A Cathepsin B-like Protease Is Required for Host Protein Degradation in *Trypanosoma brucei*  

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Identification and analysis of Clan CA (papain) cysteine proteases in primitive protozoa and metazoa have suggested that this enzyme family is more diverse and biologically important than originally thought. The protozoan parasite *Trypanosoma brucei* is the etiological agent of African sleeping sickness. The cysteine protease activity of this organism is a validated drug target as the benzyloxycarbonyl-phenylalanine-alanine diazomethane inhibitor Z-Phe-Ala-CHN₂ (where Z is benzyloxycarbonyl). Whereas the presumed target of this inhibitor was rhodesain (also brucipain, trypanopain), the major cathepsin L-like cysteine protease of *T. brucei*, genomic analysis has now identified tbcatB, a cathepsin B-like cysteine protease as a possible inhibitor target. The mRNA of tbcatB is more abundantly expressed in the bloodstream versus the procyclic form of the parasite. Induction of RNA interference against rhodesain did not result in an abnormal phenotype in cultured *T. brucei*. However, induction of RNA interference against tbcatB led to enlargement of the endosome, accumulation of fluorescein isothiocyanate-transferrin, defective cytokinesis after completion of mitosis, and ultimately the death of cultured parasites. Therefore, tbcatB, but not rhodesain, is essential for *T. brucei* survival in culture and is the most likely target of the diazomethane protease inhibitor Z-Phe-Ala-CHN₂ in *T. brucei*.

The Clan CA cysteine proteases include the papain-related proteases of plants and the lysosomal cathepsins of mammalian cells. Recent genomic and biochemical studies of protozoa and primitive metazoa suggested that there was an explosion of gene diversity in this family coincident with the evolution of the first eukaryotic cell (1). In contrast to higher plants and most mammalian cells, the cysteine proteases of primitive protozoa and metazoa function in a variety of chemical environments and cellular compartments (2, 3). Therefore, the biological importance and distribution of cysteine proteases is much greater than originally proposed for the cathepsins.

Because primitive protozoa include many of the major parasitic organisms of humans and domestic animals, parallel studies of the pathogenesis of parasitic diseases have underscored the importance of Clan CA proteases as virulence factors. Furthermore, considerable progress has been made in targeting these proteases for the development of new antiparasitic chemotherapy (2, 4–7). One notable observation in this regard was that the benzyloxycarbonyl-phenylalanine-alanine diazomethane cysteine protease inhibitor (Z-Phe-Ala-CHN₂) was lethal to *Trypanosoma brucei*, the causative agent of African sleeping sickness, in *vitro* and in *vivo* (8, 9). The presumptive target for this inhibitor was a cathepsin L-like protease, rhodesain, isolated from *T. brucei rhodesiense* and the homologue of brucipain, trypanopain, or congopain in other *Trypanosoma* species (10, 11). However, peptide diazomethanes are relatively nonselective irreversible cysteine protease inhibitors, and recent genomic analysis of *trypanosomes* suggested that a repertoire of Clan CA protease genes might be present.

To identify and validate the target of Z-Phe-Ala-CHN₂ in *T. brucei*, as well as to clarify its biological function, a genomic scan using papain as a probe was first undertaken. The previously characterized cathepsin L-like cysteine protease, rhodesain (12), was identified as expected, but a second cathepsin B-like gene product was also discovered. Subsequent expression, biochemical analysis, and localization studies suggested that the cathepsin B-like protease in *T. brucei* was a plausible target for the diazomethane inhibitor. Therefore, to clarify the roles of both the *T. brucei* cathepsin B and cathepsin L homologues, RNA interference (RNAi) was used in conjunction with radiolabeled active site probes.

**MATERIALS AND METHODS**

*Identification of Clan CA Cysteine Proteases in the T. brucei Genome*—The 159 amino acid sequence Cys-19 to Asn-178 representing the 159 amino acid sequence Cys-19 to Asn-178 representing the active site of papain, EC 3.4.22.2, was used to search (by BLAST) the *T. brucei* genomic data bases from the Sanger Center and the Institute for Genomic Research for cysteine protease homologues.

*Subcloning of TbtcB cDNA into Escherichia coli*—Reverse transcription polymerase chain reactions were carried out using the One Step with Platinum Taq kit (Invitrogen). Two micrograms of total RNA from *T. brucei* were mixed with the splice leader primer, TsSL 5′-ATTATTAGAACAGTTTCTGTACTATATTG-3′, plus the reverse primer, TbCatBBHIF, 5′-GCTAATATCTCAGATACGCCGTGTTGGG-3′, to amplify the full-length cDNA of tbcatB. To amplify the open reading frame of tbcatB for recombinant expression, the primers TbCatBBHIF, 5′-GCTAATAGCACTGACGTGACTATATTG-3′, and TbCatBBHIF, 5′-GCTAATAGCACTGACGTGACTATATTG-3′,  

1 The abbreviations used are: Z-Phe-Ala-CHN₂, benzyloxycarbonyl-phenylalanine-alanine diazomethane; RNAi, RNA interference; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole.

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bead lysis buffer (1% Triton X-100, 10 mM Tris pH 7.5, 50 mM Tris, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 7.5, 10% glycerol) and sonicated on ice five times for 2 min intervals with 3-min breaks between each interval. The crude lysate was cleared by centrifugation for 15 min at 25,000 × g. The cleared supernatant was decanted, and the pellet was resuspended in 50 ml of guanidine HCl buffer (6 M guanidine HCl, 20 mM Tris, pH 8.0, 100 mM NaH2PO4) for 2 h at room temperature. After 2 h, the lysate was cleared by centrifugation, and 10 ml of equilibrated nickel-agarose beads (Qiagen) in 50% slurry were added to the cleared lysate. The beads were incubated with the lysate for 3 h at room temperature. The mixture was poured into a 10-ml gravity column and washed with guanidine HCl buffer, pH 8.0. The recombinant 6-His tagged protein was eluted from the beads with 1 ml of wash buffer containing 50 mM NaCl, 0.2 M imidazole, 1% SDS, and 0.5 M urea. The recombinant His-282, and Asn-302 (tbcatB numbering). The tbcatB open reading frame also encodes two conserved motifs identified in the active site of lysosomal cathepsins including the residues that form the catalytic triad, Cys-42, His-282, and Asn-302 (tbcatB numbering). The tbcatB open

Identification of Clan CA Cysteine Protease Active Sites with 32P-Labeled Inhibitors—Ten million T. brucei cultured in 10 ml of complete HMI-9 medium at 37 °C and 5% carbon dioxide were incubated with 131I-labeled inhibitors for 2 h (18). After 2 h, the parasites were pelleted by centrifugation at 900 × g for 20 min with a Beckman GH 3.8A rotor and lysed in 100 µl of lysis buffer (50 mM sodium acetate, 1 mM EDTA, 1% Triton X-100, pH 5.5) for 1 h on iced. Alternatively, 10 million unlabeled parasites were pelleted and lysed using identical conditions. Lysates were cleared by centrifugation at 16,000 × g for 15 min at 4 °C in Eppendorf tubes. The protein concentration of the cleared lysates was determined by Bradford assay and equal amounts of parasite lysates were labeled with 131I-MB-074 for 1 h at 25 °C (19). Quantification of labeled enzymes was determined by PhosphorImager analysis (Molecular Dynamics).

Identification of TbtcB, a Cathepsin B-like Homologue in T. brucei—Using the highly conserved catalytic region of papain as a probe, we searched T. brucei databases for homologues to papain. From this search, cathepsin L-like and cathepsin B-like protease sequences were identified. The cDNAs encoding these two gene products were amplified from T. brucei RNA by reverse transcriptase PCR, subcloned, and sequenced. The 1.3-kb cDNA encoded a 450-amino acid polypeptide with a predicted Mr of 48,431 identical to rhodesain (or brucipain or trypanopain), the previously characterized cathepsin L-like protease identified by biochemical assays in T. brucei lysates (20). The 1.0-k cDNA encoded a cathespisin B-like protease, TbtcB, having 341 amino acids with a predicted Mr of 37,223. The TbtcB open reading frame contained each of the conserved motifs identified in the active site of lysosomal cathepsins including the residues that form the catalytic triad, Cys-42, His-282, and Asn-302 (TbtcB numbering). The TbtcB open reading frame also encodes two conserved motifs identified in cathepsin B family proteases: Gly-Cys-Xaa-Gly-Gly, which is identical to human cathepsin B (residues 70–74), and an occluding loop motif that is thought to be responsible for the exopeptidase activity of cathepsin B-like proteases (21).
The tbcatB sequence has been deposited in the GenBankTM data base under accession number AY508515.

**Examination of Rhodesain and TbcatB mRNA Expression in T. brucei**—Rhodesain has been characterized extensively with synthetic peptides demonstrating that it was the major cysteine protease activity in *T. brucei* (22–24). Consistent with it being the most abundantly expressed cysteine protease gene product in *T. brucei*, genomic analysis has indicated that there were between 10 and 20 copies of the rhodesain gene repeated in tandem (25). We examined the mRNA expression of rhodesain and tbcatB to elucidate their relative abundance in the two major developmental stages of *T. brucei*. Northern blot analysis demonstrated that the amounts of rhodesain mRNA expressed in either the bloodstream (mammalian host stage) or the procyclic (insect stage) forms of the parasite were equivalent (Fig. 2A). In shorter exposures of the autoradiography, the rhodesain mRNA could be visualized as a doublet in the RNA from both forms of the parasite. These mRNA results were surprising because studies from previous protein expression assays concluded that rhodesain protein was up-regulated in the bloodstream-form parasites (12). It is therefore reasonable to conclude that rhodesain, like its homologue in *Trypanosoma cruzi*, may be translationally regulated in procyclic parasites. We used gene-specific probes to hybridize to tbcatB mRNA for Northern blot analysis. Our results demonstrated that tbcatB mRNA was less abundantly expressed than rhodesain mRNA in both developmental stages of *T. brucei*; however, the sizes of the two transcripts were very similar. To avoid ambiguous results, we carried out the tbcatB and rhodesain blots in parallel rather than using blots that were stripped and reprobed. Northern blot analysis also demonstrated that the message for tbcatB was up-regulated in the bloodstream-form parasites (Fig. 2B). The differential expression of tbcatB observed in the bloodstream-form versus procyclic parasites suggests that in contrast to rhodesain, tbcatB is regulated at the transcriptional level. Up-regulation of tbcatB mRNA in the bloodstream-form parasites suggests that it may primarily function in the bloodstream parasites.

**Silencing of Rhodesain mRNA Expression in T. brucei**—To examine the consequences of inhibiting the activity of each protease in *T. brucei*, RNAi was used to silence the mRNA expression of rhodesain or tbcatB. We used tetracycline to induce RNAi in *T. brucei*, stably expressing the rhodesain, pZJMTbRho (or tbcatB), or pZJMTbCB transgenes. The tetracycline-induced pZJMTbRho parasites did not exhibit visible phenotypic abnormalities, signs of cell cycle defects, or changes in growth rate compared with the uninduced parasites. Total RNA from induced and control parasites was extracted and analyzed by Northern blot analysis 24 h postinduction. The rhodesain probe hybridized with two distinct messages on the Northern blot with about equal intensity. Because the Northern blot analysis was carried out on total RNA, the two transcripts recognized by the probe could have arisen if the rhodesain gene lay within a cluster of related genes as described for the cathepsin L-like genes of *Leishmania* species (26). Alternatively, the two transcripts may represent a mixture of mature rhodesain mRNAs and either immature or alternatively processed transcripts. In either case, both of these transcripts were reduced to almost undetectable levels after tetracycline-induced RNAi was done in these parasites (Fig. 3A). Immunoblot analysis was used to compare the amount of rhodesain in control parasites with the amount in RNAi-silenced parasites. Affinity-purified rabbit anti-rhodesain antibodies recognized two bands, 45 and 47 kDa, in the Western blots of control parasites. These two bands could reflect that rhodesain has become glycosylated because a cryptic glycosylation site has been identified (12) and these antibodies do not distinguish between glycosylated and non-glycosylated forms of rhodesain. Recently, it has been demonstrated that a species of mammalian cathepsin L is able to initiate translation from a second
forms of T. brucei. Total RNA from bloodstream (BF) or procyclic (PF) forms of T. brucei were cross-linked to polyvinylidene difluoride membrane and then hybridized with rhodesain-specific (A) or tbcatB-specific (B) probes. Ribosomal RNA loading controls are shown beneath each blot. Note that while the message for tbcatB is less abundant than rhodesain, it is differentially up-regulated in the mammalian bloodstream form.

downstream methionine codon and become translocated into the nucleus (27). Rhodesain is a cathepsin L-like protease that also contains several downstream methionine codons where translation can initiate. Initiation at a downstream methionine codon may also account for the second species of rhodesain detected by the antibodies. In either case, these two bands were nearly undetectable by the same antibodies in extracts where rhodesain expression was silenced in parasites by RNAi (Fig. 3B).

To confirm the effects of mRNA silencing on protease activity in vivo, we used JPM565, a derivative of E-64, the irreversible inhibitor of papain and other cysteine proteases. E-64 and its derivatives form a thioether bond with the sulphydryl group in the active center of cysteine proteases, and they are therefore ideal for active site titration because 1 mol of the inhibitor inhibits 1 mol of protease (28). Furthermore, JPM565 has a preference for cathepsin L-like proteases and can be iodinated allowing quantitative analysis to be carried out on labeled proteases (18, 29). When control parasites were incubated with 125I-JPM565, both species of rhodesain were detected by the label (Fig. 3C). The tetracycline-induced parasites showed a 65% reduction in labeling of rhodesain by 125I-JPM565 (Fig. 3D). Together these observations suggested that rhodesain was not essential for parasite replication in culture.

Knock Down of TbcatB Leads to Dysmorphism and Death of T. brucei—When the pZJMTbCB clones were induced with tetracycline, distension of the posterior endosome/lysosome compartment of the parasites was easily visualized within 12 h postinduction (Fig. 4). Distension of these RNAi-induced parasites in the region of the endosome reached a maximum by 24 h postinduction. In addition to posterior swelling, some RNAi-induced parasites displayed multiple flagella that looked similar to uninduced control parasites undergoing cytokinesis (Fig. 4A, Phase Contrast). To examine the consequences of endosome/lysosome dysfunction in the parasites, we used DAPI to stain nuclear DNA and kinetoplast DNA (which is the mitochondrial DNA equivalent in T. brucei) of control and induced parasites. In uninduced controls, the population was composed of non-dividing parasites containing one kinetoplast and one nucleus, parasites undergoing kinetoplast division and segregation containing two kinetoplasts and one nucleus, and parasites undergoing nuclear mitosis containing two kinetoplasts and two nuclei. These observations were consistent with the control parasites existing as an asynchronous population (30). The nuclei of RNAi-induced pZJMTbCB clones were stained with DAPI 24 h postinduction. These clones demonstrated a dramatic increase in the number of parasites containing two kinetoplasts and two nuclei, but no parasites containing one kinetoplast and one nucleus were detected 48 h postinduction (Fig. 4A, DAPI).

The DNA content of control or induced parasites was then analyzed by flow cytometry to verify the karyotype results observed by microscopy. In the uninduced control population, a similar number of parasites contained either non-replicating 2C or postmitotic 4C DNA. A similar profile was observed in the parental 90-13 and wild-type 221 bloodstream-form parasites consistent with uninduced parasites existing as an asynchronous population (data not shown). After 24 h of tetracycline induction, the majority of parasites observed contained 4C DNA or greater, suggesting that the tetracycline-induced population of parasites was arrested in cytokinesis after completing several rounds of DNA synthesis and mitosis (Fig. 4A, FACS). No proliferation in the pZJMTbCB clones was detected by either direct counting with a hemocytometer or by a luciferase-based proliferation assay that detects adenosine triphosphate generation by 48 h postinduction (Fig. 4B) (31). The tetracycline-induced parasites died by 72 h postinduction. These abnormalities were not observed in uninduced control parasites where normal proliferation occurred. We carried out dosage-dependent induction of the parental 90-13 strain versus pZJMTbCB clones. After 48 h of induction, the proliferation of the 90-13 strain was not affected at any of the concentrations of tetracycline, indicating that this drug does not produce any toxic effects on T. brucei. Proliferation of the pZJMTbCB clones was inhibited at tetracycline concentrations as low as 1 ng/ml, showing dosage-dependent inhibition of proliferation by RNAi. The parasites that remain at 1 ng/ml of tetracycline may represent those that inefficiently produce RNAi having a more modest reduction in mRNA for tbcatB (Fig. 4C).

The effect of RNAi on tbcatB protease in the parasites was examined by Western blot analysis. A modest but significant decrease in the amount of tbcatB protein was detected in the extracts of the induced parasites versus the control parasites (Fig. 4D). We used the cathepsin B-specific inhibitor MB-074 to confirm reduction of cathepsin B activity in the tetracycline-induced clones. MB-074 is a derivative of CA-074 that binds specifically to the occluding loop found in cathepsin B-like...
proteases but not other cysteine proteases (32). MB-074 has a tyramine group that allows it to be labeled by $^{125}$I. The labeled inhibitor was added to crude lysates of control or tetracycline-induced pZJMTbCB clones and resolved by SDS-PAGE for quantitative analysis. A 34-kDa band consistent in size with the band recognized by tbcatB antibodies was labeled by $^{125}$I-MB-074 in both extracts, but a slight decrease in the 34 kDa band was visualized in the extracts of the induced parasites (Fig. 4E). When the intensities of the labeled bands from the extracts were compared by PhosphorImager analysis, a 32% reduction of the 34 kDa protein was observed in the extracts of the RNAi-induced parasites (Fig. 4F).

**TbcatB but Not Rhodesain Is Required for Degradation of Transferrin**—Because they lack cytochromes, the bloodstream form of *T. brucei* acquires iron from the host by internalizing transferrin through receptor-mediated endocytosis. The host transferrin is then rapidly degraded in the “endosome/lysosome” located between the nucleus and kinetoplast of the parasite. Because of the observation that tbcatB RNAi led to swelling of this compartment, FITC-transferrin was used to assay the effect that knocking down tbcatB had on the ability of the parasites to degrade this key host protein. No accumulation of FITC-transferrin was detected in uninduced control pZJMTbCB clones indicating that the ability of the parasites to de-
grade transferrin was not hindered. However, when the clones were induced by tetracycline, FITC-transferrin began to accumulate throughout the parasite and within the endosome/lysosome, suggesting that their ability to efficiently and effectively degrade transferrin was hindered (Fig. 5A). A similar accumulation of FITC-transferrin was also previously reported when *T. brucei* were treated with the cysteine protease inhibitor Z-Phe-Ala-CHN₂ (8). No accumulation of FITC-transferrin was detected in control pZJMTbRho parasites or in tetracycline-induced pZJMTbRho clones, indicating that knocking down rhodesain activity in the parasites does not interfere with the ability of the parasites to degrade transferrin (Fig. 5B).

**DISCUSSION**

Scory *et al.* (8) showed that a peptide diazomethane inhibitor was lethal to *T. brucei* in culture and produced swelling of the endosome/lysosome compartment of *T. brucei*. The presumptive target of this inhibitor was rhodesain, the major proteolytic activity and cathepsin B-like protease of the parasite. We have identified a cathepsin B-like homologue, tbcatB, in the *T. brucei* genome that confirms the Clan CA cysteine protease repertoire of *T. brucei* is more complex than previously thought. The *T. brucei* genome contains several protease activities that are not related to rhodesain, including cathepsin B-like proteases (13). These activities are expressed in both the bloodstream and intracellular stages.

T. brucei is more complex than previously thought. The Clan CA cysteine protease family evolved very early to carry out a broad array of biologic functions in primitive eukaryotic cells, some of which have been retained by higher eukaryotes.
The differential regulation of tbcatB and rhodesain suggests that these two proteases may have distinct biological roles. To address this issue in *T. brucei*, we utilized RNA interference to selectively knock down rhodesain or tbcatB activity in *T. brucei*. In cultured bloodstream trypanosomes, no observable phenotype was found after nearly complete knockdown of rhodesain message, protein, and proteolytic activity. In contrast, induction of RNAi in the pZJMTbCB clones produced a modest 32% reduction in protein and protease activity but led to a distinctive dysmorphic phenotype. Dilation of the anterior end and a block in cytokinesis of the tbcatB knockdown parasites produced a tadpole-like morphology with an enlarged lysosome/endosome compartment. These parasites were able to complete multiple rounds of genomic replication and mitosis as evidenced by the appearance of multiple kinetoplasts and nuclei but were not able to complete cytokinesis. This defect in cytokinesis is reminiscent of that seen and reported for /H9251 -tubulin-depleted parasites (14). It is possible that tbcatB could also be involved in a microtubule-related event alternatively; lack of cytokinesis may be an indirect consequence of iron depletion because of the inability of the parasite to degrade transferrin. The fact that a dysmorphic and later lethal phenotype was observed with modest reduction in the total protease suggests that this enzyme may be slowly replenished or be at a critical steady-state concentration in the parasites.

Finally, the lethal tbcatB RNAi effect associated with inhibition of FITC-transferrin degradation recapitulated the phenotype seen with parasites exposed to a peptide diazomethane cysteine protease inhibitor. This suggests that tbcatB was the most probable target of that inhibitor and plays a major role in host serum protein degradation by the parasite.

*T. brucei* is the causative agent of African sleeping sickness, a major health problem in sub-Saharan Africa and one of the great neglected diseases. Identification of a specific enzyme required for the viability of bloodstream trypanosomes represents an exploitable target for the development of new chemotherapy.

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