Use of recombinant Entamoeba histolytica cysteine proteinase 1 to identify a potent inhibitor of amebic invasion in a human colonic model

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Use of Recombinant Entamoeba histolytica Cysteine Proteinase 1 To Identify a Potent Inhibitor of Amebic Invasion in a Human Colonic Model


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Cysteine proteinases are key virulence factors of the protozoan parasite Entamoeba histolytica. We have shown that cysteine proteinases play a central role in tissue invasion and disruption of host defenses by digesting components of the extracellular matrix, immunoglobulins, complement, and cytokines. Analysis of the E. histolytica genome project has revealed more than 40 genes encoding cysteine proteinases. We have focused on E. histolytica cysteine proteinase 1 (EhCP1) because it is one of two cysteine proteinases unique to invasive E. histolytica and is highly expressed and released. Recombinant EhCP1 was expressed in Escherichia coli and refolded to an active enzyme with a pH optimum of 6.0. We used positional-scanning synthetic tetrapeptide combinatorial libraries to map the specificity of the P1 to P4 subsites of the active site cleft. Arginine was strongly preferred at P2, an unusual specificity among clan CA proteinases. A new vinyl sulfone inhibitor, WRR483, was synthesized based on this specificity to target EhCP1. Recombinant EhCP1 cleaved key components of the host immune system, C3, immunoglobulin G, and pro-interleukin-18, in a time- and dose-dependent manner. EhCP1 localized to large cytoplasmic vesicles, distinct from the sites of other proteinases. To gain insight into the role of secreted cysteine proteinases in amebic invasion, we tested the effect of the vinyl sulfone cysteine proteinase inhibitors K11777 and WRR483 on invasion of human colonic xenografts. The resultant dramatic inhibition of invasion by both inhibitors in this human colonic model of amebiasis strongly suggests a significant role of secreted amebic proteinases, such as EhCP1, in the pathogenesis of amebiasis.

The intestinal protozoan parasite Entamoeba histolytica is the etiologic agent of amebic colitis and liver abscess, which cause high rates of morbidity and mortality worldwide (49). The mechanism by which Entamoeba histolytica is able to invade and damage the host’s target tissues has been the subject of intense research. Several virulence factors have been identified, including secreted cysteine proteinases (39, 42). These amebic enzymes have been implicated in the in vitro cytopathology of cell monolayers (20, 23), which correlates with the observed separation of colonic epithelial cells before invasion (51). Other correlates with invasion include the ability of cysteine proteinases to degrade extracellular matrix components (19) and colonic mucin (31, 32). Furthermore, cysteine proteinases enable E. histolytica to evade the host’s immune defenses by activating and locally depleting complement (43), and by degrading anaphylotoxins C3α and C5α (41), human immunoglobulin G (IgG) (53), human IgA (21), and interleukin-18 (IL-18) (37).

The recent completion of the Entamoeba histolytica genome project has revealed the presence of at least 40 genes encoding cysteine proteinases (25). Of all the cysteine proteinase genes, only ehcp1 and ehcp5 are unique to E. histolytica, as their orthologs are either absent (ehcp1) or nonfunctional (ehcp5) in Entamoeba dispar, a morphologically identical but noninvasive Entamoeba species (5, 6, 54). Surprisingly, only a small subset of these genes are expressed in cultured Entamoeba histolytica trophozoites (6), and only three, ehcp1, ehcp2, and ehcp5, account for more than 90% of the cysteine proteinase-specific transcripts in culture (5, 6). Quantitative studies of the expression of the major Entamoeba histolytica cysteine proteinases have shown that E. histolytica cysteine proteinase 1 (EhCP1) is one of the most highly expressed and released cysteine proteinases in cultured trophozoites (6, 8, 17). In a recent study of gene expression in a mouse model of amebic colitis, EhCP1 expression was increased almost twofold following invasion, while expression of EhCP5, the other E. histolytica-specific proteinase, was not (11). To further characterize this impor-
tant amebic cysteine protease, we have cloned, expressed, and refolded recombinant EhCP1 (rEhCP1) to obtain active protease. We now show that rEhCP1 can cleave physiologic substrates, such as the third component of complement, pro-IL-18, and IgG, components of the host immune response which must be circumvented for the amebae to invade. We designed a new inhibitor based on the marked preference of EhCP1 for arginine in the P2 position. We now show for the first time that specific inhibitors of cysteine proteinases block invasion in the human colon.

MATERIALS AND METHODS

Entamoeba cultures and purification of genomic DNA. E. histolytica strain HM1:IMSS was grown axenically in TYI-S-33 medium (9) and subcultured every 48 to 72 h. E. histolytica genomic DNA was purified from trophozoite nuclei with the DNeasy kit (QUIAGEN, Valencia, CA).

Isolation and purification of released amebic cysteine proteases. Released proteinases in conditioned medium (CM) from Entamoeba histolytica trophozoites were prepared as previously described in phosphate-buffered saline supplemented with additional cysteine and dithiotreitol which maintained proteinases in conditioned medium (CM) from Entamoeba histolytica viability (by trypan blue exclusion) (17).

First time that specific inhibitors of cysteine proteinases block invasion. A recombinant amebic cysteine proteinase, we have cloned, expressed, and refolded recombinant EhCP1 (rEhCP1) to obtain active protease. We have designed a new inhibitor based on the marked preference of EhCP1 for arginine in the P2 position. We now show for the first time that specific inhibitors of cysteine proteinases block invasion in the human colon.

Proteinase activity assay. The proteinase activity was determined by measuring the release of the fluorescent leaving group, 4-amino-7-methylcoumarin (AMC), from synthetic peptide substrates (Bachem, Torrance, CA), in a Fluoro-Ascan-Acetone fluorometer (LabSystems) (17). Enzyme activity, initial velocity, and relative fluorescence units (RFU; the amount of proteinase activity needed for the release of 1 pmol of AMC per minute) were calculated with fridge-freezer liquid chromatography on a HiTrap-Q column (Amersham Biosciences, United Kingdom).

Production of Ab and immunoblots assays. Polyclonal antibodies (Abs) were raised by immunizing rabbits subcutaneously three times with gel-purified rEhCP1 (20 to 30 nM) to 10-fold dilutions of inhibitor (500 nM to 100 nM) final concentration. These conditions blocked irreversible cysteine protease inhibitors (4, 52) were performed by adding rEhCP1 (20 to 30 nM) to 10-fold dilutions of inhibitor (500 nM to 100 μM) with 10 μM Z-Arg-Arg-AMC (K_{\text{inh}} = 2 μM) on a Flex Station with robotics (Molecular Devices) and an 0.7-s read time. The value of K_{\text{inh}} varied linearly with inhibitor concentration, K_{\text{inh}} was determined by linear regression analysis (4). If the variation was hyperbolic, indicating saturation inhibition kinetics, K_{\text{inh}} and K_S were determined from an equation describing a two-step irreversible inhibitor mechanism (k_{\text{in}} = k_{\text{out}}[I]/(K_I + K_S + [1 + [S]/K_{\text{M}}])) and nonlinear regression analysis using Prism (4).

Inhibition of rEhCP1 and released proteinases by cysteine proteinase inhibitors. K1777 is a vinyl sulfone inhibitor (N-methylpiperezine-urea-phenylalanyl-homophenylalanyl-4-vinylsulfonyl-2-benzene), which has been shown to inhibit a number of proteozoon cysteine proteinases (10, 26) and has undergone extensive toxicity testing (41). WRR483 was synthesized as a K1777 derivative with a vinyl sulfone substituent at the position 4 of the benzene ring. The irreversible cysteine protease inhibitors (4, 52) were performed by adding rEhCP1 (20 to 30 nM) to 10-fold dilutions of inhibitor (500 nM to 100 μM) with 10 μM Z-Arg-Arg-AMC (K_{\text{inh}} = 2 μM) on a Flex Station with robotics (Molecular Devices) and an 0.7-s read time. The value of K_{\text{inh}} varied linearly with inhibitor concentration, K_{\text{inh}} was determined by linear regression analysis (4). If the variation was hyperbolic, indicating saturation inhibition kinetics, K_{\text{inh}} and K_S were determined from an equation describing a two-step irreversible inhibitor mechanism (k_{\text{in}} = k_{\text{out}}[I]/(K_I + K_S + [1 + [S]/K_{\text{M}}])) and nonlinear regression analysis using Prism (4).

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Amebic infection of a human intestinal xenograft model. Human intestinal xenografts were transplanted subcutaneously into the backs of SCID mice and infected with E. histolytica trophozoites as previously detailed (28, 46, 55). Xenografts were injected with medium alone, E. histolytica trophozoites (1 × 10^6) in medium, or trophozoites preincubated with K1777 (20 μM final concentration) or WRR483 (10 μM final concentration). These conditions blocked all detectable cysteine proteinase activity in trophozoites for >24 h (data not shown). A total dose of 1.5 mg of either inhibitor was administered intraperitoneally to each mouse (10). The xenografts were harvested at 24 h, the mucus plug was removed, and sections were fixed in 4% paraformaldehyde for 1 h, extraction for fixation for histology. The presence of an intact muscularis layer and complete removal of the mucus plug were confirmed in each experimental xenograft.

Quantification of amebic invasion in human intestinal xenografts. We developed a sensitive, quantitative real-time PCR assay to measure amebic invasion in...
Xenografts infected with *E. histolytica*. A standard curve to measure the number of *E. histolytica* trophozoites invading human intestinal xenografts was generated by adding trophozoites (10^2 to 10^6) to 25 to 50 mg of human intestinal xenograft tissue and extracting the DNA with the PUREGENE DNA purification kit (Gentra Systems, Minneapolis, MN). Real-time PCRs were carried out using the SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) with 100 ng of the extracted DNA (from xenografts and standards) and 100 nM (final concentration) of both the forward primer (5'/H11032-AAATCAATTGTGAAGTTATTGGAGTGA-3'/H11032) and the reverse primer (5'/H11032-TCCTACTCCTTCTTTACTTTATCTGCT-3'/H11032), to amplify a 92-bp region of the *E. histolytica* peroxiredoxin gene (7). Real-time PCR was performed under conditions previously described (40) with a standard curve and negative controls consisting of reaction mixtures without template or containing noninfected xenograft DNA (100 ng).

The potential presence of any PCR inhibitor in each of the human intestinal xenograft DNA samples was tested by the quantitative measure of amplifiable human genomic DNA with primers specific for the detection of human *Alu* sequences (56). Xenograft DNA (0.1 ng) was amplified with 100 nM of sense (5’-ACG CCT GTA ATC CCA GCA CTI T3’) and antisense (5’-TCG CCC AGG CTG GAG TGC A 3’) human *Alu* primers, under the same PCR conditions as described above. Negative controls, positive controls, and the experimental samples were run in duplicate. The threshold cycle values from each sample were averaged, and the standard deviation was calculated.

**RESULTS**

**Expression, purification, and refolding of thio-pro-EhCP1.**

Expression of the thio-pro-EhCP1 recombinant protein in *E. coli* resulted in an overexpressed protein of ~50 kDa, corresponding to the predicted molecular mass for thio-pro-EhCP1 of 50.3 kDa (36.3 kDa for the zymogen form of EhCP1, 13 kDa for the thioredoxin peptide, and 0.9 kDa for the six-His tag) (Fig. 1). rEhCP1 was purified by nickel affinity chromatography and reacted with rabbit polyclonal anti-EhCP1 Ab and monoclonal antithioredoxin Ab (Fig. 1).

The optimal refolding conditions to produce active rEhCP1 were determined by using a 16-condition screen as previously reported (3, 38). The most effective conditions were identified by cleavage of the synthetic peptide substrate Z-Arg-Arg-AMC. Refolded rEhCP1 was unique in several ways. (i) Refolded rEhCP1 did not require preincubation at low pH, which is usually required for the self-activation from the proform to the mature form of eukaryotic cysteine proteinases. (ii) Refolded rEhCP1 could be activated in a Tris-EDTA, phosphate-EDTA, or citrate-EDTA buffer at neutral or slightly alkaline pH (7.0 to 7.5) with 2 to 5 mM dithiothreitol (DTT). (iii) Refolded rEhCP1 could be activated with 5 to 10 mM cysteine alone. rEhCP1 activation by cysteine is of importance as it would not affect the in vitro cleavage of several of its putative biological substrates, particularly *C3*, which is readily denatured in the presence of other thiol-reducing agents such as DTT (43) or 2-mercaptoethanol, each of which is widely used for the in vitro activation of cysteine proteinases.

**FIG. 2.** Substrate specificity determined by synthetic combinatorial libraries. A P1 complete diverse library and P2, P3, and P4 sublibraries of the P1-lysine fixed library were used to determine the substrate specificities of EhCP1. Activities are displayed as percentages of the maximum at each position. Amino acids are represented by the single-letter code (“n” is norleucine). Error bars represent the standard deviations from the results of duplicate experiments.
Refolded active rEhCP1 was purified by ion-exchange chromatography with a Hi-Trap-Q column (Amersham Biosciences, United Kingdom). As shown in Fig. 1, analysis of the peak fractions revealed the presence of one polypeptide band with a molecular mass of ~31 kDa shown by silver staining of each panel.

**FIG. 4.** Localization of EhCP1. EhCP1 was localized in amebic trophozoites by confocal microscopy (A) using polyclonal Ab to EhCP1 (green) and monoclonal Ab to EhCP3 (red). These results were confirmed by immunoelectron microscopy (B) where EhCP1 (15-nm gold particles) was present in large vesicles, distinct from EhCP3 (20-nm gold particles).

Arg-Arg-AMC, typical cathepsin B substrates, were readily cleaved, whereas Z-Phe-Arg-AMC, a typical cathepsin L substrate, was not (data not shown). To further define the substrate requirements of rEhCP1, we mapped the specificity of the rEhCP1 active site using two synthetic tetrapeptide combinatorial libraries (Fig. 2). An almost absolute requirement for arginine at the P2 position was found.

**pH optimum of rEhCP1.** rEhCP1 was found to have a pH optimum of 6.0, retaining most of its catalytic activity in the pH range of 5.5 to 7.5 (data not shown). A 50% or higher reduction in the catalytic activity is seen at pH values of ≤5.0 or ≥8.0.

**Biological substrate specificity of rEhCP1.** To determine if rEhCP1 had the same biological substrate specificity as the secreted native proteinases, human C3, IgG, and pro-IL-18 were incubated with equal amounts of active purified rEhCP1 or native Eh-secreted proteinases (CM). rEhCP1 cleaves C3 in a fashion identical to that of the secreted native proteinases (43), generating the α′ subunit (Fig. 3A). rEhCP1 also cleaves the human IgG heavy chain in a dose- (Fig. 3B) and time-dependent manner (53) (data not shown), similar to native proteinases. Human pro-IL-18 is also cleaved by rEhCP1, similar to the degradation seen by released amebic proteinases (CM) and rEhCP5 (Fig. 3C) (37).

**Intracellular localization of EhCP1.** In order to determine the intracellular localization of EhCP1, Entamoeba histolytica trophozoites were labeled with rabbit polyclonal anti-EhCP1 Ab and imaged by fluorescence, confocal, and electron microscopy. EhCP1 localizes within large cytoplasmic vesicles, which are distinct from those containing EhCP3 (Fig. 4A). These results were confirmed by immunoelectron microscopy (Fig. 4B).

**Inhibition of EhCP1 by vinyl sulfone inhibitors.** We first tested the inhibition of purified rEhCP1 by K11777, a vinyl sulfone inhibitor which has been shown to cure Trypanosoma cruzi infection in animals (10), has undergone extensive toxicity testing (1), and is approaching clinical trials for Chagas’ disease. The $K_{\text{inact}}/K_{\text{app}}$ of K11777 against EhCP1 was 350 (1/M · s). We next synthesized a peptidomimetic vinyl sulfone with an arginine substituted for phenylalanine in the P2 position based on the distinct specificity for arginine in P2 detected by active site mapping (Fig. 2). The resulting $K_{\text{inact}}$ for WRR483 was 849,000 (1/M · s), an increase of ~2,500-fold.
Effect of the cysteine proteinase inhibitors K11777 and WRR483 on amebic invasion in the intestinal xenograft model.

We next tested the efficacy of the inhibitors in an in vivo model of invasion of human intestine. The pathology of human colon xenografts infected for 24 h with virulent *E. histolytica* trophozoites closely mimics human disease, with undermined ulcers containing trophozoites interspersed with normal mucosa (Fig. 5, left). When colon xenografts were infected with *E. histolytica* trophozoites preincubated with the cysteine proteinase inhibitor K11777 (1) (data not shown) or WRR483 (Fig. 5, right), no invasion was seen. Amebic invasion in human colon xenografts was quantified by a real-time PCR assay that could detect as few as 10 trophozoites (data not shown). The amplification of human Alu sequences in all of the xenografts was equivalent (15.93 ± 1.3) (56). Xenografts infected with *E. histolytica* trophozoites preincubated with the specific irreversible vinyl sulfone cysteine proteinase inhibitor K11777 had a >80% reduction in the number of detectable *E. histolytica* trophozoites (Fig. 6A). Inhibition of amebic invasion with the vinyl sulfone cysteine proteinase inhibitor WRR483 was even more dramatic, with a >95% reduction in the number of detectable trophozoites in the xenograft tissue (Fig. 6B).

**DISCUSSION**

Released cysteine proteinases play a fundamental role in invasion of target tissues by *E. histolytica* trophozoites. Analysis of the *E. histolytica* genome reveals that 40 genes encode cysteine proteinases (25). Studies of the expression of the cysteine proteinase genes have shown that only three, *ehcp1*, *ehcp2*, and *ehcp5*, account for more than 90% of the cysteine proteinase-specific transcripts (5, 6) and more than 95% of secreted amebic proteinases in vitro (6, 8, 17). We focused on EhCP1 because it is unique to *E. histolytica*, there being no homologous gene in the closely related but noninvasive *E. dispar* (5). It is also one of the most highly expressed and released cysteine proteinases (16, 17). Because of the difficulty in separating the large number of similar native cysteine proteinases, we focused on one of the most highly expressed and released cysteine proteinases (16, 17). We focused on EhCP1 because it is unique to *E. histolytica*, there being no homologous gene in the closely related but noninvasive *E. dispar* (5). It is also one of the most highly expressed and released cysteine proteinases (16, 17). Because of the difficulty in separating the large number of similar native cysteine proteinases, we focused on EhCP1 as the most likely to contribute to the invasion of human tissue.

The unique substrate specificity to design a vinyl sulfone inhibitor, WRR483, from the scaffold compound K11777.

**FIG. 6.** Quantification of amebic invasion in colonic xenografts. Human intestinal xenografts were infected with 1 × 10⁶ *Entamoeba histolytica* trophozoites which had been preincubated in the presence or absence of 20 μM specific irreversible cysteine proteinase inhibitor K11777 (A) or 10 μM of WRR483 (B). At 24 h of incubation the xenografts were harvested and DNA was extracted from 100 mg of xenograft tissue. The amount of *E. histolytica* trophozoites present in each sample of tissue was determined by a real-time PCR assay using a standard curve which related the number of *E. histolytica* trophozoites to the cycle threshold. Values represent the means ± standard errors of duplicates from three xenograft experiments.
rEhCP1 cleaves a number of key physiologic substrates equivalently to the native enzymes. For example, active rEhCP1 readily cleaves the α chain of human complement component C3 (Fig. 3A), which we have shown mimics the cleavage of C3 convertases, producing an active C3b molecule (43). The cleavage site is actually one amino acid residue distal to native convertases, placing an arginine in the P2 position of EhCP1 (43). The released C3a anaphylatoxin fragment is further degraded by the amebic cysteine proteinases (41), limiting the host inflammatory response. Human IgG is also cleaved by refolded active rEhCP1 (Fig. 3B) as previously shown with purified amebic proteinases (53). This observation is consistent with recent clinical findings that systemic antiamebic IgG responses do not protect people from reinfection with *E. histolytica* (12, 13, 14). Previously, we have demonstrated that rEhCP5, the only other cysteine proteinase unique to *E. histolytica*, degraded pro-IL-18, a finding that implicates amebic cysteine proteinases in the observed lack of neutrophils in amebic lesions (37). Now we have found that rEhCP1 produces a similar dose- (Fig. 3C) and time-dependent degradation of pro-IL-18.

The intracellular localization is known for only a few amebic cysteine proteinases. EhCP5 (18), EhCP2 (35, 36), and EhCP112 (34) are membrane associated, while EhCP3 is intracellular (17, 36). By using confocal and immunoelectron microscopy, we found that EhCP1 localizes to large cytoplasmic vesicles, sites distinct from the vesicles containing EhCP3 (Fig. 4). These are likely the larger, prephagocytic, nonacidic vesicles, which were shown to contain EhCP1 in proteomic studies (35). This localization is consistent with the higher pH optimum (6.0) of EhCP1 as well. We had previously shown that EhCP1, EhCP2, and EhCP5 are passively released during phagocytosis (17). This release is likely to be part of the newly described EhRab11B-associated secretory pathway, as overexpression of EhRab11B led to increased release of all three proteinases (30).

Animal models of amebiasis have been problematic as only humans and higher primates are naturally susceptible to infection. Cysteine proteinase inhibitors such as E-64 and an EhCP5 antisense construct, which blocked expression of multiple cysteine proteinases, inhibited amebic liver abscess formation in SCID mice (50) and hamsters (2). The trophozoites had to be injected directly into the liver, however, which bypasses the normal route of infection through the colon. To avoid this limitation, we closely mimicked human infection by using the human xenograft model (55). When fetal intestinal loops are implanted under the skin of SCID mice, they develop physiologic lesions formed in the human graft (Fig. 5).

To test the proof of principle that EhCP1 and released cysteine proteinases are critical for invasion of the human colon, we evaluated the effect of inhibition of EhCP1 by the vinyl sulfone inhibitor K11777, which has a *K*_ass of 350 (1/M · 1/s) for rEhCP1. We initially chose this inhibitor because it has undergone extensive pharmacokinetic, bioavailability, and toxicity testing (1) and is approaching phase I clinical trials for treatment of Chagas’ disease. We quantified invasion by real time-PCR, with the specific amplification of a short sequence from the amebic peroxidexin gene (7, 40). Our initial results show for the first time that a specific cysteine proteinase inhibitor blocks amebic invasion of the human colon by more than 80% (Fig. 6A).

We next tested the new vinyl sulfone inhibitor WRR483, which was synthesized with arginine at the P2 position instead of phenylalanine to specifically target EhCP1. This specific inhibitor was almost 2,500-fold more potent than K11777 against rEhCP1 (849,000 1/M · 1/s). WRR483 is even more effective than K11777 at blocking amebic invasion in the human intestinal xenograft model (Fig. 5, right), as it reduced invasion by more than 95% (Fig. 6B).

Cysteine proteinases are an attractive target for agents designed to disrupt invasion by *E. histolytica*. The unique requirement of EhCP1 for arginine in the P2 position and the demonstrated ability of a specific cysteine proteinase inhibitor with these particular structural features (WRR483) to block amebic invasion of the bowel support the feasibility of this approach. We have now shown that the expression of active recombinant enzymes will enable us to dissect the role of individual cysteine proteinases in the virulence of this important protozoan parasite. Also, these recombinant enzymes will be a fundamental tool as we address the question of whether *E. histolytica* releases a higher level of cysteine proteinases facilitating invasion, in contrast to noninvasive *E. dispar*, or whether the key determinants of its invasiveness are the unique proteinases, EhCP1 and EhCP5.

We expect that our continuing efforts in specific inhibition of these unique proteinases will shed yet more light on their roles in the pathogenesis of amebiasis. In the meantime, our current studies have proven that inhibition of amebic cysteine proteinases blocks invasion of the human bowel and have identified a promising, orally available scaffold inhibitor.

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