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Cysteine protease inhibitors alter Golgi complex ultrastructure and function in *Trypanosoma cruzi*

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SUMMARY

Cruzain, the major cysteine protease of the protozoan parasite *Trypanosoma cruzi*, is a target of rational drug design for chemotherapy of Chagas' disease. The precise biological role of cruzain in the parasite life cycle and the mechanism involved in the trypanocidal effect of cysteine protease inhibitors are still unclear. Here we report biological and ultrastructural alterations caused by cysteine protease inhibitors in *T. cruzi* epimastigotes. Cruzain, a glycoprotein that transits the Golgi-endosomal pathway, localized to pre-lysosomes/lysosomes in the posterior end of untreated epimastigotes by fluorescent microscopy utilizing either a biotinylated cysteine protease inhibitor to tag the active site, or a specific anti-cruzain antibody. Radiolabeled or biotinylated cysteine protease inhibitors bound exclusively to cruzain in intact epimastigotes confirming that cruzain is accessible to, and is targeted by the inhibitors. Treatment of *T. cruzi* epimastigotes with specific cysteine protease inhibitors arrested growth, altered the intracellular localization of cruzain, and induced major alterations in the Golgi complex. Following treatment, cruzain accumulated in peripheral dilations of Golgi cisternae. There was a concomitant 70% reduction in gold-labeled cruzain transported to lysosomes. Cisternae abnormalities in the Golgi compartment were followed by distention of ER and nuclear membranes. Brefeldin A increased the number and size of cisternae in epimastigotes. Pre-treatment of epimastigotes with cysteine protease inhibitors followed by exposure to brefeldin A induced a more rapid appearance of the cysteine protease inhibitor-induced Golgi alterations. Our results suggest that cysteine protease inhibitors prevent the normal autocatalytic processing and trafficking of cruzain within the Golgi apparatus. Accumulation of cruzain may decrease mobility of Golgi membranes and result in peripheral distention of cisternae. These major alterations of the Golgi complex parallel the death of *T. cruzi* epimastigotes.

Key words: *T. cruzi*, Cysteine protease inhibitor, Cruzain, ER/Golgi complex

INTRODUCTION

The major proteolytic activity of the protozoan parasite *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, resides in cruzain (a.k.a. cruzipain, GP57/51), a thiol-dependent cathepsin L-like cysteine protease (Scharfstein et al., 1986; Bontempi et al., 1989; Cazzulo et al., 1989, 1990a,b; Eakin et al., 1992; Murta et al., 1990; Gazzinelli et al., 1993; McKerrow et al., 1995). Although comparable levels of cruzain messenger RNA are present in all developmental stages of the parasite, protease levels and activity are approximately 10-fold higher in epimastigotes, the stage of the parasite found in the insect vector, suggesting regulation at the translational/post-translational levels (Eakin et al., 1992; Tomas and Kelly, 1996). While residing in the insect gut, the epimastigote derives much of its nutrition by endocytosis and subsequent degradation of host molecules in lysosome-like vesicles (reservosomes). Cruzain is thought to play a major role in this process. Cruzain is synthesized as a pre-proenzyme with three distinct domains, the pro-domain, the catalytic domain, and the carboxyl-terminal extension (Eakin et al., 1992). Three potential N-linked glycosylation sites, one localized to the C-terminal extension and the other two within the catalytic domain, have been identified in this glycoprotein (Cazzulo et al., 1990a,b; Labriola et al., 1995). Although protein transport in trypanosomatids is poorly understood (Clayton et al., 1995), experimental evidence suggests that cruzain is processed as it transverses the ER and Golgi apparatus before reaching epimastigote lysosomes (Soares et al., 1992; Parodi et al., 1995; Labriola et al., 1995). Thus, this protease is a potentially useful marker to follow protein transport and processing through the ER-Golgi-endosomal pathway in trypanosomatids.

Micromolar concentrations of peptide-based cysteine protease inhibitors (CPI) arrest parasite replication, trypomastigote-amastigote differentiation, and trypomastigote output (Ashall et al., 1990; Meirelles et al., 1992; Harth et al.,

1993). The three-dimensional structure of cruzain was solved at the 2 Å level (McGrath et al., 1995) and structure-based synthesis of specific cruzain inhibitors was performed (Ring et al., 1993; Palmer et al., 1995; Bromme et al., 1996). It has been hypothesized that the trypanocidal effect of cysteine protease inhibitors results from the direct inhibition of the active cruzain contained within the lysosomes. We now report that three types of irreversible cysteine protease inhibitors, designed to bind at the cruzain active site, specifically inhibit cruzain in live epimastigotes. Biotinylated derivatives of these inhibitors and an anti-cruzain antibody allowed us to localize cruzain in epimastigotes. Cysteine protease inhibitors induced a specific alteration in the Golgi-lysosomal pathway consistent with altering cruzain auto-processing and blocking transport from the Golgi apparatus.

MATERIALS AND METHODS

Parasite cultures

Epimastigote cultures of the *Trypanosoma cruzi* Y strain were maintained in exponential phase of growth in LIT medium as described previously (Doyle and Weinbach, 1989).

Effect of cysteine protease inhibitors on epimastigote growth rate

Stock solutions (20 mM) of the cysteine protease inhibitors: morpholinecarbonyl-phenylalanine-homophenylalanine-vinyl sulphone phenyl, Mu-F-hF-VSPh and [^{14}C]-Mu-F-hF-VSPh (Arris Pharmaceuticals, Inc., CA; Palmer et al., 1995); and morpholineurea-phenylalanine-homophenylalanine-fluoromethyl ketone, Mu-F-hF-FMK (Smith et al., 1988) were prepared in dimethyl sulfoxide (DMSO) and stored at −70°C until used. Aliquots containing 10^6 epimastigotes/ml were incubated with or without 5-50 μM inhibitor (0.005-0.25% DMSO). Growth rates were estimated by daily parasite counts using a hemocytometer chamber (n=2). Untreated and/or DMSO-treated epimastigote cultures were followed in parallel as controls.

Autoradiography of *T. cruzi* proteins radiolabeled with a cysteine protease inhibitor

Live epimastigotes were incubated with or without 10 μM unlabeled Mu-F-hF-VSPh for 2 hours at 26°C. ^{14}C-labeled Mu-F-hF-VSPh (10 μM) was then added and parasites re-incubated as described above. Epimastigotes were either resuspended directly in sample buffer (SB) or sonicated. Sonicated epimastigotes were centrifuged for 1 hour at 14,000 ***g***, 4°C, and membrane and soluble fractions collected, TCA-precipitated, and resuspended in SB. Samples were electrophoresed and autoradiographed. Controls were recombinant cruzain (Eakin et al., 1993) incubated as described above for 1 hour at 26°C.

Fluorescence and immunofluorescence studies

Live epimastigotes were labeled with 10 μM biotinylated-Phe-Ala-FMK (ESP, Dublin, CA) for 1 hour at 26°C. Like Mu-F-hF-VSPh, this inhibitor binds irreversibly at the active site of cruzain (McGrath et al., 1995) and can be detected by virtue of biotin-streptavidin complexes. Epimastigotes were repeatedly washed with PBS, and fixed with 2% paraformaldehyde, pH 7.2. Cell membranes were permeabilized for 1 minute with 0.02% Triton X-100, washed in PBS, and stained with fluorescein-bound streptavidin (Zymed, San Francisco, CA). For immunofluorescence microscopy, fixed and permeabilized epimastigotes were incubated with an anti-cruzain polyclonal rabbit antibody raised against the catalytic domain (Eakin et al., 1995), followed by incubation with a fluorescein-conjugated anti-rabbit antibody (Zymed, San Francisco, CA). For lysosomal localization, epimastigotes were labeled for 15 minutes with 1 μg/ml Lysosensor (Molecular Probes, Eugene, OR), a fluorescent acidotropic probe which selectively accumulates within cellular compartments with low pH. Epimastigotes were observed in a Zeiss microscope equipped with UV epifluorescence and a Model MC80 photographic unit.

Ultrastructural studies of *T. cruzi* epimastigotes

Epimastigotes were treated with 20 μM Mu-F-hF-VSPh for 1 hour to 72 hours prior to fixation. Epimastigotes were fixed in 1.5% glutaraldehyde in 0.66 M sodium cacodylate buffer, pH 7.4, at room temperature for 2 hours. Epimastigotes were then embedded in EPONATE 12 (Ted Pella, Inc.), sectioned, stained and observed using a Zeiss 10C electron microscope (Stenberg et al., 1984). In some experiments, epimastigotes were treated with 20 μM Mu-F-hF-VSPh for 1 hour and/or with 5 μg/ml brefeldin A (BFA) (Molecular Probes, Eugene, OR) prior to fixation and staining with uracyl acetate.

Immunocytochemistry of *T. cruzi* epimastigotes

The techniques used for preparing ultrathin cryosections and immunogold labeled sections have been described previously (Kjeldsen et al., 1993). Briefly, epimastigotes were collected by centrifugation, washed twice with PBS, and fixed with 2% paraformaldehyde-0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2-4 hours at 4°C. Epimastigotes were then cryoprotected, frozen, sectioned, and immunolabeled. A rabbit polyclonal anti-cruzain antibody (dilution 1:500) and a goat anti-rabbit IgG-10 nm gold labeled antibody were used. Control experiments were performed by replacing the anti-cruzain antibody with normal rabbit serum (Vector Lab., Burlingame, CA). Gold-labeled cruzain was quantified in twenty lysosomes of similar diameter in treated and untreated *T. cruzi* epimastigotes (n=10). The percentage of reduction of lysosomal cruzain in treated cells was determined from the number of gold-particles in treated cells/number of gold-particles in untreated controls.

RESULTS

Cysteine protease inhibitors arrest epimastigote growth

Cysteine protease inhibitors arrested growth of epimastigotes of the *T. cruzi* Y strain at concentrations ranging from 5-50 μM. Growth patterns of epimastigote cultures treated with Mu-F-hF-VSPh, and Mu-F-hF-FMK were similar. For simplicity, only growth curves of epimastigotes treated with Mu-F-hF-VSPh are shown (Fig. 1). Both cysteine protease inhibitor-treated and control epimastigotes had a doubling time of 28 hours during the first 2 days of culture. This initial period of normal division was followed by a marked decrease in growth rate in inhibitor-treated cultures. 5 μM concentrations of the inhibitors reduced growth rates by approximately 60%. After 48 hours, epimastigote growth was completely arrested by incubation with ≥10 μM of any of the three inhibitors. The effect of CPI was irreversible and death occurred after 5 days as determined by transfer of treated epimastigotes to drug-free medium.

In vivo inhibition and labeling of cruzain

Complete inhibition of cruzain activity was observed in cytoplasmic extracts prepared from epimastigotes incubated with Mu-F-hF-VSPh (≥5 μM) for 2 hours. Incubation of live epimastigotes with radiolabeled Mu-F-hF-VSPh followed by electrophoresis and autoradiography showed that this CPI

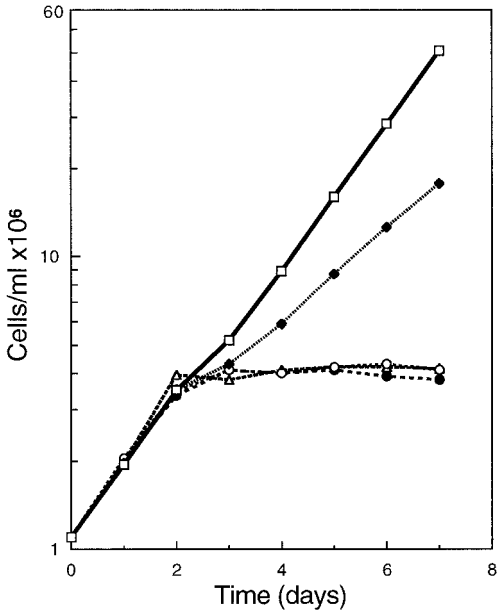

Fig. 1. Growth patterns of *T. cruzi* epimastigotes cultured with or without cysteine protease inhibitors. Aliquots containing 10^6 epimastigotes/ml were incubated in LIT medium containing DMSO and/or 5-50 μM Mu-F-hF-VSPh for up to 7 days. Control epimastigotes (□) and epimastigotes treated with 5 μM (◆); 10 μM (○); 20 μM (▽); and 50 μM (●) were counted daily by duplicate in a hemocytometer chamber.

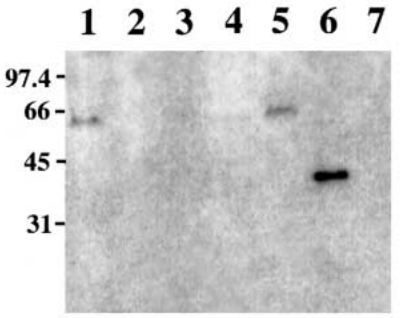

Fig. 2. Autoradiogram of *T. cruzi* proteins bound by a radiolabeled-CPI. Live epimastigotes were incubated with or without unlabeled Mu-F-hF-VSPh prior to addition of [^{14}C]-Mu-F-hF-VSPh. Epimastigotes were either resuspended in sample buffer or membrane and soluble fractions extracted prior to electrophoresis and autoradiography. Recombinant cruzain controls were treated as described above. Lane 1, epimastigotes treated with [^{14}C]-Mu-F-hF-VSPh; lane 2, epimastigotes treated sequentially with unlabeled Mu-F-hF-VSPh and labeled-CPI; lane 3, SB; lane 4, supernatant from epimastigotes incubated with [^{14}C]-Mu-F-hF-VSPh; lane 5, membranous organelles from epimastigotes incubated with [^{14}C]-Mu-F-hF-VSPh; lane 6, recombinant cruzain labeled with [^{14}C]-Mu-F-hF-VSPh; lane 7, recombinant cruzain incubated sequentially with unlabeled and labeled Mu-F-hF-VSPh. Glycosylated native cruzain has a higher molecular mass than the recombinant protease.

bound specifically to a single epimastigote protein of approximately 51 kDa, the native molecular mass of cruzain (Scharfstein et al., 1986; Cazzulo et al., 1989) (Fig. 2, lane 1). This 51 kDa protein was previously confirmed as cruzain by specific anti-cruzain antibody (Eakin et al., 1995). This radiolabeled species was not detected when parasites were pre-incubated with cold Mu-F-hF-VSPh for 2 hours (Fig. 2, lane 2). Cruzain localized to the membrane fraction of epimastigotes (Fig. 2, lane 5). Radiolabeled inhibitor also bound recombinant cruzain (Fig. 2, lane 6). Similar results were obtained for autoradiograms exposed for 30-90 days.

Localization of cruzain and CPI-induced alterations

The normal morphology of a *T. cruzi* epimastigote is shown in contrast phase (Fig. 3A) and Giemsa-stained (Fig. 3B) micrographs. The proximity of the nucleus and kinetoplast (mitochondrial DNA) with the origin of the anterior flagellum is apparent. To investigate the cellular localization of the cruzain-CPI complex, live epimastigotes were treated with a biotinylated-F-A-FMK for 1 hour. Epimastigotes were also evaluated in parallel with a fluorescent acidic probe specific for lysosomes (Fig. 3C). Both the biotinylated inhibitor (Fig. 3D) and the specific anti-cruzain antibody (Fig. 3E) localized the cysteine protease to membrane bound organelles, consistent with epimastigote lysosomes or 'reservosomes' (Soares et al., 1992). These large organelles are clustered at the posterior end of the parasite (Fig. 3C-E). In epimastigotes treated with CPI for 48 hours, the antibody localized cruzain not only to the lysosomes but also to the Golgi complex region which is anterior to the nucleus in *T. cruzi* (Fig. 3F). These results, together with the inactivation of cruzain in crude extracts of treated epimastigotes, suggested that the cysteine protease inhibitor is effectively transported into epimastigote organelles where it binds to cruzain and alters its subcellular localization. We confirmed this observation by ultrastructural analysis of epimastigotes following treatment with the cysteine protease inhibitor Mu-F-hF-VSPh (Fig. 4). The normal morphology and ultrastructure of cellular membranes and organelles in a *T. cruzi* epimastigote is shown for comparison (Fig. 4A). *T. cruzi* presents a characteristic network of microtubules subjacent to the surface membrane, and a single mitochondrion extended throughout the cytoplasm containing a condensed mitochondrial DNA, the kinetoplast. The Golgi complex consists of a single stack of cisternae (De Souza, 1984; Figuereido and Soares, 1995). The earliest changes noted were alterations in the Golgi complex including the peripheral dilation of cisternae, followed by swelling of endoplasmic reticulum and nuclear membranes, proliferation of cytoplasmic vesicles, and enlargement of the mitochondria (Fig. 4B,C). More severe morphological alterations in the nuclear membrane, endoplasmic reticulum and Golgi apparatus, together with the appearance of abnormal vesicles occupying most of the cytoplasm (Fig. 4D) were observed 72 hours post-treatment, and resulted in cell death. Similar ultrastructural alterations were observed with Mu-F-hF-FMK (not shown).

To evaluate whether these abnormalities might reflect accumulation of cruzain along a protein transport pathway, sections of untreated (Fig. 5A,C) and CPI-treated (Fig. 5B,D) *T. cruzi* epimastigotes were incubated with specific anti-cruzain antibody. Immunoelectron microscopy confirmed the accumulation of cruzain within the abnormally dilated periphery of Golgi cisternae in CPI-treated parasites (Fig. 5D). A concomitant 70% reduction in the mean number of gold particles, representing labeled cruzain, was observed in

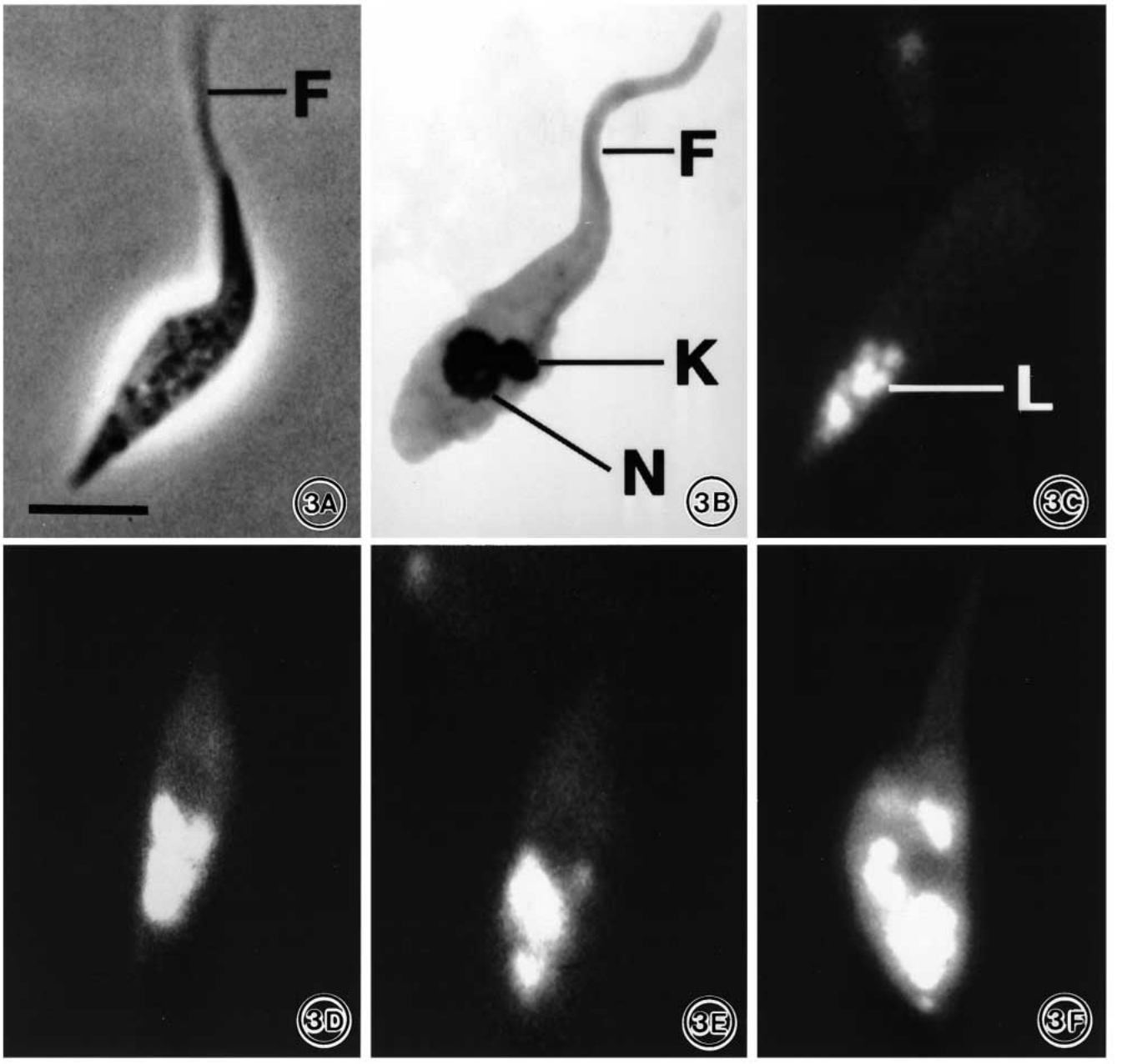

Fig. 3. Fluorescence and immunofluorescence localization of lysosomes and cruzain in *T. cruzi* epimastigotes. (A) Contrast phase micrograph of a T. cruzi epimastigote. (B) Giemsa-stained epimastigote. (C) Epimastigote lysosomes labeled with Lysosensor localized to the posterior end of the parasite. (D) Epimastigote labeled with biotinylated-F-A-FMK. (E) Epimastigote incubated with specific anti-cruzain polyclonal rabbit antibody followed by fluorescein-conjugated secondary antibody. (F) Cysteine protease inhibitor-treated epimastigote incubated with anti-cruzain polyclonal rabbit antibody followed by fluorescein-conjugated secondary antibody. F, flagellum; K, kinetoplast; L, lysosomes; N, nucleus. Bar, 5 μm.

epimastigote lysosomes 36-48 hours post-treatment with CPI [i.e. 97 gold particles in twenty lysosomes from ten CPI-treated epimastigotes (Fig. 6A) vs 386 gold particles in untreated epimastigotes (Fig. 6B)].

BFA alters synthesis and trafficking pathways of Golgi membranes, and increases the amount of membranes associated with this organelle. Epimastigotes were treated with BFA to investigate the possibility that the peripheral distention of cisternae resulted from an altered mobilization of Golgi membranes. *T. cruzi* epimastigotes normally have 5-8 cisternae (Fig. 7A); treatment with BFA increased the number of cisternae up to 16 (Fig. 7B). A rapid appearance of dilated cisternae in the Golgi complex (Fig. 7C), comparable to those observed after prolonged treatment (48 hours) with the CPI alone (Fig. 4B), occurred when *T. cruzi* epimastigotes were pretreated with Mu-F-hF-VSPh followed by BFA. These results strongly suggest that CPI alter the normal recycling of Golgi membranes.

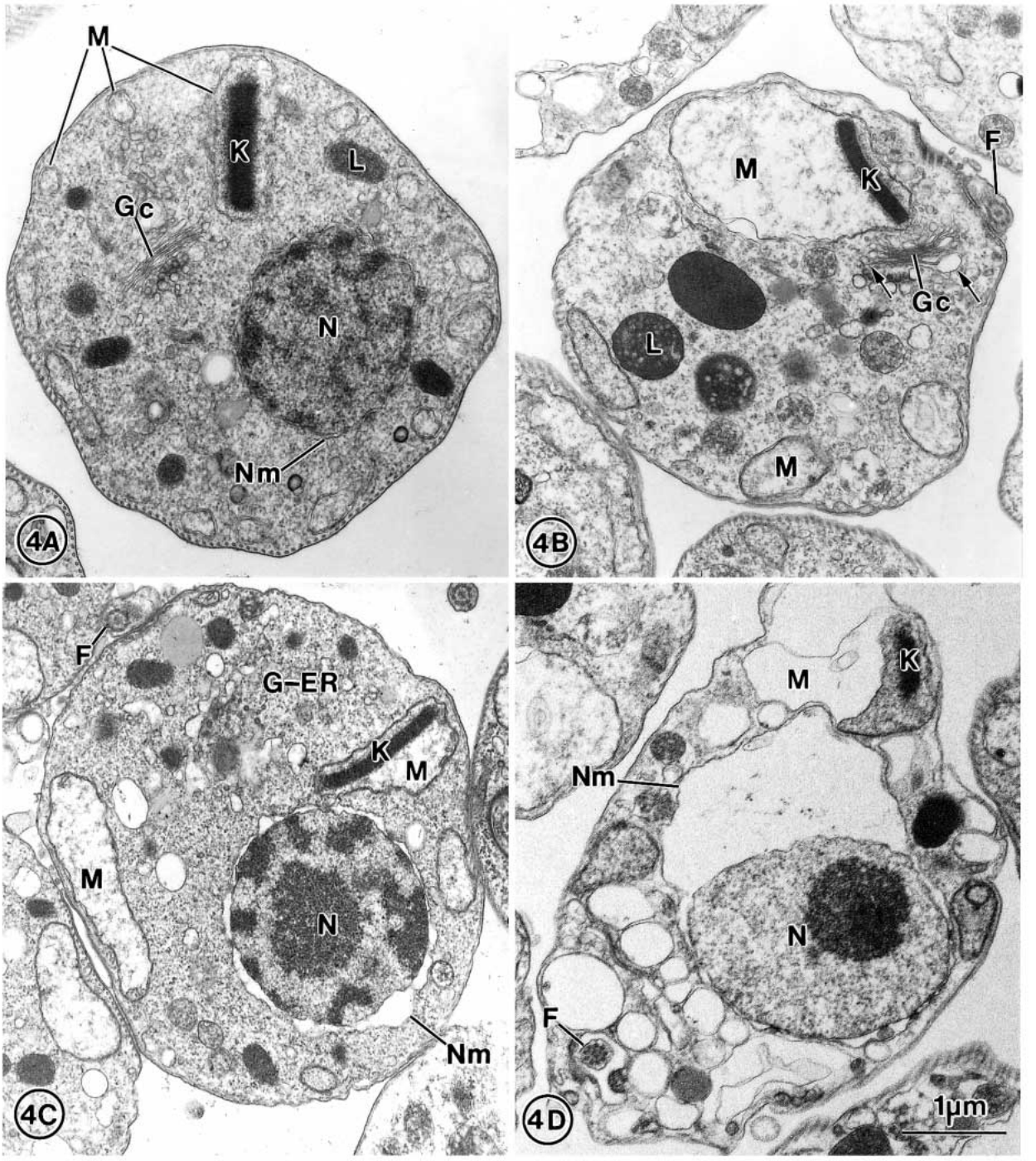

Fig. 4. Effect of cysteine protease inhibitors at the ultrastructural level. (A) Electron micrograph of a control *T. cruzi* epimastigote showing the normal structure of the nucleus (N), nuclear membrane (Nm), kinetoplast (K) which is contained within the mitochondrion (M), Golgi cisternae (Gc), and lysosomes (L). (B) T. *cruzi* epimastigote 48 hours post-treatment with 20 µM Mu-F-hF-VSPh shows an enlargement of the mitochondrion and alterations in the Golgi apparatus which contains enlarged cisternae (arrows); F, flagellum. (C) Swelling of the nuclear membrane (Nm), alterations in the Golgi complex-endoplasmic reticulum (G-ER), and appearance of cytoplasmic vesicles also occur after 48 hours of treatment with 20 µM Mu-F-hF-VSPh. (D) Major morphological alterations in nuclear, ER, and Golgi membranes are observed 72 hours post-treatment with 20 µM Mu-F-hF-VSPh.

DISCUSSION

Cysteine protease inhibitors, which differ in chemistry but share a specific dipeptide backbone bound in the S1 and S3 subsites of cruzain (McGrath et al., 1995), arrest epimastigote growth and, ultimately, result in epimastigote death. Radiolabeled Mu-F-hF-VSPh bound solely to the ~51 kDa native cruzain in vivo. Although complete inhibition of cruzain

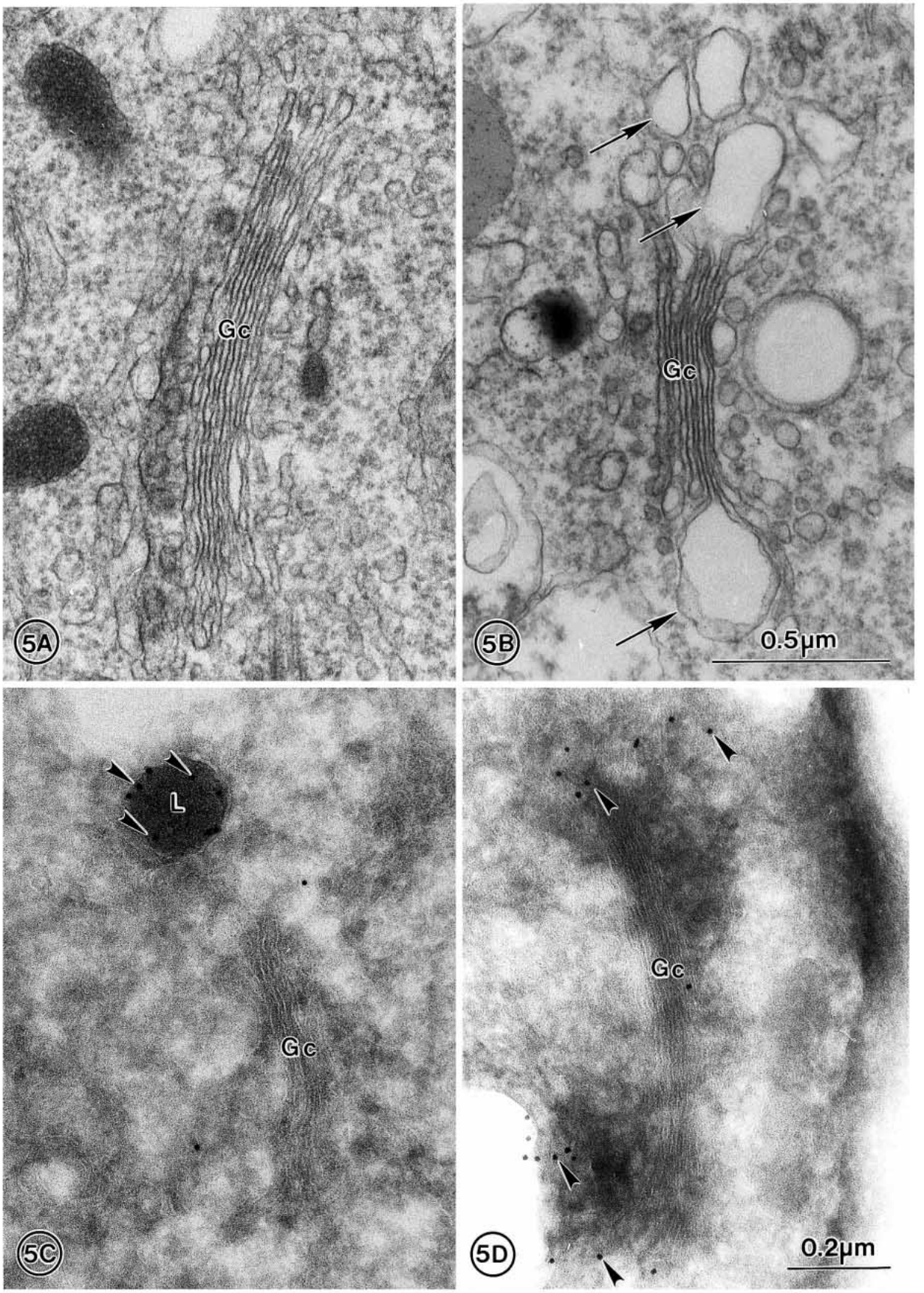
Gc
5A
Gc
5B
0.5μm
L
Gc
5C
Gc
5D
0.2μm

Fig. 5. Electron micrographs of ultrathin Epon sections (A,B) and of cryosections immunogold labeled with a specific anti-cruzain antibody (C,D) of the Golgi apparatus in untreated and CPI-treated *T. cruzi* epimastigotes. (A) Untreated epimastigote showing normal cisternae of the Golgi apparatus (Gc). (B) Epimastigote treated with 20 μM Mu-F-hF-VSPh for 48 hours. A marked enlargement of Golgi cisternae (arrows) is apparent. (C) Untreated epimastigote showing low levels of immunogold labeled cruzain in the Golgi apparatus and most immunogold label (arrowheads) localized to the lysosome (L). (D) Epimastigote treated with Mu-F-hF-VSPh for 48 hours. There is a marked increase of immunogold label (arrowheads) as a result of accumulation of cruzain within the Golgi apparatus. Bars: 0.5 μm (A,B); 0.2 μm (C,D).

activity occurred in epimastigotes treated for only 2 hours, arrest of epimastigote growth and death was evident only after 48 hours. This may reflect the cumulative inhibition of newly synthesized cruzain until a disruption of normal protein trafficking and subsequent damage to both the Golgi and ER compartments occur. Cruzain was localized to lysosomes/reservosomes in untreated epimastigotes by using either an antibody or a biotinylated inhibitor, confirming previous biochemical (Bontempi et al., 1989) and immunocytochemical studies (Souto-Padron et al., 1990; Murta et al., 1990; Soares et al., 1992). Treatment with cysteine protease inhibitors resulted in significant alterations in the localization of cruzain in *T. cruzi* epimastigotes. The accumulation of cruzain within Golgi vesicles suggested that the catalytic site of cruzain is already accessible to the inhibitors within the Golgi apparatus. A parallel decrease in the amount of cruzain within lysosomes/reservosomes was quantified immunocytochemically and suggested blockade in the transport of cruzain through the endosomal pathway.

Major morphological alterations of the nuclear membrane, endoplasmic reticulum, and Golgi complex were all induced by CPI-treatment. The distention of the perinuclear membrane compartment and the endoplasmic reticulum, and the formation of vesicles may also result from the interrupted trafficking of proteins within the Golgi complex. Prolonged exposure (72 hours) of *T. cruzi* epimastigotes to the inhibitors resulted in the formation of numerous large intracytoplasmic vacuoles probably originating from the ER and Golgi apparatus, the appearance of pycnotic nuclei, and epimastigote death.

Little is known regarding the transport of glycoproteins through the ER-Golgi-lysosome pathway in *T. cruzi* (Clayton et al., 1995). In mammalian cells, folding of glycoproteins within the ER is dependent on the presence of oligosaccharides. Similarly, *T. cruzi* glycoproteins appear to be transiently retained in the ER by anchors recognizing monoglucosylated oligosaccharides which ensure that only correctly folded glycoproteins are transported to the Golgi apparatus. Evidence in support of this mechanism comes from the treatment of *T. cruzi* epimastigotes with the inhibitor 1-deoxy-noijirimycin which induced a marked delay in the arrival of cruzain to lysosomes and in the amount accumulated within lysosomes (Labriola et al., 1995). However, the specific signals mediating the targeting of cruzain to lysosomes are still unknown as *T. cruzi* does not appear to utilize the mannose 6-phosphate pathway (Cazzulo et al., 1990b) and no structural motif corresponding to a sorting signal has been yet identified.

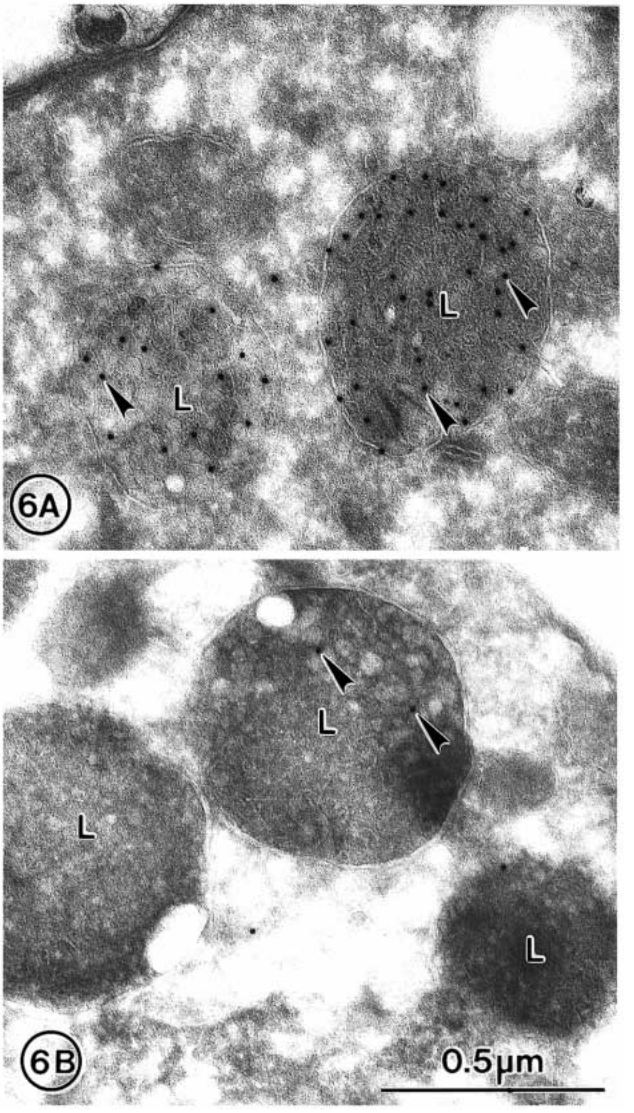

Fig. 6. Immunogold labeled cruzain within lysosomes of untreated and CPI-treated *T. cruzi* epimastigotes. (A) Lysosome (L) of an untreated epimastigote is intensely immunogold labeled (arrowheads). (B) Lysosome of a CPI-treated epimastigote showing a marked reduction in immunogold label (arrowheads).

The machinery involved in the formation, targeting, and fusion of transport vesicles in the ER and Golgi complex is better understood in mammalian cells (Rothman and Wieland, 1996). Transported proteins have distinct motifs which determine their capacity to enter different vesicle-shuttles. Coated vesicles are responsible for the anterograde and retrograde transport within the ER-Golgi system. In addition, recent evidence favors an anterograde movement of proteins flowing from cisterna to cisterna across the Golgi stack (Rothman and Wieland, 1996). Our results are consistent with the anterograde trafficking of cruzain through the Golgi complex, and with an alteration of this process by cysteine protease inhibitors resulting in an accumulation of cruzain within Golgi cisternae. Cruzain is synthesized as a pre-proenzyme containing three distinct domains. Both the

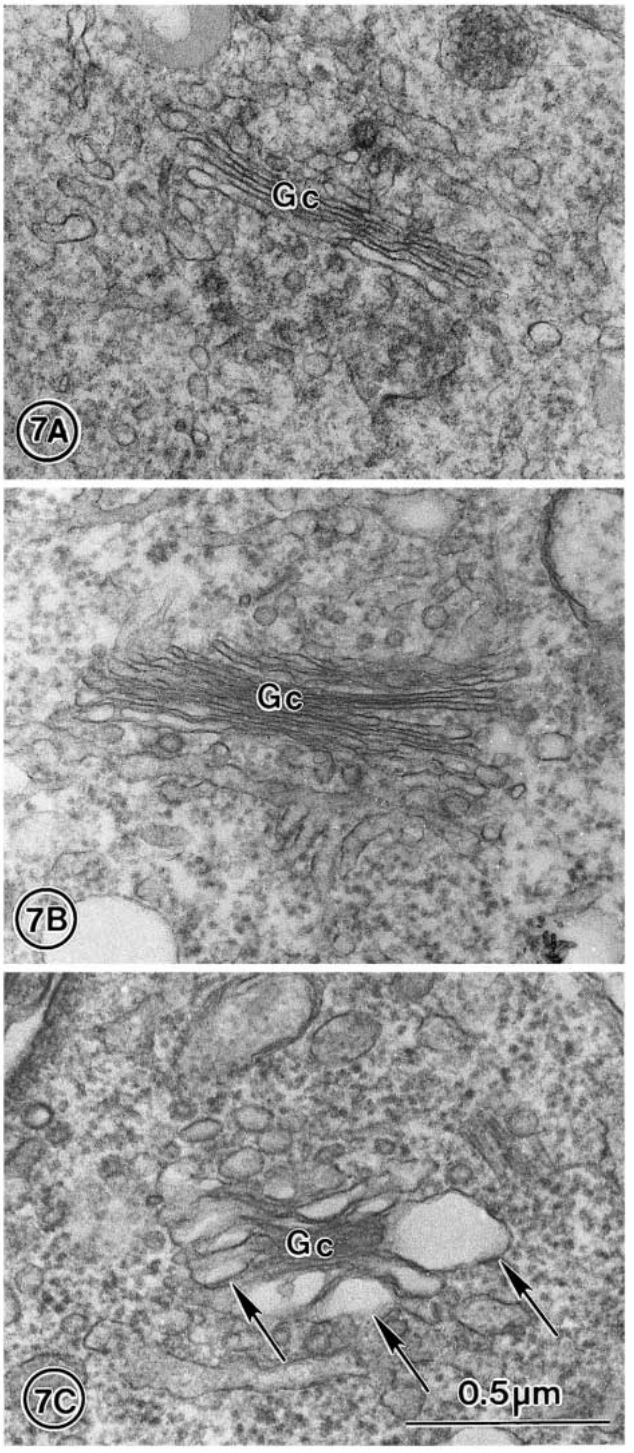

Fig. 7. Electron micrographs of *T. cruzi* epimastigotes treated with BFA with or without CPI. (A) Golgi complex (Gc) of an untreated epimastigote. (B) BFA treatment increases the number of Golgi cisternae. (C) Treatment of epimastigotes with 10 µg/ml BFA and 20 µM Mu-F-hF-VSPh for 1 hour induces the rapid appearance of large peripheral dilations of Golgi cisternae (arrows).

prodomain and the carboxy-terminal extension are autoproteolytically removed by active cruzain (Hellman et al., 1991). By analogy to the autoactivation of mammalian cathepsin L and B (McIntyre and Erickson, 1991; Mach et al., 1994), we suggest that a conformational change, possibly induced upon entering a compartment at lower pH, results in activation of some cruzain molecules in the Golgi apparatus where the prodomain unfolds from the active site. A cascade of activation would follow as 'trans' activation augments autoactivation by autoproteolysis and release of the catalytic domain from the prodomain. We propose that the prodomain is bound in or recycled to the Golgi complex by a membrane associated receptor analogous to that identified for procathepsin L and D in mammalian cells (McIntyre and Erickson, 1991). The freed catalytic domain would be shuttled in post Golgi vesicles and released into the lysosome/reservosome. Our labeling studies confirm that while the carboxy-terminal domain is still attached to the catalytic domain when cruzain reaches the lysosome, the prodomain has been removed (Hellman et al., 1991). Cysteine protease inhibitors would prevent the cleavage of the postulated Golgi-retention signal in the prodomain from the catalytic domain, and thus prevent subsequent trafficking of cruzain to the late endosome/lysosome compartment.

Two recent reports on the human proteases stromelysin-3 (ST3) and furin provide a model for self-activation within the Golgi complex. ST3 is a secreted metalloprotease which is processed to its enzymatically active form within the Golgi complex by furin (Pei and Weiss, 1995). Furin is a transmembrane serine proteinase which self-activates in the ER and concentrates in the *trans*-Golgi network. If the autoprocessing of furin is inhibited, the protease cannot be transported to its normal cellular site (Takahashi et al., 1995).

BFA and CPI, both alone and in combination, produced different effects on *T. cruzi* epimastigotes versus mammalian cells. This observation suggests there may be significant differences in Golgi complex function and protein trafficking mechanisms between these two types of eukaryotic cells. Although both ultrastructure and localization are similar, the Golgi complex is composed of numerous cisternae stacks in mammalian cells but only of a single set of 4-8 cisternae anterior to the nucleus and kinetoplast in *T. cruzi*. In contrast to the major alterations induced by CPI-treatment in *T. cruzi* epimastigotes, ultrastructural alterations in the Golgi complex or other organelles were not apparent in mammalian epithelial cells or macrophages exposed to CPI (our unpublished observation). This differential sensitivity to CPI may be partly attributed to the activation of lysosomal cysteine proteases in different cellular compartments. While our results suggest that cruzain is activated within the Golgi complex, mammalian cysteine proteases are activated within prelysosomes/lysosomes (Mach et al., 1994). BFA, a fungal macrocyclic lactone, has become a powerful tool for investigating membrane trafficking. BFA-induced alterations are complex and may differ in various mammalian cell lines (Tooze and Hollinshead, 1992). The molecular mechanism of action of BFA is still controversial but two distinct BFA-stimulated and BFA-independent membrane tubulation processes seem to occur in mammalian cells. BFA blocks anterograde protein traffic by inhibiting the formation of several types of coated transport vesicles while enhancing the number and length of membrane tubules from the Golgi complex, endosomes, and TGN (Klausner et al., 1992; de Figuereido and Brown, 1995). These events are the result of a

tight blockade of trafficking out of the endoplasmic reticulum, and of the enhanced movement of Golgi membranes back to the ER (Klausner et al., 1992; Cole et al., 1996; Lippincott-Schwartz et al., 1991; Lippincott-Schwartz, 1993). *T. cruzi* epimastigotes appeared to be more resistant to BFA than mammalian cells as the compound induced only an increase in the size and number of Golgi cisternae. These ultrastructural alterations differed from those described for mammalian cells and resembled BFA-induced changes in plants (Driouich et al., 1993). When mammalian cells were simultaneously treated with BFA and CPI, only those ultrastructural changes associated with BFA-treatment were apparent (our unpublished observation). However, when *T. cruzi* epimastigotes were exposed to both compounds, there was an enhancement of Golgi complex defect induced by CPI. Treatment of *T. cruzi* epimastigotes with CPI and BFA induced, within minutes, alterations comparable to those observed in epimastigotes exposed to the inhibitor alone for 48 hours. No increase in the number of Golgi cisternae occurred, suggesting that CPI prevented one effect of BFA presumably by immobilizing Golgi membranes. On the other hand, BFA may still have inhibited anterograde protein trafficking thus potentiating the effect of the CPI. These results are also consistent with a model of alteration of cruzain trafficking between the Golgi complex and lysosomes, and a concomitant accumulation of cruzain within Golgi vesicles. The CPI-induced accumulation of unprocessed cruzain may disrupt the normal retrograde transit of Golgi membranes back to the ER, with the consequent dilation of cisternae and the disruption of overall normal protein trafficking and processing in *Trypanosoma cruzi*.

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