tr>
<tr>
<td></td>
<td>(99%)</td>
</tr>
<tr>
<td>Sigma cocktail§ (100 μl/ml)</td>
<td>33.2±6</td>
</tr>
<tr>
<td></td>
<td>(97.5%)</td>
</tr>
</tbody>
</table>

Activity (± s.d.) is in fluorescence units/minute. Results are from 2 independent experiments (n=2).

*Inhibition is (100-relative cruzain activity). Relative activity was normalized to each corresponding control without inhibitor (0% inhibition).
‡Epimastigotes were cultured as in Table 1.
§Sigma cocktail (Sigma, cat. P8340) contains AEBSF (irreversible serine protease inhibitor); pepstatin (inhibitor of acid proteases, e.g. cathepsin D); E64; bestatin (inhibitor of leucine aminopeptidases); leupeptin (ac-leu-Leu-Arg-Ala); and aprotonin (trypsin inhibitor).
fraction (lane 2) but the profile of membrane-bound cruzain molecules (lane 6) was similar to controls (lane 8). When sensitive CA-I/72 epimastigotes were cultured in the presence of a lethal concentration of N-Pip-F-hF-VSΦ (lanes 3 and 7) a shift in the relative molecular masses (51/57 kDa) of the major soluble and membrane-bound cruzain species was observed.

Fig. 1. Ultrastructure of CPI-sensitive and -resistant epimastigotes. T. cruzi epimastigotes of the parental, CPI-sensitive CA-I/72 clone were exposed to increasing concentrations of CPI to generate CA-I/KR parasites. Note the increase in the number of Golgi cisternae and vesicles trafficking to the flagellar pocket in CA-I/KR parasites. (A) CA-I/72 epimastigote control (×35,000). (B-D) CA-I/KR epimastigotes cultured with 200 μM N-Pip-F-hF-VSΦ (×35,000). FP, flagellar pocket; G, Golgi complex; K, kinetoplast; N, nucleus.
CA-I/KR epimastigotes cultured with 200 μM N-Pip-F-hF-VS\(\beta\) secreted mostly cruzain molecules of 51/57 kDa (Fig. 4, lane 2) while CA-I/72 controls secreted a major species of approximately 45 kDa (Fig. 4, lane 1). The amino terminus of the latter was sequenced (Protein and Nucleic Acid Facility; Beckman Center; Stanford Medical Center, CA) and the amino acids identified mature cruzain without the prodomain (Eakin et al., 1993). Negligible secretion of 51/57 kDa precursors followed overnight removal of the cysteine protease inhibitor in CA-I/KR even after prolonged exposure (≥10 minutes) of the ECL-developed western blot (Fig. 4, lane 3). Analyses of \([^{35}S]\)methionine labeled and silver-stained electrophoretic profiles showed different protein patterns for CA-I/KR and control epimastigotes (data not shown).

**pH of subcellular organelles**

Detection of Alk-Pase, Ac-Pase, and T-Pase activities at the ultrastructural level allowed the determination of pH within subcellular organelles in the different *T. cruzi* stocks. Only results with Ac-Pase at pH 5 are shown. Acid phosphatase...
activity resided mainly in lysosomes of CA-I/72 controls with some product formation in vesicles proximal to the flagellar pocket (Fig. 5A). Similar results were obtained when CA-I/72 epimastigotes were treated with 10 mM CPI for a short period of time (Fig. 5B). CPI-resistant epimastigotes showed more conspicuous labeling indicative of pH 5 in trans-Golgi cisternae (Fig. 5C), within vesicles trafficking toward the flagellar pocket, and in luminal membranes of the flagellar pocket (Fig. 5D).

Endocytosis

The functionality of the endocytic pathway was analyzed with positively charged Nanogold coating the cell surface of CA-I/KR and CA-I/72 epimastigotes. Endocytosed gold particles accumulated as expected within lysosomes and endocytic vesicles proximal to the flagellar pocket in both cells (Fig. 6).

Protease activity of sensitive and resistant epimastigotes

Sixteen hours of treatment with 20 mM N-Pip-F-hF-VS showed decreased protease activity levels by 97% in sensitive CA-I/72 epimastigotes (Table 1; CA-I/72+20 mM CPI). CA-I/KR epimastigotes also had a low protease activity (3% relative to untreated CA-I/72 epimastigotes) if the drug pressure was constantly maintained for >1 year (CA-I/KR+200 mM CPI). Sixteen hours without the inhibitor was sufficient for a significant increase in protease activity in CA-I/KR epimastigotes (CA-I/KR-CPI). 98% of protease activity in CA-I/72 controls was abolished by 100 mM N-Pip-F-hF-VS overnight and E64 (Table 2; CA-I/72), but only ~15% in CA-I/KR (CA-I/72+200 mM CPI). Residual protease activity in all strains was abolished by an inhibitor cocktail (Sigma) that included AEBSF (irreversible serine protease inhibitor); pepstatin (inhibitor of acid proteases); E64 (cysteine protease inhibitor); bestatin (inhibitor of leucine aminopeptidases); leupeptin (ac-Leu-Leu-Arg-Ala); and aprotinin (trypsin inhibitor). Withdrawal (16-72 hours) of the drug-pressure in epimastigote cultures, partially reversed CA-I/KR proteolytic activity to active cruzain (Table 2, CA-I/KR-CPI; and Fig. 3, lane 1), capable of hydrolyzing the substrate Z-F-R-AMC and inhabitable by 100 mM CPI and E64.

DISCUSSION

Cysteine protease inhibitors are in preclinical development as potential chemotherapy for Chagas’ disease. The lethal mechanism of action is unusual-preventing autocatalytic processing of the enzyme target with subsequent accumulation of precursors in Golgi complex (Engel et al., 1998a). Mechanisms previously associated with decreased sensitivity or resistance to chemotherapy in parasitic protozoa include decreased drug uptake, increased export of drugs, decrease in drug activation, and alterations of the target enzyme to decrease drug-binding (reviewed by Borst and Ouellette, 1995; Ullman, 1995). In this report we describe a novel mechanism conferring drug resistance in trypanosomes, upregulation of the secretory protease activity residing mainly in lysosomes of CA-I/72 controls with some product formation in vesicles proximal to the flagellar pocket (Fig. 5A). Similar results were obtained when CA-I/72 epimastigotes were treated with 10 mM CPI for a short period of time (Fig. 5B). CPI-resistant epimastigotes showed more conspicuous labeling indicative of pH 5 in trans-Golgi cisternae (Fig. 5C), within vesicles trafficking toward the flagellar pocket, and in luminal membranes of the flagellar pocket (Fig. 5D).

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pathway. We generated *T. cruzi* epimastigotes that were resistant to 20-fold the lethal concentration of CPI necessary to cure an acute infection in experimental animals. Great difficulty was encountered in developing CPI-resistance and over a year of gradual 2-10 μM step-wise increases in drug concentration was required. However, drug-resistance was stable and only a modest decrease in replication rate occurred. Drug withdrawal for periods of up to 4 months did not reverse resistance in epimastigote cultures. CPI-resistant *T. cruzi* epimastigotes are sensitive to nifurtimox and benznidazole. Conversely, nifurtimox-resistant epimastigotes are cross-resistant to benznidazole but sensitive to CPI. Thus, resistance to these trypanocidal drugs appears to be mediated by different cellular mechanisms. Cysteine protease inhibitors may therefore be effective even in cases of Chagas’ disease refractory to conventional therapy.

Ultrastructural analyses of CPI-resistant epimastigotes showed structural hypertrophy and hyperactivity of the secretory pathway. There was a marked increase in vesicles between the Golgi complex and the flagellar pocket, the site of secretion in trypanosomes, and an increase from 5-7 to up to 16 Golgi cisternae. Enlargement in the size and quantity of membranes associated with the flagellar pocket was evident (Fig. 1B-D). In contrast to CPI-treated, sensitive parasites (Fig. 2; Engel et al., 1998a), no accumulation of cruzain in peripheral Golgi vesicles (data not shown) or lysosomes occurs in CA-I/KR epimastigotes cultured for a year with 20-fold the lethal CPI-concentration (Fig. 2). While most epimastigote cruzain is normally targeted to reservosomes (lysosomes), the protease was detected by immuno-EM mainly within the lumen of and in vesicles proximal to the flagellar pocket in CA-I/KR epimastigotes (Fig. 2). CPI-resistant *T. cruzi* cultured for two days without inhibitor retained the resistant phenotype and ultrastructure. The pH within the trans-Golgi network appears to be important for the sorting of secretory cargo and retrieval of components of the biosynthetic pathway in mammalian cells (Demaurex et al., 1998). The acidity of the lumen of the secretory pathway in mammalian cells increases gradually (lower pH) as the secretory products approach the plasma membrane. A resting pH 5.9-5.95 is maintained within the distal subcompartments of the Golgi complex (Demaurex et al., 1998). In CA-I/KR

**Fig. 6.** Endocytosis in drug-sensitive and -resistant epimastigotes. Endocytosed nanogold particles coating the cell surface accumulated within 15 minutes in lysosomes (arrowheads) and endocytic vesicles (arrows) of CPI-resistant epimastigotes. Insert shows endocytic vesicles of control, sensitive epimastigotes (>22,000). Nu, nucleus; K, kinetoplast; FP, flagellar pocket.
epimastigotes a considerably enhanced low pH (pH 5) compartment within the trans-Golgi cisternae is evident (Fig. 5C) perhaps allowing premature release of unprocessed cruzain precursor molecules from their transport receptor for secretion. Increased amounts of Ac-Pase in the flagellar pocket is also consistent with upregulation of the protein secretory pathway in drug-resistant epimastigotes.

Resistant T. cruzi epimastigotes secreted cruzain species of higher Mr (51/57 kDa) than controls into the culture supernatant (Fig. 4), and differed in the amounts of unprocessed cruzain precursors associated with membrane fraction of epimastigote extracts (Fig. 3). Amino acid sequencing confirmed that sensitive epimastigotes secrete mature cruzain (~45 kDa) into the culture supernatant. Based on previous analyses (Engel et al., 1998a) and on our western-blot analyses (Fig. 4), we hypothesize the higher Mr molecules secreted by resistant parasites correspond to inhibitor-bound, unprocessed cruzain precursors. As a consequence of inhibitor binding and cruzain secretion, CA-I/KR epimastigotes had negligible cysteine protease activity in the lysosomal/endosomal compartment as compared to the parental clone. Addition of 100 μM N-Pip-F-hF-VSΦ and E64 abolished most activity in sensitive CA-I/72 epimastigotes but not the residual protease activity in CA-I/KR parasites (Tables 1 and 2) suggesting CPI-resistant T. cruzi may use other proteases for survival (Lowndes et al., 1996; Burleigh et al., 1997; Wiser et al., 1997; Nóbrega et al., 1998).

A model for the upregulation of the secretory pathway induced by resistance to cysteine protease inhibitors and the effect of drug withdrawal in CPI-resistant T. cruzi epimastigotes versus the effect of cysteine protease inhibitors on cruzain processing and trafficking in wild-type cells is proposed in Fig. 7. Active cruzain is either targeted to lysosomes/reservosomes in sensitive parental trypanosomes or partially secreted via the flagellar pocket. Cysteine protease inhibitors bind the active site of the protease preventing autocatalytic activation and inducing accumulation of protease within enlarged Golgi cisternae (Engel et al., 1998a). Inactive but soluble cruzain is secreted by CPI-resistant parasites cultured with a cysteine protease inhibitor while removal of the inhibitor restores cruzain activity. Resistance to cysteine protease inhibitors requires upregulation of Golgi function via increased number of vesicles in the secretory pathway, and release of inactive protease precursors unable to reach their final site of biologic activity.

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