

Entamoeba histolytica Cysteine Proteinases Disrupt the Polymeric Structure of Colonic Mucin and Alter Its Protective Function

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The adherent mucous gel layer lining the colonic epithelium is the first line of host defense against invasive pathogens, such as *Entamoeba histolytica*. The mucous layer prevents the attachment of amoeba to the colonic epithelium by trapping and aiding in the expulsion of the parasite. Disruption of the mucous layer is thought to occur in invasive amebiasis, and the mechanism by which the parasite overcomes this barrier is not known. The aim of this study was to characterize the specific interactions occurring between *E. histolytica* secreted cysteine proteinases and colonic mucin as a model to examine the initial events of invasive amebiasis. *E. histolytica* secreted products were examined for mucinase activity utilizing mucin metabolically labeled with [³⁵S]cysteine as a substrate. Cysteine proteinases degraded mucin in a time- and dose-dependent manner. A significant reduction (>50%) in high-molecular-weight mucin with altered buoyant density was observed when degraded mucin was analyzed by Sepharose 4B column chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, and CsCl density gradient centrifugation. Mucinase activity was eliminated by the specific cysteine protease inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane and was independent of glycosidase activity. Moreover, the degraded mucin was 38% less effective than native mucin at inhibiting amebic adherence to target epithelial cells. These results are the first to show that *E. histolytica* cysteine proteinases alter the protective function of the mucous barrier by disrupting the structure of the MUC2 polymer. Mechanistically, the parasite achieves this via proteolytic degradation of the terminal cysteine-rich domains.

The enteric protozoan parasite *Entamoeba histolytica* is the causative agent of human amebiasis. Infection with this parasite may result in amebic colitis and liver abscess formation, causing significant morbidity and mortality. More than 500 million people are infected with the parasite worldwide, resulting in an estimated 50 million cases of diarrhea and 100,000 deaths per year (36, 38). Although less than 1% of *E. histolytica* infections result in invasive disease, amebiasis ranks second only to malaria as a cause of mortality due to a protozoan parasite.

There are three separate and distinct phases in the pathogenesis of intestinal amebiasis: (i) colonization, (ii) mucous disruption and/or depletion, and (iii) binding to and cytolysis of host colonic epithelial cells. Histopathology studies in the gerbil model of invasive amebiasis suggest that amoeba first colonize the mucous layer by adherence via the parasite surface Gal-lectin to galactose (Gal) and *N*-acetyl-D-galactosamine (GalNAc) residues present on colonic mucin (9). Following colonization, the parasite causes a disruption and/or dissolution of the mucous layer to gain access to the underlying epithelium. This phenomenon may be a result of the concerted actions of a battery of cysteine proteinases (CPs) released by the parasite into its microenvironment (17). The amoeba CPs have also been implicated in the recruitment of host inflammatory cells to the site of invasion (39). Subsequent to depletion of the mucous barrier, the parasite may come into contact with and cause lysis of host epithelial and polymorphonuclear

cells, inducing colonic ulceration and colitis. Following invasion, trophozoites are capable of migrating through the lamina propria and submucosa before they disseminate to soft organs, most often the liver, causing amebic liver abscess and death if left untreated (8).

Colonic mucin serves an important function in preventing amebic invasion of the colon. Amebic adherence to Gal or GalNAc residues of MUC2 mucin facilitates colonization of the mucous layer lining the colon via the 170-kDa Gal-lectin. This high-affinity ($K_d = 8.20 \times 10^{-11}$ M) interaction inhibits parasite adherence to and cytolysis of target cells, in turn, protecting the colonic epithelium from parasite invasion (10). In order for the parasite to gain access to the underlying epithelial cells, it must first breach the protective mucous layer. The mechanisms that enable the parasite to overcome this barrier have yet to be determined.

MUC2 is the major glycoprotein component of the colonic mucous gel layer. The MUC2 apoprotein (Fig. 1) is composed of two mucin domains termed the variable-number tandem repeat region (VNTR) and the irregular repeat region (IR). The VNTR is composed of a well-conserved 23-amino-acid tandemly repeated sequence, rich in the amino acids threonine and proline, and the actual number of repeats varies significantly among alleles. The IR comprises a much shorter mucin domain constituting a 347-amino-acid repeat region rich in serine, threonine, and proline (22, 35). Both mucin domains are heavily glycosylated with oligosaccharides bound to serine and threonine residues via O-glycosidic bonds. Twenty-one separate oligosaccharide structures in the major colonic mucin species have been identified, and characterization studies have revealed oligosaccharides ranging in chain length from 2 to 12 residues for the mature MUC2 glycoprotein (25). These mucin

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FIG. 1. A hypothetical model of a MUC2 monomer. The molecular mass of the monomer is approximately 1.5×10^6 Da, containing ~5,000 amino acids (14). The mucin domains are represented by shaded boxes and include the IR (180 kDa) and VNTR (~930 kDa). The protein core of the IR and VNTR are resistant to proteolytic attack due to steric hindrance. Less-glycosylated segments (A and B) flank the mucin domains. These regions contain D domains, which are rich in cysteine and are sites for polymerization of MUC2. The D domains are hypothesized to be targets for proteases.

domains are resistant to proteolytic attack due to their extensive glycosylation, and the action of glycosidases in these regions would be necessary to expose the protein core to proteases.

The N- and C-terminal regions, which flank the mucin tandem repeats, are composed of various cysteine-rich D domains with high sequence similarity to the polymeric serum glycoprotein, von Willebrand factor (13). The terminal D domains are poorly glycosylated compared to the mucin domains. These cysteine-rich regions play a critical role in the disulfide bond-dependent dimerization and subsequent polymerization of MUC2, which gives rise to the viscoelastic and protective nature of mucus (2, 18). Mucous gel formation has been observed to be inhibited upon disruption of the nonglycosylated regions of mucin, either by disulfide bond reduction or proteolytic digestion (5, 14). In addition, studies using rat MUC2, a homologue to human intestinal MUC2, have revealed these flanking regions to be extremely susceptible to proteolytic cleavage (18).

E. histolytica releases significant quantities of CPs (*E. histolytica* CPs [EhCPs]) into its environment (17). EhCPs are the main class of proteinase produced by the trophozoite (17, 20, 24, 26), and a direct correlation between EhCP activity and amebic virulence and invasiveness has been reported (15, 28). The EhCPs degrade extracellular matrix proteins such as laminin, collagen, and fibronectin (30), contributing to the cytopathic effect involving the detachment of host epithelial cells (16). The proteinases may play a key role in immune evasion, since they have been found to degrade immunoglobulins and complement (34). The role of EhCPs in liver abscess formation has also been investigated, and antisense inhibition of EhCPs in trophozoites resulted in decreased liver abscess formation in hamsters (1). In addition, incubation of trophozoites with the CP inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) greatly reduced liver abscess formation in severe combined immunodeficient mice (32). Although there have been numerous studies concerning the role of amebic CPs in invasive amebiasis at the mucosal and systemic levels, there have been few attempts to elucidate the primary events involved in invasion. Amebic invasion of the colonic epithelium may be facilitated by the ability of EhCPs to degrade colonic mucin, which may alter its gel-forming ability and eliminate its protective function. Herein, we examine the interactions between *E. histolytica* secretory proteinases and LS 174T cell mucin as a model for invasive amebiasis.

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MATERIALS AND METHODS

Cell cultures. The human colonic adenocarcinoma cell line LS 174T was obtained from the American Type Culture Collection (Rockville, Md.) and cultured in minimal essential medium (MEM) (Invitrogen Corporation, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, Utah), 100 μ g of streptomycin sulfate per ml, 100 U of penicillin per ml, and HEPES. Cultures were grown in plastic tissue culture flasks (15 by 10 cm) and maintained in a humidified 5% CO₂ atmosphere at 37°C as previously described (4). LS 174T cells grown to 80% confluence were used for metabolic labeling of mucin, as well as a source of nonlabeled native mucin. Chinese hamster ovary (CHO) cells were cultured in F12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U of penicillin per liter, and 100 μ g of streptomycin sulfate per ml at 37°C. Once the cells were confluent, they were harvested by 0.25% trypsin digestion for 5 min.

Cultivation and harvesting of *E. histolytica*. *E. histolytica* HM-1:IMSS trophozoites serially passaged through gerbil livers to maintain high virulence were maintained axenically in TYI-S-33 medium at 36.6°C as previously described (4). Trophozoites were harvested at the logarithmic growth phase (72 h); the trophozoites were chilled on ice for 10 min and collected by centrifugation (700 \times g for 5 min at 4°C).

Collection of amoeba secretory products. Following harvest, trophozoites were washed twice with Hank's balanced salt solution (HBSS; Invitrogen) and incubated in HBSS (2 \times 10⁷ amoeba/ml) in the absence of serum for 2 h at 36.6°C. Trophozoites were collected by centrifugation (700 \times g for 5 min at 4°C), and the supernatant contained amoeba secretory products (SPs). Amebic viability was determined to be >95% after a 2-h incubation in HBSS as determined by the trypan blue exclusion assay. Protein concentrations of SPs were determined by the method of Bradford, using bovine serum albumin (BSA) as a standard (6), and the SPs were stored at -80°C until needed.

Enzymatic assay for amebic secreted proteases. General proteolysis was detected by a colorimetric method using the universal substrate, azocasein, as previously described (29). Amoeba SPs were incubated with the protease inhibitors E-64 (20 μ M), Pefabloc SC (4 mM), Na₂EDTA (0.7 mM), and pepstatin (1 μ M) (Roche Diagnostics GmbH, Mannheim, Germany) for 20 min at 37°C prior to the assay. The change in optical density at 440 nm was monitored, and the percentage of residual activity was determined. Specific CP activity was measured against benzyloxycarbonyl-L-arginyl-L-arginine-*p*-nitroanilide (Z-Arg-Arg-*p*NA; Bachem, Torrance, Calif.) as previously described with some modifications (20). The reaction mixture consisted of 0.1 mM substrate in reaction buffer followed by the addition of secreted proteins (50 μ g). Secreted proteins were incubated with a panel of protease inhibitors prior to the assay. The cleavage rate of *p*-nitroaniline was monitored at 405 nm for 10 min at 37°C. One unit of enzyme activity was defined as the number of micromoles of substrate digested per minute per mg of protein. Secreted products were assayed for proteinase activity by substrate gel electrophoresis as previously described (17).

Metabolic labeling of LS 174T mucin. LS 174T cells were grown to 80% confluence, and culture medium was removed and replaced with fresh MEM supplemented with [³⁵S]cysteine (0.5 μ Ci/ml) (specific activity, >1,000 Ci/mmol) (Amersham Biosciences, Baie D'Urfé, Quebec, Canada). Supernatants containing [³⁵S]cysteine-labeled mucin were collected twice weekly for 2 weeks and stored at -20°C. [6-³H]glucosamine labeling of mucin was achieved by replacing MEM with fresh MEM containing [6-³H]glucosamine (2 μ Ci/ml) (specific activity, 25 to 40 Ci/mmol) (ICN, Montreal, Quebec, Canada). Native mucin was collected from cell cultures grown in MEM void of radiolabel. The purification steps for mucin were identical under all conditions (radiolabeled or native mucin) unless specified otherwise. Supernatants were concentrated by speed vacuum or lyophilization. Particulates were removed by centrifugation (750 \times g for 10 min at 4°C), and supernatants were resuspended in column buffer (0.01 M Tris-HCl, 0.001% sodium azide [pH 8.0]) (Sigma-Aldrich, St. Louis, Mo.).

Preparation of native and metabolically labeled LS 174T mucin. LS 174T supernatants were applied to a Sepharose 4B (S4B) column (50 by 2.5 cm; Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with column buffer. The column was calibrated by using the following molecular mass standards: blue dextran (2,000 kDa) (Pharmacia, Uppsala, Sweden), thyroglobulin (669 kDa), and BSA (68 kDa). Samples were eluted at a flow rate of 40 ml/h, and 4-ml fractions were collected. All purification steps were performed at 4°C. Aliquots (100 μ l) of each fraction (fractions 1 to 40) were added to individual scintillation vials containing 5 ml of liquid scintillation fluid (ICN, Costa Mesa, Calif.). The elution profile for radiolabeled mucin was determined by liquid

scintillation counting. Fractions containing void volume (V_0) mucin (fractions 11 to 18) were pooled and dialyzed for 24 h against deionized water at 4°C. Total ^3H - or ^{35}S -labeled activity was determined for each fraction. To isolate native mucin, the protein contents in the fractions were monitored (absorbance at 280 nm), the elution profile was obtained, and the protein concentration was determined.

Highly purified mucin was obtained by CsCl density gradient centrifugation. Metabolically labeled S4B V_0 mucin (2×10^6 cpm for ^{35}S -labeled mucin and 3×10^6 cpm for ^3H -labeled mucin) was resuspended in 10 ml of Dulbecco's phosphate-buffered saline (DPBS) (pH 7.2) (Invitrogen). Cesium chloride (Invitrogen) was added to the mucin suspension to achieve a starting density of 1.42 g/ml, and the suspension was dispensed equally into two centrifuge tubes (13 by 51 mm; Beckman, Palo Alto, Calif.). A gradient was established by centrifugation of the samples at $250,000 \times g$ for 48 h at 4°C. The contents of the tubes were divided into eight equal fractions, each fraction was removed from the top of the tube, and the density was determined. Total ^3H - or ^{35}S -labeled mucin activity was quantified by liquid scintillation counting and normalized for 1.0-ml fractions. For native mucin, 100 μl of each fraction was removed and the protein concentration was determined.

Mucin degradation assays. (i) S4B size exclusion chromatography. To determine mucinase activity, ^{35}S -labeled, S4B V_0 -purified mucin (10^5 cpm) was incubated with EhSPs (50 μg) in 0.5 ml of DPBS (pH 7.0) for 6 h at 37°C and fractionated by S4B chromatography (column, 30 by 0.75 cm) (Bio-Rad Laboratories). To determine the specific class of protease responsible for degrading mucin, SPs were incubated for 20 min prior to the assay with the following protease inhibitors: E-64 (20 $\mu\text{g}/\text{ml}$), Pefabloc SC (0.5 $\mu\text{g}/\text{ml}$), and pepstatin (0.7 $\mu\text{g}/\text{ml}$). Thirty fractions (0.5 ml each) were collected at a flow rate of 7 ml/h. The ^{35}S -labeled mucin elution profile was determined.

(ii) SDS-PAGE and autoradiographic analysis. [^{35}S]cysteine-labeled (2×10^4 cpm) V_0 mucin was incubated with 50 μg of SPs in 0.5 ml of reaction buffer at 37°C, and the reactions were terminated at various time points (15, 30, 60, 180, and 360 min) by boiling. SPs were also incubated with E-64 (100 μM) for 20 min prior to the assay to inhibit CP activity. The samples were concentrated and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris-Cl [pH 6.8], 10 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Digests were analyzed by SDS-PAGE (4% stacking and 7% resolving gels) under reducing conditions and visualized by autoradiography by exposing the Kodak XAR-5 film with an intensifying screen to the gel for 1 week at -70°C as previously described (3). The relative density of stacking gel mucin was determined, and the percent mucinase activity was calculated using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image>).

(iii) Buoyant density analysis. S4B V_0 mucin (10^5 cpm of ^{35}S -labeled mucin) was incubated with 100 μg of SPs or DPBS alone for 18 h at 37°C. Specificity for CPs was demonstrated by preincubating the SPs with E-64 (100 μM) for 20 min prior to the assay. The digests were concentrated and resuspended in DPBS to a final volume of 5.0 ml, and CsCl was added to achieve a starting density of 1.42 g/ml. Samples were then analyzed by density gradient centrifugation as described above for previous mucin purification steps. To differentiate amoeba cysteine protease activity from glycosidase activity, ^3H -labeled V_0 mucin (10^6 cpm) was incubated with SPs (250 μg) at 37°C for 18 h. ^3H -labeled mucin degradation was analyzed as described above for [^{35}S]cysteine-labeled mucin.

Functional analysis of degraded mucin. Amebic adherence assays to target CHO cells were performed by a modified version of a standard protocol (10). Briefly, trophozoites were first washed with M199s medium (Invitrogen) supplemented with 5.7 mM cysteine, 25 mM HEPES, and 0.5% BSA (Sigma-Aldrich). The trophozoites were resuspended to a concentration of 10^6 amoeba/ml. The trophozoite solution was incubated with medium alone, medium and S4B V_0 mucin (100 $\mu\text{g}/\text{ml}$), or mucin preincubated with SPs for 1 h at 37°C. To determine if CPs were responsible for the loss of protective function, SPs were also incubated with E-64 (100 μM) prior to the assay. Following incubation, 100 μl of the trophozoite solution (10^4 trophozoites) was added to 2×10^6 CHO cells in M199s (volume, 1 ml). The samples were pelleted by centrifugation at $600 \times g$ for 5 min at 4°C, followed by incubation at 4°C for 2 h. Rosette formation was defined as the percentage of amoeba adherent to three or more target cells, which was determined by counting >100 amoeba per tube.

Statistical analysis. Data (means \pm standard deviations [SDs]) were analyzed by the Student *t* test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Secreted protease activity. Inhibition studies employing several protease inhibitors, including E-64, Pefabloc SC, pepsta-

TABLE 1. Effects of protease inhibitors on EhCP activity^a

Inhibitor	Specificity of inhibition	% Residual activity ^b
Cystatin	Cysteine	2 \pm 0.79
E-64	Cysteine	5 \pm 0.54
Leupeptin	Cysteine/serine	5 \pm 0.43
TLCK	Cysteine/serine	2 \pm 0.25
TPCK ^c	Cysteine/serine	6 \pm 1.49
Pefabloc SC	Serine	93 \pm 1.43
Aprotinin	Serine	100 \pm 2.51
Pepstatin	Aspartic acid	100 \pm 7.57
Phosphoramidon	Metallo-	100 \pm 1.00

^a EhCP activity was measured using the synthetic substrate Z-Arg-Arg-pNA. CPs cleave substrates with arginine in the P-2 position. SPs typically contained between 8 and 13 U of activity per μg of protein, and in this study, 50 μg of protein with a total of 630 U was used.

^b Mean percent residual activity (of control) \pm SD ($n = 3$).

^c TPCK, tosylsulfonyl phenylalanyl chloromethyl ketone.

tin, and EDTA, were used to determine the major catalytic classes of enzymes released by the parasite. In addition, other protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, aprotinin, and *N* α -*p*-tosyl-L-lysine chloromethyl ketone [TLCK]) were used to confirm the results. The majority of the secreted protease activity was inhibited by E-64 ($>90\%$). Zymogram analysis of the SPs (gelatin substrate gels) revealed three major bands of protease activity at 57, 44, and 25 kDa. Proteinase activity increased with increasing concentrations of SPs, and the majority of the activity was eliminated by E-64 (data not shown). To determine the activity and specificity of the CPs, Z-Arg-Arg-pNA was used as a substrate (Table 1). SPs were incubated with a panel of protease inhibitors to determine the specificity of the enzymes for the substrate. As expected, only inhibitors of cysteine and cysteine/serine proteases eliminated enzyme activity, confirming the presence of CP activity in the SPs.

Degradation of mucin by EhCPs. (i) Analysis by S4B gel filtration. High-molecular-weight (MW) mucin polymers can be isolated by S4B column chromatography (4). As shown in Fig. 2A, [^{35}S]cysteine-labeled mucin eluted exclusively in the V_0 , whereas mucin incubated with 50 μg of SPs for 6 h resulted in a 34% decrease in high-MW V_0 mucin and a corresponding increase in degraded, lower-MW cleavage products in fractions 12 to 25. To determine if CP activity was responsible for mucin degradation, SPs were pretreated with protease inhibitors. Consistent with the data in Table 1, only E-64 eliminated mucinase activity by more than 50%, whereas Pefabloc SC and pepstatin displayed no inhibitory effect. To rule out any possibility of enzymatic activity to nonmucin components, [^{35}S]cysteine-labeled mucin was purified by CsCl density gradient centrifugation prior to treatment with SPs. As shown in Fig. 2B, SPs almost completely degraded the highly purified mucin as characterized by S4B column chromatography, demonstrating specific mucinase activity against the poorly glycosylated, [^{35}S]cysteine-labeled regions of mucin.

(ii) Analysis by SDS-PAGE. Due to its high MW and extensive glycosylation, the majority of ^{35}S -labeled mucin remains in the 4% stacking gel and the first portion of the running gel when analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3A, SPs degraded mucin in a time-dependent fashion. Mucinase activity occurred as early as 15 min and increased

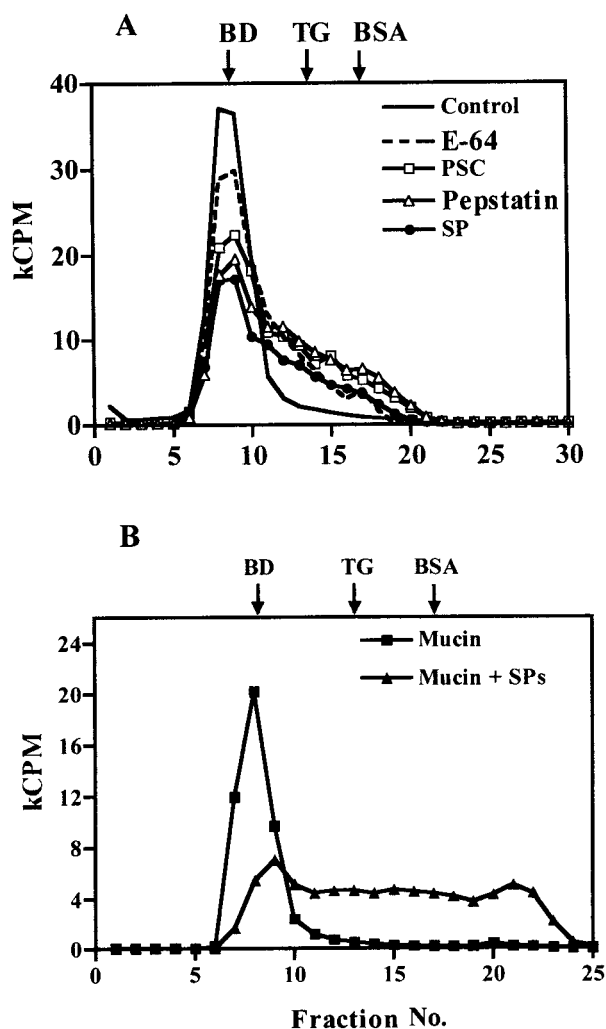


FIG. 2. (A) S4B elution profile of $[^{35}\text{S}]$ cysteine-labeled mucin degraded by *E. histolytica* SPs. The elution profiles of control, high-MW mucin and mucin incubated with SPs alone or with E-64, Pefabloc SC (PSC), or pepstatin are shown. kCPM, 1,000 cpm. (B) S4B elution profiles of CsCl mucin (fraction 6) degraded by *E. histolytica* SPs. The elution profiles of control, $[^{35}\text{S}]$ cysteine-labeled mucin alone and mucin incubated with SPs are shown. For details of the molecular mass markers, see Materials and Methods (BD, blue dextran; TG, thyroglobulin).

progressively with time, resulting in a reduction of stacking gel mucin and a corresponding increase in the appearance of degraded mucin polypeptide fragments at 85 and 120 kDa. After 6 h of incubation, there was more than a 90% decrease in ^{35}S -labeled stacking gel mucin. In contrast, highly purified mucin isolated by CsCl density gradient centrifugation was found to be slightly more resistant to degradation by the SPs (Fig. 3B). Nonetheless, almost complete degradation of mucin (>70%) occurred within 6 h of incubation with the SPs. The degradation was inhibited by 85% in the presence of E-64, clearly implying that CPs disrupt the polymeric structure of MUC2.

(iii) **Analysis by cesium chloride density centrifugation.** Purification of mucin by CsCl density gradient centrifugation separates noncovalently bound mucins from other proteins in the

high-density hexose-rich fractions (4). Figure 4A clearly shows that the majority of the $[^{35}\text{S}]$ cysteine-labeled mucin partitioned in fraction 6 and had a buoyant density of >1.42 g/ml. This partitioning is consistent with highly purified mucin, which migrates to fraction 6 on a CsCl density gradient (4). In contrast, following incubation with SPs, there was a dramatic shift in ^{35}S -labeled mucin from fraction 6 to fractions 1 to 4, a shift to a lower buoyant density (<1.40 g/ml; Fig. 4B). The appearance of ^{35}S -labeled mucin in these low-density fractions suggests that the N- and/or C-terminal cysteine-rich regions of MUC2 are altered by SPs. Evidence for this is clearly shown in Fig. 4C, where pretreatment of SPs with E-64 inhibited the degradation of MUC2 and resulted in a notable reduction in degraded mucin in fractions 1 to 3 and an increase in ^{35}S -labeled mucin activity in fractions 5 to 7. The partitioning profile of $[6\text{-}^3\text{H}]$ glucosamine-labeled mucin was similar to that of ^{35}S -labeled mucin; however, the majority of the $6\text{-}^3\text{H}$ -labeled mucin remained in fraction 6 after exposure to SPs, suggesting proteinase but not glycosidase activity (data not shown).

Functional analysis of degraded mucin. To determine if the protective function of mucin was compromised by EhCPs, amebic adherence assays to CHO cells were performed. As shown in Fig. 5, native S4B V_0 mucin was capable of inhibiting amebic adherence to CHO cells by >73% compared to the control without mucin. However, following incubation of mucin with 100 and 250 μg of SPs, amebic adherence to target cells increased 52 and 71%, respectively. To examine the role of CPs in this event, SPs (250 μg) were preincubated with E-64. Interestingly, not only was E-64 found to inhibit mucin degradation but also it helped to maintain the protective function of the mucin. This was evident from the fact that mucin incubated with E-64-treated SPs inhibited amebic adherence to CHO cells by 67%, which was similar to that of native mucin. These results directly implicate EhCPs in altering the protective function of mucin.

DISCUSSION

The majority of individuals infected with *E. histolytica* are asymptomatic carriers, but invasion does occur in a small percentage of those afflicted with the parasite (33). In order for invasion to occur, the parasite must overcome the protective mucous layer lining the colon. Histopathology studies of the human colon and rectum have revealed that the mucous layer lining these regions is separated into two striated layers. The outer layer contains most of the bacterial and fecal contents, and the inner layer contains little to no bacteria (21). These observations imply that mucin plays a role in establishing a clear barrier between luminal contents, including pathogens, and the colonic epithelium.

Previous studies have indicated that *E. histolytica* cellular lysates and SPs were ineffective at degrading human colonic mucin, and it was suggested that the parasite may cause a mechanical depletion of the mucous blanket by inducing goblet cell hypersecretion prior to invasion (31). In this study, we have shown that EhCPs are capable of degrading human colonic mucin. We have previously demonstrated that colonic mucin can be purified from cellular secretions of LS 174T cells by S4B column chromatography and CsCl density gradient centrifugation (4). Mucin collected from CsCl density gradients has been

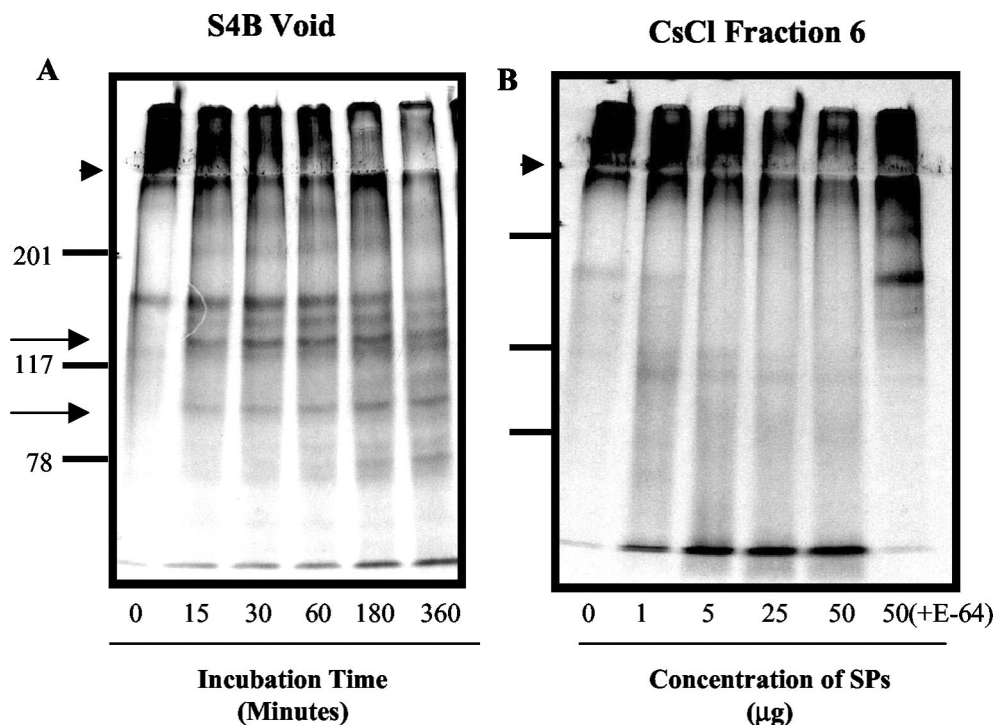


FIG. 3. (A) Time-dependent degradation of [^{35}S]cysteine-labeled S4B V_0 mucin incubated with SPs (50 μg). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. (B) Dose-dependent degradation of CsCl-purified, [^{35}S]cysteine-labeled mucin (fraction 6). In the rightmost lane, SPs were preincubated with E-64. The positions of the 4% separating gel (arrowheads) and cleavage products (arrows) are indicated.

extensively characterized and shown to be free of contaminants, such as proteoglycans or low-MW proteins (4). Metabolic labeling of LS 174T cell mucin with [^{35}S]cysteine allowed us to track the poorly glycosylated flanking regions of the molecule. This strategy allowed us to directly examine the ability of *E. histolytica* SPs to disrupt the cysteine-rich regions of highly purified mucin. Our results demonstrate that *E. histolytica* SPs were effective at degrading the poorly glycosylated regions of colonic mucin as visualized by S4B column chromatography, SDS-PAGE, and CsCl density gradient centrifugation. The parasite SPs efficiently disassembled the mucin polymer into smaller cleavage products. In addition, protease inhibition studies revealed that the CPs are responsible for most of the mucinase activity. These results are significant because the cysteine-rich regions of MUC2 are essential for mucin polymerization and gel formation. Interestingly, the cysteine-rich flanking regions of MUC2 and other gel-forming mucins are well conserved between species (19). This indicates the importance of disulfide bond-mediated mucin polymerization in mucous gel formation.

Degraded mucin was not as effective at inhibiting amebic adherence to target cells as the native mucin molecule, demonstrating that the degraded mucin had lost its inherent protective properties. This may be a consequence of the depolymerization and subsequent loss of the viscoelastic properties of the mucous gel. In vivo, mucin degradation may facilitate parasite invasion of the colonic epithelium. The mechanism by which proteolytic degradation of mucin affects amebic adherence is not known, but the polymeric form appears to be more

protective than the degraded form. Even though we did not detect significant glycosidase activity, their role as virulence factors cannot be entirely ruled out. One could speculate that differences in the lengths of the VNTRs between individuals and/or differences in glycosylation patterns may play a role in facilitating the pathogenesis of invasive amebiasis, but there is no evidence for this.

Clearly, multiple parasite virulence factors contribute to the deterioration and penetration of the mucous barrier. The role that the CPs play in the pathogenesis of invasive amebiasis is not yet fully understood. Most studies have been limited to host-parasite interactions under conditions that simulate post-invasion of the protective mucous barrier. In order to understand how invasive amebiasis occurs, it is essential to directly examine the interactions between *E. histolytica* and colonic mucin. Cysteine proteinases are known to be important virulence factors in diseases caused by various mucin-dwelling protozoa such as *Trichomonas vaginalis*, *Tritrichomonas foetus*, *E. histolytica*, and *Giardia lamblia* (23). One study has shown that of these organisms, only the trichomonads produced the necessary range of glycosidases needed for the complete breakdown of mucin (11). This may suggest that the other organisms utilize an alternative method for overcoming the mucous barrier. At least seven genes encoding CPs in *E. histolytica* have currently been identified (7, 12, 27). However, only gene products from five of these genes, *EhCP1*, *EhCP2*, *EhCP3*, *EhCP5*, and *EhCP112*, have been identified in cultured trophozoites (7, 12, 37). Bruchhaus et al. (7) have reported that the *EhCP1*, *EhCP2*, and *EhCP5* enzymes contribute to approximately 90%

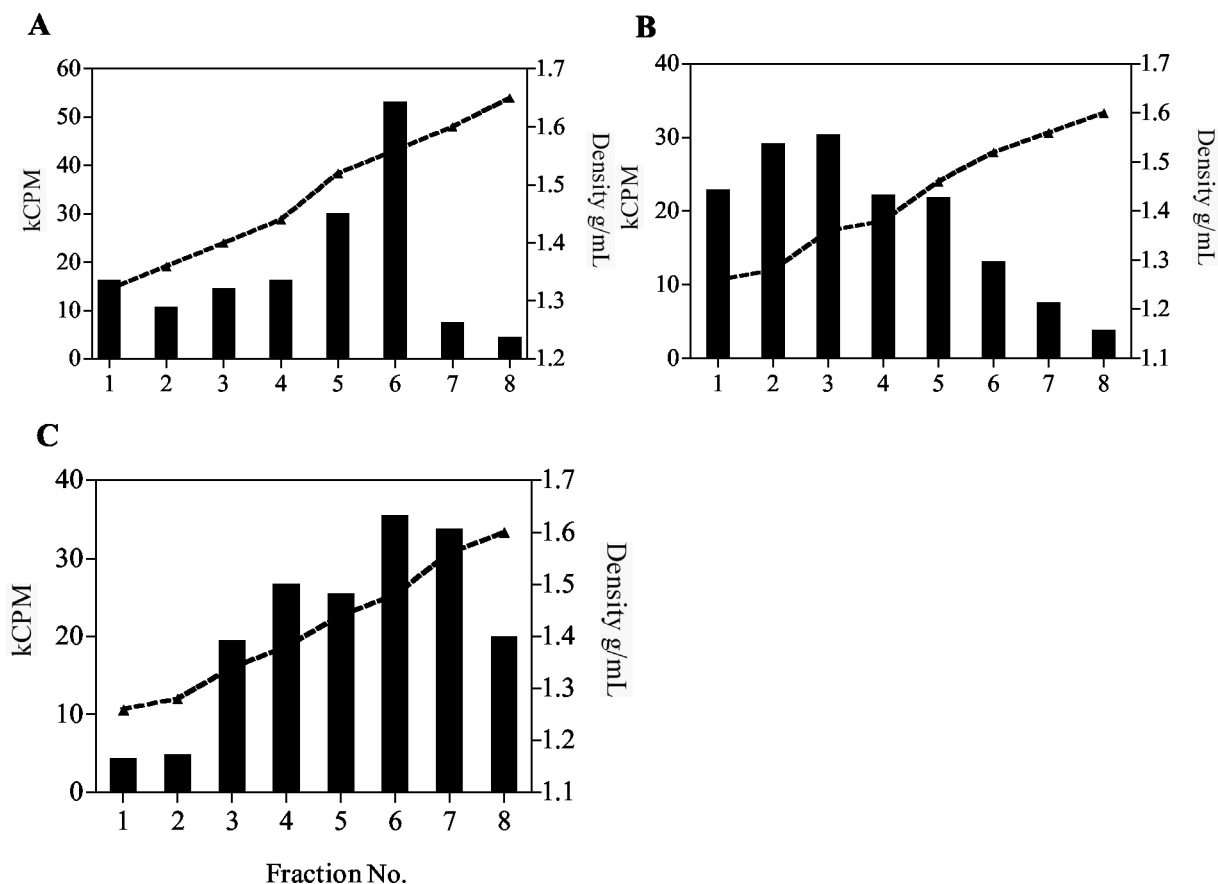


FIG. 4. (A) CsCl density gradient centrifugation of mucin degraded by *E. histolytica* SPs. Mucin partitioned in fraction 6, with a density of >1.42 g/ml (▲—▲). (B and C) Mucin incubated with SPs (B) and with SPs pretreated with E-64 (C). Each graph shows the results of one representative experiment of three separate experiments. kCPM, 1,000 cpm.

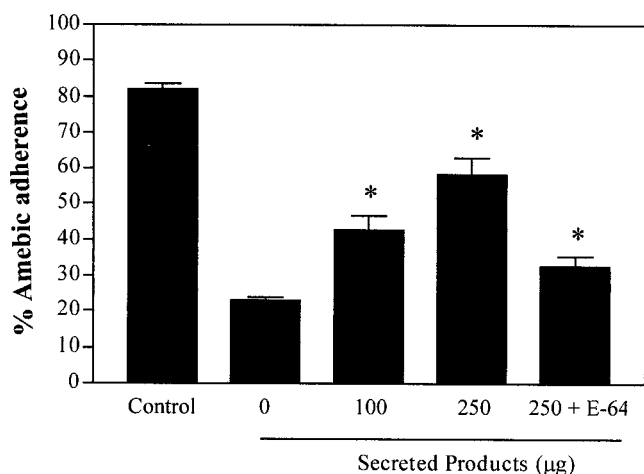


FIG. 5. EhCPs alter the protective function of LS 174T cell mucin. Note that preincubation of SPs with E-64 (100 μ M) significantly reversed amebic adherence to target cells (values that were significantly different [$P < 0.05$] from the value for the homologous control are indicated by the asterisks). The mean amebic adherence of different concentrations of SPs \pm SD (error bar) ($n = 6$) from one representative experiment of three experiments is shown.

of the total CP activity from the parasite. However, a specific CP involved in mucin degradation or amebic pathogenesis has not been identified. Clearly, future studies should focus on identifying the specific proteases involved in degrading colonic mucin. Identification of the virulence factors that play a role in the initial events of invasive amebiasis may aid in the development of new targets for chemotherapy or new vaccine candidates to prevent invasive amebiasis.

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