FEBS Letters 580 (2006) 5306–5312

Two cysteine protease inhibitors, EhICP1 and 2, localized in distinct compartments, negatively regulate secretion in *Entamoeba histolytica*

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Received 9 August 2006; revised 27 August 2006; accepted 28 August 2006

Available online 12 September 2006

Edited by Stuart Ferguson

Abstract The enteric protozoan parasite *Entamoeba histolytica* uniquely possesses two isotypes of ICPs, a novel class of inhibitors for cysteine proteases. These two EhICPs showed a remarkable difference in the ability to inhibit cysteine protease (CP) 5, a well-established virulence determinant, whereas they equally inhibited CP1 and CP2. Immunofluorescence imaging and cellular fractionation showed that EhICP1 and EhICP2 are localized to distinct compartments. While EhICP1 is localized to the soluble cytosolic fraction, EhICP2 is targeted from lysosomes to phagosomes upon erythrocyte engulfment. Overexpression of either EhICP1 or EhICP2 caused reduction of intracellular CP activity, but not the amount of CP, and decrease in the secretion of all major CPs, suggesting that both EhICPs are involved in the trafficking and/or interference with the major CP activity. These data indicate that the two EhICPs, present in distinct subcellular compartments, negatively regulate CP secretion, and, thus, the virulence of this parasite.

Keywords: *Entamoeba histolytica*; Parasitic protozoan; Cysteine protease; Inhibitor; Chagasin; Phagocytosis

1. Introduction

Cysteine proteases (CPs) represent a broad class of proteolytic enzymes widely distributed among living organisms and play major roles in various biological processes [1]. The protozoan parasite *Entamoeba histolytica*, the causative agent of human amoebiasis [2], invades the intestinal epithelium and causes amoebic dysentery, colitis, and abscesses in liver, lung, and brain [3]. Papain-family CPs (EC 3.4.22) have been known as one of the virulence factors of this parasite [4]. Among >40 CP genes present in this organism [5,6], CP1, CP2, and CP5 are most highly expressed and contribute to about 90% of the total CP activity of the amoeba [7]. It has been recently shown that expression of individual CPs is controlled independently. For instance, expression of CPs including CP4 and CP6 drastically increased (6–35-fold) upon animal intestinal infection, while expression of CP8 decreased by 5–14-fold [8]. It was also shown that heat shock at 42 °C also down-modulated expression of CP1, CP2, and CP8 by 6–9-fold while it up-regulated expression of CP6 and CP4 by 9- and 4-fold, respectively [9]. CP5 has been implicated to play a major role in the pathogenicity since antisense knockdown of CP5 expression reduced both in vitro and in vivo virulence [10,11] and CP5 gene is degenerated in a genetically related but non-pathogenic *E. dispar* species [5,7]. Since the incubation of trophozoites with a synthetic CP inhibitor, *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), inhibited the formation of liver abscess [12], CPs has been viewed as an attractive chemotherapeutic target. Although biological and pathological importance of the amoebic CPs has been extensively studied, it remains poorly understood how the activity of CPs is regulated so that these highly competent hydrolases with a wide substrate specificity elicit activity for specific and only desired biological processes [13]. A variety of classes of protease inhibitors that regulate CPs have been reported [1] (see MEROPS peptidase database (http://merops.sanger.ac.uk/)).

A novel class of proteinaceous CP inhibitors, named chagasin or inhibitor for CP (ICP), have been recently discovered in parasitic protozoa including *Trypanosoma cruzi* [14], *Leishmania mexicana* [15], *L. major*, *T. brucei, E. histolytica, Cryptosporidium parvum*, bacteria, and archaea [16]. It was shown that ICP inhibited papain-family CPs with an inhibition constant (K_i) of a nano to picomolar range [14], similar to cystatin [17]. Structural analyses by NMR revealed that chagasin and *L. mexicana* ICP consisted of eight β-strands and one small α-helix, and three mobile loops in the same side bound to CP [18,19]. Chagasin has been shown to regulate differentiation and invasion of mammalian cells [20]. In *L. mexicana*, ICP has been assumed to play a role in the protection against a hydrolytic environment in insect vectors or hosts [15]. Dissimilar to other organisms, *E. histolytica* possesses two isotypes of ICP, and significance of this apparent redundance is not understood. In this study, we describe the characterization of two EhICPs, designated EhICP1 [21] and EhICP2, which shows distinct biochemical properties and intracellular localization. We also show, using the amoeba cell lines overexpressing epitope-tagged EhICPs, that both EhICPs are involved in the negative regulation of the secretion of CPs.

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Abbreviations: E-64, *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; Z-RR-MCA, benzyloxycarbonyl-Arg-Arg-7-amino-methylcoumarin; GFP, green fluorescent protein; HA, hemagglutinin; TCA, trichlororoacetic acid; Mab, monoclonal IgG

0014-5793/$32.00
doi:10.1016/j.febslet.2006.08.081

2. Materials and methods

2.1. Parasite and culture conditions

Trophozoites of *E. histolytica* HM-1:IMSS cl-6 were cultured axenically in BI-S-33 medium at 35.5 °C [22].

2.2. Expression and purification of recombinant EhICPs

EhICP sequences were obtained from the *E. histolytica* genome database at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/E_histolytica/). A entire coding region of EhICP1 gene, or a part of EhICP2 gene lacking a putative signal sequence, was amplified by PCR with oligonucleotide primers (Supplementary Table) and the trophozoite cDNA as a template. The obtained PCR products were inserted into pET100/D-TOPO expression vector (Invitrogen). Histidine-tagged EhICPs were purified from the soluble lysate of *E. coli* strain BL21(DE3) transformed with the resulting plasmids with affinity chromatography using HisTrap HP columns (GE Healthcare Bio-Sciences). EhICP1 or EhICP2 was further purified using a MonoQ 5/5HR or MonoS 5/5HR column (GE Healthcare Bio-Sciences), respectively, preequilibrated with 50 mM ethanolamine, pH8.5, with a linear gradient of 0–1 M NaCl at a flow rate of 1.0 ml/min. The eluted recombinant EhICP1 and EhICP2 were estimated to be >95% pure by SDS–PAGE analysis (data not shown). The histidine tag at the amino terminus of recombinant EhICPs was removed by enterokinase and EK-Away resin (Invitrogen).

2.3. Production of antibody

Antisera were raised against purified recombinant EhICP1 and EhICP2 in rabbits commercially. IgGs were further purified with a ProteinG-Sepharose column (GE Healthcare Bio-Sciences).

2.4. Identification of targets of EhICPs

The trophozoite lysate (120 μg) was incubated with histidine-tagged EhICP1 (290 ng), EhICP2 (290 ng), or E-64 (280 μM) in 300 μL of buffer A (50 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 50 mM imidazole, 1 mM DTT, and 0.5% Triton X-100) at 4 °C for 2 h. After 10 μL of HisTrap HP beads (50% slurry) was added, the mixtures were rotated gently at 4 °C for 12 h. After the beads were extensively washed with buffer A containing 350 mM NaCl, the bound proteins were subjected to SDS–PAGE and immunoblot analyses with anti-CP2 and anti-CP5 IgGs, which were a generous gift by Sharon L. Reed [23], and Iris Bruchhaus, Mathias Leippe, and Egbert Tannich [11], respectively.

2.5. Production and activation of recombinant CPs

CP1, 2, and 5 genes lacking the putative signal sequences were amplified by PCR using oligonucleotide primers (Supplementary Table) designed based on available nucleotide sequences (Q01957 (CP1), Q01958 (CP2), and AAFB01000593 (CP5)) and trophozoite cDNA. PCR products were inserted into pET-15b expression plasmid (Novagen). Histidine-tagged recombinant CP proteins were produced as inclusion body in *E. coli* strain BL21(DE3). Recombinant CP proteins were purified by affinity chromatography under denaturing conditions. Refolding and activation were performed as previously described [24] with some modifications. Briefly, recombinant CP1 and CP2 (100 μg/ml, 30–40 μL) were dialyzed against refolding buffer B (100 mM Tris–HCl (pH 8.8), 300 mM NaCl, 5% glycerol, 0.1 mM EDTA, 3 mM reduced glutathione and 0.5 mM oxidized glutathione) at 4 °C for 1.5 h, and subsequently against refolding buffer C (25 mM Tris–HCl (pH 6.8), 150 mM NaCl) at 4 °C for 1.5 h. Recombinant CP5 was treated similarly except that refolding buffer B contained 50 mM Tris–HCl (pH 8.8) and refolding buffer C contained 50 mM Tris–HCl (pH 6.8) without NaCl. Refolded recombinant CPs were activated by the incubation with an equal volume of 100 mM Tris–HCl (pH 8.8), 20 mM DTT and 2% Triton X-100 at 37 °C for 30 min.

2.6. Measurement of inhibition constants of EhICPs

Activated recombinant CPs or the trophozoite lysate (a final concentration of 1.56 or 22.6 μg/ml, respectively) were incubated with various concentrations of the purified recombinant EhICP1 or EhICP2 in 150 mM sodium acetate buffer (pH 5.0), 1 mM DTT and 1 mM EDTA at 37 °C for 30 min. Subsequently, benzyloxycarbonyl-Arg-Arg-7-amino-methylcoumarin (Z-RR-MCA) (Peptide Institute Inc.) was added at a final concentration of 100 μM. Hydrolysis of the fluorescent substrate was estimated by measuring emission at 460 nm with excitation at 355 nm. The apparent equilibrium constants ($K_{i\ app}$) for inhibition were calculated by a slope of $[I]/(1 - v_i/v_o)$ versus v_o/v_i, where v_i or v_o represents the steady-state velocity with or without an inhibitor, respectively [25]. The true K_i values were calculated from $K_{i\ app} = K_i/(1+[S]/K_m)$, with a K_m value measured independently.

2.7. Estimation of the ratio of CPs to ICPs

The ratio of CPs to ICPs was determined by titration of CPs with E-64 or papain with heat-stable ICPs (data not shown) as previously shown [20] with minor modification. Briefly, to measure CP concentrations the trophozoite lysate (a final concentration, 2.50 μg/mL) was incubated with various concentrations of E-64 for 30 min at 37 °C, and the remaining CP activity was measured as described above. In order to estimate ICP concentrations, the lysate was boiled for 10 min to inactivate CPs and centrifuged at $15000 \times g$ for 10 min at 4 °C. The serially diluted supernatant was incubated with papain (a final concentration, 162.6 nM) for 30 min at 37 °C, and the remaining CP activity was measured. Molar concentration was calculated by linear regression of $[I]$ on the x-axis and v_o on the y, at which v_o = zero.

2.8. Creation of cell lines overexpressing an epitope-tagged EhICP1, EhICP2, or CP5

A full-length EhICPs and CP5 genes amplified by PCR with oligonucleotide primers (Supplementary Table) were inserted into pKT-C′GFP, pKT-C′HA or pKT-N′HA plasmid. They are the derivatives of pEhEx [26], which allow expression of a gene of interest as carboxyl-terminal fusion with GFP (pKT-C′GFP), or with three tandem repeats of the HA peptide fused at the amino (pKT-N′HA) or carboxyl terminus (pKT-C′HA). Transformation and selection of transformants were previously described [27,28].

2.9. Preparation of cell lysates and culture supernatants

Semi-confluent cultures were harvested at 24–48 h after initiation of cultures and resuspended in BI-S-33 medium without bovine serum at a concentration of 5×10^5 cells/ml. After the cultures (2.0 ml) were incubated at 35.5 °C for 2 h, culture supernatants were centrifuged at $15000 \times g$ for 10 min. Attached trophozoites were rinsed with ice-cold PBS containing 2% glucose, and collected by centrifugation at $1000 \times g$ for 10 min. The culture supernatants were dialyzed twice against PBS containing 0.2% Triton X-100 for 2 h for measurement of CP activity, and concentrated to 10-fold with trichlororoacetic acid (TCA)–acetone precipitation for Western Blot analysis.

2.10. Indirect immunofluorescence microscopy

Immunoflurorescence imaging was carried out with Carl Zeiss LSM510 conforcal laser-scanning microscope as previously described [26]. Acidic compartments of trophozoites were stained with LysoTracker RED DND-99 (Invitrogen) at 35.5 °C for 12 h. After trophozoites were incubated with gerbil erythrocytes (1×10^7/ml) at 35 °C for 10 min, they were fixed, permealized, and reacted with anti-EhICP2 IgG or anti-HA 16B12 monoclonal IgG (Mab) (Berkeley Antibody) as previously described.

2.11. Cell fractionation

Cell fractionation was performed as previously described [29] at 4 °C with modifications. Briefly, the trophozoites were gently disrupted in an isotonic buffer (200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM HEPES, protease inhibitor cocktail (Roche)) using a Dounce glass homogenizer. The lysate was centrifuged at $5000 \times g$ for 10 min at 4 °C. The supernatant was subsequently ultracentrifuged at $100000 \times g$ for 20 min at 4 °C. These supernatants and pellets were subjected to immunoblot analyses with anti-EhICP1, anti-EhICP2, anti-NifU [30], anti-CP5, and anti-Hgl IgGs the last of (a gift by Barbara J. Mann and William A. Petri, Jr.) [31].

3. Results and discussion

While *E. histolytica* possesses >40 CPs that belong to papain family, it apparently lacks common CP inhibitors including